MASARYKOVA UNIVERZITA

PŘÍRODOVĚDECKÁ FAKULTA

ÚSTAV EXPERIMENTÁLNÍ BIOLOGIE

Habilitační práce

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MASARYKOVA UNIVERZITA
PŘÍRODOVĚDECKÁ FAKULTA
ÚSTAV EXPERIMENTÁLNÍ BIOLOGIE

NOVÉ ASPEKTY STUDIA GENOMIKY MNOHOČETNÉHO MYELOMU

Poděkování

- Jirkovi, Sáře a Soně za jejich podporu a lásku
- prof. MUDr. Romanu Hájkovi, CSc., zakladateli Babákovy myelomové skupiny
- Mgr. Lence Bešše, Ph.D. a Mgr. Lence Sedlaříkové, mým naprosto skvělým studentkám
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Tuto práci věnuji svým skvělým rodičům, Jarce a Viktorovi

Obsah

1 Úvod

Babákova myelomová skupina (BMS) je výzkumné těleso, které se zabývá výzkumem monoklonálních gamapatií. Tato skupina je zapojena do International Myeloma Working Group, což je poradní orgán složený z kliniků i z vědců, který vydává doporučení na léčbu i důležitých výzkumných témat. BMS je také zapojena v rámci České republiky do České myelomové skupiny.

Jako skupina jsme se rozhodli zaměřit na několik témat. Především se jednalo o výzkum MGUS (premaligního stavu mnohočetného myelomu), zavedení detekce minimální residuální choroby (MRD), výzkum extramedulárního relapsu mnohočetného myelomu a nových markerů onemocnění, především cirkulujících mikroRNA.

Moje práce se v rámci této skupiny rozvíjela právě těmito směry. Jednak jsem se zaměřila na výzkum extramedulárního relapsu, kdy jsme se věnovali především poznání problematiky zvyšující se incidence a komplexní biologie této formy onemocnění. Dále jsme se věnovali zavedení metody detekce MRD pomocí qPCR, která je i nadále zlatým standardem hodnocení účinnosti léčebných strategií u klinických studií. V neposlední řadě jsem se věnovala novým markerům onemocnění, zejména cirkulujícím mikroRNA, které mají potenciál překonat bolestivý postup stanovení diagnózy mnohočetného myelomu, který využívá invazivní odběr kostní dřeně. Rovněž by mohly sloužit jako prognostické či prediktivní markery a mít tak roli i v tzv. „personalizované medicíně“, což je u mnohočetného myelomu z důvodu jeho rozsáhlé heterogenity velmi důležité. Také se zdá, že tyto markery mají důležitou roli v patogenezi onemocnění.

Tato témata byla pod mým vedením zpracována rovněž studenty bakalářských, magisterských a doktorských studijních programů v rámci jejich závěrečných prací.

2 Problematika

2.1 Mnohočetný myelom

Mnohočetný myelom (MM) je zhoubné lymfoproliferativní onemocnění které je charakterizováno infiltrací a akumulací patologických klonálních plazmatických buněk (PB) v kostní dřeni (KD), osteolytickými ložisky ve skeletu a přítomností monoklonálního imunoglobulinu (M-Ig) v séru a/nebo moči (Hájek *et al*., 2012).

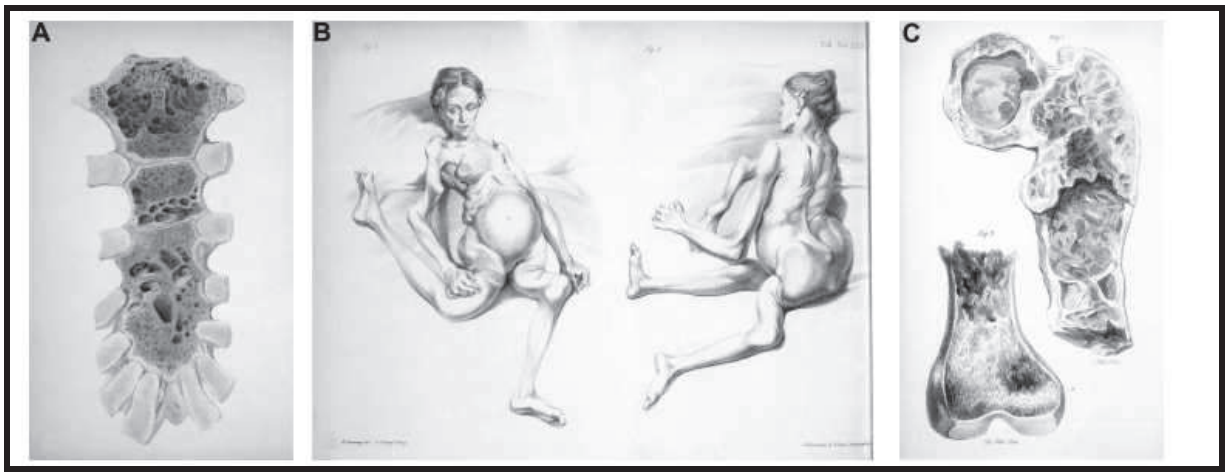
Jedná se o druhé nejčastější hematoonkologické onemocnění, které v České republice vykazuje incidenci okolo 4/100 000 obyvatel. V Evropě je ročně diagnostikováno více než 40 000 nových případů (Adam *et al.*, 2008; Hájek *et al*., 2012). Celkově MM představuje asi 10 % všech hematologických malignit, 1 až 2 % všech nádorových onemocnění a zhruba 2 % všech úmrtí v důsledku nádorových onemocnění (Avet-Loiseau *et al*., 2007). MM postihuje zejména starší osoby, medián věku při stanovení diagnózy se pohybuje kolem 65 let a je mírně častější u mužů (Adam *et al*., 2008).

Dnes je prokázané, že MM předchází monoklonální gamapatie nejasného významu (MGUS), ačkoliv okolnosti a důvody zvratu benigního MGUS do maligního MM nejsou dosud objasněny (Kyle *et al*., 2011).

Prvotní příčina vzniku MM je dodnes neznámá a průběh u jednotlivých pacientů velice heterogenní. I když se daří prodlužovat dobu remise, většina pacientů stále relabuje. Skupina vysoce rizikových pacientů (tvoří asi 10 až 15 % všech MM pacientů) relabuje obvykle do 12 měsíců od diagnózy (Shaughnessy *et al*., 2007). Zároveň je možné za vysoce rizikový MM považovat extramedulární myelom (EM), protože tito pacienti mají také výrazně horší přežití. Důsledkem akumulace genetických změn u EM pacientů je autonomní růst myelomových buněk, který je nezávislý na stromatu KD a umožňuje přechod myelomových buněk do extramedulární oblasti – nejčastěji do podkoží a měkkých tkání (Usmani *et al*., 2012).

2.2 Historie MM

První řádně zdokumentovaný případ MM byl publikován v roce 1844. Jednalo se o případ 39-leté ženy Sarah Newbury, u které se objevily příznaky únavy a bolesti kostí v důsledku mnohonásobných zlomenin (Obr. 1). Teprve po její smrti, která nastala o 4 roky později, pitva prokázala výrazné změny KD (Solly, 1844).



Obrázek 1 První popsaný pacient s mnohočetným myelomem – Sarah Newbury (z Kyle *et* Rajkumar, 2008). A) Destrukce sterna B) Pacientka se zlomeninami C) Destrukce femuru

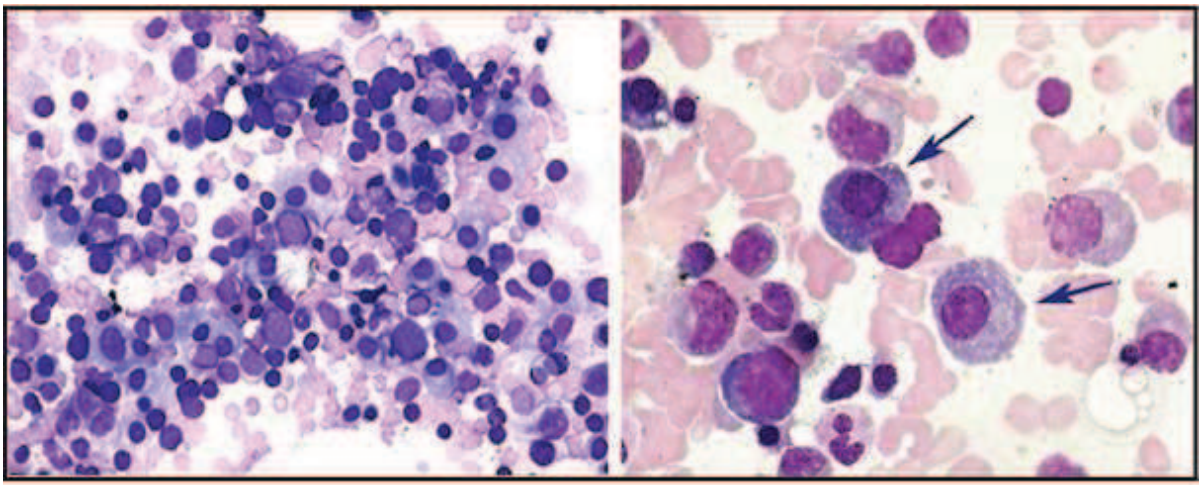
Nejznámnějším případem MM té doby byl však Thomas Alexander McBean, u kterého se ve věku 45 let objevily podobné příznaky jako u Sarah Newbury – bolesti, zlomeniny kostí. I přes veškerou „moderní" léčbu té doby, která zahrnovala například pijavice, McBean zemřel (Macintyre, 1850). Vzorek jeho moče byl poslán Dr. Bence Jonesovi, který popsal přítomnost proteinu v moči. Tento protein se do dnešní doby nazývá Bence-Jonesova bílkovina a stal se jedním ze základních markerů MM (Jones, 1848).

Myelom byl také nazýván Kahlerovou chorobou podle známého pražského lékaře Otty Kahlera, který popsal chorobu svého kolegy Dr. Loose. Pacient měl progradující bolesti kostí, proteinurii s typickou charakteristikou Bence-Jonesovy bílkoviny a v době pitvy byly nalezeny velké kulaté buňky konzistentní s buňkami MM (Kyle *et* Rajkumar, 2008).

2.3 Patofyziologie MM

Patogeneze MM je komplexní multifaktoriální proces vedoucí k nádorové transformaci populace B-lymfocytů sérií genetických změn (Hájek *et al*., 2011). Takto pozměněné buňky dále nekontrolovatelně proliferují a diferencují v buňky plazmablastické, které si zachovávají schopnost migrace a proliferace. Zralá myelomová (plazmatická) buňka je terminálním stadiem vznikajícím z pozměněných plazmablastů nahromaděných v KD, kde přežívá podstatně déle než fyziologické PB, které přibližně po dvou dnech produkce přirozených imunoglobulinů podléhají apoptóze. Proto jsou myelomové buňky označovány za buňky dlouhověké. Ačkoliv se zdá, že buňky v terminálním stádiu se již dále nemnoží, nekontrolovatelná proliferace v KD je hlavním rozdílem mezi vývojovou řadou

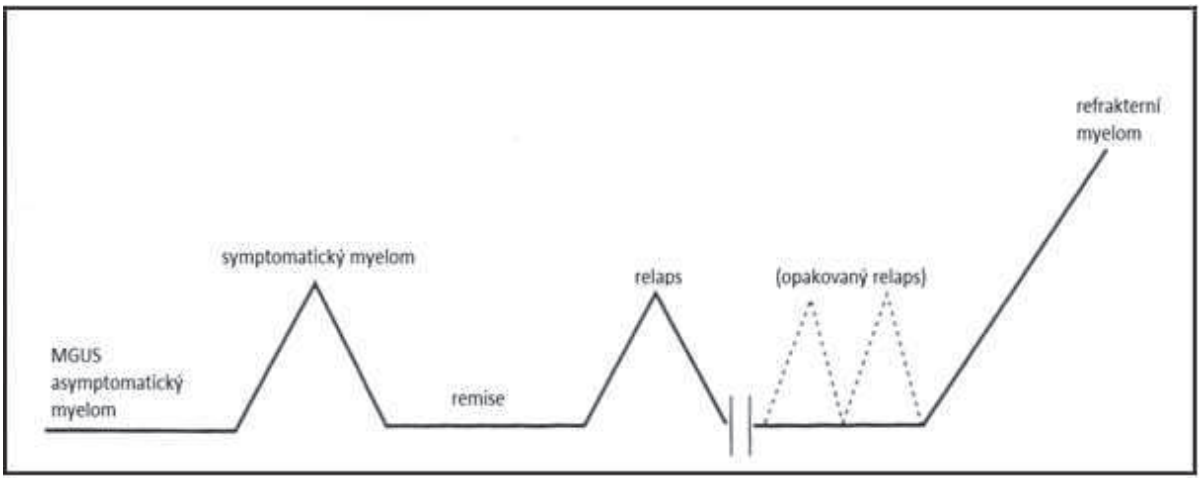
myelomových buněk a fyziologickým procesem maturace plazmablastů (Obr. 2) (Adam *et al*., 2001; Shapiro-Shelef *et* Calame, 2004).



Obrázek 2 Srovnání počtu myelomových PB (vlevo) oproti počtu fyziologických PB (vpravo) (Maslak, 2009)

Studie provedené během posledního desetiletí přinesly značný posun v porozumění biologických a molekulárních mechanismů, které se patogeneze MM účastní. Zásadní vliv má vhodné mikroprostředí KD umožňující růst nádoru, což úzce souvisí s novotvorbou cév (angiogenezí), poruchou funkce imunitního systému a interakcí buněk myelomových s buňkami stromatu (Uchiyama *et al*., 1993; Hájek *et al*., 2011). Kooperace těchto procesů indukuje sekreci mnohých cytokinů a aktivaci vnitrobuněčných signálních drah (pro proliferaci, přežití, lékovou rezistenci a nestabilitu genomu buněk MM). Příkladem může být interleukin-6 (IL-6), který aktivuje signální dráhu JAK2/STAT3 a tím také geny stimulující růst a inhibující apoptózu myelomových buněk (Shain *et al*., 2009).

Zároveň buňky MM vykazují nestabilní genom a v průběhu onemocnění v něm dále dochází k četným mutacím a chromozomovým aberacím (CHA). Poměrně často se jedná o složité komplexní změny karyotypu (Hájek *et al*., 2011). Běžným cytogenetickým nálezem jsou aneuploidie, monozomie chromozomu 13, translokace zasahující lokus pro těžký řetězec imunoglobulinu (gen IGH, oblast 14q32), ztráta krátkého raménka chromozomu 17, zisk dlouhého raménka chromozomu 1 a další. Některé z těchto změn jsou typické pro vysokorizikový MM a jsou zařazeny mezi nepříznivé prognostické faktory (Sawyer, 2011). MM typicky probíhá v určitých vývojových stadiích (Obr. 3), které je od sebe nutné odlišit pomocí diagnostických kritérií.

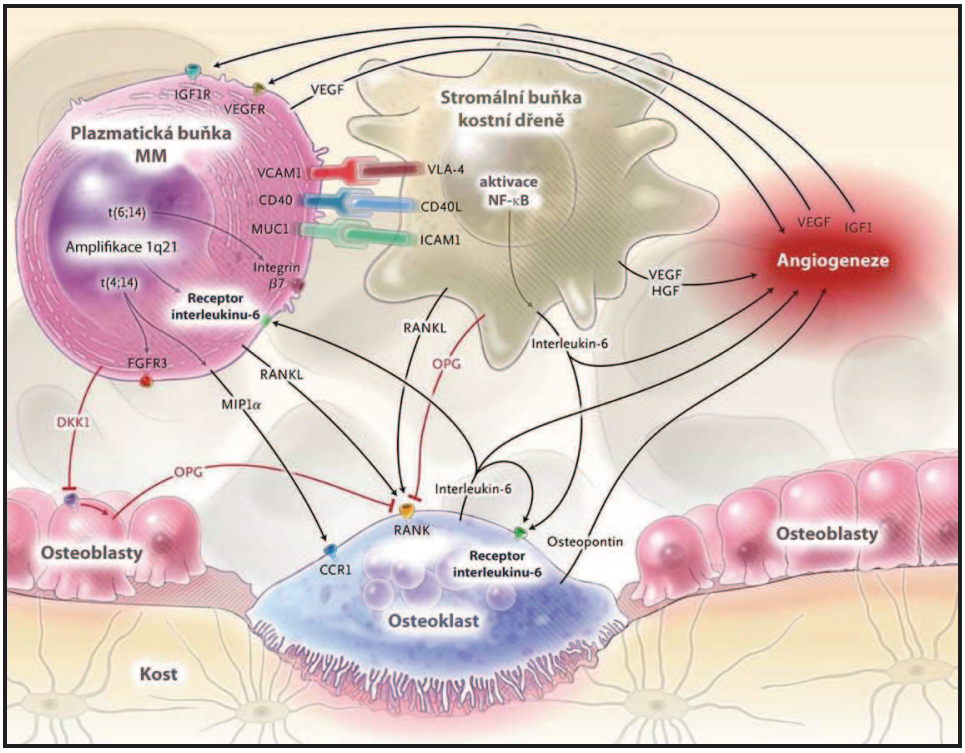


Obrázek 3 Vícestupňový transformační proces MM (Špička *et al*., 2005)

2.4 Mikroprostředí kostní dřeně u MM

Hlavním zdrojem obtíží u MM je osídlování KD myelomovými buňkami, což vede k typickým znakům MM, jako jsou osteolytické léze a patologické zlomeniny. V kostech neustále probíhá proces remodelace, kde resorpce následuje novotvorbu kostní tkáně, přičemž jsou oba děje v rovnováze. U MM je tato rovnováha porušována ve prospěch kostní resorpce (Bataille *et al*., 1991). MM je vhodný model pro studování interakcí tumoru a mikroprostředí ze tří důvodů: na rozdíl od normálních buněk se maligní PB hromadí pouze v KD, což naznačuje, že stromální buňky poskytují jedinečné mikroprostředí pro růst maligních buněk. Dalším důvodem je přítomnost mnoha adhezivních molekul na povrchu myelomových buněk (Uchiyama *et al*., 1992), normální buňky povrchové markery téměř neprodukují. Konečně třetím důvodem je růst heterogenních populací adherentních buněk odebraných z KD pacientů s MM v podmínkách *in vitro* (Caligaris-Cappio *et al*., 1991). Dochází zde k procesům osídlování („homing“) myelomovými buňkami, šíření MM malými cévami a vzniku rozpustných faktorů. Sekrecí těchto faktorů (cytokiny, chemokiny) a fyzickou interakcí podporují stromální buňky růst, přežívání, rezistenci k léčbě a pohyby myelomových buněk (Uchiyama *et al*., 1992; Pellat-Deceunynck *et al*., 1995). Mikroprostředí KD je nejen radikálně ničeno přítomností myelomových buněk a naopak také mění jejich chování. Interakce MM buněk s proteiny mezibuněčné hmoty a stromálními buňkami KD je velmi důležitá pro patogenezi a rezistenci k léčbě MM (Obr. 4).

Mikroprostředí KD bylo zpracováno do přehledové práce v časopise Klinická onkologie (Fišerová *et al*., 2012).



Obrázek 4 Interakce mezi plazmatickými buňkami a buňkami KD u MM (Palumbo *et* Anderson, 2011; upraveno)

2.5 Klinické projevy MM

Klinické projevy MM zahrnují soubor typických příznaků, z nichž ne všechny se projeví u každého pacienta. Jedná se o příznaky vyvolané produkovaným M-Ig (hyperviskozita, poruchy srážení krve, myelomová nefropatie, motorická a senzitivní polyneuropatie), poruchy imunitního systému (únava, časté infekce a horečky, celková slabost) dále bolesti kostí způsobené osteolýzou a jiné méně časté příznaky jako syndrom zvýšené kapilární propustnosti, metabolické poruchy a kožní projevy (Adam *et al*., 2008).

2.6 Léčba mnohočetného myelomu

Mnohočetný myelom je obtížně léčitelné, ale už ne nevyléčitelné onemocnění. Zatímco v 50. letech minulého století byl medián přežití pacientů se symptomatickým myelomem méně než 1 rok, v současnosti se více než ztrojnásobil, a to především u osob mladších 65 let (Turesson *et al*., 2010). Cílem primoléčby je dosažení kompletní remise a u relabujících pacientů nejméně velmi dobré parciální remise. U většiny pacientů stále dochází k relapsu po různě dlouhé a zkracující se remisi (Hájek *et al*., 2011).

Léčba MM zůstávala dlouhou dobu bez výrazného zlepšení kvality a délky života pacientů. Skutečné výsledky přineslo až zavedení klasických chemoterapeutik melfalanu a prednisonu v 60. letech (Alexanian *et al*., 1969). Melfalan s prednisonem se v rámci kombinace s terapeutiky nové éry používají dodnes.

Léčebnou odpověď a medián přežití až o 12 měsíců prodloužila transplantace multipotentních hematopoetických buněk. První slibné výsledky přinesla intravenózní infuze buněk KD provedená roku 1957 (Thomas *et al*., 1957). První klasická autologní transplantace s vysokodávkovanou chemoterapií byla zdokumentována McElwainem a Powlesem (McElwain et Powles, 1983). Ačkoliv transplantace zůstává vysoce efektivní léčebnou strategií u MM, nelze ji aplikovat plošně kvůli vysokému věku nemocných. Navíc každá transplantace musí být kombinována s účinnými terapeutiky, mezi nimiž stále větší podíl získávají nové léky.

Inhibitory proteazomu (PI) a imunomodulační léky (IMiDs) začaly novou éru v léčbě MM. Jsou to jediné léky proti myelomu s vysokou antimyelomovou aktivitu, jak v monoterapii, kde dosahují léčebné odpovědi u 1/3 předléčených nemocných, tak v kombinaci s konvenčními chemoterapeutiky, kde dosahují léčebné odpovědi u 60 — 100 % nemocných (Hájek *et al*., 2011). Na rozdíl od cytotoxických chemoterapeutik jsou založeny na modulaci signálních kaskád a molekulárních interakcí myelomových buněk s mikroprostředím nádoru, čímž se zvyšuje jejich účinnost a specifita vůči myelomu. V současnosti jsou v preklinických a klinických studiích hodnoceny třetí generace imunomodulačních agens a proteazomových inhibitorů. Očekává se zvýšení efektivity a pokles nežádoucích účinků.

2.6.1 Chemoterapeutika

Konvenční protinádorová chemoterapie u myelomu je zaměřena na podávání alkylačních cytostatik a glukokortikoidů (Hájek *et al*., 2011). Principem působení alkylujících látek je zastavení buněčného cyklu s následnou smrtí buňky. Alkylační činidla působí na rychle proliferující buňky a jsou nezávislá na fázi buněčného cyklu. Mechanismus alkylace spočívá v navázání alkylové skupiny na dusíky dvou guaninových bází sousedních řetězců DNA. Mezi řetězci se vytvoří pevná kovalentní vazba, která je příčinou zastavení replikace, a tím i buněčného cyklu (Spanswick *et al*., 2002). Mezi nejvýznamnější z nich patří melfalan a cyklofosfamid (Hájek *et al*., 2011). Glukokortikoidy jsou steroidní hormony, které mimo jiné způsobují apoptózu hematologických buněk pravděpodobně represí

transkripce genů kritických pro přežití buňky (Greenstein *et al*., 2002). Mezi nejvýznamnější kortikosteroidy patří prednison a dexametazon (Hájek *et al*., 2011).

2.6.2 Transplantace hematopoetických kmenových buněk

V České republice bylo v roce 2001 provedeno 330 autologních transplantací, přičemž 90 z nich pro nemocné s MM (Špička *et al*., 2005). První randomizovanou studií srovnávající autologní transplantační léčbu s kontrolní terapií byla analýza francouzské skupiny, jejímž výsledkem bylo signifikantní zvýšení kompletních remisí a velmi dobrých parciálních remisí o více než polovinu. Medián doby přežití byl prodloužen o 13 měsíců (Attal *et al*., 1996). Mnoho následujících studií potvrdilo pozitivní přínos autologní transplantace, a to i přesto, že nevede k vyléčení MM, zůstává pro čtvrtinu nemocných způsobem, jak dosáhnout delšího přežití. Léčebný postup s největším kurativním potenciálem představuje alogenní transplantace, která je však díky vysokým rizikům mortality a s tím spojenými restrikčními opatřeními vhodná pouze pro desetinu nemocných s MM (Reynolds *et al*., 2001). Podle toho, zda je pacient způsobilý pro transplantaci, byly zavedeny dvě formy iniciální terapie. Klasickou počáteční terapii pro pacienty před transplantací představuje kombinace léků vinkristinu, doxorubicinu a dexametazonu (VAD). Nemocní nezpůsobilí pro transplantaci jsou většinou léčeni kombinací melfalanu, prednisonu (MP) a jedním z nových léků (Alexanian *et al*., 1990; Palumbo *et al*., 2008; San Miguel *et al*., 2008).

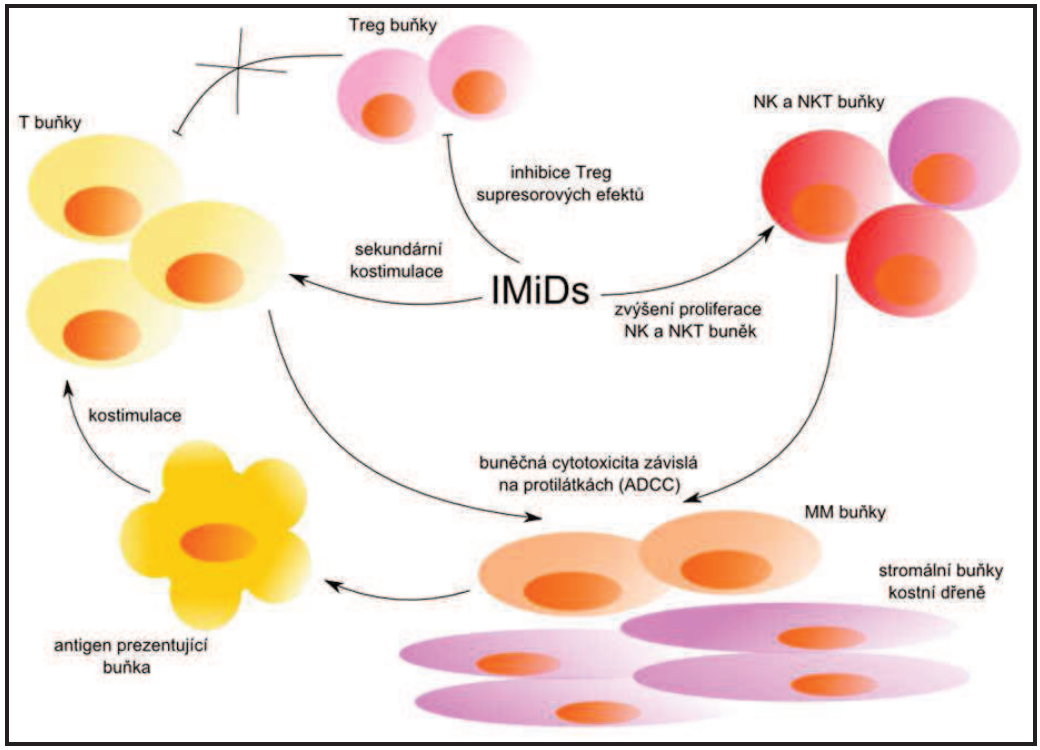
2.6.3 Nové léky

Imunomodulační léky

Mezi IMiDs patří thalidomid a jeho analogy, lenalidomid a pomalidomid. Antimyelomové účinky těchto léků, jejichž mechanismy doposud nebyly uspokojivě vysvětleny, jsou pleiotropního charakteru a zahrnují modulace imunitního systému, antiangiogenní, protizánětlivé a antiproliferativní aktivity s přímým vlivem jak na nádorové buňky, tak na jejich mikroprostředí. Jedním z hlavních mechanismů působení IMiDs je modifikace vrozené a adaptivní imunity jedince prostřednictvím buněk imunitního systému. Bylo prokázáno, že IMiDs kostimulují CD4+ a CD8+ T-lymfocyty, které za normálních okolností potřebují ke své plné aktivaci signál zajišťovaný antigen prezentující buňkou prostřednictvím povrchové molekuly CD28. Aktivace T-lymfocytů má za následek jejich zvýšenou proliferaci a produkci prozánětlivých cytokinů třídy Th-1, mezi něž patří interleukin-2 (IL-2) a interferon-γ (IFNγ) (Haslet *et al*., 1998). Zvýšená sekrece IL-2

následně způsobí aktivaci NK buněk. NK buňky jsou základní složkou vrozené imunity a mají schopnost zabíjet nádorové buňky (Obr. 5). Buněčná cytotoxicita může být indukována bud' závisle (ADCC), anebo nezávisle na protilátkách (Davies *et al*., 2001). Antiangiogenní vlastnosti IMiDs jsou dány inhibicí chemotaktických faktorů podílejících se na migraci endotelových buněk formujících nové cévy. Mezi takové faktory patří zejména tumor nekrotizující faktor α (TNFα), vaskulární endoteliální růstový faktor (VEGF) a bazický fibroblastový růstový faktor (bFGF) sekretované stromálními buňkami KD (Dredge *et al*., 2002). S antiangiogenním a především s protizánětlivým účinkem je spojena i inhibice enzymu cyklooxygenázy 2 (COX-2). COX-2 hraje roli v transformaci kyseliny arachidonové v prostaglandiny. Produkce enzymu je indukována řadou prozánětlivých stimulů, jako jsou lipopolysacharidy (LPS), TNFα a interleukin-1β (IL-1β), přes signální dráhu jaderného faktoru κB (NFκB). Protizánětlivý efekt IMiDs je zprostředkován interleukinem 10 (IL-10) (Payvandi *et al*., 2004).

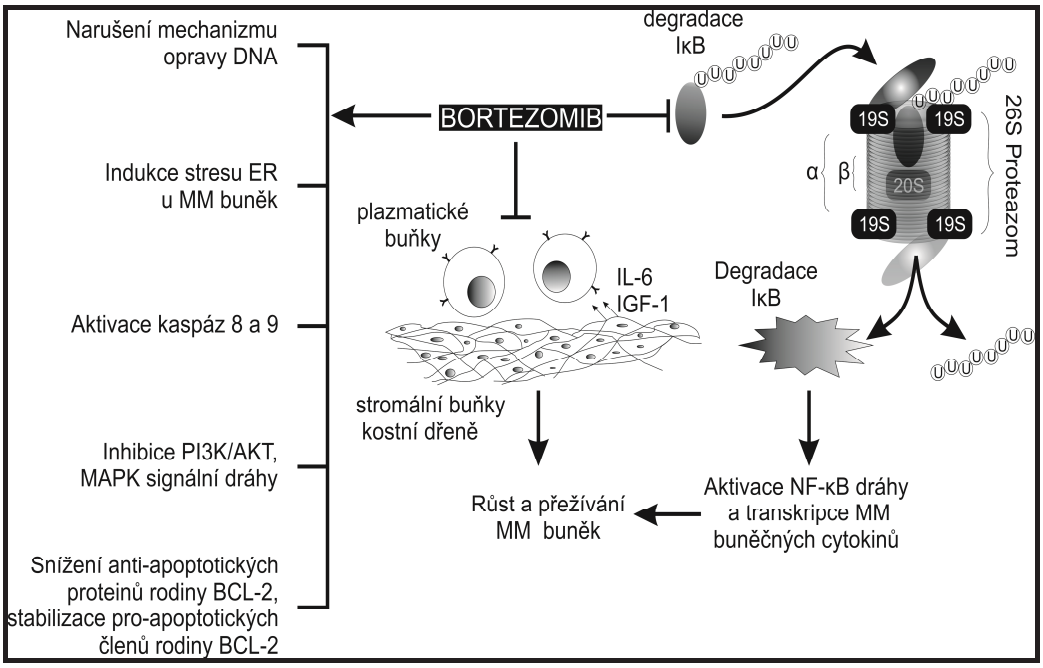
Molekulární podstata účinku IMiDs byla zpracována do přehledové práce v časopise Leukemia Research (Sedlaříková *et al*, 2012).



Obrázek 5 Pleiotropní účinek IMiDs u MM (Sedlaříková *et al*., 2012)

Inhibitory proteazomu

PI se ukázaly být velice důležitou součástí léčby pacientů s MM. Prvním PI schváleným pro léčbu MM se stal bortezomib, který vykazoval silné antimyelomové účinky (Obr. 6) (Hájek *et al*., 2011). Bohužel, navzdory jeho vysoké účinnosti se u velkého procenta pacientů s MM po čase objevuje rezistence k tomuto léku. Mechanizmus inhibice proteazomu bortezomibem spočívá v jeho kovalentní vazbě na β5 podjednotku proteazomu, případně LMP7 podjednotku imunoproteazomu. S nižší afinitou se bortezomib váže také na podjednotky β1 a β2 (Berkers *et al*., 2005). Samotná inhibice je zprostředkována farmokoforovou skupinou, v tomto případě zbytkem kyseliny borité (Groll *et al*., 2006). Jelikož je proteazom zapojen do obratu intracelulárních proteinů, patří mezi primární důsledky jeho inhibice hromadění nefunkčních proteinů a chyby v signálních drahách, které vyúsťují v narušení adheze myelomových buněk, potlačení novotvorby cév, zastavení buněčného cyklu, omezení odpovědi na poškození DNA a indukci apoptózy MM buněk (Richardson *et al*., 2005).



Obrázek 6 Účinek bortezomibu u MM (Kubiczková *et al*., 2012)

Úspěch bortezomibu vzbudil obrovský zájem o proteazomové inhibitory. Optimalizace dávek a kombinace bortezomibu s jinými protinádorovými terapeutiky sice omezily jeho vedlejší účinky a částečně potlačily rezistenci, je však jasné, že druhá generace PI může přinést daleko lepší výsledky. Carfilzomib, Marizomib a MLN9708 reprezentují druhou generaci PI a nabízejí řadu výhod v podobě zvýšené účinnosti, bezpečnosti lékového

profilu a překonání rezistence k bortezomibu díky své odlišné chemické struktuře, biologickým vlastnostem, mechanizmu účinku, i/reverzibilitě inhibice proteazomu a způsobu užívání (Lonial et Boise, 2011). Poskytují tak nové možnosti pacientům, kteří se stali rezistentními k bortezomibu.

Molekulární dráhy a působení inhibitorů proteazomu byly zpracovány do přehledových prací v časopise Klinická onkologie (Kubiczková *et al*., 2013a) a v časopise Journal of Cell and Molecular Medicine (Kubiczkova *et al*., 2014).

2.7 Diagnostika MM

Diagnóza se stanovuje na základě srovnání biochemických, cytologických, rentgenologických a histologických nálezů pomocí všeobecně uznávaných diagnostických kritérií publikovaných skupinou International Myeloma Working Group (IMWG) v roce 2003. K diagnóze symptomatického myelomu pak postačí přítomnost M-Ig, klonálních PB a kritéria CRAB (Hájek *et al*., 2011) (Tab. 1 a 2).

Tabulka 1 Diagnostická kritéria MM (IMWG, 2003; upraveno)

Symptomatický MM	Je přítomen M-Ig v séru a/nebo v moči (bez specifikace koncentrace). V KD jsou přítomny klonální PB (> 10 %). Je přítomno poškození orgánů a tkání myelomem, tak, jak je definováno v níže uvedené tabulce „CRAB“.
Extramedulární MM	Není obvykle přítomný M-Ig, jen zcela výjimečně v nízké koncentraci. Prokázané solitární extramedulární ložisko klonálních PB. Normální KD, není přítomna infiltrace PB. Není přítomna dysfunkce orgánu či tkáně způsobená myelomem.

Tabulka 2 CRAB – kritéria poškození orgánů či tkání myelomem (IMWG, 2003)

Kritérium dysfunkce orgánu	
C – calcium	Hyperkalcémie, kalcium > 2,75 mmol/l nebo o 0,25 nad normální limit
R – renal	Selhání ledvin, kreatinin > 176,8 μmol/l
A – anemia	Hemoglobin < 100 g/l nebo 20 g/l pod dolní limit
B – bone	Kostní změny, osteolytická ložiska nebo osteoporóza s kompresivními frakturami

Relaps onemocnění je diagnostikován po splnění jednoho či více následujících kritérií:
I) znovuobjevení M-Ig v moči nebo v séru;
II) počet maligních PB v KD dosáhne či přesáhne 5 %;
III) vznikají nová osteolytická ložiska nebo se zvětšují ložiska stávající;
IV) hyperkalcémie, pokles hemoglobinu, vzestup sérového kreatininu (bez jiných možných příčin než znovuobnovené aktivity MM) (Adam *et al*., 2001; Hájek *et al*., 2012).

2.8 Prognóza MM

2.8.1 Prognostické systémy

S využitím nejnovějších léčebných protokolů se nadále zvyšuje celkové přežití (OS) pacientů (5 let u 80 % pacientů, více než 10 let u 30-40 % pacientů). Průběh onemocnění je u jednotlivých pacientů vysoce variabilní – např. vysoce rizikoví pacienti s MM mají natolik negativní prognózu, že i přes využití nových strategií se předpokládaná délka života pohybuje mezi 2 až maximálně 5 roky (Hájek *et al*., 2011). Z tohoto důvodu musel být kromě diagnostických kritérií zaveden také systém pro stanovení pokročilosti onemocnění a rozdělení pacientů do skupin podle klinického stadia a prognózy.

Prvním byl prognostický systém dle Durieho a Salmona (DS) (1975) se třemi stádii, která se vyznačují odlišnými hodnotami vybraných klinických parametrů odrážejících velikost nádorové masy. Prognostický význam tohoto systému v éře nových léků se stále zvažuje, je však nadále použitelný pro určení pokročilosti nádoru a doporučuje se uvádět i toto stádium u diagnózy vzhledem k možnosti srovnání výsledků léčby s dříve diagnostikovanými případy MM (Tuchman *et* Lonial, 2011; Hájek *et al*., 2012).

V současné době se vedle DS systému používá mezinárodní prognostický systém navržený IMWG nazvaný International Staging System (ISS) (Greipp *et al*., 2005), který v době diagnózy sleduje pouze dva jednoduché a lehce měřitelné laboratorní ukazatele, sérové koncentrace albuminu a β2-mikroglobulinu (Tab. 3). ISS poskytuje dobrý základ pro budoucí pokročilejší studie, avšak pro vyhledávání vysokorizikových pacientů má určitá omezení. Identifikace pacientů s největším rizikem je zde dosaženo pouze u malé skupiny (5 - 9 %), přesnější vyhodnocení vyžaduje další cytogenetickou a molekulárně-genetickou analýzu. Je zaměřen na prognostiku na populační úrovni, ale nebere v potaz další důležité prognostické parametry, jako je míra proliferace a abnormální genom. Jeho význam a uplatnění v éře nových léků bude muset být upřesněn (Avet-Loiseau, 2010). Vysoce

rizikoví pacienti přestavují v DS systému i ISS klinické stadium III, které je v DS systému charakterizováno zejména poruchou funkce ledvin s hodnotou sérového kreatininu (≥ 2 mg/100 ml) (Durie *et* Salmon, 1975) a v ISS vysokou hladinou sérového β2-mikroglobulinu (≥ 5,5 mg/l) (Greipp *et al*., 2005).

Tabulka 3 International Staging System (Greipp *et al*., 2005; upraveno)

Klinické stadium	Kritéria	Medián přežití
I	sérový β2-mikroglobulin < 3,5 mg/l sérový albumin ≥ 3,5 g/dl	62
II	A: sérový β2-mikroglobulin < 3,5 mg/l a sérový albumin < 3,5 g/dl B: sérový β2-mikroglobulin 3,5 – 5,5 mg/l bez ohledu na hladinu sérového albuminu	44
III	sérový β2-mikroglobulin ≥ 5,5 mg/l	29

Ideální prognostický systém by měl podle Avet-Loiseau (2010) kombinovat sledování hladiny sérového β2-mikroglobulinu (jakožto odrazu nádorové masy), poruchy funkce ledvin, obecného stavu pacienta, proliferace PB a genetických změn. Je více než zřejmé, že stávající prognostické faktory nemohou být považovány za univerzální, zejména z důvodu již zmíněného nejistého uplatnění a významu v době nových léků. Identifikace rizikových skupin s vysokou prediktivní hodnotou by mohla přispět k lepšímu výběru pacientů pro personalizovanou léčbu (Decaux *et al*., 2008).

2.8.2 Cytogenetika

Detekce cytogenetických změn u MM představuje významný prognostický faktor, ať se jedná o početní odchylky či strukturní přestavby chromozomů (ztráty, zmnožení i přemístění genetického materiálu). PB se v KD v rané fázi onemocnění vyskytují v nízkých počtech a mají nízkou proliferační aktivitu. Využití konvenčních cytogenetických metod, při kterých je zapotřebí analyzovat dělící se buňky, je proto značně omezeno a některé významné změny jimi není možné odhalit. Pomocí modernějších molekulárně-cytogenetických metod (interfázní fluorescenční in situ hybridizace – iFISH, komparativní genomové hybridizace – CGH) bylo však prokázáno, že CHA je možné nalézt u téměř všech MM pacientů (medián 8 až 10 změn karyotypu u jednoho pacienta v době diagnózy). Dnes

se proto tyto metody rutinně využívají k detekci všech vyšetřovaných aberací (Kuglík *et al*., 2008; Chen *et al*., 2007).

Početní chromozomové aberace

Jedním z nejdůležitějších prognostických faktorů jsou početní aberace chromozomů (aneuploidie) a s nimi spojené výrazné rozdíly v přežívání pacientů. Jako hypodiploidní se označuje karyotyp s méně než 46 chromozomy, častými monozomiemi a IGH translokacemi. Hypodiploidie má nepříznivý dopad na prognózu a představuje hlavní nezávislý faktor pro vyhodnocení OS (pouze 10 % pacientů přežívá dobu 5 let). Abnormální hyperdiploidní karyotyp se vyznačuje zmnožením jednoho či více chromozomů, a tudíž celkovým počtem chromozomů vyšším než 46. Hyperdiploidie a trizomie představují pozitivní prognostické nálezy a mají lepší OS (medián OS 33,8 měsíců u hyperdiploidie vs. 12,6 u hypodiploidie) (Smadja *et al*., 2001; Debes-Marun *et al*., 2003). Kromě uvedených se u MM vzácně vyskytuje také pseudodiploidie a hypotetraploidie – souhrnně s hypodiploidií tyto stavy označujeme jako nonhyperdiploidní karyotyp (Wuilleme *et al*., 2005).

Částečné delece v oblasti 13q nebo monozomie chromozomu 13 jsou spojeny s negativním dopadem na prognózu i přežívání. Na tomto chromozomu je v oblasti 13q14 lokalizován tumor-supresorový gen *RB1*, který kóduje jaderný protein Rb regulující buněčný cyklus. *RB1* má důležitou úlohu v patogenezi solidních nádorů, a přestože jeho role v patogenezi MM není dosud objasněna, jeho delece je považována za významný negativní molekulární marker kvůli ztrátě kontroly buněčného cyklu (Avet-Loiseau *et al*., 2000).

Strukturní chromozomové přestavby

Hlavním typem strukturních aberací jsou IGH translokace v oblasti 14q32, které jsou často spojeny s nonhyperdiploidním karyotypem. Transkripci genu *IGH* v oblasti 14q regulují tři zesilovače, které jsou v důsledku reciproké translokace přemístěny na partnerský chromozom, kde zvyšují expresi přítomných onkogenů, jako např. *CCND1* (11q13), *FGFR3* a *MMSET* (4p16), *c-MAF* (16q23), *MAFB* (20q12) a *CCND3* (6p21) (Mohamed *et al*., 2007). Nejčastější je translokace t(11;14)(q13;q32), která je přítomna u 15-20 % pacientů a vede k nadměrné expresi cyklinu D1 (gen *CCND1*). Na rozdíl od ostatních 14q32 přestaveb je považována spíše za příznivý či neutrální prognostický faktor. U 10-15 % pacientů je detekována translokace t(4;14)(p16;q32) (často ve spojení se částečnou delecí/monozomií chromozomu 13), jejíž přítomnost má negativní dopad na přežívání. Důsledkem této přestavby je zvýšení exprese dvou genů, které jsou lokalizovány v oblasti

4p16 – *FGFR3* a *MMSET*. Translokace t(14;16), která ovlivňuje expresi transkripčního faktoru kódovaného genem *c-MAF*, je sledována u 5 -7 % pacientů a má negativní dopad na prognózu a přežívání (Fonseca *et al*., 2003; Fonseca *et al*., 2009).

Zisk/amplifikace dlouhého raménka chromozomu 1 v oblasti 1q21 je nejčastější strukturní přestavbou nacházenou u MM (40 % nově diagnostikovaných a až 70 % relabujících pacientů). Často je detekována v asociaci s delecí genu *RB1* v oblasti 13q14 a její dopad na prognózu a OS je (stejně jako u této abnormality) nepříznivý. Jedním z genů lokalizovaných v této oblasti je *CKS1B* (oblast 1q21.3, gen pro regulační podjednotku 1B kinázy CDC28), jehož zvýšená exprese zrychluje proliferaci a hraje tak roli v progresi MM (Němec *et al*., 2010; Zhan *et al*., 2007).

Delece na krátkém raménku chromozomu 17 v oblasti 17p13 je asociována s progresí MM, zkrácením OS a celkově zhoršenou prognózou v důsledku delece supresorového genu *TP53*. Ten kóduje protein p53, který reguluje buněčný cyklus, iniciuje opravu DNA či apoptózu buňky při neopravitelném poškození (Chang *et al*., 2005).

Vysokorizikový MM je z hlediska cytogenetických změn charakterizován nálezem hypodiploidie, monozomie 13 nebo delece genu *RB1*, del(17)(p13) a IGH translokace zasahující 4p16 nebo 16q23 (Tab. 4) (Fonseca *et al*., 2003). Decaux *et al*. (2008) v jejich studii později potvrdili, že skupina vysoce rizikových pacientů měla častější výskyt delece 13q14, delece 17p13, zisku/amplifikace 1q21 a IGH translokace, zatímco karyotyp pacientů s nízkým rizikem relapsu (low-risk) byl často hyperdiploidní. Přestože cytogenetické analýzy jsou pro určení prognózy stále významné, genomické techniky využívající analýzu genové exprese se pro identifikaci pacientů s vysokým rizikem ukázaly jako účinnější (Sawyer, 2011).

Tabulka 4 Srovnání mediánu OS pacientů rozdělených podle přítomnosti či nepřítomnosti specifické cytogenetické abnormality a vliv na prognózu (Fonseca *et al*., 2003; upraveno)

Cytogenetická abnormalita	Medián OS s nalezenou abnormalitou (měsíce)	Medián OS bez nalezené abnormality (měsíce)	Vliv na prognózu
t(11;14)(q13;q32	50 (37-60)	39 (36-44)	poz.
t(4;14)(p16;q32)	26 (21-33)	45 (39-50)	neg.
t(14;16)(q32;q23	16 (13-22)	41 (37-48)	neg.
del(17)(p13)	23 (20-36)	44 (39-49)	neg.
monozomie 13	35 (29-41)	51 (41-57)	neg.

2.8.3 Genomické analýzy

Zjišťování profilu genové exprese (GEP, z angl. gene expression profiling) umožnilo analýzu různých genů, které mohou být zapojeny v patogenezi MM a tím mohou přispívat k přežívání pacientů. Prvním molekulárním klasifikačním systémem byla tzv. TC klasifikace, kde T v názvu představuje translokace a C představuje cykliny D. Pacienti jsou zde rozděleni do osmi skupin na základě přítomnosti IGH translokace, specifické trizomie a odlišné exprese cyklinů D. Tímto způsobem bylo zjištěno, že nejhorší prognózu mají pacienti s IGH translokacemi zasahujícími 4p16 nebo 16q32 a deregulovanou expresí cyklinu D2 (Bergsagel *et al*., 1996).

Zhan *et al*. (2006) vytvořili klasifikaci, která je založena na kombinaci GEP, přítomnosti translokace nebo hyperdiploidie. V tomto systému byly dvě ze sedmi skupin spojeny se špatnou prognózou a vysoce rizikovými proměnnými – skupina PR vyznačující se zvýšenou expresí genů řídících buněčnou proliferaci a progresi buněčného cyklu a skupina MS se zvýšenou expresí genů *MMSET* a *FGFR3* (tj. translokace zasahující 4p16).

První validovaný prognostický GEP model byl vytvořen dle hypotézy, že extrémní změny v genové expresi určité podmnožiny genů jsou spojeny s kratším přežíváním pacientů, a profil genové exprese těchto genů tak může představovat významný nezávislý prognostický znak. Skupina Dr. Shaughnessyho z University of Arkansas na základě této hypotézy identifikovala panel 70 genů, jejichž exprese je pozměněna (snížena nebo zvýšena) právě u skupiny pacientů s vysokorizikovým MM (zde 13-14 % všech MM pacientů). Zajímavým rysem tohoto modelu je vysoké zastoupení genů ležících na chromozomu 1 – téměř 50 % z 19 genů se sníženou expresí a 30 % z 51 genů vykazujících její zvýšení. V souladu s dříve publikovanými cytogenetickými nálezy je poloha genů se zvýšenou expresí v oblasti dlouhého raménka chromozomu 1, tedy v oblasti s častým výskytem amplifikace genetického materiálu, která je zároveň spojena s nepříznivým dopadem na prognózu a přežívání (Shaugnessy *et al*., 2007). Procento buněk s amplifikací oblasti 1q21 by podle některých studií mohlo být spojeno s progresí onemocnění (Hanamura *et al*., 2006). Kromě této spojitosti má skupina vysokorizikových pacientů souvislost s dalšími známými klinickými a prognostickými parametry – vysoká hladina sérového β2-mikroglobulinu a kreatininu, delece chromozomu 13 a jiné cytogenetické abnormality s negativním vlivem indikující kratší přežívání této skupiny pacientů. Zjednodušený model využívá pouze 17 genů z původních 70 (s přesností 97,7 %) (Shaughnessy *et al*., 2007).

Model využívající pro předpověď přežívání nově diagnostikovaných pacientů jen 15 genů byl vytvořen francouzskou skupinou The Intergroupe Francophone du Myélome (IFM). U pacientů s vysokým rizikem je sledováno zvýšení exprese u genů účastnících se řízení rozličných fází celého buněčného cyklu (např. geny řídící kontrolu buněčného cyklu, replikaci, opravu a sbalování DNA, mitózu a vytvoření dělícího vřeténka). Ve spojení s dalšími prognostickými faktory (IGH translokace a sérový β2-mikroglobulin) může být využit k identifikaci nejvíce rizikové skupiny pacientů (Decaux *et al*., 2008).

Sledování homozygotních delecí (ztráta obou alel) u genů zapojených do patogeneze MM může také sloužit k nalezení specifických GEP s prognostickým významem. Na základě hypotézy, že změny na úrovni DNA musí být spojeny se změnami na úrovni genové exprese, bylo identifikováno celkem 97 genů spojených s nepříznivým dopadem na přežívání pacientů. Z tohoto seznamu byly vybrány tři páry genů (*BUB1B* versus *HDAC3*, *CDC2* versus *FIS1*, *RAD21* versus *ITM2B*), které jsou po vzájemném srovnání schopny odlišit jedince s horší prognózou (Dickens *et al*., 2010).

Moreaux *et al*. (2011) získali pomocí srovnání exprese genů v lidských myelomových liniích (HMCL, z angl. human myeloma cell lines) a klasifikace do skupin dle Zhan *et al*. (2006) sedm genů, jejichž pozměněná exprese by mohla být znakem pro negativní prognózu (*TEAD1*, *CLEC11A*, *LRP12, MMSET, FGFR3, NUDT11* a *KIAA1671*). Tyto geny využili k vytvoření jednoduchého systému bodování pacientů od 0 do 7 a jejich rozdělení do tří skupin s odlišným dopadem na přežívání. S nejhorší prognózou je spojena třetí skupina, která vykazuje změnu exprese u 5 nebo více z těchto genů. Avšak žádný z nich nebyl v doposud publikovaných GEP modelech využit. Celkově mají dodnes prezentované modely pouze málo společných genů (zejména kvůli vysoké heterogenitě onemocnění spojené s heterogenitou v expresi genů), což pro přesné určení prognózy a rozdělení pacientů do skupin, které by byly navzájem srovnatelné, představuje značnou komplikaci.

Doporučení pro stávající modely rozdělující pacienty do odlišných rizikových skupin je následující: vyšetření klinického stadia ISS dle sérového albuminu a β2-mikroglobulinu, FISH vyšetření cytogenetických abnormalit t(4;14), t(14;16), del(17) a zisk/amplifikace 1q21, histologie a doplňkové vyšetření např. GEP (Munshi *et al*., 2011). Dalšími kritérii jsou v případě relabujících pacientů typ odpovědi na terapii a délka bezpříznakového období (Hájek *et al*., 2012).

Tématika vysoce rizikového MM byla zpracována do přehledové práce v časopise Clinical Lymphoma, Myeloma and Leukemia (Paszeková *et al*., 2014).

2.9 Flow cytometrie

Flow cytometrie u MM patří mezi hlavní vyšetřovací metody a identifikace imunofenotypu a stanovení počtu PB se používá v diferenciální diagnostice (Kovářová *et al*., 2008). Své uplatnění nachází i při stanovení množství cirkulujících PB u pacientů s nově diagnostikovaným onemocněním, tato hodnota je nezávislým prognostickým faktorem pro celkové přežití (Nowakowski *et al*., 2005). Dále se uplatňuje také při určování pravděpodobnosti progrese asymptomatické monoklonální gamapatie, v hodnocení minimální residuální nemoci nebo účinnosti léčby MM (Kovářová *et al*., 2008).

Vzhledem k tomu, že většina MM pacientů relabuje i v současné době, jsou analýza odpovědi na léčbu a detekce minimální residuální choroby velice důležité. Zde se ukazuje velká výhoda vícebarevné flowcytometrie, která poskytuje rychlé a přesné informace o stavu pacienta (Silvennoinen *et al*., 2014).

PB jsou charakterizovány především expresí cytoplazmatického imunoglobulinu a povrchových membránových antigenů, jako jsou membránový glykoprotein 1 plazmatických buněk (PC-1), antigen 1 rakoviny prostaty (PCA-1), CD38 a CD138. Po dlouhou dobu byl fenotyp myelomových buněk považován za totožný s fenotypem normálních PB. Posléze se ovšem začaly odhalovat odlišnosti v expresi některých povrchových znaků (Paiva *et al*., 2010).

CD138 (Syndekan-1) je transmembránový heparan sulfátový proteoglykan typicky exprimovaný na PB a je považován za nejvíce specifický marker pro PB (Lin *et al*., 2004). K jeho expresi dochází jak na fyziologických PB, tak i patologických, a to již ve stádiu prekurzorů. Pokud dojde ke ztrátě exprese tohoto markeru a jeho uvolnění do cytoplazmy, dochází k apoptóze PB (Kovářová *et al*., 2008).

Dalším zásadním znakem pro identifikaci všech typů PB je CD38. Tento marker je nespecifický a může být detekován na hematopoietických kmenových, T a B buňkách. Neoplastické PB jej typicky exprimují s nižší intenzitou než normální (Lin *et al*., 2004).

Dalším důležitým markerem je CD45. Rané PB tento znak exprimují, ale v průběhu diferenciace na zralé PB jej ztrácí a stávají se CD45- (Paiva *et al*., 2010).

Pro určení rozdílu mezi patologickými a normálními PB hraje klíčovou roli exprese markeru CD19 a CD56 (Kovářová *et al*., 2008). CD56 je adhezivní molekula, jejíž ztráta může fungovat jako transmigrační signál a umožnit uvolnění maligních buněk z KD (Paiva *et al*., 2010). Zatímco zralé MM buňky jsou jasně odlišné od normálních díky fenotypu

CD19-CD56+, popřípadě v menší míře se vyskytující i CD19- CD54- a CD19+56+, normální PB vykazují fenotyp CD19+CD56- (Paiva *et al*., 2010).

Další markery, jejichž exprese je omezena především na maligní PB jsou B lymfocytární marker CD20, kostimulační molekula T-lymfocytů CD28, CD117 a CD200. Naopak markery spojované s fyziologickými PB jsou CD81 a CD27, jejichž exprese se pojí s lepší prognózou pacientů (Kovářová *et al*., 2008).

Klonální PB u MM tedy vykazují zvýšené hladiny CD56, CD86, CD126 a sníženou hladinu u CD38 a CD40 (Pérez-Andrés *et al*., 2005). Analýza cytoplazmatické exprese lehkých řetězců κ a λ je potřebná k potvrzení klonality PB (Kovářová *et al*., 2008).

2.10 Detekce minimální reziduální choroby

I když je v současné době MM již léčitelným onemocněním, velkým problémem je relaps onemocnění, ke kterému dochází u velké většiny pacientů. Minimální residuální choroba (MRD, minimal residual disease) je stav, kdy u pacientů v klinické remisi stále přetrvávají klonogenní buňky, jejichž proliferace vede ke klinickému relapsu. Současné přístupy pro zhodnocení přítomnosti nádorových buněk jsou založeny na morfologickém hodnocení vzorku KD a elektroforetických metodách, které detekují změny v séru a hladině paraproteinu v moči. Citlivost těchto metod je velice limitovaná. I u pacientů dosahujících kompletní remise nakonec dochází k relapsu onemocnění jako důsledku přítomnosti MRD. Většinou standardních metod jsou tyto klonogenní buňky nezachytitelné, ale včasný záchyt by však měl přímý vliv na přežívání pacientů.

V současné době se k detekci MRD využívá flow cytometrie a PCR, které mají uplatnění pro zjištění účinnosti léčby, porovnání efektivity různých léčebných strategií, monitorování pacientů a relapsu onemocnění. Flow cytometrie je jednodušší, rychlejší, levnější, ale méně citlivá. PCR je citlivější a umožňuje provádět retrospektivní studie s využitím zamražené DNA. Detekce MRD u MM pomocí PCR využívá amplifikace tumor-specifického molekulárního markeru, který je detekován v nádorových ale ne ve zdravých buňkách. U MM je takovýmto markerem přestavba těžkého řetězce imunoglobulinu, ke které dochází v pre-B lymfocytech a dále je tato oblast modifikovaná v terminálních centrech pomocí mechanismu somatické hypermutace. Při detekci MRD pomocí PCR je využíváno především právě takto vzniklé hypervariabilní oblasti těžkého řetězce imunoglobulinu, jejíž sekvence slouží pro návrh primerů a sond specifických pro pacienty, které jsou pak v následné PCR reakci schopny detekovat přítomnost MRD s citlivostí až 10^{-6}. PCR produkt

je pak možné kvalitativně vyhodnotit jako přítomnost residuální choroby. Včasný záchyt klonogenních buněk a zvýšení citlivosti detekčních metod povede k lepšímu přežívání pacientů, rychlejší detekci relapsu a rychlejšímu nástupu účinku léčby. Sledování residuální choroby na molekulární úrovni pomocí PCR se v současné době využívá u leukémií i různých typů lymfomů pro zjištění prognózy onemocnění a je rovněž důležitým faktorem při volbě terapie. U MM je však situace složitější z důvodu vysoké heterogenity tohoto onemocnění.

Z toho důvodu se detekci MRD na bázi PCR prozatím podařilo zavést a optimalizovat pouze na několika pracovištích. Naše skupina byla první skupinou v ČR, která zavedla detekci MRD na bázi PCR. V rámci studie byly zavedeny jednotlivé kroky detekce MRD na bázi PCR u pacientů s MM, a to: kvalitativní PCR s využitím již známých rodin primerů a práce se sekvencemi a detekce specifických přestaveb IgH pomocí bioinformatického nástroje IMGT/V-QUEST, kdy se podařilo stanovit nádorově specifický marker u 80 % pacientů. Dále design ASO primerů pro následné kvalitativní zhodnocení přítomné MRD. A identifikace a návrh specifických sond pro kvantitativní zhodnocení přítomnosti myelomového klonu jednotlivých pacientů v 50 % případů. Citlivost metody dosahovala až 10^{-6}.

Další snahou optimalizace PCR detekce MRD u MM bude standardizace celého postupu práce vzhledem k odběru vzorků (při diagnóze, mezi cykly terapie, u pacientů v remisi), definice prognostické hladiny MRD a zhodnocení její prediktivní hodnoty pro přežití. Na základě takto získaných informací by bylo vhodné vyvinout databázi genetických map, primerů, sond a protokolů pro detekci myelomových klonů a MRD. Tato metoda by tak měla velkou šanci stát se nezávislým prognostickým faktorem s širokým využitím v multicentrických studiích.

Tato práce byla opublikována v časopise Biomedical Papers (Sedlaříková *et al*., 2014).

3 Extramedulární forma mnohočetného myelomu

Extramedulární MM (EM) představuje agresivní formu tohoto onemocnění s velmi špatnou prognózou (Varettoni *et al*., 2010; Usmani *et al*., 2012, Pour *et al*., 2013). EM bývá diagnostikován v době stanovení diagnózy nebo v průběhu MM (Varettoni *et al*., 2010). Zdá se, že EM je spojen se sekundárními změnami v myelomovém klonu, progresí agresivního onemocnění, špatnými prognostickými faktory a rezistencí k léčbě (Katodritou *et al*., 2009; Sheth *et al*., 2009). I když je EM v současné době velice aktuálním tématem, první zprávy pocházejí ze 40. a 50. let minulého století, kdy Churg *et* Gordon (1942) a Hayes (1952) uvedli výskyt EM u pacientů s MM. Z jejich poznatků vyplývá, že EM byl přítomen ještě před érou vysokodávkovaných cytostatik a není tedy novou diagnózou.

3.1 Incidence EM

Existují různé zprávy o incidenci EM. Prvními zmínkami o tomto onemocnění jsou pitevní protokoly, které ukázaly, že přibližně u 70 % pacientů s MM se vyskytovalo extraskeletální ložisko (Churg *et* Gordon, 1942; Hayes *et al*., 1952). Tyto nálezy byly popsány ještě před zavedením chemoterapie, ale i tak se výskyt EM zdá být velmi vysoký. Thomas *et al*. (1957) uvádí 40% výskyt EM v játrech při pitvě v sérii 64 pacientů s MM.

S vývojem lepších zobrazovacích systémů byl EM diagnostikován v průběhu života a uváděný výskyt nálezu byl mnohem nižší. V nedávných studiích byl výskyt EM popsán u 6 – 20 % pacientů v průběhu onemocnění (Varretoni *et al*., 2010) a až 37 % u pacientů po alogenní transplantaci (Perez-Simon *et al*., 2006), ale existují i zprávy udávající nízkou incidenci a to kolem 9 - 14 % (Alegre *et al*., 2002). Novější studie (Wu *et al*., 2009; Varretoni *et al*., 2010) uvádějí, že EM se v 68 až 85 % objevuje při diagnóze a vyskytuje se jako infiltrace měkkých tkání spojená s kostními lézemi.

Další rozsáhlá studie, kterou zveřejnila skupina z University of Arkansas, analyzovala pacienty mezi lety 2000 - 2010, kteří vykazovali EM v době diagnózy MM nebo při progresi onemocnění/relapsu. Výzkumná skupina analyzovala 936 pacientů podle protokolu „total therapy“ (TT), 240 pacientů podle „non – TT“ protokolu a 789 pacientů léčených mimo protokol (n = 1965). Celkově byl primární EM (v době diagnózy) zdokumentován u 2,41 % TT pacientů, 4,35 % u non-TT pacientů a 4,5 % pacientů léčených mimo protokol. Výskyt sekundárního (při progresi onemocnění/relapsu) EM u pacientů po 5 letech od transplantace autologními kmenovými buňkami byl dokumentován v případě 3,43

% TT pacientů, 5,2 % non – TT pacientů a 7,24 % pacientů léčených mimo protokol (Usmani *et al*., 2012).

3.2 Místa výskytu a typy EM

Většina autorů rozlišuje dvě skupiny EM: první skupina je charakterizována přímým rozšířením extramedulárního nálezu z kosterního nádoru, zatímco druhá je výsledkem hematogenního šíření (Blade *et al*., 2011). EM může ovlivnit jakoukoliv tkáň, ale mezi nejčastěji zasažené orgány patří pohrudnice, lymfatické uzliny, měkké tkáně, játra, kůže, plíce, urogenitální trakt, prsa a pankreas (Varettoni *et al*., 2010). Usmani *et al*. (2012) jako primární EM označují extramedulární ložisko nalezené při diagnóze MM a sekundární jako extramedulární ložisko diagnostikováno v době relapsu MM. Jako nejčastější místa výskytu primárního EM uvádí hrudní stěnu, lymfatické uzliny, kůži, měkké tkáně a paraspinální prostor, zatímco sekundární EM byl nejčastěji nalezen v játrech.

3.3 Prognóza EM

Přítomnost EM je spojen s agresivním typem onemocnění, což vede ke zkrácení OS a přežití bez progrese (PFS). Varretoni *et al*. (2010) ukázali změny celkového přežití u MM pacientů v letech 1971 až 1999, které se měnilo následovně: 32 měsíců v letech 1971-1993, 45 měsíců v letech 1994-1999 a 54 měsíců v letech 1994-1999. Nicméně pacienti s EM měli kratší PFS než pacienti s MM bez EM relapsu (18 vs. 30 měsíců, $p = 0,003$), ale medián OS nebyl statisticky významně odlišný. Incidence EM měla negativní prognostický dopad na OS a PFS i po úpravě na věk, pohlaví a stupeň stádia nemoci (Varettoni *et al*., 2010).

Existuje stále více důkazů, že EM je spojen se sekundárními změnami, progresí agresivního onemocnění, špatnými prognostickými faktory a rezistencí k léčbě (Oriol, 2011). Nová data také naznačují, že EM představuje subklon nádoru, který získal sekundární mutace, zejména delece genu TP53, což způsobilo, že se tento klon stal více rezistentní k léčbě (Oriol, 2011).

Usmani poukázal na kratší OS u pacientů s primárním EM než u pacientů s MM (31 % vs. 59 % po 5 letech, $p < 0,001$), jakož i kratší PFS ve všech třech léčebných skupinách (50% vs. 21% po 5 letech, $p < 0,001$). Kumulativní incidence EM 5 let po transplantaci (kdy data o primárním a sekundárním EM byla spojena) byla zvýšena u vysoce rizikových pacientů definovaných pomocí metody GEP (10,8 % vs. 2 %, $p < 0,001$) a u pacientů s před transplantačními cytogenetickými abnormalitami (7 % vs. 4,1 %, $p = 0,004$). Nízká hladina

hemoglobinu a trombocytů stanovená před transplantací byla spojena se zvýšeným výskytem EM ve všech třech skupinách pacientů (8,9 % vs. 3,4 % a 8,6 % vs. 3,4 % , $p < 0,001$).

3.4 Molekulární mechanismy EM relapsu

V současné době existuje jen velmi málo publikací, které se snaží charakterizovat molekulární mechanismy EM. Většina vědeckých pracovišť publikuje své poznatky na základě testování malého množství vzorků, z těchto důvodů nejsou přesné molekulární mechanismy stále známy.

Je zřejmé, že při EM relapsu je výrazně snížená závislost PB na mikroprostředí KD, protože PB jsou schopny přežívat i mimo KD. Na přežívání PB mimo KD se podílejí zejména změny v signální dráze chemokinového CXC receptoru 4 (CXCR4), která je důležitá pro usídlení a expanzi buněk MM. Během adheze PB ke stromatu KD, dochází k expresi molekul very late antigen-4 (VLA-4), CD56 a CD44, které interagují s receptory na povrchu endotelových buněk KD, jako např. adhezivní molekula vaskulárních buněk 1 (VCAM-1). Existuje také několik chemokinů a chemokinových receptorů, jako jsou CCR1, CCR2 a CXCR4, které jsou důležité pro migraci a adhezi buněk MM. Zdá se tedy, že existuje několik mechanismů, které umožňují extramedulární šíření PB, jako je snížení exprese adhezivních molekul, snížená regulace chemokinových receptorů, změny týkající se angiogeneze VEGF, matrixové metaloproteinázy (MMP-9) a dalších faktorů a mutace v NF-κB signální dráze (Blade *et al*., 2011). V poslední době bylo prokázáno, že thalidomid indukuje sníženou regulaci CXCR4 a jeho ligandu, které jsou kritické pro usídlení buněk MM – je tedy možné, že thalidomid by mohl usnadnit extramedulární růst buněk MM prostřednictvím ztráty stromálních buněčných interakcí. Thalidomid také snižuje regulaci CD56, dalšího faktoru důležitého pro usídlení MM buněk v KD (Ali *et al*., 2007).

EM je pravděpodobně dále spojen s biologickými změnami v samotném klonu buněk MM. Sheth *et al*. (2009) ukázali asociaci mezi expresí *TP53*, *CD56* a *Ki-67* a EM relapsem. Inaktivace p53 je spojena s více agresivním onemocněním, s rezistencí na chemoterapii a horším OS pacientů s MM (Neri *et al*., 1993) a navíc byla popsána akumulace p53 v jádru PB EM. CD56 je adhezivní molekula nervových buněk, která hraje důležitou roli v buněčné adhezi a migraci. U EM byla zjištěna zvýšená exprese *Ki-67*, snížená regulace CD56 a delece *TP53* (Shethet *et al*., 2009). Také další výzkumná skupina potvrdila sníženou regulaci CD56 ve vzorcích EM (Dahl *et al*., 2002), nicméně v jiné studii byla CD56 společně s CCND1 nadměrně exprimovány v EM vzorcích ve srovnání s MM vzorky (Kremer *et al*.,

2005). Dále lze předpokládat, že aktivační mutace genu *RAS* hrají roli v šíření EM nádorů (Rasmussen *et al*., 2005).

Ve velké studii 764 nově diagnostikovaných pacientů s MM byly identifikovány komplexní genomové přestavby u 1,3 % pacientů a tito pacienti vykazovali medián OS 12 měsíců (Magrangeas *et al*., 2011). Zejména oblasti 1q a 16q měli největší počet kopií přestaveb. K chromotrypsis (tříštění chromozómů) dochází při diagnóze, což u těchto pacientů značí špatnou prognózu. Je možné, že pacienti s chromotrypsis při diagnóze představují odlišnou biologickou entitu na rozdíl od pacientů, kteří přežijí více než 10 let (Wirk *et al*., 2013).

Existuje pouze několik studií, které pečlivě analyzovaly morfologii a cytogenetiku PB v extramedulárních ložiscích. Většina článků ukazuje, že tyto buňky mají nezralou nebo plasmablastickou morfologii (Katodritou *et al*., 2009). Jiné skupiny neukázaly žádné rozdíly mezi cytogenetickými abnormalitami nalezenými v PB buňkách KD a v extramedulárních ložiscích (Rosinol *et al*., 2009).

Tématika extramedulárního relapsu byla zpracována do práce Pour *et al*. (2013) v časopise Haematologica, který se zabýval incidencí, přežitím a aberacemi u pacientů s EM relapsem. Přítomnost EM relapsu byla prospektivně hodnocena u všech pacientů léčených na IHOK FN Brno pro relaps MM od roku 2005 do roku 2008. Z analýzy byli vyloučeni všichni pacienti, kteří měli EM ložiska nebo prokázanou infiltraci parenchymatózních orgánů klonálními PB již při stanovení diagnózy MM. Celkem bylo hodnoceno 226 relabovaných pacientů. Medián věku činil 60,8 let (27,9 - 83,5 let), medián sledování pacientů byl 3,7 let (0,1 – 22 let).

EM relaps byl verifikován pomocí zobrazovacích metod (ultrazvuk, počítačová tomografie (CT), magnetická rezonance (MRI). Pokud se jednalo o ložisko dostupné odběru v lokální anestezii, bylo provedeno i cytologické hodnocení aspirátu z ložiska nebo histologické hodnocení bioptického vzorku k průkazu klonálních PB. Za EM relaps byl považován nález patologických měkkotkáňových hmot na MRI či CT nejčastěji v oblasti související s osovým skeletem u pacientů s jinými projevy relapsu/progrese MM, nebo nález klonálních PB v cytologickém nebo histologickém hodnocení vzorku z ložiska.

Pacienti s EM relapsem byli rozděleni do dvou skupin: 1) EM-S (soft-tissue related) - pacienti s EM relapsem, u nichž nebyla prokázána souvislost měkkotkáňových ložisek s kosterní tkání či difuzní infiltrací parenchymatózních orgánů. 2) EM-B (bone-related) - pacienti s EM relapsem s ložiskem klonálních PB, které vyrůstalo ze skeletu.

Zhodnoceno bylo celkové přežití pacientů, doba výskytu a léčba předcházející EM relapsu v obou skupinách pacientů. Léčba pacientů byla velice heterogenní, nicméně všichni pacienti byli v průběhu choroby léčeni thalidomidem nebo bortezomibem. Pro léčbu EM relapsu byl standardně použit léčebný režim, který obsahoval nový lék, který nebyl dosud použit standardně v kombinaci s cytostatikem a kortikoidy. Pokud byl k dispozici štěp a stav pacienta to umožňoval, byl podán vysokodávkovaný melfalan. Pro léčbu EM byly tedy použity thalidomid (v 33 %), bortezomib (v 38 %) nebo lenalidomid (v 5 %) obsahující režimy a u 42 % patientů byl použit i vysokodávkovaný melfalan s podporou autologního štěpu.

Hodnoceno bylo 226 relabovaných MM pacientů. EM relaps byl prokázán u celkem 24 % (55/226) nemocných. U 58 % (32/55) z těchto pacientů nebyla prokázána souvislost EM s kosterní tkání. Zaznamenali jsme postižení téměř všech orgánů. Nejčastěji bylo pozorováno solidní nádorové ložisko zasahující do kůže a podkoží u 40 % (22/55) pacientů. Celkem 42 % (23/55) pacientů mělo EM myelomová ložiska související s kosterní tkání, nejčastěji byla takto postižena páteř a to u celkem 33% (18/55) pacientů.

EM relaps/progrese se vyskytl překvapivě časně v průběhu choroby. V prvním relapsu bylo zaznamenáno EM postižení u 53 % (29/55) pacientů, ve druhém relapsu u 33 % (18/55). A později v průběhu choroby pouze u 14 % (8/55) pacientů. Mezi skupinami EM-S and EM-B nebyl pozorován rozdíl v době, kdy se EM postižení objevilo, $p = 0,868$. U obou skupin bylo více než 50 % pacientů postiženo již v prvním relapsu.

Před vznikem EM relapsu/progrese bylo konvenčními chemoterapeutickými režimy léčeno 20 % (11/55) pacientů, režim obsahující thalidomid byl použit u 38 % (21/55) pacientů, režim s bortezomibem mělo 29 % (16/55) pacientů a vysokodávkovaný melfalan s autologní transplantací periferních kmenových buněk byl před vznikem EM postižení použit jako léčebná varianta u celkem 53 % (29/55) pacientů. Ve skupině pacientů EM-B byly pozorované numerické rozdíly v podaném typu léčby (konvenční terapie 26 % vs 16 %; bortezomibem 22 % vs 34 %), rozdíly však nebyly statisticky signifikantní ($p = 0,669$).

Medián OS všech 226 pacientů činil 89,5 měsíců a rozdíl mezi nemocnými bez EM relapsu/progrese (76 %; 171/226) a s EM relapsem (24 %; 55/226) byl statisticky významný (medián 109 vs. 38 měsíců; $p < 0,001$). Ve skupině 55 nemocných s EM měla signifikantně kratší medián OS podskupina nemocných s EM-S (medián 30 vs. 45 měsíců, $p = 0,002$).

Medián OS od stanovení diagnózy EM relapsu/progrese činil u všech 55 pacientů 8 měsíců a byl významně nižší v podskupině nemocných s EM-S (4 vs. 12 měsíce, $p = 0,006$). U pacientů s EM byla zaznamenána léčebná odpověď (ORR, z angl. overall response rate)

pouze u 24 % (13/55) nemocných, z toho kompletní remise u 5 % (3/55) a u 19 % (10/55) parciální remise. Doba do progrese u těchto pacientů však činila pouhých 5,4 měsíců. Léčebné výsledky nezávisí na podané léčbě a jsou velmi špatné, nebyl nalezen signifikantní rozdíl ($p = 0,412$), výpovědní hodnota je však limitována velikostí souboru.

Dalším tématem této části byly možné změny v expresi 15 vybraných genů u pacientů s EM relapsem ve srovnání s pacienty s vysokorizikovým MM podle publikace Dr. Shaughnessyho, která vycházela z toho, že EM relaps je možbé považovat za nejvíce rizikovou formu MM. Devět pacientů s EM relapsem, u kterých byl k dispozici jak nádor (TU) tak KD, a 9 pacientů s vysokorizikovým (HR) MM bylo zahrnuto do této části práce. Jako vysokorizikoví byli identifikováni ti pacienti, kteří prodělali relaps během 2 let od zjištění diagnózy. Pacienti s EM relapsem byli identifikováni v rámci předchozí studie.

Statisticky významné rozdíly byly nalezeny mezi třemi skupinami vzorků – PB KD u vysokorizikových pacientů (HR) oproti PB KD u pacientů s EM, PB KD u HR pacientů oproti PB TU u pacientů s EM a PB KD u pacientů s EM oproti PB TU u pacientů s EM. V prvním srovnání genové exprese PB KD u HR pacientů proti PB KD u pacientů s EM byl významný rozdíl v expresi stanoven pouze u 4 genů. Ve druhém srovnání byly nalezeny také 4 geny se signifikantními změnami exprese mezi PB KD u HR pacientů oproti PB TU u pacientů s EM. Třetí srovnání genové exprese mezi PB KD u pacientů s EM oproti PB TU u pacientů s EM odhalilo největší podíl statisticky významných rozdílů a to hned u 9 z 15 genů.

Ikdyž je tento soubor pacientů velice malý, ukazuje na rozdíly v genové expresi mezi skupinami myelomových pacientů a s nimi související heterogenitu MM. EM relaps představuje pokročilý stav maligní transformace onemocnění a ztráta závislosti na mikroprostředí KD značí další změny genomu. Vzorky EM z TU nacházející se mimo prostředí KD vykazují odlišnou genovou expresi oproti vzorkům získaným u EM pacientů přímo z KD, zejména z důvodu vyšší agresivity tohoto „klonu“ buněk. Jelikož byl nalezen poměrně velký počet statisticky významných rozdílů v expresi mezi skupinami PB z KD a PB z TU u pacientů s EM, zdá se, že se opravdu jedná o odlišné klony.

Tato část práce byla zpracována v článku v časopise Biomedical Papers (Ševčíková *et al*., 2015).

4 MikroRNA

MikroRNA (miRNA) jsou krátké, nekódující, 21-25 nukleotidů dlouhé jednořetězcové molekuly RNA, které regulují genovou expresi u rostlin i živočichů (Clarke *et al*., 2007)

Aby mohla být krátká RNA označena za miRNA, musí splňovat následující kritéria: musí být jednoznačně identifikovatelná pomocí Northern blotu, musí se vyskytovat v kmenové části vlásenkové, asi 70 nukleotidů dlouhé prekurzorové struktury, sekvence krátké RNA a jejího prekurzoru musí být fylogeneticky konzervovaná a inhibice klíčových ribonukleáz v biogenezi miRNA musí vést k poklesu hladin krátké RNA a k akumulaci její prekurzorové struktury (Ambros, 2000; Slabý *et* Svoboda, 2012).

MiRNA jsou negativní regulátory a fungují dvěma způsoby v závislosti na stupni komplementarity mezi miRNA a cílovou mRNA. Za prvé, miRNA se mohou vázat s dokonalou nebo téměř dokonalou komplementaritou k protein-kódujícím mRNA sekvencím a tím indukují RNA-zprostředkovanou interferenční (RNAi) dráhu. Stručně řečeno, mRNA transkripty jsou štěpeny ribonukleásami v multiproteinových RNA-indukovaných komplexech (miRISC), což vede k degradaci cílových mRNA. Tento mechanismus miRNA-zprostředkovaného umlčování exprese genů je běžný u rostlin (Llave *et al*., 2002), ale vyskytuje se i v savčích buňkách (Yekta *et al*., 2004). Nicméně u většiny živočišných buněk převládá druhý mechanismus genové regulace, který nezahrnuje štěpení cílové mRNA, ale pouze její umlčení. Tyto miRNA se vážou s neúplnou komplementaritou na 3' nepřekládané oblasti (UTRs) jejich mRNA cíle a cíleně potlačují genovou expresi post-transkripčně, zřejmě na úrovni translace, prostřednictvím komplexu RISC, který je podobný (nebo možná totožný) s komplexem dráhy RNAi (Olsen *et* Ambros, 1999). miRNA pak využitím tohoto mechanismu snižují hladiny proteinů bez ovlivnění hladiny mRNA. Nicméně se ukazuje, že i neúplně komplementární miRNA mohou také indukovat mRNA degradaci (Bagga *et al*., 2005; Lim *et al*., 2005).

MiRNA byly objeveny u *Caenorhabidtis elegans* roku 1993 (Lee *et al*., 1993). Množství popsaných miRNA u všech organismů roste exponenciálně a jejich počet je obsažen v internetové databázi miRBase. Doposud bylo popsáno 28 645 miRNA (nejnovější verze 21, červen 2014, www.mirbase.org).

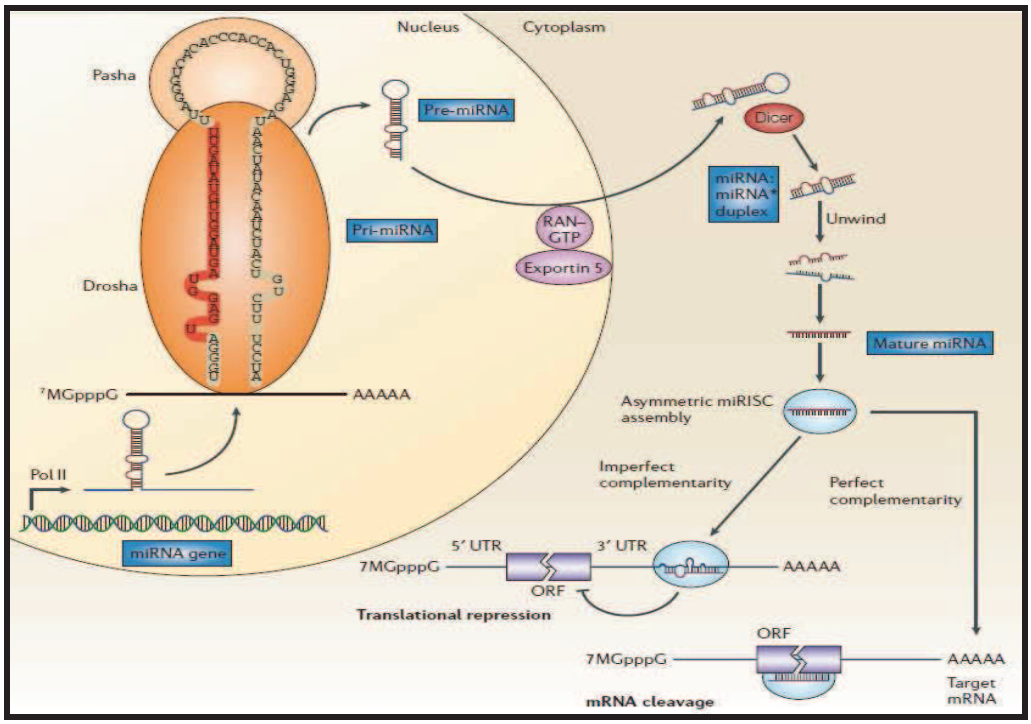
Přibližně polovina z anotovaných lidských miRNA je mapována do fragilních míst chromozomů, což jsou oblasti genomu, které jsou spojeny s různými lidskými nádory. Nedávné důkazy naznačují, že miRNA mohou fungovat jako nádorové supresory

a onkogeny, a proto jsou označovány jako "oncomirs". Faktory, které jsou potřebné pro biogenezi miRNA, jsou rovněž spojovány s různými typy nádorů a mohou samy fungovat jako nádorové supresory a onkogeny. Dále bylo zjištěno, že subtypizace a klasifikace nádorů do určitých podskupin pomocí jejich miRNA profilů je přesnější než pomocí expresních profilů protein-kódující genů. Právě rozdíly v expresi určitých miRNA v různých typech nádorů by se mohly stát velice užitečným nástrojem při diagnóze a léčbě rakoviny. Genové terapie využívající miRNA by mohly být posléze využity k zamezení progrese nádoru. Příkladem slibných kandidátů pro léčbu rakoviny mohou být let-7, která negativně reguluje Ras nebo miR-15 a miR-16, které negativně regulují BCL-2 (Esquela-Kerscher *et* Slack, 2006).

V současnosti začíná být význam miRNA plně doceňován, probíhá klinické testování miRNA jako nových léků pro léčbu hepatitidy C (Miravirsen). Miravirsen je specifický inhibitor miR-122, což je specifická jaterní miRNA, která je nezbytně nutná pro replikaci viru hepatitidy C. Miravirsen tedy odstraňuje molekulu, kterou virus potřebuje k replikaci. Předpokládá se, že tato terapie zabrání vzniku resistence viru k tomuto novému typu terapie. Virus hepatitidy C je známý vysokou rychlostí vzniku mutací (www.santaris.com).

4.1 Biogeneze miRNA

MiRNA jsou převážně transkribovány pomocí RNA-polymerázy II jako pri-miRNA (primární miRNA), dlouhé prekurzory, které na koncích obsahují čepičku a polyadenylový konec (Obr. 7). Pri-miRNA jsou zpracovány v jádře pomocí enzymu Drosha a proteinu Pasha (také známý jako DGCR8), do zhruba 70nukleotidových pre-miRNA, které se skládají do nedokonalých struktur vlásenek se smyčkou (Basyuk *et al*., 2003; Lee *et al*., 2003). Pre-miRNA jsou pak exportovány do cytoplazmy pomocí GTPdependentního transporterového proteinu exportin 5 (Lund *et al*., 2004; Yi *et al*., 2003) a podstupují další zpracování. Během dalších procesů je z pre-miRNA odstraněna vlásenka pomocí enzymu Dicer a zůstává pouze dvouřetězcová RNA o velikosti zhruba 22 nukleotidů (duplex 5p a 3p) (Ketting *et al*., 2001). Následně je duplex miRNA:miRNA* začleněn do miRISC komplexu. Zralý řetězec (vedoucí řetezec) miRNA je přednostně udržen ve funkčním miRISC komplexu a negativně reguluje své cílové geny, kdežto druhý řetězec je uvolněn a degradován (Esquela-Kerscher *et* Slack, 2006). O osudu řetězců rozhoduje stabilita párování na 5‘ konci duplexu miRNA: miRNA* (Slabý *et* Svoboda, 2012).



Obrázek 7 Biogeneze miRNA (Esquela-Kerscher *et* Slack, 2006).

4.2 Cirkulující miRNA

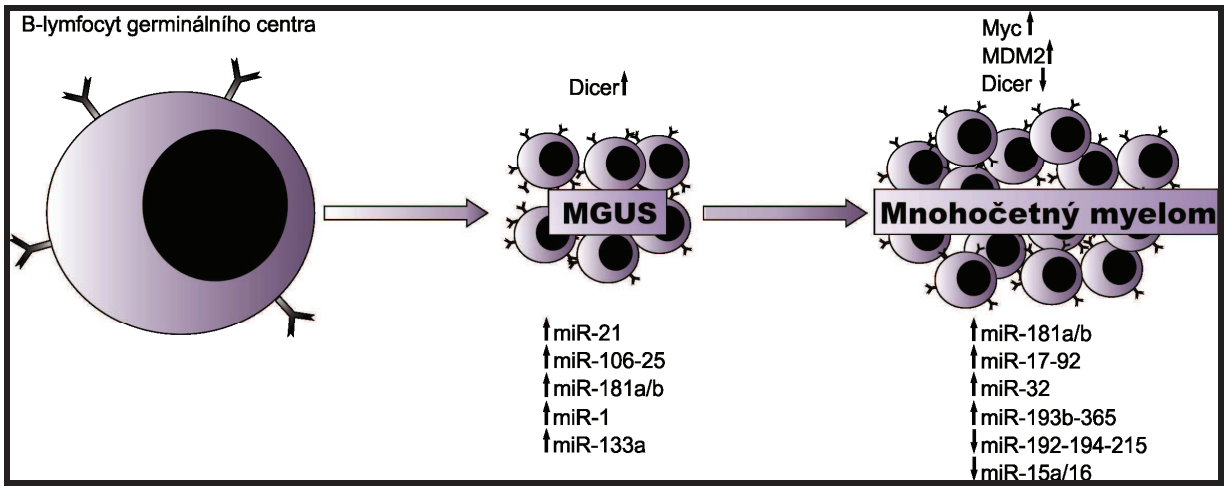
Nová skupina cirkulujících miRNA byla nalezena prakticky ve všech lidských tělních tekutinách, jako je plazma, sérum, sliny, moč aj. Tyto miRNA jsou vysoce stabilní a odolné vůči působení RNáz. Je možné, že se tyto extracelulární miRNA účastní mezibuněčné komunikace, což by znamenalo, že miRNA mohou obsahovat specifickou informaci a mohou být exportovány dovnitř nebo ven z buněk jako odpověď na biologické podněty (Wang *et al*., 2012). Hladiny specifických cirkulujících miRNA by mohly sloužit jako biomarkery různých patologických stavů, včetně nádorových onemocnění, u kterých specifické profily cirkulujících miRNA jsou schopny odlišit zdravé jedince od pacientů (Wittmann et Jäck, 2010) nebo přímo korelují s progresí a stádiem nádoru (Menéndez *et al*., 2012). Jako markery mají tyto cirkulující miRNA několik předností: jsou jednoduché, snadno dostupné a snadno měřitelné standardními laboratorními metodami (Wang *et al*., 2012). Zejména u solidních nádorů byly cirkulující miRNA popsány jako molekulární marker nádorové diagnostiky i prognózy (Ng *et al*., 2009; Wittmann *et* Jäck, 2010).

4.3 MikroRNA u mnohočetného myelomu

První abstrakta zabývající se úlohou miRNA v patogenezi MM byla prezentována v roce 2005 na setkání Americké hematologické společnosti (ASH). Jako první byly popsány

expresní profily miRNA u myelomových linií a vzorků pacientů a bylo zjištěno, že jak buněčné linie, tak maligní, CD138+ plazmatické buňky pacientů mají odlišnou expresi některých miRNA (miR-125b, miR-133a, miR-1 nebo miR-124a) ve srovnání s PB zdravých dárců (Masri *et al*., 2005). Další práce, ve které byla použita kvantitativní PCR (qRT-PCR), popisuje zvýšenou expresi let-7a, miR-16, miR-17-5p a miR-19b a naopak sníženou expresi miR-372, miR-143 a miR-155 u MM pacientů a buněčných linií ve srovnání se zdravými kontrolami (Bakkus *et al*., 2007). Exprese miR-15 a miR-21 se v této studii významně nelišila mezi zdravými dárci a nemocnými, což je v rozporu s pozdější studií, která identifikovala miR-21 jako onkogen s antiapoptotickou funkcí (Löffler *et al*., 2007). Pomocí chromatinové imunoprecipitace bylo zjištěno, že se STAT3 podílí na regulaci exprese miR-21 v IL-6 závislých PB po přídavku IL-6. Zdá se, že u těchto buněk je transkripce miR-21 kontrolována pomocí IL-6 a zprostředkovaná aktivací STAT3, což napomáhá přežívání maligních buněk. Navíc ektopická exprese miR-21 za nepřítomnosti IL-6 vedla ke snížení apoptózy buněk, což potvrzuje účast miR-21 v procesu apoptózy, která je zprostředkovaná pomocí STAT3 (Löffler *et al*., 2007).

V pilotní studii, zabývající se úlohou miRNA v maligní transformaci PB, byla pomocí miRNA mikročipů a následné qRT-PCR srovnávána exprese miRNA jak u zdravých dárců, tak u osob s MGUS, pacientů s MM a u buněčných linií (Obr. 8). Byly identifikovány specifické profily miRNA popisující PB v MM, MGUS a MM liniích, tak transformaci z MGUS do MM. U MGUS bylo nalezeno 48 miRNA, u MM pacientů již 96 odlišně exprimovaných miRNA ve srovnání se zdravými dárci. U obou skupin, MM i MGUS, byla pozorována zvýšená exprese miR-21, klastru miR-106-25 a miR-181a/b, nicméně pouze u MM byla stanovena zvýšená exprese miR-32 a klastru miR-17-92. Zdá se tedy, že se tyto miRNA podílejí na progresi onemocnění a napomáhají transformaci z MGUS do MM (Pichiorri *et al*., 2008).



Obrázek 8 Schématické znázornění transformace plazmatické buňky. Reprezentativní miRNA a geny významně deregulované u jedinců s MGUS a MM ve srovnání se zdravými jedinci. (Kubiczkova *et al*., 2012)

V návaznosti na získané poznatky byla provedena (u PB zdravých dárců a MM pacientů) srovnávací analýza expresního profilu miRNA a expresního profilu kódujících genů (Gene Expression Profiling – GEP), která prokázala souvislost mezi globální zvýšenou expresí miRNA a špatnou prognózou high-risk MM pacientů (Zhou *et al*., 2010). Další studie by mohly podpořit tuto souvislost, jelikož bylo pozorováno, že vyšší viabilita MM buněk souvisí s vyřazením z funkce Argonaut (EIF2C2/AGO2) komplexu, který je hlavním regulátorem maturace a funkce miRNA a jehož exprese je zvýšená u high-risk MM (Diederichs *et* Haber, 2007; Liu *et al*., 2004). EIF2C2/AGO2 se navíc podílí na diferenciaci B-lymfocytů (O'Carroll *et al*., 2007) a je znám jako marker nádorové progrese u MM (Shaughnessy *et al*., 2007). V této studii byla také navržena hypotéza, že miRNA mohou působit synergisticky a tím významně přispívat k progresi MM.

Jiná miRNA mikročipová srovnávací studie odhalila zvýšenou expresi klastru miR-193b-365 u PB MM pacientů (Unno *et al*., 2009). Dále byly porovnány expresní miRNA profily PB MM pacientů s profily normálních PB a byla zjištěna významně zvýšená exprese miR-222, miR-221, miR-382, miR-181a a miR-181b a snížená exprese miR-15a a miR-16 (Roccaro *et al*., 2009). Gutiérrez *et al*. (2010) ve své práci porovnali miRNA expresní profil PB 60 MM pacientů s PB zdravých dárců a pozorovali sníženou expresi 11 miRNA (miR-375, miR-650, miR-214, miR-135b, miR-196a, miR-155, miR-203, miR-95, miR-486, miR-10 a miR-196b), z nichž pouze miR-155 byla již dříve popsána v souvislosi s lymfoidními buňkami.

Nedávno publikovaná práce popisuje 40 miRNA se sníženou expresí v PB MM pacientů ve srovnání se zdravými dárci, z nichž 6 miRNA (miR-214, miR-135b, miR-196a,

miR-155, miR-203 a miR-486) se shoduje s miRNA publikovanými skupinou Gutiérrez *et al*.(2010). Navíc výsledky klastrovací analýzy 54 MM pacientů poukázaly na 3 miRNA, a to miR-296, miR-194 a let-7f, jejichž zvýšená exprese souvisí s lepším přežíváním pacientů (Corthals *et al*., 2011).

Stanovené expresní profily PB MM pacientů nejsou jednotné, nicméně některé miRNA byly potvrzeny ve více studiích.

4.4 Resistence na léky a miRNA u mnohočetného myelomu

Přítomnost miRNA je také spojována s rezistencí vůči některým lékům. Jak bylo výše zmíněné, bortezomib patří do skupiny inhibitorů proteasomu. Jedná se o dipeptid kyseliny borité vykazující protinádorové účinky (Adams *et al*., 1999). Bortezomib byl schválen k léčbě MM v relapsu i pro léčbu nově diagnostikovaných pacientů (Hájek, 2009). V roce 2009 byly popsány expresní dráhy miRNA, které souvisí s léčebnou odpovědí k bortezomibu. Srovnání expresních profilů linií rezistentních a citlivých k bortezomibu odhalilo 22 deregulovaných miRNA, z toho zvýšenou expresi měly miR-155, miR-342-3p, miR-181a, miR-181b, miR-128 a miR-20b, naopak snížená exprese byla pozorována u let-7b, let-7i, let-7d, let-7c, miR-222, miR-221, miR-23a, miR-27a a miR-29a. Mezi predikované cíle těchto miRNA patří geny zapojené do buněčného cyklu, buněčného růstu, apoptózy a ubikvitinace. Následně, pro stanovení klinického významu uvedených miRNA, byly korelovány expresní profily miRNA PB pacientů rezistentních a citlivých k bortezomibu s jejich odpovědí na léčbu. Bylo zjištěno, že pacienti citliví k terapii bortezomibem měli stejný profil deregulovaných miRNA jako linie citlivé k bortezomibu a stejně tak profil pacientů rezistentních k bortezomibu inklinoval k profilu stanovenému na liniích (Neri *et al*., 2009).

V další studii, zabývající se změnou expresních profilů miRNA během získané lékové rezistence, byly srovnány modelové expresní profily miRNA mezi MM buněčnými liniemi (RPMI-8226 a U266) se získanou rezistencí k doxorubicinu a melfalanu a jejich parentálními liniemi. Výsledky expresní analýzy byly validovány pomocí qRT-PCR a významné změny byly pozorovány u miR-21 a miR-181a a miR-181b. Exprese miR-21 byla zvýšená u obou klonů linií rezistentních k melfalanu. Překvapivě bylo zjištěno, že exprese miR-181a a miR-181b byla snížená u U266 doxorubicin rezistentní linie, ale zvýšená u RPMI-8226 doxorubicin rezistentní linie. Zdá se, že změny vedoucí k lékové rezistenci jsou náhodné a efekt miRNA je závislý na kontextu (Munker *et al*., 2010).

4.5 Mechanismus deregulace miRNA u MM

Nové studie, navazující na předchozí objevy, částečně vysvětlují mechanismus deregulace miRNA u MM. Srovnávací mikročipová analýza miRNA a analýza počtu kopií (Copy Number Variations – CNV) DNA nebo GEP MM linií objasnily deregulaci 16 miRNA, jejichž geny leží v oblastech chromozomů, které jsou často předmětem různých alelových změn u MM. Mezi nejčastější změny patřily zisky chromozomů. Bylo zjištěno, že miR-548-1 se vyskytovala s nejvyšší četností (94 %) v oblastech zisku chromozomu, zatímco miR-130b, miR-185, miR-648 a miR-649 (všechny leží v oblasti 22q11.21) jsou zastoupeny v oblastech ztráty chromozomu. Mezi další často deregulované miRNA patřily miR-22 ležící v oblasti 17p13.3, miR-106b a miR-25 v oblasti 7q22.1, miR-15a v oblasti 13q14.3, miR-21 v oblasti 17q23.1 a miR-92b, která se nachází v oblasti 1q22 (Lionetti *et al*., 2009a). Klastr miR-15a/16-1 byl dále podrobněji studován a bylo zjištěno, že u pacientů s delecí chromozomu 13 zcela chybí miR-15a a miR-16, nicméně u pacientů bez delece chromozomu 13 byla exprese miR-15a a miR-16 také významně snížená (Roccaro *et al*., 2009).

Další studie, srovnávající CNV s čipy mapující jednonukleotidové polymorfismy (Single Nucleotide Polymorphism – SNP) ukázala, že exprese miR-15a a miR-16 není závislá na statutu chromozomu 13, ale obecně je u MM pacientů exprese zmíněných miRNA zvýšená oproti normálním PB (Corthals *et al*., 2010).

Byla také nalezena korelace mezi šesti intragenovými miRNA a geny, uvnitř kterých se miRNA nacházejí. Tyto geny jsou deregulovány u MM linií a pacientů, a některé jsou důležité v patogenezi MM, jako mesoderm specific transcript (*MEST*) a miR-335 nebo Ena/vasodilator-stimulated phosphoprotein-like (*EVL*) a miR-342-3p (Ronchetti *et al*., 2008). V jiné práci byla nalezena souvislost mezi 32 intragenovými miRNA a geny, uvnitř kterých leží, některé z těchto genů jsou opět významně deregulovány u MM pacientů. Studie potvrdila již výše zmíněné korelace, navíc byla zjištěna souvislost mezi genem coatomer protein complex, subunit zeta 2 (*COPZ2*) a miR-152 (Lionetti *et al*., 2009a). Získané výsledky naznačují, že změna počtu kopií genu souvisí se zvýšenou expresí jeho intragenových miRNA, což by částečně vysvětlovalo mechanismus změněné exprese miRNA u MM.

Jelikož je myelom velmi heterogenní onemocnění, pro které jsou charakteristické komplexní cytogenetické aberace, je velmi pravděpodobné, že tyto aberace ovlivňují také expresi miRNA. V nedávné studii bylo rozděleno 60 MM pacientů do různých

cytogenetických podskupin na základě traslokačních partnerů IgH genu a statutu RB genu a tyto podskupiny pacientů byly srovnány s jejich expresí 365 miRNA. Výsledky klastrovací analýzy poukázaly na zvýšenou expresi miR-1 a miR-133a, které souvisí s translokací t(14;16) (Gutiérrez *et al*., 2010). Změněná exprese jiných miRNA byla dále popsána v souvislosti s translokacemi t(4;14), t(11;14) nebo t(14;16) (Gutiérrez *et al*., 2010; Lionetti *et al*., 2009b). Nově bylo popsáno 5 miRNA, které byly zvýšený u pacientů s t(11;14) a to miR-122a, miR-33, miR-489, miR-519 a miR-555 (Corthals *et al*., 2011).

Další možností deregulace miRNA je změna v jejich zpracování nebo maturaci. Již dříve zmíněná studie EIF2C2/AGO2 komplexu uvádí, že úbytek AGO2 souvisí se zástavou růstu a apoptózou u MM buněk (Zhou *et al*., 2010). V souladu s tím bylo prokázáno, že změněná hladina enzymu Dicer, ale ne enzymu Drosha, může souviset s progresí MM. Autoři pozorovali podobnou hladinu enzymu Dicer u PB zdravých dárců a pacientů s MGUS, která je však významně zvýšená oproti SMM (Smouldering MM) a MM pacientům. Navíc bylo pozorováno, že skupina pacientů s vyšší hladinou enzymu Dicer měla delší dobu do progrese (Sarasquete *et al*., 2011).

Zmíněné výsledky jsou však v rozporu s nedávno provedenou studií, ve které nižší exprese genu DICER1 u skupiny MM pacientů souvisí s delší dobou do progrese nemoci (Corthals *et al*., 2011). Zdá se tedy, že regulační mechanismy ovlivňující jak miRNA maturaci tak jejich funkci se mohou podílet na změněné expresi miRNA, další studie určitě pomohou objasnit zmíněné nesrovnalosti.

4.6 miRNA ovlivňující kritické geny u MM

Mnoho vědeckých skupin se zabývalo otázkami, jak důležité jsou z funkčního hlediska změny v expresi miRNA a jak tyto změny souvisí s patogenezí MM. Pro zodpovězení těchto otázek jsou využívány různé přístupy od predikce cílových genů pomocí in silico modelů až po pokusy s transgenními zvířaty.

Je známo, že kódující geny, které se podílejí na procesu kancerogeneze u MM, jsou cílem pro deregulované miRNA. Bylo prokázáno, že klastr miR-17-92, nacházející se v oblasti 13q31-32, ovlivňuje expresi genu PTEN, genu pro transkripční faktor E2F1 a BIM (Ventura *et al*., 2008; Xiao *et al*., 2008). U transgenních myší se zvýšenou expresí tohoto klastru v lymfocytech byly pozorovány lymfoproliferativní a autoimunitní onemocnění a časná úmrtí. Dále bylo zjištěno, že purifikované myší CD4+ lymfocyty se zvýšenou expresí miR-17-92 obsahovaly snížené množství proteinů Pten a Bim, což naznačuje, že

miR-17-92 klastr ovlivňuje tyto nádorové supresory (Xiao *et al*., 2008). Brzy nato byla publikována další studie, ve které bylo prokázáno, že zmíněný klastr je nezbytný pro vývoj B-lymfocytů. Nepřítomnost miR-17-92 vedla ke zvýšené hladině pro-apoptotického proteinu BIM a tím k zástavě vývoje z pro-B do pre-B stádia (Ventura *et al*., 2008). Zdá se tedy, že zvýšená exprese miR-17-92 negativně reguluje zmíněné nádorové supresory a přispívá k transformaci PB a progresi MM.

Predikce in silico také ukázala, že cílem miR-21 a klastru miR-106-25 jsou mezi jinými nádorové supresory PTEN, BIM a p21, a proto je pravděpodobné, že se tyto miRNA mohou podílet na vývoji plně rozvinutého myelomu (Pichiorri *et al*., 2008).

Jiná miRNA, miR-19a/b, ovlivňuje dráhu STAT-3/IL-6, která je důležitá v patogenezi MM. Bylo prokázáno, že miR-19a/b přímo ovlivňuje suppressor of cytokine signaling-1 (SOCS-1, negativní regulátor IL-6), a tím přispívá k jeho časté deregulaci u MM buněk (Pichiorri *et al*., 2008). Také miR-21 zmíněná výše působí jako onkogen a podílí se na regulaci této dráhy (Löffler *et al*., 2007).

Jak již bylo zmíněno dříve, miR-15a a miR-16-1 leží v oblasti chromozomu 13q14.3, která je deletována u více než 50 % pacientů s MM. Tato delece je považována za primární mutaci, která se podílí na patogenezi MM (Fonseca *et al*., 2004). miR-15a/16 jsou považovány za nádorové supresory podílející se na proliferaci MM buněk *in vitro* i *in vivo* tím, že inhibují AKT serin/treonin proteinovou kinázu (*AKT3*), ribosomální protein S6, MAP kinázy a NFκB aktivátor *MAP3KIP3* (Roccaro *et al*., 2009). Dále bylo prokázáno, že miR-15a/16 nejen regulují expresi genů buněčného cyklu, jako jsou cykliny D1 a D2, dále *CDC25A*, ale rovněž ovlivňují expresi genů spojených s apoptózou: *BCL-2* nebo *MCL-1* (Aqeilan *et al*., 2010). Navíc ektopická exprese miR-15a/16 negativně reguluje angiogenezi pomocí VEGF (Roccaro *et al*., 2009). Nedávno byla popsána úloha miR-15a/16 v mikroprostředí kostní dřeně. Bylo zjištěno, že exprese miR-15a/16 je v MM buňkách po ovlivnění cytotoxickými látkami vyšší. Nicméně, po interakci těchto buněk se stromálními buňkami kostní dřeně odvozenými od MM (MM-BMSC) pacienta, byla pozorována snížená exprese miR-15a/16 u myelomových buněk. Důvodem byla zvýšená produkce IL-6 stromálními buňkami, který inhiboval expresi zmíněných miRNA. Zdá se tedy, že mikroprostředí je důležité pro přežití MM buněk a chrání je před působením léků pomocí sekrece IL-6, který inhibuje expresi miR-15a/16 (Hao *et al*., 2011).

Nově publikované práce se dále zaměřují na vztah miRNA k nádorovému supresoru p53. Výsledky screeningové metody umožňující identifikovat miRNA, které negativně regulují signalizaci p53 pomocí přímé interakce s genem *TP53* naznačily, že miR-25 a miR-

30d mohou ovlivňovat p53. Navíc byla exprese miR-25 a miR-30d zvýšená v PB MM pacientů a u miR-25 zvýšená exprese korelovala se sníženou expresí mRNA TP53 (Kumar *et al*., 2011). Také miR-181a byla popsána jako negativní regulátor exprese genu TP53, což potvrzuje spojitost mezi p53 a aberantní miRNA expresí (Pichiorri *et al*., 2010). Je známo, že miR-34a je transkripčním cílem p53 zprostředkovávajícím apoptózu (Lodygin *et al*., 2008). U MM pacientů byla pozorována hypermetylovaná miR-34a v oblasti 1p36. Jelikož se krevních nádorových onemocnění nevyskytuje mutace TP53 tak často, jako u solidních nádorů, mohla by hypermetylace miRNA částečně vysvětlit dysregulaci p53 signalizace (Chim *et al*., 2010). V další studii byla nalezena snížená exprese miR-192, miR-194 a miR-215 u části nových diagnóz MM pacientů. Další pokusy *in vitro* prokázaly, že při použití molekulárních inhibitorů MDM2 mohou být tyto miRNA transkripčně aktivovány pomocí p53 a posléze modulovat expresi MDM2. Je tedy patrné že miR-192, miR-194 a miR-215 ovlivňují MDM2/TP53 regulační osu a kontrolují rovnováhu mezi MDM2 a p53. Navíc miR-215 a miR-192 ovlivňují signální dráhu IGF a tím zabraňují zvýšené migraci PB do KD (Pichiorri *et al*., 2010).

Během posledních let bylo provedeno mnoho studií srovnávajících globální profil CD138+ PB MM pacientů a zdravých dárců pomocí různých high-throughput screeningových metod, od oligonukleotidových čipů až po qRT-PCR profilování. Každá z metod má své silné a slabé stránky poskytující rozdílné výsledky, ke kterým navíc přispívá velká heterogenita onemocnění. Obecně bylo doposud ve většině prací u myelomu identifikováno více miRNA se zvýšenou expresí u PB než se sníženou expresí. Výjimkou je práce Guttiérez *et al*.(2010), která popisuje více miRNA se sníženou expresí.

Dále můžeme říci, že ani identifikace jednotlivých miRNA není jednotná, což může být způsobeno několika faktory. Za prvé je v každé studii rozdílný soubor pacientů a kontrol a rozdílná velikost souboru. Jak již bylo zmíněno, je MM velmi heterogenní onemocnění a každý pacient má jinou kombinaci genetických mutací a cytogenetických aberací, což se může projevit na rozdílné subklasifikaci do skupin ve srovnání se zdravými dárci. Za druhé, pacienti mohou mít v různých stádiích onemocnění odlišné profily miRNA. Na příklad miR-15 byla popsána jako zvýšená u nových diagnóz, ale snížená u relapsů (Pichiorri *et al*., 2008; Zhou *et al*., 2010). V neposlední řadě se na odlišnostech podílejí rozdíly ve zpracování vzorku, purifikaci PB, extrakci miRNA a dále rozdílné mikročipové platformy a různé verze čipů.

Dnes již víme, že změněná exprese miRNA u MM může být z příčin genetických, cytogenetických nebo epigenetických. Byly také popsány specifické miRNA charakterizující

progresi MM, lepší prognózu nebo rezistenci k lékům. Mechanismus deregulace není zatím přesně známý, víme již, že v pozadí stojí jak změna v cílovém genu pro miRNA, tak změny v počtu kopií lokusů, ve kterých se nachází miRNA, defekty v biogenezi miRNA a epigenetické změny. Snahou dalších studií by mělo být objasnění komplexity regulace miRNA a identifikace terapeutických cílů.

Tato část práce byla zpracována do kapitoly v knize MikroRNA v onkologii (Kubiczková *et al.* in: Slabý *et al*., 2012).

4.7 Cirkulující mikroRNA u MM

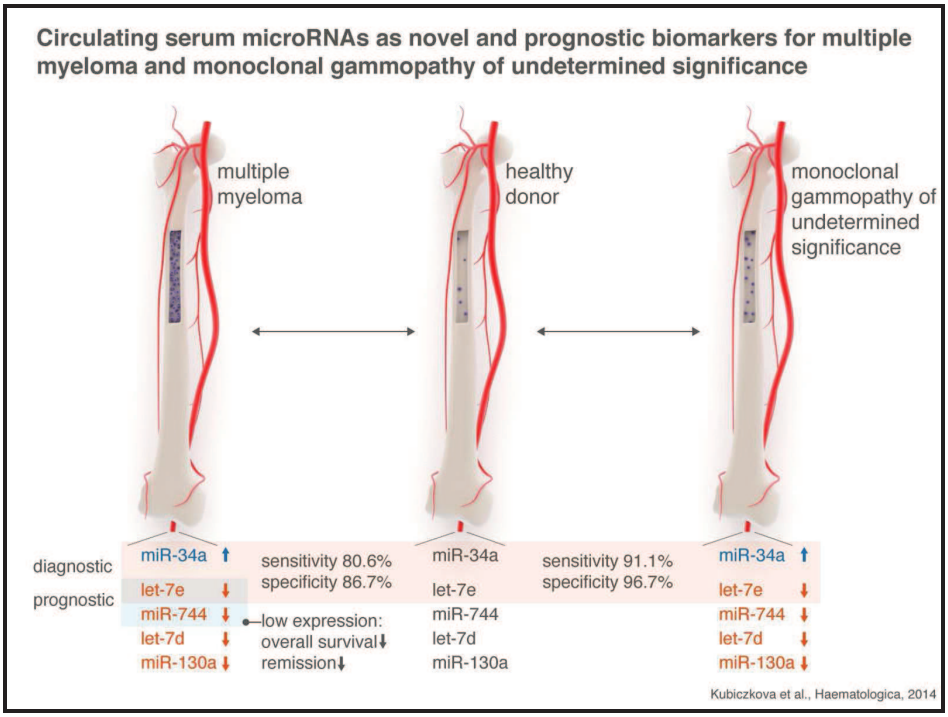
Naše práce se v současné době soustřeďuje na cirkulující miRNA u MM. Vzhledem k tomu, že většina vyšetření pro detekci relapsu nebo onemocnění se provádí z kostní dřeně, je nutné najít markery, které by byly snadno dostupné, možnost odběru by minimálně zatěžovala pacienta a byly by opakovatelně odebíratelné.

Naše první práce se zaměřila na několik miRNA, které by mohly být důležité z hlediska patogeneze MM – miR-29a, miR-142-5p, miR-410 a miR-660. Pro tyto pokusy bylo použito vzorků séra pacientů s MM (91 pacientů) při diagnóze ve srovnání se zdravými dárci (bez hematologických malignit). Pomocí real-time PCR upravené pro miRNA byly stanoveny hladiny jednotlivých miRNA ve vzorcích MM pacientů a bylo zjištěno, že hladiny miR-29a, 660 a 142-5p jsou zvýšeny v séru pacientů s MM, ale jen hladina miR-29a je schopna odlišit pacienty s MM od zdravých dárců se specificitou 70 % a senzitivitou 88 %.

Naše práce o cirkulující formě miR-29a jako markeru mnohočetného myelomu byla mezi prvními pracemi o cirkulujících miRNA v oblasti výzkumu MM a byla opublikována v časopise Leukemia & Lymphoma (Ševčíková *et al*., 2012). Role miR-29a v hematologických malignitách byla shrnuta v přehledovém článku v časopise Biomedical Papers (Fišerová *et al*., 2014).

Naše další práce se zaměřila na odlišení pacientů s MM a s MGUS od zdravých dárců a využila jiný postup. Nejdříve byl vytvořen expresní profil miRNA pomocí Taq Man Low Density Arrays, který byl potom ověřen pomocí real-time PCR. Expresní profil označil 14 deregulovaných miRNA, které byly detekovány na větším souboru pacientů. Multivariační analýza ukázala, že kombinace miR-34a a let-7e odliší MM pacienty od zdravých kontrol se specificitou 80,6 % a sensitivitou 86,7 % a od MGUS se specificitou 91,1 % a sensitivitou 96,7 %. Další analýzy prokázaly korelaci hladiny let-7e a miR-744 s přežitím pacientů s MM.

Tato práce o cirkulujících formách mikroRNA u MM a MGUS byla opublikována v časopise Haematologica (Kubiczková *et al*., 2013b). Byla vybrána pro tzv. grafický abstrakt daného čísla časopisu (Obr. 9).



Obrázek 9 Grafický abstrakt článku Kubiczková *et al*., 2014

Dále jsme se zabývali také cirkulujícími formami miRNA u Waldenstromovy markoglobulinemie (Kubiczková-Bešše *et al*., 2014), která byla opublikována v časopise American Journal of Hematology. Tato práce potvrdila naše výsledky u pacientů s MM a MGUS na nezávislé skupině pacientů ve srovnání s pacienty s Waldenstromovou makroglobulinémií.

V současnosti připravujeme manuskript o roli mikroRNA v EM relapsu pacientů s MM (Bešše *et al*., in preparation).

5 Závěr

Mnohočetný myelom je krevní nádorové onemocnění. Jde o velice heterogenní nemoc, což je z hlediska výzkumu i kliniky problematické. Naše práce se zaměřuje na nové aspekty studia funkční genomiky této choroby a současně se snaží o lepší pochopení patogeneze MM včetně extramedulárního relapsu tohoto onemocnění.

Extramedulární progrese myelomu je krajně nepříznivou variantou MM. Bohužel se jeho incidence výrazně zvyšuje, což je pravděpodobně dáno prodlužujícím se přežíváním pacientů a podle nových studií i novými léky, které sice dramaticky prodlužují přežití i kvalitu života pacientů, ale zdá se, že mění myelomové buňky a umožňují jejich přežití mimo kostní dřeň. I naše výsledky ukazují, že klon PB v KD a EM ložisku je odlišný. Změna biologického chování myelomových buněk ve smyslu umožnění vzniku extramedulárního ložiska není způsobena pouhou změnou exprese genů či CD markerů na povrchu buňky, ale zdá se, že se jedná se o změny komplexní, zahrnující bezesporu i změny v mikroprostředí celé KD.

Dále je naší snahou přispět ke zlepšení diagnostiky onemocnění jako takového, i diagnostiky v rámci monoklonálních gamapatií a aplikovat získané poznatky a metodiku v rámci ČR. V současné době se pozornost obrací na cirkulující miRNA. Ty se zdají být vhodnými kandidáty pro biomarkery onemocnění díky své vysoké stabilitě a souvislosti s onemocněním. Především u MM má možnost využití cirkulujících miRNA jako biomarkerů potenciál překonat bolestivý postup stanovení diagnózy MM, který využívá invazivního odběru KD.

I když je v současnosti MM již léčitelným onemocněním, relaps zůstává stale velkým problémem u většiny pacientů. To je dáno přítomností MRD, kdy v klinické remisi nadále přetrvávají klonogenní buňky, jejichž proliferace vede k relapsu onemocnění. V současné době je zlatým standardem pro detekci MRD alelově specifická PCR, kterou se nám za tímto účelem povedlo zavést jako první skupině v rámci ČR.

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Přílohy

Význam mikroprostředí kostní dřeně v patogenezi mnohočetného myelomu

Fišerová B, Kubiczková L, Sevčíková S, Hájek R.

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Význam mikroprostředí kostní dřeně v patogenezi mnohočetného myelomu

Implication of Bone Marrow Microenvironment in Pathogenesis of Multiple Myeloma

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Souhrn

Mnohočetný myelom je hematoonkologické onemocnění charakterizované maligní proliferací plazmatických buněk. Tyto buňky se hromadí v kostní dřeni, kde potlačují fyziologickou krvetvorbu a zároveň interagují s celou škálou cytokinů, růstových faktorů a adhezivních molekul. Je zřejmé, že právě mikroprostředí kostní dřeně hraje velkou roli v patogenezi onemocnění, ale i v rezistenci k léčbě.

Klíčová slova

mnohočetný myelom – kostní dřeň – IL-6

Summary

Multiple myeloma is a hematooncological disease characterized by malignant proliferation of plasma cells. These cells accumulate in the bone marrow where they suppress physiological hematopoiesis; at the same time, these cells interact with a wide variety of cytokines, growth factors and adhesion molecules. It is obvious that the bone marrow microenvironment plays an important role in disease pathogenesis as well as treatment resistance.

Key words

multiple myeloma – bone marrow – IL-6

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Úvod

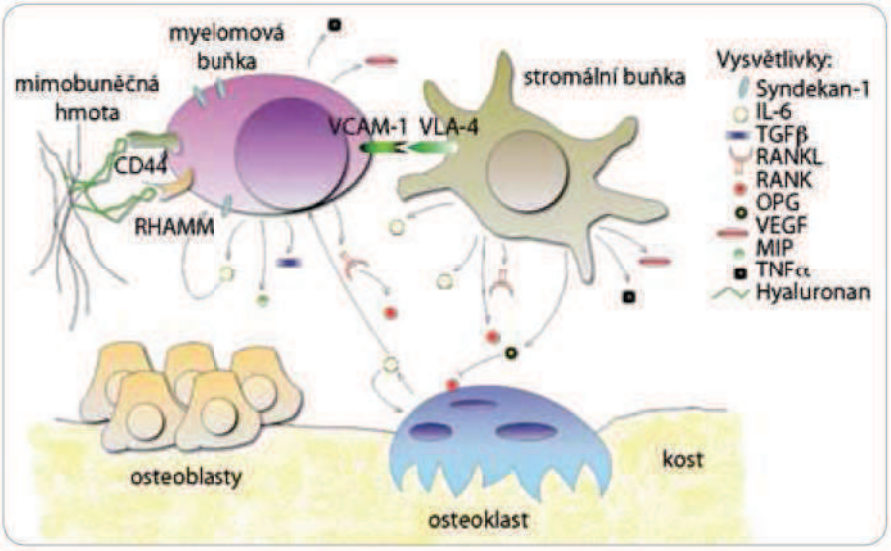
Mnohočetný myelom (MM) je maligní hematologické onemocnění charakterizované monoklonální expanzí plazmatických (myelomových) buněk. Toto onemocnění se týká hlavně starších pacientů s mediánem věku stanovení diagnózy 69 let, incidence v ČR je 4/100 000. Myelomové buňky jsou lokalizovány v kostní dřeni, kde narušují fyziologický proces hematopoezy a zároveň narušují strukturu kosti, což vede ke vzniku osteolytických lézí, které jsou pro pacienty s MM hlavním zdrojem obtíží [1,2].

MM je vhodným modelem pro studium interakcí tumoru a mikroprostředí ze tří důvodů: na rozdíl od normálních buněk se maligní plazmatické buňky hromadí zejména v kostní dřeni, což znamená, že stromální buňky poskytují jedinečné mikroprostředí pro růst maligních buněk. Dalším důvodem je přítomnost mnoha adhezivních molekul na povrchu myelomových buněk [3], normální buňky povrchové markery téměř neprodukují. Třetím důvodem je bezesporu možnost kultivace heterogenních populací adherentních buněk odebraných z kostní dřeně pacientů s MM v podmínkách *in vitro* [4].

Interakce adhezivních molekul

Adhezivní molekuly umožňují přímé propojení mezibuněčné hmoty s myelomovými buňkami a buňkami navzájem. Po navázání dalších molekul se spouští řada dějů, které mohou ovlivňovat vývoj buněčných složek nebo aktivovat signalizační kaskády. Je také podporována aktivace osteoklastů a růst maligních buněk, které jsou ještě více zadržovány v kostní dřeni [5] (obr. 1).

Jedním ze specifických povrchových markerů myelomových buněk je syndekan–1 (CD138). Je to transmembránový proteoglykan, který se může přímo vázat na proteiny mezibuněčné hmoty, u MM se váže na kolagen typu I [6]. V kostní dřeni je syndekan–1 detekován pouze na buňkách z B lymfoidní linie a jeho exprese se mění se stupněm diferenciace. U myší byl nalezen na povrchu pre-B buněk, u zralých B buněk se nevyskytoval a opět byl produkován u plazmatických buněk [7]. U pacientů s MM se vyskytuje pouze na povrchu myelomových buněk [8], inhibuje osteoklastogenezi a pozitivně ovlivňuje diferenciaci osteoblastů [9]. U myelomových buněk, které procházejí apoptózou, se však rychle ztrácí [10]. Jelikož je syndekan–1 produkován na povrchu životaschopných myelomových buněk, byly vyvinuty specifické protilátky, které dnes umožňují identifikaci a purifikaci myelomových buněk ze vzorků pacientů [8].



Obr. 1. Interakce v mikroprostředí kostní dřeně mnohočetného myelomu. Myelomové buňky interagují s ostatními buňkami a mimobuněčnou hmotou prostřednictvím adhezivních molekul (CD44, RHAMM, VCAM-1) a po adhezi je podporováno vylučování cytokinů a růstových faktorů. Některé faktory působí i autokrinně (IL-6) a tím se ještě více stimuluje jejich produkce.

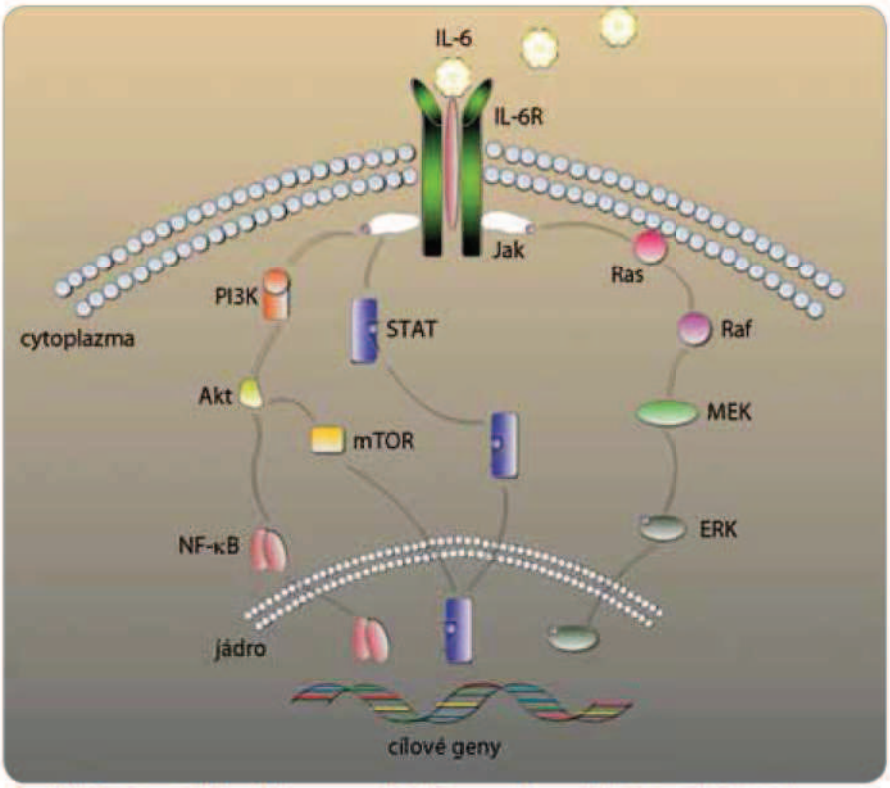
Za interakci myelomových buněk s hyaluronanem jsou zodpovědné dva receptory, a to CD44 [11] a RHAMM [12]. Standardní forma receptoru CD44 se na povrchu myelomových buněk vyskytuje zřídka, ovšem některé nestandardní receptorové varianty jsou zde velmi časté. Byly detekovány například varianty 3v, 4v, 6v a 10v, které nejsou přítomné u zdravých jedinců [13]. Receptor RHAMM napomáhá pohybu myelomových buněk po hyaluronovém substrátu. U MM jsou vylučovány tři formy: RHAMMFL, což je běžný typ, a dále dva deleční mutanti, RHAMM–48, (delece 48 bp) a RHAMM–147 (delece 147 bp). Oba deleční mutanti jsou přítomni jen u B buněk a plazmatických buněk MM, ale ne u zdravých jedinců. Výskyt delečních mutantů mění intracelulární signalizaci v buňkách MM [12].

Cytokiny, růstové faktory

Myelomové i stromální buňky produkují látky, které ovlivňují vývoj MM. Mezi tyto látky se řadí cytokiny a chemokiny, které mohou působit jako promotory nádorového vývoje, růstové faktory nebo chemoatraktanty. Váží se na receptory, a tím aktivují různé signální kaskády. Pacienti s MM vykazují typické rozpustné faktory v mikroprostředí kostní dřeně: IL-16, IL-2R, MCP–1, HGF, IL-1RA, MIG, IP–10, EGF [14].

Stěžejním cytokinem MM je interleukin-6 (IL-6), který je produkován mnoha buňkami včetně osteoblastů, monocytů, makrofágů a stromálních buněk kostní dřeně. Za fyziologických podmínek je jeho hladina nízká nebo nedetekovatelná, ale bylo prokázáno, že u MM pacientů s osteolytickými lézemi je hladina IL-6 zvýšená [15].

U MM je IL-6 hlavním cytokinem, který zprostředkovává růst, přežívání a lékovou rezistenci myelomových buněk (obr. 2). I když některé myelomové buňky produkují IL-6 autokrinně [16], primárně je produkován stromálními buňkami kostní dřeně a působí parakrinně na růst a diferenciaci myelomových buněk [17]. Produkce IL-6 ve stromálních buňkách je tedy indukována buď adhezí myelomových buněk [18], nebo prostřednictvím jiných cytokinů, jako tumor nekrotizující



Obr. 2. IL-6 a signální kaskády v mnohočetném myelomu. Navázáním IL-6 na jeho receptor se aktivují čtyři signální dráhy důležité pro vývoj, růst a lékovou rezistenci v MM. Fosforylace Jak spustí dráhu Ras/Ras/MEK/ERK a STAT. ERK a STAT se přesunou do jádra, kde aktivují cílové geny. Jak také spouští signální dráhu PI3K/Akt, Akt dále může aktivovat mTOR, který působí na STAT a NF-κB, který se opět přesouvá do jádra a přepisuje dané geny.

faktor α (TNFα) [19] a vaskulární endoteliální růstový faktor (VEGF) [20]. Zmíněné faktory následně aktivují např. signální dráhu jaderného faktoru κB (NF–κB), která má vliv na přežívání a růst myelomových buněk [21].

Osteolýza, další patologický proces podporovaný přítomností IL-6, je navozena hned několika mechanizmy. Za prvé, IL-6 indukuje produkci RANKL v mezenchymálních buňkách kostní dřeně a osteoblastech. Vazbou RANKL na RANK je navozeno dozrávání osteoklastů a aktivace signálních drah [22]. Za druhé, IL-6 indukuje zvýšení hladin proteinů zapojených do procesu kostní resorpce, např. peptidů vázajících paratyroidní hormon PTHrP [23]. Za třetí, IL-6 inhibuje osteogenezi zprostředkovanou Wnt, ještě více ruší homeostázu v kosti a posouvá rovnováhu směrem k degradaci kosti [24].

Hladina IL-6 odráží stupeň rozvoje monoklonálních gamapatií, jak bylo prokázáno ve studii, do které bylo začleněno 131 pacientů. Ze skupiny 22 nemocných s MGUS, což je prekancerózní stadium předcházející MM, byl IL-6 detekován pouze u jednoho jedince, u MM to bylo už 35 % pacientů a u nejagresivnějšího stadia zvaného plazmocytární leukemie se IL-6 vyskytoval ve vysoké koncentraci u všech pacientů. Navíc se hodnoty IL-6 lišily i mezi MM pacienty, podíl pacientů s vyšší hladinou IL-6 byl jiný při diagnóze (37 %), během intenzivního vývoje (60 %) a během stabilní fáze (13 %) [25]. Z druhé strany bylo prokázáno, že agresivní mimokostní stadia MM mohou být nezávislá na hladině IL-6 [26]. V klinických studiích byl testován účinek anti-IL-6 mAB (např. CNTO 328), který ale neprokázal zásadní vliv na léčbu MM.

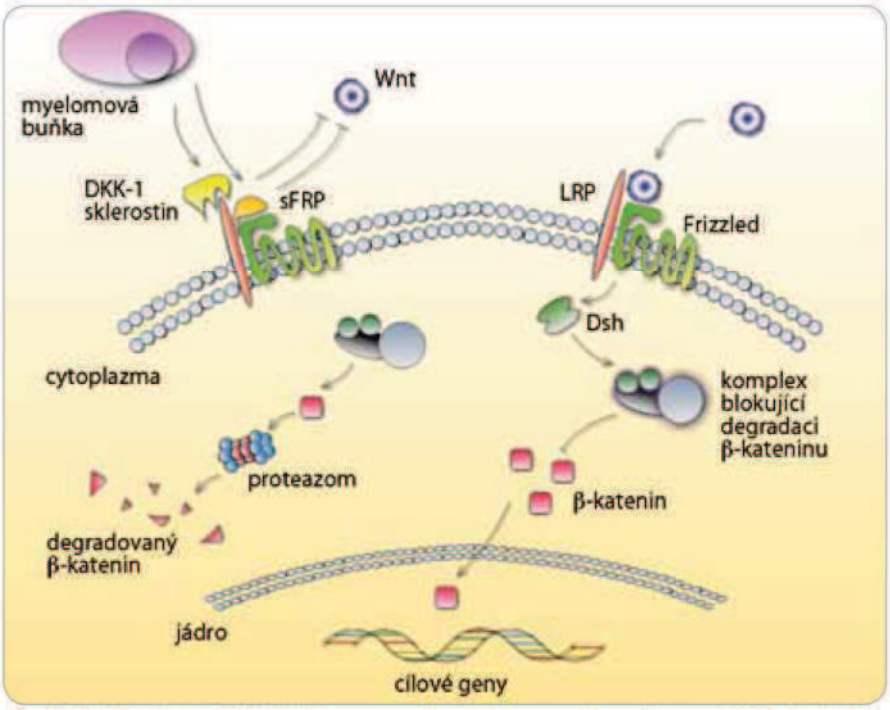
RANKL, ligand z rodiny TNF, je produkován nezralými osteoblasty, stromálními buňkami a T lymfocyty [27,28]. Osteoklasty a jejich prekurzory produkují jeho receptor RANK. Vazbou RANKL na RANK se aktivují signální dráhy, které jsou důležité při diferenciaci osteoklastů z jejich prekurzorových buněk, RANKL také reguluje diferenciaci, funkci a přežívání osteoklastů [22,29]. Osteoprotegerin (OPG) je antagonistou RANKL, inhibuje vazbu RANK–RANKL, inhibuje diferenciaci a aktivaci osteoklastů, a tím brání degradaci kosti [30].

U MM je RANKL hlavním osteoklastogenním faktorem podílejícím se na lytické kostní nemoci. Jeho vysoká produkce ve stromálních buňkách pacientů s MM má důležitou úlohu v patogenezi MM [31]. Některé studie ukázaly, že RANKL je produkován i myelomovými buňkami [32]. V mikroprostředí kostní dřeně MM se interakcí stromálních a myelomových buněk produkce RANKL zvyšuje a OPG snižuje, což podporuje kostní resorpci a osteolýzu [33]. Hladiny těchto molekul korelují s klinickou aktivitou MM a závažností kostní nemoci [34]. Denosumab, monoklonální protilátka proti RANKL, byla schválena FDA k ochraně kostí před dalším poškozením u pacientů s nádory prostaty a prsu s kostními metastázami. V současné době probíhají klinické studie fáze III u pacientů s MM.

Myelomové buňky také vylučují transformující růstový faktor β (TGFβ), pleiotropní cytokin, který za normálních podmínek mimo jiné inhibuje imunitní odpověď tím, že brání proliferaci a diferenciaci B lymfocytů a sekreci Ig [35]. Na rozdíl od účinku na B lymfocyty TGFβ nesnižuje proliferaci myelomových buněk, a při vysokých koncentracích dokonce podporuje vylučování IL-6 těmito buňkami. Tímto zčásti zprostředkovává růst myelomu a podporuje patogenezi [36]. Navíc je TGFβ důležitý v nerovnovážné kostní remodelaci u MM, je aktivní při zvýšené kostní resorpci a inhibuje osteoblasty. Inhibice TGFβ podporuje diferenciaci osteoblastů, které pak inhibují růst a přežívání myelomových buněk. Naopak potlačení diferenciace osteoblastů urychluje ztrátu kostní tkáně [37].

Signální dráhy důležité v mikroprostředí kostní dřeně MM

V patogenezi MM je mnoho regulátorů, proteinových kináz a růstových faktorů,



Obr. 3. Dráha Wnt a její inhibitory v mnohočetném myelomu. V pravé části obrázku je zobrazena situace bez inhibitorů, kdy po navázání Wnt není β-katenin degradován. Přesunutím do jádra se spouští přepis genů pro normální vývoj osteoblastů. V levé části obrázku myelomová buňka produkuje inhibitory DKK-1 a sklerostin, které se vážou na receptor LRP, a sFRP, který blokuje vazbu na Frizzled receptor. B-katenin není přenesen do jádra, ale degradován v proteazomu, a tak je potlačena osteoblastogeneze.

pomocí kterých buňky komunikují. Tyto procesy nejsou jednoduché, právě přímým kontaktem strukturních a buněčných složek nebo podporou autokrinní a parakrinní produkce cytokinů se v mikroprostředí kostní dřeně aktivuje široké spektrum signálních drah [18,21,38,39].

Wnt/β–katenin

Důležitou roli při růstu, vývoji a fungování osteoblastů hraje signální dráha Wnt/β–katenin (obr. 3). Glykoproteiny Wnt se váží na koreceptory LRP–5 nebo LRP–6 a Frizzled receptor a aktivují Wnt dráhu. Přenos signálu stabilizuje β–katenin, který je translokován do jádra a zde stimuluje expresi genů zodpovědných za diferenciaci osteoblastů. Bez přítomnosti signálu je β–katenin fosforylován a degradován v proteazomu.

Existují dvě funkční skupiny antagonistů Wnt signalizace, a to sFRP a Dickkopf (DKK), po jejichž navázání se naruší funkce osteoblastů [40,41]. Významným inhibitorem u MM je DKK–1, který se váže na LRP–5. Je vylučován myelomovými buňkami a v jejich přítomnosti také stromálními buňkami a osteoblasty. DKK–1 se vyskytuje především u pacientů s osteolytickými lézemi a jeho hladina koreluje s rozšířením osteolytických ložisek [24,42,43]. Kromě potlačení diferenciace osteoblastů také podporuje osteoklastogenezi zvýšenou expresí RANKL a sníženou expresí OPG [44]. V současnosti probíhají klinické studie fáze I/II, které testují anti-DKK–1 monoklonální protilátku (BHQ880) u MM.

Další skupina inhibitorů sFRP blokuje vazbu k receptoru Frizzled. Myelomové buňky produkují sFRP–2 a sFRP–3 a ty významně potlačují diferenciaci osteoblastů a tvorbu kostí [45,46]. Existuje však i studie, která ukazuje, že hladiny DKK–1 a sFRP u pacientů s MM nepotlačují diferenciaci lidských osteoblastů. To znamená, že nemusí být jedinými faktory zodpovědnými za inhibici osteoblastů [47].

Sklerostin je produkován osteocyty a působí na dráhu Wnt podobně jako DKK–1, tedy navázáním na LRP–5 [48]. Inhibuje aktivitu osteoblastů a indukuje jejich apoptózu, je negativním regulátorem tvorby kostní tkáně [49]. Teprve nedávno bylo potvrzeno, že sklerostin je vylučován i myelomovými buňkami, a tak přispívá k patogenezi MM [50].

Dráha NF–κB

Obecně je tato dráha důležitá pro proliferaci, přežívání a vývoj nádorových buněk. U MM působí aktivace signalizace NF–κB pozitivně na růst, rezistenci k lékům a přežívání myelomových buněk v mikroprostředí kostní dřeně [51]. Aktivace může probíhat jak klasickým způsobem, tak i alternativně. Bylo prokázáno, že mikroprostředí kostní dřeně u MM spouští tuto signalizaci prostřednictvím adheze i vylučováním cytokinů a chemokinů. Například růstový a anti-apoptotický faktor IL-6, jehož produkce je vyšší při adhezi myelomových a stromálních buněk, může indukovat dráhu NF–κB [21]. Také TNFα aktivuje NF–κB: za prvé u stromálních buněk, čímž podporuje vylučování IL-6 těmito buňkami, a za druhé u myelomových buněk, kde podporuje adhezi buněk a zvyšuje produkci intracelulární adhezivní molekuly 1 (ICAM-1) a vaskulární adhezivní molekuly 1 (VCAM-1) [19]. Aktivace je u MM možná oběma způsoby. Již dříve bylo zjištěno, že klasická dráha může být blokována inhibicí IKKβ proteinu [51], ovšem to neplatí u alternativního způsobu aktivace. Proto byla také zjišťována inhibice jiné molekuly, a to IKKα, která se vyskytuje u obou způsobů aktivace. Růst buněk byl sice zpomalen, ale aktivita signalizace NF–κB byla vyšší než u kontroly, což naznačuje, že inhibiční efekt IKKα je nezávislý na aktivitě NF–κB [52].

Dráha PI3K/Akt

Fosfatidylinositol–3–kináza (PI3K)/Akt je jedna z nejčastěji aktivních drah u lidských nádorů. Mnoho proteinových kináz a transkripčních faktorů, které se účastní této signalizace, ovlivňuje rezistenci myelomových buněk k léčbě. Dráha PI3K/Akt reguluje průběh buněčného cyklu a apoptózu, indukuje syntézu DNA a působí na přežívání a migraci myelomových buněk. Je propojena s dráhou NF–κB přes kinázu Akt, která, podobně jako IKK u dráhy NF–κB, fosforyluje a degraduje IκBα. To vede k přesunutí NF–κB do jádra, kde může induko-

vat transkripci anti-apoptotických genů. Tedy Akt může inhibovat apoptózu aktivací NF–κB [53].

Aktivátory této dráhy jsou IL-6 a růstový faktor podobný inzulinu 1 (IGF-1); aktivace PI3K je důležitá při proliferaci a anti-apoptotické odpovědi myelomových buněk na tyto cytokiny [54]. IL-6 nejen spouští dráhu PI3K/Akt, ale tato interakce má navíc regulační účinky na buněčný cyklus, chrání buňky před apoptózou způsobenou léky a ovlivňuje růst MM [55].

Důležitou součástí této dráhy je kináza mTOR, která se u savců vyskytuje ve dvou rozdílných komplexech, mTORC1 a mTORC2, které ovšem mají rozdílné funkce. Akt aktivuje mTORC1, který fosforyluje další molekuly regulující syntézu proteinů, a tak kontroluje buněčný růst. Naopak mTORC2 v odpovědi na růstové faktory aktivuje Akt a reguluje přežívání buněk [56]. V souvislosti s MM byl identifikován DEPTOR, který za fyziologických podmínek inhibuje mTORC1 a mTORC2. I když jeho vysoká produkce u MM inhibuje mTORC1, překvapivě také vede k aktivaci dráhy PI3K/mTORC2/Akt. Tento nepřímý způsob aktivace je důležitý ku příkladu u myelomových buněk, kterým chybí mutace aktivující PI3K [57]. V současnosti probíhají klinické studie, ve kterých jsou testovány kombinace lenalidomidu a everolimu (RAD001), inhibitoru mTOR jak v solidních nádorech, tak u refraktorního MM.

Dráhy Ras/Raf/MEK/MAPK a Jak2/STAT3

Dalšími důležitými drahami jsou Ras/Raf/MEK/MAPK a Jak2/STAT3, které mohou být aktivovány IL-6. Inhibice IL-6R sice blokuje fosforylaci STAT3, ale neovlivňuje aktivaci dráhy MAPK, z čehož vyplývá, že v mikroprostředí kostní dřeně se dráha STAT3 aktivuje prostřednictvím IL-6 a dráha MAPK mechanizmy nezávislými na IL-6 [58].

K aktivaci dráhy Ras/Raf/MEK/MAPK, která stimuluje angiogenezi, proliferaci buněk a jejich apoptózu, jsou potřebné jak adhezivní interakce, tak vylučování růstových faktorů. Hlavními aktivačními faktory této dráhy jsou IL-6 a IGF-1, které aktivují Ras [59,60]. Následně dojde k aktivaci i ostatních složek – Raf, MEK a ERK. Tuto dráhu spouštějí také další faktory, jako VEGF [61]. Bylo dokázáno, že inhibicí aktivity ERK se sníží produkce VEGF, která vede ke snížené tvorbě nových cév v kostní dřeni indukované myelomovými buňkami [62].

Dráha Jak2/STAT3 má vliv na přežívání myelomových buněk, aktivace této dráhy indukuje proliferaci a inhibici apoptózy. STAT3 přímo přispívá k malignímu rozvoji MM tím, že chrání myelomové buňky před apoptózou a podporuje přežívání [63]. Stimulace buněk IL-6 vede k signalizaci přes IL-6R a spouští fosforylaci STAT3 přes Jak, STAT3 je přenesen do jádra, kde aktivuje transkripci daných anti-apoptotických genů. Aktivace pomocí IL-6 tedy reguluje přežívání myelomových buněk vylučováním anti-apoptotických proteinů z rodiny Bcl–2, například Bcl–XL, Mcl–1 [63,64]. Bylo zjištěno, že v myelomových buňkách je STAT3 neustále aktivován a inhibice této dráhy indukuje apoptózu *in vitro* [58,65].

Rezistence k lékům způsobená mikroprostředím MM

Rezistentní fenotyp způsobený mikroprostředím MM může být dvojího typu: léková rezistence zprostředkovaná interakcemi cytokinů (cytokine mediated drug resistance – CM–DR), nebo léková rezistence zprostředkovaná adhezivním kontaktem buněk (cell adhesion–mediated drug resistance – CAM–DR). Oba mechanizmy mají zásadní význam v patogenezi MM. Výraz CAM–DR byl poprvé použit ve studii MM, kde byla pozorována zvýšená produkce α_4, β_1 a β_7 integrinů, které jsou vylučovány myelomovými buňkami. Adheze maligních buněk k fibronektinu může přispět ke vzniku rezistence *de novo*, chrání buňky před apoptózou způsobenou léky [38].

S adhezí myelomových buněk k fibronektinu je také asociována zvýšená hladina $p27^{kip1}$. Tento protein je důležitý pro udržení rezistentního fenotypu, má význam v buněčném cyklu, kde zadržuje buňky ve fázi G_1. Bylo prokázáno, že přerušením adheze se hladina $p27^{kip1}$ sníží, buňky pokračují v S fázi buněčného cyklu a opět se stávají citlivými k lékům. Pokusy s inhibicí produkce $p27^{kip1}$ neměly vliv na adhezi, ale zvrátily lékovou rezistenci. To dokazuje, že vyšší produkce tohoto proteinu přispívá k CAM–DR [66].

U MM je také nadměrně vylučována adhezivní molekula P–selektin a její ligand PSGL–1. Kromě jiných funkcí, jako jsou adheze a osídlování myelomových buněk v mikroprostředí kostní dřeně, je PSGL–1 důležitý v rozvoji lékové rezistence myelomových buněk *in vivo* a *in vitro*. Inhibice interakcí tohoto ligandu k selektinu podporuje citlivost myelomových buněk k bortezomibu, inhibitoru proteazomu [67].

Hladina HSP70 je také zmíněnými interakcemi zvýšena, navíc podporuje vyšší produkci IL-6, který pak napomáhá přežívání myelomových buněk pomocí aktivace signálních drah. Inhibice HSP70 potlačuje adhezi myelomových buněk k fibronektinu a způsobuje apoptózu rezistentních buněk. Fenotyp lékové rezistence je tak změněn a buňky se stávají citlivými k lékům. Tato studie ukázala, že inhibice HSP70 může způsobit apoptózu buněk, které vykazují rezistenci *de novo* i rezistenci získanou během léčby [68].

S rezistencí je také asociována dráha NF–κB, jejíž aktivace je stimulována adhezí k fibronektinu [39]. Neustálá aktivita této dráhy vzdoruje inhibujícím účinkům bortezomibu, i v jeho přítomnosti přispívá jinými mechanizmy k rezistenci. V této studii bylo navíc zjištěno, že pokud se myelomové buňky kultivují se stromálními buňkami pacienta s MM, aktivita dráhy NF–κB, a tím i rezistence k bortezomibu, je ještě více podporována [69]. Pozdější výsledky potvrdily, že aktivace dráhy NF–κB je významně vyšší ve stromálních buňkách a tyto buňky se významně liší od fyziologicky normálních buněk [70].

Závěr

V posledních letech bylo provedeno mnoho studií týkajících se mikroprostředí kostní dřeně u pacientů s MM, které dostávají toto téma do popředí zájmu a ukazují na jeho význam v patogenezi nemoci. Nicméně mechanizmy probíhající v tomto mikroprostředí nebyly doposud zcela objasněny, proto je nutné pokračovat v dalším výzkumu. Na základě nových poznatků o aktivitě

buněk kostní dřeně a procesech podílejících se na vzniku myelomových buněk bude možno identifikovat nové potenciální cíle a markery pro nové léky a stanovit efektivnější léčbu pro pacienty.

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TGF-beta - an excellent servant but a bad master

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TGF-β – an excellent servant but a bad master

Lenka Kubiczkova, Lenka Sedlarikova, Roman Hajek and Sabina Sevcikova*

Abstract

The transforming growth factor (TGF-β) family of growth factors controls an immense number of cellular responses and figures prominently in development and homeostasis of most human tissues. Work over the past decades has revealed significant insight into the TGF-β signal transduction network, such as activation of serine/threonine receptors through ligand binding, activation of SMAD proteins through phosphorylation, regulation of target genes expression in association with DNA-binding partners and regulation of SMAD activity and degradation. Disruption of the TGF-β pathway has been implicated in many human diseases, including solid and hematopoietic tumors. As a potent inhibitor of cell proliferation, TGF-β acts as a tumor suppressor; however in tumor cells, TGF-β looses anti-proliferative response and become an oncogenic factor. This article reviews current understanding of TGF-β signaling and different mechanisms that lead to its impairment in various solid tumors and hematological malignancies.

Keywords: TGF-β, SMAD proteins, Oncogene, Suppressor, Solid tumors, Leukemia, Multiple myeloma

Introduction

Although our understanding of molecular mechanisms that underlie cancer development and progression has increased, cancer remains a significant health concern in many developed countries. There is a strong requirement for new diagnostic and treatment options as well as elucidation of how cells acquire the six essential phenotypes, or hallmarks, necessary to become fully malignant [1]. Pharmacological targeting of cancer hallmarks may offer new possibilities of effectively treating development and/or metastases of human tumors (reviewed in [2]). Transforming Growth Factor-β (TGF-β) is a key player in cell proliferation, differentiation and apoptosis. The importance of this regulation is apparent from the role of TGF-β in development and consequences of aberrant TGF-β signaling in cancer [3]. Nevertheless, it is still not elucidated how malignant cells overcome the cytostatic functions of TGF-β or how TGF-β stimulates the acquisition of cancer hallmarks of developing and progressing human cancers. In this paper, we review different molecular and cellular mechanisms that lead to impairment of TGF-β signaling in various solid tumors and hematological malignancies.

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History of TGF-β discovery

In the early 1980s, it had become apparent that cell growth is controlled by many polypeptides and hormones. A new hypothesis of 'autocrine secretion' was postulated, which suggested that polypeptide growth factors are able to cause malignant transformation of cells [4]. A new polypeptide called SGF (Sarcoma Growth Factor) was discovered in cultures of transformed rat kidney fibroblasts [5]; soon it became apparent that this factor is a mixture of at least two substances with different functions. They were called Transforming Growth Factor-α (TGF-α) and Transforming Growth Factor-β (TGF-β) [6]. TGF-β was further described by Roberts and Sporn as a secreted polypeptide capable of inducing fibroblast growth and collagen production [7]. Soon after its discovery, TGF-β was found to inhibit cell proliferation as well; thus, a dual role of this cytokine was recognized [8,9].

TGF-β family and isoforms

The TGF-β superfamily is composed of a large group of proteins, including the activin/inhibin family, bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), the TGF-β subfamily, and the glial cell line-derived neurotrophic factor (GDNF) family. This review will focus solely on the TGF-β family.

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The TGF-β proteins have been discovered in a variety of species, including invertebrates as well as vertebrates. TGF-β superfamily is fundamental in regulation of various biological processes, such as growth, development, tissue homeostasis and regulation of the immune system [10,11].

Beta-type subfamily growth factors are homodimeric or heterodimeric polypeptides with multiple regulatory properties depending on cell type, growth conditions and presence of other polypeptide growth factors. Since their expression is also controlled by distinct promoters, their secretion is temporal and tissue specific [12].

There are three known isoforms of TGF-β (TGF-β1, TGF-β2 and TGF-β3) expressed in mammalian tissues; they contain highly conserved regions but diverge in several amino acid regions. All of them function through the same receptor signaling pathways [13,14].

TGF-β1, the most abundant and ubiquitously expressed isoform, was cloned from human term placenta mRNA [15]. In mouse development, Tgf-β1 mRNA and/or protein have been localized in cartilage, endochondral and membrane bone and skin, suggesting a role in the growth and differentiation of these tissues [16].

TGF-β2 was first described in human glioblastoma cells. It was found that TGF-β2 is capable of suppressing interleukin-2-dependent growth of T lymphocytes. Thereby, it was named glioblastoma-derived T cell suppressor factor (G-TsF). Physiologically, TGF-β2 is expressed by neurons and astroglial cells in embryonic nervous system [17]. It is also important in tumor growth enhancing cell proliferation in an autocrine way and/or reducing immune-surveillance of tumor development [18]. Their mature forms, which consist of the C-terminal 112 amino acids, TGF-β1 and TGF-β2 share 71% sequence similarity [19].

The third isoform, TGF-β3, was isolated from a cDNA library of human rhabdomyosarcoma cell line; it shares 80% of amino acid sequence with TGF-β1 and TGF-β2. Studies on mice demonstrated essential function of Tgf-β3 in normal palate and lung morphogenesis and implicate this cytokine in epithelial-mesenchymal interaction [20,21]. Its mRNA is present in lung adenocarcinoma and kidney carcinoma cell lines; interestingly, umbilical cord expresses very high level of TGF-β3 [19].

TGF-β synthesis and activation

Mature dimeric form of TGF-β, composed of two monomers stabilized by hydrophobic interactions and disulphide bridge, initiates intracellular signaling [22]. The three TGF-βs are synthesized as pro-proteins (pro-TGF-βs) with large amino-terminal pro-domains (called latency associated proteins – LAPs), which are required for proper folding and dimerization of carboxy-terminal growth-factor domain (mature peptide) [23]. This complex is called 'small latent complex' (SLC). After folding and dimerization, TGF-β dimer is cleaved from its propeptides in trans-Golgi apparatus by furin type enzymes; however, it remains associated with its pro-peptide through noncovalent interactions, creating 'large latent complex' (LLC). Most cultured cell types release latent TGF-β into extracellular matrix as LLC which in addition includes a 120–240 kDa glycoprotein called latent TGF-β binding protein (LTBP) [24]. LTBP is composed primarily of two kinds of cysteine-rich domains: EGF-like repeats (most of which are calcium-binding) and eight-cysteine domains [25]. LTBP participates in the regulation of latent TGF-β bioavailability by addressing it to the extracellular matrix (ECM) [26]. Non-active TGF-β stays in ECM; its further activation is a critical step in the regulation of its activity (Figure 1).

A number of papers have reported TGF-β activation by retinoic acid and fibroblast growth factor-2 (FGF-2) in endothelial cells [27,28], or by endotoxin and bleomycin in macrophages [29]. Further, a variety of molecules is involved in TGF-β activation. Proteases including plasmin, matrix metaloproteases MMP-2 and MMP-9, are TGF-β activators *in vitro* [30,31]. Other molecules involved in the mechanism of activation are thrombospondin-1 [32], integrins, such as αVβ6 or αVβ8 [33,34], or reactive oxygen species (ROS).

Moreover, latent TGF-β present in conditional medium is activated by acid treatment (pH 4.5) *in vitro* [35]. *In vivo*, a similar pH is generated by osteoclasts during bone resorption. Since the bone matrix deposited by osteoblasts is rich in latent TGF-β, the acidic environment created by osteoclasts *in vitro* might result in latent TGF-β activation [36].

TGF-β receptors

In most cells, three types of cell surface proteins mediate TGF-β signaling: TGF-β receptor I (TβRI), II (TβRII) and III (TβRIII) [13,37]. Out of these three receptors, TβRIII, also called betaglycan, is the largest (250–350 kDa) and most abundant binding molecule. This cell-surface chondroitin sulfate / heparan sulfate proteoglycan is expressed on both fetal and adult tissues and most cell types [38]. Endoglin (CD105) was shown to act as type III receptor for TGF-β as well [39]. Endoglin is a membrane, an RGD-containing glycoprotein, which is expressed in a limited set of cell types, primarily vascular endothelial cells, several hematopoietic cell types, bone marrow stromal cells and chondrocytes. Its expression strongly increases in active vascular endothelial cells upon tumor angiogenesis [40-42]. Moreover, in normal brain, it was found to be expressed in the adventitia of arteries and arterioles, and it is expressed on several types of tumor cells, such as invasive breast cancers and cell lines or renal cell carcinoma [43-45]. Although betaglycan and endoglin are co-receptors not directly

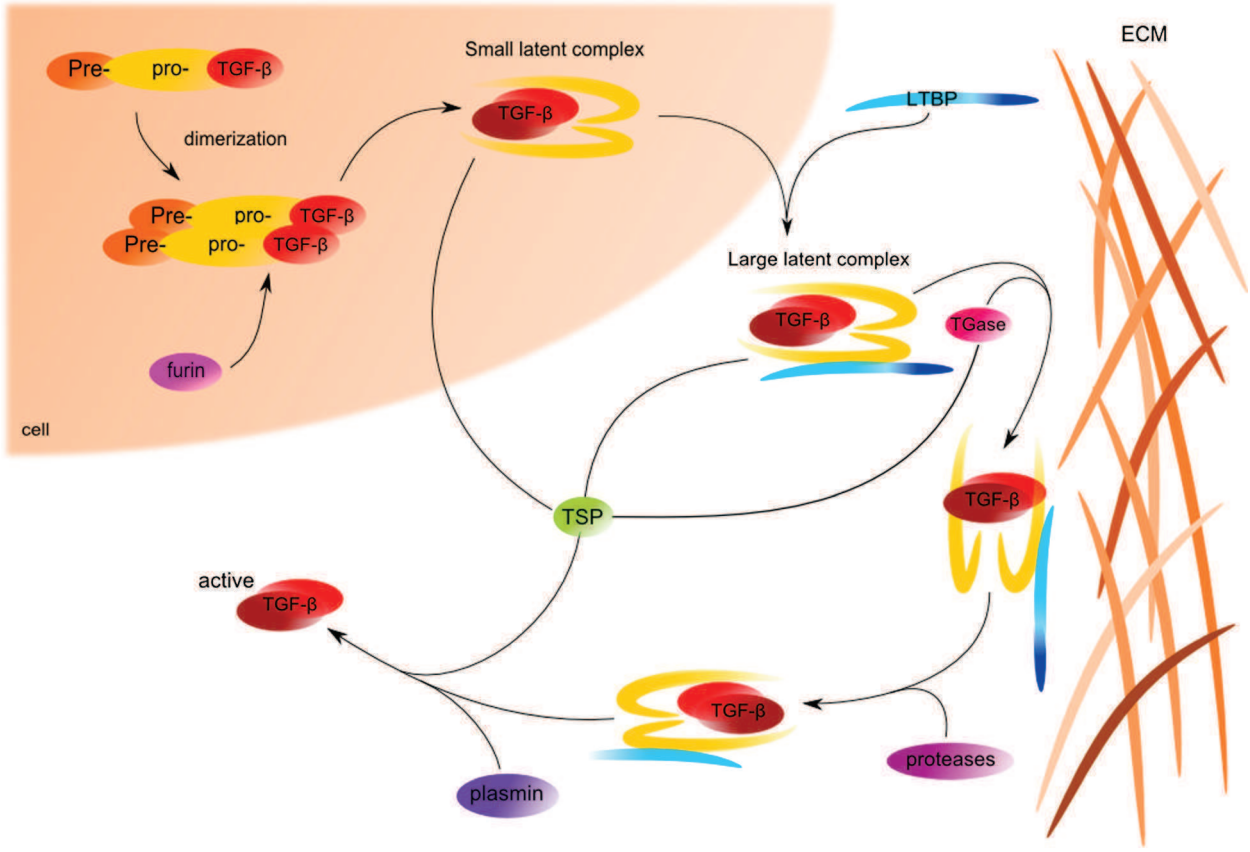

Figure 1 TGF-β synthesis and activation. TGF-βs are synthesized as inactive precursors that contain pre-region (Signal peptide) and pro-region (N terminal peptide - LAP). Processing of inactive form starts with proteolytic cleavage that removes signal peptide from pre-pro-TGF-βs form. After dimerization, TGF-βs are cleaved by proteases (eg. Furin) into C-terminal mature peptides and N-terminal LAP (Latency Associated Peptide). TGF-βs with LAP form small latent complexes (SLP) that are transported to extracellular matrix where can further covalently bind to latent TGF-β binding protein (LTBP) to form a large latent complexes (LLC). LTBP is able to connect inactive TGF-β forms to ECM proteins. This interaction is further supported by covalent transglutaminase-induced (TGase) crosslinks. Activation of TGF-β starts with release of LCC from ECM by proteases. Then, the mature protein is cleaved from LTBP, which is provided *in vitro* by acidic condition, pH or plasmin or *in vivo* by thrombospondin (TSP). Once the active TGF-β family member is released from the ECM, it is capable of signaling.

involved in intracellular TGF-β signaling due to lack of kinase domain, they can control access of TGF-β to TGF-β receptors and consequently modulate intracellular TGF-β activity [46,47]. Betaglycan binds all three isoforms of TGF-β, with higher affinity for TGF-β2; however, endoglin binds TGF-β1 and -β3 with constant affinity and has only weak affinity for TGF-β2 [39,48].

TβRI and TβRII mediate signal transduction. Both receptors are transmembrane serine/theronine kinases, which associate in a homo- or heteromeric complex and act as tetramers. They are organized sequentially into an N-terminal extracellular ligand-binding domain, a transmembrane region, and a C-terminal serine/threonine kinase domain. The type II receptors range from 85 to 110 kDa, while the type I receptors are smaller and their size ranges from 65 to 70 kDa [49]. Moreover, TβRI contains a characteristic, highly conserved 30 amino acids long GS domain in the cytoplasmic part, which needs to be phosphorylated to fully activate TβRI [36]. TβRII contains 10 bp polyadenine repeat in the coding region of the extracellular domain. This region is frequently a target of changes leading to frameshift missense mutations or early protein terminations that result in truncated or inactive products [50].

TGF-β receptors activation

Bioactive forms of TGF-βs are dimers held together by hydrophobic interactions and, in most cases, by an intersubunit disulfide bond as well. The dimeric structure of these ligands suggests that they function by bringing together pairs of type I and II receptors, forming heterotetrameric receptor complexes [51]. Binding of TGF-β to extracellular domains of both receptors also induces proper conformation of the intracellular kinase domains. These receptors are subject to reversible post-translational modifications (phosphorylation, ubiquitylation and

sumoylation) that regulate stability and availability of receptors as well as SMAD and non-SMAD pathway activation.

Receptor phosphorylation activates TGF-β signaling pathway – the ligand binds to TβRII first, followed by subsequent phosphorylation of a Gly-Ser regulatory region (GS-domain) within TβRI. This leads to incorporation of TβRI and formation of a large ligand-receptor complex that consists of dimeric TGF-β ligand and two pairs of TβRI and TβRII [52]. The TGF-β receptor complex is extremely stable upon solubilization [53]. TGF-β1 and TGF-β3 bind to TβRII without participation of type I receptor, whereas TGF-β2 interacts only with combination of both receptors (reviewed in [54]). Although ligand binding may induce autophosphorylation of TβRII cytoplasmic domain, signaling in the absence of TβRI has not been reported [49]. TβRIII betaglycan promotes binding of TGF-β2 to TβRII, since the affinity of TGF-β2 to TβRII is low in the absence of betaglycan [46]. Endoglin binds TGF-β1, TGF-β3 but not TGF-β2 in the presence of the TβRI and TβRII. In some cell types, endoglin was found to inhibit TGF-β signaling – for example in chondrocytes, it enhances TGF-β1-induced SMAD1/5 phosphorylation but inhibits TGF-β1-induced SMAD2 phosphorylation [55].

Ubiquitylation and ubiquitin-mediated degradation define stability and turnover of receptors. Ubiquitylation occurs through sequential actions of E1, E2 and E3 ubiquitin ligases that provide specificity in the ubiquitylation process [56]. The E3 ubiquitin ligases such as Smurf1 and Smurf2 (SMAD ubiquitylation-related factor 1 and 2) regulate the stability of TβRI and heteromeric TGF-β receptor complex [57,58].

Sumoylation, similarly to ubiquitylation, requires E1, E2 and E3 ligases which results in SUMO polypeptide attachment. Although sumoylation has not been observed for any other transmembrane receptor kinases, it was shown to modify TβRI function by facilitating the recruitment and phosphorylation of SMAD3 [59].

TGF-β receptors are also constitutively internalized via clathrin-dependent or lipid-raft-dependent endocytic pathways (reviewed in [60]).

TGF-β signaling

SMAD proteins

The SMAD proteins are the only known latent cytoplasmic transcription factors that become directly activated by serine phosphorylation at their cognate receptors. SMADs can be classified into 3 groups based on their function: the receptor-regulated SMADs (R-SMADs), SMAD1, SMAD2, SMAD3, SMAD5 and SMAD8; the common SMAD (Co-SMAD), SMAD4, and the inhibitory SMADs (I-SMADs), SMAD6 and SMAD7 (reviewed in [61]).

R-SMADs and Co-SMAD consist of a conserved MH1 domain (Mad-homology-1) and C-terminal MH2 domain (Mad-homology-2), which are connected by a 'linker' segment. The C-terminal domain promotes transcriptional activity, when fused to a heterologous DNA binding domain [62]. On the contrary, I-SMADs contain only the highly conserved MH2 domain. The MH1 domain is responsible for binding to DNA; however, the MH2 domain contains hydrophobic patches also called hydrophobic corridors that allow binding to nucleoporins, DNA-binding cofactors and various cytoplasmic proteins, as well as interaction with receptors. Both domains can interact with sequence-specific transcription factors. SMAD3 and SMAD4 bind with their MH1 domain to SMAD-binding elements (SBE) on DNA, whereas the common splice form of SMAD2 does not bind to DNA (reviewed in [63]).

I-SMADs function as intracellular antagonists of R-SMADs. Through stable interactions with activated serine/threonine receptors, they inhibit TGF-β family signaling by preventing the activation of R- and Co-SMADs. I-SMADs regulate activation of R-SMADs via binding with their MH2 domain to TβRI, thereby competing with R-SMADs and preventing R-SMADs phosphorylation [64]. SMAD6 is also able to compete with SMAD4 for heteromeric complex formation with activated SMAD1 [65]. Whereas SMAD6 appears to preferentially inhibit BMP signaling, SMAD7 acts as a general inhibitor of TGF-β family signaling. Another possible mechanism of inhibition signaling transduction by I-SMADs is facilitated by HECT type of E3 ubiquitin ligase Smurf1 and Smurf2 [57,58].

Canonical signaling

The SMAD pathway is the *canonical signaling* pathway that is activated directly by the TGF-β cytokines (Figure 2). TβRI recognizes and phosphorylates signaling effectors – the SMAD proteins. This phosphorylation is a pivotal event in the initiation of TGF-β signal, followed by other steps of signal transduction, subjected to both positive and negative regulation.

R-SMAD binding to the type I receptor is mediated by a zinc double finger FYVE domain containing protein SARA (The SMAD Anchor for Receptor Activation). SARA recruits non-activated SMADs to the activated TGF-β receptor complex [66]. However, TMEPAI (TransMembranE Prostate Androgen-Induced gene/protein), a direct target gene of TGF-β signaling, perturbs recruitment of SMAD2/3 to TβRI and thereby participates in a negative feedback loop to control the duration and intensity of SMADs activation [67]. Receptor-mediated phosphorylation of SMAD2 decreases the affinity of SMAD2 to SARA, leading to dissociation from SARA [68]. Afterwards, phosphorylated complex of

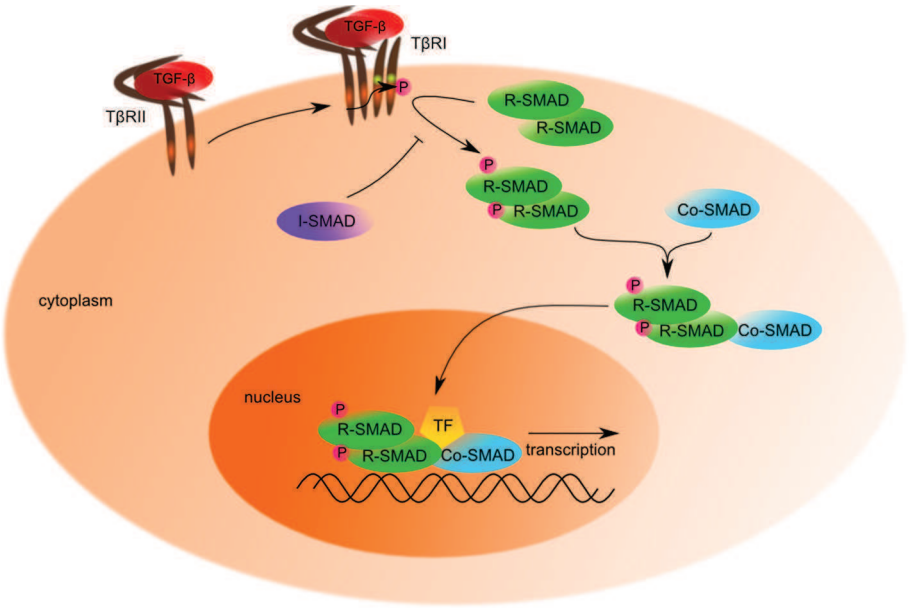

Figure 2 TGF-β canonical signaling pathway. After ligand binding, TGF-β receptors dimerize and phosphorylate intracellular SMAD proteins. Complex of SMAD2 and/or SMAD3 becomes phosphorylated by TβRI and forms a complex with SMAD4 which is subsequently transported into the nucleus where it binds with specific transcription factors (TF) and induces a transcription of TGF-β dependent genes.

SMAD2/3 forms a higher-order complex with SMAD4 and moves to the nucleus. At this point, Smurf1 interacts with R-SMADs in order to trigger their ubiquitylation and degradation and hence their inactivation [69]. Further, it was found that Smurf1 and Smurf2 facilitate the inhibitory effect of I-SMADs. Smurf2 binding in the nucleus to SMAD7 induces export and recruitment to the activated TβRs, where it causes degradation of receptors and SMAD7 via proteasomal and lysosomal pathways [57]. Smurf1 (specific for BMP-SMADs) also interacts with SMAD7 and induces SMAD7 ubiquitylation and translocation into the cytoplasm [58].

For proper translocation to the nucleus, the SMADs contain a nuclear localization-like sequence (NLS-like; Lys-Lys-Leu-Lys) that is recognized by importins [70]. Interestingly, the nuclear translocation of SMADs was also described *in vitro* to occur independently of added importin-like factors, because SMAD proteins can directly interact with nucleoporins, such as CAN/Nup214 [71,72]. Complex of SMAD2/3 and SMAD4 is retained in the nucleus by interactions with additional protein binding partners and DNA. Dephosphorylation and dissociation of SMAD transcriptional complexes are thought to end this retention, allowing export of R-SMADs out of the nucleus [73].

Different protein binding partners provide another venue for regulatory inputs controlling the activity of SMADs. Each SMAD-partner combination targets a particular subset of genes and recruits either transcriptional co-activators or co-repressors. Members of many DNA-binding protein families participate as SMADs cofactors, such as FOX, HOX, RUNX, E2F, AP1, CREB/ATF, Zinc-finger and other families. The SMAD cofactors differ in various cell types, thereby determining the cell-type dependent responses [63]. By association with DNA-binding cofactors, SMADs reach target gene specificity and target specificity. Stimulation of various cells by TGF-β leads to rapid activation or repression of a few hundred genes; possibly, the pool of activated SMAD proteins is shared among different partner cofactors [74,75].

On chromatin level, SMADs can recruit histone acetyltransferases. Several studies revealed that TGF-β proteins influence transcription of different genes through interaction of the MH1 domain of SMADs with sequence-specific transcription factors and co-activators CBP and p300. CBP and p300 interact with SMAD1, SMAD2, SMAD3 and SMAD4 *in vitro* and *in vivo*, and the interaction between the SMADs and CBP/p300 is stimulated in response to TGF-β [76-79]. Moreover, histone deacetylases and chromatin remodeling complexes are also involved in SMAD regulation. In this way, SMADs functionally interact with a variety of transcription factors and regulate diverse signaling pathways as well (reviewed in [80]).

SMADs act as sequence specific transcription factors; however, they can regulate cell fate by alternative mechanisms. Recent data indicate that R-SMADs

associate with the p68/Drosha/DGCR8 miRNA processing complex to regulate miRNA processing in a ligand-dependent and RNA-sequence specific manner. So far, more than 20 TGF-β/BMP-regulated miRNAs (T/B-miRs) have been described [81,82].

Non-SMAD signaling

Diversity of TGF-β signaling in cells is determined not only by various ligands, receptors, SMAD mediators or SMAD-interacting partners, but also by the ability of TGF-β to activate other signaling pathways (Figure 3). TGF-β can indirectly participate in apoptosis, epithelial to mesenchymal transition, migration, proliferation, differentiation and matrix formation (reviewed in [83]). It activates various branches of mitogen-activated protein kinases (MAPK) pathway, such as ERK1/ERK2, Jun-N terminal kinase (JNK) and p38 and PI3K kinases [84]. In response to TGF-β, both SMAD-dependent and SMAD-independent JNK activations are observed [85]. SMAD-independent activation of p38 was observed in mouse mammary epithelial NMuMG cells with mutant TβRI [86].

Other pathways influenced by TGF-β are the growth and survival promoting pathway AKT/PKB, the small GTP-binding proteins RAS, RHOA, RAC1 as well as CDC42 and mTOR [87-89]. TGF-β participates in mediating activation of protein tyrosine kinases FAK, SRC and ABL, particularly in mesenchymal or dedifferentiated epithelial cells [90-92]. TGF-β also influences NF-κB signaling and Wnt/β-catenin pathway [93].

Role of TGF-β in tumors

In tumors, TGF-β can be either a proto-oncogene or a tumor suppressor, depending on cell context and tumor stage [94]. Cancer cells often evade growth inhibition effects of TGF-β, while leaving intact TGF-β-mediated cellular responses that promote tumor progression.

Importantly, the use of mouse models has enabled the elucidation of the dual role of TGF-β in cancer (reviewed in [95]). As homozygous deletions of *Tgf-β1, Tgf-β2, Tgf-β3, TβRI* and *TβRII* are lethal in mice, manipulation of TGF-β pathway was achieved mainly through transgene expression or conditional null mutations *in vivo* [96]. The dual role of TGF-β was shown on a set of experiments with mice skin cancer. The first study demonstrated that TGF-β1 expression targeted to keratinocytes inhibits benign tumor outgrowth; however, later it enhances malignant progression rate and phenotype of the benign papillomas [97]. Study on transgenic mice overexpressing a dominant negative TβRII in the basal cell compartment and in follicular cells of the skin complemented previous results. In non-irritated epidermis of transgenic mice, proliferation and differentiation were normal; however, during tumor promotion, transgenic mice showed an elevated level of proliferation in the epidermis [98]. Furthermore, using mice with inducible expression of TGF-β1 in epidermis confirmed the dual role of TGF-β [99,100].

TGF-β as a tumor suppressor

The most critical effect of TGF-β on target cells is suppression of proliferation. Its growth inhibitory function

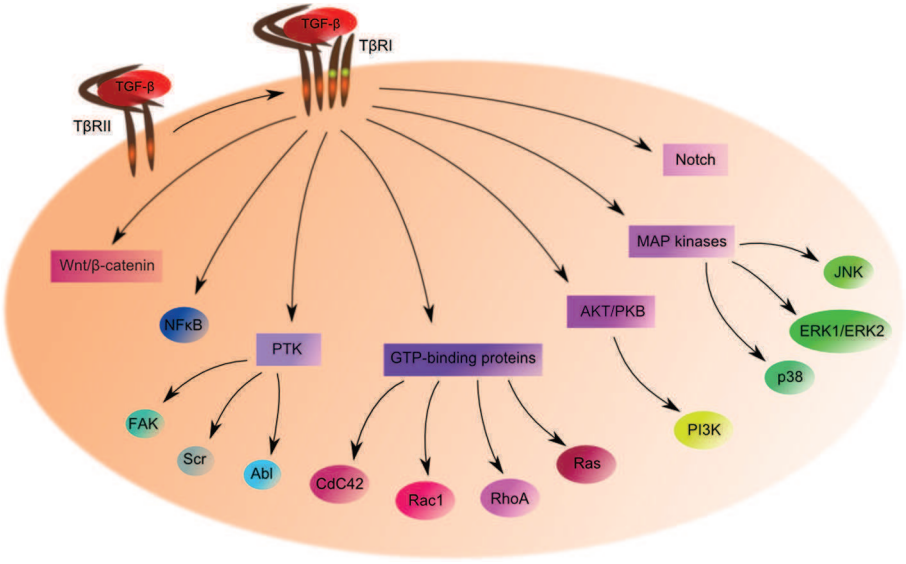

Figure 3 TGF-β non-canonical signaling pathway. After ligand binding, several different branching signaling pathways can be activated in malignant cells, such as Notch signaling, MAP kinases, AKT/PKB pathway, GTP-binding proteins pathway, PTK pathway, NF-κB and Wnt/β-catenin pathway.

is based on the ability to suppress expression and function of c-Myc and cyclin-dependent kinases (CDKs) and to enhance expression of the CDK inhibitors $p15^{INK4B}$ [101] [102] and $p27^{KIP1}$ [103].

Cellular responses to TGF-β depend on cell type and physiological conditions. TGF-β stimulates various mesenchymal cell types, including fibroblasts; however, it is a potent inhibitor of epithelial, endothelial, neural cells and hematopoietic cells, including immune cells [10]. Central function of TGF-β is inhibition of cell cycle progression by regulating transcription of cell cycle regulators (Figure 4). Anti-proliferative responses can be induced at any time during cell cycle division; yet, they are effective only in G1 phase. Once a cell is committed to enter replication, it will continue to double its DNA, divide and then arrest when entering the following G1 phase. At this point, TGF-β mediates cell cycle arrest by suppressing expression and function of c-Myc, members of the Id family inhibitors and CDKs and enhancing expression of CDK inhibitors, such as $p15^{INK4B}$, $p21^{CIP1}$ and $p27^{KIP1}$ [104,105].

TGF-β induces the expression of the CDK inhibitor $p15^{INK4B}$ in a variety of cell types. $p15^{INK4B}$ is a member of the INK4 family of CDK inhibitors, which binds to CDK4 and CDK6 subunits, inactivates their catalytic activity and prevents cyclin D-CDK4/6 complex formation [101,106]. Furthermore, TGF-β can induce expression of $p21^{CIP1}$ in several cell types [107,108]. Other CDK inhibitory responses, observed in several cell types after exposure to TGF-β, are inhibition of CDK4 expression and down-regulation of CDC25A expression [109].

Low levels of c-Myc allow for TGF-β induced transcription of $p15^{INK4B}$ and $p21^{CIP1}$ genes. Decreased expression of c-Myc in keratinocytes is mediated by SMAD3 in association with transcription factors E2F4 and E2F5, p107 co-repressor and SMAD4 [110]. On the other hand, down-regulation of Id proteins in epithelial cells is due to activated SMAD3 that induces activating transcription factor (ATF) expression and then together with ATF directly represses the Id promoter [104].

TGF-β as a tumor promoter

TGF-β acts as tumor suppressor in normal epithelium; it inhibits cell proliferation and induces apoptosis. Yet, during tumor progression, sensitivity to these effects of TGF-β is frequently lost and, in later stages, TGF-β signaling has pro-oncogenic function. Several activities have been described to TGF-β that would favor tumor progression [111].

Mutations in signaling components

Malignant cells become resistant to suppressive effects of TGF-β either through mutation and/or functional inactivation of TGF-β receptors or by downstream alterations in the SMAD-signaling pathway. During late stages of tumor progression, TGF-β acts as tumor promoter and is often over-expressed in many cancers. Elevated plasma level of TGF-β1 was observed in hepatocellular

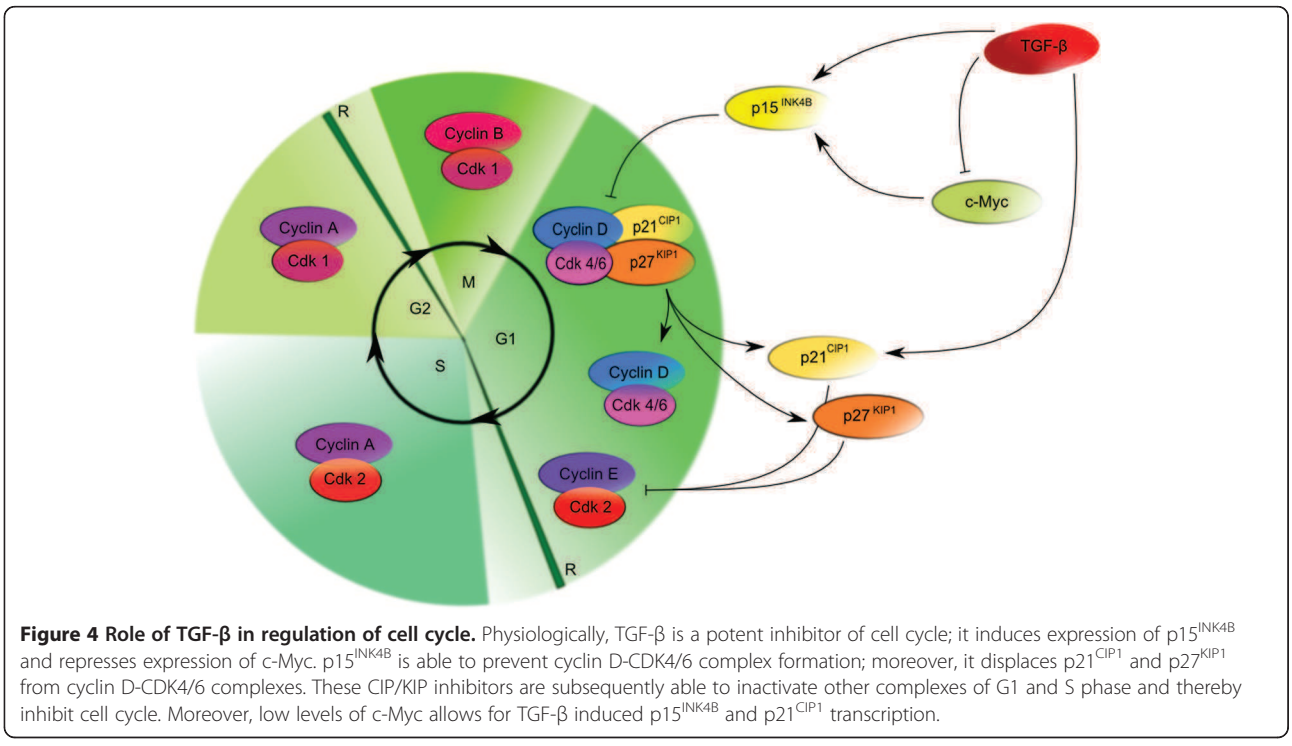

Figure 4 Role of TGF-β in regulation of cell cycle. Physiologically, TGF-β is a potent inhibitor of cell cycle; it induces expression of $p15^{INK4B}$ and represses expression of c-Myc. $p15^{INK4B}$ is able to prevent cyclin D-CDK4/6 complex formation; moreover, it displaces $p21^{CIP1}$ and $p27^{KIP1}$ from cyclin D-CDK4/6 complexes. These CIP/KIP inhibitors are subsequently able to inactivate other complexes of G1 and S phase and thereby inhibit cell cycle. Moreover, low levels of c-Myc allows for TGF-β induced $p15^{INK4B}$ and $p21^{CIP1}$ transcription.

carcinoma, colon, HCC, prostate, lung and breast cancers and correlates with poor prognosis [112].

Mutations in downstream TGF-β signaling components cause variable attenuations or complete loss of expression; these mutations, which have been detected in many common tumors, affect TGF-β signal transmission that potentially results in human cancer development and progression. In particular, TβRI, TβRII, SMAD2 and SMAD4 are frequently lost, mutated or attenuated (gene/LOH/expression). Inactivation of TβRII leads to increased tumor spreading and metastasis in a variety of carcinomas, including colon [113], breast [114], pancreatic [115], intestinal [116] or head and neck squamous cell carcinoma (HNSCC) [117]. Also, deregulated expression or aberrant function of Smurf1 and 2 was described. Several human carcinoma cell lines such as colon HT-29, breast MDA-MB-231, gastric MKN-1 and ovarian OVCAR-5 display high levels of one or more E3 ligases, including Smurf2 [118,119]. Moreover, in esophageal squamous carcinoma, high expression levels of Smurf2 associated with low levels of SMAD2 phosphorylation were detected [120]. Furthermore, TGF-β pathway is modulated by epigenetic mechanisms, such as transcriptional repression of *TβRII*, DNA methylation of *TβRI* and *TβRII* and histone modifications [121-123].

TGF-β in tumor microenvironment and metastases

Tumor metastases accounts for the majority of cancer associated deaths. Recent evidence strongly suggests that tumor microenvironment is essential in this process. It consists of tumor cells and a variety of immune cells, which infiltrate into tumors. This dynamic microenvironment is not only important for cross-talk with tumor cells or escape of tumor from host immune surveillance, but it also induces formation of new blood vessels and invades the vasculature. Areas of hypoxic tissue are thought to drive genomic instability and alter DNA damage repair [124]. Recent studies suggest that TGF-β is one of the critical regulators of inflammation; it is thought that tumor metastasis is a coordinated process between tumor cells and host cells through inflammation [125]. However, it seems that different mechanisms are implemented in different tumor type.

TGF-β as a proto-oncogene is important in stromal-epithelial cross-talk, as was shown for the first time in mouse experiments, where deletion of the TβRII in stromal fibroblasts resulted in transformation of adjacent epithelia of prostate and forestomach. Moreover, in this model, hepatocyte growth factor (HGF) was up-regulated and complementary activation of the HGF receptor MET was detected in tissues where TβRII had been ablated, which implicates this paracrine signaling network as a potential mechanism for regulation of carcinoma development [126]. Further experiment performed on these mice revealed that mice fibroblasts have up-regulated expression of growth factors and increased proliferation of mammary cancer cells [127]. Together, it indicates that TGF-β responses mediated by stromal fibroblasts can regulate carcinoma initiation and progression of adjacent epithelium *in vivo* and *in vitro.*

Interestingly, it was found that TGF-β in breast cancer favors metastasis to lungs. TGF-β stimulation of mammary carcinoma cells in tumor microenvironment, before they enter circulation, primes these cells for seeding of lungs through a transient induction of angiopoetin-like4 (Angptl4) via canonical signaling pathway [128]. TGF-β is involved in regulation of chemokines and chemokine receptors which take part in inflammatory cells recruitment. The loss of TβRII in breast cancer cells can enhance recruitment of $F4/80^{+}$ cells to tumor microenvironment and increase the expression of pro-inflammatory genes, including *CXCL1, CXCL5* and *PTGS2* (cyclooxygenase-2). Further, *in vitro* treatment of carcinoma cells with TGF-β suppressed the expression of *CXCL1, CXCL5* and *PTGS2* [129].

Different mechanism was observed in gastric carcinoma, where SMAD-dependent TGF-β pathway, in collaboration with PKC-δ expression and phosphorylation and integrin expression and activation, regulates cell invasion and cell spreading [130].

Beside the effects already mentioned, TGF-β is broadly implemented in induction of epithelial-to-mesenchymal transition [131]. The NBT-II cell line, derived from a chemically induced rat bladder carcinoma, forms epithelial colonies that can be converted into migratory mesenchymal cells within a few hours by adding Tgf-β and other factors, such as Fgf1, Fgf7, Fgf10, Egf, Igf1, Igf2 or Hgf [132].

TGF-β as a regulator of immune cells

The tumor microenvironment is filled with various inflammatory cells, including myeloid cell subpopulations, T cells and B cells. TGF-β is one of the most potent endogenous negative regulators of hematopoiesis. It modulates proliferation, differentiation and function of all types of lymphocytes, macrophages and dendritic cells, thus regulating the innate, non-antigen-specific as well as antigen-specific immunity [133].

TGF-β is involved in normal B cells maturation and differentiation, such as regulation of expression of cell-surface molecules, inhibition of IgM, IgD, CD23 and the transferrin receptor and induction of MHC class II expression on pre-B cells and mature B cells [134].

In T cells, TGF-β regulates maturation; for example, it is released by regulatory T cells and inhibits the Ag-specific proliferation of naive CD4+ cells from T cell receptor (TCR) [135]. TGF-β1 also inhibits aberrant T cell expansion by maintaining intracellular calcium

concentration levels low enough to prevent mitogenic response by Ca^{2+}-independent stimulatory pathways [136].

In myeloid cells, such as macrophages and monocytes, TGF-β1 is mostly suppressive, it inhibits cell proliferation and down-regulates production of reactive oxygen and nitrogen intermediates; however, it is able to enhance some other activities of myeloid cells. TGF-β1 can be recognized by monocytes and macrophages as a chemotactic factor; it induces direct monocytes migration *in vitro* [137].

TGF-β pro-metastatic and pro-inflammatory effects are regulated via nuclear factor kappa B (NF-κB), the master regulator of inflammation and a regulator of genes that controls cell proliferation and cell survival. TGF-β1 is a negative regulator of NF-κB activation, as was shown in the gut; it directly stimulates IκB-α promoter transcriptional activity *in vitro*. However, SMAD7 maintains high NF-κB activity by blocking TGF-β1 signaling [138].

Targeting the TGF-β signaling pathway

As the signaling pathway deregulations are responsible for cancer initiation and progression, interrupting the tumor promoter properties of TGF-β signaling would be an attractive therapeutic strategy, without altering physiologic tumor suppressor functions exhibited in early stages of tumorigenesis. Strategies such as using monoclonal TGF-β-neutralizing antibodies, large molecule ligand traps, reducing translational efficiency of TGF-β ligands using antisense technology and antagonizing TGF-β receptor I/II kinase function by small molecule inhibitors are the most prominent methods being explored today [139,140]. Furthermore, studies have shown that combined treatment with tumor cell vaccines and antisense TGF-β therapy reduced tumor size and increased survival benefit [141,142]. Preclinical studies also show that TGF-β inhibition can augment therapeutic efficacy of cytotoxic agents [143]. However, as there are still potential limitations and risks of TGF-β targeted therapy, caution must be given as to when, how and how much therapy would be beneficial or how much toxicity will be induced by chronically administered therapy. However, daily administration of a high dose of neutralizing TGF-β antibody in adult mice for 12 weeks and a lifetime exposure to soluble TβRII (sTβRII) in transgenic mice did not significantly affect their health. This suggests that anti-TGF-β treatments are likely to be safe [144].

TGF-β in solid tumors

Brain tumors

TGF-β has a suppressive role in physiological development of the central nervous system (CNS): all TGF-β isoforms and receptors necessary for TGF-β signal transduction are detected in developing as well as adult CNS [145].

The most aggressive type of primary brain tumors, glioblastoma multiforme (GBM), is characterized by poorly differentiated and highly proliferating cells that originate from glial cells [146,147]. Here, the release from cytostatic TGF-β effect is explained by a broad range of inactivating mutations in the TGF-β signaling pathway. Several studies describe mutations in TβRI and TβRII in adenomas and gliomas [148,149] as well as correlation between higher expression of TβRI and TβRII with more aggressive glioma cell lines and tumors [150,151]. Moreover, high levels of TGF-β indicate that TGF-β is able to induce its own expression and thereby create a malignant autocrine loop and control glioma-cell proliferation [152]. Alterations of SMAD protein levels and activation were reported in brain tumor cell lines and patient samples. In glioma cell lines, SMAD3 level and SMAD2 nuclear translocation was lower in 9 out of 10 cell lines [153]. Kjellman *et al.* reported that SMAD2, SMAD3 and SMAD4 mRNA levels were reduced in GBM samples in comparison to normal brain samples, astrocytomas and anaplastic astrocytomas [150]. Nevertheless, these data are controversial to a study in which higher phospho-SMAD2 (p-SMAD2) level correlated with higher grade of glioma [154]. Further analysis of cell lines and patient samples would elucidate such discrepancies.

Urogenital tumors

TGF-β is a crucial molecule in the genesis of urogenital tumors, such as urinary bladder carcinoma, renal cell carcinoma, ovarian and prostate cancers [155].

The TGF-β pathway is involved in urinary bladder cancer progression. The amount of secreted TGF-β1 correlates with more aggressive phenotype of cell lines. In addition, deregulated TGF-β signaling led to enhanced migration and invasiveness of bladder cancer cells [156]. Silencing of TβRI expression by siRNA led to significant inhibition of TGF-β-induced signal transduction and thereby reduced invasiveness of bladder cancer cells [157].

Clear cell renal cell carcinoma (CCRCC) is the most common malignancy of the kidney; it accounts for 2-3% of all malignant diseases in adults [158]. In CCRCC patient samples, sequential loss of TβRIII and TβRII expression was associated with renal cell carcinogenesis and progression [155]. Cross-talk between Notch signaling and TGF-β pathway contributes to aggressiveness of CCRCC. Recently, it was described that inhibition of Notch signaling leads to attenuation of basal TGF-β-induced signaling in CCRCC cells; it also influenced genes involved in cancer migration [159].

Ovarian cancer

In advanced ovarian tumors, low expression of TGF-β1 mRNA is connected to better prognosis. It was found that TGF-β1 mRNA expression was significantly lower in tumors of patients who had optimal surgery than in patients with suboptimal surgery. TGF-β1 mRNA expression was also significantly lower in tumors with high sensitivity to chemotherapeutics than in those with low sensitivity [160].

Alterations in the *TβRI* gene occur in ovarian cancer and account, at least in part, for the frequent loss of TGF-β responsiveness of these cancer cells. Presence of *TβRI 6*A* allele in about 27% of human ovarian cancers suggests that it acts as a low penetrating tumor marker in the development of ovarian cancer [161-163].

Mutations in the *TβRII* allele that cause loss or decrease in TβRII protein level are also present, BAT-RII mutations (mutations in polyadenine tract in exon 3) were found in 22% of ovarian tumors [161]. Although this mutation is connected to microsatellite stability, in ovarian cancers this association remains controversial [164].

Mutations in SMAD4 are not very common in ovarian cancer but were reported in primary cultures or cell lines [165]. Reduced expression or loss of SMAD4 protein leads to decreased ability to bind DNA; SMAD4 inactivation is involved in the acquisition of a more aggressive tumor [161].

It has been suggested that SMAD4 and SMAD3 are involved in metastatic potential of ovarian cancers [166,167]. In ovarian cancer cell lines, TGF-β supported metastatic activity at least partly through activation of MMPs [168]. Deregulation in TGF-β/SMAD4 signaling leads to epigenetic silencing of a putative tumor suppressor, RunX1T1, during ovarian carcinogenesis [169]. Recently, genome-wide screening done by ChIP-seq of TGF-β-induced SMAD4 binding in epithelial ovarian cancer revealed that SMAD4-dependent regulatory network was strikingly different in ovarian cancer compared to normal cells and was predictive of patients survival [170].

Prostate cancer

In prostate cancer, high level of TGF-β1 expression is linked to tumor progression, cell migration and angiogenesis [171]. In some prostate cell lines, even low level of TGF-β1 induced its own expression in an autocrine manner. However, only in benign cells, higher concentration of TGF-β1 leads to recruitment of protein phosphatase 2A (PP2A) by activated TβRI, which terminates the induction of TGF-β1. On the contrary, in malignant cells, incorrect recruitment of PP2A by TβRI is responsible for protruded production of TGF-β1 [172].

When compared to other types of cancer, such as breast and colon, down-regulation of TβRs is found more often than mutations in SMADs. Kim *et al.* compared protein levels of TβRI and TβRII in benign and malignant prostate tissues and observed that loss of receptors expression correlated with more advanced tumor [173]. Decreased level of receptor protein is accompanied with decreased mRNA expression; thereby, loss of receptor expression is a potential mechanism to escape the growth-inhibitory effect of TGF-β [174]. However, mutations are present in only some cases of prostate cancer, which suggests that other mechanisms are involved. For example, in a study by Turley *et al.*, loss of TβRIII expression correlated with disease progression [175]. In some cases of prostate cancer, insensitivity to TGF-β is caused by promoter methylation in TβRI [176].

So far, mutations in SMAD2 proteins were not found in prostate cancer. However, studies *in vitro* revealed that SMAD2 functions as a tumor suppressor of prostate epithelial cells. It is possible that tumor suppressor function of SMAD2 could be lost during differentiation of normal tissues or during prostatic carcinogenesis [177-179].

Breast cancer

In normal mammalian breast development, all TGF-βs isoforms are functionally equivalent; they are all involved in establishing proper gland structures and apoptosis induction. However, they have distinct roles in mammary growth regulation, morphogenesis and functional differentiation [180-182].

In breast cancer, results evaluating TGF-β as a prognostic factor are controversial. On the one hand, analysis demonstrated TGF-β1 expression to be significantly higher in patients with a favorable outcome as compared to patients with a poor prognosis [183]. On the other hand, several studies showed that TGF-β over-expression is related to worse outcome [184,185]. Elevation of TGF-β has been shown to participate in breast cancer metastasis [186].

Alterations of TGF-β signaling molecules are relatively rare, except for TβRII down-regulation. No specific mutations were found in the coding or in the regulatory region of the TβRII gene promoter in breast cancer [187,188]. However, the loss of TβRII expression has been linked to tumor progression and metastasis, principally in HER2-negative patients [114]. In addition, resistance of breast cell lines to TGF-β may be due to reduced expression of TβRII [189]. Mutations of TβRII are rare among breast cancer patients, while changes in receptor expression may take part in tumor progression [187]. Opposite to TβRII, intragenic mutations occur in TβRI and are associated with metastatic breast cancer [190].

Although the role of TβRIII remains unclear, it seems that this receptor is a suppressor of breast cancer. Loss of TβRIII through allelic imbalance is a frequent genetic

event during human breast cancer development that increases metastatic potential; moreover, decreased TβRIII expression correlates with decreased recurrence-free survival in breast cancer patients [191].

Mutations in downstream signaling pathway including SMAD proteins are not very common in breast cancer; however, inactivating mutations or loss of expression in SMAD4 have been described [164,192].

Tumors of the digestive tract

Gastric cancer

Resistance to TGF-β is a hallmark of gastric cancer. The relationship between TGF-β resistance and up-regulated level of miR-106b-25 cluster (miR-106b, miR-93, and miR-25) has been recently elucidated [193]. The cluster is an intronic part of the *Mcm7* gene and thus is regulated by E2F1. Conversely, miR-106b and miR-93 control E2F1 expression thus establishing negative feedback that prevents E2F1 self-activation. Over-expression of miR-106b, miR-93 and miR-25 decreases response of gastric cancer cells to TGF-β since they interfere with synthesis of TGF-β downstream effectors that promote cell cycle arrest and apoptosis, such as $p21^{CIP1}$ and BIM, respectively [193] (Figure 5).

Mutations in TβRII that lead to insensivity of cell lines to TGF-β mediated growth inhibition have been previously described [194]. It has been shown that conditional loss of TGF-β signaling due to dominant negative mutation in TβRII leads to increased susceptibility to gastrointestinal carcinogenesis in mice [195].

Epigenetic changes in TβRI are another important mechanism of escape from TGF-β physiological function. Hypermethylation of a CpG island in the 5' region of the TβRI was found in 80% of gastric cancer cell lines and 12.5% of primary tumors. Treatment with demethylating agent increased expression of TβRI and transient transfection of TβRI into TGF-β resistant cell line restored TGF-β responsiveness [123].

Effects of TGF-β on gastric cancer invasiveness and metastasis are mediated by activation of JNK and ERK pathways which support expression of fascin-1, an actin-binding protein. Moreover, signaling pathway based on SMAD proteins is not involved in this process because transitional repression of SMADs did not alter fascin-1 expression [196].

Nevertheless, impaired signaling based on SMAD proteins also occurs in gastric cancer. Shinto *et al.* found a correlation between expression level of p-SMAD2 and patients prognosis. P-SMAD2 protein expression level was significantly higher in patients with diffuse form of carcinoma and metastatic tumors and is associated with worse outcome [197]. TGF-β signaling is also abrogated by decreased expression of SMAD3. Low or undetectable level of SMAD3 was observed in 37.5% of human gastric cancer tissues. In cell lines, which showed deficient expression of SMAD3, introduction of *SMAD3* gene led to growth inhibition caused by TGF-β [198].

Sonic hedgehog (Shh), a member of the hedgehog signaling pathway, promotes invasiveness of gastric cancer through TGF-β-mediated activation of the ALK5–SMAD3 pathway. Higher concentrations of N-Shh (human recombinant form of Shh) enhanced cell motility and invasiveness in gastric cancer cells; moreover, treatment of cells with N-Shh led to enhanced TGF-β1 secretion, TGF-β-mediated transcriptional response, expression of ALK5 protein and phosphorylation of SMAD3. Effect of Shh on cell motility was not observed after treatment of cells with anti-TGF-β blocking antibody or TGF-β1 siRNA [199].

Hepatocellular carcinoma

Reduced TβRII expression was observed in approximately 25% of hepatocellular carcinoma (HCC) patients; this event is associated with aggressive phenotype of HCC and intrahepatic metastasis. TβRII down-regulation also correlated with an early recurrence time and higher grade of tumor suggesting that TβRII down-regulation is a late event in HCC development. In addition, TGF-β is a tumor suppressor in the majority of HCCs expressing TβRII [200].

Mutations in intracellular signaling components have been observed: SMAD2 mutations occur in 5% of HCC, while loss of SMAD4 expression was found in 10% of HCC [201,202].

Several studies of HCC indicated that over-expression of SMAD3 promotes TGF-β-induced apoptosis [203,204]. Pro-apoptotic activity of SMAD3 requires both input from TGF-β signaling and activation of p38 MAPK, which occurs selectively in liver tumor cells. SMAD3 represses transcription of an important apoptotic inhibitor, BCL-2, by directly binding to its promoter [203].

Therapeutic options for patients with HCC are still limited; however, it was recently described that blocking the TGF-β signaling pathway with LY2109761, a kinase inhibitor of TβRI, is associated with inhibition of molecular pathways involved in neo-angiogenesis and tumor growth. LY2109761 interrupts the cross-talk between cancer cells and cancer-associated fibroblasts, leading to significant reduction of HCC growth and dissemination. Currently, LY2109761 is being tested in clinical trial phase II [205-207].

Colorectal cancer

In colorectal cancer (CRC), TGF-β1 inhibits proliferation of less aggressive tumor cells but stimulates growth of tumor cells at later stages by autocrine manner. High level of TGF-β1 correlates with tumor progression [208]. In colorectal cell lines, TGF-β induces proliferation by

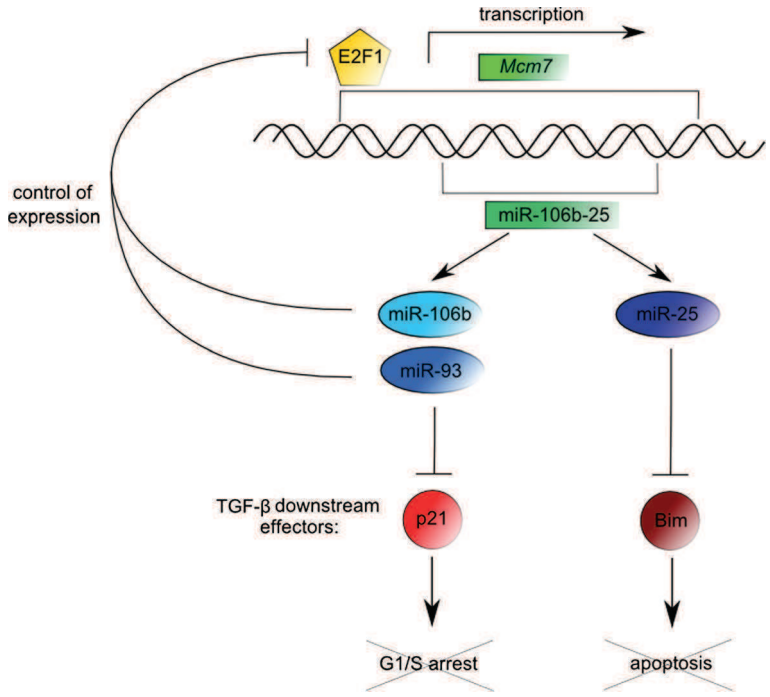

Figure 5 The E2F1/miR-106b-25/p21 pathway. In gastric cancer, miR-106b-25 cluster is activated by E2F1 in parallel with its host gene, Mcm7. In turn, miR-106b and miR-93 regulate E2F1 expression, establishing a miRNA negative feedback loop. Over-expressed miR-106b, miR-93, and miR-25 inhibit the synthesis of $p21^{CIP1}$ and Bim (TGF-β downstream effectors) and therefore prevent cell cycle inhibition and apoptosis.

RAS-independent manner [209]. In a recent study, TGF-β, TβRI, TβRII, SMAD4, pSMAD2/3 and E-cadherin were found to be closely related to TNM stage of CRC. Therefore, TGF-β, TβRII, SMAD4, pSMAD2/3 and E-cadherin come into view as valuable independent biomarkers of prognosis in CRC patients [140].

Inactivating mutations in SMAD2 and SMAD4 are frequent especially in pancreatic and colorectal carcinomas, although they do not stand for the most frequent tumor changes. Most of SMAD2 mutations have been found in the MH2 protein domain, thereby preventing complex formation with SMAD3 and SMAD4. Alterations of SMAD2 are present in about 6% of colorectal carcinoma cases [210]. SMAD3 mutation is a very rare event in human solid tumors; however, a missense mutation in *SMAD3* gene (leading to reduced activity of SMAD3 protein) was found in human colorectal cell lines [211]. Inactivation of SMAD4 is a genetically late event in gastrointestinal carcinogenesis. It was identified with less frequency in advanced colon cancers and in 16% of colon carcinomas [212,213]. Nevertheless, recent studies revealed that some of the TGF-β induced pathways are SMAD4 independent [214]. Proteomic screen of *SMAD4* wt and *SMAD4* deficient cell lines detected different protein levels in cell lines pointing to SMAD4 dependent and independent TGF-β responses in colon carcinoma cells [215]. Another study indicated that novel genetic variant -4 T(10) in the *SMAD4* gene promoter affects its activity. Obtained preliminary results indicate that *SMAD4* gene promoter haplotype -462 T(14)/-4 T(10) represents a potentially relevant genetic marker for pancreatic and colorectal cancer [216]. This downstream inactivation of TGF-β signaling components promotes colon adenoma to carcinoma progression.

Mutations of TβRII are frequent alterations of the TGF-β signaling pathway (reviewed in [217]). They are present in approximately 30% of CRC cases and were reported in cancer cell lines, sporadic colon cancers and patients with hereditary non-polyposis colorectal cancer with microsatellite instability and in a smaller percentage in microsatellite stable cancers [123,218,219]. TβRII mutations occur in >90% of microsatellite unstable (MSI) colon cancers and most principally affect a polyadenine tract in exon 3 of TβRII, the BAT-RII; however, non-BAT point mutations in TβRII were found with less frequency also in microsatellite stable cancers [164,219]. Interestingly, it has been recently published that restoration of TβRII in cancer cell lines with microsatellite instability (MSI), bearing mutated TβRII, promoted cell survival and motility. Therefore, it is plausible that such mutations contribute to favorable outcome in MSI patients [220].

In contrast to TβRII, mutations in TβRI are less common. They are rare in colon as well as pancreatic cancer.

Decreased TβRI allele expression is associated with higher risk of colon cancer development [221]. Recently, it has been described that TβRIII mRNA expression is not significantly altered in human colorectal cell lines; however, protein levels of TβRIII are frequently increased, suggesting a distinct role for TβRIII in colon cancer. Thus, enhanced expression of TβRIII is possibly involved in cancer progression [222].

Other mechanisms, such as crosstalk between TGF-β and Wnt/β-catenin pathways, are involved in colon cancer progression [214]. It has been shown that SMAD4 restoration is associated with suppression of Wnt/β-catenin signaling activity, decrease of β-catenin/Tcf target genes expression and with induction of functional E-cadherin expression [223].

Recently, the role of microRNA in colon cancer has been established. Elevated levels of miR-21 and miR-31 promote motility and invasiveness of colon cancer cell line and enhance the effect of TGF-β. It seems that miR-21 and miR-31 act as downstream effectors of TGF-β [224].

Pancreatic cancer

Pancreatic cancer has the poorest prognosis among GI cancers due to aggressiveness, frequent metastases and resistance to treatment. SMAD4, also called DPC4 (deleted in pancreatic carcinomas), suggests close relationship between loss of this gene and pancreatic cancer. Mutation or deletion of SMAD4 is a well-characterized disruption in the TGF-β pathway – it occurs late in neoplastic progression, at the stage of histologically recognizable carcinoma. In pancreatic cancers, SMAD4 is homozygously deleted in approximately 30% of cases, inactivated in 20%, while allelic loss of the whole 18q region was found in almost 90% of cases [225]. These mutations are present mostly in the MH2 domain; however, missense, nonsense or frameshift mutations are present within the MH1 domain as well [226,227].

Dual role of SMAD4 was established in a mouse model. *Smad4* or *TβRII* deletion in pancreatic epithelium did not affect pancreatic development or physiology. However, when activated K-Ras was present in cells, loss of *Smad4* or *TβRII* or *Smad4* haploinsufficiency led to progression to high-grade tumors. Thus, it is possible that Smad4 mediates the tumor inhibitory action of TGF-β signaling, mainly in the progressive stage of tumorigenesis [115].

In concordance with colorectal cancer, mutations in TβRII were found in cancers with microsatellite instability; however, mutations in TβRII and also in TβRI are less common [217]. Frequency of mutations in *TβRII* is about 4% and even less for *TβRI* [228]. Interestingly, polymorphism within the *TβRI* gene, which is less effective in mediating anti-proliferative signals than wild type, was described [229].

High level of TGF-β was found in serum of patients with pancreatic adenocarcinoma suggesting that TGF-β could possibly become a marker for monitoring disease activity [230].

As previously mentioned in HCC, targeting TβRI/II kinase activity in pancreatic cancer with the novel inhibitor LY2109761 also suppressed pancreatic cancer metastatic processes. LY2109761 suppressed both basal and TGF-β1–induced cell migration and invasion and induced anoikis. *In vivo*, LY2109761, in combination with gemcitabine, significantly reduced the tumor burden, prolonged survival and reduced spontaneous abdominal metastases [231].

Lung cancer

In non-small cell lung carcinoma (NSCLC), elevated expression of TGF-β correlates with disease progression [232]. Furthermore, significantly higher serum concentrations of TGF-β1 cytokine were found in lung cancer patients. Presumably, elevated expression and higher levels of serum TGF-β represent an important prognostic factor that could serve as a complementary diagnostic test in lung cancer detection [233].

Defective expression of TβRII was observed in primary NSCLC, where TβRII acts as a tumor suppressor. Down-regulation of TβRII on transcriptional level could be explained by aberrant methylation of the *TβRII* promoter [234]. Moreover, reduced expression of TβRIII has been found in NSCLC cells compared to normal human bronchial epithelial cells [235].

Downstream components of TGF-β signaling pathways are important in NSCLC development. Jeon *et al.* observed a correlation between better tumor-related survival and absence of SMAD6. Moreover, SMAD6 contributes to lung cancer progression by limiting TGF-β-mediated growth inhibition of cell lines, which was proven by knockdown of SMAD6 that resulted in increased apoptosis in lung cancer cell line [236].

TGF-β signaling is also required for lung adenocarcinoma (LAC) progression. In a study on LAC cell line A549, knockdown of TβRII resulted in suppression of cell proliferation, invasion and metastasis and induced cell apoptosis [237].

TGF-β in hematological malignancies

Leukemia

Myeloid leukemia

TGF-β is a potent inhibitor of human myeloid leukemia cells [238]. In acute myeloid leukemia (AML), t(8;21) translocation results in the formation of a chimeric transcription factor AML1/ETO. Jakubowiak *et al.* used transient transfection assays and a reporter gene

construct that contained SMAD and AML1 consensus binding sequences and demonstrated that AML1/ETO represses basal promoter activity function and blocks response to TGF-β1. AML1/ETO possibly binds to SMAD3, instead of activating TGF-β1 signaling pathway. It represses TGF-β1-induced transcriptional activity and blocks TGF-β1 signaling, thus contributing to leukemia genesis [239].

In addition, in AML, dominant negative mutations in *SMAD4* were found. They are characterized by a missense mutation in the MH1 domain and a frameshift mutation in the MH2 domain of SMAD4. Mutated SMAD4 lacks transcriptional activity [240].

The t(3;21) translocation fusion product AML1/EVI-1 likely interacts with SMAD3 through the first zinc finger domain, represses SMAD3 activity by preventing SMAD3 from interacting with DNA, thereby repressing TGF-β-mediated growth suppression in hematopoietic cells. This way, AML1/EVI-1 contributes to leukemogenesis [241].

In acute promyeloytic leukemia (APL), t(15;17) translocation in which the retinoic acid receptor α (RARα) gene on 17q12 fuses with a nuclear regulatory factor PML on 15q22 results in the fusion protein PML-RARα [242]. PML is normally found in 2 isoforms, a nuclear isoform and a cytoplasmic isoform. Cytoplasmic isoform is required for association of SMAD2/3 with SARA and for the accumulation of SARA and TGF-β receptors, resulting in SMAD phosphorylation (Figure 6). The PML-RARα oncoprotein antagonizes with cytoplasmic PML function by withdrawing cytoplasmic PML from the SMAD/SARA/TβRI/TβRII complex resulting in defects in TGF-β signaling [243].

In chronic myeloid leukemia (CML), t(9;22) (the so-called Philadelphia chromosome) results in the formation of BCR-ABL fusion gene [244]. The fusion protein is an active tyrosine kinase which enhances resistance of malignant cells to TGF-β-induced growth inhibition and apoptosis. BCR-ABL protein targets AKT and transcription factor FOXO3 and thus impairs the cytostatic effect of TGF-β1 [245]. In addition, by improving proteasomal degradation, BCR-ABL blocks TGF-β1-induced expression of $p27^{KIP1}$. Thus, BCR-ABL kinase promotes activation of cyclin-dependent kinase and cell cycle progression [246].

In CML, expression of EVI-1, a proto-oncogene that is expressed at very low levels in normal hematopoietic cells, is increased. [247]. EVI-1 binds to the MH2 domain of SMAD3 repressing its DNA-binding ability and transcriptional activity and this way attenuates TGF-β signaling [248].

Moller *et al.* showed that BCR-ABL up-regulates TGF-β signaling when expressed in Cos-l cells. In Cos-1 cells, the expression of BCR-ABL up-regulates TGF-β-mediated transcriptional activity by interaction between TβRI and kinase domain of BCR-ABL, which leads to increased activity of SMAD3 promoter and increased SMAD2 and SMAD3 protein expression level [249].

Lymphoid leukemia

In children T-cell acute lymphoblastic leukemia (ALL), SMAD3 protein is absent or significantly decreased, however SMAD3 mRNA is present in T-cell ALL and normal T-cells at similar level. The level of SMAD3 is decisive for the T-cell response to TGF-β. A reduction in SMAD3 interplays with other oncogenic events, such as alterations in the retinoblastoma pathway, to precede T-cell leukemogenesis. It was proven that the loss of Smad3 can work in tandem with a loss of $p27^{KIP1}$, which is also frequently altered in human T-cell ALL, to promote T-cell leukemogenesis in mice [250].

The t(12;21) translocation found in ALL generates the TEL-AML1 chimeric protein. Loss of sensitivity to TGF-

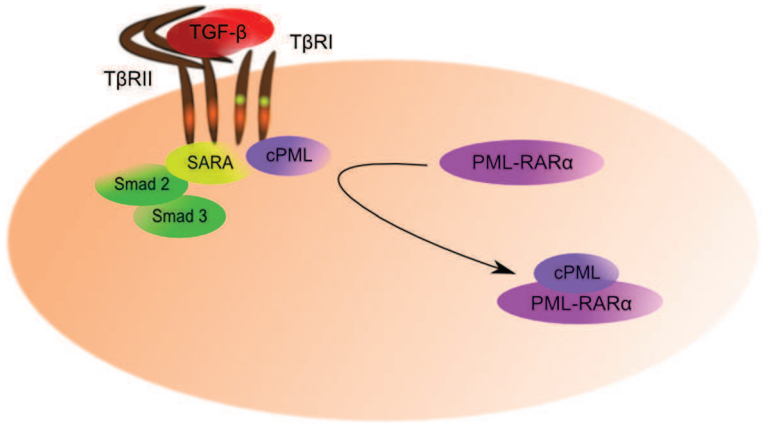

Figure 6 TGF-β signaling in APL. Cytoplasmic isoform of PML (cPML) protein interacts with SMAD2/3 and SARA and is required for accumulation of SARA-SMAD2/3 and TGF-β receptors in early endosome. However, the PML-RARα oncoprotein physically interacts with cPML and thus leads to impaired TGF-β signaling.

β could be an important component of the function of TEL-AML1; it was shown that TEL-AML1 blocks the ability of TGF-β to suppress proliferation via activation of $p27^{KIP1}$. The exact mechanism needs to be elucidated; however, a possible alternative is that TEL-AML1, in addition to binding SMAD3, binds co-repressors NcoR and SIN3A and this complex is able to transcriptionally activate the key cell cycle negative regulators, including $p27^{KIP1}$[251].

Scott *et al.* showed that mRNA of downstream components of TGF-β pathway, such as $p21^{CIP1}$ and $p15^{INK4B}$, are absent in ALL cell lines with high frequency, while $p27^{KIP1}$ mRNA levels are not reduced. These findings suggest epigenetic silencing of TGF-β signaling in molecular pathogenesis of ALL and possibly $p15^{INK4B}$ and $p21^{CIP1}$ are inactivated by this mechanism. In ALL, $p15^{INK4B}$ mRNA absence is often connected with promoter methylation, whereas reduced $p21^{CIP1}$ expression happens independently of promoter methylation, indicating that within the same malignancy, epigenetic silencing of TGF-β signaling is methylation-dependent or independent [252].

In adult acute T-cell leukemia, TGF-β signaling is inactivated through the activity of viral oncoprotein Tax. This oncoprotein compromises trans-activation of TGF-β responsive promoters by inhibiting the ability of SMAD proteins to mediate TGF-β-induced transcriptional activation by interfering with transcriptional factor CBP/p300 [253]. Another model of its function is that Tax interacts with the MH2 domains of SMADs 2, 3 and 4 in order to inhibit formation of the SMAD3/4 complex, disturb the interplay of the SMAD proteins with transcriptional factor CBP/p300, prevent binding of the SMAD complex to its target DNA sequence and thus inhibit TGF-β signaling [254]. The Tax repressor effect is mediated by activating JNK leading to increased phosphorylation of c-Jun, which is followed by formation of SMAD3/c-Jun complex that inhibits the ability of SMAD3 to bind DNA [255].

In hairy-cell leukemia (HCL), higher levels of TGF-β1 were observed in bone marrow (BM), serum and plasma from peripheral blood. The main source of this cytokine in active and latent form is hairy cell (HC). HCs produce TGF-β1, which is stored in BM near bone marrow fibroblasts; it activates them to synthesize collagen and reticulin fibers. TGF-β1 is important in fibrosis and is directly involved in the pathogenesis of BM reticulin fibrosis in HCL [256].

Lymphoma

Peripheral and cutaneous T-cell lymphoma

In cutaneous T-cell lymphoma and Sézary syndrome, reduced levels of TβRI and TβRII correlate with decrease in TβRI and TβRII mRNA levels. This leads to the loss of TGF-β growth inhibitory responses [257].

Knaus *et al.* detected a single point mutation (Asp-404-Gly [D404G]) in the kinase domain of TβRII in advanced lymphoma. This dominant negative mutation prevents cell surface expression of normal TβRII. The ability of the mutant receptor to prevent function of normal TGF-β receptors is a new mechanism for loss of responsiveness to the TGF-β in tumorogenesis. Since TβRI is not able to bind TGF-β in the absence of TβRII, no TβRI is detected on the surface of these cells. This mutant receptor binds to normal receptor in an intracellular compartment, likely the endoplasmic reticulum, and blocks development of the normal receptor on the cell surface [258]. In addition, a 178-bp deletion in exon 1 in the gene for TβRI was reported to be responsible for loss of TβRI expression on the cell surface in anaplastic large cell lymphoma cell line JK. This deletion was confirmed to be present also in patients' samples. Also, loss of TβRI is followed by loss of its tumor suppressive properties in human T-cell lymphoma [259].

Non-Hodgkin´s lymphomas (NHL)

ATL, adult T-cell leukemia/lymphoma is a rare form of Non-Hodgkin's lymphoma (NHL). Zinc-finger E-box binding homeobox 1 (ZEB1) is a candidate tumor suppressor gene since mRNA of ZEB1 was found to be down-regulated in ATL. Physiologically, ZEB1 binds phosphorylated SMAD2/3 to enhance TGF-β signaling, and it can counteract the SMAD7-mediated inhibition of TGF-β1 function. Down-regulation of ZEB1 mRNA together with over-expression of inhibitory SMAD7 mRNA in ATL leads to loss of responsiveness to TGF-β-mediated growth arrest. Therefore, ZEB1 has an important role in regulation of TGF-β1 signaling pathway by binding to R-SMADs and also I-SMADs [260].

SMAD1 protein level is elevated and it is phosphorylated in response to TGF-β1 signaling in NHL. This suggests a role of SMAD1 in mediating the effects of TGF-β in NHL [261].

In B-cell lymphoma, Bakkebo *et al.* found that phosphorylation of SMAD1/5 is surprisingly an important event for the TGF-β-mediated anti-proliferative effects. TβRI was highly expressed in these cells and likely is important for signaling through SMAD1/5 pathway. Also, the regulation of TGF-β-mediated proliferation is at least partly dependent on activated p38 MAPK [262]. In B-cell lymphoma, the cell line resistant to TGF-β1 did not possess functional TβRII. This led to the absence of nuclear translocation of phosphorylated SMAD3 and SMAD2, the lack of nuclear expression of $p21^{CIP1}$ and the down-regulation of c-Myc. Chen *et al.* found that methylation of promoter (CpG methylations at −25 and −140) plays an important role in TβRII gene silencing [263].

In diffuse large B-cell lymphoma (DLBCL), miR-155, which is over-expressed in aggressive type of B-cell lymphoma, targets SMAD5 by binding to the 3′ UTR of the SMAD5 gene. Treatment of DLBCL cell line with TGF-β1 resulted in phosphorylation of SMAD2/3 but also of SMAD1/5 indicating an active non-canonical signaling. Over-expression of miR-155 in this cell line significantly limited the cytostatic effect of cytokine due to impaired TGF-β1-mediated induction of $p21^{CIP1}$. In miR-155-overexpressing and SMAD5 knockdown DLBCLs, the disruption of $p21^{CIP1}$ induction was independent of the inhibitory effects of TGF-β1 thus creating a link between miR-155, TGF-β pathway and lymphomagenesis [264].

In small lymphocytic lymphoma/chronic lymphocytic leukemia (SLL/CLL), the CLL cells are resistant to the growth-inhibitory effects of TGF-β in spite of TβRII expression which is similar as in normal B cells. Therefore, the loss of responsiveness to TGF-β is most likely due to altered binding of TGF-β to the receptor complex or downstream signaling pathway [265].

Lagneaux *et al.* attributed the loss of responsiveness of CLL cells to TGF-β especially to decreased cell-surface expression of TβRI. CLL cells resistant to TGF-β1 showed no surface TβRI able to bind TGF-β1, but the expression of TβRII was normal. On the other hand, both TGF-β1-sensitive and TGF-β1-resistant CLL cells contained normal levels of TβRI and TβRII mRNAs. The absence of functional TβRI on the surface of CLL cells, in spite of normal mRNA level, could be explained by point mutations in the *TβRI* gene [266,267].

In CLL, Schiemann *et al.* found mutations in the signal sequence of TβRI (Leu12Gln substitution together with an in-frame single Ala deletion) which leads to reduced gene transcription stimulated by TGF-β [268]. In addition, CLL cells exhibited an increased expression of the TGF-β co-receptor, TβRIII, which is normally not expressed entirely in hematopoietic cells [269]. On the other hand, Lotz *et al.* found over-expression of TGF-β in CLL cells; all primary cells in this study were sensitive to the growth-inhibitory effects of this cytokine [270].

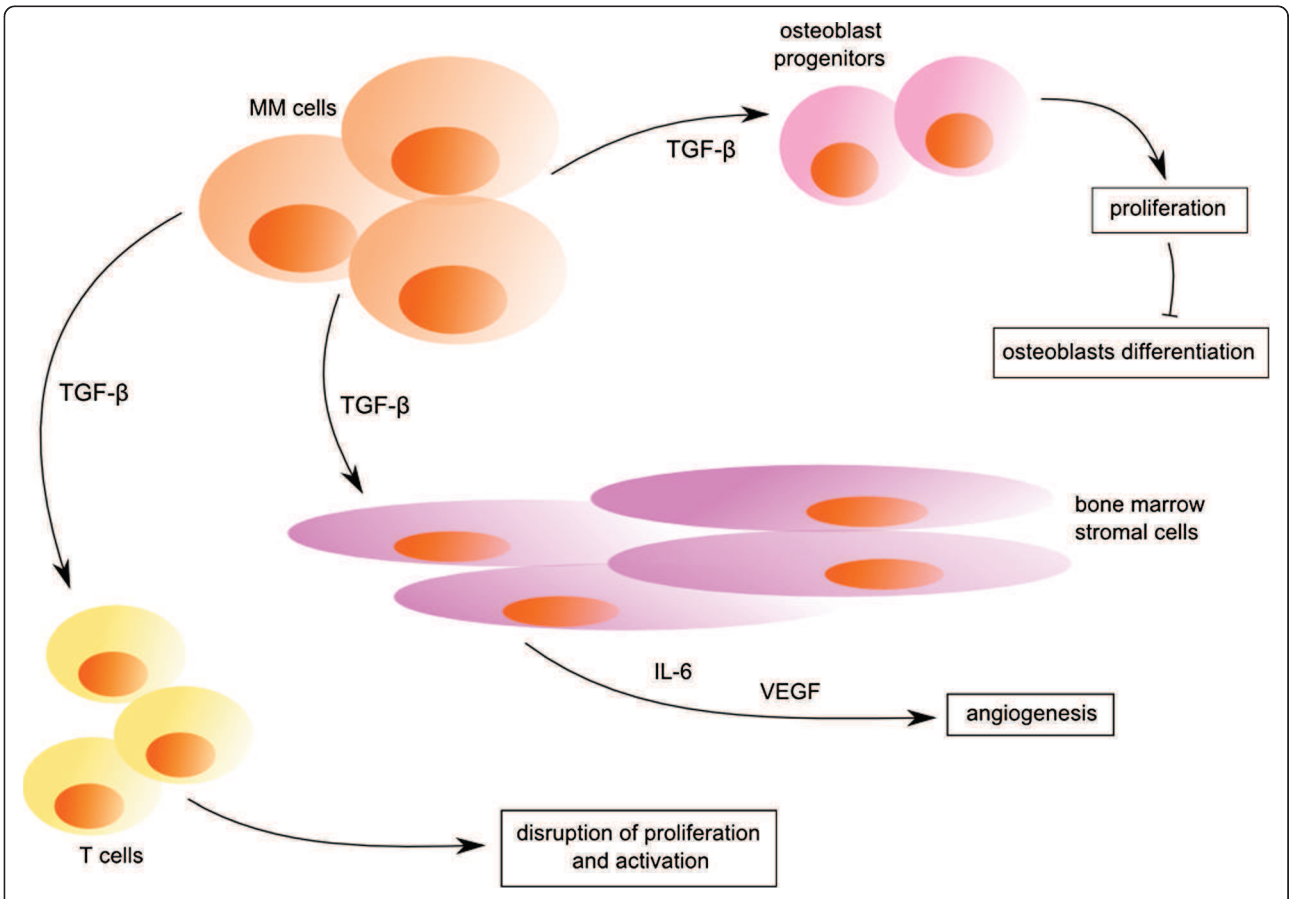

Figure 7 TGF-β signaling in the bone marrow microenvironment of multiple myeloma. Myeloma cells are able to produce TGF-β cytokine which influence cells of bone marrow microenvironment such as osteoblast progenitors, bone marrow stromal cells. Moreover, it disrupts T cell proliferation and activation.

In Burkitt´s lymphoma, TGF-β-mediated growth arrest is associated with transcriptional repression of the *E2F-1* gene. On the other hand, over-expression of the *E2F-1* gene overcomes the TGF-β-mediated G1 arrest. So, the transcriptional repression of the *E2F-1* gene is required for growth arrest suggesting that TGF-β can effectively exert tumor suppression also in cells without c-Myc, $p15^{INK4B}$ and $p21^{CIP1}$ regulation [271]. Inman and Allday reported that in Burkitt´s lymphoma, cells express normal levels of TβRI RNA and protein, but decreased levels of TβRII RNA, leading to lack of responsiveness to TGF-β1 [272].

Multiple myeloma

In multiple myeloma (MM), higher levels of TGF-β are secreted by myeloma cells as well as bone marrow stromal cells (BMSC). TGF-β secretion escalates with the stage of B cell differentiation (Figure 7). Increased production of TGF-β is followed by increased interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) secretion by BMSC, related to tumor cell proliferation. TGF-β is the major inducer of IL-6 and VEGF, two important cytokines of MM. On the other hand, TGF-β inhibits proliferation and Ig secretion of normal B cells [273].

After treatment with TβRI kinase inhibitor (SD-208), decreased production of IL-6 and VEGF and also attenuated tumor cell growth was observed. Mechanism of action of SD-208 is blocking nuclear accumulation of SMAD2/3 and related production of IL-6. This leads to inhibition of MM cell growth, survival, drug resistance and migration [274].

In MM, no mutations in *TβRI* or *TβRII* genes were described; MM cells contain TβRI and TβRII proteins in the cytoplasm. Resistance to the growth-inhibitory functions of TGF-β signaling develops, possibly due to defective trafficking of TβRI and TβRII to the cell surface in these cells [275,276]. Possibly, the loss of TβRII expression on the cell surface is the result of gene silencing by hypermehylation correlating to poor survival [277]. TβRIII expression is diminished on mRNA and protein level in MM, enhancing cell growth, proliferation, mobility, heterotrophic cell–cell adhesion and contributing to disease progression [278].

Serum level of TGF-β is an important prognostic factor in MM. Higher levels of this cytokine mean lower levels of normal Ig resulting in immune impairment [279]. TGF-β secreted from MM cells disrupts proliferation, activation and IL-2 responsiveness in T cells. TGF-β is important in this immune-suppression, and its intensity of suppression is tumor burden dependent [280].

In MM patients, TGF-β represses bone formation in bone lesions. Initially, TGF-β enhances proliferation of osteoblast progenitors and promotes mineralization of bone matrix. Then, TGF-β inhibits subsequent phases of differentiation of osteoblasts and represses mineralization of matrix. This effect can be abrogated by inhibitors of TβRI kinase domain (reviewed in [281]).

Conclusion

TGF-β signaling is complex and finely regulated fundamental pathway, which has an important role during human development and adult life. It is broadly intertwined with other signaling pathways. Moreover, it is involved in cancerogenesis of solid tumors as well as hematological malignancies. Paradoxically, TGF-β is both a tumor suppressor and tumor promoter. The tumor suppressor activities are widely described as anti-proliferative and apoptotic effects. During cancer progression, tumor frequently avoids tumor suppressive activities of TGF-β either by acquiring mutations of signaling components or by inhibiting its anti-proliferative response. This 'switch' helps the tumor to use TGF-β as an oncogenic factor inducing tumor motility, invasion, metastasis and epithelial-to-mesenchymal transition. Advances in the study of molecular mechanisms that elucidate oncogenic activities of TGF-β lead to a strong desire to target TGF-β signaling in cancer therapy. However, the exact mechanisms involved in the malignant transformation of TGF-β needs to be clarified. Only then, it will be possible to develop successful therapeutic strategies as well as provide new therapeutic targets to restore the normal TGF-β function.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

L.K. wrote the original manuscript, L.S. and R.H. cooperated on revising the manuscript. S.S. revised the manuscript critically and approved the final version of the manuscript. All authors read and approved the final manuscript.

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Invited review

Mechanism of immunomodulatory drugs in multiple myeloma

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ABSTRACT

Multiple myeloma is the second most common hematological cancer in the world. It is characterized by accumulation of malignant plasma cells in the bone marrow, osteolytic lesions and monoclonal immunoglobulins in blood/urine. With the introduction of immunomodulatory drugs into the treatment protocol, the outcome of multiple myeloma patients has dramatically improved with more than 30% of patients surviving for 10 years thus shifting multiple myeloma to a treatable condition.

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Contents

1. History of multiple myeloma treatment

Multiple myeloma (MM) is a plasma cell malignancy. The World Health Organization ranks MM among immunosecretory peripheral neoplasms of B lymphocytes [1]. MM has complex pathophysiology characterized by accumulation of clonal malignant plasma cells in the bone marrow accompanied by production of monoclonal immunoglobulins or light or heavy chains, resulting in clinical manifestation of the disease. Clinically, MM manifests by osteolysis, impaired immune system, hypercalcemia, peripheral neuropathy and renal insufficiency [2,3].

For MM, first 'treatment attempts' included rhubarb pill and infusion of orange peel which were given to the first documented patient by Dr. Solly in 1844 [4]. Six years later, William Macintyre

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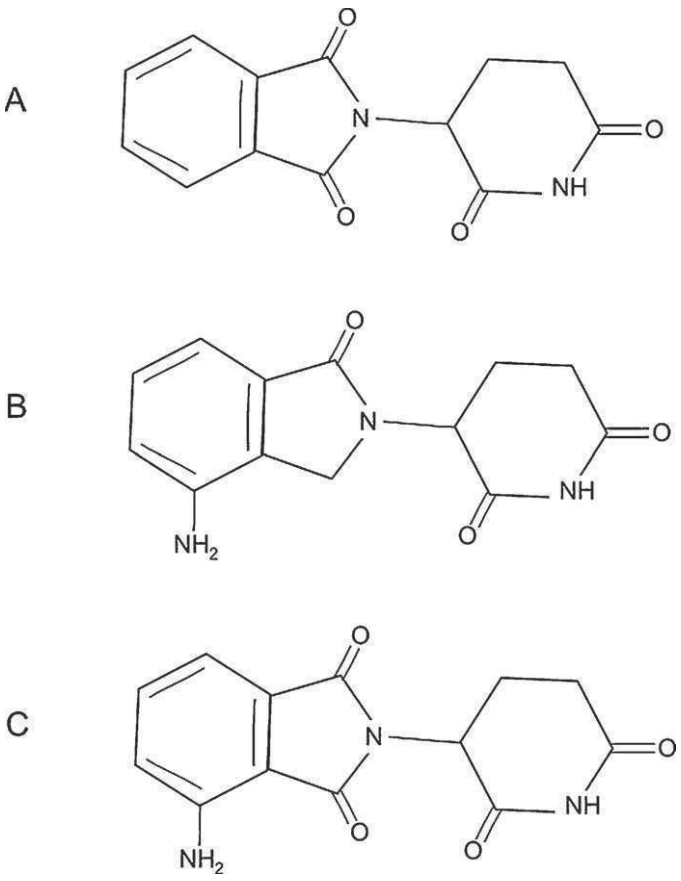

Fig. 1. Structure of IMiDs. (A) Structure of thalidomide, (B) structure of lenalidomide and (C) structure of pomalidomide.

described phlebotomy as 'maintenance therapy' for MM [5]. In the middle of the 20th century, Loge and Rundles used urethane to decrease the number of myeloma cells and pain in the bones [6].

The first truly successful treatment strategy was melphalan in combination with prednisone (MP) which has been used since the 60s of the 20th century [7]. Next, polychemotherapy regimens were introduced and achieved good treatment responses – for example, combination of vincristine, adriamycin and dexamethasone (VAD) treatment used as induction chemotherapy before autologous transplantation [8]. Unfortunately, these regimens were not curative.

In the first decade of the 21st century, a major effort was put into creating novel therapies, such as proteasome inhibitors and immunomodulatory drugs (IMiDs) that successfully and dramatically altered the therapeutic landscape for MM treatment. Moreover, several new highly efficient drugs (pomalidomide, carfilzomib, bendamustin), which will increase treatment options very soon, will play a key role in overcoming resistance to previous treatment or increase survival of patients [3].

In this review, we will focus on the role and molecular mechanism of IMiDs in the treatment of multiple myeloma.

2. Immunomodulatory drugs

Immunomodulatory drugs (IMiDs) are a group of new therapeutic agents, thalidomide-derivatives lenalidomide and pomalidomide (Fig. 1). The development of thalidomide, lenalidomide and pomalidomide represents a paradigm shift in the treatment of MM. These drugs possess pleiotropic properties against MM, including the ability to modulate host immune responses, impact cytokine secretion, angiogenesis, inflammation and they also have a direct effect on MM cells via induction of apoptosis (reviewed in [9]).

Thalidomide and lenalidomide have been approved by the FDA for treatment of MM. While pomalidomide has not been approved yet, it is widely expected that the approval will be granted in 2012.

2.1. Thalidomide

Thalidomide, 2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione ($C_{13}H_{10}N_2O_4$), is a synthetic derivative of glutamic acid. It contains one chiral centre and is a racemic mixture of two optically active enantiomers, S and R, with the ability of rapid chiral interconversion at physiological pH. The S enantiomer is responsible for the teratogenic and anti-tumor properties of thalidomide, while R enantiomer has sedative effects (reviewed in [10]).

Despite its infamous history as a human teratogen, thalidomide is an effective anti-inflammatory and anti-tumor agent [10]. Singhal and colleagues were the first to describe its effect in the treatment of MM, where it induces apoptosis of myeloma cells, down-regulates expression of adhesion molecules thereby disrupting mutual interactions between myeloma cells and bone marrow stromal cells; thalidomide also possesses anti-angiogenic and anti-inflammatory properties and stimulates host immune cells [11–14].

Thalidomide was first synthesized by Chemie Grünenthal in 1954 and was broadly used as a sedative and hypnotic agent for morning sickness during pregnancy. In 1961, its teratogenic effect on children whose mothers took thalidomide was first described. Thalidomide caused limb hypoplasia (phocomelia), absence of ears, deafness, malformations of gastrointestinal system and heart, facial and palatal defects. Thalidomide was taken off the market immediately, but affected development of nearly 10,000 children all around the world (reviewed in [10]).

A lucky chance led to the discovery of its anti-inflammatory properties in the treatment of erythema nodosum leprosum, a cutaneous complication of leprosy [15]. Thus, thalidomide was introduced back into the market, but its use has been limited by obligatory program STEPS (System for Thalidomide Education and Prescribing Safety) in order to minimize its teratogenic potential [16].

The use of thalidomide for the treatment of graft versus host disease after allogeneic transplantation of the bone marrow revealed more of its effects [17]. In addition, D'Amato showed that malformations caused by thalidomide are the consequences of its anti-angiogenic properties; such a mechanism of inhibition of growth of tumoral vessels might be utilized even in treatment of cancer [12]. Later, its effect on relapsed and refractory MM was examined, leading to the discovery of its strong anti-tumor activity [11].

2.2. Lenalidomide

Lenalidomide, 3-(4-amino-l-oxo-l,3-dihydro-2*H*-isoindol-2-yl) piperidine-2,6-dione ($C_{13}H_{13}N_3O_3$), has preserved chiral centre and occurs as a racemate, in a mixture of two optically active enantiomers, S and R. Lenalidomide is generated by adding an amino group to position 4 of the phthaloyl ring of thalidomide and removing a carbonyl group from the 4-amino substituted phthaloyl ring (reviewed in [18].

Clinical experience with thalidomide led to initiation of research of its analogs with more favorable toxic profile and increased effectiveness (reviewed in [9]). Lenalidomide (Revlimid, formerly CC-5013, Celgene) is ranked among these derivatives. Unfortunately, its teratogenic potential is preserved; for that reason, its use comes under the safety program as well [9].

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Similarly to thalidomide, lenalidomide inhibits angiogenesis and adhesion of MM cells to bone marrow cells, reduces secretion of growth factors, induces apoptosis of MM cells, inhibits production of inflammatory cytokine TNF-α and supports cytotoxic activity of NK cells and T cells [19–21]. However, this IMiD mediates activation of Wnt/β-catenin signaling which is a mechanism of inducible chemoresistance to lenalidomide at the transcriptional and post-transcriptional levels [22].

2.3. Pomalidomide

Chemical formula of pomalidomide is 4-amino-2-(2,6-dioxo-3-piperidyl)isoindoline-1,3-dione ($C_{13}H_{11}N_3O_4$). This compound is derived from thalidomide by adding an amino group to position 4 of the phthaloyl ring and exists in two forms, enantiomers S and R (reviewed in [18]).

Another analog of thalidomide is pomalidomide (Actimid, Celgene), which has a pleiotropic effect on myeloma cells (reviewed in [9]). It induces apoptosis of MM cells, inhibits angiogenesis, has strong immunomodulatory abilities and is most effective in TNF-α mRNA degradation when compared to thalidomide and lenalidomide [19,20,23].

3. Mechanisms of immunomodulatory drugs in multiple myeloma

There are several mechanisms of IMiDs in multiple myeloma. They have direct antitumor, immunoregulatory and anti-angiogenic activity. We shall also discuss anti-inflammatory properties and effects these drugs have on the bone marrow microenvironment in multiple myeloma.

3.1. Direct antitumor effects

The direct antitumor effect of IMiDs on myeloma cells was discovered in the study of Hideshima et al. In that study, myeloma cell lines were treated with IMiDs, and this treatment led to CDK inhibition and cell cycle arrest in G_1 phase [23].

The IMiDs-induced cell cycle arrest of clonal plasma cells in G_1 phase is mediated via increased levels of $p21^{WAF-1}$ protein through epigenetic modifications. Reduction of histone methylation in the promoter of $p21^{WAF-1}$ gene and increased histone acetylation make transcriptional factors (such as Sp1, Sp3, Egr1 and Egr2) accessible to DNA [24]. Increased $p21^{WAF-1}$ expression subsequently induces $p21^{WAF-1}$ and CDK 2, 4 or 6 complex formation, leading to inhibition of their kinase activity [25]. Afterwards, pRb hypophosphorylation occurs preventing the cell from G_1/S phase transition. In the study of Gandhi et al., lenalidomide induced p15 and p27 tumor-suppressor gene expression in myeloma cells [26].

Myeloma cells are protected from apoptosis by anti-apoptotic proteins that are regulated via NF-κB transcriptional factor [27]. In these cells, IMiDs prevent NF-κB from activation which leads to decreased expression of anti-apoptotic proteins, for example cellular inhibitor of apoptosis 2 (cIAP2), FLICE inhibitory protein (FLIP), X-linked inhibitor of apoptosis protein (XIAP) which prevents caspase 8 activation, TRAIL/Apo2L and Fas sensitivity. In addition, IMiDs directly induce activation of caspase 8 and subsequently activation of caspase 3 [28,29].

IMiDs block IGF-1 production by decreased NF-κB transcriptional activity. IGF-1 induces phosphorylation of transcriptional factor FKHRL-1 thereby disrupting its pro-apoptotic activity and also increasing levels of apoptotic inhibitors cIAP2, FLIP and XIAP. IMiDs reduce IGF-1 effects which lead to increase in sensitivity to TRAIL/Apo2L in myeloma cells [30].

Another study showed that lenalidomide downregulates levels of interferon regulatory factor 4 (IRF4) in MM. It was associated with decreased MYC levels, as well as G_1 phase cell cycle arrest, decreased cell proliferation and cell death. So, lenalidomide-induced IRF4 inhibition partially mediates anti-proliferative and pro-apoptotic effects of the compound [31].

The direct target of IMiDs is still unknown but the requirement of cereblon (CRBN) expression has been shown to be important for anti-myeloma activity of these agents. IMiDs are able to bind CRBN which leads to its cytotoxic activity. In the case of CRBN absence, cells show IMiDs resistance (reviewed in [32]). In addition, dysregulation of Wnt/β-catenin pathway might be a possible cause of lenalidomide resistance and phosphorylated β-catenin as a possible substrate of CRBN. In the presence of IMiDs, CRBN is not able to form ubiquitin ligase which results in accumulation of β-catenin. In conclusion, CRBN inhibition may have a key role in the treatment of MM [22].

3.2. Immunomodulatory effects of IMiDs

3.2.1. Co-stimulation of T cells

Activation of T cells is mediated via the T cell receptor (TCR), but also requires a secondary co-stimulation signal mostly mediated by antigen presenting cells (APCs). IMiDs are able to co-stimulate partially activated $CD3^+$ T cells [13,33]. This stimulation is equal for both $CD4^+$ and $CD8^+$ cells (Fig. 2). IMiDs improve their proliferation and augment production of Th1 type cytokines, IL-2 and interferon γ (IFN-γ). Subsequently, secretion of IL-2 and IFN-γ increases number of natural killer cells (NK cells), improves their function and mediates lysis of myeloma cells. One of the mechanisms of augmenting IL-2 production via IMiDs is mediated by increase of activation protein-1 (AP-1) transcriptional activity. AP-1 is a key factor of IL-2 production [34]. IL-2 and IFN-γ production is also mediated via JAK/STAT signaling pathway, where activated STAT proteins induce expression of target genes. On the other hand, these genes can be negatively regulated by the suppressor of cytokine signaling (SOCS). SOCS1 is a negative regulator of IL-2 and IFN-γ. In immune cells, the SOCS1 expression is significantly inhibited by IMiDs [35].

Activation of T cells can be abrogated by various factors. Lenalidomide overcomes blockage of cytotoxic T lymphocyte antigen 4-immunoglobulin (CTLA4-Ig), disrupting cell proliferation and cytokine secretion [36]. So, lenalidomide triggers tyrosine phosphorylation of CD28 on T cells (B7-CD28 pathway) followed by activation of NF-κB and facilitation of G_1 to S phase transition. In addition, phosphoinositide 3 kinase (PI3K) is activated during CD28 phosphorylation and leads to PI3K/Akt signaling pathway activation and facilitation of nuclear factor of activated T cells 2 (NFAT2) translocation, resulting in IL-2 secretion [37].

Pomalidomide does not provide co-stimulative signal by itself, but is able to increase signal from another cell or molecule. Pomalidomide also augments promotor of IL-2 gene activity and so IL-2 production [34].

3.2.2. Enhancement of NKT and NK cells

Natural killer T cells (NKT) are T cells with NK cell surface markers. They have direct cytotoxic anti-tumor properties. Dendritic cells (DC) with NKT ligand, α-galactosylceramide, are responsible for activation and expansion of NKT cells [38]. When these cells are exposed to lenalidomide, increased NKT expansion via DC occurs. IFN-γ secretion by NKT cells leading to partial activation of NK cells and proliferation is associated with this exposure as well [39].

NK cells are irreplaceable elements of innate immunity. They protect the organism by killing tumor cells and virus-infected cells. IMiDs enhance NK cell proliferation in the presence of IL-2, which facilitates killing of myeloma cells [13]. In addition, lenalidomide improves antibody-dependent cell-mediated cytotoxicity (ADCC) and thus increases granzyme B and Fas ligand (FasL) expression

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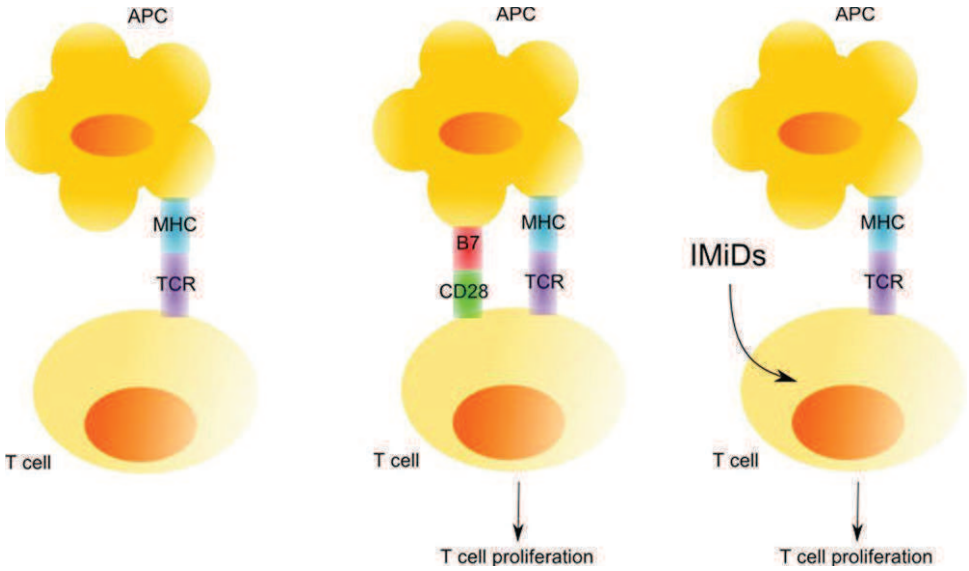

Fig. 2. Co-stimulatory activity of IMiDs. APCs activate T cells by binding peptides of major histocompatibility complex (MHC) to the TCR. The B7-CD28 secondary co-stimulatory pathway is also required for effective T cell activation. IMiDs are able to enhance T cell stimulation in the absence of the secondary signals.

in NK cells leading to tumor cells apoptosis. Increased monocyte chemotactic protein-1 (MCP-1) expression is associated with this process as well. MCP-1 attracts T cells in order to migrate to the tumor. Granulocyte macrophage colony-stimulating factor (GM-CSF) expression is also increased during this process, improving anti-tumor response [21].

3.2.3. Inhibition of regulatory T cells

Regulatory T cells (Tregs) play an active role in establishing and maintaining immunological unresponsiveness to self antigens and negative control of various immune responses to non-self antigens. Regulatory function for Tregs is provided by a master molecule FoxP3. At present, several studies showed that Tregs were expanded both in hematological malignancies and solid tumors suppressing the function of naïve T cells [40]. The study of Galustian and colleagues showed that lenalidomide and pomalidomide inhibit proliferation of Tregs via decreased FoxP3 mRNA expression. Another possible mechanism was prevention of CD134 expression on the surface of Treg cells. This process abrogates T cells activation (Fig. 3). Unlike its analogs, thalidomide had no effect on Treg cells in this study [41].

On contrary, several other studies presented different results. Muthu Raja et al. found that patients responding to lenalidomide and dexamethasone combination had increased number of Tregs and concluded that this treatment strategy was not able to enhance the immune anti-tumor response [42]. Similarly, Gupta and colleagues discovered a decrease in the frequency of Tregs with reduced expression of FoxP3 in previously untreated MM patients. However, the immunosuppressive potential of Tregs was preserved proposing normal Tregs function. After treatment with thalidomide, an increase in the number of Tregs was observed in this study [43]. These conflicting results might be caused by different identification approaches of Treg cells. At this point, data about Tregs in MM are contradictory, so no clear conclusions can be presented [44].

3.3. Anti-angiogenic activity of IMiDs

IMiDs have been shown to have anti-angiogenic effects which are independent of their immunomodulatory effects (Fig. 4). While anti-angiogenic properties of thalidomide prevail, lenalidomide and pomalidomide have an immunomodulatory potential [12,20].

IMiDs modulate factors affecting endothelial cell migration, especially TNF-α, VEGF and bFGF which are secreted by bone marrow stromal cells. According to Dredge and colleagues, IMiDs reduce Akt phosphorylation and thus interfere in the PI3K/Akt signaling pathway affecting the expression of these factors and restraining angiogenesis [45].

VEGF expression induces VE-cadherin tyrosine phosphorylation via Src kinase. It disrupts contact of endothelial cells leading to necessary migration during angiogenesis [46]. Lenalidomide blocks Src kinase activity and disrupts subsequent VE-cadherin tyrosine phosphorylation during angiogenesis [47].

In addition, myeloma cells produce VEGF after IL-6 stimulation. Endothelial cells and bone marrow stromal cells respond to VEGF production via IL-6 secretion thereby closing the paracrine loop [48]. Gupta et al. observed that thalidomide and lenalidomide decreased expression of these factors and thus inhibit growth of new vessels and myeloma cells nutrition [49].

Lu et al. found an inhibitory effect of lenalidomide on expression of endothelial cells hypoxia-inducible factor 1α (HIF-1α) which has a key role in hypoxia-mediated effects, together with angiogenesis and metastasis promoting aggressive tumor phenotype. The inhibition of HIF-1α expression is at least partially mediated via PI3K/Akt signaling pathway suppression. In this study, pomalidomide seemed to have less impressive inhibitory effect on HIF-1α expression and thalidomide had almost none [50].

3.4. Anti-inflammatory properties of IMiDs

Cyclooxygenase 2 (COX-2) catalyzes conversion of arachidonic acid into several inflammatory prostaglandins (PG). For example, PG-E_2 contributes to inflammation, tumor-induced angiogenesis and production of IL-6 [51]. IMiDs have been shown to inhibit the expression of COX-2 via reducing the half-life of COX-2 mRNA and thereby reducing levels of PG-E_2 [52]. In addition, lenalidomide increases IL-10 production which also plays a partial role in COX-2 mRNA degradation.

Thalidomide has an inhibitory effect on TNF-α, inflammatory cytokine produced by monocytes and macrophages [53]. TNF-α inhibition is mediated via its mRNA degradation [54]. Unlike thalidomide, lenalidomide inhibits TNF-α production more effectively, but the most effective inhibitor of TNF-α is pomalidomide [19].

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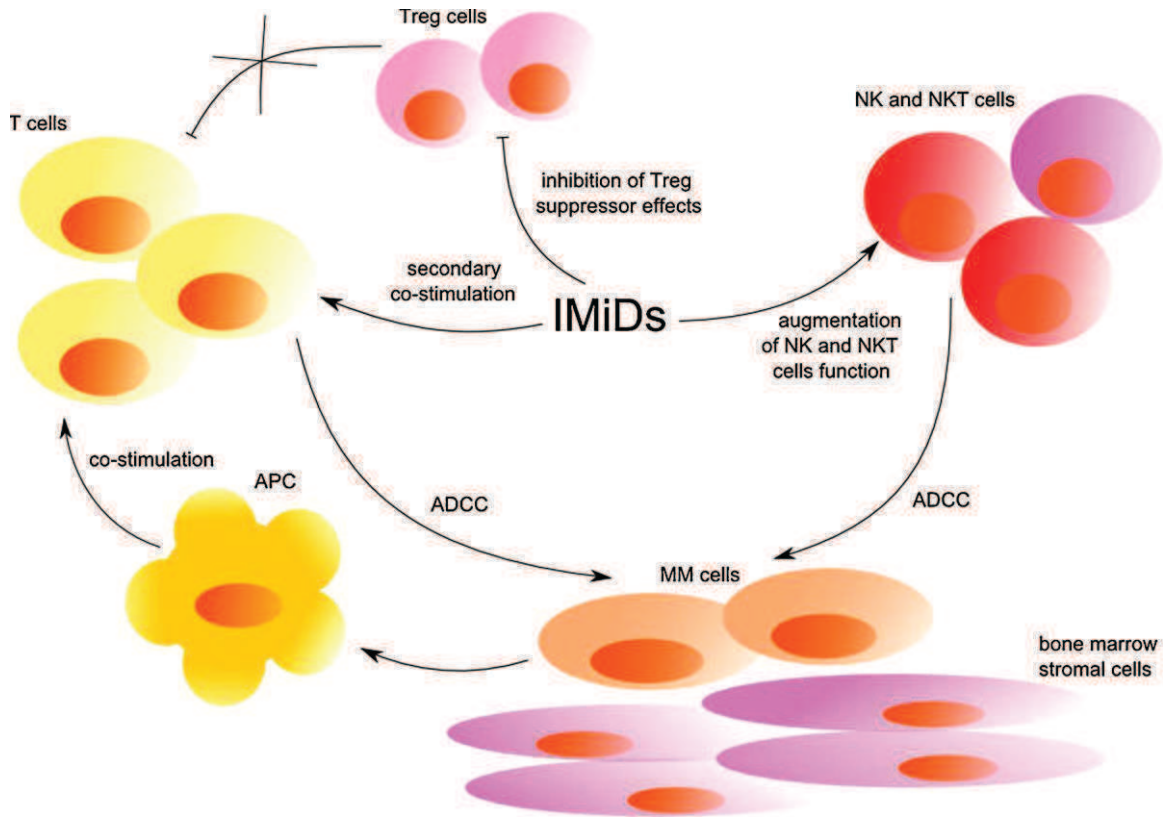

Fig. 3. Overview of the immunomodulatory effects of IMiDs. These effects include co-stimulation of T cells, inhibition of regulatory T cells with suppressor effects on the host immune system and enhancement of NK and NKT cell function and proliferation.

Anti-inflammatory and anti-angiogenic properties of thalidomide are partially controlled by NF-κB transcriptional factor. In the cytoplasm, NF-κB is bound to IκB and thereby inhibited. Stimulation by IL-1β or TNF-α leads to IκB dissociation, and then, the expression of inflammatory genes is activated. Thalidomide abrogates IκB phosphorylation, dissociation and resultant inflammatory cytokine secretion [55].

However, IMiDs inhibit not only TNF-α and IL-1β production, but also IL-6, IL-12 and TGF-β production. These cytokines enhance growth and survival of myeloma cells, drug resistance, cell migration and adhesive molecule expression. On the other hand, IMiDs increase anti-inflammatory cytokine IL-10 production [56].

IL-6 production is partially mediated via JAK/STAT signaling pathway and modulated by SOCS1 expression. In 75% of MM

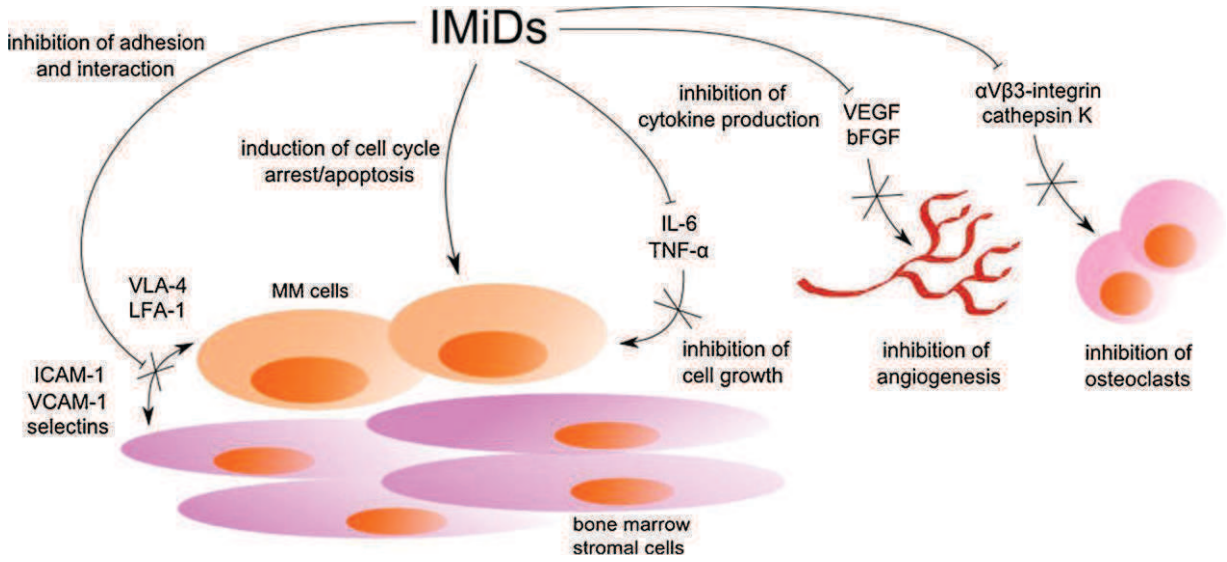

Fig. 4. Overview of non-immunomodulatory effects of IMiDs. These effects consist of anti-angiogenic activity, inhibition of cell growth and enhancement of MM cells apoptosis, inhibition of osteoclasts activity and reduction of myeloma cells/stromal cells interactions.

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cases, promoter of SOCS1 gene is silenced by CpG dinucleotide hypermethylation leading to uncontrolled IL-6 production, myeloma cells growth and suppression of host immune system. Pomalidomide particularly demethylates promoter of SOCS1 gene in MM cells, induces SOCS1 transcription and thus inhibits IL-6 production [35].

3.5. Effects of IMiDs in the bone marrow of multiple myeloma

Osteolytic lesions are the most striking symptom of MM. IMiDs prevent osteoclast maturation and thus inhibit bone structure disruption [57]. Mechanism of inhibition lies in reduced expression of the cysteine protease cathepsin K, a protease associated with matrix degradation and bone resorption, and in reduced levels of αVβ3-integrin, a marker associated with osteoclast differentiation mediating interactions between cells and the extracellular matrix [58]. These mechanisms are consequences of downregulated levels of transcriptional factor PU.1, which is a key mediator of osteoclastogenesis – it regulates the differentiation of myeloid cells to osteoclast precursor cells [59]. In addition, Breitkreutz et al. observed that lenalidomide decreases MIP-1α secretion, which is one of the most important factors for growth and survival of osteoclasts. This agent also inhibits RANKL, a key mediator of osteoclastogenesis [57].

Adhesive molecules, induced by TNF-α, facilitate interaction between clonal plasma cells and bone marrow stromal cells [14]. It has been shown that for example very late antigen 4 (VLA-4) interactions with fibronectin are important for migration and homing of MM cells into the bone marrow milieu. This molecule is expressed on myeloma cells as well as on physiological plasma cells promoting terminal B cell differentiation and secretion of immunoglobulins (reviewed in [60]). Nevertheless, IMiDs inhibit production of TNF-α thereby decreasing VLA-4 and lymphocyte function-associated antigen 1 (LFA-1) expression. VLA-4 and LFA-1 are adhesive molecules on the surface of plasma cells. IMiDs modulate expression of adhesive molecules, such as ICAM-1, E-selectin, L-selectin and VCAM-1, on the surface of bone marrow cells [61]. This goes along with consequent inhibition of adhesion-mediated cell signaling as well as cytokine production in the bone marrow milieu [49].

Thalidomide significantly inhibits SDF-1α and CXCR4 receptor expression on MM cells. Their interaction is important for myeloma cell adhesion and migration. However, this mechanism has not been fully clarified [62]. Reduced contact between myeloma cells and the bone marrow leads to decreased IL-6 and VEGF production supporting survival of MM cells [14,49]. This mechanism also overcomes cell adhesion mediated drug resistance to apoptosis of tumor cells [48].

4. Conclusion

The introduction of the novel drugs, especially IMiDs, turned multiple myeloma into a chronic disease. The possibility of actually curing the patients is within our reach and combinations of these drugs with the upcoming drugs from the pipeline may bring this in the very new future. Even though multiple myeloma is a very heterogenous disease, IMiDs have been able to attack the malignant cells by various mechanisms, ranging from direct anti-myeloma effect, immunomodulatory effects to anti-angiogenic effect and many others.

Conflict of interest

The authors have nothing to declare.

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LETTER TO THE EDITOR

Serum miR-29a as a marker of multiple myeloma

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MicroRNAs (miRNAs) are short non-coding RNAs about 21–25 nucleotides in length; they have been reported to be involved in the initiation and progression of solid tumors as well as hematological malignancies [1]. Moreover, circulating miRNAs have been observed in serum. These circulating serum miRNAs are very stable, resistant to RNAse treatment, and differ between healthy subjects and patients with colorectal and lung cancer as well as diabetes [2]. It has further been described that elevated expression levels of some serum miRNAs of patients with colorectal cancer decrease after surgery [3]. A recent paper described circulating serum miRNAs as markers of renal cell carcinoma [4]. Multiple myeloma (MM) is the second most common hematological malignancy worldwide [5]. MM is characterized by malignant proliferation of plasma cells (PCs) that accumulate in the bone marrow and displace normal hematopoiesis [6]. Currently, there are no specific markers for MM prediction. With the introduction of novel drugs and a very real possibility of targeted therapy in the near future, such markers are becoming increasingly important. If a specific marker were to be found in the peripheral blood, it would be easily accessible and could be obtained at various time points of treatment and even at the time of remission for frequent monitoring.

For our study, we chose four miRNAs based on their possible relationship to MM pathogenesis. miR-410 is encoded by the 14q32.31 locus, which is frequently involved in MM translocations, and it has previously been described as a prognostic marker in neuroblastoma [7]. Aberrant expression of miR-660 has also been linked to MM [8]. miR-142-5p has been found to be aberrantly expressed in MM and monoclonal gammopathy of unknown significance (MGUS) [9,10], and miR-29a to be up-regulated in MM PCs compared with normal PCs [9]. We decided to investigate whether these miRNAs (miR-29a, miR-142-5p, miR-410 and miR-660) are present in the serum of MM patients with MM. To the best of our knowledge, we are the first to report the presence of serum miRNAs in MM patients with MM. Our data show that serum miR-29a is differentially expressed in MM patients with MM versus healthy donors.

Serum samples from 91 MM patients with MM obtained at the time of diagnosis (prior to any treatment) were included in this study. These MM patients with MM (age range 41–88 years) were diagnosed between 2001 and 2010 at the Faculty Hospital Brno, and were included in this study only after signing the informed consent form approved by the ethical committee. Serum was separated after clotting and centrifugation of whole blood and stored at − 80 °C. As controls, blood donors with no tumor diagnosis were included (age range 45–64 years). Clinical parameters of the MM patients with MM and healthy donors are described in Table I.

Total RNA enriched for small RNAs was isolated from 200 μL of serum by miRNeasy Mini Kit (Qiagen, Germany) according to modified manufacturer's protocol. Each sample was mixed with 800 μL of QIAzol solution and 1.25 μL of 0.8 μg/μL MS2 RNA carrier (Roche, Switzerland). Extracted RNA was eluted in 30 μL of RNase free water. Quantification and purity measurements of RNA/miRNA were performed by Nanodrop ND-1000 spectrophotometer (Thermo Scientific, USA); samples with absorbance A260/280 > 1.8 were stored at − 80 °C for further processing.

Reverse transcription (RT) was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, USA) and small RNA-specific RT primers for hsa-miR-16, hsa-miR-29a, hsa-miR-142-5p, hsa-miR-410 and hsa-miR-660 (ID: 000391, 002112, 002248, 001274 and 001515; all Applied Biosystems) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the TaqMan Small RNA Assay on a 7500 Real Time PCR System (Applied Biosystems) using 1.4 μL of RT product according to the manufacturer's instructions. All experiments were run in duplicate. Average threshold cycle and standard deviation (SD) values were calculated.

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Table I. Basic characteristics of patients and healthy donors.

	Patients (*n*)	Healthy donors (*n*)
Total	91	30
Sex		
Male	49	14
Female	42	16
ISS		
Stage 1	28	—
Stage 2	32	—
Stage 3	26	—
Not classified	5	—
Durie–Salmon		
I	10	—
II	14	—
III	58	—
Not classified	9	—
Type of monoclonal Ig		
IgD	2	—
IgA	22	—
IgG	46	—
IgM	2	—
LC only	16	—
Biclonal	1	—
Non-secretory	2	—
Age according to ISS stage (years), median/mean (range)		
Total	63.9/64.9 (41–88)	—
Stage 1	60.4/60.7 (41–81)	—
Stage 2	65.3/65.5 (48–82)	—
Stage 3	70/69 (47–83)	—
Not classified	55.9/63.2 (49–88)	—
Age according to sex (years), median/mean (range)		
Total	—	55.5/55.4 (45–64)
Male	—	55.5/56.4 (51–64)
Female	—	55.5/54.6 (45–58)

ISS, International Staging System; Ig, immunoglobulin; LC, light chain.

Analysis of the qRT-PCR data was performed using SDS 2.0.1 software (Applied Biosystems) (settings: automatic baseline, threshold 0.2). Expression data were normalized to the expression of miR-16 reference miRNA. Statistical differences between miRNA levels in patients with MM and healthy donors were evaluated using the non-parametric Mann–Whitney *U*-test. Sensitivity, specificity and area under the curve (AUC) for serum miRNA levels were determined using receiver operator characteristic (ROC) analysis. All calculations were performed using MedCalc software version 12.2.1.0. *p*-values of less than 0.05 were considered statistically significant. Results were processed using the non-parametric Mann–Whitney *U*-test. Correlation was assessed using the Spearman correlation coefficient.

We used qRT-PCR to test differences in serum miRNA expression between samples from an independent cohort of MM patients with MM and samples from healthy donors. In total, 91 MM patients with MM and 30 healthy donors were included in this study. Expression levels of serum miR-142-5p, miR-660, miR-410 and miR-29a were analyzed (Table II). For normalization of expression data, miR-16 was used as previously reported [10]. We chose miR-16 as a reference as it was the most stable in our preliminary experiments (data not shown). The expression of miR-142-5p, miR-660 and miR-29a in serum was significantly increased in patients with MM compared to healthy donors ($p = 0.0183$, $p = 0.0062$ and $p < 0.0001$, respectively) [Figures 1(A)–1(C)]. As the difference in miR-410 expression level between MM and healthy donor sera did not reach statistical significance ($p = 0.2918$), miR-410 was excluded from further analysis. ROC curve analysis revealed that the serum level of miR-29a might serve as a useful biomarker for differentiating serum of patients with MM from that of controls, with an AUC of 0.832 (95% confidence interval [CI], 0.753–0.894). At a cut-off value of 0.0103 for the relative expression of miR-29a normalized to miR-16 levels, the sensitivity was 88% and specificity was 70% [Figure 1(D)]. Expression of miR-29a was not significantly different between patients at various Durie–Salmon ($p = 0.223$) or International Staging System (ISS) stages ($p = 0.677$). Although there were statistically significant differences between the ages of MM patients with MM and those of healthy volunteers ($p > 0.01$), there was no correlation between age and level of miR-29a within the group of MM patients with MM ($p = 0.442$), as well as within the group of healthy donors ($p = 0.411$).

Markers of diagnosis, remission and relapse of MM are obtained from the bone marrow after purification of the target cell population [6]. As bone marrow sampling is an invasive procedure, it cannot be repeated as often as needed. On the other hand, markers from peripheral blood are easily accessible and can be used for frequent monitoring.

Table II. Expression levels* and basic characteristics of miRNAs.

	Expression level				
miRNA	HD	MM	FC	*p*-Value	Putative target genes
miR-29a	0.0089 0.0080–0.0119	0.0199 0.0129–0.0302	2.2371	< 0.0001	IGF1, NQO2, CDK2, AKT2, MYCN, MMP8, BCL11A, PTEN, CCNA2
miR-142-5p	0.0031 0.0024–0.0041	0.0046 0.0027–0.0075	1.4839	0.0183	CENTB2, RAD50, ELK4, IGF1, MCL1, MYCN, MAPK6, PTEN
miR-410	0.0002 0.0001–0.0003	0.0002 0.0001–0.0004	1.0000	0.2918	CDC85A, CREB1, FGF2, FGF7, IGF, SMAD7
miR-660	0.0033 0.0029–0.0039	0.0040 0.0032–0.0049	1.2121	0.0062	E2F3, GDA, BCL2L11, CDK2

*Presented as median and interquartile range.
HD, healthy donors; MM, multiple myeloma; FC, fold change.

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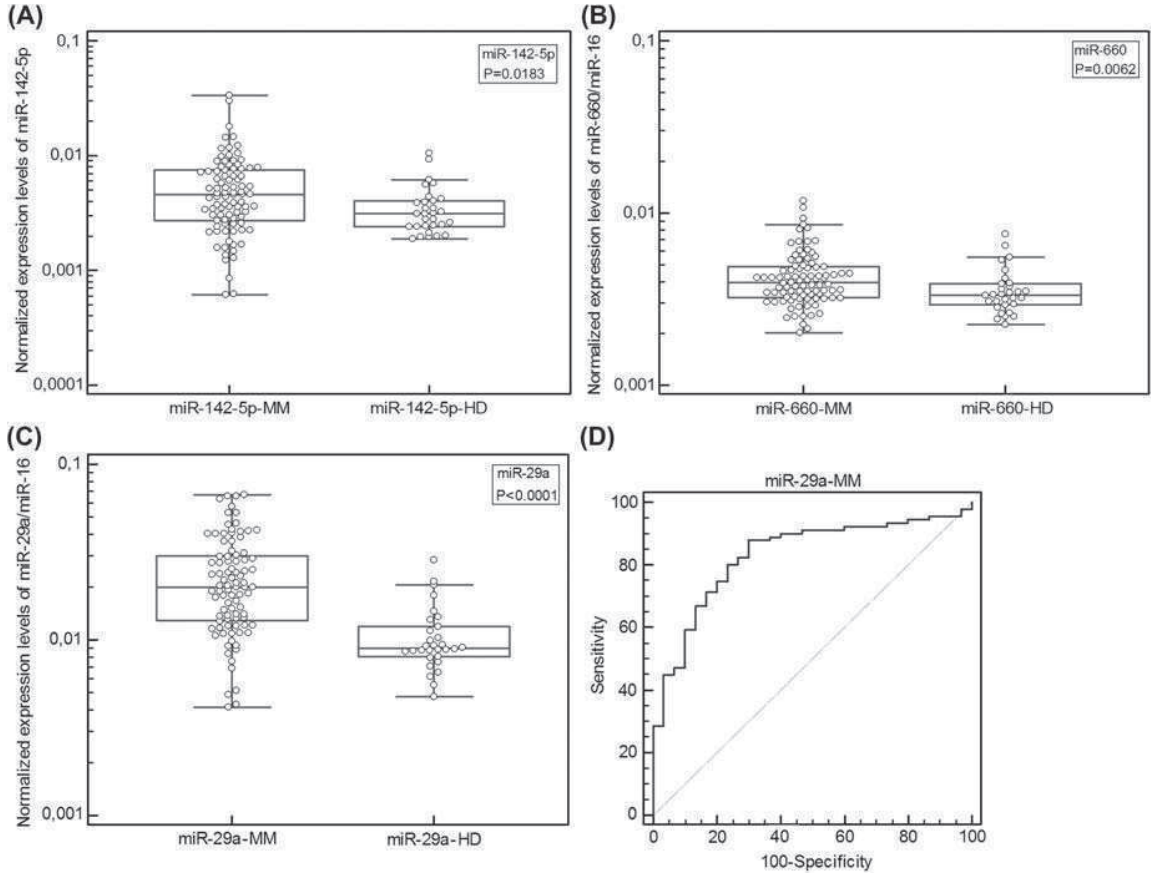

Figure 1. Comparison of serum miRNA expression levels and ROC analysis. (A–C) Comparison of serum miR-142-5p (A), miR-660 (B) and miR-29a (C) expression levels (log10 scale on *y*-axis) in patients with multiple myeloma (MM) ($n = 91$) and healthy donors (HD) ($n = 30$). Expression of each miRNA was normalized to expression of miR-16. Lines represent mean value, 25–75% quartile and min–max values. Statistically significant differences were determined using Mann–Whitney *U*-test. (D) Receiver operating characteristic (ROC) curve analysis of serum miR-29a in patients with MM showed 0.832 AUC, 88% sensitivity and 70% specificity at cut-off value > 0.0103.

Unfortunately, at this point, they are not perfect. Also, a good marker for prediction of early relapse is missing.

Circulating serum miRNA might represent a novel putative, easily accessible and stable marker. Further investigation is needed to estimate whether these circulating miRNAs are directly associated with changes occurring in MM, as they may also reflect indirect pathological effects of the disease, such as bone lesions or renal failure in MM [11,12]. Also, a possible relationship of miRNA levels found in PCs of patients with MM and corresponding sera is not clearly understood.

Our results show that miR-142-5p, miR-660 and miR-29a are up-regulated in the serum of MM patients with MM. Further analytical characteristics of miR-29a (sensitivity 88%, specificity 70%) proved that it is potent in discriminating MM serum from healthy donor serum. To our knowledge, this is the first report concerning circulating serum miR-29a differentially expressed in MM patients with MM. Although our observations are promising, further large-scale studies and validations are needed to establish miR-29a as a marker of the disease.

Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/lal.

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Soft-tissue extramedullary multiple myeloma prognosis is significantly worse in comparison to bone-related extramedullary relapse

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Soft-tissue extramedullary multiple myeloma prognosis is significantly worse in comparison to bone-related extramedullary relapse

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ABSTRACT

Even in the era of new drugs, multiple myeloma patients with extramedullary relapse have a poor prognosis. Our goal was to analyze the frequency and outcome of extramedullary relapse occurring in relapsed multiple myeloma patients. In total, we analyzed the prognosis of 226 relapsed multiple myeloma patients treated between 2005 and 2008 and evaluated them for presence of extramedullary relapse. We found evidence of extramedullary relapse in 24% (55 of 226) of relapsed multiple myeloma patients. In 14% (32 of 226) of patients, the lesions were not adjacent to the bone, while extramedullary relapse adjacent to the bone was documented in 10% (23 of 226) of cases. Patients without extramedullary relapse had significantly longer overall survival than patients with extramedullary relapse (109 *vs.* 38 months; $P<0.001$). Moreover, patients with soft tissue-related extramedullary relapse had significantly poorer overall survival compared to bone-related extramedullary relapse patients (30 *vs.* 45 months; $P=0.022$). Also, overall survival from diagnosis was as low as five months for soft tissue-related extramedullary relapse patients when compared to 12 months overall survival for bone-related extramedullary relapse. This is the first study that shows a significant difference in prognosis for different types of extramedullary relapse. If the extramedullary myeloma infiltration was not bone-related, overall survival after relapse was extremely short (5 months).

Introduction

Multiple myeloma (MM) is characterized by malignant proliferation of clonal plasma cells that usually produce a unique monoclonal immunoglobulin. MM comprises approximately 1% of all cancers and is the second most common hematologic malignancy.[1] Unfortunately, the etiology of MM is still unknown.[2] Current treatment strategies combine novel agents, such as thalidomide, bortezomib, lenalidomide, with conventional chemotherapy, corticosteroids and autologous stem cell transplantation and significantly prolong long-term patient outcome. More than 30% of patients undergoing intensive treatment live for more than ten years, and there is a clear but limited possibility for some patients with low-risk disease to be cured.[3] However, relapse is still a frequent event; whenever it occurs, there is no chance for a cure with current treatment options. Even in relapsed disease, remissions can be obtained in the majority of patients[4,5] although these tend to be shorter than first treatment responses.[6,7] Extramedullary myeloma is not frequent but is always associated with a significantly shorter overall survival, even in the era of novel agents.[3,8-12]

Extramedullary myeloma (EM) is a type of MM defined by the presence of extraskeletal (i.e. soft tissue or visceral) clonal plasma cells infiltrates.[13] EM can be present either at the time of initial diagnosis (primary EM) or at the time of relapse (secondary EM).[3]

Clinically, three types of extramedullary lesions can be described: a) tumor mass adjacent to bone and extending into soft tissues; b) soft tissue or visceral tumor that is not connected to the bone; or c) diffuse infiltration of organs by plasma cells without any obvious focal lesion. However, the majority of studies do not discriminate between these three types of EM lesions.

There are very few large studies focusing on incidence of EM. Primary EM is found in approximately 4-16% of MM patients at the time of diagnosis. Secondary EM is found in 6-20% during further MM disease course.[14] Unfortunately, the prognosis of EM patients is generally poor, and there is no effective treatment for EM.[15]

The primary objective of this study was to analyze the frequency and outcome of secondary EM occurring in relapsed MM patients. In addition, we identified the clinical difference and outcome of bone-related and bone-unrelated subtypes of EM.

Methods

All consecutive relapsed MM patients (in total 226 patients) treated in our department at the University Hospital Brno, Czech Republic, between 2005 and 2008 were included in this analysis and prospectively evaluated for the presence of EM. They were included in this study only after signing the informed consent form approved by the Ethical Committee of the hospital.

The median age of patients was 60.8 years (range 27.9-83.5), and the median follow up was 3.7 years (range 0.1-22) after diagnosis. Other base-line patients' characteristics are shown in Table 1. Out of 226 MM patients, we excluded 6.2% (15 of 226) of patients with pri-

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mary EM, i.e. evidence of extramedullary disease at the time of MM diagnosis.

EM was diagnosed using imaging methods, such as ultrasound, computed tomography (CT) or magnetic resonance (MR). Biopsies were carried out if the lesion was accessible to confirm the presence of clonal plasma cells on cytological or histological examination. EM was defined as the presence of a pathological soft tissue mass by imaging in patients with other findings compatible with MM progression/relapse or a finding of clonal plasma cells in biopsy or aspirate from the extramedullary lesion. Patients with clinical suspicion of extramedullary progression underwent further examinations. All patients with new neurological symptoms had MRI of spine or brain if there were no contraindications. All patients with leptomeningeal infiltration had lumbar punction with flow-cytometric evaluation of cerebrospinal fluid. If there were some contraindications, CT was performed. Ultrasound was performed in patients with hepatic lesion and also if soft tissue infiltration was suspected.

Patients with EM were divided into two groups: 1) soft tissue-related EM (EM-S), i.e. the presence of soft tissue or visceral masses not linked to skeletal involvement by MM, or diffuse organ infiltration by malignant plasma cells; 2) bone-related EM (EM-B), i.e. a plasma cell mass adjacent to a bone lesion.

Overall survival (OS), time to EM, and previous treatments were analyzed for both groups of patients. The treatment was rather heterogeneous but all patients had received either thalidomide or bortezomib prior to relapse. EM therapy consisted of a novel agent that had not been used previously, in combination with corticosteroids and chemotherapy. Patients with a good performance status and available stem cell autograft received high-dose chemotherapy with melphalan followed by stem cell rescue. Regimens used for EM treatment included thalidomide in 33% of patients, bortezomib in 38% and lenalidomide in 5%. Forty-two percent of patients were treated with high-dose melphalan and stem cell transplantation. Chromosomal abnormalities were evaluated using FISH as reported previously.[16-18]

Statistical analysis

Kaplan-Meier estimates were used for survival analysis. Differences between survival times in patient subgroups were tested using the log rank test. Differences in categorical variables were analyzed using the M-L χ^2 test. The level of statistical significance was 5% for all tests.

Results

We found evidence of EM in 24% (55 of 226) of evaluable relapsed MM patients. In 14% (32 of 226) of these patients, the lesions were not adjacent to bone and thus were classified as EM-S, while EM-B was documented in 10% (23 of 226) of cases. In the EM-S group, the most common site of EM was skin and subcutaneous tissue (69%), while extramedullary masses extending from vertebrae (78%) were most common in the EM-B group. Histological evaluation was performed in 66% of proven EM cases. In other cases, MRI (22%), CT (4%) and ultrasound (2%) were used for proven EM cases (Table 2). EM occurred early in the course of the disease: for 53% of patients (29 of 55 patients) at first relapse, 33% (18 of 55) at second relapse, 14% (8 of 55) at third and higher relapse. In both groups, more than half of patients were diagnosed with EM during the first relapse (Table 3). Time from diagnosis to EM relapse was similar for both EM-S and EM-B groups and the difference was not statistically significant (21 *vs.* 23 months).

Conventional chemotherapy was used in 20% (11 of 55) of patients prior to EM relapse. Thalidomide-containing regimens, bortezomib-containing regimens and high-dose chemotherapy with autologous stem cell transplantation had been given to 38% (21 of 55), 29% (16 of 55) and 53% (29 of 55) of patients, respectively. Differences in the treatment regimens of EM-B and EM-S groups prior to relapse were not statistically significant (Table 4).

Median OS for all the 226 MM patients followed was 89 months with median follow up of 44.4 months (range 6.5-264). There was no difference in incidence of EM in gender (P=0.54) or median age at the time of EM diagnosis (P=0.132).

Overall survival was significantly longer for patients without EM than for patients with EM (109 *vs.* 38 months; P<0.001) (Figure 1A). However, there were no differences

Table 1. Patients' characteristics.

Characteristics	N.
Gender M/F	115/111
Median age in years (range)	60.8 (27.9-83.5)
Durie-Salmon stage I/II/III	35/41/148
Durie-Salmon stage A/B	187/37
Isotope: IgG/IgA/LC/other	135/50/25/16
Light chain: kappa/lambda	142/74
ISS stage I/II/III	57/65/37
Median follow-up in years after diagnosis (range)	3.7 (0.1-22)
EM-S	32
EM-B	23

ISS: International Staging System. In total, 226 MM patients were analyzed. EM-S: extramedullary relapse - soft tissue; EM-B: extramedullary relapse - bone related.

Table 2. Organ involvement of 55 patients with extramedullary relapse of multiple myeloma

Involved organ	N. (%)	Biopsy (%)
Bone related - in total (EM-B)	23 (41.8)	8 (14.6)
Bone related - spine	18 (32.7)	4 (7.3)
Bone related - other	5 (9.1)	4 (7,3)
Soft-tissue related - in total (EM-S)	32 (58.2)	28 (51%)
Skin	22 (40)	22 (40)
Central nervous system	2 (3.6)	2 (3.6)
Retroperitoneal tumor mass	4 (7.3)	2 (3.6)
Lungs	2 (3.6)	0
Lymph nodes	1 (1.8)	1 (1.8)
Liver	1 (1.8)	1 (1.8)

Table 3. Occurrence of extramedullary lesions in relapse of MM.

	EM-B, N. (%)	EM-S, N. (%)
1st relapse	12 (52.2)	17 (53.1)
2nd relapse	7 (30.4)	11 (34.4)
3rd relapse	2 (8.7)	3 (9.4)
4th relapse	1 (4.3)	1 (3.1)
6th relapse	1 (4.3)	0 (0.0)

in overall response rate (ORR) or complete response rate (CR) in first-line treatment between these two groups (P=0.201). Also, TTP after first-line treatment was similar in patients with and without EM (20 months vs. 16 months; P=0.112). Interestingly, we did not find any difference between TTP after first-line treatment between the EM-B and EM-S groups of patients (14 months *vs.* 18 months; P=0.128).

Overall response rate after EM relapse was as low as 24% (13 of 55) in EM patients, with 5% (3 of 55) and 19% (10 of 55) of patients achieving complete and partial responses, respectively. Time to next progression (TTP) was only 5.4 months in EM patients.

When we analyzed the two groups of EM patients, we found that the EM-S group of patients had significantly poorer survival compared to EM-B patients (30 *vs.* 45 months; P=0.022) (Figure 1B). Analysis of OS from diagnosis of EM confirmed the poorest outcome for patients with EM-S when compared to EM-B (median OS 5 *vs.* 12 months; P=0.006) (Figure 2).

Results of cytogenetic analysis using FISH at the time of diagnosis were available for 22% (38 of 171) of patients without EM and 24% (13 of 55) of EM patients. The following chromosomal abnormalities with presumed impact on the prognosis of MM[16,17] were studied: RB1 deletion, p53 deletion, IgH gene disruption, translocation t(4;14), amplification 1q21 and hyperploidy. No differences were shown in the incidence of any of these chromosomal abnormalities between EM patients and those without EM (Table 5).

Discussion

Even in the era of new drugs, extramedullary relapse remains incurable. EM has been mostly studied in MM patients treated with high-dose chemotherapy, and most published studies evaluated patients at first relapse.[9,19-21] The incidence of EM has been estimated to be 10-15% of all MM relapses.[8,9,13] Recently, Usmani *et al.* analyzed 1965 MM patients for presence of EM at the time of MM diagnosis and at the time of relapse. The presence of extramedullary disease at the time of diagnosis was reported between 2.41% and 4.5% depending on the type of therapy. At the time of relapse/disease progression, extramedullary involvement was noted in 3.43-7.24% of patients.[3] In our cohort of 226 relapsed MM patients, the incidence of EM, after excluding all patients with known extramedullary disease at the time of diagnosis, was 24%, including 14% of EM-S and 10% of EM-B, respectively. However, the absence of an exact EM definition and consequently different format of EM calculation and reports make correct comparison difficult, even between trials.

In our study, we systematically analyzed all consecutive

Table 4. Treatment of patients before diagnosis of extramedullary relapse.

Treatment	EM-B, n. (%)	EM-S, n. (%)
Conventional regimens	6 (26.1)	5 (15.6)
Thalidomide	9 (39.1)	12 (37.5)
Bortezomib	5 (21.7)	11 (34.4)
Autologous transplantation	13 (56.5)	16 (50.0)
Interferon alfa	11 (47.8)	11 (34.4)

Table 5. Cytogenetic aberrations of MM patients. We found cytogenetic aberrations in 51 patients at the time of diagnosis, in 38 patients without and 13 patients with extramedullary relapse. There was no statistical difference in incidence of aberrations between both groups.

	EM relapse n. (%)	No EM relapse n. (%)	P level
Deletion RB1 positive	13 (54)	37(62)	0.744
Deletion p53 positive	6 (33)	25 (8)	*
IgH gene disruption positive	10 (80)	18 (61)	0.417
Translocation t(4;14) positive	12 (33)	26 (31)	1.00
Gain 1q21 positive	9 (56)	28 (50)	1.00
Hyperdiploidy positive	4 (0)	14 (36)	*

*N: number of patients evaluated; %: percent of patients with positive aberration out of n patients; * analysis not performed due to low number of patients.*

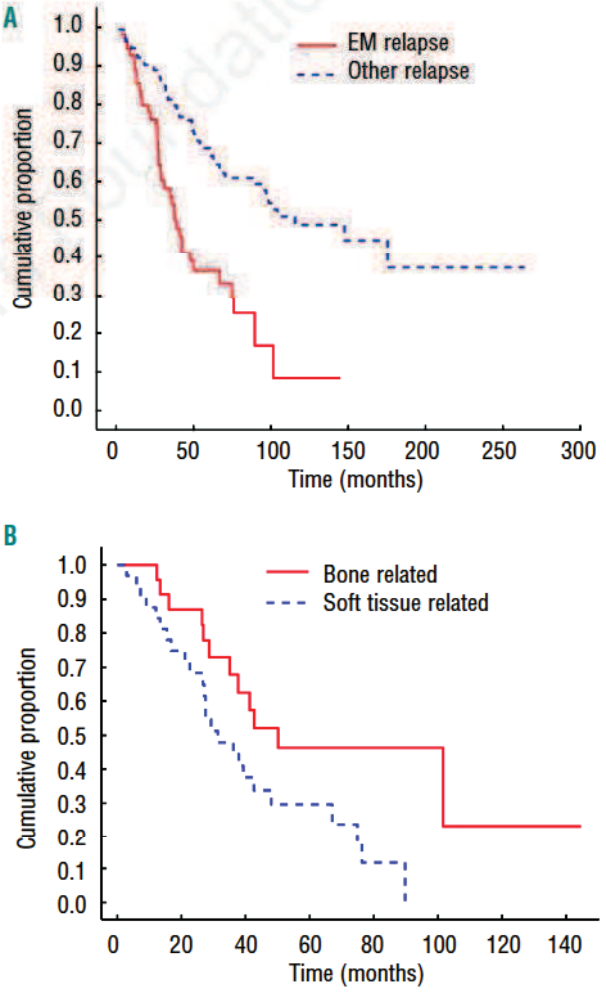

Figure 1. Overall survival and survival from diagnosis of EM patients. (A) Overall survival from diagnosis in patients with extramedullary relapse (EM) is significantly shorter than in other MM patients (38 *vs.* 109 months P>0.001). (B) Comparison of survival from diagnosis in patients with bone related extramedullary relapse (EM-B) is significantly longer than in patients with soft-tissue related extramedullary relapse (EM-S) (30 *vs.* 45 months; P=0.022)

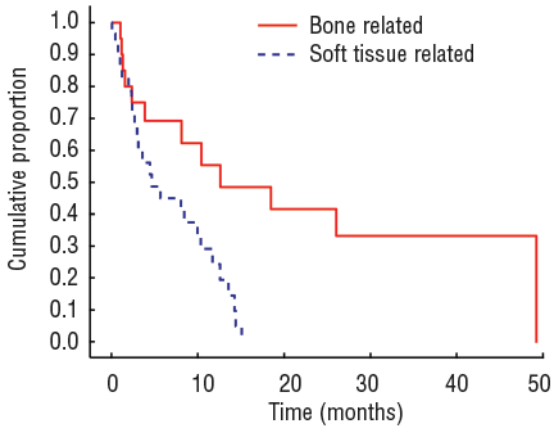

Figure 2. Overall survival from diagnosis of patients with bone related extramedullary relapse (EM-B) is significantly longer than in patients with soft-tissue related extramedullary relapse (EM-S) (4 *vs.* 12 months; *P*=0.006).

patients who relapsed during a 3-year period, from 2005 to 2008; up to 53% of EM occurred in the first relapse. We were not able to detect any association between EM relapse and any novel agent (thalidomide or bortezomib). In this single center experience, EM-B was observed only in 2% (4 of 113) of patients who underwent initial therapy with classical treatment protocol without any novel agents in the period 1996-2002 (4 x VAD and melphalan 200 mg/m^2).[22]

The most important finding in our analysis is the significant difference in prognosis for the two different types of EM. In accordance with the results of most research groups, we noted survival of approximately 12 months in our EM patients if the extramedullary mass was adjacent to the bone. However, if the extramedullary myeloma infiltration was not bone-related, the overall survival was extremely short and not longer than four months.

Based on these results, it is possible to divide patients with EM relapse into two different prognostic groups: 1) 'bone-related' extramedullary myeloma (EM-B), i.e. myeloma mass is adjacent to a distinct bone. The patients with this type of EM have an OS that is less than 50% shorter than patients who relapse without the EM component; 2) 'bone-unrelated' extramedullary myeloma (EM-S), i.e. myeloma mass of soft tissue with no relation to the bone. Such patients have the worst prognosis with a limited OS of less than 4-6 months. This would suggest that the biological behavior of these two types of EM is probably different.

Clear identification of one type of EM from another is now easily available with widespread use of PET/CT and/or whole body MRI and should be standardized in every future published cohort of EM patients. Our analysis covers only the relapsed setting, and the prognostic significance of the subtype of EM remains to be validated in primary EM. Thus, we believe that all studies analyzing EM should include details about primary or secondary EM as well as division of patients into EM-S and EM-B groups.

We completely excluded patients with primary EM from our analysis. We strongly believe that it is important to do so as we found that OS and TTP are similar after first-line treatment in all MM patients. However, survival rapidly decreases if EM develops during the course of the disease. Similarly, the presence of EM at the time of diagnosis was a very strong negative prognostic factor in the Arkansas analysis; it was the only parameter that was significantly important in all groups of patients. Also, patients with primary EM had the worse prognosis.[3] According to these data and our results, it is clear that the prognosis of MM patients is similar until the development of EM. But after this event, survival is very poor for patients with EM regardless of whether it is primary or secondary EM.

Our cohort of relapsed EM patients is one of the largest ever published. While no patient with skin and subcutaneous plasmocellular masses was seen in our center prior to 2005, as many as 22 cases were diagnosed between 2005 and 2008. It is likely that the recent changes in treatment strategies are associated with the increased incidence of EM, although the trend is unlikely to be associated with any particular drug. Our results suggest that the incidence of soft tissue EM is increasing in the era of novel drugs. These data need to be confirmed in prospective studies and comparative studies with historical controls. Many analyses, including ours, show that survival of MM patients has improved significantly after the implementation of novel therapeutic regimens.[2,11,12,22,23] However, patients with EM generally do not benefit from these agents and their survival remains extremely poor. Thus, EM remains one of the major ongoing issues in the care of MM patients.

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Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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Inhibitory proteazomu v léčbě mnohočetného myelomu

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Inhibitory proteazomu v léčbě mnohočetného myelomu

Proteasome Inhibitors in Treatment of Multiple Myeloma

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Souhrn

Mnohočetný myelom, maligní onemocnění plazmatických buněk, zůstává stále velmi obtížně léčitelným hematoonkologickým onemocněním, pro které je nutné hledat nové možnosti terapie ovlivňující jak plazmocyty samotné, tak i mikroprostředí kostní dřeně. Klonální plazmocyty se vyznačují zvýšenou regulací ubikvitin-proteazomové kaskády, což zvyšuje jejich citlivost k působení inhibitorů proteazomu. Léčebné přístupy využívající inhibitory proteazomu patří do éry nových léků a v posledních letech se ukázaly být velice důležitou součástí léčby pacientů s mnohočetným myelomem. Prvním inhibitorem proteazomu schváleným pro léčbu mnohočetného myelomu se stal bortezomib, který vykazoval silné antimyelomové účinky. Bohužel, navzdory jeho vysoké účinnosti se u velkého procenta pacientů s mnohočetným myelomem pacientů po čase objevuje rezistence k jeho působení. Ve snaze překonat rezistenci k bortezomibu a vyvinout inhibitor proteazomu s lepším toxickým profilem byly vyvinuty inhibitory proteazomu druhé generace – carfilzomib, marizomib a MLN9708, které by mohly nadějně změnit průběh mnohočetného myelomu v onemocnění chronické.

Klíčová slova

mnohočetný myelom – inhibitory proteazomu – bortezomib – carfilzomib – marizomib – MLN9708

Summary

Multiple myeloma, a plasma cell malignancy, still remains a hard-to-treat hematological disease that desperately needs new therapy targeting plasmocytes but also the bone marrow microenvironment. Clonal plasmocytes are characterized by increased regulation of ubiquitin-proteasome pathway which augments their sensitivity to proteasome inhibitors. Treatment strategies based on proteasome inhibitors belong to the era of new drugs, and they have become increasingly important for treatment of multiple myeloma in recent years. Bortezomib became the first proteasome inhibitor approved for the treatment of multiple myeloma and showed remarkable anti-myeloma activity. However, despite its high efficiency, a large proportion of patients have became bortezomib resistant. The second generation of proteasome inhibitors – carfilzomib, marizomib and MLN9708 – were developed in an effort to overcome bortezomib-resistance and find proteasome inhibitors with a better toxic profile. These drugs brought a chance that multiple myeloma would become a chronic disease.

Key words

multiple myeloma – proteasome inhibitors – bortezomib – carfilzomib – marizomib – MLN9708

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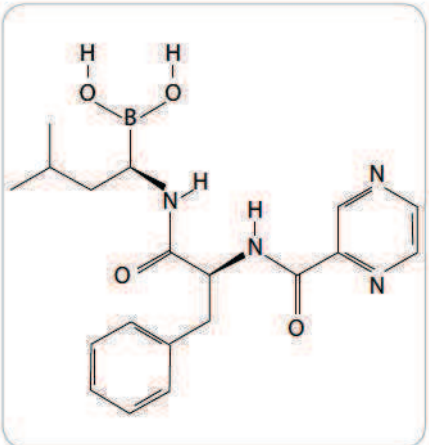
Úvod – inhibitory proteazomu

Proteazom jako nová buněčná struktura byl poprvé identifikován na počátku 70. let skupinou doktora Harrise [1]. Mnoho dalších objevů bylo učiněno později, koncem let 70. a počátkem let 80. v laboratoři prof. Avrama Hershka, a bylo zjištěno, že jeho funkce spočívá v ATP-dependentní degradaci intracelulárních proteinů a jeho specifita je dána interakcí pouze s takovými proteiny, které jsou označeny polyubikvitinovým řetězcem anebo obsahují specifickou sekvenci aminokyselin [2,3]. Za tento významný objev získali prof. Ciechanover, Hershko a Rose v roce 2004 Nobelovu cenu za chemii.

Proteazom je útvar cylindrického tvaru. Skládá se ze čtyř homologních prstenců tvořených sedmi podjednotkami α nebo β, které jsou nad sebou uspořádány v pořadí α-β-β-α [4]. V této struktuře lze rozeznat tři různé typy proteolytických podjednotek – β1, β2 a β5. Každá z nich obsahuje aktivní místo na svém N-konci, kde se nachází zbytek aminokyseliny threoninu (Thr1) [5]. Ačkoli proteazom obsahuje více katalytických míst, k inhibici jeho funkce postačuje pouze zablokování podjednotky β5, která vykazuje chymotrypsinovou aktivitu [6]. V hematopoietických buňkách je hlavním typem proteazomu jeho inducibilní izoforma – imunoproteazom, jehož výskyt koreluje s hladinou cytokinů. Je charakterizován nahrazením proteolyticky aktivních podjednotek β1, β2 a β5 jejich ekvivalenty β1i (LMP2), β2i (MELC1) a β5i (LMP7) [7].



Obr. 1. Struktura bortezomibu (The PubChem Project).

Nejčastějšími proteiny určenými k degradaci jsou špatně sbalené proteiny a proteiny s krátkým biologickým poločasem, které mají většinou regulační funkci [8]. Produkty proteolytické reakce jsou pak oligopeptidové řetězce s průměrnou délkou 8–12 aminokyselin [9].

Použití inhibitorů proteazomu (IP) patří v současné době mezi jedny z nejúspěšnějších strategií pro léčbu mnohočetného myelomu (MM), nádorového onemocnění způsobeného maligní transformací B lymfocytů s charakteristickou klonální proliferací a akumulací plazmatických buněk v kostní dřeni [10]. IP jsou zpravidla krátké peptidy, ke kterým je kovalentně připojen farmakofor – skupina atomů, která se váže do katalytických míst proteazomu, a tím zabraňuje jeho správné funkci. Konečným důsledkem inhibice proteazomu v myelomových buňkách je indukce apoptotických drah, překonání rezistence ke konvenční chemoterapii a senzitizace vůči dalším terapeutikům.

V tomto přehledovém článku se zaměříme na mechanizmus účinku a roli IP v léčbě mnohočetného myelomu.

První generace inhibitorů proteazomu

Bortezomib

Bortezomib, známý také pod označením PS-341 a komerčním názvem Velcade (Millenium Pharmaceuticals), je prvním a jediným IP, který byl doposud oficiálně schválen pro klinickou praxi. Po chemické stránce se jedná o dipeptidylový derivát kyseliny borité se sumárním vzorcem $C_{19}H_{25}BN_4O_4$ (obr. 1).

Poprvé byl bortezomib syntetizován v polovině 90. let minulého století společností Myogenics [11]. O jeho vysoké specifitě, účinnosti a oxidační stabilitě vypověděly výsledky studií in vitro u 60 rakovinných linií [12]. První klinická studie s Velcade pro léčbu hematologických malignit byla zahájena v listopadu 1999. Tým pod vedením dr. Orlowského ve studii s nízkými dávkami léku, sloužícími pouze k ověření jeho bezpečnosti, zaznamenal úplné vymizení příznaků (CR) MM u 47leté pacientky. U ostatních osmi pacientů z celkového počtu jedenáct došlo alespoň k minimální léčebné odpovědi (MR) nebo stabilizaci onemocnění [13]. Výsledek to byl natolik převratný, že po ověření v dalších fázích klinických studií vedl k urychlenému schválení bortezomibu pro léčbu relabujícího a refrakterního myelomu v roce 2003 v USA a o rok později také v České republice [14,15].

Mechanizmus účinku

Mechanizmus inhibice proteazomu bortezomibem spočívá v jeho kovalentní vazbě na β5 podjednotku, případně LMP7 podjednotku imunoproteazomu. S nižší afinitou se bortezomib váže také na podjednotky β1 a β2 [16]. Rozdílnost v afinitě je dána odlišnými interakcemi postranních řetězců inhibitoru s jednotlivými podjednotkami [17].

Vazebná konformace je zaujímána v podobě antiparalelního β skládaného listu, který je stabilizován vodíkovými můstky mezi atomy hlavního řetězce agens a konzervovanými zbytky katalytických míst (Gly47N, Thr21N, Thr21O a Ala49O). Samotná inhibice je zprostředkována farmokoforovou skupinou, v tomto případě zbytkem kyseliny borité. Atom boru zde kovalentně interaguje s nukleofilem, kterým je volný elektronový pár kyslíku Thr1O. Vzniklá elektronová mezera je stabilizována vazbou Gly47N na hydroxyl boru. Tetrahedrická struktura proteolyticky inaktivního produktu je dále zpevněna vazbou druhého hydroxylu na aminoskupinu v katalytickém místě [17]. Vzniklý adukt je charakterizován nízkým stupněm disociace, a proto, i když se jedná o reverzibilní reakci, zůstává po několik hodin prakticky vysoce stabilní.

Jelikož je proteazom zapojen do obratu intracelulárních proteinů, patří mezi primární důsledky jeho inhibice hromadění nefunkčních proteinů a chyby v signálních drahách, které vyúsťují v narušení adheze myelomových buněk, potlačení novotvorby cév, zastavení buněčného cyklu, omezení odpovědi na poškození DNA a indukci apoptózy [18].

Původní hypotézou hlavního biologického účinku bortezomibu na myelomové buňky byla inhibice transkripčního faktoru NF-κB, a tím zabránění tran-

skripce genů, které blokují apoptózu. Hlavní zástupce NF-κB se nachází v cytoplazmě v podobě inaktivního komplexu s vlastním inhibitorem I-κB, jehož degradace proteazomem je klíčem k aktivaci samotného transkripčního faktoru. Model proteazomové inhibice počítal se zastavením degradace I-κB, a tedy konstitutivní inhibicí NF-κB [19]. Ačkoli se tento předpoklad nepotvrdil, existuje ještě alternativní nekanonická dráha aktivace NF-κB, která by eventuálně mohla být bortezomibem blokována [20].

Klíčovou událostí v navození apoptózy myelomových buněk bortezomibem zůstává aktivace iniciačních kaspáz 8 a 9, které předávají apoptotický signál efektorovým kaspázám štěpícím obsah buňky zevnitř. Iniciační kaspázy mohou být aktivovány nejrůznějšími způsoby. U bortezomibem navozené inhibice proteazomu byla pozorována zvýšená aktivita c-Jun N-terminální kinázy (JNK), která souvisí s apoptózou odehrávající se přes Fas receptor. Fas patří do rodiny TNF receptorů, které po navázání ligandu spouštějí proapoptotický signál, který, pokud není interferován, vyústí v buněčnou smrt. Zablokováním přirozeného obratu intracelulárních proteinů dochází také k disproporcionaci apoptotických proteinů rodiny Bcl-2 ve prospěch jejich proapoptotických členů, což vyústí v permeabilizaci vnější mitochondriální membrány a taktéž v aktivaci efektorových kaspáz. Další cestou je aktivace proteinu p53 s výraznými proapoptotickými účinky a jeho následná stabilizace štěpením příslušného ubikvitin-ligačního enzymu MDM2 [21,22].

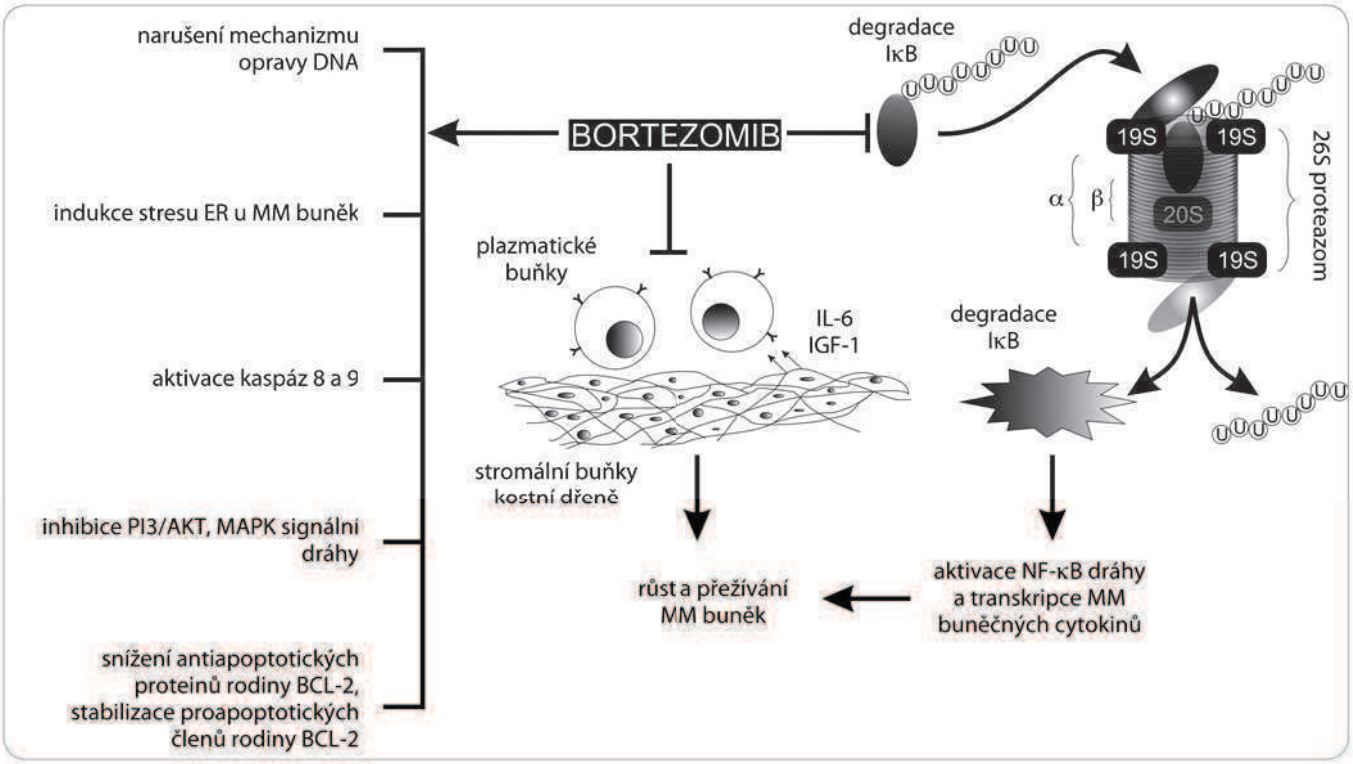
Dále bortezomib zabraňuje opravám poškozené DNA, indukuje stres endoplazmatického retikula (ER) v buňkách MM, snižuje adhezi nádorových buněk k buňkám kostní dřeně inhibicí signalizační dráhy MAPK, zabraňuje nádorové angiogenezi a podílí se na apoptóze osteoklastů a diferenciaci osteoblastů [23,24] (obr. 2).

Klinické studie

Klinické studie fáze II, SUMMIT a CREST, potvrdily antimyelomový účinek bortezomibu, který byl pozorován ve studiích preklinických fází a v první fázi klinického výzkumu [25,26]. Do studie SUMMIT bylo zařazeno 202 pacientů, z nichž 193 bylo následně hodnoceno. Léčebné odpovědi dosáhlo 35 % z nich, přičemž kompletní remise byla pozorována v sedmi případech, a to ve velmi krátké době (medián odpovědi 1,3 měsíce). U dalších 24 % pacientů byla pozorována stabilizace nemoci. Navíc, 74 pacientů s nedostatečnou odpovědí na monoterapii bortezomibem dostalo v kombinaci dexametazon a v 13 případech bylo dosaženo následného zlepšení léčebné odpovědi [25].

Mezinárodní randomizovaná klinická studie fáze III APEX srovnávala účinnost bortezomibu v porovnání s dexametazonem. Do studie bylo zapojeno 669 pacientů s relabujícím MM, z nichž bylo nakonec vyhodnoceno 627. Již při prvních výsledcích bylo zřejmé, že bortezomib je signifikantně mnohem účinnější než dexametazon v léčebné odpovědi (38 % vs 18 %), kompletní remisi (6 % vs < 1 %) i v jednoročním přežití (80 % vs 66 %) [18].

Klinická studie fáze III VISTA byla podkladem pro schválení bortezomibu pro



Obr. 2. Mechanizmus působení bortezomibu. Bortezomib blokuje proces degradace IκB, která je klíčem k aktivaci NFκB. Dále bortezomib snižuje adhezi plazmatických buněk ke stromálním buňkám kostní dřeně a navozuje apoptózu aktivací kaspáz 8 a 9. Bortezomib v buňce narušuje mechanizmus opravy DNA, indukuje apoptózu narušením membrány endoplazmatického retikula, inhibuje MAPK signální dráhy, snižuje hladiny antiapoptotických proteinů a stabilizuje hladiny proapoptotických proteinů.

primoléčbu. Cílem její pozdější analýzy bylo potvrdit, že kombinace bortezomibu s melfalanem a prednisonem zvyšuje celkové přežití nemocných [27].

Minulý rok vyšla studie francouzské skupiny, která porovnávala použití bortezomibu subkutánně (s.c.) proti intravenóznímu podávání (i.v.) s překvapivými výsledky. Ve studii bylo hodnoceno celkem 222 nemocných randomizovaných v poměru 2 : 1 (s.c. : i.v.). Z výsledků studie vyplývá, že všechny sledované parametry související s léčebnou účinností léků byly velmi podobné a nezávislé na použití aplikační cesty. Ale četnost nežádoucích účinků při podkožním podání byla pro určité typy toxicit nejméně o 10 % nižší (gastrointestinální, dýchací potíže a potíže související s mediastinem a hrudníkem, průjem), především však byla nižší četnost výskytu periferní polyneuropatie, která při intravenózním podání bortezomibu znamená závažný problém. Periferní neuropatie stupně 2 a výše byla pozorována u 38 % při podkožním podání oproti 53 % u nemocných s intravenózním podáním. Pro polyneuropatie stupně 3–4 byly poměry rovněž ve prospěch podkožního podání (6 % vs 16 %). Při podkožním podání nelze konstatovat, že by poškození nervů přestalo být problémem, ale jde o významnou redukci četnosti nežádoucího účinku výhradně změnou cesty podání [28]. V EU se přechází na podání bortezomibu jednou týdně, lze tedy očekávat – v kombinaci s podkožním podáním – další redukci četnosti polyneuropatie. Evropská komise EMA již odsouhlasila na základě těchto výsledků možnost podkožního podání bortezomibu v běžné praxi.

Toxicita, rezistence a limity

Původní hypotéza, že biologické účinky inhibice proteazomu bortezomibem jsou nezávislé na typu buňky s fatálním dopadem pro lidský organizmus, byla vyvrácena. Faktem však zůstává, že působení bortezomibu na ostatní buňky organizmu se odráží v podobě relativně vysoké míry nežádoucích účinků. Mezi ty hematologické patří trombocytopenie, kdy s každým podáním bortezomibu dochází k poklesu počtu trombocytů. Dále anémie, dyspeptické poruchy a zejména periferní neuropatie, které bývají hlavním důvodem pro ukončení léčby [13,15,29]. Periferní neuropatie se vyskytuje s incidencí 35–55 % po podání bortezomibu a je pravděpodobně způsobena účinkem bortezomibu na proteázu HtrA2/Omi – stresem indukovanou proteázu důležitou pro přežití nervových buněk a formaci neuritů [30].

Navzdory vysoké účinnosti bortezomibu disponuje až 60 % pacientů primární rezistencí nebo se u nich v průběhu léčby vyvine rezistence sekundární [25]. Doposud bylo identifikováno několik málo molekulárních mechanizmů, pomocí kterých tyto rezistence mohou vznikat. Jedním z nich jsou mutace v propeptidovém lokusu pro β5 podjednotku proteazomu a její nadměrná syntéza [31]. Se zvýšenou rezistencí nádorových buněk také koreluje zvýšená hladina antiapoptotických proteinů rodiny Bcl-2 a proteinů tepelného šoku Hsp 27, 70 a 90 [32,33].

Studie Zhang et al (2011) odhalila, že u buněčných linií rezistentních k bortezomibu zůstala enzymatická aktivita proteazomu inhibovaná i po další léčbě bortezomibem stejně jako u buněčné linie citlivé k tomuto léku. Tyto výsledky naznačují, že mechanizmus rezistence se objevuje až později v signalizační kaskádě [34].

V poslední době se navíc ukázalo, že přírodní produkty obsahující vicinální dioly jsou schopné inhibovat účinek bortezomibu vazbou na zbytek kyseliny borité. Mezi tyto produkty patří zelený čaj obsahující epigalokatechin-3-galát a vitamin C [35,36].

Druhá generace inhibitorů proteazomu

Úspěch bortezomibu vzbudil zájem vědecké obce o proteazomové inhibitory. Optimalizace dávek a kombinace bortezomibu s jinými protinádorovými terapeutiky sice omezily jeho vedlejší účinky a částečně potlačily rezistenci, bylo však jasné, že druhá generace inhibitorů proteazomu může přinést daleko lepší výsledky. Carfilzomib, Marizomib a MLN9708 reprezentují druhou generaci IP a nabízejí řadu výhod v podobě zvýšené účinnosti, bezpečnosti lékového profilu a překonání rezistence k bortezomibu díky své odlišné chemické struktuře, biologickým vlastnostem, mechanizmu účinku, i/reverzibilitě inhibice proteazomu a způsobu užívání [37].

Carfilzomib

Carfilzomib (známý též jako PR-171, Kyprolis, Onyx Pharmaceuticals) je tetrapeptidový epoxyketon se sumárním vzorcem C40H57N5O7 (obr. 3). V preklinických výzkumech byl identifikován jako vysoce potentní IP. Bylo prokázáno, že dokáže navodit apoptózu u bortezomib-naivních i u bortezomibem předléčených myelomových buněk bez zvýšené toxicity a je schopen překonat primární i sekundární rezistenci [38,39]. Ačkoli mechanizmus překonání rezistence nebyl doposud zcela objasněn, jedním z možných vysvětlení může být odlišný způsob a typ vazby, který dovoluje obejít důsledky mutací v genech pro proteazomové podjednotky. Jiným vysvětlením může být jeho vyšší selektivita vůči katalytickým podjednotkám imunoproteazomu, jehož význam doposud nebyl dostatečně reflektován [39–41].

V červenci 2012 schválila FDA carfilzomib pro léčbu pacientů s MM, kteří už mají za sebou více než dvě linie léčby včetně bortezomibu a nějakého imunomodulačního léku a u kterých došlo k progresi onemocnění do 60 dnů od ukončení předchozí léčby (www.onyx.com). Předpokládá se, že nejpozději do dvou let od schválení v USA bude lék schválen i pro Českou republiku.

Mechanizmus působení

Inhibice chymotrypsinové podjednotky proteazomu carfilzomibem je z mechanického hlediska ireverzibilní reakcí snižující aktivitu proteazomu na méně než 20 %. Znovunastolení proteazomové aktivity v buňce je možné pouze nasyntetizováním jednotlivých podjednotek a jejich sestavením do nových proteazomů [38].

Carfilzomib se primárně navazuje na β5 katalytickou podjednotku proteazomu a LMP7 podjednotku imunoproteazomu s vyšší selektivitou než bortezomib [7]. Navázáním epoxybutanového farmakoforu, který vykazuje vysokou specifitu vůči hydroxylové a především

aminové skupině N-terminálního Thr1, vzniká šestičlenný morfolinový kruh. Tato intermolekulární cyklizace probíhá dvoustupňovým mechanizmem, kdy tedy kyslík hydroxylové skupiny Thr1 nukleofilně atakuje uhlík epoxyketonu za vzniku hemiacetalu. Druhým krokem je nukleofilní atak α-amino dusíku Thr1 na C2 uhlík epoxidového kruhu, což má za následek vytvoření morfolinového aduktu [42,43].

V buňkách MM vystavených působení carfilzomibu byla pozorována indukce vnější i vnitřní apoptotické kaskády s výrazným zvýšením hladiny kaspázy 3, 7, 8 a 9. Programovaná buněčná smrt byla asociována s aktivací JNK, depolarizací mitochondriální membrány, vylitím cytochromu c, počátečním poklesem fosforylovaného eIF2 v souvislosti se stresem endoplazmatického retikula navozeným akumulací nefunkčních proteinů a zvýšením hladiny proapoptotického proteinu Noxa, který je členem rodiny proteinů Bcl-2 [7,38].

Klinické studie

První klinická studie s carfilzomibem pro léčbu hematologických malignit byla zahájena v září 2005. Studie 29 pacientů prokázala snášenlivost a klinickou aktivitu terapeutika. Objektivní léčebné odpovědi bylo dosaženo u dvou z deseti pacientů s MM [44]. Pokračováním fáze I bylo vyhodnocení bezpečnosti a účinnosti carfilzomibu v kombinaci s lenalidomidem a dexametazonem, standardními léky pro pacienty s relabujícím myelomem. Ačkoli se jedná teprve o první zhodnocení studie, Niesvizky et al (2009) zaznamenali klinický přínos v 78 % případů [45]. U šesti pacientů došlo ke kompletní remisi a nebyly pozorovány závažné vedlejší účinky. Výsledky by měly být potvrzeny v rámci fáze III začínající klinické studie ASPIRE. Nedávno byly publikovány výsledky otevřené multicentrické studie fáze II s carfilzomibem v monoterapii pro relabující/refrakterní myelom. Do studie bylo zařazeno 129 pacientů, z nichž 47,6 % dosáhlo léčebné odpovědi. Ve třech případech došlo ke kompletní remisi [46].

V klinické studii fáze I/II u nově diagnostikovaných pacientů s MM léčených kombinací carfilzomibu, lenalidomidu a nízko dávkovaného dexametazonu dosáhlo 62 % pacientů téměř kompletní remise [47]. Odpověď na léčbu se zlepšovala v průběhu času a tento léčebný režim byl vyhodnocen jako vysoce účinný a dobře tolerovatelný.

Toxický profil carfilzomibu není zdaleka tak bohatý jako u bortezomibu. Periferní neuropatie byly pozorovány u relativně nízkého procenta pacientů a nejčastějšími vedlejšími účinky byly únava, anémie, trombocytopenie a nauzea [46].

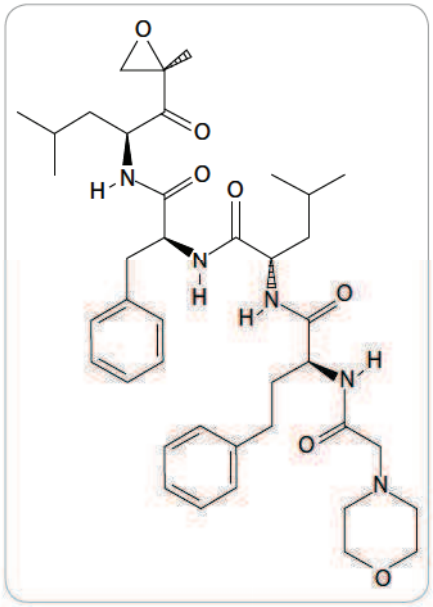
Marizomib

Marizomib, známý také jako NPI-0052 nebo Salinosporamid A, je prvním přírodním proteazomovým inhibitorem, který byl zařazen do klinického výzkumu MM. Jedná se o produkt obligátní mořské bakterie, aktinomycéty Salinispora tropica [48]. Po chemické stránce je marizomib bicyklo γ-laktam-β-lakton se sumárním vzorcem C15H20ClNO4 (obr. 4). Na rozdíl od předešlých IP ve své struktuře neobsahuje peptidový řetězec. V preklinických výzkumech s buněčnými liniemi MM bylo prokázáno překonání rezistence na konvenční terapie a léčbu bortezomibem spolu s vyšší účinností. Kombinace bortezomibu s marizomibem by mohla umožnit používat takovou koncentraci jednotlivých léků, která nepůsobí na pacienty toxicky a zlepšuje společný antimyelomový účinek jednotlivých léků [49].

Mechanizmus působení

Marizomib se přednostně navazuje do β5 a β1 katalytického místa proteazomu a s nižší afinitou také na β2 podjednotku. Za ireverzibilnost vazby zodpovídá chloretylová skupina substituující β-lakton. Skupina se navazuje do S2 vazebné kapsy aktivního místa a chlor se chová jako odstupující atom, čímž umožní vytvořit stabilní komplex konečného produktu po acylaci Thr1O β-laktonem inhibitoru [49].

Na rozdíl od bortezomibu, který aktivuje kaspázu 8 i 9, je apoptotický účinek marizomibu zprostředkován především kaspázou 8, v menší míře pak kaspázou 9, ale s odlišným mechanizmem aktivace než u bortezomibu, čímž překonává rezistenci myelomových buněk s mu-

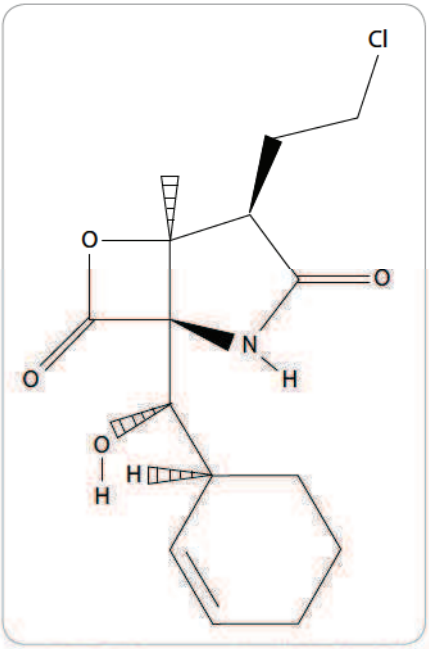


Obr. 3. Struktura carfilzomibu (The PubChem Project).

tacemi v genech pro proteiny rodiny Bcl-2. Dochází tedy k uvolnění cytochromu c a proteinu Smac z mitochondrií do cytosolu, generaci kyslíkových radikálů a aktivaci výše zmíněných kaspáz. Studie Chauhana et al (2005) prokázala, že marizomib je schopen indukovat apoptózu u myeloomových buněk dokonce i v přítomnosti myelomových růstových faktorů, IL-6 a IGF-1 [32]. Navíc se podílí na zablokování sekrece IL-6 stromálními buňkami kostní dřeně, aniž by došlo k ovlivnění jejich životaschopnosti. Marizomib významně blokuje migraci buněk MM indukovanou VEGF, a potvrzuje tak i své antiangiogenní účinky. U marizomibu byla pozorována jako u jednoho z mála IP i inhibice kanonické NF-κB dráhy a následné sekrece cytokinů [33].

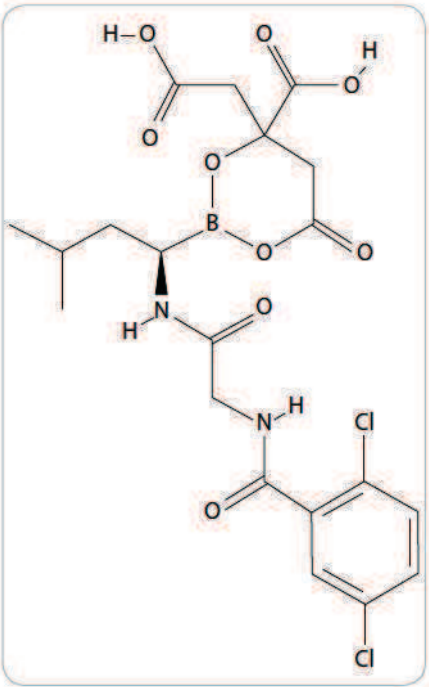
Klinické studie

V současné době se marizomib nachází v doposud nevyhodnocené I. fázi klinické studie [50]. Další klinické studie I právě nabírají pacienty. Jedná se o studii pacientů s relabujícím nebo refrakterním myelomem léčených marizomibem (NPI-0052-101) a studii pacientů trpících některou z pokročilých malignit (NPI-0052-102). Mezi zatím nejčastěji pozorované nepříznivé účinky tohoto léku patří únava, nespavost, ne-



Obr. 4. Struktura marizomibu (The PubChem Project).

volnost, průjem, zácpa, bolesti hlavy, halucinace, změny v kognitivních funkcích, ztráta rovnováhy nebo horečky. Významný výskyt periferní neuropatie však nebyl pozorován. Tato data naznačují, že marizomib by mohl být bezpečným lékem bez zkřížené rezistence s ostatními IP a aktivním u pacientů refrakterních k bortezomibu [51,52].



Obr. 5. Struktura MLN9708/MLN2238 (The PubChem Project).

MLN9708

MLN9708 je analog kyseliny borité a první orálně podávaný IP druhé generace, který v preklinických studiích prokázal větší potenciál účinku proti buňkám MM *in vivo* než bortezomib [53]. Jde o reverzibilní typ IP, který ve vodných roztocích či plazmě okamžitě hydrolyzuje na MLN2238, jeho biologicky aktivní formu (obr. 5) [54]. Je tedy schopen rozsáhlejší distribuce do krve ve stabilní formě a má vyšší farmakodynamický účinek v tkáních [51].

Mechanizmus působení

MLN9708 (MLN2238), stejně jako bortezomib, inhibuje především chymotrypsinovou proteolytickou (β5) podjednotku 20S proteazomu. Navíc je schopen ve vyšších koncentracích inhibovat kaspázovou (β1) a trypsinovou (β2) proteolytickou podjednotku a indukovat akumulaci ubikvitinovaných proteinů [53,55]. K rozpadu 20S podjednotky proteazomu po léčbě MLN9708 dochází šestkrát rychleji než po léčbě bortezomibem.

MLN9708 je zodpovědný za kaspázově dependentní indukci apoptózy myelomových buněk. Po podání dochází k aktivaci kaspáz 8, 9 a 3; dále k navýšení hladiny proteinů p53, p21, NOXA, PUMA, E2F a naopak ke snížení hladiny cyklinu D1 a CDK6. Léčba pomocí MLN9708 vedla také k indukci exprese Bip a CHOP, proteinů stresové odpovědi ER a k účinné inhibici kanonické i nekanonické dráhy NF-κB ovlivňujíce tak sekreci cytokinů důležitých pro růst a přežívání myelomových buněk stromálními buňkami kostní dřeně. Takto jsou narušeny cytoprotektivní účinky mikroprostředí kostní dřeně. Chauhan et al (2011) dále pozorovali snížení počtu buněk nesoucích VEGFR2 a PECAM, což naznačuje inhibici nádorem indukované angiogeneze [53].

Lee et al (2011) ve své studii na myších modelech prokázali, že na rozdíl od bortezomibu, MLN9708 pravděpodobně také zmírňuje osteolýzu kostí, nejčastější příznak MM [54]. Profilování miRNA v buňkách MM léčených MLN9708 prokázalo zvýšenou expresi miR-33b. Zvýšená exprese této miRNA je asociovaná se sníženou schopností migrace a životaschopností buněk MM stejně jako se zvýšenou apoptózou a citlivostí myelomových buněk k léčbě MLN9708. Navíc zvýšená exprese miR-33b vede k negativní regulaci onkogenu PIM-1. Ve studii Tiana et al (2012) bylo tedy naznačeno, že miR-33b funguje jako nádorový supresor, který se podílí na apoptóze myelomových buněk vyvolané léčbou MLN9708, což vede k inhibici růstu nádoru a prodloužení přežití lidských myelomových xenoimplantátových modelů [56].

Klinické studie

V současné době hodnotí několik klinických studií fáze I bezpečnost MLN9708 u různých populací pacientů léčených různými dávkami tohoto léku. Dvě probíhající studie (C16004 a C16003) hodnotí účinek MLN9708 v monoterapii u pacientů s relabujícím či refrakterním myelomem, kteří byli dříve léčeni některým z IP. Mezi nejčastější nepříznivé účinky léčby MLN9708 patří únava, trombocytopenie, nevolnost, průjmy, zvracení a méně často neutropenie. Je však důležité, že po léčbě MLN9708 trpí pouze 10 % pacientů periferní neuropatií.

Dále probíhají studie účinnosti tohoto léku v různých kombinacích u nově diagnostikovaných pacientů. Jde např. o studii účinku MLN9708 v kombinaci s melfalanem a prednisonem (C16006) a v kombinaci s lenalidomidem a nízkými dávkami dexametazonu (C16005 a C16008) [46]. Bylo rovněž zjištěno, že MLN9708 v kombinaci s lenalidomidem vykazuje synergistickou antimyelomovou aktivitu a tato kombinace léků má potenciál pro klinické studie, neboť jde o vysoce účinný perorální léčebný režim bez známek periferní neuropatie [53].

Budoucnost inhibitorů proteazomu

Doposud byly identifikovány čtyři významné mediátory přímé antimyelomové aktivity IP, a to transkripční faktor NF-κB, pro- a antiapoptické faktory, protein p53 a proteiny stresové odpovědi ER. Účinek IP by však měl být chápán jako komplexní děj zahrnující na mnoha místech všechny tyto hlavní mechanizmy,

neboť ani samotná inhibice NF-κB ani samotná mutace zajišťující inaktivitu proteinu p53 nezastaví apoptózu myelomových buněk indukovanou IP. Identifikace podrobných mechanizmů působení IP má velký potenciál pro odhalení možných molekulárních cílů pro budoucí léčiva. Příkladem mohou být dnes známé specifické deubikvitinační enzymy, které mohou zastavit degradaci určitých molekul bez nutné inhibice proteazomu.

Na otázku, jak zajistit vyšší selektivitu, účinnost a výrazně omezit vedlejší účinky dnešních IP, mohou nabídnout odpovědi specifické inhibitory imunoproteazomu.

S ohledem na heterogenní podstatu MM lze do budoucna počítat se zavedením genetického screeningu jednotlivých genových sad kódujících klíčové molekuly, na jehož základě by byla zahájena optimální léčba s predikcí stupně účinnosti IP u jednotlivých nemocných.

Závěr

Důležitou a vysoce efektivní strategií při hledání léčebných přístupů k MM je poznání důležité role, kterou v léčbě tohoto heterogenního onemocnění hraje inhibice proteazomu bortezomibem. Toto poznání vedlo k vývoji IP druhé generace, které se liší ve svých chemických strukturách (boronáty, epoxyketony, salinosporamidy), účinnosti a toxických profilech, a poskytují tak nové možnosti pacientům, kteří se stali rezistentními k bortezomibu. Hlavní cesta dalšího výzkumu ve vývoji nových léčiv by se tedy měla ubírat směrem lepšího porozumění mechanizmům účinku IP a především mechanizmu, kterým buňky získávají rezistenci k těmto lékům.

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High-Risk Multiple Myeloma: Different Definitions, Different Outcomes?

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Review

High-Risk Multiple Myeloma: Different Definitions, Different Outcomes?

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Abstract

Multiple myeloma (MM) is a clonal plasma cell malignancy. Although MM is still not completely curable, it can be maintained at the level of a long-term chronic condition. Irrespective of the treatment strategy, relapse is still a major problem for most patients. Approximately 10% to 15% of all MM patients relapse early and have poor prognosis and outcome. Currently, there are many ways of identifying these high-risk patients using cytogenetics or molecular biology. Despite these various approaches to definition of high risk patients, a clear definition of high-risk MM has not been widely accepted. In this review, we discuss and compare various approaches, and their strengths and weaknesses in early identification of high-risk MM patients.

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Introduction

Multiple myeloma (MM) is a malignant B-lymphoproliferative disease characterized by infiltration of clonal plasmocytes in the bone marrow, osteolytic lesions of the skeleton, and presence of monoclonal immunoglobulin (M-protein) in serum and/or urine.[1] MM accounts for 10% of all hematologic malignancies.[2] It is the second most common hematologic cancer and represents 1% of all cancer diagnoses and 2% of all cancer deaths.[3] Despite new advances in the treatment of MM, it remains mostly an incurable disease.

MM progresses from a premalignant stage called monoclonal gammopathy of undetermined significance (MGUS).[4] MGUS is a plasma cell proliferative disorder characterized by plasma cell content of less than 10% in the bone marrow, M-protein in serum < 30 g/L, no end organ damage including bone lesions, and no evidence of other B-cell proliferative disorder.[5] Smoldering myeloma (SM), also called asymptomatic myeloma, is an intermediary between MGUS and MM. SM has M-protein in serum ≥ 30 g/L and/or bone marrow plasma cells $\geq 10\%$, and no related organ or tissue impairment or symptoms. Symptomatic MM is a disease characterized by neoplastic proliferation of a single clone of plasma cells producing M-protein, inducing end organ damage, including bone lesions, anemia, renal insufficiency, and hypercalcemia (CRAB symptoms).[5] The comparison of the stages is shown in Table 1.[5]

Extramedullary MM arises outside the bone marrow when the clonal plasma cells are capable of leaving the bone marrow niche and infiltrate virtually any organ. Extramedullary disease can accompany newly diagnosed disease or relapse and has dismal outcome for patients.[6] It is considered a poor prognostic marker in newly diagnosed and in relapsed patients and is more prevalent in genomically defined high-risk MM.

Generally, MM can be divided into 2 subgroups that are approximately equally distributed.[7] Hyperdiploid MM is characterized mostly by numerical gains (eg, multiple trisomies) and few structural changes, and nonhyperdiploid tumors are characterized by many chromosomal rearrangements (eg, translocations involving region 14q32) and sometimes chromosome loss.

MM is a heterogeneous disease at the genetic level and in terms of clinical outcome.[8] The etiology is still unclear and pathogenesis is a complex multifactorial process.[1] It is known that there are some changes in the microenvironment of the bone marrow that allow the tumor to grow while the function of the immune system is decreased. The outcome for patients with MM is highly variable.[9] Although the median overall survival time is 3 to 4 years, the range is from less than 6 months to more than 10 years. Many reports have described a huge number of prognostic factors in MM.[10] In this list, there are many factors that have been confirmed by several studies: the most important parameters are probably $ß_2$-microglobulin, proliferation index, and genetic abnormalities (Table 2).[10]

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Table 1 Stages of MM

MGUS	• M-protein in serum <30 g/L • Bone marrow clonal plasma cells <10% • No related organ or tissue impairment (no end organ damage, including bone lesions) • No evidence of other B-cell proliferative disorders
Asymptomatic (smoldering) myeloma	• M-protein in serum ≥30 g/L and/or • Bone marrow clonal plasma cells ≥10% • No related organ or tissue impairment (no end organ damage, including bone lesions) or symptoms
Symptomatic MM	• M-protein in serum and/or urine • Bone marrow clonal plasma cells • Related organ or tissue impairment (end organ damage, including bone lesions)
Extramedullary MM	• No M-protein in serum and/or urine • Extramedullary tumor or clonal plasma cells • Normal bone marrow • Normal skeletal survey • No related organ or tissue impairment (end organ damage, including bone lesions)

Abbreviations: MGUS = monoclonal gammopathy of undetermined significance; MM = multiple myeloma; M-protein = monoclonal immunoglobulin.
Adapted from International Myeloma Working Group 2003. Criteria for the classification of monoclonal gammopathies, multiple myeloma and related disorders: a report of the International Myeloma Working Group. Br J Haematol 2003; 121:749-57.

Clinically, relapse is defined as ≥ 25% increase in the serum or urine protein ≥ 0.5 mg/dL; however, the presence of 'biochemical relapse' alone is not an indication for additional systemic therapy. Patients should also have some form of symptomatic relapse before initiation of therapy.[11]

The overall objective of creating a strong staging system for distinguishing patients with different risk is the identification of risk groups that could in particular have improved outcome because they would be considered for different treatment decisions. Novel therapies might benefit patients for whom other therapies fail.[12] In high-risk patients, preliminary reports show high response rates with use of novel drugs, such as bortezomib, lenalidomide, and thalidomide, suggesting that the effect of adverse prognostic factors might be overcome when using this type of therapy. The use of genetic information for risk stratification and treatment selection continues to be investigated in clinical trials and will probably have greater significance for clinical research and patient care in the near future.

Discussion

MM Stratification Systems

First, Durie and Salmon introduced a staging system of 3 different stages, each presented by different levels of selected clinical features that were significantly correlated with measured myeloma cell mass—extent of bone lesions, hemoglobin, and level of serum and/or urine M-protein, serum calcium, and serum creatinine.[13] Creatinine level further defined lower risk (with relatively normal renal function; serum creatinine value < 2.0 mg per 100 mL) and higher risk patients (with abnormal renal function; serum creatinine value ≥ 2.0 mg per 100 mL) in each of the 3 stages. The Durie Salmon system was created predominantly to identify some level of tumor burden at the time of diagnosis, but according to Tuchman and Lonial,[14] its utility in the setting of prognosis in the era of new drugs is a bit limited. However, it is still considered a means of measuring tumor mass and should be mentioned to compare patient's outcome with previously diagnosed cases of MM.[15]

Table 2 Summary of the Most Common Parameters That Accompany Poor Prognosis

Parameter	Poor-Prognosis Values
Plasma Cell Leukemia	
17p Deletion	Present
International Staging System	Stage 3
ß$_2$-Microglobulin	≥5.5 mg/L
Gene Expression Profiling	University of Arkansas 70-gene model or Intergroupe Francophone du Myélome 15-gene model

Adapted from Avet-Loiseau. Ultra high-risk myeloma. Hematology Am Soc Educ Program 2010; 2010:489-93.

In an effort to ensure a more objective classification of patients, the International Myeloma Working Group (IMWG)[9] described the International Staging System (ISS) based on ß$_2$-microglobulin and albumin levels (Table 3).[9] These clinical parameters, chosen because of their wide availability and simplicity of their identification in blood tests, classify MM patients into 3 groups with different overall survival (62 months, 44 months, and 29 months for stages 1, 2, and 3, respectively). ISS has been validated in young and older patients, in patients treated with conventional chemotherapy, autologous stem cell transplantation, or novel agents at diagnosis and relapse, and even though it is more than a decade old, it still represents the most widely used staging system for patients with MM.[16] ISS provides useful information regarding the baseline biological characteristics of the disease. Because of its simplicity and reproducibility, the ISS has demonstrated its value in comparing outcome of clinical trials. However, it has some important limitations, eg, ISS identifies just 3 large prognostic groups, but MM patients are described as a very heterogenic group that cannot be included in only 3 prognostic categories. Identification of the highest risk patients is achieved in only a small number of patients (from 5% to 9%) and better identification of these patients requires a more refined cytogenetic and molecular genetic classification. Another limitation is its focus on prognostication at the population level, so it is not applicable for individualized treatment decisions.[10]

High-Risk Definition Using Cytogenetics

Because only dividing cells can be analyzed, the low proliferative activity of tumor cells early in the disease is a significant limitation of conventional cytogenetics in MM.[8] This limitation has been partly overcome by the use of fluorescence in situ hybridization (FISH), multicolor FISH, comparative genomic hybridization (CGH), and spectral karyotyping. The study of Kapoor et al[17] reinforced the importance of using conventional cytogenetics and interphase FISH (iFISH) for risk assessment. These methods remain independent prognostic tools despite the introduction of novel agents and are now a part of risk stratification models. Most large

Table 3 International Staging System

Stage	Criteria	Median Survival (Months)
1	• Serum $ß_2$-microglobulin <3.5 mg/L • Serum albumin ≥3.5 g/dL	62
2	• Serum $ß_2$-microglobulin <3.5 mg/L, but serum albumin <3.5 g/dL or • Serum $ß_2$-microglobulin from 3.5 to <5.5 mg/L irrespective of the serum albumin level	44
3	• Serum $ß_2$-microglobulin ≥5.5 mg/L	29

Adapted from Greipp et al. International staging system for multiple myeloma. J Clin Oncol 2005; 23:3412-20.

series have used iFISH, although this technique has some weaknesses in MM.[10] It allows analysis of only a limited number of chromosomal abnormalities and requires plasma cell identification and purification. Still, iFISH was able to detect genomic changes in almost 90% of patients at the time of diagnosis, which is approximately 3 times more frequent than with conventional cytogenetics banding methods.

Chromosomal abnormalities in MM are complex, highly variable, and long chromosomes are altered numerically and structurally.[8] Their complexity is reflected in a median number of 8 to 10 karyotypic changes per patient at diagnosis. Smadja et al[18] were the first to describe the importance of chromosome ploidy number, and they identified the significant difference in survival between hyperdiploid and nonhyperdiploid patients. The nonhyperdiploid group is associated with poorer overall survival and with presence of structural abnormalities, typically translocations involving the immunoglobulin heavy chain locus (IgH) located at 14q32. Based on a multivariate analysis of several prognostic factors, nonhyperdiploidy was shown as the most important independent factor for overall survival. The hyperdiploid group has better overall survival and is associated with numerical aberrations (multiple trisomies of chromosomes 3, 5, 7, 9, 11, 15, 19, and 21).

There are 5 main IgH translocations involving 11q13 (*CCND1* [cyclin D1]), 4p16 (*FGFR3* [fibroblast growth factor receptor 3] and *MMSET* [multiple myeloma SET domain]), 16q23 (*MAF* [v-maf musculoaponeurotic fibrosarcoma oncogene homolog]), 20q12 (*MAFB*), and 6p21 (*CCND3* [cyclin D3]).[19] These translocations are mostly found in the nonhyperdiploid group and are characterized by overexpression of translocated genes.[20] IgH translocations are considered to be primary events and rather negative prognostic factors.[21] However, the most frequent translocation t(11;14)(q13;q32), which is found in 15% to 20% of patients, is usually found as neutral with regard to prognosis—in most series it seems to be associated with favorable outcome, but this effect is not strong enough to be statistically significant and there is great heterogeneity in MM patients with this translocation.[22] The second most frequent translocation is t(4;14)(p16;q32) which occurs in 10% to 15% of patients and results in overexpression of 2 protein-coding genes located at 4p16—*FGFR3* and *MMSET*.[23] It has been associated with poor survival and is often associated with changes of chromosome 13.[24] The t(14;16)(q32;q23) and t(14;20)(q32;q12) are 2 less frequent but presumably clinically important translocations that involve the *MAF* genes.[22] Both of them appear to be associated with poor survival because *MAF* and *MAFB* are known oncogenes and their deregulation might play a role in MM oncogenic transformation.[25] The prognostic value of t(14;16) was further analyzed in a retrospective study that compared the outcome of patients with and without this translocation.[26] Even though the incidence is low, the results did not confirm poor prognostic value of t(14;16) in contrast to other prognostic parameters; its role in the distinction of high-risk MM remains unclear.

Rearrangements of chromosome 1 are the most common structural aberrations, mostly involving (mainly interstitial) deletions of 1p and amplifications of 1q[27]; some cases showed more than 1 abnormality.[21] Deletions of 1p are associated with poor prognosis[28]; patients with 1q21 gain or amplification detected using FISH have unfavorable prognosis and significantly shorter survival.[29] An association between 1q21 gain and del(13) was found, but no association with translocations t(4;14), t(11;14), or del(17p), and it can be considered as another independent prognostic factor.

Chromosome 13 abnormalities are found in approximately 45% to 50% of cases; most of these cases are complete monosomy 13 (90%), and the remaining 10% represent del(13).[30] Initially it appeared that these abnormalities have an important effect on patients' survival—partial or complete loss of chromosome 13 seemed to be connected with aggressive clinical course and an unfavorable prognosis.[31] However, subsequent analyses showed that this abnormality alone is not a negative prognostic factor and its assumed effect comes from known close association with other high-risk genetic features, such as t(4;14),[32] del(17p) or high serum level of $ß_2$-microglobulin.[3] This observation further demonstrates that the presence of t(4;14) is sufficient for shortening survival and should be considered the most relevant cytogenetic prognostic marker for MM patients.

Deletion or inactivation of the *TP53* gene occurring at 17p13 is more frequent in advanced MM stages and has been identified as a clinical indicator of very poor prognosis because patients with del(17p) have more aggressive disease, higher prevalence of extramedullary disease, and overall shorter survival.[33] When compared with patients lacking *TP53* abnormality, del(17p) is also frequently associated with mutations of the other *TP53* allele.[34] Because the position of these mutations might determine the disease outcome, further and larger analysis of MM is needed.

High-risk cytogenetic markers were defined in several studies[3,17,35,36] as the presence of any one or more of these abnormalities—hypodiplody, monosomy of chromosome 13 or deletion 13q, deletion of *TP53* (locus 17p13), IgH translocations t(4;14)(p16;q32) or t(14;16)(q32;q23), and plasma cell labeling index of 3% or greater. Decaux et al,[37] confirmed that the high-risk group of patients was significantly associated with deletion of 13q, deletion of 17p, gain of 1q, and translocation t(4;14). The low-risk group was significantly correlated with hyperdiploid status determined using FISH. Considering that the prognostic value of cytogenetic abnormalities depends strongly on their coexistence with each other, eg, deletion of 13q and its association with t(4;14), performing a systematic FISH analysis on all patients with newly diagnosed MM is still of great importance. However, some chromosomal abnormalities, such as t(4;14) or del(17p), have been shown to be major prognostic markers and very useful in refining

Table 4 Clinical and Genetic Features of TC Molecular Subgroups of MM

TC Group	Recurrent Translocation	Gene(s) at Breakpoint	Dysregulated Cyclin D	Multiple Trisomies (n)
6p21	6p21	*CCND3*	D3	7
11q13	11q13	*CCND1*	D1	40
D1	None	None	D1	81
D1 + D2	None	None	D1 + D2	21
D2	None	None	D2	45
None	None	None	None	6
4p16	4p16	*FGFR3/MMSET*	D2	42
maf	16q23, 20q11	*MAF, MAFB*	D2	19

Abbreviations: D1-3 = cyclins D1-D3; maf = v-maf musculoaponeurotic fibrosarcoma oncogene homolog; MM = multiple myeloma; TC = Translocation and Cyclin D.
Adapted from Bergsagel et al. Cyclin D dysregulation: an early and unifying pathogenic event in multiple myeloma. Blood 2005; 106:296-303.

identification of high-risk patients, yet they need to be evaluated in the context of other parameters (especially ß$_2$-microglobulin level).[3] Decaux et al[37] claims that, although powerful, cytogenetic abnormalities target only small subsets of patients and do not account for heterogeneity of the clinical outcome.

High-Risk as Defined Using Genomics

Gene expression profiling (GEP) has enabled analysis of gene expression patterns that can be involved in MM pathogenesis and might contribute to survival of MM patients.

The first molecular classification system was the Translocation and Cyclin D (TC) classification,[38] based on GEP of mRNA spikes involving 5 IgH translocations, specific trisomies, and overexpression of cyclin D genes (*CCND1-3*). The patients were divided into 8 groups (Table 4)[38] defined according to early, perhaps initiating oncogenic events, with differences in GEP and clinical features; this classification suggests that dysregulation of cyclin D is an early and unifying pathogenic event in myeloma. Better survival was observed in the TC 11q13 group and substantially shortened survival was noted for patients in the TC 4p16 and TC 16q23 (*MAF*) groups.

Zhan et al[20] developed a GEP-based classification that divides MM into 7 different groups (Table 5)[20] based on the presence of translocations, gene expression, or hyperdiploidy. In this system, 2 groups were connected with high-risk variables and poor prognosis—the proliferation (PR) group, characterized by overexpression of cell cycle progression and cell proliferation genes, and the MMSET (MS) group, with overexpression of *MMSET* and *FGFR3* genes involving the t(4;14). However, the associations between classes and survival are likely to be dependent on the type of therapy used.

The first validated GEP model for prognosis predication in patients was published by Shaughnessy et al[39] from the University of Arkansas, in the United States. The hypothesis of this work was that expression extremes of a subset of genes that correlates with survival might be representative of the effects of DNA copy changes in myeloma disease progression. They identified a set of 70 genes with expression level changes that allowed the identification of a small cohort of 13% to 14% of patients at high-risk for early disease-related death. A remarkable feature of the high-risk signature was the significant overrepresentation of genes located on chromosome 1—nearly 50% of 19 underexpressed genes and 30% of 51 overexpressed genes are located on chromosome 1. Most upregulated genes are mapped to 1q, and downregulated genes to 1p. This is in accord with the previously published suggestion that progression of disease can also be associated with the percentage of cells with 1q21 amplification.[40] It also suggests that gain of DNA material on 1q and loss of 1p are important determinants of high-risk MM.[39] Results have shown that the low-risk myeloma group had a pattern similar to MGUS and normal plasma cells, and the high-risk group revealed a pattern similar to human myeloma cell lines.

The high-risk group had a strong association with known clinical prognostic variables—higher level of ß$_2$-microglobulin in serum, creatinine, C-reactive protein, and serum lactate dehydrogenase (LDH), chromosome 13 deletion, and other cytogenetic abnormalities.[39] When applied to samples from relapsed patients, 76% exhibited a high risk score. This increase in the frequency of the high-risk label from 13% at diagnosis gives molecular evidence of disease evolution that influences outcome after relapse. The model

Table 5 Genetic Signatures of Expression-Defined Subgroups

Group	Features
PR	Overexpression of numerous cell cycle- and proliferation-related genes (eg, *CCNB2*, *CCNB1*, *MCM2*, *CDCA2*, *BUB1*, *CDC2*, and *TYMS*) and cancer-testis antigen genes; higher GEP-defined proliferation index
LB	Low bone disease (lower expression of genes involved in bone disease and low incidence of magnetic resonance imaging-defined focal bone lesions)
MS	t(4;14)(p16;q32) → overexpression of *MMSET* and *FGFR3* genes
HY	Hyperdiploidy, often associated with trisomies of chromosomes 3, 5, 7, 9, 11, 15, 19, and 21
CD-1	t(11;14)(q13;q32) → overexpression of *CCND1* genes
CD-2	t(6;14)(p21;q32) → overexpression of *CCND3* genes
MF	t(14;16)(q32;23) and t(14;20)(q32;q11) → overexpression of *MAF/MAFB* genes

Abbreviations: CD = cyclin; GEP = gene expression profiling; HY = hyperdiploid; LB = low bone; MF = MAF-MAFB; MS = MMSET; PR = proliferation.
Adapted from Zhan et al. The molecular classification of multiple myeloma. Blood 2006; 108:2020-8.

was then modified to only 17 genes to identify high-risk patients with 97.7% accuracy of the 70-gene model. Unlike other classifications, this high-risk signature model reflects not only tumor cell proliferation, but also other features that indicate shorter survival, such as drug resistance.

Decaux et al[37] developed a 15-gene model that predicts survival in patients with newly diagnosed MM. The Intergroupe Francophone du Myélome (IFM) suggested that their 15-gene model improves ISS prognostication and can be more discriminating than the FISH model for stratifying MM patients according to survival. In their report, they state that myeloma cells from high-risk patients overexpress genes involved in cell cycle progression and its surveillance, whereas the low-risk patient group is more heterogeneous and includes the hyperdiploid gene signature. High-risk MM patients were characterized by overexpression of genes involved in multiple phases of the cell cycle. The list of genes contains cell-cycle regulated genes that are involved in essential cell cycle processes, such as cell-cycle control, DNA replication, DNA repair, DNA packaging, mitosis, and spindle-assembly checkpoint (SAC). Their hypothesis is that the SAC activity network is perturbed in plasma cells of high-risk patients, thereby maintaining mild chromosomal instability that will facilitate tumorigenesis and drug resistance, leading to a targeted therapeutic model in which SAC inactivation might be an efficient way to provoke plasma cell death. In low-risk patients, they identified 3 significant gene sets. Two of them are related to hyperdiploidy in MM. When compared with other prognostic factors ($ß_2$-microglobulin level in serum $\geq$ 5.5 mg/L and/or t(4;14)), their results identified subsets with different survival in low- and high-risk groups. This indicates that there are different biologic features associated with survival, and the combination of these 3 could make an independent prognostic tool to identify the highest risk patients. It was also confirmed that the high-risk group is associated with poor prognosis markers (deletion of 13q and 17p, gain of 1q, and translocation t(4;14)).

Dickens et al[41] used high-density single-nucleotide polymorphism (SNP) arrays to identify homozygous deletions (HZD) in genes relevant to pathogenesis and outcome in MM. This loss of material can be used to find expression signatures and specific genes with prognostic significance. When combined with global gene expression data, they were able to identify key pathologically relevant features. The resulting list of genes with HZD has significantly overrepresented deletions within the 'cell death network' including 15 genes important in cell cycle regulation, apoptosis, and regulation of transcription. Deletion of any of these genes means shorter survival; therefore, it is considered to be an independent marker of poor prognosis. These changes at the DNA level need to be associated with changes at the level of gene expression—this analysis generated a list of 97 genes annotated as cell death and connected with poor outcome. From this list, a more applicable 6-gene cell death signature was derived (*BUB1B* vs. *HDAC3, CDC2* vs. *FIS1, RAD21* vs. *ITM2B*) that was able to identify similar subset of patients with poor prognosis with 100% specificity. If any 1 of these pairs has a ratio of $\geq$ 1, then the test is positive for poor prognosis at presentation and at relapse. Although the 97-gene signature is more sensitive for identification of poor prognosis, the genes selected for the 6-gene signature are highly specific so no patients would be incorrectly classified as such.

In their report, Dickens et al[41] also made a comparison of the 3 signatures: the 70-gene signature from University of Arkansas group,[39] the 15-gene signature from the IFM,[37] and their own 97-gene cell death signature. The genes in each of the signatures are different, except 1 shared gene, *BIRC5* (National Center for Biotechnology Information: location 17q25, member of the inhibitor of apoptosis gene family, which encodes negative regulatory proteins that prevent apoptotic cell death; gene expression is high in most tumors). There were 37 cases identified as poor prognosis using all 3 signatures. Overall, the 97-gene cell death signature identified 89 cases with poor prognosis, the IFM 15-gene signature identified 64, and the Arkansas group 70-gene signature identified 90 of them. Thus, it seems that the sensitivity of the 97- and 70-gene signatures are almost equal. The fact that there are no shared genes highlights the complexity of the biologic behavior of the tumor. Also, use of GEP analyses will possibly require more work.[15]

Moreaux et al[42] derived a high-risk signature from analyzing GEP and identification of 248 heterogeneity genes in 40 human myeloma cell lines (HMCLs) and divided them into 6 groups. The HMCLs used differed in their dependence on the addition of exogenous interleukin (IL)-6 to grow in vitro (24 of 40 referred to as $HMCLs^{serum+IL6}$ and the remaining 16 as $HMCLs^{serum}$). Each of the 6 groups is represented by genes that are known markers of MM, such as *c-MAF*, *CCND1*, *FRZB*, *MMSET*, *FGFR3*, and cancer-testis antigen genes. When applied on primary MM cells of newly diagnosed patients, the resulting clusters overlapped with 7 groups of molecular classification previously described by Zhan et al (Table 6).[20,42] Considering this overlapping, the same nomenclature was used to identify HMCL groups. These data suggest that HMCLs have kept the molecular heterogeneity of MM cells of newly diagnosed patients.

Assuming that some heterogeneity genes could be used as predictors for patients' survival, Moreaux et al[42] found 7 of the 248 HMCL heterogeneity genes with bad prognostic value (*TEAD1*, *CLEC11A*, *LRP12*, *MMSET*, *FGRF3*, *NUDT11*, and *KIAA1671*). Most of them were overexpressed in the MS and PR groups, which were previously described as high-risk.[20] Using these 7 genes, a simple staging was built, scoring patients from 0 to 7, resulting in creation of the HMCL score containing 3 groups with different

Table 6 Overlap Between Clustering of Primary MM Cells Based on HMCL Heterogeneity Gene Signature[42] and 7-Group Molecular Classification[20]

HMCL Heterogeneity Gene Signature	7-Group Molecular Classification
Cluster 1	100% of patients of MS group
Cluster 2	71% of patients of LB group
Cluster 3	100% of patients of MF group
Cluster 4	46% of patients of PR group and 29% of patients of HY group
Cluster 5	92% of patients of HY group
Cluster 6	89% of patients of CD-1 and CD-2 groups

Abbreviations: CD = cyclin; HMCL = human myeloma cell line; HY = hyperdiploid; LB = low bone; MF = MAF/MAFB; MM = multiple myeloma; MS = MMSET; PR = proliferation.

outcome. Group 1 comprised patients with no or 1 bad prognosis gene, group 2 patients expressed from 2 to 4 bad prognosis genes, and group 3 at least 5 of them. Of course, group 3 was associated with the worst prognosis. These 7 HMCL genes share no gene with the 70-gene signature from the University of Arkansas and the 15-gene signature from IFM, and the HMCL score was shown to be more potent in some of the independent patient cohorts they used.

Conclusion

According to Decaux et al,[37] an ideal prognostic model would probably combine ß$_2$-microglobulin level that reflects tumor burden, creatinine level that reflects renal insufficiency, general patient condition (eg, age older than 50 years, presence of primary tumor or other serious disease, long-term corticosteroid use, weight loss, and chronic inflammation of lungs or kidney), a marker of plasma cell proliferation, and genetic changes. It is still recommended to determine patient's stage using a prognostic system based on clinical parameters (Durie Salmon or ISS system) because it enables comparison of outcomes with previously diagnosed cases. After the International Myeloma Workshop Consensus Panel 2, Munshi et al[15] presented recommendations for current risk stratification in MM. For newly diagnosed patients, they suggest to investigate ISS stage using serum albumin and ß$_2$-microglobulin levels (ISS stage), bone marrow cytogenetic examination for t(4;14), t(14;16), and del(17p) using purified plasma cells in FISH analysis, serum LDH level, immunoglobulin type A, histology for plasmablastic disease, and additional investigation of cytogenetics, GEP, labeling index, magnetic resonance imaging/positron emission tomography scan as an emerging tool for bone disease evaluation, and DNA copy number alteration using CGH/SNP array. Patients at relapse are often characterized by changes in these risk factors; their levels are usually rising and patients should be reclassified as high-risk. Additional risk stratification criteria for relapsed patients include type of response and length of response to previous therapy. FISH analysis is mandatory for baseline risk stratification, but should only be repeated at relapse/progression for patients who were not initially classified as high-risk. If a high-risk feature has been already present at diagnosis, then there is no need to test for it again at relapse, although investigation for additional changes should be performed.

Unlike cell-based staging systems that have the longest history and have been successfully validated, genomic tools (either transcriptional or DNA-based) are still evolving and their prognostic significance might be treatment-specific. Overall, the presented GEP models only share a few common genes (mainly because of high clinical and biologic heterogeneity of the disease), which represents a significant complication for accurate assessment of prognosis and for categorization of patients into groups that would be mutually comparable. It is clear that the established prognostic factors do not have a universal value, especially because of their uncertain stability in the era of novel drugs. Identifying risk groups with high predictive power could notably improve patient care—patients predicted to have poor outcome might be considered for early administration of experimental therapy regimens which might ameliorate the adverse influence of these prognostic features. Because of this promising future use, further research for multifunctional prognostic markers or stabilization of existing models is needed. The general agreement is that the risk stratification should be global and not divided for old vs. new therapy.

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Disclosure

The authors have stated that they have no conflicts of interest.

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Circulating serum microRNAs as novel diagnostic and prognostic biomarkers for multiple myeloma and monoclonal gammopathy of undetermined significance

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Circulating serum microRNAs as novel diagnostic and prognostic biomarkers for multiple myeloma and monoclonal gammopathy of undetermined significance

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ABSTRACT

Multiple myeloma still remains incurable in the majority of cases prompting a further search for new and better prognostic markers. Emerging evidence has suggested that circulating microRNAs can serve as minimally invasive biomarkers for multiple myeloma and monoclonal gammopathy of undetermined significance. In this study, a global analysis of serum microRNAs by TaqMan Low Density Arrays was performed, followed by quantitative real-time PCR. The analyses revealed five deregulated microRNAs: miR-744, miR-130a, miR-34a, let-7d and let-7e in monoclonal gammopathy of undetermined significance, newly diagnosed and relapsed multiple myeloma when compared to healthy donors. Multivariate logistical regression analysis showed that a combination of miR-34a and let-7e can distinguish multiple myeloma from healthy donors with a sensitivity of 80.6% and a specificity of 86.7%, and monoclonal gammopathy of undetermined significance from healthy donors with a sensitivity of 91.1% and a specificity of 96.7%. Furthermore, lower levels of miR-744 and let-7e were associated with shorter overall survival and remission of myeloma patients. One-year mortality rates for miR-744 and let-7e were 41.9% and 34.6% for the 'low' expression and 3.3% and 3.9% for the 'high' expression groups, respectively. Median time of remission for both miR-744 and let-7e was approximately 11 months for the 'low' expression and approximately 47 months for the 'high' expression groups of myeloma patients These data demonstrate that expression patterns of circulating microRNAs are altered in multiple myeloma and monoclonal gammopathy of undetermined significance and miR-744 with let-7e are associated with survival of myeloma patients.

Introduction

Multiple myeloma (MM) accounts for more than 10% of hematologic cancers.[1] In MM, malignant bone marrow plasma cells (BMPCs) undergo massive clonal expansion resulting in high levels of monoclonal immunoglobulin (mIg, M-protein) in blood and/or urine. This is often accompanied by other clinical symptoms, such as osteolytic lesions, increased calcium level, renal insufficiency and anemia.[1,2] MM evolves from a pre-malignant condition called monoclonal gammopathy of undetermined significance (MGUS) which progresses to MM at a rate of 1% per year.[3] Although there are serum markers used for diagnosis of MGUS and MM, such as levels of FLC or mIg,[4-6] recently a lot of attention has been paid to circulating microRNAs that could serve as new diagnostic and/or prognostic tools.[7-9]

MicroRNAs (miRNAs) are a class of short, non-coding, single stranded RNAs with regulatory function.[10,11] MiRNAs play crucial roles in a variety of basic biological processes; they even contribute to tumor formation and development.[12] In tumors, different miRNAs expression profiles compared to healthy tissues were described and resulting miRNAs signatures correlated with patients' survival and prognosis. Such observations highlighted miRNAs as promising biomarkers for diagnosis and even possible targets for therapies.[13]

So far, a number of studies, using BMPCs as the source of miRNAs, found several deregulated miRNAs in MM and MGUS, and implicated miRNAs in signaling pathways deregulated in MM pathogenesis.[14-17] Some of these miRNAs have a therapeutic potential *in vitro* and *in vivo*, such as miR-34a.[18] Nevertheless, obtaining a marker from the bone marrow (BM) is an invasive procedure for patients; therefore, there is still a need for a minimally invasive test that can be easily repeated. There is now a greater possibility of using miRNAs as biomarkers after the discovery that they are present in various body fluids.[19]

Moreover, they are very stable, as they are protected from degradation by association either with secreted membrane vesicles (exosomes, apoptotic vesicles) or with RNA-binding proteins (nucleophosmin, Argonaut 2 (Ago2)).[19-21] It was shown that the miRNAs profile of body fluids reflects physiological or pathological conditions and can be used for patient

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classification.[22] In this study, a new serum miRNAs expression profile, potent enough to distinguish newly diagnosed MM and MGUS patients from healthy controls, was created based on TaqMan Low Density Arrays (TLDA). This profile was validated by quantitative real-time PCR (qPCR) on a larger cohort of newly diagnosed and relapsed MM as well as MGUS patients. Moreover, miRNAs levels were correlated with clinical, biochemical and cytogenetic characteristics and survival data.

Methods

Patients and healthy donors

Peripheral blood (PB) serum samples from 103 newly diagnosed MM patients, 18 MM patients in relapse, 57 MGUS and 30 healthy donors (HD) from the Faculty Hospital Brno, Czech Republic, were obtained for this study. PB serum samples were collected as follows: centrifugation 3500 rpm/15 min/20°C. Samples were frozen as 0.5 mL aliquots, stored at -80°C and thawed only once. For 70 MM and 36 MGUS samples, BMPCs were obtained for routine interphase fluorescence *in situ* hybridization analysis (I-FISH), as described previously.[23] Patients' and donors' characteristics are described in Table 1 and in the *Online Supplementary Table S1*. For 6 newly diagnosed MM patients, BMPCs and exosomal and non-exosomal fraction from PB serum were collected. All patients signed an informed consent form approved by the hospital ethical committee before enrollment into this study.

MiRNA extraction

Total RNA enriched for miRNAs was extracted from all serum samples using miRNeasy Kit (Qiagen) modified for circulating miRNAs according to the manufacturer's instructions. MiRNA/RNA quantity was assessed on a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific) as measurement of each sample 2 times with mean SD=0.292 ng/μL. All samples fit into the Nanodrop ND-1000 validated measuring range.

Exosomes precipitation

Exosomes were collected using ExoQuick Exosome Precipitation Solution (System Biosciences). Serum samples were centrifuged for 3500 rpm/10 min/4°C, 250 μL of serum was combined with 63 μL of ExoQuick, incubated for 30 min/4°C and centrifuged for 2 min/13000 rpm. Exosomal and non-exosomal fraction was used for miRNA/RNA extraction, as described above.

TaqMan Low Density Arrays

Megaplex profiling using human TaqMan Low Density miRNA Arrays A+B, v3.0 (TLDA) (Life Technologies) was performed to evaluate the expression of 667 miRNA (see *Online Supplementary Methods*). QPCR was performed on the ABI7900HT system; raw data were analyzed using SDS software v.2.3, RQ Manager v1.2.1 (Life Technologies).

Candidate miRNA confirmation by qPCR and quantification of miRNA

Individual TaqMan miRNA assays for 6 miRNA (hsa-miR-222-002276, hsa-miR-744-002324, hsa-miR-130a-000454, hsa-miR-34a-000426, hsa-let-7e-002406, hsa-let-7d-002283, Life Technologies) were used for qPCR on a 7500 Real-Time PCR System. QPCR and reverse transcription was performed following the manufacturer´s recommendations (see *Online Supplementary Methods*). Absolute quantification to determine the copy number of each miRNA per 1 ng of total miRNA/RNA was performed, as described previously[24] (*Online Supplementary Appendix* and *Online Supplementary Figure S1*). For determination of assay precision see the *Online Supplementary Methods*.

Interphase fluorescence in situ hybridization analysis

Interphase fluorescence *in situ* hybridization analysis (I-FISH) was performed as a part of routine diagnostic procedure on $CD138^+$ BMPCs, as previously described[25] (*Online Supplementary Methods*).

Statistical analysis

TaqMan Low Density Arrays data were analyzed according to the manufacturer's recommendations; multiple testing correction was applied using Benjamini-Hochberg correction for assessment of adjusted *P* values. For determination of the relative expression levels of target miRNAs see the *Online Supplementary Appendix*. Normalized expression data from the screening phase of the study were statistically evaluated using the R statistical computing language using the Bioconductor package and LIMMA model combined with hierarchical clustering (HCL).[26-28] Other statistical methods used are described in the *Online Supplementary Methods*. $P<0.05$ was considered statistically significant. Data were statistically analyzed with IBM SPSS Statistics, v.20 and R v.2.15.3 with survival ROC package.

Results

Low density arrays study

Screening of 667 miRNAs using TLDA was performed on 4 newly diagnosed MM patients, 4 HD and 5 MGUS samples to identify differentially expressed circulating miRNAs that could serve as putative biomarkers. Fourteen miRNAs were significantly deregulated (all $P<0.003$, adjusted $P<0.05$) between MM patients and HD: 7 miRNAs were up-regulated (miR-222, miR-218, miR-34a, miR-1274A, miR-138, miR-10b*, miR-1243), 7 miRNAs were down-regulated (miR-191, miR-130a, let-7d, miR-103, let-7e, miR-744, miR-151-5p) in MM patients (Figure 1). Out of these, miR-222, miR-744, miR-34a, miR-130a, let-7d and let-7e were further validated, as their position at the top of the list, fold change and favorable expression levels ($Ct<30$) were taken into account (*Online Supplementary Appendix* and *Online Supplementary Table S2*). However, no significant change in miRNAs expression was observed between MM and MGUS samples (*data not shown*). Therefore, we used the same 6 miRNAs to look for the difference between MGUS samples and healthy donors.

Validation of candidate miRNAs using qPCR

Since qPCR is more sensitive and more quantitative over a wider dynamic range than TLDA, we employed miRNA specific assays (miR-222, miR-744, miR-130a, miR-34a, let-7d and let-7e) on a larger cohort of 103 newly diagnosed MM patients and 30 HD to confirm the pattern of candidate miRNAs expression between MM/HD samples and also on 57 MGUS and 18 relapsed MM samples.

To accurately determine expression differences between groups, miRNAs were normalized as amount of miRNA copy numbers per 1 ng of total RNA/miRNA using absolute quantification approach. Standard curves for all 6 validated miRNAs were obtained (*Online Supplementary Appendix* and *Online Supplementary Figure S1*), and individual assays imprecision was also assessed (*Online Supplementary Appendix* and *Online Supplementary Figure*

Table 1. Patients' and healthy donors' base-line characteristics used for RT-PCR.

	MM	MGUS	HD
N. of patients/donors	103	57	30
Gender: males-females	49.5%-50.5%	66.7%-33.3%	46.7%-53.3%
Age median (min-max) [years]	66 (47-83)	67 (54-80)	55 (45-64)
ISS stage: I-II-III	34%-28%-38%	ND	ND
Durie-Salmon stage: I-II-III	10.9%-17.8%-71.3%	ND	ND
Durie-Salmon substage: A-B	79.6% - 20.4%	ND	ND
Ig isotype: IgG-IgA-IgM-IgD-LC only-	52.4%-27.2%- 1.9%-2.9%-10.7%	81.8%-3.6%-12.7%-0%-1.8%-	ND
NonSecr.-Biclonal	-3.9%-1.0%	0%-0%	
Light chains: kappa-lambda	59.2%-36.9%	53.7%-46.3%	ND
N. of previous treatment lines			
None (first-line treatment)	103 (100%)	57 (100%)	ND
First-line based treatment: thalidomide-Bortezomib -lenalidomide	76%-18%-8%	ND	ND
Biochemical parameters median (min-max)			
Hemoglobin (g/L)	108 (62.7-157)	138 (104-166)	ND
Thrombocytes (count x10^9)	215 (37.6-561)	233 (112-483)	ND
Calcium (mmol/L)	2.41 (1.85-4.94)	2.34 (2.04-2.67)	ND
Albumin (g/L)	39.0 (22.1-50.4)	43.8 (30.6-53.3)	ND
Creatinine (umol/L)	92.0 (48.0-884.0)	86.0 (50.0-779.0)	ND
β2-microglobulin (mg/L)	3.82 (1.10-42.6)	2.11 (1.21-35.0)	ND
Lactate dehydrogenase (ukat/L)	3.16 (1.15-18.69)	3.43 (1.92-7.88)	ND
C-reactive protein (mg/L)	4.0 (0-174.3)	3.1 (0-280.6)	ND
Monoclonal Ig (g/L)	26.65 (0-88.5)	8.7 (0-26.6)	ND
Plasma cell infiltration of bone marrow (%)	27.0 (10.0-94.0)	2.0 (0-8.4)	ND
Chromosomal abnormality			
13q14 deletion	30 (44.1%)	9 (23.7%)	ND
17p13 deletion	9 (13.2%)	1 (2.6%)	ND
Translocation t(4:14)	9 (18.4%)	3 (12.5%)	ND
1q21 gain	24 (37.5%)	3 (9.4%)	ND
Hyperdiploidy	29 (45.3%)	5 (15.2%)	ND

ND: not determined.

S2). As the difference in miR-222 expression between MM and HD was not significant (P=0.3022) it was excluded from further studies.

A significant decrease was observed in expression of miR-744, miR-130a, let-7d and let-7e (all P<0.001) in the MM group. However, miR-34a was significantly increased (P<0.0001) when compared to the HD group (*Online Supplementary Appendix* and *Online Supplementary Table S3*). These data confirm the results of the screening phase (for the correlation between TLDA and qPCR data, see *Online Supplementary Appendix* and *Online Supplementary Figure S1*). Similarly, expression of miR-744, miR-130a, let-7d and let-7e was decreased in MGUS samples (all P<0.0001) and the expression of miR-34a was increased in MGUS when compared to HD (P<0.0001) (*Online Supplementary Appendix* and *Online Supplementary Table S3*).

Receiver operating characteristics (ROC) curve analysis revealed that serum levels of all validated miRNAs can be used to distinguish MM and MGUS patients from HD (*Online Supplementary Appendix* and *Online Supplementary Table S4*). Moreover, multivariate logistical regression analysis showed that the combination of miR-34a and let-7e could improve the stratification power characterized with area under the curve (AUC) of 0.898, sensitivity of 80.6% and specificity of 86.7% for MM, and with AUC 0.976, sensitivity of 91.1% and specificity of 96.7% for MGUS (Figure 2).

MiRNA expression pattern correlates with biochemical parameters but not with PCs infiltration

To determine the correlation of miRNAs expression levels with clinical parameters, stage (ISS, Durie-Salmon (DS)) and percentage of BMPC infiltration, Spearman bivariate correlation was performed. All studied miRNAs significantly correlated with higher levels of hemoglobin: miR-744, miR-130a, let-7d and let-7e positively; miR-34a negatively. Moreover, levels of miR-744, miR-130a, let-7d and let-7e showed a significant positive correlation with thrombocyte count and a significant negative correlation with levels of creatinine and beta(β)2-microglobulin. Expressions of miR-744, let-7d, let-7e showed a significant positive correlation and miR-34a significantly negatively correlated with levels of albumin, and miR-744 and let-7e a significant negative correlation with C-reactive protein (CRP) level. Only let-7e expression showed a significant negative correlation with level of monoclonal immunoglobulin (Ig).

Similar data were obtained for MGUS patients, where levels of all studied miRNAs showed a significant positive correlation with hemoglobin level. In addition, levels of miR-744, miR-130a, let-7d and let-7e were significantly associated with levels of albumin and inversely correlated with levels of creatinine and β2-microglobulin. Also, levels of miR-744, miR-130a and let-7d showed a significant negative correlation with CRP levels. In contrast to MM patients, none of the studied miRNAs in MGUS correlated

with thrombocyte count (Table 2).

In MM, expression levels of miR-744, let-7d and let-7e were linked to advanced ISS stage; this trend was also observed for miR-130a, although it did not reach statistical significance. Also, only let-7e correlated with DS stage; levels of miR-744, miR-130a, let-7d and miR-34a were associated with DS sub-stage. However, none of the studied miRNAs in MM and MGUS correlated with percentage of PC infiltration in BM, which confirms previous observed findings[28] (*Online Supplementary Appendix* and *Online Supplementary Table S5*).

Level of circulating let-7e correlates with del(13q14) in PCs

Little is known about the origin of circulating miRNAs and their relationship with BMPCs. Therefore, the expression levels of five miRNAs were correlated with typical chromosomal MGUS and MM aberrations, such as gain of 1q21, 13q14 deletion, 17p13 deletion, translocation t(4;14) and hyperdiploidy (HY) status (HY of chromosomes 5, 9 and 15). We found that presence of del(13q14) in MM showed a significant correlation with lower levels of let-7e, and we also observed a trend for lower levels of miR-744 to be linked with this aberration (*Online Supplementary Appendix* and *Online Supplementary Table S6*).

Derivation of evaluated miRNAs

To further investigate potential derivation of all studied miRNAs, we measured their levels in exosomal and exosome-depleted supernatant of 6 newly diagnosed MM patients. Concentration of miR-744, miR-130a, let-7d and let-7e (all $P<0.05$) was found to be significantly higher in the exosome pellet compared to the exosome-depleted supernatant. However, there was no significant difference between these two fractions for miR-34a (*Online Supplementary Figure S4A*). For the same patients, we obtained miRNAs from BMPCs, and we observed levels of miR-744, miR-34a, let-7d and let-7e to be significantly

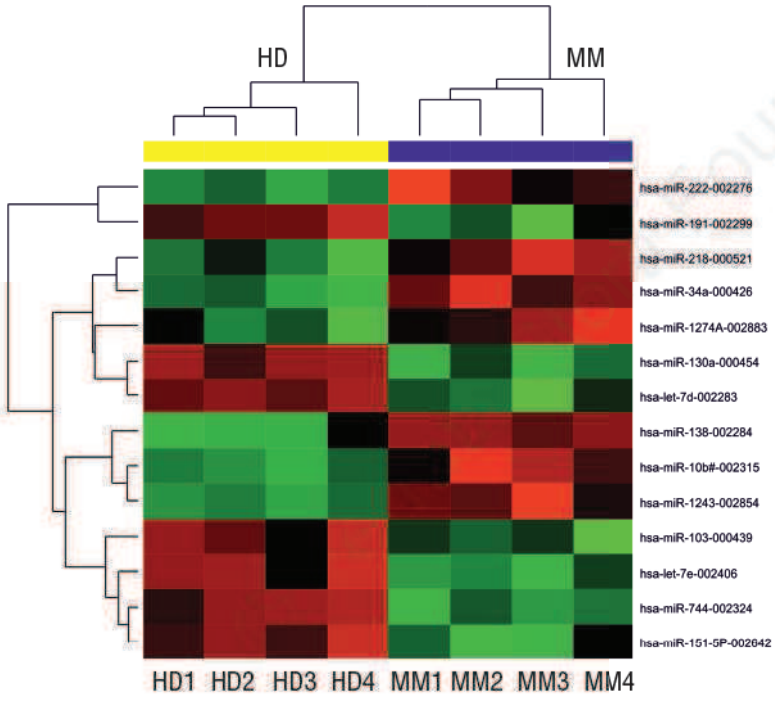

Figure 1. Hierarchical clustergram discriminating serum of MM patients and healthy donors according to differentially expressed miRNAs (yellow color indicates serum samples of HD, blue MM patients; adjusted $P<0.05$).

Table 2. Correlation of serum microRNAs in MM and MGUS with biochemical parameters. For correlation of the data, Spearman coefficient was adopted; significant coefficients of correlation ($P<0.05$) are marked with bold .

	Multiple myeloma					MGUS				
rS	miR-744	miR-130a	miR-34a	let-7d	let-7e	miR-744	miR-130a	miR-34a	let-7d	let-7e
Monoclonal Ig (g/L)	**-0.175**	-0.011	0.135	-0.087	**-0.199**	0.059	0.062	0.116	0.000	0.051
Hemoglobin (g/L)	**0.543**	**0.283**	**-0.258**	**0.387**	**0.585**	**0.383**	**0.465**	**0.270**	**0.290**	**0.424**
Thrombocytes (count x10^9)	**0.555**	**0.390**	-0.190	**0.427**	**0.515**	-0.007	-0.092	-0.127	0.000	0.024
Albumin (g/L)	**0.355**	0.093	**-0.204**	**0.302**	**0.355**	**0.464**	**0.341**	0.221	**0.401**	**0.309**
Creatinine (µmol/L)	**-0.415**	-0.354	-0.007	**-0.310**	**-0.406**	**-0.369**	**-0.330**	-0.090	**-0.468**	**-0.367**
β2-microglobulin (mg/L)	**-0.575**	**-0.236**	0.170	**-0.439**	**-0.571**	**-0.451**	**-0.279**	**-0.211**	**-0.484**	**-0.277**
Lactate dehydrogenase (µkat/L)	**-0.207**	-0.095	0.141	**-0.202**	-0.176	-0.115	-0.115	-0.180	-0.061	-0.216
C-reactive protein (mg/L)	**-0.221**	-0.086	0.122	-0.178	**-0.253**	**-0.331**	**-0.349**	-0.019	**-0.299**	-0.224

higher in BMPCs than in exosomal fraction (all $P<0.05$) (*Online Supplementary Figure S4B*). Interestingly, levels of miR-130a were comparable in BMPCs and exosomes ($P=0.8438$). However, there was no correlation found between miRNAs from BMPCs and from exosomal fraction (*data not shown*).

Dynamics of miRNA levels during disease progression

As deregulated miRNAs expression in MGUS and MM patients was observed at the time of diagnosis, the next step was to check if this profile changes during disease progression. For 18 MM patients, who had not undergone PBMC transplantation, serum samples at the time of diagnosis and in relapse (after 2 lines of treatment) were collected. All of the miRNAs in MM samples differed significantly from HD at the $P<0.0001$ (miR-744: FC=0.270; miR-130a: FC=0.487; miR-34a: FC=10.083; let-7d: FC=0.243; let-7e: FC=0.300). Moreover, a significant increase of miR-34a (FC=3.560; $P<0.0001$) and decrease of let-7d (FC=0.460; $P=0.0182$) was found in relapsed samples compared to samples at the time of diagnosis. For miR-744 and let-7e, a trend toward lower expression was observed; however, no change in expression between diagnostic and relapsed sample was observed for miR-130a (*Online Supplementary Appendix* and *Online Supplementary Figure S5*).

Analyses of overall survival and time to progression

Furthermore, miRNAs expression was verified as a possible indicator of survival. Univariate Cox proportional hazards survival model with one explanatory variable showed prognostic impact for serum miR-744 (HR 0.670 [HR95%CI: 0.548; 0.819]; $P<0.0001$) and for let-7e (HR: 0.611 [HR95%CI: 0.450; 0.829]; $P=0.002$) for the MM cohort of patients. To characterize the prognostic significance of this miRNA, a multivariate Cox proportional hazards survival model was used. The variables in the multivariate model were the only variables which remained statistically significant when potential predictors were combined with miRNA expression and forced into the model. The results showed that neither miR-744 nor let-7e is independently associated with overall survival (OS) when combined with other factors (miR-744: $P=0.902$; let-7e: $P=0.472$) (*Online Supplementary Appendix* and *Online Supplementary Table S7*). Survival cut-off points were established based on time-dependent ROC analysis (*data not shown*), which showed suitable AUC for a 0.5-1.5 year time period for miR-744 and a 1.5 year time point for let-7e.

To determine the prognostic impact of defined miR-744 and let-7e expression cut-off values, we compared OS between the 'low' and the 'high' expression subgroups (Figure 3A and B). For miR-744, worse 1-year OS was indicated in the 'low' expression subgroup of patients (43 of 103) in comparison with the 'high' expression group (60 of 103) ($P<0.0001$). One-year mortality rate for the 'low' miR-744 expression group was 41.9% (95%CI: 28.8%; 57.9%), and for the 'high' expression group it was 3.3% (95%CI: 0.8%; 12.7%), respectively. Similarly for let-7e, worse 1-year OS was indicated in the 'low' expression subgroup of patients (52 of 103) in comparison with the 'high' expression group (51 of 103) ($P=0.001$). One-year mortality rate for the 'low' let-7e expression group was 34.6% (95%CI: 23.4%; 49.2%) and for the 'high' expression group 3.9% (95%CI: 1.0%; 14.8%). In the same way, the Cox model showed prognostic impact for serum miR-744 (HR: 0.690 [HR95%CI: 0.584; 0.817]; $P<0.0001$) and let-7e (HR: 0.552 [HR95%CI: 0.424; 0.718]; $P<0.0001$) in time to progression (TTP) for the MM patient cohort. Only MM patients who had an event after first-line of therapy were taken into account (86 of 103). We compared TTP between miR-744 'low' and 'high' expression subgroups and between let-7e 'low' and 'high' expression subgroups using the cut-off value defined by time-dependent ROC analysis. The analysis showed suitable AUC for a 1-2 year time period for miR-744 and a 1-year time point for let-7e (Figure 3C and D). Shorter TTP was indicated in patients in the 'low' miR-744 expression subgroup (37 of 86) in comparison with the 'high' expression group of patients (49 of 86) ($P<0.0001$), and median time of remission was approximately 11.5 months (95%CI: 6.49; 16.50) for the 'low' expression and approximately 47.5 months (95%CI: 24.63; 70.37) for the 'high' expression groups, respectively. For let-7e, shorter TTP was indicated in the 'low' expression subgroup of patients (43 of 86) in comparison with the 'high' expression subgroup of patients (43 of 86) ($P<0.0001$), and median time of remission was approximately 11.5 months (95%CI: 7.17; 15.83) for the 'low' expression and approximately 47.5 months (95%CI: 31.61; 63.39) for the 'high' expression groups, respectively.

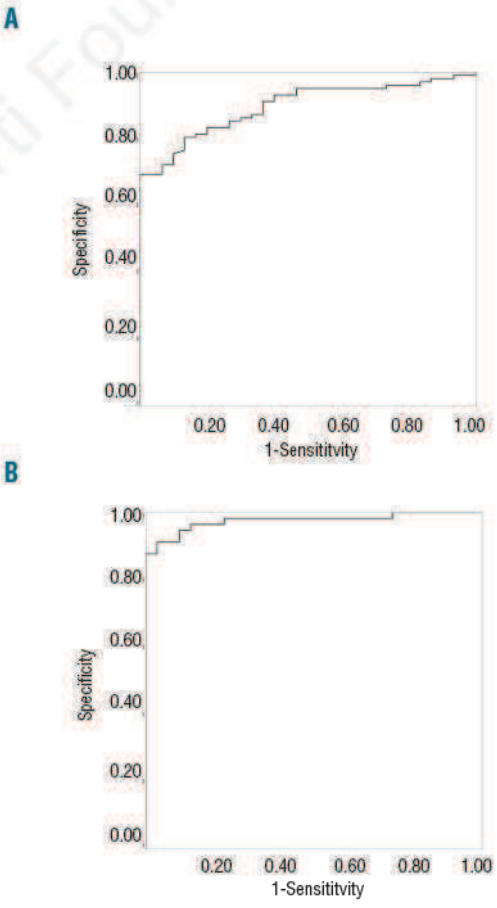

Figure 2. ROC curves curves for combination of serum miR-34a and let-7e yielded in (A) AUC of 0.898, sensitivity 80.6% and specificity 86.7% in discriminating MM from HD and in (B) AUC of 0.976, sensitivity 91.1% and specificity 96.7% in discriminating MGUS from HD.

Biochemical and stage characteristics (ISS, DS, DS sub-stage) of presented groups of MM patients and *P*-values are provided in the *Online Supplementary Appendix* for both the 'high' and the 'low' miR-744 and let-7e expression groups (*Online Supplementary Appendix* and *Online Supplementary Tables S8A* and *B, S9A* and *B*). The miR-744 and let-7e 'low/high' expression groups were significantly different in levels of hemoglobin, thrombocytes, albumin, creatinine, β2-microglobulin and lactate dehydrogenase ($P<0.05$). Significant differences between groups in ISS and DS sub-stage distribution ($P<0.05$) were also observed.

Interestingly, a significant association between group of patients with lower expression of let-7e and occurrence of del(13q14) ($P=0.031$) was found. There was no difference between the 'high' and the 'low' miR-744 and let-7e expression groups in terms of the occurrence of the other analyzed cytogenetic abnormalities (*data not shown*).

Discussion

It has been shown that miRNAs are present as circulating molecules in human body fluids and thus may serve as a new class of powerful and minimally invasive biomarkers.[7,8,29-31] However, the studies differ regarding deregulated miRNAs, the array platforms used and the normalization methods adopted. The origin of circulating miRNAs and their function is still unclear as circulating miRNAs may not always be directly associated with malignant cells but may also reflect indirect effects, could be secreted by non-malignant cells, or actively taken up by malignant cells.[32,33]

In this study, TLDAs were used to identify circulating miRNAs that are differently expressed in MM serum samples and could reflect this pathological condition. Fourteen differently expressed miRNAs between MM and HD serum samples were identified. Out of these, five miRNAs (miR-744, miR-130a, let-7d, let-7e and miR-34a) were cho-

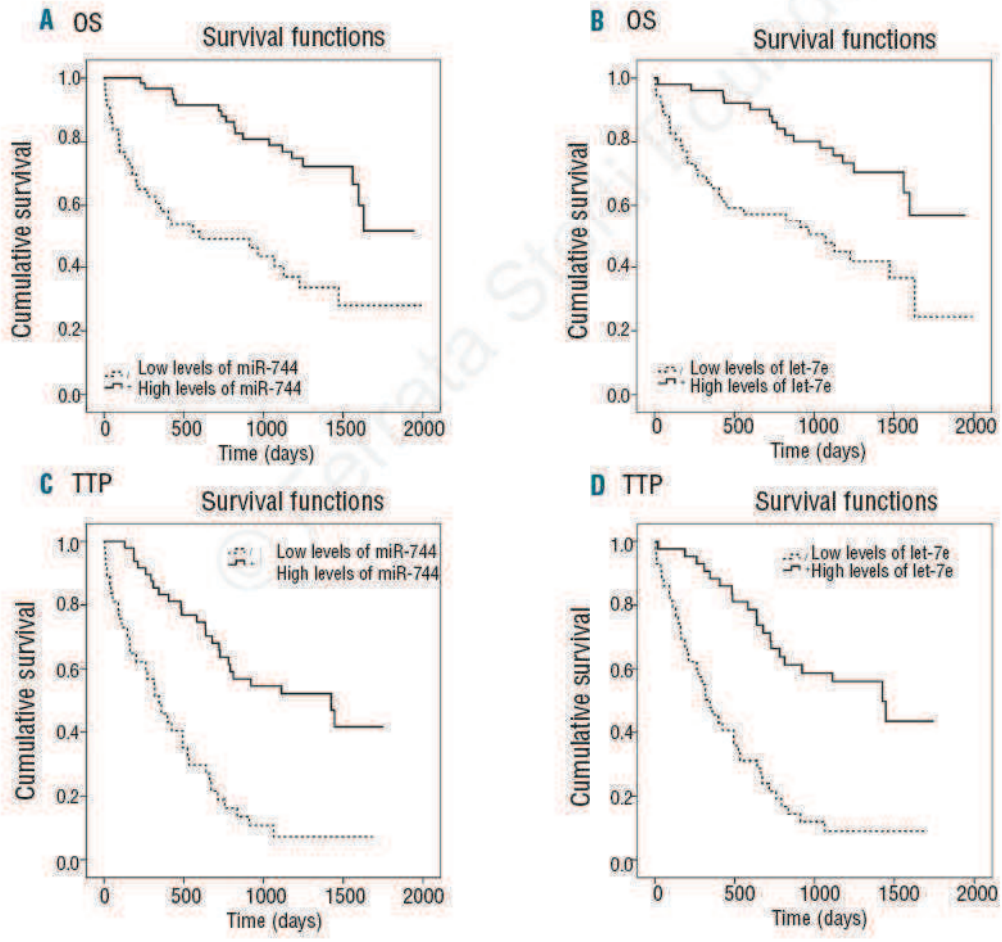

Figure 3. Kaplan-Meier curves of miR-744 and let-7e and their association to (A) (B) OS (C) (D) TTP. The thresholds of cut-off points were determined using a time-dependent ROC analysis. For miR-744 OS, the cut-off value was derived from 0.5-1.5 years survival, for let-7e it was derived from 1.5 years survival. Similarly for TTP, the cut-off values were derived for miR-744 from 1-2 years progression, for let-7e from 1 year progression. Log2 scale of amount of copies/1ng of miRNA/RNA was used for miRNA expression in this analysis. The Y axis represents survival probability and the X axis represents time of follow up in days.

sen and confirmed as significantly deregulated on a bigger cohort of MM and MGUS patients using an absolute quantification approach. At this point, since no miRNA is accepted as a standard for serum samples, this is probably the most accurate method of quantification of serum miRNAs in MM. Therefore, to accurately determine the expression differences between groups, miRNA levels were normalized to amount of miRNAs copy numbers per 1 ng of total RNA/miRNA.

Analytical characteristics of the five miRNAs (miR-744, miR-130a, miR-34a, let-7d and let-7e) showed that they can all discriminate MGUS and MM from HD. However, the combination of serum miR-34a and let-7e (the highest sensitivity of 91.1% and specificity of 96.7% for MGUS, and 80.6% sensitivity and 86.7% specificity for MM) proved to be an even more powerful discriminating tool.

In the group of MM patients, most of the five miRNAs were observed to be associated with some of the clinical parameters, ISS or DS sub-stage. Particularly in the cases of miR-744, let-7d and let-7e, lower levels were associated with advanced ISS stage. As lower levels of miR-744, miR-130a and let-7d are related to the advanced DS sub-stage, they might reflect the renal impairment that often develops in MM patients. This observation is further supported by the relation of lower miRNA levels to higher creatinine and β2-microglobulin levels. Lactate dehydrogenase (LDH) level helps to assess tumor burden, and the level of β2-microglobulin reflects the tumor mass.[6,34] Furthermore, anemia associated with MM is caused by inadequate erythropoietin levels consequent to renal impairment and the effect of inflammatory cytokines.[35] C-reactive protein (CRP) as well as albumin levels are known to be hallmarks of tumor activity.[36,37] Taking all these facts into consideration, we can anticipate that serum miRNA levels are associated with tumor mass and disease activity. Interestingly, such correlation pattern with biochemical parameters was observed also for MGUS.

However; as no correlation with infiltration of BMPCs in MM and MGUS was observed, which is in concordance with previously presented data from another group,[29] our observations further suggest that circulating miRNAs reflect other MM pathological effects as well. To further investigate potential derivation of all studied miRNAs, we estimated their levels in exosomal and exosome-depleted fractions and in BMPCs. Four miRNAs were observed to be present primarily in exosomes, which is consistent with previous observations that exosome fraction is highly enriched in miRNAs.[38] Moreover, all of the studied miRNAs were found to be abundantly present in BMPCs when compared to levels in exosomes. Interestingly, levels of miR-130a were comparable in exosomal fraction and in BMPCs, suggesting their involvement in intercellular communication. However, as we did not find any linear dependence between miRNA levels in exosomal fraction/exosome-depleted fraction and miRNA levels in BMPCs, it is not clear whether they originate from BMPCs.

Different miRNAs expression was confirmed also in 18 paired MM samples taken at diagnosis and at relapse with higher levels of miR-34a and lower levels of let-7d, suggesting that deregulated levels of miRNAs reflect patient condition and are associated with more advanced disease.

To the best of our knowledge, the possibility of a prognostic serum miRNAs marker in MM has not yet been investigated. In this study, lower levels of miR-744 and let-7e were found to be significantly associated with the worse OS and TTP of MM patients. It should be mentioned that this is related to a short-time period (1-2 years). For-miR-744, the observation could be partially explained by the fact that the gene for miR-744 lies in the 17p12 region, close to the *TP53* gene (17p13). Deletions at chromosome 17p13.1-17p12 were previously found to be associated with poor survival.[39] Also, low *TP53* gene expression, which is highly correlated with loss of heterozygosity of the TP53 locus, was associated with shorter event-free survival and OS.[40]

However, we were not able to prove the relationship between low levels of miR-744 and deletion of *TP53*, and thus we cannot say that absence of the 17p13.1-17p12 region can fully explain the lower levels of miR-744.

As patients were not equally distributed across ISS stage, we assume that miR-744 and let-7e impact on OS and TTP could be explained by ISS heterogeneity. However, no differences in DS stage between groups with low/high expression of miR-744 were observed, but they were observed between groups with 'low/high' expression of let-7e. Interestingly, the miR-744 'low' expression group of patients was associated with presence of 1q21 amplification or t(4;14), which have been previously described as unfavorable prognostic factors for MM.[41,42]

The 'low/high' miR-744 and let-7e groups of MM patients were also observed to be clinically heterogeneous, which was demonstrated by different levels of albumin, creatinine, β2-microglobulin, LDH, hemoglobin and thrombocyte count between groups. As mentioned above, all listed parameters are known to be markers of tumor mass and disease activity.[34,35,43] Although our initial findings concerning clinical data, such as OS and TTP, show that these miRNAs are not an independent factor, but rather a hallmark of a complex pathological process that accompanies MM, they both reflect disease status and thus can serve as new auxiliary peripheral blood prognostic markers for MM.

In conclusion, we have identified for the first time a profile of five serum miRNAs which are deregulated in MM and MGUS sera. Levels of miR-744, miR-130a, let-7d and let-7e were significantly decreased whereas miR-34a was increased in MM and MGUS. Deregulated levels of miRNAs were observed in advanced MM suggesting that they are stable markers of MM. Moreover, levels of miR-744 and let-7e might be useful as a marker of patients' survival. Even though additional larger-scale studies are needed to address other biological characteristics of these miRNAs, it is obvious that circulating serum miRNAs have diagnostic and prognostic implications for MGUS and MM patients.

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Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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Detection of tumor-specific marker for minimal residual disease in multiple myeloma patients

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Detection of tumor-specific marker for minimal residual disease in multiple myeloma patients

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Aim. Multiple myeloma (MM) is a malignant lymphoproliferative disease of terminally differentiated B lymphocytes, characterized by expansion of monoclonal plasma cells. It is the second most common hematological cancer in the world. The introduction of novel drugs is slowly turning MM into a chronic disease. The aim of treatment is hematological remission and eradication of clinical manifestation. Nevertheless, most MM patients eventually relapse. For this reason, research is focused on more accurate monitoring of remission and relapse by molecular biology techniques. One of these techniques is allele-specific PCR and quantitative real-time PCR based on specific detection of VDJ immunoglobulin heavy chain gene rearrangement of clonal cells. The hypervariable region of IgH rearrangement is used as a marker for detection of minimal residual disease (MRD) in MM as this sequence is used for allele-specific primers and probe design. This technique is a complementary tool for flow cytometry in MRD detection in MM. The aim of this study was to introduce detection of MRD by PCR in the Czech Republic.

Results. We successfully introduced qualitative and quantitative detection of a tumor marker for MRD assessment of MM by PCR in our laboratory.

Key words: multiple myeloma, minimal residual disease, tumor-specific marker, ASO PCR, RQ-PCR, IgH gene rearrangement

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INTRODUCTION

Multiple myeloma (MM) is a plasma cell malignancy that is ranked among B lymphoproliferative neoplasias by the World Health Organization[1]. MM is a complex disease characterized by accumulation of clonal malignant plasma cells (PC) in the bone marrow (BM) together with production of monoclonal immunoglobulins or light/heavy chains, resulting in clinical manifestation of the disease. Osteolysis, hypercalcemia, anemia, immune system impairment and renal insufficiency are among the most common clinical manifestations of MM (ref.[2,3]).

With the introduction of new drugs and use of autologous stem cell transplantation, MM is slowly turning into a chronic disease. The aim of the treatment is hematological remission and eradication of clinical manifestations. Nevertheless, most MM patients eventually relapse[3]. This implies that not all clonogenic malignant cells had been killed and that the residue of malignant cells persisting even after treatment contributes to recurrence of the disease.

For this reason, more accurate monitoring of remission and relapse by molecular biology techniques is important. One of these techniques is allele-specific (ASO) PCR and a real-time quantitative PCR (RQ-PCR) based on analysis of junctional regions of rearranged immunoglobulin heavy chain (IGH) gene[4]. The hypervariable region of IgH rearrangement is used as a tumor marker for detection of minimal residual disease in MM. Determination of such marker and its sequence analysis further allows for allele-specific (ASO) primers and probe design[5].

MRD detection using PCR has major advantages because of its sensitivity, accuracy, reproducibility, need of small amount of DNA and widespread and irreplaceable use in retrospective studies. On the other hand, PCR methods are more complex, expensive, take more time and allow detection of only one clone that was present at the time of diagnosis[6]. However, detection of tumor marker by PCR has a wide application for clinical evaluation of patients, for early relapse detection or for quantification of tumor contamination in healthy hematopoietic cells for autologous transplantation[7].

The use of flow cytometry (FC) for MRD detection appears to have prognostic significance as well[6,7]. One current approach is MRD detection using an 8-color polychromatic FC. This technique is also able to differentiate the expression of immunoglobulin light chain (IgL) κ or λ (ref.[8,9]). MRD detection via FC is applicable in approximately 90% of MM patients, which is important for routine practice[6]. The significance of FC use in MRD detection was shown in the large studies of Paiva et al. In these studies, patient treatment response was evaluated

via immunofixation, serum free light chain, multiparameter FC immunophenotyping and FC together with assessment of high-risk cytogenetics[10,11].

Unfortunately, there has never been a comparative study of PCR *vs*. FC in a large cohort of patients treated with widely used treatment regimens. Nevertheless, according to some smaller studies - both techniques have comparable prognostic significance. Nowadays, they are considered to be complementary tools for MRD monitoring[6,12].

Currently, new technologies for the detection of tumor-specific marker are emerging, such as droplet digital PCR or next generation sequencing (NGS) (ref.[13,14]). However, the qualitative and quantitative detection of tumor-specific marker by ASO-PCR is still the golden standard technique[7]. Nevertheless, the technique of MM-specific marker detection using ASO-PCR has not been established in the Czech Republic so far. Therefore, the aim of this work was to introduce allele-specific qualitative and quantitative detection of MM-related marker by PCR on BM and peripheral blood samples of MM patients in our laboratory for further MRD assessment.

MATERIAL AND METHODS

Patients and samples

Frozen genomic DNA (gDNA) derived from mononuclear cells from BM (BMMC) of 10 newly diagnosed and relapsed MM patients diagnosed between 2006 and 2007 at the Faculty Hospital Brno was included in the study (Table 1). For 6 patients, gDNA samples of mononuclear cells from peripheral blood (PBMC) at the time of relapse were available, for 2 other patients, gDNA samples of BMMC at the time of relapse were available. gDNA was isolated using phenol-chloroform extraction, and stored at -20 °C. Also, gDNA from PBMC of 10 healthy donors was included in the study. This gDNA was isolated using QIAamp DNA Mini Kit (Qiagen). All samples were included only after patients signed the informed consent approved by Ethical committee of the hospital.

Amplification and sequencing of tumor-specific IgH gene rearrangement

To identify tumor-related IgH rearrangements, 500 ng of gDNA was PCR-amplified using sets of primers for IgH variable (V), diversity (D), and joining (J) gene segments with 2mM dNTP, 20 mM $MgCl_2$, 5x Buffer and GoTaq Flexi DNA Polymerase (Promega) (ref.[15,16]). The reaction was carried out for initial denaturation at 94 °C for 1 min and then 33 (40) cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 30 s, with a final extension of 10 minat 72 °C (ref.[17]). PCR products were then run on 2% agarose gels to find clonal products. Clonal PCR products were excised and purified using MinElute Gel Extraction Kit, QIAquick PCR Purification Kit or QIAquick Gel Extraction Kit (all Qiagen) and further sequenced. Purified PCR fragments were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit on ABI3130 DNA Sequencer (Applied Biosystems). The relevant VH family or JH consensus primers were used as sequencing primers to obtain the sequence information (Table 2) (ref.[15,16,18]).

ASO primers design and nested PCR amplification of tumor-specific IgH gene rearrangement

Patient-specific ASO primers of CDRIII region were designed using IMGT/V-QUEST (http://www.imgt.org/) and PrimerBlast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesized by Eurofins MWG Operon (Ebersberg, Germany) (ref.[19]). Nested PCR amplification using ASO primers for each IgH sequence identified earlier were performed using 2mM dNTP, 20 mM MgCl2, 5x Buffer and GoTaq Flexi DNA Polymerase (Promega). PCR was performed as initial denaturation at 94 °C for 1 min, and then 33 cycles of amplification at 94 °C for 30 s 58-62 °C (dependent on specific ASO primer Tm) for 30 s, and 72 °C for 30 s, with final extension of 10 min at 72 °C. PCR products were run on 2% agarose gels[17]. PCR products from ASO PCR were also sequenced as described previously, to ensure the detection of the same sequence.

Table 1. Patient's baseline characteristics.

Total number of patients	10
Sex: male/female	5/5
Average age at diagnosis (range) [years]	64 (52-81)
ISS stage: I-II-III (%)	60-30-10
Durie-Salmon stage: I-II-III (%)	40-0-60
Ig isotype: IgG-IgD-LC only (%)	70-10-20
Monoclonal Ig (g/l)	34.7 (0-79.8)
Plasma cells infiltration of bone marrow (%)	31.1 (10.0-81.6)

Table 2. Sequences of the consensual primers used for IgH rearrangement detection.
(Symbols: R = A/ G, Y = T/C, S = G/C, K = G/T).

Primer	Sequence (5'-3')
VH1FS	CAGGTGCAGCTGGTGCARYCTG
VH2FS	CAGRTCACCTTGAAGGAGTCTG
VH3FS	GAGGTGCAGCTGGTGSAGTCYG
VH4aFS	CAGSTGCAGCTGCAGGAGTCSG
VH4bFS	CAGGTGCAGCTACARCAGTGGG
VH5FS	GAGGTGCAGCTGKTGCAGTCTG
VH6FS	CAGGTACAGCTGCAGCAGTCAG
VHFR2-1	CTGGGTGCGACAGGCCCCTGGACAA
VHFR2-2	TGGATCCGTCAGCCCCCAGGGAAGG
VHFR2-3	GGTCCGCCAGGCTCCAGGGAA
VHFR2-4	TGGATCCGCCAGCCCCCAGGGAAGG
VHFR2-5	GGGTGCGCCAGATGCCCGGGAAAGG
VHFR2-6	TGGATCAGGCAGTCCCCATCGAGAG
VHFR2-7	TTGGGTGCGACAGGCCCCTGGACAA
JHD	ACCTGAGGAGACGGTGACCAGGGT

Design of probes for RQ-PCR

For RQ-PCR analysis of tumor-related sequence H-chain V- region (VH) family-specific consensus reverse probes (called L-VH1 to L-VH6) derived from the germline sequence FR3 and designed for use in RQ-PCR in childhood ALL were used[20]. Because of the high rate of somatic hypermutations occurring in MM, novel probes were required. The new specific IgH probes were designed according to recommendations of Ladetto et al.[17] (Eurofins MWG Operon, Ebersberg, Germany) and labeled at the 5‘ end with 6-carboxy-fluorescein (FAM) and 6 carboxytetramethyl rhodamine (TAMRA) at the 3‘ end. RQ-PCR reaction was performed in 25 μL with 500 ng of patient's gDNA using 1x TaqMan Gene Expression MasterMix (Life Technologies), 10 pmol of each patient's specific ASO primer, 5 pmol specific IgH probe. Reactions were incubated in a 96-well optical plate at 50 °C for 2 min, 95 °C for 10 min, followed by 42 cycles at 95 °C for 15 s and 60 °C for 1 min. All reactions were run in triplicate on 7500 Real-Time PCR System. Standards for RQ-PCR were obtained by cloning the tumor-specific IgH region with the TOPO TA cloning Kit (Invitrogen). A variable number of white-positive colonies (colonies with correct plasmid insertion) were grown overnight in Luria-Bertani broth containing 50 mg/mL ampicillin. Plasmid DNA was purified using QIAprep Spin Miniprep Kit (Qiagen). Standard curves were prepared by ten-fold serial dilutions of plasmid in gDNA obtained from healthy donors according to the European Study Group on MRD Detection in ALL (ESG-MRD-ALL criteria) (ref.[21]). Then, the quantitative analysis of tumor-specific sequence was related to the reference human RNase P gene (Applied Biosystems).

Monoclonality of the specific sequence was verified by sequence analysis of PCR product from single colonies cloned with specific VDJ gene rearrangement of IgH.

RESULTS

gDNA samples of ten patients obtained from BMMC were used for introduction of tumor-specific marker identification by PCR. Fifty percent of the patients (5/10) were suitable both for qualitative and quantitative tumor marker detection. In 30% (3/10) of patients, the sequence was not clear and they were assessed as oligoclonal. This technique was unsuccessful in 20% (2/10) of our patients (Table 3).

Tumor-specific IgH gene rearrangement amplification and sequence analysis

The tumor-specific marker was established for 80% (8/10) patients by PCR with consensual primers (derived from FR1 and FR2 conservative regions). IGHV3 allele was present in 30% (3/10) of patients, IGHV2 in 10% (1/10) and IGHV4 in 10% (1/10) of patients. Sequences were analyzed using bioinformatic tool IMGT/V-QUEST for CDR2/3 hypervariable region evaluation. Only productive IgH rearranged sequences (in frame junctions, no stop codon) were used for further work.

Monoclonality of the specific sequence was verified by direct sequence analysis of cloned PCR product from

Table 3. A summary of individual steps of PCR detection of tumor-specific marker in MM (nd – not done).

Patient	ASO PCR			qPCR		
	alelle	ASO primers	PCR II	ASO probes	standard curves	qPCR
1	IGHV2	done	done	done	done	quantifiable
2	IGHV3	done	done	done	done	quantifiable
3	oligoclonal	done	done	nd	nd	nd
4	IGHV4	done	done	done	done	out of quantitative range
5	IGHV3	done	done	done	done	quantifiable
6	oligoclonal	done	done	nd	nd	nd
7	oligoclonal	done	done	nd	nd	nd
8	IGHV3	done	done	done	done	quantifiable
9	nd	nd	nd	nd	nd	nd
10	nd	nd	nd	nd	nd	nd

Table 4. Results of RQ-PCR detection of tumor-specific marker in MM.

Patient	Status	ASO probe	RNase P	Malignant cells./ 10^6 healthy cells	Conclusion
1	1. relapse	14.4	43 348	662,1	positive, quantifiable
2	diagnosis	4 239	37 390	226 726,9	positive, quantifiable
	1. relapse	1.7	29 462	114,4	positive, quantifiable
4	1. relapse	3.9	3 331,6	2 327	positive, out of quantitative range
5	diagnosis	21 963	59 797	734 598,4	positive, quantifiable
8	1. relapse	68	3 348,1	40 606,7	positive, quantifiable

plasmid in bacteria in 5 patients. In 3 remaining oligoclonal patients, we identified ≥ 3 different clones.

ASO primer design, testing and qualitative PCR for tumor-specific marker detection

ASO primers were designed for 5 patients with successfully obtained molecular marker and for 1 clone of 3 oligoclonal patients (Supplementary table S1). In 7 patients, ASO primers were designed according to patient's specific gDNA sequence and in 1 case, we used plasmid sequence with cloned VDJ region for primer design, because of higher quality of the sequence. Qualitative cross-reaction was performed to verify specificity of designed ASO primers to only one patient. Six out of 8 pairs of primers were specific for only one patient.

Qualitative PCR with designed ASO primers was performed on gDNA diagnostic samples from 5 patients with monoclonal sequence. The original clone was present in follow-up samples of 3 patients at the time of relapse. For the other 2 patients, samples were not available. Moreover, PCR was performed on follow-up samples at the time of relapse for 3 oligoclonal patients as well, where 1 of the original clones was successfully detected in 1 oligoclonal patient relapse sample.

ASO probe design and RQ-PCR for tumor-specific marker detection

Quantitative detection of MRD was performed on 5 monoclonal patients. In these patients, we were able to clone monoclonal sequence into plasmids in order to obtain standard curves (Supplementary table S2, Fig. S1). These standard curves fulfilled ESG-MRD-ALL criteria[21]. The detection was not performed for oligoclonal patients, as the plasmids carried different inserts of the VDJ rearrangements.

For RQ-PCR analysis of tumor-related marker, we started with specific consensus probes derived from the germline sequence of the FR3 region and designed for use in RQ-PCR in childhood ALL (ref.[20]). These consensus probes were used in 2/5 patients (LVH2 and LVH3 probes) and were fully complementary with patient's sequences. Because of high rate of somatic hypermutations occurring in MM, we designed new probes according to recommendations of Ladetto et al. for 3/5 patients (Supplementary table S3) (ref.[17]).

Quantification was based on human RNase P reference gene. The RQ-PCR reaction was successful for all 5 patients. Four samples were assessed as positive (concordant with qualitative PCR results) and quantifiable (Table 4). In one patient, MRD was assessed as positive, out of quantitative range. The RQ-PCR sensitivity was up to 10^{-6}

DISCUSSION

The aim of the work was to introduce MM-related marker identification for further MRD detection by PCR in our laboratory as a complementary tool for MRD assessment by FC. For the purpose of method introduction, we used retrospective patient samples from the time of diagnosis and relapse in order to confirm presence of clonogenic cells and their tumor-specific marker.

MRD monitoring is important for identification of patients at increased risk of relapse and plays a key role in treatment response assessment in clinical trials[7]. Unlike MRD detection by FC which can be applied in approximately 90% of patients with MM thus allowing routine examination, approaches based on PCR are more complex and can be applied in approximately 75% of patients with MM because of the extensive heterogeneity of the disease and presence of several MM clones at the time of diagnosis[6]. Although PCR is less applicable than FC, it is a powerful technique for treatment efficacy assessment and risk stratification in MM (ref.[17,22-25]). In this study, with the PCR detection of clonogenic cells, we were able to obtain qualitative assessment in 80% of patients and quantitative data, important for serial monitoring, in 50% of patients (Table 3).

We preferentially used FR1 or FR2 derived primers, as we need to obtain sequence of variable regions CDR2/3. Therefore, FR1/2 derived primers allowed us to obtain sequence that was shorter and more suitable for our analysis compared to using L derived primers[15,26]. Further, we did not use FR3 derived primers because the sequences obtained after such amplification are too short which increases the risk of false positive results. For comparison, Owen et al. used FR3 derived primers although this approach allowed detection of specific IgH rearrangement only in 56% of patients[27]. However, all of the above mentioned approaches are possible, as described previously by van Dongen et al.[16].

In our case, the successfully identified VDJ rearrangements were in accordance with average frequency of VH variants[16,28]. Although it is possible to perform PCR reaction with several primer families, in approximately 20% cases we were not able to identify specific VDJ rearrangement sequence. This was most likely due to the presence of somatic hypermutations and subsequent loss of primer binding sites in patient-specific sequence given by extensive heterogeneity in MM clones[4,7,15]. On average, there are 8% of mutated nucleotides of VDJ rearrangement sequence in MM patients[29,30]; however only 2% in chronic lymphocytic leukemia (CLL) and 4% in follicular lymphoma[7].

As the average homology of a patient's sequence with germline sequence is 92.2%, the annealing ability of primers derived from consensus regions of IgH is limited[30.] In our case, the primer design was successful for all patients with monoclonal sequences (5/10), and for one of the clones of all patients with oligoclonal sequence (3/10). In 1/10 case, we used sequence obtained from the plasmid with cloned hypervariable region for the primer design, as was previously described by Voena et al.[15.]

We also verified specificity of designed primers to only one patient by qualitative cross-annealing reaction, as was shown in 6/8 designed pairs of primers. Specific ASO primers are used for tumor marker identification and its further detection during the patient follow-up. However, we cannot exclude annealing of specific ASO primers on

different MM patient. The CDR2 and CDR3 regions are relatively short; therefore, there is a high probability of similar motifs repetition. Nevertheless, the crucial thing is that designed ASO primers are not able to amplify healthy DNA; primers have to be specific for MM (ref.[31]).

The method used for MRD quantification is RQ-PCR with ASO primers and probes. The fluorescence signal is provided by probe derived from the more conservative region of the VDJ rearrangement (FR3). First, the specific probe is chosen from previously designed probes for use in real-time PCR in childhood ALL (ref.[20]). Since these probes can be used for several patients, there was a significant reduction in the price of this method compared to methods using patient-specific probes[17,32].

Concerning the probes, we confirmed results of Ladetto et al., who analyzed the effect of mismatch between probe sequence and sequence of the patient. Probe was unsuccessful every time in the case of at least three mismatches. In contrast, probe was always successful in the case of no or one mismatch. And in the case of two mismatches, the success of probe annealing was in the type of substitution: G/C (strong interaction); A/T (weak interaction) (ref.[17]).

In our study, the RQ-PCR was successful for all 5 monoclonal patients. Four samples were assessed as positive (concordant with qualitative PCR results) and quantifiable. In one patient, MRD was assessed as positive, but out of quantitative range. The rate of ASO RQ-PCR is considerably variable because of significant heterogeneity of MM cells but approximately ranging from 30% to over 80% (ref.[22,23,32,33]). In the remaining 3 oligoclonal patients, we were not able to perform the quantification because of the oligoclonal nature of the disease. In these types of samples, it is not very feasible to prepare standard curves necessary for the MRD quantification.

The main advantage of MRD detection by RQ-PCR is its high sensitivity. The sensitivity is dependent on the specific ASO probe hybridisation and therefore also on the clone-specific IGHV sequence used for ASO primer and probe design[17]. Therefore, for this reason, we cannot reach the same sensitivity for all patients. In our work, we were able to reach RQ-PCR sensitivity of at least 10^{-4} and up to 10^{-6}. This result is in accordance with other studies since most studies dealing with RQ-PCR detection of MRD also reach sensitivity between 10^{-4} and 10^{-6} (ref.[7,21,33]).

Despite all the risks and complications, PCR based MRD detection has wide application, such as quantification of tumor contamination in healthy hematopoietic cells for autologous transplantation or following the dynamics of clonogenic cells and activity of the tumor load[34,35]. Its quantification is useful for treatment response and prognostic assessment as well as for early relapse detection[17,22,23,36,37].

CONCLUSION

Standard techniques used for remission evaluation are able to give only superficial information about the treatment efficiency because of their limited sensitivity. There is a need for more sensitive methods to gather more detailed insight and detection of small tumor cell residues. One of these methods is PCR detection of tumor-specific marker for MRD. This approach has some limitations because of significant heterogeneity of tumor plasma cells and presence of somatic hypermutations in MM. For this reason, it is only successful in some patients and also molecular biological approaches of MM-related marker detection are provided only in a limited number of laboratories. However, we successfully managed to establish this method at both qualitative and quantitative level in MM patients for the first time in the Czech Republic.

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Supplementary data

Table S1. Characteristics of used designed ASO primers for qualitative PCR detection of tumor-specific marker in MM.

Patient	Clonality	Alelle	Primer	Sequence (5'-3')	Length (mer)	T_m (°C)
1	monoclonal	IGHV2	forward	CACTTATTGATTGGGATGGT	20	56
			reverse	TAGTCAAAGTGCAGTCGCT	19	56
2	monoclonal	IGHV3	forward	ATGAGTAGTGACGGGGGTA	19	58
			reverse	TGTTGAAACTCTCGCACAGT	20	58
3	oligoclonal	IGHV3	forward	TAGTGGAGGTGAAACCCAAT	20	58
			reverse	TGCTTCATCTCCAGTGCCA	19	58
4	monoclonal	IGHV4	forward	TTACACTGGGAGCACCAAC	19	58
			reverse	AGATCGTAATCCGATCTCGC	20	60
5	monoclonal	IGHV3	forward	AAATCACCACCCACGGAGAT	20	60
			reverse	TCCGACATCATACGCACAGT	20	60
6	oligoclonal	IGHV3	forward	AGCTATATCACATGATGGAAGT	22	60
			reverse	TAGAACCCCCACTCCCGA	18	58
7	oligoclonal	IGHV1	forward	ACCCTAACGTTGGTGATACAA	21	60
			reverse	TAATCATAGTAATCTCTCGCAC	22	60
8	monoclonal	IGHV3	forward	TACTGGTGGTGGTAGCACAT	20	60
			reverse	CAATTATCATCCGCTTTCGC	20	58

Table S2. Standard curves parameters for RQ-PCR detection of tumor-specific marker in MM.

Patient	Probe	Slope	Correlation coefficient	Quantitative range	Sensitivity
1	ASO probe	-3.33	0.9988	1.00E-06	1.00E-05
	RNase P	-3.56	0.9995	1.00E-06	1.00E-05
2	ASO probe	-3.18	0.9959	1.00E-06	1.00E-06
	RNase P	-3.47	0.9991	1.00E-06	1.00E-06
4	ASO probe	-3.33	0.9959	1.00E-05	1.00E-05
	RNase P	-3.55	0.9985	1.00E-06	1.00E-06
5	ASO probe	-3.07	0.9985	1.00E-04	1.00E-04
	RNase P	-3.47	0.9990	1.00E-04	1.00E-04
8	ASO probe	-3.35	0.9974	1.00E-06	1.00E-05
	RNase P	-3.56	0.9995	1.00E-06	1.00E-05

Table S3. Sequences of designed probes for RQ-PCR detection of tumor-specific marker in MM.

Patient	Sequence (5'-3')	Length (mer)
2	5´FAM: CTCTGGAGATGGTGAATCTGCC-TAMRA	22
4	5´FAM: CCGTGTTTGCGGCGGTCACA -TAMRA	20
5	5´ FAM: GCCGTGTCCTCGACCCTCA-TAMRA	19

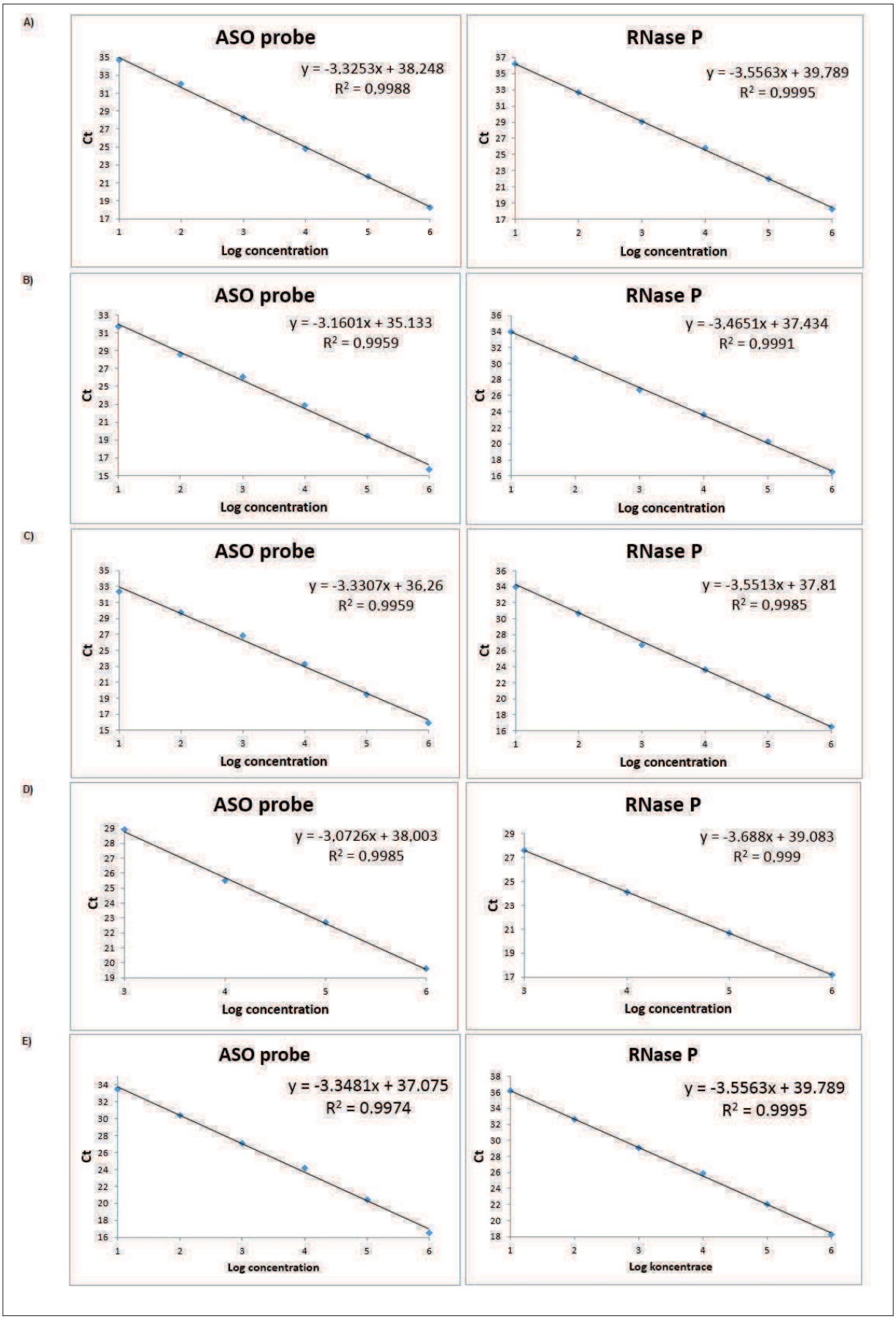

Fig. S1. Standard curves for quantitative real-time PCR detection of tumor-specific marker in MM.

A) Patient 1, B) Patient 2, C) Patient 4, D) Patient 5, E) Patient 8.

Proteasome inhibitors - molecular basis and current perspectives in multiple myeloma

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Proteasome inhibitors – molecular basis and current perspectives in multiple myeloma

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Abstract

Inhibition of proteasome, a proteolytic complex responsible for the degradation of ubiquitinated proteins, has emerged as a powerful strategy for treatment of multiple myeloma (MM), a plasma cell malignancy. First-in-class agent, bortezomib, has demonstrated great positive therapeutic efficacy in MM, both in pre-clinical and in clinical studies. However, despite its high efficiency, a large proportion of patients do not achieve sufficient clinical response. Therefore, the development of a second-generation of proteasome inhibitors (PIs) with improved pharmacological properties was needed. Recently, several of these new agents have been introduced into clinics including carfilzomib, marizomib and ixazomib. Further, new orally administered second-generation PI oprozomib is being investigated. This review provides an overview of main mechanisms of action of PIs in MM, focusing on the ongoing development and progress of novel anti-proteasome therapeutics.

Keywords: multiple myeloma • new-generation proteasome inhibitors • bortezomib

Introduction

The degradation of cellular proteins is a tightly regulated and complex process that plays a central role in regulating cellular function and maintaining homoeostasis in every eukaryotic cell [1]. The ubiquitin-proteasome pathway (UPP) represents the major pathway for intracellular protein degradation. More than 80% of cellular proteins are degraded through this pathway, including those involved in the regulation of numerous cellular and physiological functions, such as cell cycle, apoptosis, transcription, DNA repair, protein quality control and antigens [2, 3].

Proteasome as a new cell structure was described by Harris group in the beginning of 1970s as a hollow cylinder and single-torus proteins [4]. Later, it was elucidated that the function of proteasome is

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an ATP-dependent degradation of intracellular proteins, and its specificity is determined by interaction only with such proteins that are labelled by polyubiquitin chain or contain a specific amino acid sequence [1, 5]. For this important discovery, Ciechanover, Hershko and Rose received the Nobel Prize in chemistry in 2004.

Human 26S proteasome is formed by 20S proteolytic core region and 19S regulatory particle. 20S proteasome is an abundant, barrel-shaped molecule consisting of four highly homologous rings that enclose a central catalytic chamber with proteolytic active sites. Each of the rings contains seven subunits α and β, which are arranged one above the other in the order of α-β-β-α (Fig. 1). While the outer two α-rings surround a small opening through which only denatured polypeptide substrates may pass, two central β-rings contain multiple proteolytic sites that function together in protein degradation [6, 7]. Each of these two β rings comprises three proteolytic sites - β1 (caspase-like, C-L), β2 (trypsin-like, T-L) and β5 (chymotrypsin-like, CT-L) [8, 9]. Exposing cells to few stimuli, such as interferon-γ, tumour necrosis factor-α (TNF-α) and bacterial lipopolysaccharides, induces the synthesis of other catalytic subunits that are together incorporated into alternative proteasome form – immunoproteasome, which is preferentially expressed in cells of lymphoid origin and plays a role in major histocompatibility complex class I antigen presentation and other constitutive proteolytic activities [10–12]. In the 20S immunoproteasome (i20S), proteolytically active subunits β1, β2 and β5 are substituted by their equivalents β1i (LMP2) β2i (MECL-1) and β5i (LMP7; Fig. 1) [13]. Although proteasome contains multiple catalytic sites, to inhibit its function at the constitutive or immunoproteasome level, it is sufficient to block only the β5/LMP7 subunit (CT-L) [14, 15]. Proteins intended for degradation are incorrectly folded proteins and proteins with short half-life and mostly regulatory function that are being cut into oligopeptide chains with an average length of 8–12 amino acids [16, 17].

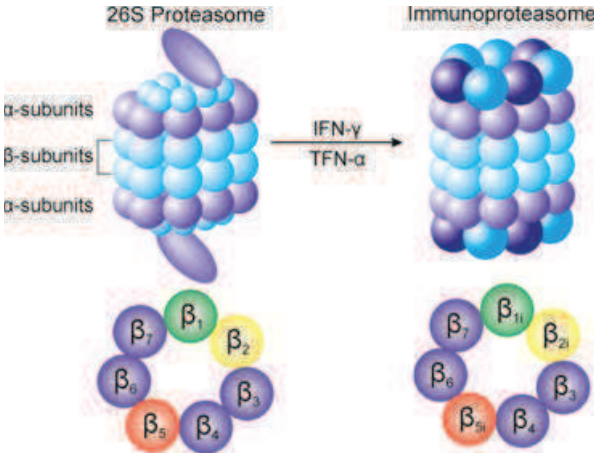

Fig. 1 Structure of 26S proteasome and immunoproteasome and its catalytic subunits. 26S proteasome consists of regulatory particle and proteolytic core region containing four subunits (arranged as α-β-β-α). In cells of hematopoietic origin, various stimuli, such as interferon (IFN)-γ and tumour necrosis factor (TNF)-α induce synthesis of immunoproteasome. Arrangement of proteolytically active subunits: β1 (β1i) – caspase-like subunit, β2 (β2i) – trypsin-like subunit, β5 (β5i) – chymotrypsin-like subunit of proteasome and immunoproteasome is displayed.

Proteasome inhibitors in multiple myeloma

It has become evident that defects within the UPP pathway are associated with a number of diseases, including cancer; thus, inhibitors of this pathway should prevent malignant cells from proliferation [18, 19]. The biggest group of proteasome inhibitors (PIs) is short peptides containing covalently attached pharmacophore – a group of atoms that binds to the catalytic sites of proteasome and thus prevents proper proteasome function [20]. Inhibition of proteasome is particularly useful for the treatment of multiple myeloma (MM), a haematological malignancy caused by malignant transformation of B-lymphocytes into pathological clonal plasma cells (PCs) that accumulate in the bone marrow (BM) and secrete high amounts of monoclonal immunoglobulin (Ig) [21]. As both normal and malignant PCs are highly secretory cells, they require a well-developed endoplasmic reticulum (ER), expansion of secretory apparatus and production of chaperone proteins that ensure proper Ig translation and folding [22]. A stress signalling pathway called the unfolded protein response (UPR) ensures that the PCs can handle the proper folding of proteins and prevent the aggregation of accumulating misfolded proteins. These proteins are then transported out of the ER and degraded by proteasome [23, 24]. It was shown that treatment of MM cells with PIs results in the accumulation of misfolded Ig within the ER, because of inhibition of proteasome function [25]. Such stress activates the UPR pathway, which is mediated by activation or translational repression of several transcription factors, such as XBP-1, ATF6 and PERK/eIF2α [25, 26]. Generally, UPR allows the cell to survive reversible environmental conditions, such as chemical insult or nutrient deprivation. However, during prolonged stress caused by PIs, UPR activation leads to cell cycle arrest [27] and induction of apoptosis [28]. PIs initiate UPR leading to apoptosis preferentially in cells with high Ig production; thus, partial inhibition of proteasome *in vivo*, which is not toxic to patients' normal cells, is sufficient to kill MM PCs [29].

Further, the therapeutic success of PIs in MM relies on their pleiotropic effects, which decrease both growth and survival of MM cells and the interaction between MM cells and BM microenvironment (MM cells adhesion, formation of new blood vessels and cytokine circuits; Fig. 2). Treatment with PIs has been associated with reports of increased bone formation markers and decrease in markers of bone resorption, which are the consequences of enhanced osteoblastogenesis and reduced number of osteoclasts [30, 31].

The ability of PIs to kill MM PCs and restore proper bone formation led to their use in clinics as one of the therapeutic approaches. Since the approval of first-class PI bortezomib for the treatment of MM, response rates and median survival of MM patients have considerably improved [32, 33]. Further, new-generation PIs, such as

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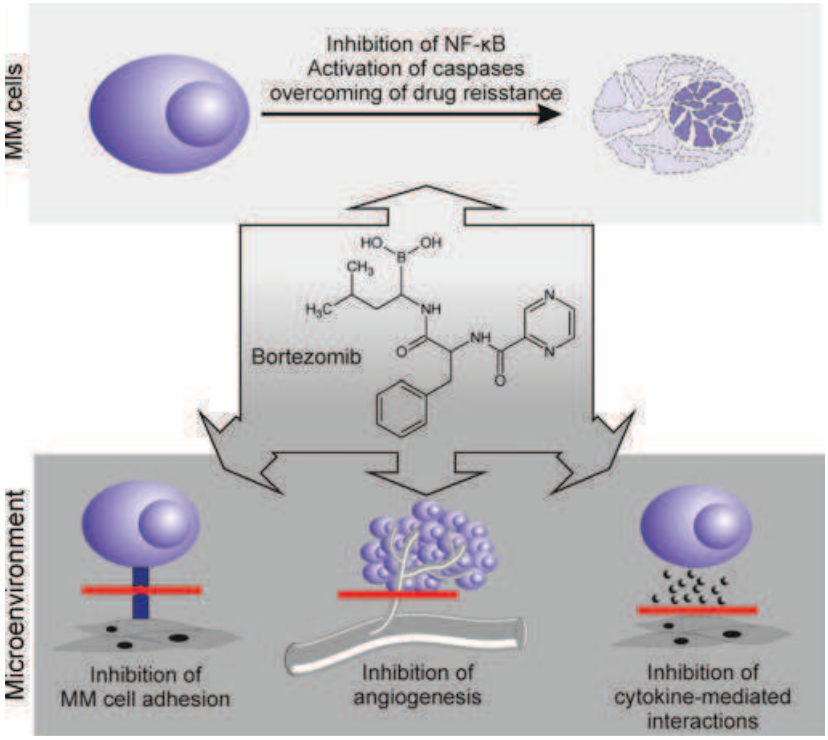

Fig. 2 Effects of proteasome inhibitors (PIs; bortezomib) on multiple myeloma (MM) cells and bone marrow (BM) microenvironment. Bortezomib affects MM cell survival and signalling pathways eventually leading to apoptosis. Also, bortezomib influences surroundings of MM cells as it inhibits adhesion of MM cells to BM microenvironment, angiogenesis and cytokine-mediated interactions.

Table 1 Characteristics of proteasome inhibitors evaluated for multiple myeloma treatment

Inhibitor of proteasome	Active moiety	Proteasome target	Key celullar effects	Binding	References
Bortezomib	Boronate	Preferentially CT-L/LMP7, C-L/LMP2 subunit, less T-L/MECL-1 subunit	NF-κB, caspase-8, 9, p21, p27, p53, Bid and Bax, caveolin-1, p-H3, EZH2, miR-29b, miR-15a	Reversible	[87]
Carfilzomib	Epoxyketone	Preferentially CT-L/LMP7 subunit	Caspases-3, 7, 8 and 9, JNK, eIF2, NOXA	Irreversible	[74]
Marizomib	β-lactone	Preferentially CT-L/LMP7 subunit, T-L/MECL-1 subunit, less C-L/LMP2 subunit	Caspase-8, NF-κB	Irreversible	[119]
Ixazomib	Boronate	Preferentially CT-L/LMP7 subunit, less C-L/LMP2 and T-L/MECL-1 subunit	Caspase-8, 9 and 3, p53, p21, NOXA, PUMA, E2F, cyclin D1 and CDK6, Bip, CHOP, miR-33b	Reversible	[82, 87]
Oprozomib	Epoxyketone	CT-L/LMP7 subunit	Caspases-8, -9, -3, PARP, JNK, NF-κB	Irreversible	[89, 120]
Delanzomib	Boronate	CT-L/LMP7 subunit	NF-κB	Reversible	[90]

carfilzomib, ixazomib, marizomib and oprozomib, are based on different chemical moieties than bortezomib and have modified pharmacologic properties, potentially resulting in better clinical outcome and reduced toxicity for MM patients (Table 1). Some of them are currently approved for the treatment of MM, the others are being investigated in multiple ongoing clinical studies (Table 2).

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Table 2 Clinical development of the drugs and ongoing pivotal trials in MM (according to myeloma.org and clinicaltrials.gov)

	Bortezomib	Carfilzomib	Marizomib	Ixazomib	Oprozomib	Delanzomib
Stage of development	Phase III	Phase III	Phase I	Phase III	Phase I/II	Phase I/II
Pivotal ongoing trials	Clinical trials for use with transplant, induction, consdolidation and maintenance therapy	Various phase III clinical trials including a trial comparing carfilzomib *versus* bortezomib	**NCT00461045:** Clinical trial of NPI-0052 in patients with relapsed or relapsed/ refractory MM	**NCT01850524**: MLN9708 in patients with newly diagnosed MM **NCT01564537**: MLN9708 in relapsed/ refractory MM	**NCT01832727:** Multicentre, open-label study of oprozomib and dexamethasone in patients with relapsed and/or refractory MM	Studies have been terminated
Approval	**EMA**: front-line, non-transplant, relapse					
	FDA: all settings	**FDA:** relapse	Not approved	Not approved	Not approved	Not approved

Bortezomib

Bortezomib (Velcade), formerly known as PS-341 (Millennium Pharmaceuticals, Cambridge, MA, USA), is the first-class PI that was approved in 2003 for treatment of refractory MM. In 2005, it was approved for treatment of MM patients who had received at least one prior therapy and in 2008 for the treatment of MM patients in first line [34–36]. Further, in 2012, FDA approved subcutaneous administration of bortezomib in all approved indications [37]. Chemically, it is a peptide boronate with molecular formula C19H25BN4O4 (Fig. 3A).

Bortezomib was synthesized for the first time in the mid-90s of the last century by Myogenics/ProScript (today Millennium Pharmaceuticals). An *in vitro* study on 60 cancer cell lines confirmed its high specificity, efficiency and oxidative stability [38]. Further, it was shown to potently inhibit cell proliferation in different MM cell lines, either drug sensitive or drug resistant [39]. The first clinical trial using bortezomib in the treatment of haematological malignancies was launched in November 1999. In this study, Orlowski *et al.* showed that low doses of bortezomib, used originally to verify its safety, led to complete remission in a 47-year-old MM patient. Moreover, further eight patients of 11 enrolled in the study showed at least minimal response or stable disease [40]. This result was important as it led to accelerated approval of bortezomib for the treatment of relapsed and refractory MM, after verification in further phases of clinical trials.

Mechanism of action

Central mechanism of bortezomib function is its covalent binding with high affinity to CT-L ($\beta5$) subunit of proteasome or LMP7 subunit of immunoproteasome; however, its binding to C-L ($\beta1$) and T-L ($\beta2$) subunits with lower affinity has been observed as well [41]. The differences in its affinity are because of different interactions of its side chains with each of the subunits [42]. When bound, bortezomib adopts an anti-parallel β sheet conformation, which is stabilized by direct hydrogen bond between the conserved residues (Gly47N, Thr21N, Thr21O, and Ala49O) of the β-type subunits and main chain atoms of the drug. The actual inhibition is mediated by a pharmacophore group, in this case boronic acid derivative. The boronic acid moiety of the drug ensures increased specificity for the proteasome. The boron atom covalently interacts with the nucleophilic oxygen lone pair of Thr1O$^{\gamma}$, while Gly47N, stabilizing the oxyanion hole, is hydrogen-bridged to one of the acidic boronate hydroxyl groups. The tetrahedral boronate adduct is further stabilized by a second acidic boronate hydroxyl moiety, which hydrogen-bridges the N-terminal threonine amine atom, functioning as a catalytic proton acceptor. Then, the resulting adduct is characterized by a low degree of dissociation, and therefore remains stable for several hours, even if it is a reversible reaction [42].

Today, the mechanism of action and molecular targets of bortezomib are well characterized. The downstream biological effects of proteasome inhibition are multifactorial, with direct effects on both MM cells and MM cell microenvironment, and key signalling pathways influenced by bortezomib are described further in this review.

NF-κB pathway

The initial rationale to use bortezomib in cancer was its inhibitory effect on inflammation-associated transcription factor, nuclear factor-κB (NF-κB) through stabilization of its inhibitor I-κB [43]. NF-κB not only regulates various immune and inflammatory responses, but it is also involved in several tumour-related processes, such as suppression of apoptosis and induction of angiogenesis, proliferation and migration. NF-κB is present in the cytoplasm as an inactive complex with its inhibitor I-κB and is activated by proteasomal degradation of I-κB [44]. As PIs inhibit function of proteasome, they prevent degradation of I-κB, subsequent translocation of NF-κB to the nucleus and

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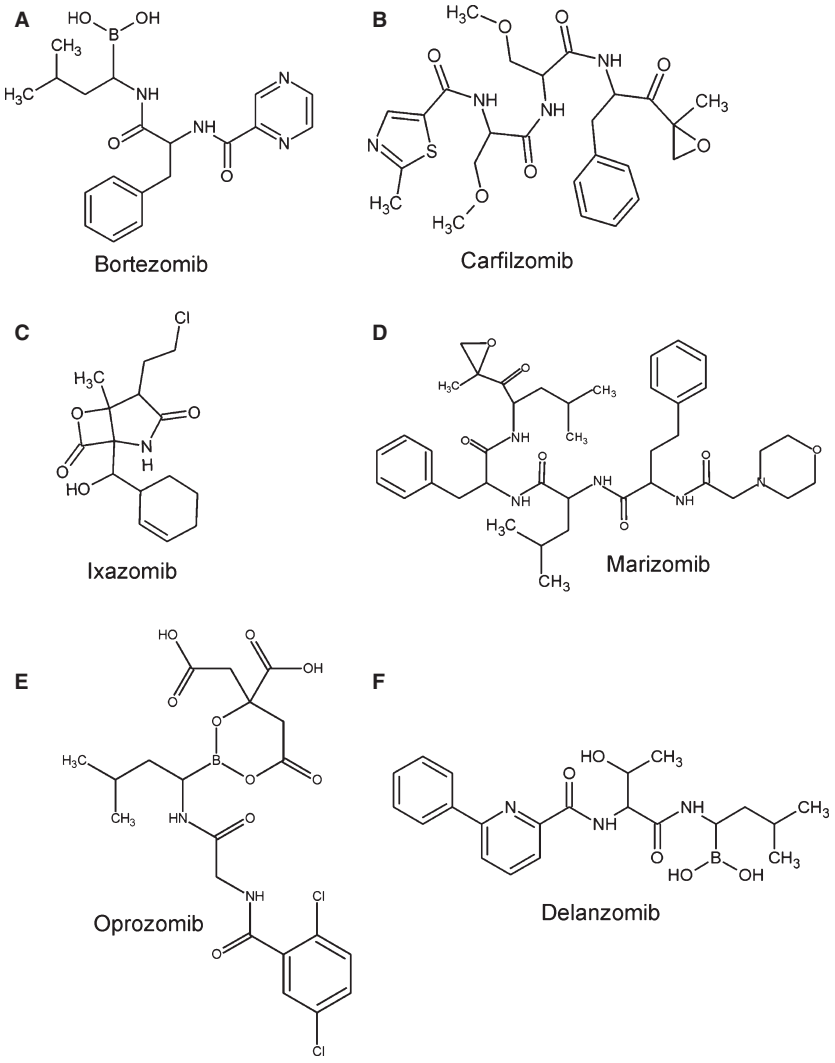

Fig. 3 Chemical structures of proteasome inhibitors. (**A**) Bortezomib, (**B**) Carfilzomib, (**C**) Ixazomib, (**D**) Marizomib, (**E**) Oprozomib, (**F**) Delanzomib.

binding to the promoters of target genes (such as anti-apoptotic genes, interleukin-6 *etc.*) [45]. It was elucidated that MM cell adhesion to BM stromal cells (BMSCs) induces NF-κB-dependent up-regulation of interleukin-6 (IL-6) expression by BMSCs [46, 47]. Therefore, inhibition of NF-κB could prevent IL-6 expression, which triggers terminal differentiation of normal B-cells and stimulates growth of MM cells [48].

Although pre-clinical and clinical studies with bortezomib showed down-regulation of transcriptional targets of NF-κB, further studies demonstrated that bortezomib is able to induce I-κB down-regulation that occurred at a transcriptional or post-transcriptional level in MM cell lines [49]. This study further showed that effect of bortezomib is cell dependent, as it triggered NF-κB activation *via* the canonical pathway, associated with down-regulation of I-κB in peripheral blood mononuclear cells, but significantly inhibited NF-κB in BMSCs. Further, it was demonstrated that bortezomib promotes non-proteasomal degradation of I-κB, as it activates two upstream NF-κB-activating kinases (RIP2 and IKKβ) and therefore is able to directly or indirectly (*via* RIP2) activate IKKβ, which subsequently phosphorylates I-κB leading to its degradation [49]. A hypothesis that instead of I-κB stabilization, bortezomib induces I-κB degradation was confirmed by a later study in which I-κB degradation by bortezomib occurred early before induction of apoptosis and could be prevented by calpain inhibitors. Therefore, in the presence of calpain inhibitors, the apoptosis-inducing activity of bortezomib was dramatically enhanced [50].

As bortezomib inhibits inducible NF-κB activity in MM cells, but enhances constitutive NF-κB activity *via* activation of the canonical pathway, bortezomib-induced cytotoxicity cannot be completely attributed to inhibition of canonical NF-κB activity in MM because inhibition of both canonical and non-canonical pathways is necessary to efficiently block total activity [49, 51].

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Apoptotic pathway

Inhibition of proteasome promotes programmed cell death of MM cells, as bortezomib is a potent activator of three distinct apoptotic pathways: the intrinsic pathway mediated by caspase-9 activation, the extrinsic pathway mediated by caspase-8 and death receptors (DR) activation and thirdly, activation of ER stress response pathway that involves caspase-2 (Fig. 4) [52–55].

In the first case, bortezomib induces Bax (pro-apoptotic member of the Bcl-2 family) accumulation, its translocation from cytosol to mitochondria, conformational change and oligomerization. Such changes lead to inhibition of anti-apoptotic Bcl-2, release of cytochrome c/Smac from mitochondria and activation of caspase-9 [56, 57]. Further, it was elucidated that bortezomib induces caspase-dependent apoptosis by promoting up-regulation of NOXA (pro-apoptotic BH3 member of Bcl-2 family), and down-regulation of apoptosis inhibitors, such as XIAP, Bcl-2 or c-FLIP *via* NF-κB blockade [58]. Bortezomib-induced cell death is also linked to the accumulation of ASF1B, Myc, ODC1, BNIP3, Gadd45α, p-SMC1A, SREBF1 and p53 [59]. Also, bortezomib induces p53-dependent apoptosis in MM cells, as it activates and stabilizes the tumour suppressor p53 protein *via* cleavage of the ubiquitin-ligation enzyme MDM2. Another mechanism of bortezomib-mediated apoptosis is *via* activation of extrinsic apoptotic pathway, as was demonstrated by an increased activity of c-Jun N-terminal kinase (JNK) and increase in death-inducing receptors Fas and DR5 that further enhanced Fas-mediated signalling and caspase-8 activation [58, 60, 61]. It was further elucidated that bortezomib activates caspase-2, which is associated with ER stress-initiated apoptosis. As caspase-2 functions upstream rather than downstream of mitochondria, it stimulates release of cytochrome c, changes within mitochondrial membrane and further caspase-9 activation [55].

Cell cycle and migration

In replicating cells *in vitro*, bortezomib seems to cause cell cycle arrest at the transition of G_2/M phase, which further leads to apoptosis in inhibited cells [62]. Bortezomib has also been shown to stabilize the cyclin-dependent kinase (CDK) inhibitors, such as p21 and p27 as it inhibits their degradation by proteasome, leading to disruption of cell cycle progression, that eventually cause apoptosis as well [39, 61]. This effect is partly mediated through inhibition of the NF-κB pathway [38].

Bortezomib *in vitro* triggered inhibition of VEGF and IL-6 secretion by MM patient-derived endothelial cells (MMECs); therefore, it inhibited function of BM milieu relevant to angiogenesis. The observation was also confirmed using an *in vivo* model [63]. Notably, bortezomib is a potent inhibitor of cell migration, as it is able to decrease caveolin-1 expression and prevent phosphorylation of caveolin-1 in MM cell lines. Caveolin-1 is a protein involved in cell motility or migration in a number of tissues and its activation requires VEGF-triggered thyrosine phosphorylation. As bortezomib also decreases VEGF secretion in the BM microenvironment, it prevents activation of caveolin-1 [64].

Using GEP70 and GEP80 models, Shaughnessy *et al.* found proteasome 26S subunit, non-ATPase4 (*PSMD4*) and two other

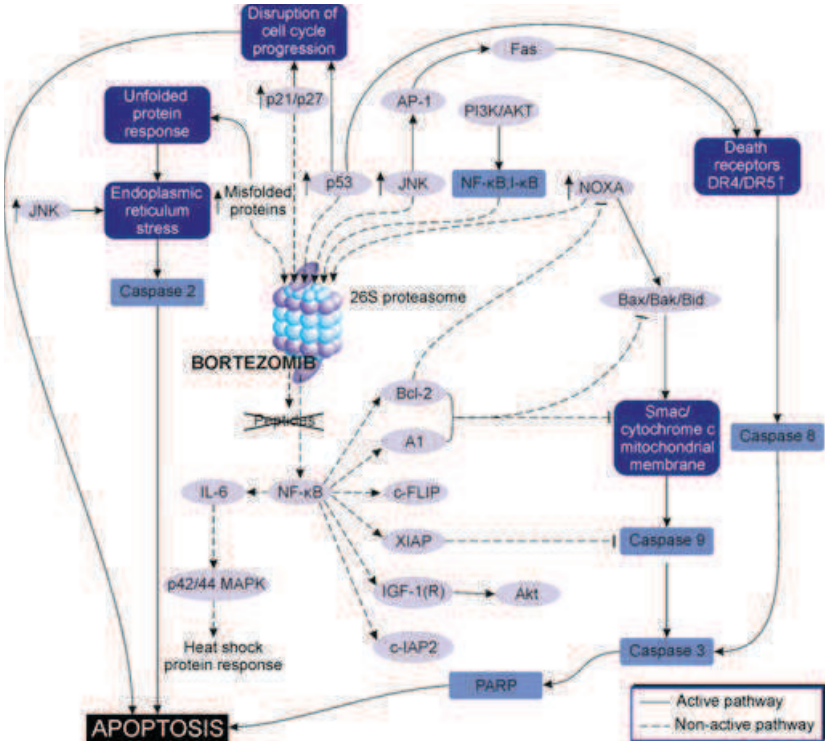

Fig. 4 Mechanism of antitumour activity of bortezomib in multiple myeloma (MM) cell. Inhibition of proteasome with bortezomib impairs turnover of multiple proteins resulting in their accumulation in the cell and disruption of multiple signalling pathways within the cell. Consequently, bortezomib-activated signalling pathways lead to disruption of cell cycle and apoptosis.

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proteasome genes to be up-regulated by bortezomib but not by immunomodulatory agents, dexamethasone or melphalan. Function of PSMD4 is binding and selecting ubiquitin-conjugates for destruction. Further analysis revealed that expression levels of PSMD4 (mapped to 1q21 region) are highly sensitive to copy number, as patients with high PSMD4 expression had four copies of 1q21 and patients with low expression had two copies. Authors anticipated that observed up-regulation of PSMD4 could be caused by preferential killing of normal PCs with two copies of 1q21, as more than 90% of PCs of MM patients tend to have more than two copies of 1q21. Both higher PSMD4 expression levels and higher 1q21 copy numbers affected clinical outcome adversely [65].

Effect of bortezomib on MM side population

Bortezomib was also shown to reduce tumourigenicity of MM as it targets the side population (SP) fraction of MM cells. The MM SP cells show high tumourigenic potential and self-renewal capability and are further characterized by up-regulation of polycomb-related genes, such as *EZH2* and *EPC1*. As bortezomib can reduce levels of p-histone H3 and EZH2, it effectively increased apoptosis and induced G2/M arrest in MM SP [66].

Bortezomib and microRNA

It was shown *in vitro* that bortezomib influences also microRNA (miRNA) expression, as the treatment of MM cell lines with bortezomib led to a dose-dependent increase in apoptotic cells and up-regulation of miR-29b. In this study, enforced expression of miR-29b also strongly increased bortezomib-induced growth inhibition, and thus potentiated its anti-MM activity. Moreover, phosphatidylinositol-3-kinase (PI3K)/AKT pathway played a major role in the regulation of miR-29b-Sp1 (transcription factor Specificity protein 1) loop and induction of apoptosis in MM cells [67]. Two other miRNAs, miR-15a and miR-16, are down-regulated in primary MM cells, their expression inversely correlated with the expression of VEGF and their ectopic overexpression *in vivo* resulted in inhibition of tumour growth and angiogenesis [68]. *In vitro* treatment of MM cells by bortezomib led to up-regulation of miR-15a in MM cells, although it was inhibited by MM-BMSCs. Interestingly, as MM-BMSCs are able to suppress miR-15a expression, they provide survival support and protect MM cells from bortezomib-induced apoptosis, as they block repression of bortezomib downstream targets (VEGF, cyclin D, Bcl-2; Fig. 5) [69].

Further effects

Bortezomib prevents repair of damaged DNA, reduces adhesion of MM cells to BM cells by inhibiting the mitogen-activated protein kinase (MAPK) signalling pathway and inhibits tumour angiogenesis [39, 70]. Angiogenesis in the BM environment plays an important role in MM pathogenesis and disease progression. It was shown in preclinical models using MMECs that bortezomib inhibited cell proliferation, chemotaxis, adhesion and capillary formation, which further supported its angiogenic inhibitory activity *in vivo*. Furthermore, it inhibited the expression and secretion of several pro-angiogenic factors, including VEGF [63].

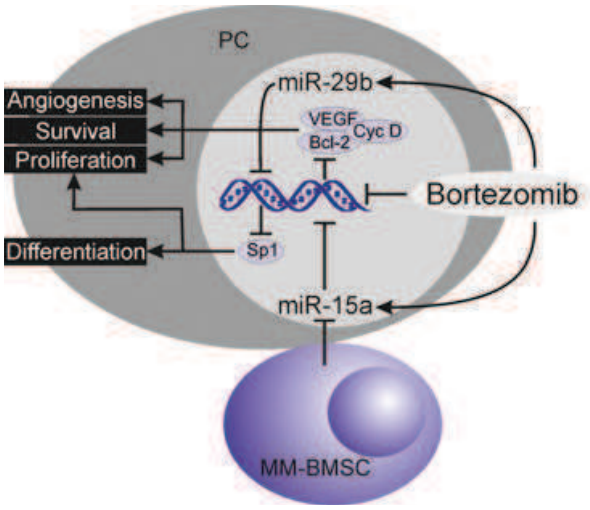

Fig. 5 Effect of bortezomib on miR-15a and miR-29b. Bortezomib up-regulates expression of miR-15a and miR-29b, which supports bortezomib-mediated effects on cell differentiation, proliferation and survival. However, expression of miR-15a is suppressed by bone marrow stromal cells (BMSCs) that protect multiple myeloma (MM) cells from bortezomib-induced apoptosis, as they block repression of bortezomib downstream targets (VEGF, cyclin D, Bcl-2-B-cell lymphoma, Sp1–transcription factor Specificity protein 1).

Bortezomib also participates in osteoclasts apoptosis and osteoblasts differentiation. It was elucidated that it promotes matrix mineralization and calcium deposition by osteoprogenitor cells and primary mesenchymal stem cells (MSCs) *via* Wnt-independent activation of β-catenin/TCF (transcription factor) signalling and nuclear accumulation of β-catenin. Both these factors are required for promoting MSCs differentiation into osteoblasts [71].

Second-generation of proteasome inhibitors

The phenomenal success of bortezomib in MM treatment increased the interest of scientific community in PIs. Optimization of the doses of bortezomib and its combination with other anticancer therapeutics reduced its negative side effects and partially suppressed resistance. Further, new generation of PIs was developed and was expected to bring even better results. Carfilzomib, ixazomib and marizomib represent the second-generation PIs and offer many benefits in terms of increased overall effectiveness, reduced negative off-target effects and overcoming resistance to bortezomib because of their different chemical structure, biological properties, mechanism of action, irreversibility/reversibility of proteasome inhibition and usage [72].

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Carfilzomib

Carfilzomib, an irreversible inhibitor of proteasome (also known as PR-171, Kyprolis; Onyx Pharmaceuticals, San Francisco, CA, USA), is a tetrapeptide epoxyketone with molecular formula C40H57N5O7 (Fig. 3B). Peptide epoxyketones are the most advanced and specific PIs known to date, as were shown in pre-clinical studies, where carfilzomib targeted haematological-specific immunoproteasome. Its promising characteristics and potential to overcome drug resistance led in 2012 to its approval by FDA for MM treatment (www.fda.gov).

Mechanism of action

Carfilzomib binds to CT-L (β5) catalytic subunit of the proteasome or LMP7 subunit of immunoproteasome with higher selectivity than bortezomib [73]. In this case, inhibition is an irreversible process that decreases proteasomal activity to less than 20%; therefore, restoration of proteasome activity in cells is only possible by new synthesis of individual subunits and their further compilation into new proteasome [74].

Carfilzomib forms a unique six-atom ring structure with β5 subunit leading to intramolecular cyclization and morpholino adduction. This intermolecular cyclization is a two-step mechanism. In the first step, oxygen from hydroxyl group of Thr1 nucleophilically attacks carbon of epoxyketone, which subsequently leads to formation of hemiacetal. The second step is a nucleophilic attack of the α-amino nitrogen of Thr1 to C2 carbon-epoxide ring, resulting in the formation of the morpholine adduct [75, 76].

Compared to bortezomib, carfilzomib has only little off-target activity outside the proteasome, can induce apoptosis of bortezomib-naïve and even bortezomib-pre-treated MM cells without increased toxicity and is also more effective in xenograft models, which is consistent with its higher affinity for the proteasome [74, 77].

In MM cells exposed to carfilzomib, induction of both external and internal apoptotic cascade was observed, with significant elevations of caspases-3, -7, -8 and -9. Programmed cell death has been associated with activation of JNK, mitochondrial membrane depolarization and cytochrome c release. Moreover, an initial decrease in phosphorylated eIF2 was observed, in connection with the ER stress that is induced by accumulation of non-functional proteins and increased levels of NOXA (pro-apoptotic member of Bcl-2 family) [15, 74].

Recently, it has been elucidated that carfilzomib promotes MSCs differentiation into osteoblasts with a mechanism similar to that used by bortezomib. It was also shown that carfilzomib does not affect β-catenin gene expression, implying that it induces activation of β-catenin/TCF activity by blocking β-catenin degradation [78].

Ixazomib

Ixazomib (MLN9708, Takeda/Millenium Pharmaceuticals), an analogue of boric acid, is the first orally administered, reversible PI, which has demonstrated greater potential activity against MM cells than bortezomib in *in vivo* pre-clinical studies [79]. This second-generation PI with chemical formula C20H23BCl2N2O9 is immediately hydrolyed in aqueous solution or plasma to MLN2238, a biologically active form (Fig. 3C) [80]. Therefore, it is capable of a wider distribution in blood in a stable form and has greater pharmacodynamic effects in tissues [81].

Mechanism of action

Ixazomib (its active form MLN2238), just like bortezomib, inhibits particularly the CT-L (β5) subunit of the 20S proteasome. Moreover, in higher concentrations, it is able to inhibit C-L (β1) and T-L subunit (β2) and induce accumulation of ubiquitinated proteins [79, 82]. It has a shorter 20S proteasome dissociation half-life than bortezomib and an improved pharmacokinetic and pharmacodynamic profile. Both ixazomib and bortezomib showed time-dependent reversible proteasome inhibition; however, proteasome dissociation half-life for ixazomib was determined to be about 6-fold faster than that of bortezomib (half-life of 18 and 110 min. respectively) [82].

Ixazomib is responsible for caspase-dependent induction of apoptosis and inhibition of cell cycle in MM cells. Administration of the drug leads to activation of caspase-8, -9 and -3, increased levels of p53, p21, pro-apoptotic proteins NOXA, PUMA, transcription factor E2F and *vice versa* reduced levels of cyclin D1 and CDK6. Treatment with ixazomib also induced expression of Bip and CHOP – heat shock protein and transcription factor connected with ER, which expression is induced by cellular stress and is involved in mediating apoptosis. Further, ixazomib effectively inhibits the canonical and non-canonical NF-κB pathways in MM supporting cells, thus influencing cytokines important for growth and survival of MM cells secreted by BMSCs. In this way, cyto-protective effects of BM microenvironment on MM cells are disrupted. It was also shown that ixazomib inhibits tumour-associated angiogenic activity, as the number of VEGFR2- and PECAM-positive cells (cells containing two distinct markers of angiogenesis) was reduced [79]. Study on mouse models revealed that unlike bortezomib, ixazomib possibly relieves bone osteolysis, the most common symptom of MM [80].

MicroRNA profiling of MM cells treated with ixazomib showed increased expression of miR-33b. Increased expression of this miRNA is associated with reduced migration and viability of MM cells as well as with increased apoptosis and sensitivity of MM to ixazomib. Moreover, overexpression of miR-33b led to negative regulation of oncogene PIM-1. Therefore, Tian *et al.* proposed that miR-33b acts as a tumour suppressor which is involved in the apoptosis of MM cells induced by ixazomib treatment, leading to inhibition of tumour growth and increased survival of human MM xenograft models [83].

Marizomib

Marizomib, also known as NPI-0052 or Salinosporamid A (Nereus Pharmaceuticals), is a secondary metabolite of obligate marine bacterium, actinomycetes *Salinispora tropica*; it is the first natural PI, which has been included in MM clinical research [84]. Chemically, marizomib is bicycle β-lactone-γ-lactam with molecular formula C15H20ClNO4 (Fig. 3D). Unlike all others PIs, it does not contain a

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peptide chain in its structure; therefore, it is structurally distinct from bortezomib and carfilzomib. In pre-clinical studies with MM cell lines, marizomib was shown to be highly effective [85]. The combination of bortezomib with marizomib would allow using individual drugs in such concentrations that are non-toxic for patients and improve combined anti-myeloma effect of drugs [86].

Mechanism of action

Unlike bortezomib and carfilzomib, which are selective for the CT-L activity of the proteasome, marizomib inhibits all three enzymatic activities of the proteasome as it binds irreversibly with high affinity to the CT-L (β5) and T-L (β2) catalytic sites as well as with lower affinity to the C-L (β1) subunit [87]. It contains a β-lactone ring that is uniquely substituted with a chloroethyl group playing a role in its irreversible properties. This group binds to the S2 binding pocket of the active site, and as chlorine behaves as a leaving group, it is eliminated to render a stable cyclic ether end product following acylation of the catalytic enzyme active site Thr1O^{γ} by the β-lactone of the inhibitor [42].

Comparably to bortezomib, marizomib inhibits the canonical NF-κB pathway and related secretion pathways, such as IL-6, TNF-α and IL-1β. [87]. On the other hand, unlike bortezomib, which activates both caspase-8 and -9, the apoptotic effect of marizomib is mainly mediated by caspase-8 activation and, to a lesser extent, by caspase-9. As the mechanism of caspases activation by marizomib is different than in bortezomib-mediated activation and relies primarily on caspase-8 activation, it allows to overcome the resistance of MM cells to apoptosis also with Bcl-2 mutations, leading to overexpression of Bcl-2. It was shown that overexpression of Bcl-2 in MM cells confers drug resistance and partially protects MM cells against bortezomib; however, caspase-9 activation by marizomib is minimally affected by Bcl-2 overexpression [87]. Apoptotic signal leads to the release of cytochrome c and Smac proteins from mitochondria to the cytoplasm, generation of oxygen radicals and activation of caspases. Moreover, marizomib is able to induce apoptosis in MM cells even in the presence of MM growth factors IL-6 and insulin growth factor-1 (IGF-1) and is involved in blocking IL-6 secretion in BMSCs without affecting their viability. Notably, marizomib significantly blocks MM cells migration induced by VEGF and thus confirms its anti-angiogenic effect [87].

Novel proteasome inhibitors

Oprozomib

Oprozomib (ONX0912; Onyx Pharmaceuticals), a new orally bioavailable and selective peptide epoxyketone PI, represents a derivate of carfilzomib, which irreversibly inhibits proteasome resulting in longer duration of inhibition compared with bortezomib (Fig. 3E). Just like bortezomib and carfilzomib, oprozomib is highly selective for CT-L (β5) subunit of proteasome; however, in contrast with bortezomib, it specifically inhibits only N-terminal threonine active proteasome subunits [88].

The orally bioavailable oprozomib inhibits proteasome with the same efficacy as intravenously delivered carfilzomib, although in higher concentrations of the drug. It is able to activate JNK and inhibit NF-κB pathways [88, 89]. Further *in vitro* study using MM cell lines showed that oprozomib inhibits growth, migration and induces apoptosis of MM cell lines and its activity is associated with activation of caspase-8, -9 and -3, and poly(ADP) ribose polymerase (PARP). Oprozomib, suchlike carfilzomib, directly inhibits osteoclasts differentiation and function *in vitro*. Conversely, it directly stimulates transforming growth factor-β (TGF-β) and MAPK signalling pathways leading to increased activity of UPR, which results in enhanced osteoblasts differentiation and matrix mineralization. Therefore, in MM, oprozomib in a similar way as carfilzomib, shifts the BM microenvironment from catabolic to anabolic state. Efficacy of oprozomib was also tested *in vivo* using mouse models. Comparably to carfilzomib, oprozomib inhibited MM growth, prolonged survival of mouse models, decreased tumour burden and inhibited bone resorption [31, 88]. Moreover, oprozomib in combination with low-dose bortezomib showed a synergistic anti-MM activity. However, mechanisms mediating combined anti-MM activity of both PIs remain to be defined [88].

Delanzomib

Delanzomib (CEP-18770; Teva Pharmaceuticals, North Wales, PA, USA) is an orally active, reversible, boronic acid-based PI. Suchlike bortezomib, it exhibits high potency primarily against CT-L (β5) and then C-L (β1) activity (Fig. 3F).

An *in vitro* study showed that it effectively decreases NF-κB activity and expression of several NF-κB downstream effectors; furthermore, it has strong anti-angiogenic activity and potently represses receptor activator of NF-κB ligand (RANKL)-induced osteoclastogenesis [90]. Further *in vitro* study on MM cell lines compared delanzomib with bortezomib in terms of specificity and activity profiles. While the two PIs show comparable proteasome inhibitory effects on cell lines, *ex vivo* study on pre-clinical mouse model of human MM showed that delanzomib induced an improved response in MM tumours and is active also against bortezomib-resistant cells [91].

Delanzomib was shown in pre-clinical and clinical studies to be effective in combination with melphalan or bortezomib with favourable cytotoxicity profile [92, 93]. Although it showed promising effect and favourable toxicity in the initial studies, its further research has been suspended because of unmanageable toxicity (Teva, personal communication).

Mechanism of resistance and cross-resistance of PIs

Despite high efficacy of bortezomib, there are still MM patients that are primarily resistant or develop secondary resistance to bortezomib during treatment [94]. So far, several molecular mechanisms of resistance development have been identified. One of them is Ala49Thr

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mutation in the β5 subunit (PSMB5) of the proteasome, which is situated in a binding site for bortezomib and leads to excessive synthesis of PSMB5. Higher levels of PSMB5 at the RNA or protein level were shown to be connected with resistance to bortezomib [95, 96]. High-throughput RNA screen of MM cell lines further revealed a panel of genes – their suppression enhanced bortezomib sensitivity. These genes included proteasome subunits α- and β-type (*PSMA5*, *PSMB2*, *PSMB3* and *PSMB7*) as well as *Aurora kinase A*, *CDK5* and modulators of the aggresome pathway. Moreover, the authors confirmed that *CDK5* knockdown sensitized MM cell lines to bortezomib and other PIs [97]. In addition, factors downstream of proteasome enzymatic complex can mediate resistance to bortezomib, as was observed in a study where increased resistance of tumour cells to the treatment correlated with elevated levels of anti-apoptotic proteins from the Bcl-2 family and heat shock proteins Hsp27, Hsp70 and Hsp90 [87, 98]. Zhang *et al.* revealed that in bortezomib-adapted cell lines, the treatment continued to inhibit proteasome enzymatic activity. However, it did not lead to induction of UPR and accumulation of pro-apoptotic proteins p53, Mcl-1S and NOXA because the cells displayed increased expression of factors protecting them from bortezomib-mediated ER stress [99]. More recently, other signalling pathways have been described to mediate bortezomib resistance. It was evaluated that interactions between Notch receptors on MM cells and Notch ligand DII expressed on MM-BMSCs could contribute to bortezomib resistance. Activation of Notch signalling leads to up-regulation of CYP1A, a cytochrome P-450 enzyme involved in the metabolism of a variety of xenobiotic compounds [100]. Blockade of Notch pathway and inhibition of CYP1A expression was able to increase sensitivity of MM cells to bortezomib *in vitro* [101]. Further *in vitro* study on MM cell line with no mutation in β5 subunit revealed evidence that increased IGF-1 signalling through enhanced IGF-1 secretion and IGF-1R activation was also associated with resistance to bortezomib [102]. Newly, the focus is on POMP (proteasome maturation protein), which is involved in addition of catalytically active β subunits to the hemiproteasome ring initially formed by structural α subunits. In bortezomib-resistant cell lines the levels of POMP mRNA are enhanced compared to their drug-sensitive counterparts. POMP overexpression, that is influenced by NF erythroid-2 (NRF-2), contributes to PI resistance in MM [103].

It was described that bortezomib-resistant cells display a marked cross-resistance to β5-targeted cytotoxic peptides, but not to other classes of therapeutic drugs, therefore cross-resistance of bortezomib-resistant cells is restricted to (peptide) drugs that primarily target the proteasome β5-subunit [96]. Therefore, drugs with similar mechanism of action as bortezomib, such as ixazomib and delanzomib, will unlikely overcome bortezomib resistance. However, new drugs that are based on epoxyketone pharmacophore, such as carfilzomib or oprozomib, differ in terms of their chemical structure and mechanism of action. Moreover, carfilzomib is a more selective inhibitor of the CT-L activity of proteasome and immunoproteasome and shows prolonged irreversible inhibition of proteasome – thus it could overcome resistance to bortezomib [77, 104]. In fact, carfilzomib was shown in pre-clinical experiments to overcome bortezomib resistance [74]. Nevertheless, new study using mouse MM model and gene expression profiling revealed that bortezomib-resistant cells show cross-resistance to ixazomib and carfilzomib as well. Results of this study also suggested that resistance to one drug class reprograms resistant clones for increased sensitivity to a distinct class of drugs, such as inhibitors of histone deacetylases [105]. Taken together, the results from pre-clinical studies are contradictory so far; although irreversible PIs demonstrate an ability to overcome some forms of bortezomib-mediated resistance, further studies using *e.g.* combination of irreversible PIs with other chemotherapeutic agents may identify strategies to enhance efficacy or decrease toxic effects. Further, there is a need for additional analyses from currently ongoing studies, which include bortezomib-refractory patients who are treated with either analogues of boric acid PIs, such as ixazomib, or epoxyketones, such as carfilzomib.

Induction of neuropathy

Peripheral neuropathy (PN) is a significant and most common dose-limiting toxicity of PIs. The pathophysiology and molecular basis of bortezomib-induced PN is not completely understood and current knowledge is limited. Damage of mitochondria and ER seems to play a key role in bortezomib-induced PN genesis, as bortezomib can activate the mitochondrial-based apoptotic pathway [106]. Generally, dipeptide boronates inhibit active proteasome subunits, but also serine proteases. Although inhibition of proteasome by bortezomib has originally been shown to be several orders of magnitude stronger than inhibition of serine proteases [107], it has been revealed that bortezomib inhibits also an ATP-dependent serine protease in mitochondria – HtrA2/Omi. As HtrA2 protects neurons from apoptosis, it is now believed that its inhibition is the cause of PN in MM [108]. In contrast with bortezomib, peptide epoxyketones, such as carfilzomib and oprozomib, specifically inhibit only N-terminal threonine active proteasome subunits. This difference may be responsible for the favourable toxicity profiles and relatively low rates of PN associated with epoxyketone PIs.

Further proposed mechanism of bortezomib-induced PN genesis is dysregulation of neutrophins, as bortezomib inhibits activation of NF-κB and thus blocks the transcription of nerve growth factor-mediated neuron survival [109].

A GEP study compared patients with grade 2–4 late-onset bortezomib-induced PN, patients developing early-onset grade 2–4 bortezomib-induced PN and patients who did not develop bortezomib-induced PN. Results suggested that patients with early-onset grade 2–4 bortezomib-induced PN have dysregulated genes involved in control of transcription, apoptosis and AMPK-mediated signalling. Out of them, AMPK-mediated signalling is of particular interest, because this enzyme stimulates the signalling pathways that replenish cellular ATP supplies in response to low glucose, hypoxia, ischaemia or heat shock, which might be triggered in MM cells in response to bortezomib. On the other hand, patients with late-onset grade 2–4 bortezomib-induced PN showed 27 differentially expressed genes when compared to the first group, with the enrichment of expression of genes involved in transcription regulation as well as in the development and function of the nervous system, including *SOD2* and *MYO5A* [110]. Moreover, the authors suggested an interaction

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Table 3 Key features of different proteasome inhibitors

Inhibitor of proteasome	IC 50 for CT-L activity	IC 50 for C-L activity	IC 50 for T-L activity	Half-life (minutes)	Application
Bortezomib	7.9 ± 0.5 nM	53 ± 10 nM	590 ± 67 nM	110	Intravenous
Carfilzomib	<5 nM	2400 nM	3600 nM	<30	Intravenous
Marizomib	3.5 ± 0.3 nM	430 ± 34 nM	28 ± 2 nM	10–15	Intravenous
Ixazomib	3.4 nM	31 nM	3500 nM	18	Oral
Oprozomib	36 nM/82 nM	ND	ND	30–90	Oral
Delanzomib	3.8 nM	ND	ND	ND	Oral

between MM-related factors and the patient's genetic background in the development of bortezomib-induced PN, as several of single nucleotide polymorphisms (SNPs) were associated with early-onset bortezomib-induced PN (SNPs located in *caspase 9*, *RDM1*, *ALOX12*, *IGF1R* and *LSM1* genes). In addition, SNPs associated with late-onset bortezomib-induced PN were preferentially located in DNA repair genes (SNPs in *ERCC3*, *ERCC4*, *ATM*, *BRCA1*, *EXO1* and *MRE11A* genes) [110].

Apart of mechanisms mentioned above, another possible mechanism for different rates of PN induced by PIs is their diverse proteasome dissociation half-life, pharmacokinetics and pharmacondynamics (Table 3). It was shown that in the group of boronic acid PIs, ixazomib has a six-fold faster proteasome dissociation half-life than bortezomib, greater overall tumour pharmacodynamic effect than bortezomib and prolonged overall survival in a mouse model. All of these features might stand for lower toxicity [82]. Further, as was tested on a fruit fly model, the group of epoxyketone PIs exerts significantly milder impact on neuromusculatory system than bortezomib [111].

Proteasome inhibitors in other diseases

Besides MM, bortezomib has been described to be effective in several other lymphoid malignancies; it demonstrated clinical potential in the treatment of mantle cell lymphoma (MCL) and non-Hodgkin lymphoma and is effective also in treatment of newly diagnosed Waldenström macroglobulinaemia (WM) [112–114]. In 2007, FDA granted approval to single-agent bortezomib for the treatment of relapsed/refractory MCL patients [115] and currently it is tested in multiple clinical trials as a component of cytotoxic chemotherapeutic regimens [116]. Further experience with second-generation PIs, such as carfilzomib is limited. Carfilzomib in combination with other drugs was tested in B-cell lymphomas (DLBCL, MCL) to overcome bortezomib resistance with promising results [117]. Also, it is under investigation in WM patients. Although the data are still immature, the biggest advantage of this drug seems to be the lack of neurotoxicity [118].

Conclusion

The inhibition of proteasome has become an impressively successful strategy in MM treatment for the past 10 years, because of the particular sensitivity of MM cells to the mechanism of action of such agents. Today, therapies based on bortezomib belong to the standards of care for both relapsed/refractory and previously untreated MM patients, dramatically improving outcome of these patients. Although the exact mechanisms of action of PIs are not yet fully defined, four major mediators of direct anti-MM activity have been identified: transcription factor NF-κB, pro- and anti-apoptotic factors, p53 protein and UPR leading to ER stress response. However, the effect of PIs should be understood as a complex process involving many signalling pathways as neither the inhibition of NF-κB nor inactivating mutations of p53 are able to evoke apoptosis of MM cells induced by PIs. Detailed studies of mechanism of PIs action have a great potential for the discovery of possible molecular targets of new-generation drugs. Continuing development of new-generation PIs will likely offer further opportunities and better regimens in terms of treatment efficacy, acceptable tolerability, administration and quality of life of MM patients. Nowadays, PIs are the most effective group of anti-MM drugs, and they will surely be a cornerstone in all combination regimens used in MM treatment even in the future.

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Conflicts of interest

The authors confirm that there are no conflicts of interest.

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The miR-29 family in hematological malignancies

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The miR-29 family in hematological malignancies

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Background. MicroRNAs are short non-coding regulators of gene expression. The human miR-29 family consists of three members: miR-29a, miR-29b and miR-29c. Members of this family were found to be aberrantly expressed in various types of tumors, including hematological malignancies. This family was described to have both oncogenic and tumor suppressor features influencing various pathological processes, such as tumor growth and apoptosis. This review summarizes current knowledge about the miR-29 family in selected hematological malignancies.
Conclusion. Recent research of miR-29 family in hematological malignancies has proven its oncogenic as well as tumor suppressive potential. Nevertheless, the level of current evidence is not sufficient, and data remain inconclusive.

Key words: microRNA, miR-29 family, hematological malignancy, circulating miRNA

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INTRODUCTION

MicroRNAs (miRNAs) are short, non-coding and highly conserved RNAs, approximately 22 bp in size[1]. The genes for miRNAs represent 1-2% of all known eukaryotic genes[2]. They regulate gene expression, both at transcriptional and translational levels. A single miRNA molecule can control expression of various genes and *vice versa*, one gene can be regulated by various miRNAs[3]. MiRNAs act in a wide range of physiological biological processes, such as cell proliferation, differentiation, apoptosis and hematopoiesis[4-6]. As all of these processes, as well as miRNAs levels, are dysregulated in solid tumors and hematological malignancies, it was confirmed that there is an association between miRNAs and cancer[7]. Despite the fact that miRNAs were discovered in 1993 (ref.[8]), it was only in 2002 that miR-15a and miR-16-1 were identified as potential cancer genes in the pathogenesis of chronic lymphocytic leukemia (CLL) highlighting the direct link between miRNAs deregulation and hematological malignancy[9].

Genes for miRNA are frequently located at fragile sites and genomic regions involved in cancers, such as minimal regions of loss of heterozygosity, minimal regions of amplification (minimal amplicons), or common breakpoint regions, explaining the contribution of miRNAs to cancerogenesis[10]. Such localization may lead to upregulation of miRNAs levels or their downregulation during pathological processes. Further, depending on the mRNA target which miRNAs bind and regulate, they can act either as oncogenes (also called oncomirs) or as tumor suppressors[11]. Due to overexpression of miRNA targeting tumor suppressor gene, anti-oncogenic mechanisms can be inhibited, whereas defects of miRNA repressing oncogene can lead to gain of oncogenic features. Both these roles have been demonstrated in tumors[12-14]. In general, miRNAs can affect specific cell development (e.g. B cell) or alter expression of components in miRNA biogenesis in hematological malignancies.

Canonical model of the miRNAs biosynthetic pathway involves several steps as shown in Fig. 1. In the nucleus, RNA polymerase II transcribes miRNA genes into long primary precursors – pri-miRNAs. These are recognized and cleaved by microprocessor complex including ribonuclease Drosha and dsRNA-binding protein Pasha (or DGCR8) (ref.[15,16]). Secondary precursors are short, about 70 nucleotides stem-loop structures, known as pre-miRNAs that are further actively transported to cytoplasm by exportins, Ran-GTP dependent transporters. In the cytoplasm, pre-miRNAs are processed near the terminal loop by RNase III type endonuclease Dicer, which is in complex with dsRNA-binding protein TRBP (TAR RNA binding protein), and this generates mature miRNA/miRNA* duplexes[17,18]. Subsequently, one strand of mature miRNA (so-called guide strand), which is less stable at the duplex 5'end, is incorporated into the Argonaut protein, a central part of multiprotein complex miRISC (miRNA-induced silencing complex). The other strand, called the passenger strand (miRNA*), is released from the duplex and degraded. The question of gene fate now lies in the RISC complex with incorporated mature miRNA because this is the site where gene mRNA and miRNA pair. If the complementarity between the seed sequence of miRNA (2-8 nucleotides at 5'end) and the 3'UTR of the target mRNA is perfect, mRNA is cleaved and degraded. In the other case, low degree of complementarity leads to inhibition of mRNA translation (Fig. 1) (ref.[19]).

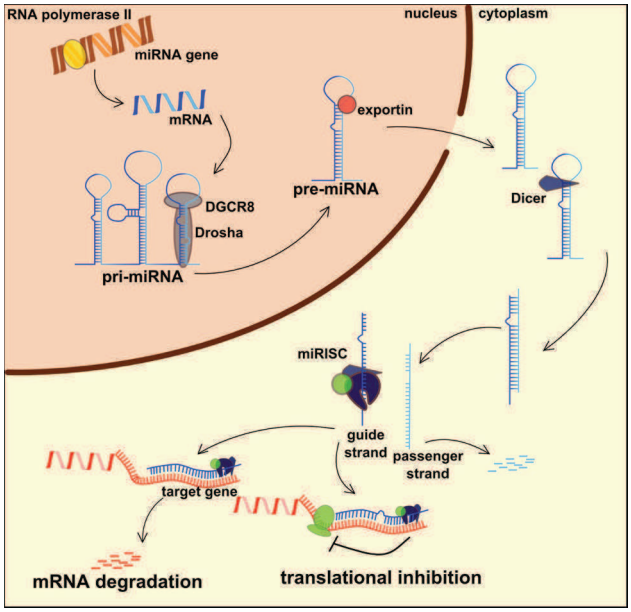

Fig. 1. MiRNA biogenesis. miRNA genes are transcribed in the nucleus, into long primary precursors – pri-miRNAs. Then, they are cleaved by Drosha and Pasha. Secondary precursors are short, stem-loop structures, known as pre-miRNAs that are further actively transported to cytoplasm by exportins. In the cytoplasm, pre-miRNAs are processed near the terminal loop by RNase III type endonuclease Dicer, and this generates mature miRNA/miRNA* duplexes.

THE MIR-29 FAMILY

In the human miR-29 family, the precursors are transcribed into two clusters, miR-29a/miR-29b-1 from chromosome region 7q32, and miR-29b-2/miR-29c from chromosome region 1q32. As the only difference between miR-29b-1 and miR-29b-2 is their localization in different parts of genome, they both form identical mature miR-29b. The first discovered member of the family was miR-29a in HeLa cells in 2001 (ref.[20]), followed by miR-29b and miR-29c (ref.[21,22]).

The members of the miR-29 family have identical seed sequence, similar expression patterns as well as function. The only differences among miR-29 members were reported in their expression levels in various cancerous tissues as some studies claim that miR-29a or miR-29c do not follow the same expression pattern as miR-29b (ref.[23]). It was described that in lung cancer, only miR-29b-2 was differentially expressed[24]. Further, miR-29b was found to be differentially expressed in cholangiocytes and in brain malignancies[25,26]. Another study demonstrated that expression of miR-29a and miR-29c in cervical cancer was decreased[27]. These results suggest that miR-29 is not tissue-specific.

MIR-29 UNDER PHYSIOLOGICAL CONDITIONS

The miR-29 family regulates several signaling pathways that are involved in various physiological and pathological processes. Physiologically, it takes part in regulation of cell cycle and proliferation[27-30], senescence[31,32], differentiation[33,34], apoptosis[25,28,30,35,36], metastasis[37,38], DNA methylation[39-41] and immune regulation[42,43], as well as regulation of extracellular matrix (ECM).

MiR-29 in cell cycle, proliferation and differentiation

Progression through the eukaryotic cell cycle is driven by cyclin-dependent kinases (CDKs), which are regulated by interaction with oscillatory expressed proteins called cyclins[44]. In cell cycle progression, from G1 to S phase, cyclin D1 binds CDK6 and CDK4 which then phosphorylate and inactivate Rb protein. These CDKs are essential for response to mitogenic stimuli, therefore the loss of CDK6 affects production of terminally differentiated cells (Fig. 2) (ref.[45]). It was demonstrated that 3'UTR of CDK6 contains 2 conserved sequence motifs with perfect homology to miR-29 seed sequence; therefore, CDK6 was suggested as a direct target of miR-29 (ref.[29]).

In terms of its role in cell differentiation, miR-29b has multiple functions in osteoblastogenesis - to control collagen expression during ECM maturation is one of them. However, this process does not happen in immature cells. Instead, miR-29b helps to maintain the differentiated phenotype in osteoblasts through regulating collagen. On the other hand, miR-29b downregulates negative regulators of signaling pathways to promote osteoblastogenesis. Both these miR-29b roles regulate osteoblast differentiation[46]. In another study, miR-29a and miR-29c were shown to be induced by the Wnt pathway that is critical in osteoblast differentiation. During the late phases of osteoblast differentiation, the expression of these miRNAs is upregulated and increased. Beside this, miR-29a and miR-29c downregulate osteoblast differentiation by targeting osteonectin, an essential protein for bone remodeling[33].

Apart from osteoblast differentiation, miR-29 was reported to play a role in muscle cells development. The miR-29 family enhances myogenic differentiation through its involvement in the NF-κB-YY1 regulatory loop. In myogenesis, downregulated transcription factors NF-κB and Yin Yang 1 (YY1) decrease miR-29 levels and this in turn induces differentiation by targeting YY1 (ref.[34]).

Another experiment showed that miR-29 together with miR-142 also regulates monocytic and granulocytic

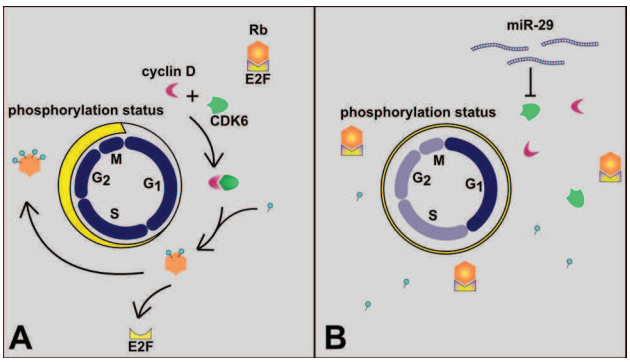

Fig. 2. MiR-29 function in cell cycle. (A) Cell cycle without miR-29 influence, (B) MiR-29 inhibits Cdk6 which cannot bind with cyclin D and phosphorylate Rb; therefore, cell is not differentiated.

(myeloid) differentiation. Targeting CDK6 by miR-29, as well as targeting cyclin T2 (CCNT2), a component of positive transcription elongation factor b (P-TEFb), by either miR-29 or miR-142 increases myeloid differentiation[47].

Aging processes and senescence

MiR-29 might be a pro-aging miRNA as it accumulates during aging and its upregulation is associated with DNA damage response. MiR-29 is part of the signaling pathway involving Ppm1d/Wip1 phosphatase, a key DNA damage response regulator, and the p53 tumor suppressor (Fig. 3) (ref.[31]). Further effects of miR-29 during cellular senescence were described in association with B-Myb which is an oncogene and a transcription factor for various genes involved in proliferation[48]. Besides these functions, B-Myb is able to induce senescence by inhibition of its transcription[49]. One of the options for repressing abundant B-Myb mRNA is through binding of Rb-E2F complexes to B-Myb promoter[50]. The other option of B-Myb repression at the posttranscriptional level involves miRNAs. MiR-29 together with miR-30 directly targets B-Myb 3'UTR and reduce its expression in cells undergoing senescence[32]. These facts are also consistent with miR-29 suppressor function in cancer.

ECM regulation

ECM regulation includes formation of extracellular matrix key proteins, e.g. various collagen (COL) isoforms, elastins, metalloproteinases, etc (ref.[23,46]). In osteoblasts, miR-29 regulates essential proteins of bone ECM. It mediates translational inhibition and decreases COL1A1, COL5A3, COL4A2 synthesis; furthermore, it maintains differentiated phenotype in mature cells[46]. The broad spectrum of collagens and other related genes, e.g. matrix metallopeptidase 2, which are miR-29 targets, was confirmed and even extended in a study done on rats. There were 20 genes for collagen predicted as miR-29 targets which makes this miRNA unique because no other miRNA targeted more than 11 collagen genes[23].

Regulation of these proteins by miR-29 is implicated in the development of fibrosis in many organs[51-53] and systemic sclerosis[54]. Not only in mice developing liver fibrosis, but also in patients with hepatic fibrosis, the miR-29 family was significantly downregulated and inhibited collagen expression in hepatic stellate cells[52]. A significant decrease was also found in the lungs of idiopathic pulmonary fibrosis patients[53].

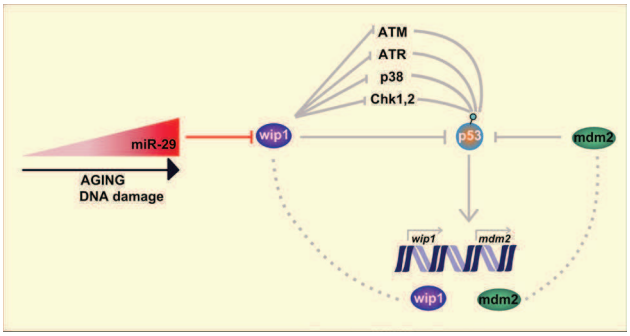

Fig. 3. Inhibition of p53 pathway by miR-29. Upregulation of miR-29 with increasing age and DNA damage inhibits Wip1 phosphatase. After that, Wip1 phosphatase cannot repress DNA damage responsefactors and p53. Wip1 and mdm2 are not transcribed by p53 which is not phosphorylated. Cell cycle is arrested.

MIR-29 IN HEMATOLOGICAL MALIGNANCIES

Despite the range of physiological processes the miR-29 family is involved in, most studies concentrate on its pathological function and tumor suppressive or oncomir (oncogenic miRNA) effects in various cancers. In terms of solid tumors and hematological malignancies, both these roles have been proven; they are believed to depend on cellular context or tissue specificity. Although expression of miR-29 was found to be altered in cancer, its role in pathogenesis of hematological malignancies is still poorly understood[55]. There is, however, a predominance of publications supporting the tumor suppressor role of the miR-29 family. By targeting oncogenes, the miR-29 family helps prevent carcinogenesis; therefore, in cancer, its levels are downregulated (Table 1) (ref.[12,56,57]).

Furthermore, it was observed that miR-29 is associated with some cytogenetic aberrations. MiR-29, among other miRNAs, was found to be down-regulated in acute myeloid leukemia (AML) patients with 11q23 balanced translocation compared to AML patients without this translocation[56]. Further, Garzon et al. observed that miR-29a and miR-29b are downregulated in primary AML samples with monosomy of chromosome 7. However, forced expression of these miRNAs had first anti-proliferative effects and later anti-apoptotic effects in AML cell lines and primary AML blasts, thus inhibiting cell growth and induced apoptosis by targeting Mcl-1 (Myeloid cell leukemia-1) (ref.[28]). In AML patients with monosomy of chromosome 7 or deletion of 7q, a link between miR-29a and oncogene Ski was described as the nuclear oncogene Ski is upregulated and miR-29a located on 7q32 is downregulated in these AML patients. Further, it was shown that miR-29a targets Ski, as their expression is inversely correlated, which suggest the tumor suppressive role of miR-29a (ref.[58]). Although previous study reported also tumor suppressor miR-29 family to be upregulated in AML patients with mutations in the nucleophosmin (NPM) gene when compared to wild type NPM (ref.[59]); however, this was not confirmed and miR-29 downregulation was described in AML patients independently of the NPM status[60].

A genome-wide profiling study on CLL (chronic lymphocytic leukemia) revealed that miR-29 precursors are upregulated[61]. Afterwards, another study demonstrated downregulated miR-29a in aggressive CLL compared to indolent CLL (ref.[35]). Interestingly, miR-29a was found to be the second and miR-29c the fifth most represented miRNA among the most expressed miRNAs in CLL (ref.[62]).

Despite some knowledge about miR-29 in other hema-

tological malignancies, little is known about this miRNA in mantle cell lymphoma (MCL) and further research in this field is needed. One report showed notably decreased miR-29 levels in MCL patients, which were associated with higher levels of its target CDK6 and with shorter overall survival of MCL patients. Therefore, the use of miR-29 as a prognostic marker and pathogenetic factor in MCL was suggested[29].

Besides studying direct miR-29 family effects and its participation in regulation, there is an effort to apply miR-29 as novel biomarkers. The analysis of miR-29a together with miR-142-3p indicated that these miRNAs could be used as AML molecular diagnostic markers. Because of their key role in regulation of normal myeloid differentiation, miR-29a and miR-142-3p abnormal expression was shown to be involved in AML development, as it directly affected target genes important in AML (ref.[60]). Recently, the first evidence of miR-29a as an unfavorable prognostic marker in AML was indicated, as downregulation of miR-29a was shown to be associated with advanced clinical features and poor prognosis in pediatric AML patients[63].

Apoptosis in hematological malignancies

Majority of studies show that miR-29 family effects in cancer are anti-apoptotic. However, in studied hematological malignancies, the miR-29 levels were lower than in physiological conditions. Therefore the tumor suppressive impact of miR-29 on cancer cells is poor (Table 1). MiR-29 family was described to target genes involved in regulation of apoptosis, such as Bcl-2 (B-cell leukemia/lymphoma) family members and a key anti-apoptotic protein Mcl-1 that are often dysregulated in malignant cells (Table 2). Constitutive Mcl-1 expression can cause malignant transformation as was demonstrated in transgenic mice[64]. MiR-29b negatively regulates Mcl-1 protein expression; low miR-29b levels upregulate Mcl-1 expression and thus induce anti-apoptotic signals and may play a role in tumor development. On the other hand, experiments with enforced miR-29b expression showed sensitivity to cell death which might be valuable in cancer therapy[25]. In multiple myeloma (MM), a plasma cell malignancy, the miR-29b tumor suppressor effects are implicated as well. It was shown that miR-29b is downregulated in MM; however, its overexpression can downregulate Mcl-1 expression and is associated with caspase-3 activation. By targeting critical oncogenic pathways, miR-29b inhibits growth and induces apoptosis of MM cells[12].

Another miR-29 family target is Tcl1 (T-cell leukemia/lymphoma 1) gene), a significant oncogene involved in CLL pathogenesis. Tcl1 operates as a coactivator of the Akt oncoprotein that is important in the anti-apoptotic pathway in B- and T-cells[65,66]. Pekarsky *et al.* demonstrated that miR-29 family members are partly natural Tcl1 inhibitors and that downregulated miR-29 levels in aggressive CLL might be a causal event in disease pathogenesis[35]. Another study suggested that the downregulation of miR-29 upregulates Tcl1 in aggressive CLL, and thus develops aggressive phenotype[14].

Amodio et al. recently identified new miR-29b target Sp1, a transcription factor that participates in cell cycle regulation and apoptosis[67]. In MM, Sp1 is involved in cell survival and promotes MM cell growth[68]. Sp1 is downregulated by miR-29 but it was demonstrated that the forced expression of miR-29b in cell lines inhibited cell growth and triggered apoptosis *in vitro* and *in vivo* in a murine model. Besides this, miR-29b-Sp1 regulatory loop was described. Not only miR-29b influences Sp1 but also Sp1 negatively regulates miR-29b. Upregulated Sp1 transcriptionally inhibits miR-29b and silenced Sp1 increases miR-29b levels. All of this may prevent the tumor formation in a model of MM (ref.[67]).

A study done by Garzon et al. describes the effects of miR-29 on both apoptosis and proliferation in AML cells. The forced expression of miR-29a and miR-29b led to cell growth inhibition and induction of apoptosis. After the transfection of the miRNAs, the first observed effect was inhibition of apoptosis. It was confirmed that Mcl-1 and other anti-apoptotic genes are miR-29 targets and that this miRNA also upregulates proapoptotic genes. The anti-proliferative effect was observed later after the transfection, which means that the miR-29-dependent proliferation is not a result of apoptosis. For studying miR-29

Table 1. MiR-29 involvement in cancer.

Target	MiR-29 function	MiR-29 regulation	Cancer development
Oncogene	Tumor suppressor	upregulated	no
		downregulated	induced
Tumor suppressor	Oncomir	upregulated	induced
		downregulated	no

Table 2. Oncogenes as miR-29 targets in hematological malignancies.

Target	Function	miR-29 member	References
Mcl-1	Cell survival, proliferation, apoptosis	miR-29b	12,25,64
Tcl-1	Apoptosis	not specified	35
Sp1	Cell growth, survival	miR-29b	67
SKI	Nuclear corepressor complexes	miR-29a	58
CDK6	Cell cycle	not specified	29

impact on proliferation, CDK6, a miR-29 target, was chosen. Transfection of miR-29b into AML cells indirectly led to decreased Rb phosphorylation through CDK6, which resulted in decreased proliferation[28].

Tumor initiation and growth

Although tumor suppressor effect of miR-29a was elucidated, some studies show that the miR-29 family has also tumor promoting effects, but this oncomir function still remains poorly understood. The first example of a miRNA initiating AML *in vivo* was reported by Han et al. They showed that miR-29a was highly expressed in human AML and its overexpression led to higher incidence of AML. MiR-29a can induce AML by converting myeloid progenitors into self-renewing leukemia stem cells, thus showing oncogenic potential[13]. The same was demonstrated in CLL where miR-29a was overexpressed in indolent CLL in comparison to normal B cells. However, a hypothesis of solely miR-29 initiating leukemia was not confirmed[14].

As for MM, one of its main characteristics is bone disease which is a result of imbalance between osteoblasts and osteoclasts bone formation caused by MM cells. It was found that miR-29b expression decreases during osteoclast differentiation *in vitro* and suppresses its targets c-Fos and metalloproteinase 2. miR-29b-based treatment of MM-related disease was suggested when the results showed enforced miR-29b expression disrupting osteoclast differentiation and overcoming osteoclast activation[69].

CIRCULATING MIR-29

In 2008, the discovery of miRNA present in body fluids was reported. MiRNAs were found in almost all body fluids, e.g. serum, plasma, saliva, urine, etc (ref.[70-73]). Interestingly, under unfavorable conditions, such as boiling, storage at room temperature, low or high pH or repeated cycles of freeze-thawing, plasma miRNAs were found to be unconventionally stable. Possibly, there are two mechanisms by which circulating miRNAs are protected from degradation. The first possibility is to form ribonucloeprotein complexes of miRNA and RNA-binding protein, e.g. Ago2 (ref.[74]), NPM-1 (ref.[75]) or high-density lipoproteins (HDLs) (ref.[76]). The other option is packaging in small vesicles. Depending on the size and form of release, these small vesicles can be exosomes, which are released from endosome membrane, or microvesicles, that are shed directly from plasma membrane, or even apoptotic bodies[77-80]. Current evidence shows that the majority of circulating miRNAs are bound to proteins rather than found in vesicles. This aside, it seems that cells can actively select which miRNAs will be released from cells and which will stay within the cell[81]. However, little is known about circulating miRNA origins and factors in their regulation and other underlying mechanisms need to be determined.

Our own data showed the presence of circulating serum miR-29a in MM patients. Serum levels of miR-29a were able to distinguish MM patients from healthy donors. Although further analysis is required, it is possible that circulating miRNAs represent a novel and easily accessible putative marker[82]. Such a marker would be important and highly clinically relevant in diseases such as MM, where frequent testing of bone marrow is not ethically permissible.

The question of comparing established biomarkers and circulating miRNAs was investigated in patients with CLL (ref.[83]). A set of 3 miRNAs, including miR-29a, was able to distinguish healthy controls from CLL patients. Furthermore, another set of miRNAs, including miR-29a, was compared with IgV_H and zeta-associated protein (ZAP) status, an established clinical risk stratifier in CLL (ref.[84]). This miRNA set could separate ZAP-70^+ and ZAP-70^- samples but did not correlate with IgV_H mutation status[83].

CONCLUSION

First dismissed as a type of junk RNA, miRNAs were demonstrated to be pivotal in gene regulation. In recent years, miRNAs have also been discovered to be important players in cancer pathogenesis and understanding of their significance has broadened. MiRNAs function both as tumor suppressors and oncomirs.

MiR-29 is involved in various physiological processes, such as proliferation, differentiation, apoptosis and senescence. It has also been shown that the miR-29 family is deregulated in hematological malignancies as well as in solid tumors. The analyses are influenced by heterogeneity of the diseases, detection methods used, various genetic background of patients/control groups, and different disease stage. In some cases, small data sets may impair data validation. However, its specific role in hematological malignancies remains unclear.

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Combination of serum microRNA-320a and microRNA-320b as a marker for Waldenstrom macroglobulinemia

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Combination of serum microRNA-320a and microRNA-320b as a marker for Waldenström macroglobulinemia

To the Editor: IgM monoclonal gammopathies are a group of diseases characterized by increased level of IgM immunoglobulin produced by one clone of B cells. These diseases range from benign (monoclonal gammopathy of undetermined significance, MGUS) to malignant, such as Waldenström macroglobulinemia (WM) or to a lesser extent multiple myeloma (MM) [1,2]. The criteria that differentiate WM from IgM-MGUS are based on the extent of bone marrow (BM) involvement, amount of serum concentration of the M-protein, presence or absence of symptomatic disease or more recently, *MYD88* (L265P) or *CXCR4* mutations [3–6]. Despite that, new criteria for the differential diagnosis between these conditions are still needed, circulating microRNAs (miRNAs) being one of them. Circulating miRNAs are present in different body fluids; they reflect physiological or pathological conditions and can be used for patient classification [7,8]. Thus, we aimed to investigate the ability of serum miRNAs to distinguish WM from IgM-MGUS as well as IgM-MM patients and healthy donors (HD).

For this purpose, circulating miRNAs were isolated from serum samples and screening of 667 miRNAs using TaqMan Low Density arrays was performed on five WM patients, five IgM-MGUS, five IgM-MM patients, and five HD samples to identify differently expressed circulating miRNAs in WM (Supporting Information Fig. S1). Out of deregulated miRNAs, miR-320b, miR-320a, miR-151-5P, and let-7a were further validated by quantitative real-time PCR (qPCR) on a larger cohort of 21 WM, 15 IgM-MGUS, 10 IgM-MM, and 18 HD serum samples (Supporting Information Table S1), as they were present at the top of the list of deregulated miRNAs between IgM-MGUS, HD, and WM and showed highest fold change and most favorable expression (Ct $<$ 30). In addition, some of the miRNA levels were correlated with clinically important parameters and *MYD88* (L265P) mutation status.

MiR-320a and miR-320b showed different expression between WM and all other groups of samples ($P < 0.05$). Let-7a and miR-151-5P were significantly decreased in WM samples as compared with HD (all $P < 0.05$) and IgM-MGUS samples (all $P < 0.05$) but not with IgM-MM ($P = 0.285$ and $P = 0.286$, respectively). As only miR-320a and miR-320b remained statistically significant, only these two miRNAs were chosen for further analyses (Supporting Information Table S2).

Receiver Operating Characteristic curves (ROC) were used to evaluate diagnostic effectiveness of miR-320b and miR-320a and to estimate the appropriate cutoff (Supporting Information Table S3). MiR-320b was more potent than miR-320a to distinguish WM from HD with sensitivity of 85.7% and specificity of 94.4% using cutoff value of 1,072 copies per 1 ng of miRNA/RNA. However, combination of miR-320b with miR-320a improved sensitivity up to 90.5% with specificity of 94.4% using cutoff value −0.6253 obtained from nominal logistic regression model (Fig. 1A). More importantly, miR-320b discriminated WM from IgM-MGUS with specificity of 73.3% and sensitivity of 85.7% using cutoff value of 1,072 copies per 1 ng of total miRNA/RNA, and combination of miR-320b with miR-320a reached specificity of 73.3% and increased sensitivity up to 90.5% with cutoff defined as −0.2373 (Fig. 1B). Furthermore, miR-320b distinguished also WM from IgM-MM with sensitivity of 71.4% and specificity of 80.0% with cutoff 904 copies per 1 ng of total miRNA/RNA, and together with miR-320a, the two-miRNA based combination yielded sensitivity of 81.0% and specificity of 100.0% with cutoff value defined as −1.4322 (Fig. 1C).

Additionally, associations between miR-320a and miR-320b expression levels and important disease parameters of IgM-MGUS, WM, and IgM-MM were investigated. In IgM-MGUS group, a positive correlation was found between levels of miR-320b and albumin ($P < 0.05$; $r_s = 0.521$) and moderate positive correlation between levels of miR-320b and calcium ($P < 0.06$; $r_s = 0.509$). In WM group, levels of miR-320a negatively correlated with β_2-microglobulin ($P < 0.05$; $r_s = -0.468$) and with percentage of lymphoplasmacytic cells infiltration in the BM ($P < 0.05$; $r_s = -0.573$). Next, in IgM-MM group, miR-320b and miR-320a negatively correlated with levels of M-Ig ($P < 0.05$; $r_s = -0.782$ and $r_s = -0.636$, respectively), and there was a positive correlation between levels of miR-320a and lactate dehydrogenase ($P < 0.05$; $r_s = 0.818$). All associations are present in Supporting Information Table S4. We also evaluated mutation status of *MYD88* (L265P) in all available PB samples (Supporting Information Fig. S2). Levels of miR-320a were significantly lower in *MYD88* (L265P) positive patients ($P = 0.032$), and there was an identical trend for miR-320b, although not significant ($P = 0.079$).

Both miR-320a and miR-320b were observed in higher concentrations in cellular fractions in comparison with cell-free fractions, and interestingly, more copies of these microRNAs were observed in the CD19-fraction. Additionally, both miR-320a and miR-320b were present in exosomes as well as in exosome-depleted samples; however, their levels tend to be increased in exosomal fractions (Supporting Information Fig. S3).

The investigation of molecular features of IgM-monoclonal gammopathies is essential to identify specific risk markers for disease development. Routinely, differentiation of IgM-MGUS from WM is possible only by trepanobiopsy, as neither BM aspiration nor flow-cytometry provide sufficient data for diagnosis. Therefore, in this study, we focused on the role of circulating serum miRNAs as biomarkers of WM. Combination of miR-320a and miR-320b served as the best indicator for WM as it was able to distinguish WM from HD, but more importantly WM from premalignant IgM-MGUS and malignant IgM-MM. Our data suggest that such miRNAs combination might be a novel effective tool for WM discrimination which, however, needs further validation and study. Although the amount of patients with mutant *MYD88* (L265P) was small, it can be hypothesized that mutation in *MYD88* might be connected to lower miRNA levels; however, underlying biology again needs to be elucidated. It still remains an open question, if studied miRNAs are actively or passively secreted from tumor cells; nevertheless, it is plausible to assume that they are actively transported in vesicles, as they are present primarily in exosomes. Association of low levels of these miRNAs with WM diagnosis suggests their involvement in the disease; however, the origin of such miRNAs in circulation still remains unclear. Nonetheless, considering the minimally invasive character of serum sampling, reproducibility, and easy detection of circulating miRNA, they may provide a convenient and inexpensive method to establish the diagnosis of WM or to predict the evolution of IgM-MGUS.

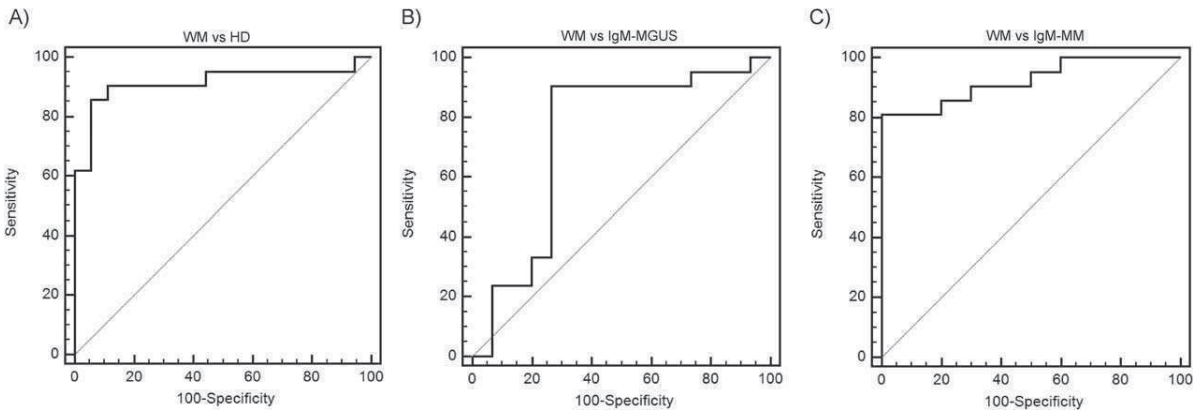

Figure 1. Receiver operating characteristics (ROC) curves. ROC curves for combination of miR-320a and miR-320b distinguishing (A) WM from HD with area under the curve (AUC) = 0.921, (B) WM from IgM-MGUS with AUC = 0.743, (C) WM from IgM-MM with AUC = 0.924.

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Additional Supporting Information may be found in the online version of this article.

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MikroRNA u mnohočetného myelomu

Kubiczková L, Ševčíková S, Hájek R.

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18. MikroRNA u mnohočetného myelomu

Lenka Kubiczková, Sabina Ševčíková, Roman Hájek

Mnohočetný myelom (MM) je maligní lymfoproliferativní onemocnění charakterizované infiltrací kostní dřeně patologickými plazmocyty, osteolytickými lézemi skeletu a přítomností monoklonálního imunoglobulinu v séru a/nebo moči. Incidence MM je 4/100 000 obyvatel v ČR. Incidence vzrůstá s věkem, s mediánem 65 let při diagnóze [1]. MM je považován za obtížně léčitelné, nicméně již vyléčitelné onemocnění s pětiletým mediánem přežití nižším než 40 % [2]. Jedna skupina MM pacientů (10–15 %) je považována za vysoce rizikovou (high-risk), jelikož u nich dochází k rapidní progresi onemocnění, pacienti dosahují kratší remise s následným vývojem refraktorní nemoci [3,4].

Pro MM je typická genomická nestabilita. Cytogenetická analýza MM buněk ukazuje na časté mutace a chromozomální aberace. Aneuplodie je velice častá, mezi nejčastější změny patří reciproké chromozomální translokace IgH lokusu, monosomie chromozomu 13, ztráta krátkého raménka chromozomu 17 a zisk dlouhého raménka chromozomu 1. Ve srovnání s ostatními hematologickými malignitami, které jsou charakterizovány malým počtem genetických aberací, jsou tyto změny u MM časté, některé jsou navíc používány jako prognostické markery [5,6]. Právě heterogenita tohoto onemocnění je pravděpodobně spojena s molekulární charakteristikou maligního klonu [3].

V poslední době přichází do popředí zájmu také problematika miRNA v patogenezi a progresi MM. Z metodického hlediska jsou dva základní přístupy ke studiu miRNA u MM, a to screeningový (spíše translační) a funkční (základní výzkum). V této kapitole se nejdříve budeme věnovat výsledkům globálního profilování exprese miRNA a dále konkrétním miRNA a jejich souvislosti s patogenezí MM.

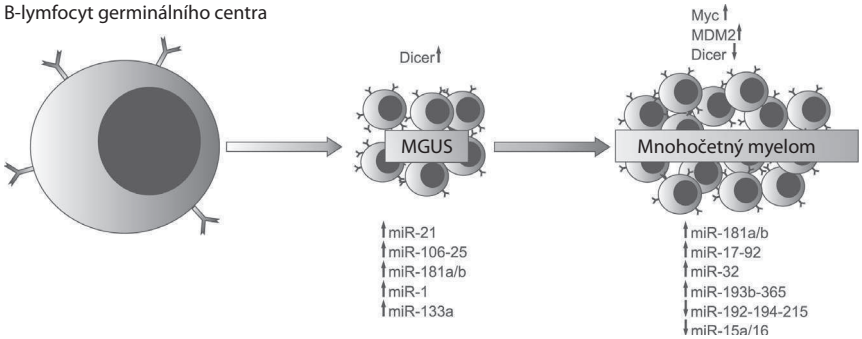
18.1. MikroRNA v patogenezi mnohočetného myelomu

První abstrakta zabývající se úlohou miRNA v patogenezi MM byla prezentována v roce 2005 na setkání Americké hematologické společnosti. Jako první byly popsány expresní profily miRNA u myelomových linií a vzorků pacientů a bylo zjištěno, že jak buněčné linie, tak maligní, CD138+ plazmatické buňky (PC – plasma cells) pacientů mají odlišnou expresi některých miRNA (miR--125b, miR-133a, miR-1 nebo miR-124a) ve srovnání s PC zdravých dárců [7].

Další práce, ve které byla použita kvantitativní PCR (qRT-PCR), popisuje zvýšenou expresi let-7a, miR-16, miR-17-5p a miR-19b, a naopak sníženou expresi miR-372, miR-143 a miR-155 u MM pacientů a buněčných linií ve srovnání se zdravými kontrolami [8]. Exprese miR-15 a miR-21 se v této studii významně nelišila mezi zdravými dárci a nemocnými, což je v rozporu s pozdější studií, která identifikovala miR-21 jako onkogen s antiapoptotickou funkcí [9]. Pomocí chromatinové imunoprecipitace bylo zjištěno, že se STAT3 podílí na regulaci exprese miR-21 v IL-6 závislých PC po přídavku IL-6. Zdá se, že u těchto buněk je transkripce miR-21 kontrolována pomocí IL-6 a zprostředkovaná aktivací STAT3, což napomáhá přežívání maligních buněk. Navíc ektopická exprese miR-21 za nepřítomnosti IL-6 vedla ke snížení apoptózy buněk, což potvrzuje účast miR-21 v procesu apoptózy, která je zprostředkovaná pomocí STAT3 [9].

V pilotní studii zabývající se úlohou miRNA v maligní transformaci PC byla pomocí miRNA mikročipů a následné qRT-PCR srovnávána exprese miRNA jak u zdravých dárců, tak u osob s monoklonální gamapatií nejasného významu (MGUS – monoclonal gammopathy of undetermined significance), pacientů s MM a u buněčných linií. Byly identifikovány specifické profily miRNA popisující jak PC v MM, MGUS a MM liniích, tak transformaci z MGUS do MM. U MGUS bylo nalezeno 48 miRNA, u MM pacientů již 96 odlišně exprimovaných miRNA ve srovnání se zdravými dárci. U obou skupin, MM i MGUS, byla pozorována zvýšená exprese miR-21, klastru miR-106-25 a miR-181a/b, nicméně pouze u MM byla stanovena zvýšená exprese miR-32 a klastru miR-17-92. Zdá se tedy, že se tyto miRNA podílejí na progresi onemocnění a napomáhají transformaci z MGUS do MM (obr. 18.1.) [10].

V návaznosti na získané poznatky byla provedena (u PC zdravých dárců a MM pacientů) srovnávací analýza expresního profilu miRNA a expresního profilu kó-



Obr. 18.1. Schematické znázornění transformace plazmatické buňky. Reprezentativní mikroRNA a geny významně deregulované u jedinců s MGUS a MM ve srovnání se zdravými jedinci.

Tab. 18.1. Deregulované mikroRNA v plazmatických buňkách pacientů s mnohočetným myelomem (MM). Převzato z [38].

MikroRNA	Lokus	Exprese u MM PCs	Cílové geny	Literatura
miR-181a/b	1q32.1	zvýšená	PCAF, HOXA11, TCL	[10,11,17]
miR-1	20q13.33	zvýšená zvýšená u MM pacientů s t(14;16)/t(14;20) zvýšená u MM pacientů s t(14;16)		[10,18,27]
miR-15b	3q25.33	zvýšená snížená u MM relapsů/ refraktorních MM		[10,11,16,17]
miR-221	Xp11.3	zvýšená zvýšená u pacientů s t(4;14) snížená u pacientů s delecí RB		[10,11,17,18, 27]
miR-222	Xp11.3	zvýšená u MGUS zvýšená zvýšená u pacientů s (4;14)	P27, PTEN	[10,11,17,27]
miR-106b-25	7q22.1	zvýšená	P21, BIM, E2F1, PCAF	[10,11,16]
miR-17-92	13q31.3	zvýšená u miR-17, miR-19a, miR-19b, miR-20a, miR-92a zvýšená u miR-17, miR-18a, miR-19a, miR-20a, miR-92a zvýšená u miR-92a snížená u miR-19a, miR-19b, miR-20a u pacientů s delecí RB	P21, BIM, E2F1, PTEN	[10,11,16,18]

dujících genů (GEP – gene expression profiling), která prokázala souvislost mezi globální zvýšenou expresí miRNA a špatnou prognózou high-risk MM pacientů [11]. Další studie by mohly podpořit tuto souvislost, jelikož bylo pozorováno, že vyšší viabilita MM buněk souvisí s vyřazením z funkce Argonaut (EIF2C2/AGO2) komplexu, který je hlavním regulátorem maturace a funkce miRNA a jehož exprese je zvýšená u high-risk MM [12,13]. EIF2C2/AGO2 se navíc podílí na diferenciaci B-lymfocytů [14] a je znám jako marker nádorové progrese u MM [15]. V této studii byla také navržena hypotéza, že miRNA mohou působit synergisticky, a tím významně přispívat k progresi MM.

Jiná miRNA mikročipová srovnávací studie odhalila zvýšenou expresi klastru miR-193b-365 u PC MM pacientů [16]. Dále byly porovnány expresní miRNA profily PC MM pacientů s profily normálních PC a byla zjištěna významně zvýšená exprese miR-222, miR-221, miR-382, miR-181a a miR-181b a snížená exprese miR-15a a miR-16 [17]. Gutierrez et al. ve své práci porovnali miRNA expresní profil PC 60 MM pacientů s PC zdravých dárců a pozorovali sníženou expresi 11 miRNA (miR-375, miR-650, miR-214, miR-135b, miR-196a, miR-155, miR-203,

miR-95, miR-486, miR-10 a miR-196b), z nichž pouze miR-155 byla již dříve popsána v souvislosi s lymfoidními buňkami [18].

Nedávno publikovaná práce popisuje 40 miRNA se sníženou expresí v PC MM pacientů ve srovnání se zdravými dárci, z nichž 6 miRNA (miR-214, miR-135b, miR-196a, miR-155, miR-203 a miR-486) se shoduje s miRNA publikovanými skupinou Gutierreze et al. Navíc výsledky klastrovací analýzy 54 MM pacientů poukázaly na tři miRNA, a to miR-296, miR-194 a let-7f, jejichž zvýšená exprese souvisí s lepším přežíváním pacientů [19].

Stanovené expresní profily PC MM pacientů nejsou jednotné, nicméně některé miRNA byly potvrzeny ve více studiích, jak je znázorněno v tab. 18.1.

18.2. Rezistence na léčbu a mikroRNA v mnohočetném myelomu

Přítomnost miRNA je také spojována s rezistencí vůči některým lékům. Bortezomib (Velcade, dříve PS-341, Millennium Pharmaceuticals, Inc.) patří do skupiny inhibitorů proteazomu. Jedná se o dipeptid kyseliny borité, vykazující protinádorové účinky [20]. Bortezomib byl schválen k léčbě MM v relapsu i pro léčbu nově diagnostikovaných pacientů [21]. V roce 2009 byly popsány expresní dráhy miRNA, které souvisejí s léčebnou odpovědí k bortezomibu. Srovnání expresních profilů linií rezistentních a citlivých k bortezomibu odhalilo 22 deregulovaných miRNA, z toho zvýšenou expresi měly miR-155, miR-342-3p, miR-181a, miR-181b, miR-128 a miR-20b, naopak snížená exprese byla pozorována u let-7b, let-7i, let-7d, let-7c, miR-222, miR-221, miR-23a, miR-27a a miR-29a. Mezi predikované cíle těchto miRNA patří geny zapojené do buněčného cyklu, buněčného růstu, apoptózy a ubikvitinace. Následně, pro stanovení klinického významu uvedených miRNA, byly korelovány expresní profily miRNA PC pacientů rezistentních a citlivých k bortezomibu s jejich odpovědí na léčbu. Bylo zjištěno, že pacienti citliví k terapii bortezomibem měli stejný profil deregulovaných miRNA jako linie citlivé k bortezomibu a stejně tak profil pacientů rezistentních k bortezomibu inklinoval k profilu stanovenému na liniích [22].

V další studii, zabývající se změnou expresních profilů miRNA během získané lékové rezistence, byly srovnány modelové expresní profily miRNA mezi MM buněčnými liniemi (RPMI-8226 a U266) se získanou rezistencí k doxorubicinu a melfalanu a jejich parentálními liniemi. Výsledky expresní analýzy byly validovány pomocí qRT-PCR a významné změny byly pozorovány u miR-21 a miR-181a a miR-181b. Exprese miR-21 byla zvýšená u obou klonů linií rezistentních k melfalanu. Překvapivě bylo zjištěno, že exprese miR-181a a miR-181b byla snížená u U266 doxorubicin rezistentní linie, ale zvýšená u RPMI-8226 doxorubicin rezistentní linie. Zdá se, že změny vedoucí k lékové rezistenci jsou náhodné a efekt miRNA je závislý na kontextu [23].

18.3. Mechanizmus deregulace mikroRNA v mnohočetném myelomu

Nové studie, navazující na předchozí objevy, částečně vysvětlují mechanizmus deregulace miRNA u MM. Srovnávací mikročipová analýza miRNA a analýza počtu kopií (CNV – copy number variations) DNA nebo GEP MM linií objasnily deregulaci 16 miRNA, jejichž geny leží v oblastech chromozomů, které jsou často předmětem různých alelových změn u MM. Mezi nejčastější změny patřily zisky chromozomů. Bylo zjištěno, že miR-548-1 se vyskytovala s nejvyšší četností (94 %) v oblastech zisku chromozomu, zatímco miR-130b, miR-185, miR-648 a miR-649 (všechny leží v oblasti 22q11.21) jsou zastoupeny v oblastech ztráty chromozomu. Mezi další často deregulované miRNA patřily miR-22 ležící v oblasti 17p13.3, miR-106b a miR-25 v oblasti 7q22.1, miR-15a v oblasti 13q14.3, miR-21 v oblasti 17q23.1 a miR-92b, která se nachází v oblasti 1q22 [24]. Klastr miR-15a/16-1 byl dále podrobněji studován a bylo zjištěno, že u pacientů s delecí chromozomu 13 zcela chybí miR-15a a miR-16, nicméně u pacientů bez delece chromozomu 13 byla exprese miR-15a a miR-16 také významně snížená [17].

Další studie, srovnávající CNV s čipy mapujícími jednonukleotidové polymorfizmy (SNP – single nucleotide polymorphism), ukázala, že exprese miR-15a a miR-16 není závislá na statutu chromozomu 13, ale obecně je u MM pacientů exprese zmíněných miRNA oproti normálním PC zvýšená [25].

Byla také nalezena korelace mezi šesti intragenovými miRNA a geny, uvnitř kterých se miRNA nacházejí. Tyto geny jsou deregulovány u MM linií a pacientů a některé jsou důležité v patogenezi MM, jako MEST a miR-335 nebo EVL a miR--342-3p [26]. V jiné práci byla nalezena souvislost mezi 32 intragenovými miRNA a geny, uvnitř kterých leží; některé z těchto genů jsou opět významně deregulovány u MM pacientů. Studie potvrdila již výše zmíněné korelace, navíc byla zjištěna souvislost mezi genem COPZ2 a miR-152 [24]. Získané výsledky naznačují, že změna počtu kopií genu souvisí se zvýšenou expresí jeho intragenových miRNA, což by částečně vysvětlovalo mechanizmus změněné exprese miRNA u MM.

Jelikož je myelom velmi heterogenní onemocnění, pro které jsou charakteristické komplexní cytogenetické aberace, je velmi pravděpodobné, že tyto aberace ovlivňují také expresi miRNA. V nedávné studii bylo 60 MM pacientů rozděleno na základě translokačních partnerů IgH genu a statutu RB genu do různých cytogenetických podskupin a tyto podskupiny pacientů byly srovnány s jejich expresí 365 miRNA. Výsledky klastrovací analýzy poukázaly na zvýšenou expresi miR-1 a miR-133a, které souvisejí s translokací t(14;16) [18]. Změněná exprese jiných miRNA byla dále popsána v souvislosti s translokacemi t(4;14), t(11;14) nebo t(14;16) [18,27]. Nově bylo popsáno pět miRNA, které byly zvýšeny u pacientů s t(11;14), a to miR-122a, miR-33, miR-489, miR-519 a miR-555 [19].

Další možností deregulace miRNA je změna v jejich zpracování nebo maturaci. Již dříve zmíněná studie EIF2C2/AGO2 komplexu uvádí, že úbytek AGO2 souvisí

se zástavou růstu a apoptózou u MM buněk [11]. V souladu s tím bylo prokázáno, že změněná hladina enzymu Dicer, ale ne enzymu Drosha, může souviset s progresí MM. Autoři pozorovali podobnou hladinu enzymu Dicer u PC zdravých dárců a pacientů s MGUS, která je však oproti doutnajícímu myelomu a MM pacientům významně zvýšená. Navíc bylo pozorováno, že skupina pacientů s vyšší hladinou enzymu Dicer měla delší dobu do progrese [28].

Zmíněné výsledky jsou však v rozporu s nedávno provedenou studií, ve které nižší exprese genu DICER1 u skupiny MM pacientů souvisí s delší dobou do progrese nemoci [19]. Zdá se tedy, že regulační mechanizmy ovlivňující jak miRNA maturaci, tak jejich funkci se mohou podílet na změněné expresi miRNA, další studie určitě pomohou objasnit zmíněné nesrovnalosti.

18.4. MikroRNA ovlivňující kritické geny u mnohočetného myelomu

Mnoho vědeckých skupin se zabývalo otázkami, jak důležité jsou z funkčního hlediska změny v expresi miRNA a jak tyto změny souvisí s patogenezí MM. Pro zodpovězení těchto otázek jsou využívány různé přístupy od predikce cílových genů pomocí *in silico* modelů až po pokusy s transgenními zvířaty.

Je známo, že kódující geny, které se podílejí na procesu kancerogeneze u MM, jsou cílem pro deregulované miRNA. Bylo prokázáno, že klastr miR-17-92, nacházející se v oblasti 13q31-32, ovlivňuje expresi genu PTEN, genu pro transkripční faktor E2F1 a BIM [29,30]. U transgenních myší se zvýšenou expresí tohoto klastru v lymfocytech byly pozorovány lymfoproliferativní a autoimunitní onemocnění a časná úmrtí. Dále bylo zjištěno, že purifikované myší CD4+ lymfocyty se zvýšenou expresí miR-17-92 obsahovaly snížené množství proteinů PTEN a BIM, což naznačuje, že miR-17-92 klastr ovlivňuje tyto nádorové supresory [29]. Brzy nato byla publikována další studie, ve které bylo prokázáno, že zmíněný klastr je nezbytný pro vývoj B-lymfocytů. Nepřítomnost miR-17-92 vedla ke zvýšené hladině pro-apoptotického proteinu BIM, a tím k zástavě vývoje z pro-B do pre-B stadia [30]. Zdá se tedy, že zvýšená exprese miR-17-92 negativně reguluje zmíněné nádorové supresory a přispívá k transformaci PC a progresi MM.

Predikce *in silico* také ukázala, že cílem miR-21 a klastru miR-106-25 jsou mezi jinými nádorové supresory PTEN, BIM a p21, a proto je pravděpodobné, že se tyto miRNA mohou podílet na vývoji plně rozvinutého myelomu [10].

Jiná miRNA, miR-19a/b, ovlivňuje dráhu STAT-3/IL-6, která je důležitá v patogenezi MM. Bylo prokázáno, že miR-19a/b přímo ovlivňuje SOCS1 (negativní regulátor IL-6), a tím přispívá k jeho časté deregulaci u MM buněk [10]. Také miR-21, zmíněná výše, působí jako onkogen a podílí se na regulaci této dráhy [9].

Jak již bylo zmíněno dříve, miR-15a a miR-16-1 leží v oblasti chromozomu 13q14.3, která je deletována u více než 50 % pacientů s MM. Tato delece je pova-

žována za primární mutaci, která se podílí na patogenezi MM [31]. MiR-15a/16 jsou považovány za nádorové supresory podílející se na proliferaci MM buněk *in vitro* i *in vivo* tím, že inhibují AKT serin/treonin proteinovou kinázu (AKT3), ribozomální protein S6, MAP kinázy a NFκB aktivátor MAP3KIP3 [17]. Dále bylo prokázáno, že miR-15a/16 nejen regulují expresi genů buněčného cyklu, jako jsou cykliny D1 a D2, dále CDC25A, ale rovněž ovlivňují expresi genů spojených s apoptózou: BCL2 nebo MCL1 [32]. Navíc ektopická exprese miR-15a/16 negativně reguluje angiogenezi pomocí VEGF [17]. Nedávno byla popsána úloha miR-15a/16 v mikroprostředí kostní dřeně. Bylo zjištěno, že exprese miR-15a/16 je v MM buňkách po ovlivnění cytotoxickými látkami vyšší. Nicméně po interakci těchto buněk se stromálními buňkami kostní dřeně odvozenými od MM (MM-BMSC) pacienta, byla pozorována snížená exprese miR-15a/16 u myelomových buněk. Důvodem byla zvýšená produkce IL-6 stromálními buňkami, který inhiboval expresi zmíněných miRNA. Zdá se tedy, že mikroprostředí je důležité pro přežití MM buněk a chrání je před působením léků pomocí sekrece IL-6, který inhibuje expresi miR-15a/16 [33].

Nově publikované práce se dále zaměřují na vztah miRNA k nádorovému supresoru p53. Výsledky screeningové metody umožňující identifikovat miRNA, které negativně regulují signalizaci p53 pomocí přímé interakce s genem TP53, naznačily, že miR-25 a miR-30d mohou ovlivňovat p53. Navíc byla exprese miR-25 a miR-30d zvýšená v PC MM pacientů a u miR-25 zvýšená exprese korelovala se sníženou expresí mRNA TP53 [34]. Také miR-181a byla popsána jako negativní regulátor exprese genu TP53, což potvrzuje spojitost mezi p53 a aberantní miRNA expresí [35]. Je známo, že miR-34a je transkripčním cílem p53 zprostředkovávajícím apoptózu [36]. U MM pacientů byla pozorována hypermetylovaná miR-34a v oblasti 1p36. Jelikož se u krevních nádorových onemocnění nevyskytuje mutace TP53 tak často jako u solidních nádorů, mohla by hypermetylace miRNA částečně vysvětlit dysregulaci p53 signalizace [37]. V další studii byla nalezena snížená exprese miR-192, miR-194 a miR-215 u části nových diagnóz MM pacientů. Další pokusy *in vitro* prokázaly, že při použití molekulárních inhibitorů MDM2 mohou být tyto miRNA transkripčně aktivovány pomocí p53 a posléze mohou modulovat expresi MDM2. Je tedy patrné, že miR-192, miR-194 a miR-215 ovlivňují MDM2/TP53 regulační osu a kontrolují rovnováhu mezi MDM2 a p53. Navíc miR-215 a miR-192 ovlivňují signální dráhu IGF, a tím zabraňují zvýšené migraci PC do kostní dřeně [35].

Závěr

Během posledních let bylo provedeno mnoho studií srovnávajících globální profil CD138+PC MM pacientů a zdravých dárců pomocí různých high-throughput screeningových metod, od oligonukleotidových čipů až po qRT-PCR profilování. Každá z metod má své silné a slabé stránky poskytující rozdílné výsledky, ke kterým navíc přispívá velká heterogenita onemocnění. Obecně bylo doposud ve většině prací u myelomu identifikováno více miRNA se zvýšenou expresí u PC než se

sníženou expresí. Výjimkou je práce Guttiereze et al., která popisuje více miRNA se sníženou expresí (tab. 18.1.).

Dále můžeme říci, že ani identifikace jednotlivých miRNA není jednotná, což může být způsobeno několika faktory. Za prvé je v každé studii rozdílný soubor pacientů a kontrol a rozdílná velikost souboru. Jak již bylo zmíněno, je MM velmi heterogenní onemocnění a každý pacient má jinou kombinaci genetických mutací a cytogenetických aberací, což se může projevit na rozdílné subklasifikaci do skupin ve srovnání se zdravými dárci. Za druhé, pacienti mohou mít v různých stadiích onemocnění odlišné profily miRNA. Například miR-15 byla popsána jako zvýšená u nových diagnóz, ale snížená u relapsů [10,11]. V neposlední řadě se na odlišnostech podílejí rozdíly ve zpracování vzorku, purifikaci PC, extrakci miRNA a dále rozdílné mikročipové platformy a různé verze čipů.

Dnes již víme, že změněná exprese miRNA u MM může mít příčiny genetické, cytogenetické nebo epigenetické. Byly také popsány specifické miRNA charakterizující progresi MM, lepší prognózu nebo rezistenci vůči lékům. Mechanizmus deregulace není zatím přesně znám, víme již, že v pozadí stojí jak změna v cílovém genu pro miRNA, tak změny v počtu kopií lokusů, ve kterých se nachází miRNA, defekty v biogenezi miRNA a epigenetické změny. Snahou dalších studií by mělo být objasnění komplexity regulace miRNA a identifikace terapeutických cílů.

Poděkování

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