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ÚSTAV LÉKAŘSKÉ CHEMIE A BIOCHEMIE

REGULACE GENOVÉ EXPRESE V NÁDOROVÉ TKÁNI

Disertační práce

Plzeň 2018 **Vlastimil Kulda**

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Regulation of gene expression in tumour tissue

Disertační práce

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Abstrakt

Deregulace genové exprese způsobená genetickými a epigenetickými změnami hraje důležitou roli v patogenezi nádorových onemocnění. Tato disertační práce je komentovaným souborem deseti publikovaných prací zabývajících se problematikou molekulární biologie nádorů, na jejichž vzniku se autor významným způsobem podílel. Všechny práce obsažené v souboru spojuje téma stanovení molekul, které se podílejí na regulaci genové exprese (mikroRNA) nebo změn na úrovni DNA, které ovlivňují expresi genů (methylace promotoru, přítomnost fúzního genu). MikroRNA jsou krátké jednovláknové RNA molekuly, které regulují genovou expresi na posttranskripční úrovni urychlením degradace mRNA nebo inhibicí translace. Jedná se o základní mechanismus s vlivem na všechny buněčné procesy včetně patogeneze různých nemocí. MikroRNA mohou působit jako onkogeny snižováním exprese tumor supresorových genů nebo naopak jako tumor supresory snižováním exprese onkogenů, ale dnes už víme, že síť mikroRNA – RNA interakcí je mnohem komplexnější. Námi publikované výsledky, které jsou součástí této práce, se týkají kolorektálního karcinomu (CRC), karcinomu prostaty, dlaždicobuněčných karcinomů hlavy a krku (HNSCC), karcinomu žaludku a nemalobuněčného karcinomu plic (NSCLC). U pacientů s CRC jsme prokázali prognostický význam miR-21. Ve tkáni karcinomu prostaty jsme popsali vyšší expresi miR-20a u hůře diferencovaných nádorů s vyšším Gleason skóre a ukázali, že přítomnost fúzního genu TMPRSS2-ERG znamená horší prognózu. Novým poznatkem u HNSCC bylo nalezení vztahu exprese miR-34a a p16-pozitivity jako markeru HPV infekce. U pacientů s pokročilými stádii karcinomu žaludku jsme našli mikroRNA s prognostickým významem (miR-150, miR-224 a miR-342). Na souboru NSCLC pacientů s pokročilým epidermoidním karcinomem léčených paliativní chemoterapií jsme našli vztah miR-34a, miR-224 a miR-342 k celkovému přežití, ale zároveň ukázali, že vysoká hladina jedné konkrétní mikroRNA může být totiž za jistých okolností spojena s nepříznivou prognózou, za jiných podmínek naopak s prognózou velmi dobrou. Výsledky založené na stanovení exprese mikroRNA v biologickém materiálu (nativní nádorová tkáň, formalínem fixovaná v parafínu zalitá tkáň, krevní plazma) ukazují, že tyto regulační molekuly mohou odrážet klinickopatologické vlastnosti nádorů a najít uplatnění jako biomarkery.

Abstract

Deregulation of gene expression caused by genetic or epigenetic changes plays an important role in pathogenesis of cancer. The thesis is a commented collection of ten publications dealing with the molecular biology of tumours. The author has significantly contributed to all of them. All the articles contained in the thesis are linked to the topic of assessment of molecules involved in gene expression regulation (microRNAs) or DNA alterations that affect gene expression (promoter methylation, presence of a fusion gene). MicroRNAs are short single-stranded RNA molecules involved in posttranscriptional regulation of gene expression by triggering mRNA degradation or inhibiting translation. It is a basic mechanism with an impact on all cellular processes including the pathogenesis of various diseases. MicroRNAs can either act as oncogenes by decreasing the expression of tumour-suppressor genes or as tumour-suppressor genes by decreasing the expression of oncogenes. However, the network of microRNA – RNA interactions is much more complex. Our published results that are part of this thesis are focused on colorectal carcinoma (CRC), prostate cancer, head and neck squamous cell carcinoma (HNSCC), gastric cancer and non-small cell lung cancer (NSCLC). In patients with CRC, we demonstrated the prognostic significance of miR-21. In prostate cancer tissue, we have described higher miR-20a expression in less differentiated tumours with higher Gleason score and showed that the presence of the TMPRSS2-ERG fusion gene is related to worse prognosis. A new finding in HNSCC was a relationship between the miR-34a expression and p16-positivity as an HPV infection marker. We found microRNAs (miR-150, miR-224 and miR-342) with prognostic significance in patients with advanced gatric cancer. In the group of NSCLC patients with advanced squamous cell carcinoma treated with palliative chemotherapy, we found a relationship of miR-34a, miR-224 and miR-342 to overall survival, but also showed that the high level of one particular microRNA may under certain circumstances be associated with an unfavourable prognosis, under other conditions, the patient's outcome is good. Results on the basis of the microRNA expression estimation in biological material (native tumour tissue, formalin fixed paraffin embedded tissue, blood plasma) indicate that these regulatory molecules can reflect the clinico-pathological features of the tumours and could become clinically applicable biomarkers.

Poděkování

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Prohlášení

Prohlašuji, že jsem tuto disertační práci vypracoval samostatně a veškeré převzaté údaje řádně citoval. V textové části jsou použity části předchozích článků, kde jsem hlavním autorem nebo spoluautorem a jejichž kompletní znění je k dispozici v přílohové části práce. Souhlasím s uložením elektronické verze své práce v databázi Univerzity Karlovy, Lékařské fakulty v Plzni.

V Plzni dne 15.6.2018

Vlastimil Kulda

Obsah

Seznam použitých zkratek

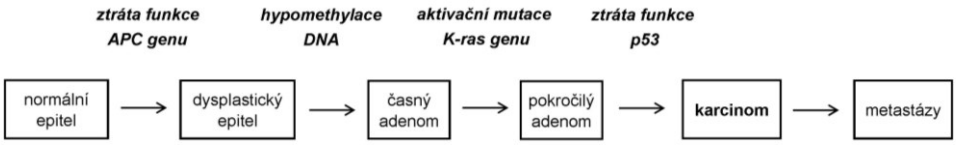
5-FU	5-fluorouracil
ABL	Abelson murine leukemia viral oncogene homolog 1 *(gen)*
ADT	androgen deprivační terapie
APC	adenomatous polyposis coli *(gen)*
AR	androgenní receptor
ARE	androgen responsivní element
ARF4	ADP-ribosylation factor 4 *(gen)*
BCR	breakpoint cluster region *(gen)*
BRCA1	breast cancer 1 *(tumor supresorový gen)*
CLM	jaterní metastázy kolorektálního karcinomu (colorectal liver metastases)
CML	chronická myeloidní leukémie
CRC	kolorektální karcinom (colorectal carcinoma)
CRISPR	clustered regularly interspaced short palindromic repeats
DD3	differential display code 3 *(gen)*
DFI	bezpříznakové období (disease free interval)
DGCR8	DiGeorge syndrome chromosomal region 8 *(protein)*
DHT	dihydrotestosteron
ECM	extracelulární matrix
ERG	ETS-related gene *(gen)*
ETS	E26 transformation-specific *(rodina transkripčních faktorů)*
FAP	familiární adenomatózní polypóza
FFPE	formalínem fixovaná do parafínu zalitá tkáň (formalin-fixed paraffin embedded)
GAPDH	glyceraldehyd-3-fosfátdehydrogenáza *(gen)*
GUSB	beta-glukuronidáza *(gen)*
H&E	hematoxylin a eosin
HNSCC	dlaždicový karcinom hlavy a krku (head and neck squamous cell carcinoma)
HPC1	hereditary prostate cancer 1 *(gen)*
HPRT1	hypoxanthin-guanin-fosforibosyltransferáza *(gen)*

HPV	human papilloma virus
LNA	locked nucleic acids
MMP	matrixové metaloproteinázy
NLK	Nemo-like kinase *(gen)*
NSCLC	nemalobuněčný plicní karcinom (non-small cell lung cancer)
OS	celkové přežití (overall survival)
PCA3	prostate cancer antigen 3 *(gen)*
PCR	polymerázová řetězová reakce (polymerase chain reaction)
PDCD4	programmed cell death 4 *(gen)*
PIN	prostatická intraepiteliální neoplázie
PSA	prostatický specifický antigen
PTEN	phosphatase and tensin homologue *(gen)*
RECK	reversion-inducing cysteine-rich protein with Kazal motifs *(gen)*
RNU6B	U6B small nuclear RNA
RT-qPCR	reverzní transkripce a kvantitativní polymerázová řetězová reakce
SCC	dlaždicobuněčný karcinom (squamous cell carcinoma)
TIMPs	tkáňové inhibitory metaloproteináz (tissue inhibitors of metalloproteinases)
TP53	tumor protein p53 *(gen)*
TPM1	tropomyosin 1 *(gen)*
TMPRSS2	transmembrane protease, serine 2 *(gen)*
TNM	tumor – nodus – metastasis *(staging systém zhoubných nádorů)*
TUG1	taurine-upregulated gene 1 *(gen)*

1 Úvod

Etiopatogeneze nádorových onemocnění je velice komplexní a neobyčejně různorodá. Obrovskou heterogenitu zastřešuje široká definice nádoru jako onemocnění, kde změna genetické informace a deregulace práce s ní vede k nekontrolovanému buněčnému dělení. Úplné pochopení všech molekulárních mechanismů zodpovědných za transformaci normální buňky v nádorovou a za následnou progresi tumoru zůstává stále výzvou pro další výzkum.

Z historického pohledu je milníkem konec dvacátého století, kdy rychlý rozvoj metod molekulární biologie vedl k postupnému odhalování genetických změn přítomných u neoplázií, postulování konceptu onkogenů a tumor supresorů a formulování základních modelů karcinogeneze. Jako příklad je možno uvést průkaz aktivačních mutací K-ras genu u nádorově transformovaných buněk (Santos et al. 1984) nebo poznání role proteinu p53 (Winchester 1983) a detekci jeho mutací v mnoha nádorech (Baker et al. 1989; Hollstein et al. 1991). Svou roli hrál i výzkum dědičných onemocnění vedoucích pravidelně ke vzniku nádorů jako je třeba familiární adenomatózní polypóza (FAP), kde byla nalezena ztráta funkce genu APC (Nishisho et al. 1991). Vše vyústilo ve formulaci prvního uceleného modelu vícekrokové karcinogeneze u kolorektálního karcinomu (CRC) dnes známého jako Vogelsteinův model (Vogelstein et al. 1988; Fearon a Vogelstein 1990).



Obrázek 1 - Vogelsteinův model patogeneze CRC

převzato a upraveno (Fearon a Vogelstein 1990)

Slabým místem Vogelsteinova modelu je, že popsané změny nejsou přítomny u všech individuálních nádorů. Dnes v době dostupného celogenomového sekvenování je možné říct, že nejčastěji mutovaným genem u nádorů všeobecně je gen TP53 kódující protein p53, jehož abnormality jsou přítomny asi u 50 % nádorů (Hainaut a Pfeifer 2016). Sekvenační studie ukázaly, že průměrně je v maligních nádorech přítomno 33–66 somatických mutací, které mohou mít vliv na funkci produktu genu (Vogelstein et al. 2013). Existují velké rozdíly mezi různými typy nádorů, dětské nádory (meduloblastom) a hematologické malignity jsou na jednom kraji spektra s minimem přítomných mutací (do 10), naopak melanom a plicní nádory patří k těm, kde je přítomno mutací daleko víc, v průměru 200. Jsou to nádory, na jejichž vzniku se ve velké míře podílejí mutageny (UV záření u melanomu, kouření cigaret u plicních nádorů). Zajímavá je studie ukazující na 10x víc přítomných mutací u karcinomů plic kuřáků ve srovnání s nekuřáky (Govindan et al. 2012).

Výzkum dále ukázal, že existuje asi 140 genů, jejichž mutace nebo aberantní exprese přináší selektivní růstovou výhodu buňkám a tak se mohou tyto geny (označují se jako „driver genes“) významným způsobem podílet na nádorové transformaci a další progresi onemocnění. Tyto geny můžeme zařadit do jedné z kategorií tumor supresor nebo protoonkogen. Typický nádor obsahuje mutace 2 až 8 „driver“ genů. Ostatní mutace v genech, které pravděpodobně nemají vliv na vlastní kancerogenezi, se označují jako „passenger“ (Vogelstein et al. 2013). Co komplikuje pochopení a výzkum, je skutečnost, že „driver“ geny nejsou u individuálních nádorů zcela shodné.

V roce 2000 Hanahan a Weinberg ve slavném článku *The hallmarks of cancer* publikovaném v časopise Cell přišli s novým přístupem, jak nahlížet na komplexitu celé problematiky. Místo jednotlivých genů podílejících se na kancerogenezi upřednostnili definování základních vlastností, které jsou pro nádory charakteristické (Hanahan a Weinberg 2000). Popsali šest klíčových schopností, které zhoubný nádor různými strategiemi postupně získává v průběhu rozvoje onemocnění:

- soběstačnost v růstových (proliferačních) signálech
- necitlivost k signálům inhibujícím růst
- vyhnutí se apoptóze
- neomezený potenciál replikace DNA
- podpora angiogeneze
- aktivace invazivity a metastazování

V roce 2011 v revidované verzi článku stejní autoři (Hanahan a Weinberg 2011) přidali do schématu další čtyři body:

- vyhnutí se destrukci imunitním systémem
- genomová instabilita a zvýšená mutageneze
- deregulace energetického metabolismu
- s nádorem asociovaný zánět (nádorové mikroprostředí)

Obrázek 2 - Hallmarks of cancer

převzato a upraveno (Hanahan a Weinberg 2011)

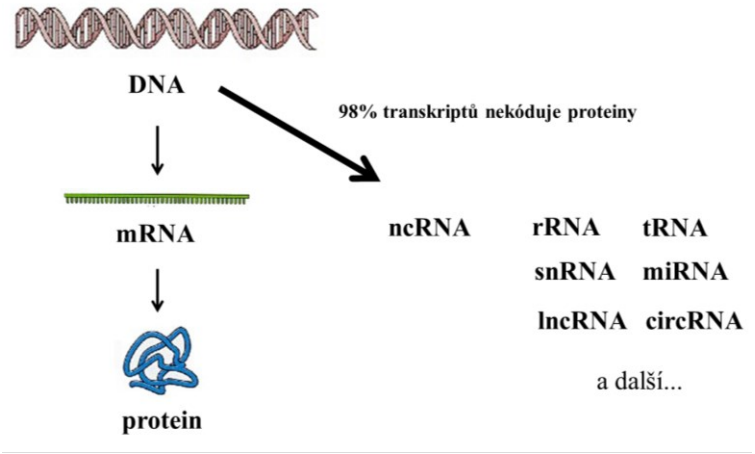
Asi největším přínosem konceptu *Hallmarks of cancer* je, že jasně ukazuje, jak je problematika kancerogeneze komplexní, zdůrazňuje nádorovou heterogenitu a roli nádorového mikroprostředí. Tvoří tak logický rámec pro další práci v dnešní době neustále se rozvíjejících nových metod jako je např. CRISPR technologie editování genomu (Jinek et al. 2012), která nabízí do budoucna slibné možnosti nejen pro další výzkum, ale i možné léčebné zásahy (Moses et al. 2018).

Pokud si položíme otázku, jaké změny vedly nádorovou tkáň k získání vlastností uvedených v konceptu *Hallmarks of cancer*, odpovědí bude: **deregulace genové exprese** v jednotlivých buňkách tkáně způsobená genetickými a epigenetickými změnami.

Disertační práce je komentovaným souborem deseti publikovaných prací zabývajících se problematikou molekulární biologie nádorů, na jejichž vzniku se autor významným způsobem podílel. Všechny práce obsažené v souboru spojuje téma stanovení molekul z kategorie možných biomarkerů, které se významně podílejí na regulaci genové exprese (mikroRNA) nebo změn v DNA, které ovlivňují expresi genů (methylace promotoru, přítomnost fúzního genu).

2 Způsoby regulace genové exprese

Velmi obecně je možné definovat genovou expresi jako proces, kterým se informace uložená v genu převede ve funkční genový produkt. Genovým produktem může být protein nebo rozmanité funkční RNA molekuly. Exprese protein kódujících genů je dvoukroková. Prvním krokem je transkripce, při které vzniká mRNA, druhým krokem je translace na ribosomech, kdy informace v mRNA vede ke vzniku proteinu. Dnes víme, že naprostá většina transkriptů nekóduje proteiny. Exprese genů kódujících tyto funkční molekuly je na rozdíl od exprese genů kódujících proteiny jen jednokroková (transkripce). Nejde jen o ribosomální RNA (rRNA) a transferovou (tRNA), dnes je známo už mnoho dalších typů RNA molekul, jako jsou mikroRNA (miRNA), short interfering RNA (siRNA), piwi-interacting RNA (piRNA), malé jaderné RNA (snRNA), malé jadérkové RNA (snoRNA), dlouhé nekódující RNA (lncRNA) a cirkulární RNA (circRNA) označované souhrnně jako ncRNA (Eddy 2001; Mattick 2003). Při analýzách transkriptomu ale pořád platí otázka, co už je „odpad“ a co je ještě nějaká funkční RNA molekula (Brosius a Raabe 2016).



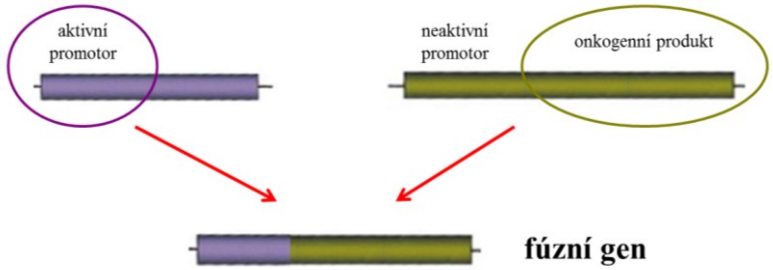
Obrázek 3 - Genová exprese

Jak je genová exprese regulována? Kde všude může docházet k regulacím?

VŠUDE, na VŠECH úrovních:

- transkripce
- posttranskripční úpravy RNA (splicing, editing)
- export RNA z jádra a lokalizace v buňce
- stabilita RNA (rychlost degradace)
- translace
- posttranslační úpravy
- aktivita proteinu

Do genové exprese mohou zasahovat i změny na úrovni struktury chromosomů (translokace, delece, inverze), které vedou ke vzniku fúzních genů, při kterých se pod regulaci aktivního promotoru dostane gen, který by jinak nebyl aktivní.



Obrázek 4 - Příklad vzniku fúzního onkogenu

Učebnicovým příkladem fúzního onkogenu je tzv. filadelfský chromosom, který vzniká translokací mezi chromosomy 9 a 22 – t(9;22)(q34;q11). Protoonkogen ABL se tak spojí s částí genu BCR a výsledkem je produkce tyrosinkinázy zasahující do buněčných signálních drah a podílející se na patogenezi chronické myeloidní leukémie (CML), vzácněji i jiných leukémií (Westbrook 1988).

Fúzních genů podílejících se na tumorigenezi bylo už v roce 2007 popsáno více než 350 (Mitelman et al. 2007), přestaly být doménou jen hematologických malignit a jsou nalézány i u solidních tumorů (Parker a Zhang 2013). V kapitole 4.10 se věnuji naší práci týkající se fúzního genu TMPRSS2-ERG u karcinomu prostaty.

Na regulaci genové exprese se podílejí i epigenetické mechanismy. Nechci se zabývat přesnou definicí epigenetiky, ale zjednodušeně lze říct, že epigenetika se zabývá (dědičnými) změnami ve funkci genů, které nejsou způsobeny pořadím nukleotidů v DNA. Do epigenetiky se řadí následující oblasti:

- methylace DNA
- remodelace chromatinu a modifikace histonů
- *RNA interference (mikroRNA)*

V kapitole 4.9 se věnuji naší práci týkající se methylace promotoru tumor supresorového genu RECK. K methylaci DNA dochází v tzv. CpG ostrůvcích za účasti enzymů DNA methyltransferáz. Methylace v oblasti promotoru brání expresi promotorem ovládaného genu. U nádorových onemocnění je sice pozorována globální demethylace DNA, ale methylace promotorů je jeden ze způsobů vyřazení z funkce tumor supresorových genů (Kulis a Esteller 2010).

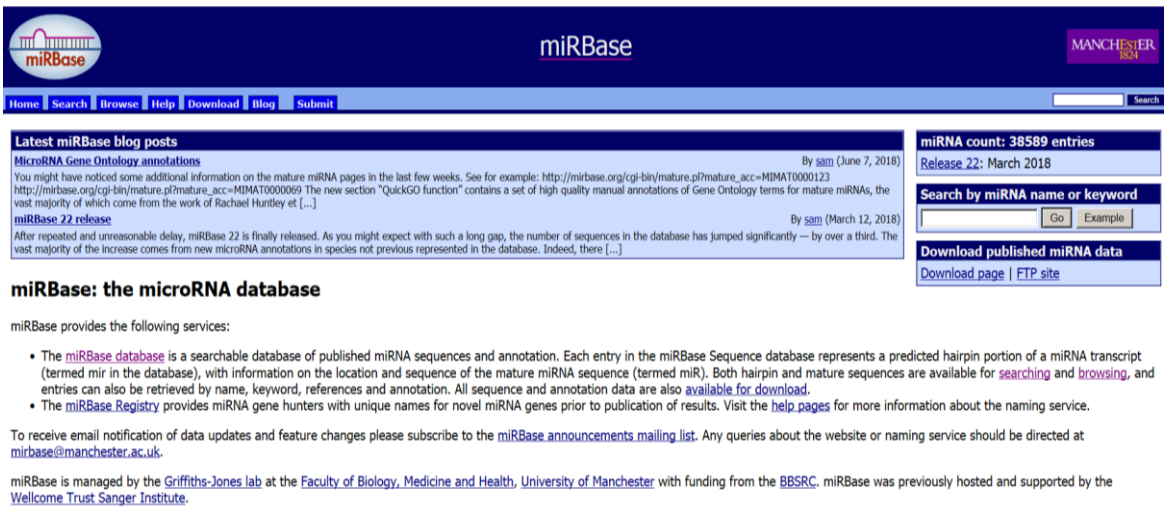
V samostatné kapitole se budu věnovat problematice mikroRNA, které se týká většina publikovaných článků komentovaných v této práci.

3 MikroRNA

Ještě koncem devadesátých let dvacátého století si málokdo uvědomoval, jaký vliv na řízení genové exprese mají molekuly RNA. MikroRNA (miRNA) jsou krátké (délka kolem 22 nukleotidů) jednovláknové nekódující RNA molekuly, které negativně regulují genovou expresi na posttranskripční úrovni prostřednictvím inhibice translace nebo snížením stability cílové mRNA. Jedna konkrétní miRNA může cílit na stovky různých RNA transkriptů, ale i obráceně, jedna konkrétní mRNA může být cílem pro mnoho různých miRNA. Je tedy zřejmé, o jak komplexní regulační síť se jedná. Odhaduje se, že exprese až 50 % genů je ovlivněna touto cestou (Zhang et al. 2007).

První zástupce této třídy molekul lin-4 byl původně popsán u modelového organismu *Caenorhabditis elegans* jako gen ovlivňující časování vývoje. Aniž by věděly, jak velmi významnou kategorii regulačních molekul vlastně objevily, týmy Victora Ambrose a Garyho Ruvkuna popsaly, že gen lin-4 nekóduje protein, ale jeho transkript reguluje hladinu lin-14 vazbou na 3' netranslatovanou oblast mRNA tohoto genu (Wightman et al. 1993; Lee et al. 1993). V roce 1998 Andrew Fire and Craig C. Mello objasnili fenomén RNA interference (Fire et al. 1998), za tyto objevy dostali v roce 2016 Nobelovu cenu za fyziologii nebo lékařství.

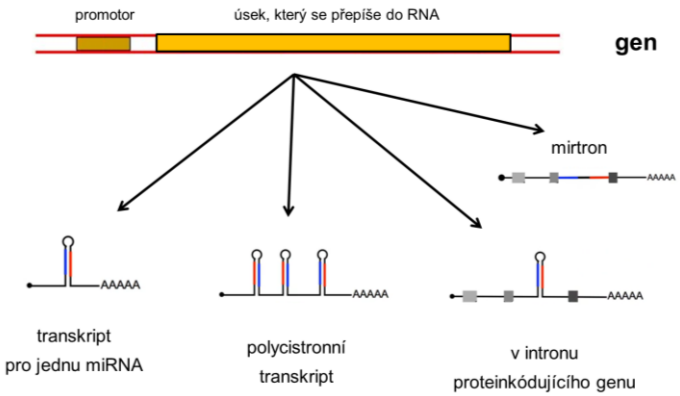
Chronologicky druhou popsanou mikroRNA byla v roce 2000 let-7 (Reinhart et al. 2000), opět hrající roli při vývoji hlístice *Caenorhabditis elegans* (Pasquinelli a Ruvkun 2002). Od svého objevu do dnešní doby bylo identifikováno už více než 2500 lidských mikroRNA, názvosloví má svá systematická pravidla stanovená hned na začátku práce na tomto poli (Ambros et al. 2003), specifické názvy zůstaly jen dvěma výše uvedeným mikroRNA. Přehled známých mikroRNA (názvy, sekvence, cílové geny) udržuje databáze miRBase dostupná na adrese www.mirbase.org (Griffiths-Jones et al. 2006).



Obrázek 5 - Databáze miRBase

3.1 Biogeneze a funkce mikroRNA

Odhaduje se, že mikroRNA geny představují 1 % genomu. Uspořádání genů kódujících mikroRNA může být velice různorodé. Mohou být kódovány samostatnými geny transkribovanými často polycistronním způsobem. Mohou být v intronech (vzácně i exonech) jiných genů, pak je jejich transkripce řízena promotorem hostitelského genu (Olena a Patton 2010). Zajímavou skupinou jsou mirtrony, kdy krátký intron dá po splicingu vzniknout přímo pre-miRNA. Jde tak o alternativní cestu k níže popsané klasické biogenezi mikroRNA (Westholm a Lai 2011; Curtis et al. 2012).

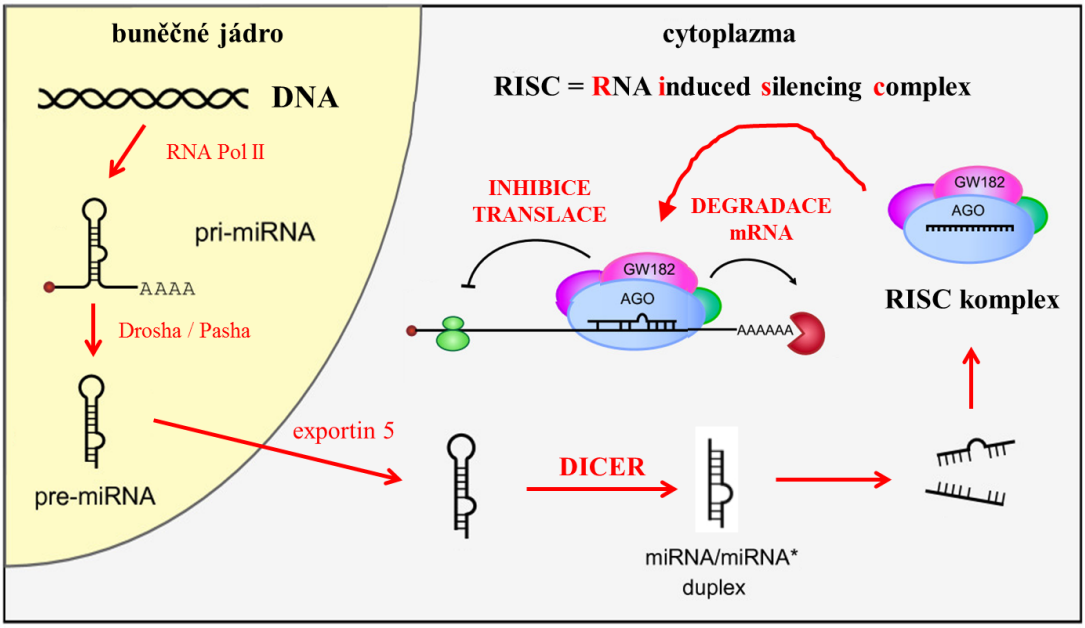


Obrázek 6 - Několik způsobů uspořádání genů pro mikroRNA

Vznik zralých mikroRNA v eukaryotických buňkách je realizován v několika krocích. V buněčném jádře dochází k transkripci za účasti RNA polymerázy Pol II (stejný typ provádí i transkripci mRNA). Vzniká dlouhý primární transkript (tisíce nt) se specifickou strukturou označovaný jako pri-miRNA. Tento transkript může podstoupit stejné modifikace jako je běžné u mRNA (čepička na 5' konci a polyadenylace 3' konce). V pri-miRNA jsou úseky komplementární k sobě navzájem, intramolekulární párování bazí vytváří na molekule vlásenky (hairpin structure, stem loop). Ještě v jádře je pri-miRNA štěpena „mikroprocesorovým komplexem“ na mnohem kratší (70-100 nt) prekurzory označované jako pre-miRNA, jsou to vlásenkové úseky původních pri-miRNA. „Mikroprocesorový komplex“ se skládá z enzymu Drosha (ribonukleáza III třída 2) a kofaktoru Pasha (dsRNA vázající protein nazývaný také DGCR8). Pre-miRNA je následně exportována do cytoplazmy transportním proteinem exportin 5. V cytoplazmě je endoribonukleázou Dicer dále štěpena, je odstraněna smyčka vlásenky a zůstane tzv. miRNA/miRNA* duplex, dvouvláknový úsek RNA, který je rozeznán výkonným proteinovým komplexem miRISC (miRNA induced silencing complex), jedno z vláken je do tohoto komplexu zabudováno, druhé je uvolněno a degradováno. Biogeneze mikroRNA je popsána v mnoha publikacích (Bartel 2004; Kim et al. 2009).

Které z dvojice vláken z miRNA/miRNA* duplexu bude zabudováno do miRISC komplexu (*guide strand*, *leading strand*, miR) a které bude uvolněno (*passenger strand*, miR*), je také regulováno (Meijer et al. 2014). Funkci negativního regulátoru vykonává

miRISC komplex se zabudovaným jedním vláknem mikroRNA, které navádí tento komplex specificky na cílové geny.



Obrázek 7 - Biogeneze a funkce mikroRNA

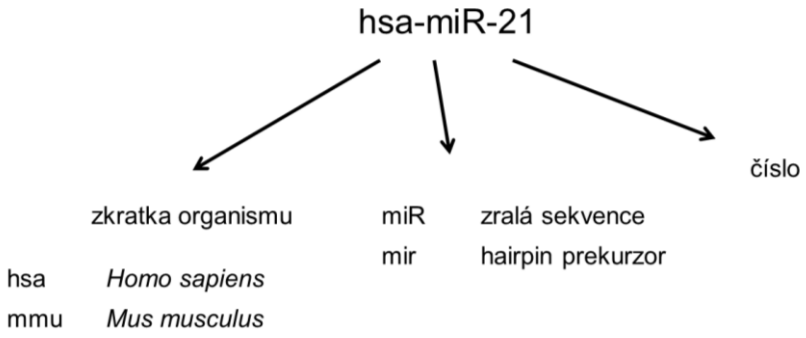
Popisují se dva mechanismy, jakými miRISC komplex ovlivňuje cílovou mRNA: urychlení degradace mRNA a represe translace. Klasický pohled na rozhodnutí, který mechanismus se uplatní, připisuje význam stupni komplementarity sekvencí. Úplná komplementarita vede k degradaci mRNA, neúplná komplementarita k inhibici translace. V tomto případě dochází k vazbě většinou na 3' netranslatovanou oblast mRNA (Singh et al. 2008; Krol et al. 2010). Neúplná komplementarita komplikuje *in silico* predikci cílových molekul, používáno je mnoho algoritmů (Wagner et al. 2014).

Ukazuje se, že mikroRNA nemusí být nutně jen negativní regulátory genové exprese, byl popsán i fenomén RNA aktivace (RNAa), kdy mikroRNA přímým působením na promotor aktivuje transkripci genu (Ramchandran a Chaluvally-Raghavan 2017).

3.2 Názvosloví mikroRNA

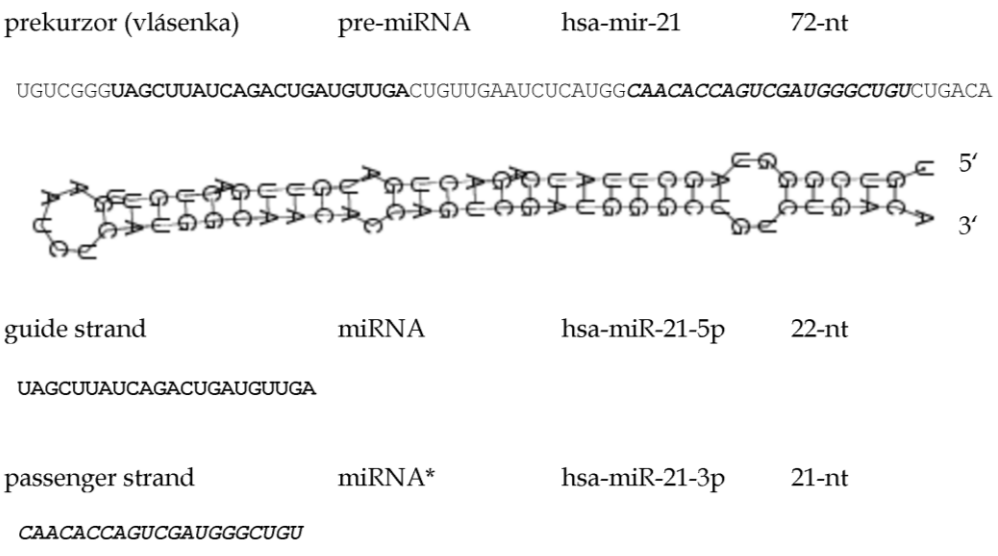
Velkou výhodou oblasti mikroRNA je systematické názvosloví důsledně dodržované téměř od začátku rozmachu práce na tomto poli. Celé systematické jméno se skládá ze 3-4 písmen udávajících druh, např. „hsa“ (Homo sapiens) značí lidskou mikroRNA. Následuje kmen „miR“ pro maturované sekvence nebo „mir“ pro prekurzory (pre-miRNA) a číselný identifikátor. Jsou respektovány principy homologie. Ortologní sekvence (stejné nebo velmi podobné sekvence u různých druhů) mají stejné číslo, tj. hsa-miR-21 a mmu-miR-21 jsou ortology. Paralogní sekvence (různé pozice podobných sekvencí v genomu jednoho druhu) jsou označovány písmeny za pořadovým číslem, např. hsa-miR-20a a hsa-miR-20b. Různé pre-miRNA v genomu dávající vznik identickým sekvencím zralých

mikroRNA jsou označovány číselnou příponou za pořadovým číslem (např. hsa-mir-16-1 a hsa-mir-16-2).



Obrázek 8 - Názvosloví mikroRNA

Dřívější nomenklatura byla nejednoznačná v jedné věci, „hlavní“ mikroRNA pocházející z vlásenky se označovala miR-21, „druhá“ mikroRNA pocházející z téže vlásenky, o které se předpokládalo, že je degradována a nezabudovává se do miRISC komplexu, se označovala hvězdičkou (miR-21*). Dnes víme, že z jedné vlásenkové pre-miRNA mohou pocházet dvě zralé mikroRNA a obě mohou vykonávat funkci v rámci miRISC komplexu, záleží na čase a místě, která je ta „hlavní“. Proto se přistoupilo k jednoznačnému označování příponami -3p nebo -5p, podle toho, z kterého konce vlásenkové pre-miRNA struktury maturovaná mikroRNA pochází.



Obrázek 9 - Sekvence a principy názvosloví na příkladu miR-21

3.3 Role mikroRNA v tumorigenezi

Brzy po objevu mikroRNA a jejich všeobecné funkce v regulaci genové exprese bylo jasné, že tyto molekuly budou zasahovat do všech buněčných procesů a tedy i do patogeneze různých nemocí včetně nádorových onemocnění.

V souvislosti s tumorigenezí se snahou ukázat na význam deregulovaných mikroRNA byl dokonce zaveden termín oncomirs (Esquela-Kerscher a Slack 2006; Cho 2007).

MikroRNA mohou působit jako onkogeny snižováním exprese tumor supresorových genů nebo naopak jako tumor supresory snižováním exprese onkogenů (Hagan a Croce 2007; Croce 2008; Iorio a Croce 2009). Dnes víme, že je těžké nebo dokonce nemožné zařadit konkrétní mikroRNA do jedné z kategorií onkogen – tumor supresor. Jedna mikroRNA může totiž cílit na stovky genů a mezi nimi mohou být jak onkogeny, tak tumor supresory. Záleží na kontextu (čase a místě působení), který vliv bude dominantní (Fabbri et al. 2007). Na poli mikroRNA – RNA interakcí je mnoho hráčů, hypotéza kompetujících endogenních RNA (ceRNA) je podrobněji rozebrána v kapitole 4.6.

4 Komentář k vybraným publikovaným pracím

Na výzkumu biomarkerů na Lékařské fakultě v Plzni se podílí více pracovišť ve spolupráci s Fakultní nemocnicí Plzeň. Kromě Ústavu lékařské chemie a biochemie, Ústavu biologie a Biomedicínského centra LF UK v Plzni jsou do projektů zapojeni i lékaři z Chirurgické kliniky, Urologické kliniky, Kliniky pneumologie a ftizeologie, Onkologického a radioterapeutického oddělení, Šiklova ústavu patologie a nelze opomenout Laboratoř pro imunoanalýzu FN Plzeň, se kterou jsem intenzivně spolupracoval.

Naše pracoviště začalo jako jedno z prvních v České republice v roce 2008 měřit expresi mikroRNA u onkologických onemocnění. Navázali jsme na zkušenosti z kvantifikace genové exprese protein kódujících genů na úrovni mRNA (Pesta et al. 2005; Cerna et al. 2006; Safranek et al. 2009). Od té doby uplynulo deset let a za tu dobu jsem byl svědkem následujícího posunu v několika rovinách:

- cíle práce
- analyzovaný biologický materiál
- soubor pacientů
- technické provedení real-time qPCR
- způsob normalizace výsledků

Po roce 2000, kdy začaly být dostupné přístroje na real-time kvantitativní PCR a zatím nebyly rozvinuté high-throughput metody typu microarray technik a sekvenování transkriptomu, bylo vesměs cílem srovnávání exprese v nádorové a v okolní zdravé tkáni a hledaní genů, jejichž exprese je v nádorové tkáni deregulovaná. Jak už to v medicíně a v molekulární biologii často bývá, data nevykazují normální rozdělení, ze statistických testů byl na testování hypotéz většinou používán neparametrický Wilcoxonův párový test. Dále jsme hodnotili vztah exprese ke klinickopatologickým charakteristikám nádoru (histologický podtyp, TNM klasifikace, stage, grade).

Postupně došlo k posunu v cílech naší práce, hodnotili jsme prognostický význam hladin exprese pomocí statistických metod pro analýzu přežívání (Coxův regresní model, Kaplan-Meierova metoda). Dalším krokem byla snaha o design souboru pacientů tak, aby výsledky analýz přežívání zároveň ukázaly na prediktivní význam (odpověď na konkrétní léčbu).

Druhou rovinou, kde došlo k vývoji v čase, byl analyzovaný biologický materiál. Začínalo se na nativní tkáni odebrané chirurgy během operačního výkonu, která byla po odběru ihned zamražena a skladována na -70°C do doby zpracování. Velmi brzy poté, co se ukázalo, že je možné izolovat nukleové kyseliny v dostatečném množství a kvalitě pro molekulárně biologické testy i z formalínem fixované do parafínu zalité tkáně (FFPE) rutinně připravované patology (Roberts et al. 2009; Mittempergher et al. 2011), zavedli jsme metodiku a začali používat řezy z FFPE bločků jako výchozí materiál, což s sebou

přineslo několik výhod. Tkáň zalitá v bločku byla hodnocena patologem a oblast s nádorovou tkání mohla být na řezu obarveném standardním barvením hematoxylinem a eosinem (H&E) označena a z dalších řezů odkrojených mikrotomem z bločku požadovaná část makrodisekcí oddělena. Výhodou FFPE bločků je i možnost retrospektivních studií. Dále je třeba zmínit, že FFPE tkáň je v klinické medicíně rutinně připravována a není třeba zavádět novou metodiku odběru vzorků.

V současné době jsme přešli opět o krok dál směrem k možnému uplatnění v klinické praxi a výchozím biologickým materiálem je pro nás krevní plazma. První naše výsledky týkající se cirkulujících mikroRNA u pacientů s kolorektálním karcinomem byly zatím prezentovány jen na konferencích (Příloha 11).

K postupnému vývoji došlo i u struktury souborů pacientů, kterých se naše studie týkají. V začátcích jsme pracovali s velmi heterogenními soubory, pacienty spojovala jen anatomická lokalita nádoru, jednalo se o různé histologické podtypy, různá klinická stádia od časných po pokročilá. U prospektivních studií ani nebyla jiná možnost, úzce vymezená vstupní kritéria by znemožnila získat potřebné vzorky v rozumné době od začátku studie. Např. o nemalobuněčném plicním karcinomu je známo, že dva hlavní histologické podtypy (adenokarcinom a epidermoidní karcinom) se liší prognózou a pozadím na molekulární úrovni (Skrzypski et al. 2013; Tian 2017). Využití FFPE bločků umožnilo začít pracovat s více homogenními soubory vzhledem k otázkám, které chceme řešit.

Pokrok se odehrál i v technickém provádění real-time kvantitativní PCR, nejen na úrovni přístrojového vybavení, kde jsme začínali na přístroji Rotor-Gene 2000 (Corbett Research), pracovali s iCycler (Bio-Rad), nyní používáme Stratagene Mx3005P (Agilent Technologies) a k dispozici máme i Rotor-Gene 6000 (Corbett Research). U kvantitativní PCR v reálném čase je měření amplifikované DNA založeno na fluorescenčních metodách. Původně jsme pracovali s barvivem SYBR Green, které má vysokou fluorescenci po interkalaci do dvouvkláknové DNA (Morrison et al. 1998), je univerzální, ale nevýhodou je jeho nespecifita. Pro kvantifikaci mRNA protein kódujících genů jsme přešli na UPL sondy (Universal Probe Library, Roche), což jsou v principu hydrolyzační sondy podobné specifickým TaqMan sondám (Navarro et al. 2015), ale využívají technologie locked nucleic acids (LNA) a jsou semiuniverzální, tj. není třeba syntetizovat sondu „na míru“. ProbeFinder Assay Design Software navrhne po zadání požadavku, co chceme kvantifikovat, specifické primery a z dostupné knihovny číslo sondy, kterou je třeba použít. Kity na stanovení mikroRNA, které používáme, TaqMan MicroRNA Assays (Applied Biosystems) a nově TaqMan Advanced miRNA Assays (Thermo Fischer Scientific), obsahují specifické TaqMan sondy.

Samostatnou kapitolou by mohlo být pojednání o tom, jak udávat výsledky stanovení genové exprese a o volbě referenčních genů. V dobách, kdy jsme začínali, jsme používali velmi pracný absolutní způsob kvantifikace. Bylo třeba připravit standardy ligováním produktů amplifikace do plasmidů a klonováním v bakteriálních buňkách získat materiál

pro přípravu standardů, které byly naředěny v rozsahu $10^3 – 10^8$ kopií na µl a zpracovávány paralelně se vzorky. Ze sestrojené kalibrační křivky bylo možné odečíst absolutní množství kopií v analyzovaných vzorcích. Postupně jsme zcela přešli na relativní způsob udávání hodnot exprese za použití $2^{-\Delta\Delta Ct}$ metody (Livak a Schmittgen 2001; Schmittgen a Livak 2008), kdy je exprese analyzovaného genu vztažena k expresi referenčního genu/kombinace referenčních genů. Klíčovou otázkou ale je, jaký referenční gen použít. Odpovím velice jednoduše, ideální univerzální referenční gen neexistuje. Při stanovování genové exprese protein kódujících genů jsme dříve nejčastěji používali glykolytický enzym glyceraldehyd-3-fosfátdehydrogenázu (GAPDH). Ukázalo se, že u nádorů je tento gen často zvýšeně exprimován (Guo et al. 2013) a jeho exprese sama o sobě může mít prognostický význam (Puzone et al. 2013) a tedy asi nebude zrovna vhodným referenčním genem. Používali jsme i hypoxanthin-guanin-fosforibosyltransferázu (HPRT1) a beta-glukuronidázu (GUSB). Volbě referenčních genů se v dnešní době věnuje mnoho prací (Kozera a Rapacz 2013; Chapman a Waldenström 2015; De Spiegelaere et al. 2015).

Ve vhodném referenčním genu pro stanovení exprese mikroRNA panuje poměrně shoda, většina prací používá jako endogenní referenční gen malou jadernou RNA RNU6B, i když také není ideální (Schwarzenbach et al. 2015).

Publikované výsledky, které jsou součástí této práce (Přílohy 1 – 10) a jsou komentovány v následujících podkapitolách, se týkají kolorektálního karcinomu (CRC), karcinomu prostaty, nemalobuněčného karcinomu plic (NSCLC) a jeho histologických podtypů (adenokarcinom a dlaždicobuněčný karcinom) a karcinomů hlavy a krku (HNSCC).

4.1 Relevance of miR-21 and miR-143 expression in tissue samples of colorectal carcinoma and its liver metastases (Příloha 1)

Kulda V, Pesta M, Topolcan O, Liska V, Treska V, Sutnar A, Rupert K, Ludvikova M, Babuska V, Holubec L, Cerny R: **Relevance of miR-21 and miR-143 expression in tissue samples of colorectal carcinoma and its liver metastases.** *Cancer Genet Cytogenet* 2010, 200(2): 154–160.

Jednalo se o první práci týkající se stanovení mikroRNA prováděnou na Lékařské fakultě v Plzni (Kulda et al. 2010). CRC patří celosvětově mezi nejčastější maligní nádory a tak je zároveň i velmi studovaný (Siegel et al. 2018). Cílem byla analýza exprese miR-21 a miR-143 ve vzorcích tkáně kolorektálního karcinomu (CRC) a jeho jaterních metastáz (CLM) a vyhodnocení vztahu naměřených hodnot k prognóze pacientů (bezpříznakové období – DFI, celkové přežití – OS). Novým poznatkem v naší práci bylo, že jsme ukázali na prognostický význam těchto mikroRNA a potenciál použití miR-21 jako biomarkeru.

Zvolili jsme mikroRNA, o kterých již bylo před zahájením naší práce publikováno, že ovlivňují geny účastnící se patogeneze CRC. O miR-21 již bylo známo, že cílí na některé tumor supresorové geny jako tropomyosin 1 (TPM1), programmed cell death 4 (PDCD4), maspin, phosphatase and tensin homologue (PTEN) a reversion-inducing cysteine-rich protein with Kazal motifs (RECK) a tak byl předpoklad, že bude mít funkci onkogenu (Si et al. 2007; Zhang et al. 2008; Zhu et al. 2007, 2008). O miR-143 bylo známo, že cílí na K-ras (Chen et al. 2009) a tak jsme naopak předpokládali její tumor supresorové působení.

Studovaná skupina zahrnovala 46 pacientů s CRC. Ve všech případech se jednalo o adenokarcinomy. Vyšetřovány byly párové vzorky resekované tkáně (nádor – normální střevní sliznice).

Tabulka 4.1 - 1 - Histologické podtypy (N = 46)

Histologie	N (%)
Tubulární	41 (89%)
Mucinózní	3 (6,5%)
Anaplastický	2 (4,5%)

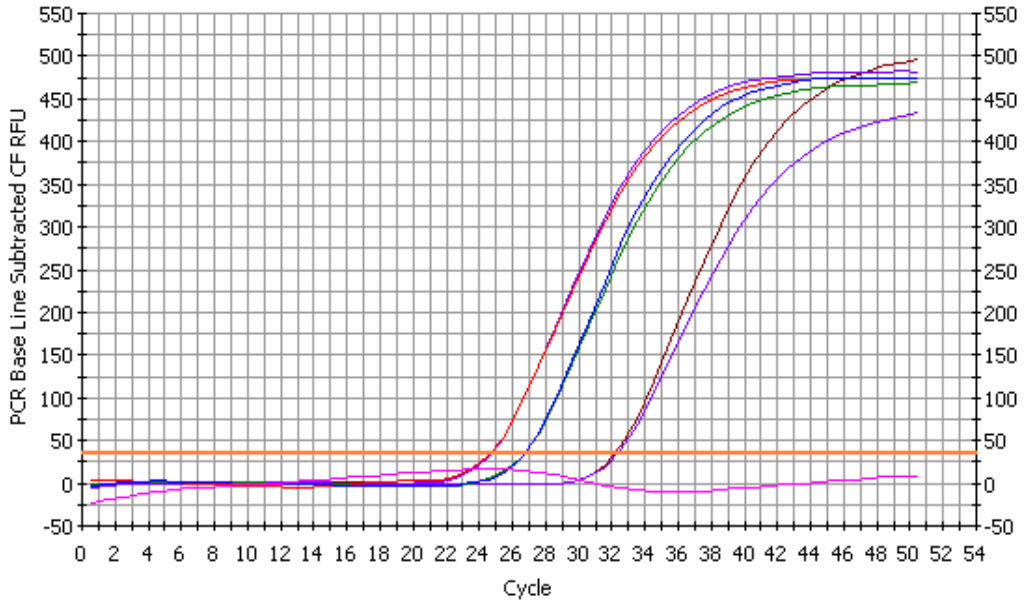
Tabulka 4.1 - 2 - Anatomická lokalizaze tumoru (N = 46)

Lokalizace tumoru	N (%)
Sigmoideum	16 (35%)
Rektum	15 (32,5%)
Levé kolon	3 (6,5%)
Pravé kolon	12 (26%)

Tabulka 4.1 - 3 - Charakteristika pacientů (N = 46)

Skupina			T				N		M		Stage			
			1	2	3	4	0	≥1	0	1	I	II	III	IV
muži	31	n	1	8	19	3	21	10	29	2	6	14	9	2
ženy	15	n	1	3	11	0	12	3	15	0	4	7	4	0
Σ	46	n	2	11	30	3	33	13	44	2	10	21	13	2
	100	%	4%	24%	65%	7%	72%	28%	96%	4%	22%	46%	28%	4%

Celková RNA byla izolována z 50 mg tkáně (fastRNA Pro Green Kit, Q-BIOgene), stanovení miR-21 a miR-143 bylo provedeno kvantitativní RT PCR metodou (TaqMan MicroRNA Assays, Applied Biosystems) na přístroji iCycler (Bio-Rad) v technických duplikátech.



Obrázek 10 - Ukázka záznamu kvantifikace (iCycler, miR-143)

Zabývali jsme se také problematikou normalizace výsledků, v této práci jsme srovnali tři způsoby normalizace, které jsme převzali z jiných publikovaných prací (Choong et al. 2007; Corney et al. 2007; Ng et al. 2009; Peltier a Latham 2008):

- k celkové RNA
- U6RNA (RNU6B)
- miR-191

Získané výsledky byly podobné, avšak ne zcela shodné. Pro prezentaci výsledků jsme se rozhodli využít v té době často využívaný způsob normalizace naměřených hodnot exprese k celkové RNA. Prokázali jsme vyšší expresi miR-21 ($p<0,0001$) a nižší expresi miR-143 ($p<0,0001$) v CRC nádorové tkáni ve srovnání s normální střevní tkání (Wilcoxonův párový test).

Tabulka 4.1 - 4 - Rozdíly v expresi miR-21 mezi nádorovou a normální tkání

Skupina	miR-21				
	N	25%	medián	75%	*P*-value
Kolorektální karcinom	46	6,650	7,165	8,030	**< 0,0001**
Kontrolní střevní tkáň	45	5,650	6,200	6,770	

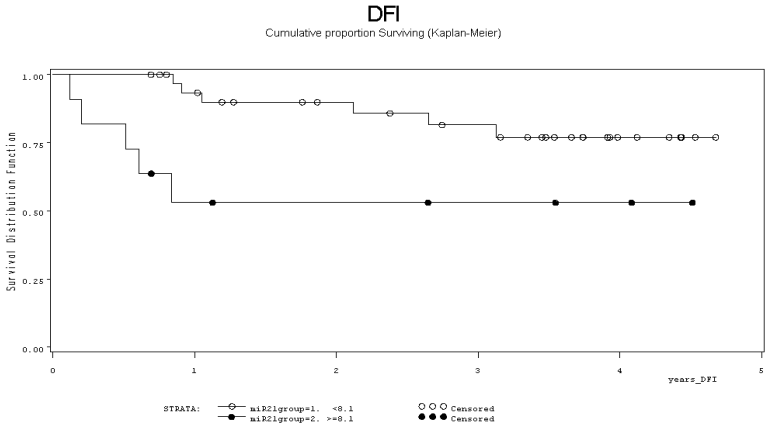
Tabulka 4.1 - 5 - Rozdíly v expresi miR-143 mezi nádorovou a normální tkání

Skupina	miR-143				
	N	25%	medián	75%	*P*-value
Kolorektální karcinom	46	10,250	11,350	12,750	**< 0,0001**
Kontrolní střevní tkáň	44	11,700	14,250	16,000	

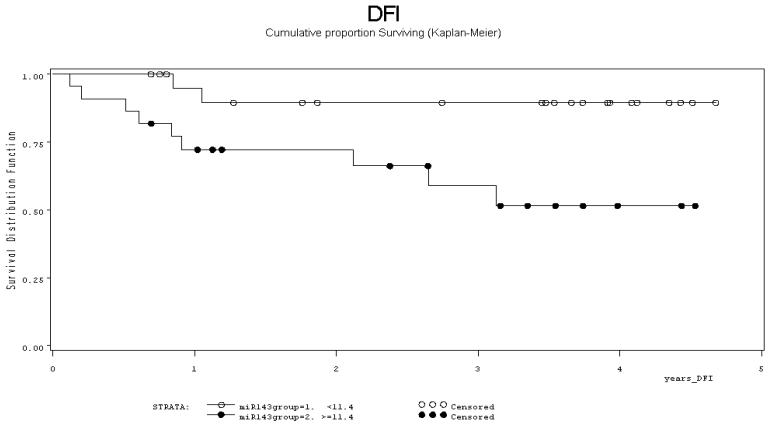
Tyto výsledky jsou v souladu s prací dalších autorů (Bandrés et al. 2006; Slaby et al. 2007). Toto pro nás bylo potvrzením správného metodického přístupu. Hlavním novým přínosem naší práce bylo, že jsme ukázali na prognostický význam těchto mikroRNA. Zaznamenali jsme vztah exprese miR-21 a miR-143 k DFI (Coxův regresní model a Kaplan-Meier analýza).

Tabulka 4.1 - 6 - Vztah exprese miR-21 a miR-143 k DFI (Kaplan-Meier)

miRNA	Hodnoty nad cut off		Cut off	Hodnoty pod cut off		Log-rank *P*-value	Wilcoxon *P*-value
	N	medián DFI (roky)		N	medián DFI (roky)		
miR-21	11	0,84	8,1	33	3,16	0,0145	0,0026
miR-143	22	2,25	11,4	22	3,51	0,0151	0,0191



Obrázek 11 - Kaplan-Meier křivky pro DFI (miR-21)



Obrázek 12 - Kaplan-Meier křivky pro DFI (miR-143)

U pacientů s vyšší expresí miR-21 v nádorové tkáni bylo kratší DFI (Wilcoxon; p=0,0026). Překvapivý výsledek jsme zjistili u vztahu miR-143 k DFI. Přestože se ukazuje, že v nádorové tkáni CRC je celkový efekt miR-143 tumor supresorový (tomu nasvědčují i naše výsledky ukazující nižší expresi v nádorové tkáni), u pacientů s nižší expresí miR-143 bylo nalezeno delší DFI (Wilcoxon; p=0,0191). To může souviset se skutečností, že konkrétní miRNA reguluje stovky genů, mezi kterými jsou onkogeny i tumor supresory, a celkový efekt závisí na „čase a místě“ působení dané mikroRNA.

V kapitole 4.3 je podrobněji rozebráno, k jakému pokroku ve znalostech o miR-21 došlo v průběhu následujících let.

4.2 Importance of miR-20a expression in prostate cancer tissue (Příloha 2)

Pesta M, Klecka J, **Kulda V**, Topolcan O, Hora M, Eret V, Ludvikova M, Babjuk M, Novak K, Stolz J, Holubec L: **Importance of miR-20a expression in prostate cancer tissue.** *Anticancer Res* 2010, 30(9): 3579–3583.

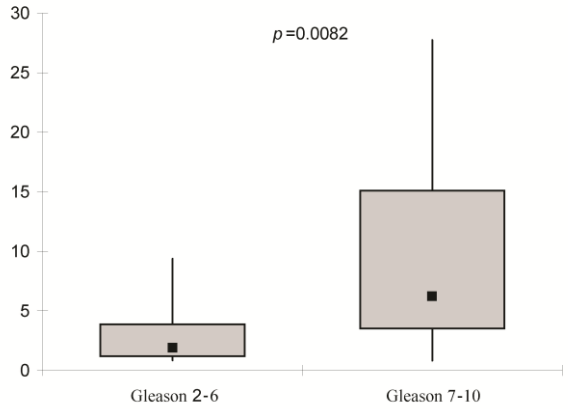
Dalším typem nádoru, u kterého jsme se zabývali deregulací exprese vybraných mikroRNA molekul, byl karcinom prostaty (Pesta et al. 2010). Karcinom prostaty je nejčastěji diagnostikovaným zhoubným nádorem u mužů v rozvinutých zemích, což pravděpodobně souvisí se zavedenými screeningovými testy využívajícími stanovení sérových hladin prostatického specifického antigenu (PSA), které odhalí i klinicky němé nádory (Zhang et al. 2017).

Cílem práce bylo stanovit expresi miR-20a, let-7a, miR-15a a miR-16 ve vzorcích tkáně karcinomu prostaty a benigní hyperplázie prostaty odebrané rutinní biopsií a hledat vztah ke klinickopatologickým chararakteristikám. Východiskem byla publikace vyhodnocující expresi molekul mikroRNA u buněčných linií karcinomu prostaty pomocí microarray technik (Porkka et al. 2007). K výběru mikroRNA, které jsme stanovovali, přispěla i rešerše literatury, kdy jsme vybrali ty mikroRNA, o kterých bylo publikováno, že hrají roli u jiných onkologických onemocnění (Roush a Slack 2008; Johnson et al. 2005; Hayashita et al. 2005; Calin et al. 2008).

Do této studie vstoupilo 138 pacientů, kteří podstoupili biopsii prostaty od května 2006 do září 2008, u všech byla indikací k biopsii zvýšená sérová hladina PSA a pozitivní nález při vyšetření *per rectum*. Histologické vyšetření rozdělilo vzorky na 53 karcinomů a 85 benigních hyperplázií.

Přínosem této práce bylo, že jsme ukázali, že stanovení je možno provést i z velmi malého množství výchozího biologického materiálu (část rutinně odebíraného bioptického vzorku). Celková RNA byla izolována z asi 10 mg tkáně (fastRNA Pro Green Kit, Q-BIOgene). Stanovení miR-20a, let-7a, miR-15a a miR-16 bylo provedeno kvantitativní RT PCR metodou (TaqMan MicroRNA Assays, Applied Biosystems) na přístroji iCycler (Bio-Rad) v technických duplikátech. Hodnoty exprese byly normalizovány k RNU6B za použití $2^{-\Delta\Delta Ct}$ metody.

Novým poznatkem zjištěným v naší práci bylo, že exprese miR-20a je vyšší ve více dediferencovaných karcinomech prostaty dle Gleason skóre, které je rutinně používáno v klinické praxi pro grading karcinomu prostaty (McKenney 2017). Hodnotí se architektonika žlázek podle stupnice s pěti stupni, G1 značí dobře diferencovaný a G5 velmi špatně diferencovaný typ. Výsledná hodnota skóre se získá součtem nejhoršího a nejčastějšího nálezu, tj. může nabývat hodnot 2–10 (Kurfürstová a Král 2013). Ve skupině vzorků hodnocených patologem vyšším Gleason skóre (7–10) byla signifikantně vyšší exprese miR-20a ve srovnání se vzorky s nižším Gleason skóre (2–6).



Obrázek 13 - Rozdíl v expresi miR-20a dle Gleason skóre

Naše závěry ukazující na onkogenní působení miR-20a v tkáni karcinomu prostaty jsou v souladu s výsledky dalších autorů. Sylvestre et al. popsali zvýšenou expresi a pozorovali antiapoptotickou aktivitu miR-20a na buněčné linii PC3 (Sylvestre et al. 2007). Volinia et al. našli zvýšenou expresi miR-20a v tkáni karcinomu prostaty (Volinia et al. 2006). Na naše výsledky navázali v roce 2014 Qiang et al., kteří také ukázali na korelaci exprese miR-20a a Gleason skóre a s tím spojený prognostický význam. Dále prokázali, že miR-20a podporuje invazivitu a migraci buněk karcinomu prostaty cílením tyrosin kinázy ABL2 (Qiang et al. 2014). Hart el al. analýzou mikroRNA expresních profilů získaných sekvenováním a následnou validací RT-qPCR metodou ukázali na zvýšenou expresi miR-20a, miR-148a, miR-200b a miR-375 a sníženou expresi miR-143 a miR-145 v tkáni karcinomu prostaty ve srovnání s normální prostatickou tkání. O miR-20a dále zjistili, že její exprese se zvyšuje s vyšším stádiem onemocnění (Hart et al. 2014).

4.3 Diagnostic and prognostic value of microRNA-21 in colorectal cancer: an original study and individual participant data meta-analysis (Příloha 3)

Zhang H, Li P, Ju H, Pesta M, **Kulda V**, Jin W, Cai M, Liu C, Wu H, Xu J, Ye Y, Zhang G, Xu E, Cai J, Lai M, Xia D, Yang J, Wu Y: **Diagnostic and prognostic value of microRNA-21 in colorectal cancer: an original study and individual participant data meta-analysis.** *Cancer Epidemiol Biomark Prev* 2014, 23(12): 2783–2792.

Po publikování prvních prací na poli mikroRNA biomarkerů vzniklých na našem pracovišti jsme navázali spolupráci, jejímž výsledkem byla metaanalýza publikovaná v roce 2014 v časopise Cancer Epidemiology, Biomarkers & Prevention (Zhang et al. 2014) zabývající se diagnostickou a prognostickou hodnotou miR-21 u pacientů s kolorektálním karcinomem (CRC). Závěrem metaanalýzy je, že cirkulující miR-21 je vhodná pro časnou detekci CRC a hladina exprese miR-21 v nádorové tkáni má prognostický význam.

Dle mého osobního názoru je miR-21 vůbec nejstudovanější mikroRNA v souvislosti s onkologickými onemocněními, o čemž svědčí více než 2000 publikací indexovaných v databázi PubMed nalezených po zadání klíčových slov „miR-21“ a „cancer“ (červenec 2018). Velké naděje jsou vkládány do možného klinického využití miR-21, ať už jako biomarkeru nebo jako terapeutického cíle.

Deregulace miR-21ve smyslu zvýšené exprese není typická jen pro CRC, ale ukazuje se být společným znakem pro různé typy nádorů, ať už je to karcinom prsu (Adhami et al. 2018), karcinomy plic (Arab et al. 2017), gliomy (Qu et al. 2016), karcinom žaludku (Simonian et al. 2018), karcinom pankreatu (Wald et al. 2017) nebo renální karcinom (Lokeshwar et al. 2018). Metaanalýza hodnotící exosomální cirkulující miR-21 jako potenciální všeobecný marker pro časnou detekci nádorových onemocnění udává senzitivitu 75 % a specificitu 85 % (Shi 2016), což staví miR-21 do pozice kandidáta na součást panelu screeningových vyšetření pro záchyt onkologických onemocnění.

Výzkum probíhá i na poli využití strategie snižovat hladinu onkogenní miR-21 jako součást léčby zhoubných nádorů (Javanmardi et al. 2017), zatím převážně na nádorových buněčných liniích (Nedaeinia et al. 2016; Wagenaar et al. 2015), ale paralelně probíhá i výzkum, jakým způsobem molekuly blokující funkci specifických mikroRNA podávat a jak kombinovat „anti-miRNA molekuly“ s jinými modalitami terapie (Hu et al. 2017; Lee et al. 2017).

4.4 MicroRNA profile in site-specific head and neck squamous cell cancer (Příloha 4)

Kalfert D, Pesta M, **Kulda V**, Topolcan O, Ryska A, Celakovsky P, Laco J, Ludvikova M: **MicroRNA profile in site-specific head and neck squamous cell cancer.** *Anticancer Res* 2015, 35(4): 2455–2463.

Cílem této práce (Kalfert et al. 2015) bylo stanovení exprese let-7a, miR-21, miR-34a, miR-200c a miR-375 ve vzorcích tkáně dlaždicových karcinomů hlavy a krku (HNSCC, head and neck squamous cell carcinoma) a vyhodnocení vztahu ke klinickopatologickým vlastnostem a lokalitě nádoru. HNSCC zahrnuje širokou skupinu nádorů od dutiny ústní po larynx, incidence je velmi závislá na geografické oblasti, celosvětově představuje asi 6 % maligních nádorů (Jou a Hess 2017). Kouření, konzumace alkoholu a papilomavirová infekce (HPV) jsou prokázanými etiologickými faktory (Ramqvist a Dalianis 2011). Studovaná skupina zahrnovala 51 pacientů s HNSCC (41 mužů, 10 žen).

Tabulka 4.4 - 1 - Charakteristika pacientů (N = 51)

Lokalizace tumoru	**N**	**%**
larynx	24	47 %
orofarynx	23	45 %
hypofarynx	4	8 %
Kouření	**N**	**%**
ano	38	74,5 %
ne	13	25,5 %
Exprese p16 (marker HPV infekce)	**N**	**%**
negativní	34	66,7 %
pozitivní	17	33,3 %

Vyšetřovány byly vždy párové vzorky (nádor – normální tkáň). RNA byla izolována z formalinem fixovaných parafinových bločků (FFPE, formalin-fixed paraffin-embedded). Stanovení exprese výše uvedených mikroRNA bylo provedeno RT real-time qPCR metodou (TaqMan MicroRNA Assays, Applied Biosystems). Pro normalizaci výsledků byla použita exprese RNU6B.

Ve srovnání s nenádorovou tkání byla v nádorové tkáni bez ohledu na lokalizaci tumoru prokázána vyšší exprese miR-21 a miR-200c, naopak nižší exprese miR-375.

Tabulka 4.4 - 2 - Rozdíly v expresi: nádor – kontrolní zdravá tkáň

Skupina	N	miR-21				miR-200c				miR-375			
		25%	medián	75%	*P*-value	25%	medián	75%	*P*-value	25%	medián	75%	*P*-value
HNSCC	51	6,62	20,11	52,53	**< 0,0001**	4,78	7,56	13,09	**0,0335**	0,02	0,06	0,29	**0,0020**
kontrolní tkáň	51	2,10	5,99	20,75		2,13	4,62	9,60		0,09	0,53	1,76	

Byly nalezeny statisticky signifikantní rozdíly v expresi let-7a, miR-200c, miR-34a dle lokality nádoru (orofaryngeální vs. laryngeální karcinomy).

Tabulka 4.4 - 3 - Rozdíly v expresi dle lokality nádoru

Skupina	N	let-7a				miR-200c				miR-34a			
		25%	medián	75%	*P*-value	25%	medián	75%	*P*-value	25%	medián	75%	*P*-value
orofarynx	23	1,96	3,40	5,37	**0,0242**	5,98	9,09	15,48	**0,0378**	0,64	1,01	1,64	**0,0178**
larynx	24	0,84	1,72	3,05		4,35	6,75	8,75		**0,19**	**0,43**	**0,90**	

Výsledky podporují hypotézu o onkogenní funkci miR-21 a ukazují, že stejný histologický typ nádoru v různých lokalitách může mít odlišné molekulárně biologické pozadí.

Exprese miR-34a signifikantně korelovala s orofaryngeálním původem a p16 pozitivitou jako markerem HPV infekce. Není překvapivé, že miR-34a je deregulovaná i u dalšího typu nádoru, kde etiologickým faktorem je HPV infekce, a to u karcinomu děložního hrdla (Ribeiro et al. 2015; Zhu et al. 2018; Pardini et al. 2018).

Dnes už je známo, že vztah mezi deregulovanou expresí miR-34a a HPV infekcí je zprostředkován osou: virový onkoprotein E6 → protein p53 → miR-34a (Chen a Zhao 2015). Onkoprotein E6 se váže na tumor supresorový protein p53 a tím inhibuje jeho funkci transkripčního faktoru aktivujícího mimo jiné promotor genu kódujícího miR-34a. Převažující působení miR-34a je tumor supresorové, cílí např. na antiapoptotický gen Bcl-2 (Li et al. 2013) a geny zodpovědné za proliferaci jako jsou např. cyklin D1 a cyklin dependentní kinázu 6 (Sun et al. 2008).

4.5 Tissue microRNAs as predictive markers for gastric cancer patients undergoing palliative chemotherapy (Příloha 5)

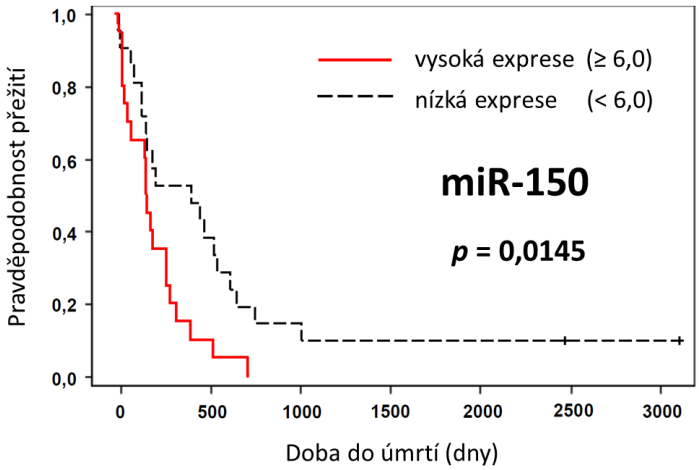
Smid D, **Kulda V**, Srbecka K, Kubackova D, Dolezal J, Daum O, Kucera R, Topolcan O, Treska V, Skalicky T, Pesta M: **Tissue microRNAs as predictive markers for gastric cancer patients undergoing palliative chemotherapy.** *Int J Oncol* 2016, 48(6): 2693–2703.

Cílem této studie (Smid et al. 2016) bylo nalezení mikroRNA, které by mohly predikovat efekt paliativní chemoterapie založené na 5-fluorouracilu (5-FU) u pacientů s karcinomem žaludku v pokročilém stádiu. Práce probíhala v rámci grantu IGA MZ „Stanovení prediktivních faktorů pro léčebný efekt chemoterapie u nemocných s karcinomem žaludku“ (2013-2015, NT14227).

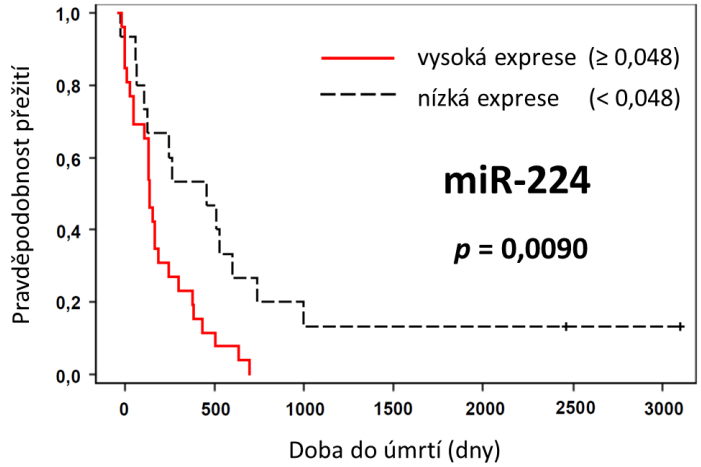
Karcinom žaludku patří celosvětově k nejčastějším maligním nádorům (Torre et al. 2015). Přestože v Evropě dochází dlouhodobě ke snížení výskytu, prognóza zůstává stále nepříznivá. K záchytu nádorů žaludku dochází totiž často až v pokročilých stádiích onemocnění, kdy radikální chirurgická operace není možná a indikována je pouze paliativní chemoterapie, jejíž účinnost v individuálních případech je velice variabilní a často selhává (Waddell et al. 2014). Zdá se, že odpověď na léčbu závisí mimo jiné na míře exprese určitých mikroRNA. Analýza těchto mikroRNA by mohla predikovat efekt chemoterapie, tedy odlišit nemocné, kteří budou mít z podané chemoterapie prospěch, od těch, kterým onkologická léčba ve výsledku pouze zhorší kvalitu života. Jinými slovy řečeno, postupovat podle přístupů personalizované medicíny (Cidon et al. 2013).

Jednalo se o retrospektivní studii zahrnující 54 pacientů (30 mužů, 24 žen) s karcinomem žaludku ve čtvrtém stádiu, kteří nepodstoupili resekci a léčeni byli pouze chemoterapeutickým režimem obsahujícím 5-FU. Vyšetřovaným biologickým materiálem byly gastroskopicky odebrané bioptické vzorky nádorové tkáně, RNA byla izolována z FFPE tkáně. Stanovení exprese vybraných 29 mikroRNA bylo provedeno kvantitativní RT PCR metodou (TaqMan MicroRNA Assays, Applied Biosystems) s normalizací na RNU6B. MikroRNA pro stanovení jsme vybrali na základě rešerše literatury, zvolili jsme ty, u nichž byla popsaná deregulace u karcinomu žaludku (Katada et al. 2009; Yao et al. 2009; Chen et al. 2014; Wang et al. 2013; Yan et al. 2014), a ty, u kterých bylo možné očekávat vliv na mechanismus účinku 5-FU (Boni et al. 2010; Amankwatia et al. 2015).

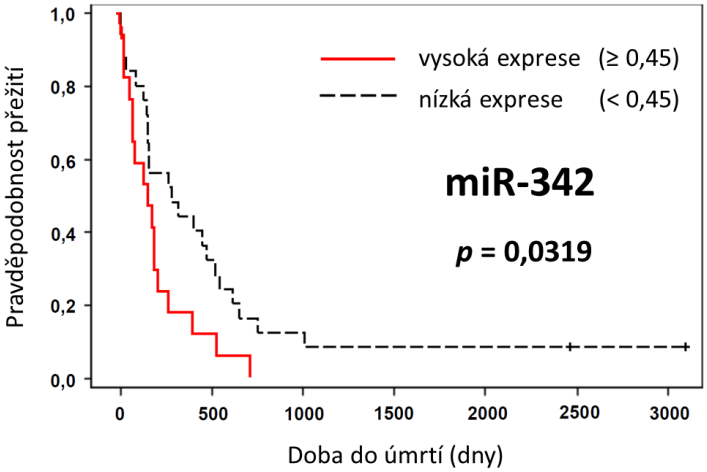
Vztah hladin exprese k celkovému přežití pacientů byl hodnocen nejprve pomocí Coxova regresního modelu. Coxův model ukázal, že zvýšená exprese miR-150, miR-181b, miR-192, miR-221, miR-224, miR-342, miR-375 a snížená exprese miR-520h byly spojeny s horším osudem pacientů. Pro tyto mikroRNA byly hledány optimální cut-off hodnoty a vztah k přežívání pacientů byl analyzován Kaplan Meierovou metodou. Jako nejzajímavější výsledek zde uvedu vztah vyšší exprese miR-150 ($p=0,0145$), miR-224 ($p=0,0090$) a miR-342 ($p=0,0319$) ke kratšímu OS.



Obrázek 14 - Kaplan-Meier křivky pro OS (miR-150)



Obrázek 15 - Kaplan-Meier křivky pro OS (miR-224)



Obrázek 16 - Kaplan-Meier křivky pro OS (miR-342)

4.6 Predictive relevance of miR-34a, miR-224 and miR-342 in patients with advanced squamous cell carcinoma of the lung undergoing palliative chemotherapy (Příloha 6)

Kulda V, Svaton M, Mukensnabl P, Hrda K, Dvorak P, Houdek Z, Houfkova K, Vrzakova R, Babuska V, Pesek M, Pesta M: **Predictive relevance of miR-34a, miR-224 and miR-342 in patients with advanced squamous cell carcinoma of the lung undergoing palliative chemotherapy.** *Oncol Lett* 2018, 15(1): 592–599.

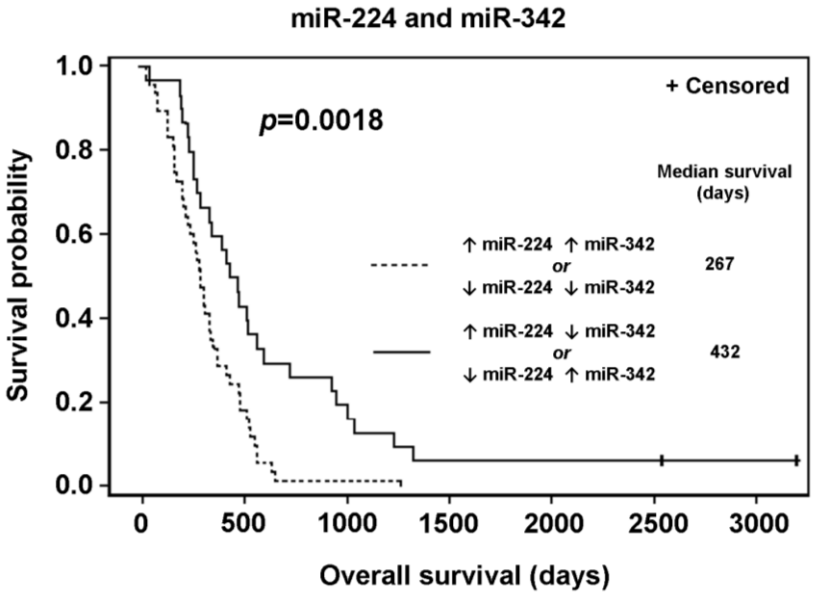
Cílem této práce (Kulda et al. 2018) bylo stanovení prediktivního významu mikroRNA molekul u pacientů s pokročilým nemalobuněčným karcinomem plic, kteří podstoupili paliativní chemoterapii. Zaznamenali jsme vztah miR-34a, miR-224 a miR-342 k celkovému přežití (OS). Hlubší analýza odhalila překvapivý výsledek, že pacienti s vysokou expresí zároveň obou miR-224 a miR-342 mají podobné celkové přežití jako ti s nízkou expresí obou těchto mikroRNA, a to signifikantně kratší než u těch, kteří mají vysokou expresi jedné z těchto mikroRNA a současně nízkou expresi druhé. Ukazuje se, že síť interakcí mezi molekulami mikroRNA a jejich cílovými transkripty je velice komplexní (ceRNA hypotéza).

Přibližně 85 % karcinomů plic tvoří nemalobuněčný karcinom plic (NSCLC), který má dva hlavní histologické podtypy, epidermoidní (dlaždicobuněčný) karcinom (SCC, squamous cell carcinoma) a adenokarcinom (Travis 2011). Chemoterapie je základní modalitou paliativní léčby pokročilých neoperovatelných karcinomů plic. Odpověď na chemoterapii se ale velmi liší pacient od pacienta, proto jsou hledány biomarkery, které by umožnily předpovědět efekt léčby. MikroRNA mají pro svou patofyziologickou roli a stabilitu v biologických vzorcích potenciál stát se cennými prediktivními markery. Vzorky bioptické tkáně tvoří vhodný materiál pro profilování mikroRNA s cílem předpovědět účinek paliativní chemoterapie.

Jednalo se o retrospektivní studii, soubor zahrnoval 81 pacientů (74 mužů, 7 žen) s pokročilým stádiem (3B, 4) epidermoidního karcinomu plic léčených paliativní chemoterapií, která byla založena na platinovém derivátu v kombinaci s paclitaxelem nebo gemcitabinem. Všichni pacienti byli buď kuřáci nebo bývalí kuřáci. Exprese 17 vybraných mikroRNA byla měřena pomocí real-time RT-qPCR v nádorové tkáni makrodisekované z FFPE bioptických vzorků pomocí TaqMan® MicroRNA Assays (Applied Biosystems, Foster City, CA, USA) v technických duplikátech na přístroji Stratagene Mx3005P (Agilent Technologies, Santa Clara, CA, USA). Hodnoty exprese byly normalizovány k RNU6B.

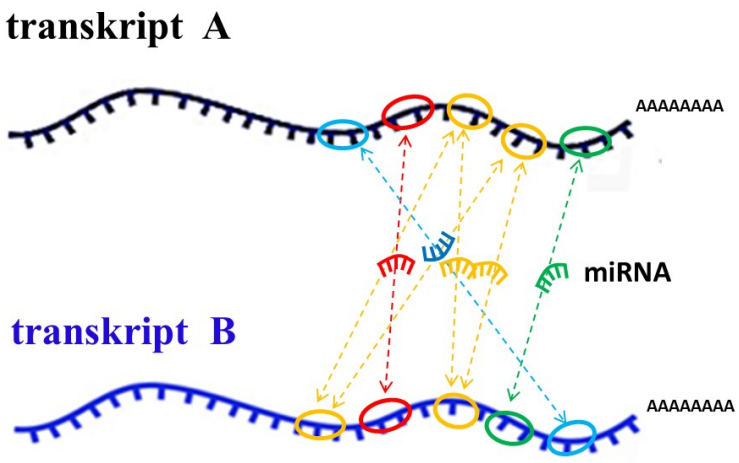
Coxův regresní model ukázal na vztah nízké hladiny miR-342 a vysoké hladiny miR-34a a miR-224 ke kratšímu OS v podskupině kuřáků. Recenzentem jsme byli vyzváni, abychom vyhodnotili i vztah *kombinací* jednotlivých markerů k OS. Nečekaný výsledek jsme zaznamenali u kombinace miR-224 a miR-342. Ukázalo se, že s lepší prognózou je spojen

vzor, kdy právě jedna z dvojice má vyšší expresi a druhá z dvojice nízkou. Signifikantně kratší OS bylo spojeno s vysokou expresí obou mikroRNA nebo naopak s nízkou expresí obou mikroRNA.



Obrázek 17 - Kaplan-Meier křivky pro OS (kombinace miR-224 a miR-342)

Ukázali jsme, že výsledek stanovení jedné jediné mikroRNA nemusí být postačující bez znalosti exprese dalších hráčů na poli mikroRNA – RNA interakcí. Vysoká hladina jedné konkrétní mikroRNA může být totiž za jistých okolností spojena s nepříznivou prognózou, za jiných okolností naopak s prognózou velmi dobrou. Tento výsledek podle našeho názoru naznačuje, že pro případné klinické použití molekul mikroRNA jako biomarkerů bude potřeba stanovení profilu více molekul mikroRNA, které mají význam pro danou klinickou otázku.



Obrázek 18 - ceRNA hypotéza

Komplexní síť vztahů mikroRNA – cílové RNA molekuly formuloval Pandolfi a jeho tým v tzv. ceRNA (competing endogenous RNA) hypotéze (Salmena et al. 2011). Vedlo ho k tomu zjištění, že i transkribované pseudogeny, přestože nevedou k tvorbě funkčního proteinu, mohou zasahovat do kancerogeneze tím, že sdílí s jinými transkripty místa, na která se vážou specifické mikroRNA molekuly, čímž se omezí jejich dostupnost pro regulaci hladiny jiných transkriptů (Poliseno et al. 2010). Jedna mikroRNA může cílit na stovky různých transkriptů, v případě omezeného poolu dané mikroRNA spolu jednotlivé transkripty (ceRNA molekuly) soutěží o tuto mikroRNA. Jako ceRNA molekuly působící v těchto sítích mohou vystupovat mRNA, transkripty pseudogenů, long non-coding RNA (lncRNA), cirkulární RNA (circRNA), obecně snad libovolné transkripty. MikroRNA jsou v ceRNA hypotéze slova, pomocí kterých tyto transkripty spolu komunikují.

Pandolfiho hypotéza je potvrzována a využívána mnohými autory (Yang et al. 2016). Bylo např. zjištěno, že taurine-upregulated gene 1 (TUG1) z kategorie lncRNA působí jako onkogen v patogenezi karcinomu prostaty tím, že jako ceRNA negativně reguluje hladinu miR-26a (Yang et al. 2018). Vznikají práce, které analýzou expresních profilů a bioinformatickými přístupy mapují interakce nastíněné ceRNA hypotézou (Jin et al. 2017; Tian et al. 2018).

4.7 Vliv exprese vybraných protein kódujících genů a mikroRNA na riziko relapsu plicních adenokarcinomů stadia 1 (Příloha 7)

Svaton M, **Kulda V**, Mukensnabl P, Topolcan O, Pesek M, Dvorak P, Fiala O, Rousarova M, Hrda K, Pesta M: **Vliv exprese vybraných protein kódujících genů a mikroRNA na riziko relapsu plicních adenokarcinomů stadia 1.** *Studia pneumologica et phtiseologica* 2017, 77(3): 93-103.

Tuto práci (Svaton et al. 2017) publikovanou v českém recenzovaném časopise uvádím jako příklad studie, kde byla na začátku jasně položená jednoduchá klinická otázka a tomu přesně odpovídal pečlivě sestavený homogenní soubor pacientů. Šlo o pacienty s jedinou histologickou podskupinou nemalobuněčného karcinomu plic (NSCLC) – adenokarcinomem. Jednalo se jen o časná stádia (1A a 1B) léčená radikální chirurgií bez podané adjuvantní chemoterapie. Cílem bylo posoudit vztah exprese vybraných protein kódujících genů a mikroRNA k prognóze a pokusit se tak najít možný marker rizika recidivy onemocnění, který by pomohl identifikovat paciety s nepříznivou prognózou, kteří by mohli mít prospěch z podání adjuvantní chemoterapie. U žádné z celkového panelu stanovených mRNA a mikroRNA jsme ale neprokázali statisticky významný vztah mezi jejich expresí a prognózou.

Adjuvantní chemoterapie je součástí léčby radikálně operovaných pacientů s plicními karcinomy stádií 2 a 3 (Péchoux et al. 2013). Pro stádium 1 nebyl pro celkovou populaci pacientů přínos adjuvantní chemoterapie prokázán (Buffoni et al. 2016), ale je známo, že i zde není riziko recidivy onemocnění po chirurgickém odstranění nádoru zanedbatelné (Liu et al. 2015).

Naším cílem bylo posoudit vztah exprese vybraných DNA opravných genů (ERCC1, BRCA1), ABC transportérů (ABCC1, ABCC10, ABCG2, ATP7B, SLC22A1, SLC29A1) a mikroRNA (miR-15b, miR-21, miR-27a, miR34a, miR-99a, miR-106a, miR-107, miR-143, miR-150, miR-192, miR-211, miR-218, miR-221, miR-224, miR-342 a miR-375) k době bez recidivy onemocnění (DFI) a celkovému přežití (OS) u pacientů s plicními adenokarcinomy nízkých stádií a pokusit se tak najít možný marker rizika recidivy onemocnění.

Jednalo se o retrospektivní studii, soubor zahrnoval 42 pacientů (31 mužů a 11 žen), kteří podstoupili radikální operační výkon v letech 2003-2011 pro plicní adenokarcinom stádií 1A nebo 1B bez adjuvantně podávané chemoterapie. V průběhu sledování se recidiva objevila u 19 pacientů. Bioptické vzorky byly odebrány během chirurgické resekce tumoru a zpracovány standardními laboratorními metodami na Šiklově ústavu patologie Fakultní nemocnice Plzeň. FFPE bločky byly skladovány při pokojové teplotě do doby analýzy. Celková RNA byla izolována soupravou miRNeasy FFPE Kit (Qiagen, Hilden, Germany) z 15 μm širokých řezů, a to jen z částí, na kterých patolog vyznačil nádorovou tkáň.

Kvantitativní stanovení exprese mRNA vybraných genů bylo provedeno pomocí real-time RT-qPCR s užitím Universal Probe Library (UPL) sond (Roche, Mannheim, Germany) v technických duplikátech na přístroji Stratagene Mx3005P (Agilent Technologies, Santa Clara, CA, USA). Hodnoty exprese byly normalizovány k celkové RNA a k expresi glyceraldehyd-3fosfátdehydrogenázy (GAPDH). Stanovení exprese mikroRNA bylo provedeno pomocí TaqMan® MicroRNA Assays (Applied Biosystems, Foster City, CA, USA) v technických duplikátech na přístroji Stratagene Mx3005P (Agilent Technologies, Santa Clara, CA, USA), normalizováno k expresi RNU6B.

Na celkovém souboru pacientů jsme neprokázali žádný statisticky významný vztah mezi expresí stanovovaných mRNA/mikroRNA a DFI/OS. Jediným statisticky signifikantním nálezem byl vztah mezi hladinou mRNA BRCA1 a OS u podskupiny kuřáků/exkuřáků. BRCA1 (breast cancer 1) se řadí mezi tumor supresorové geny, podílí se na opravách dvojitých zlomů DNA procesem homologní rekombinace (Roy et al. 2011; Prakash et al. 2015). U NSCLC už byl dříve popsán prognostický význam exprese BRCA1, kdy vysoká exprese BRCA1 znamenala kratší OS (Rosell et al. 2007), k podobným závěrům vedou i novější publikace (Lafuente-Sanchis et al. 2016).

4.8 MikroRNA u karcinomu prostaty (Příloha 8)

Pesta M, **Kulda V**: **MikroRNA u karcinomu prostaty.** In: Slabý O, Svoboda M: MikroRNA v onkologii. Galén, Praha, 2012, ISBN 978-80-7262-587-1, str. 169–182.

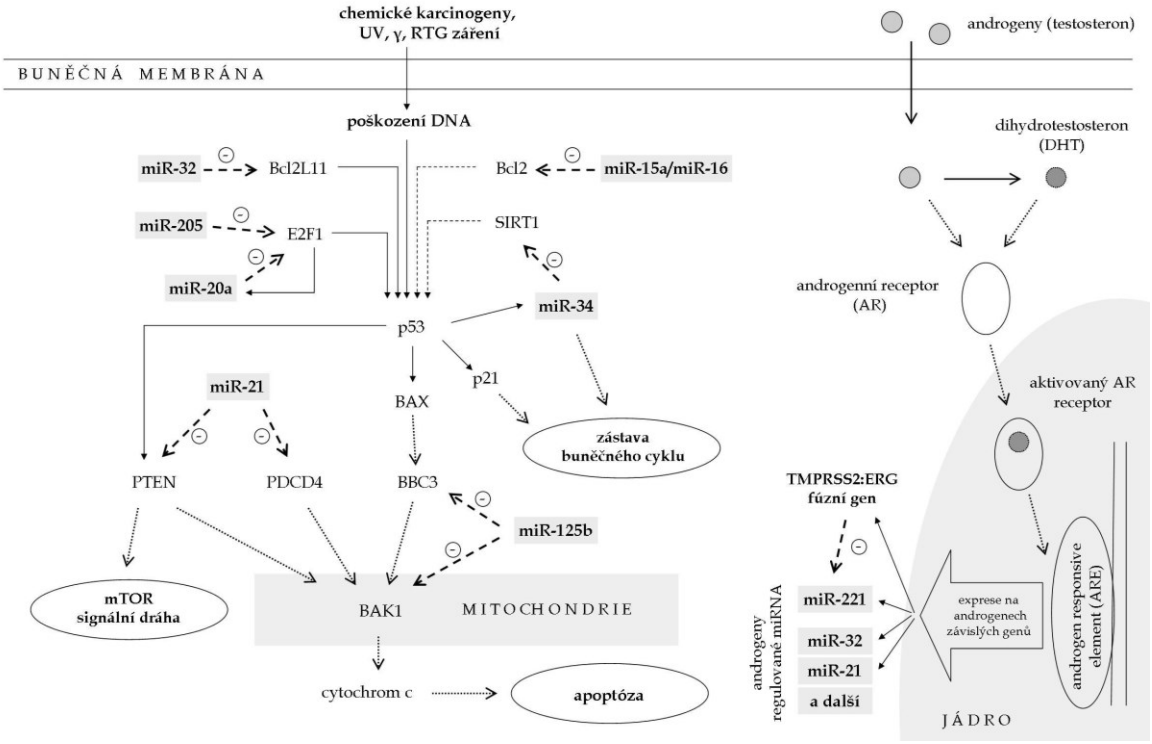
Lékařská fakulta v Plzni patřila kolem roku 2010 k několika málo pracovištím v České republice, kde byl tým pracovníků s praktickými zkušenostmi s výzkumem mikroRNA u onkologických onemocnění s poznatky publikovanými v mezinárodních odborných časopisech (Kulda et al. 2010; Pesta et al. 2010). Byli jsme požádáni, abychom přispěli do v té době vznikající monografie *MikroRNA v onkologii* (Slabý a Svoboda 2012) kapitolou pojednávající o mikroRNA v nádorové biologii karcinomu prostaty.

Karcinom prostaty patří celosvětově mezi nejčastější maligní nádory diagnostikované u mužů. Přestože patogeneze není na molekulární úrovni tak přesně zmapována jako např. patogeneze kolorektálního karcinomu, v posledních letech se naše znalosti v této oblasti značně prohloubily. Kromě mapování genetických změn jsou intenzivně studovány i změny epigenetické, které také přispívají k iniciaci a progresi tohoto onemocnění. V současnosti jsou intenzivně zkoumány tři typy epigenetických změn, a to methylace DNA, remodelace chromatinu a regulace genové exprese molekulami mikroRNA. Nynější model patogeneze karcinomu prostaty předpokládá postupnou kumulaci změn ve tkáni prostaty zahrnující ztrátu exprese genu HPC1, změny týkající se genu pro androgenní receptor (AR) a dalších (snížení exprese tumor supresorových genů PTEN a NKX3.1 a ke zvýšení exprese onkogenu cMYC). Výsledkem je změna normální tkáně prostaty ve tkáň označovanou jako prostatická intraepiteliální neoplázie (PIN). Průběh onemocnění je zásadně ovlivněn vznikem fúzního genu TMPRSS2-ERG v lézích typu PIN a dále vznikem androgen nezávislého růstu nádoru. K výše uvedeným genetickým změnám přispívají také změny epigenetické. Pozornost se soustřeďuje na roli mikroRNA ve vztahu ke schopnosti nádorových buněk zabránit apoptóze, vést k deregulaci kontroly proliferace a dále na ovlivnění androgenní signalizace. S rostoucím porozuměním zapojení molekul mikroRNA v patogenezi karcinomu prostaty se zkoumá možnost jejich využití jako biomarkerů a také jako cílů protinádorové léčby.

Kapitola v knize shrnuje poznatky platné v době jejího vzniku, je členěna do podkapitol:

- MikroRNA a apoptóza u karcinomu prostaty
- MikroRNA a androgenní signalizace
- Vybrané mikroRNA se vztahem ke karcinomu prostaty (miR-21, miR-15a/miR-16, miR-20a, miR-32, miR-34, miR-200, miR-221/miR-222, miR-125b)
- MikroRNA a léčba
- Využití molekul mikroRNA jako biomarkerů u karcinomu prostaty

Pro knihu jsme vytvořili obrázek znázorňující roli různých mikroRNA v patogenezi karcinomu prostaty vycházející z přehledového článku (Catto et al. 2011).



Obrázek 19 - Vztah mikroRNA molekul k apoptóze a androgenní signalizaci v patogenezi karcinomu prostaty

Levá část obrázku
Zabránění apoptóze je klíčová událost v karcinogenezi. Řada mikroRNA působí zásahem do buněčných signálních drah přímo souvisejících s buněčným cyklem a apoptózou. miR-125b inhibuje proapoptotické geny BAK1 a BBC3. miR-21 cílí na tumor supresorové geny se vztahem k apoptóze PTEN a PDCD4. Některé mikroRNA mají vztah k proteinu p53. Prostřednictvím miR-34 je vykonávána alespoň část efektů p53 vedoucích k zástavě buněčného cyklu. Klastr miR-15a/miR-16 indukuje apoptózu represí antiapoptotického proteinu Bcl-2. miR-32 brání apoptóze inhibicí exprese proapoptoticky působícího BCL2L11. miR-20a a miR-205 jsou zapojeny do řízení hladiny transkripčních faktorů rodiny E2F.

Pravá část obrázku
Androgeny prostupují buněčnou membránou dovnitř buňky. Účinkem enzymu 5-alfa-reduktázy se mění testosteron na mnohem více účinný dihydrotestosteron (DHT). Jak testosteron, tak DHT se vážou na androgenní receptor (AR), který vazbou androgenů mění konformaci a dimerizuje. Aktivovaný androgenní receptor působí jako specifický transkripční faktor, který se váže na sekvenci DNA nazývanou androgen responsivní element (ARE), a tak podporuje transkripci androgeny řízených genů, mezi něž patří mimo jiné i některé geny pro miRNA (miR-21, miR-32, miR-221) a v patogenezi karcinomu prostaty důležitý fúzní gen TMPSSS2-ERG.

4.9 Significance of methylation status and the expression of RECK mRNA in lung tissue of patients with NSCLC (Příloha 9)

Pesta M, **Kulda V**, Topolcan O, Safranek J, Vrzalova J, Cerny R, Holubec L: **Significance of methylation status and the expression of RECK mRNA in lung tissue of patients with NSCLC.** *Anticancer Res* 2009, 29(11): 4535–4539.

V této práci (Pesta et al. 2009) jsme se zaměřili na vztah mezi methylací promotoru genu pro RECK (reversion-inducing cysteine-rich protein with Kazal motifs), expresí mRNA RECK genu, bezpříznakovým obdobím (DFI) a celkovým přežitím (OS) u pacientů s nemalobuněčným plicním karcinomem (NSCLC).

RECK je mebránově vázaný glykoprotein, který negativně reguluje aktivitu matrixových metaloproteináz (MMP) a bylo o něm před začátkem naší práce publikováno, že má tumor supresorové působení (Eisenberg et al. 2002; Noda et al. 2003). MMP jsou enzymy degradující komponenty extracelulární matrix (ECM), tím se podílejí na růstu, šíření a metastazování nádorů (Egeblad a Werb 2002; Alaseem et al. 2017). Ukázalo se, že MMP působí i na jiné substráty než jen na komponenty ECM (kolageny, elastin, želatin), mohou specificky působit i na některé receptory nebo cytokiny (Van Lint a Libert 2007; Schlage a auf dem Keller 2015) a tak zasahovat do regulací všech možných buněčných procesů. Výzkum MMP a jejich přirozených inhibitorů, tkáňových inhibitorů metaloproteináz (TIMPs, tissue inhibitors of metalloproteinases) probíhal také na našem pracovišti (Pesta et al. 2005; Sutnar et al. 2007; Safranek et al. 2007, 2009; Pesta et al. 2011), to byl také jeden z důvodů, proč jsme se rozhodli zabývat dalším hráčem inhibujícím aktivitu MMP, proteinem RECK.

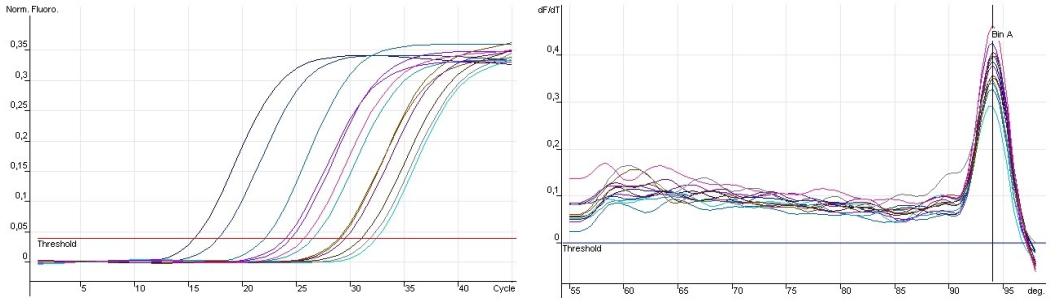
O regulaci exprese RECK bylo publikováno, že se na ní podílejí epigenetické mechanismy jako acetylace histonů (Chang et al. 2004) a methylace promotoru tohoto genu (Cho et al. 2007; Chang et al. 2007).

Methylační statut promotoru a hladina exprese mRNA byly stanovovány v párových vzorcích tkáně (nádor – normální plicní tkáň) 50 pacientů (33 mužů, 17 žen) s NSCLC operovaných v letech 2005–2007.

Tabulka 4.9 - 1 - Histologické podtypy NSCLC (N = 50)

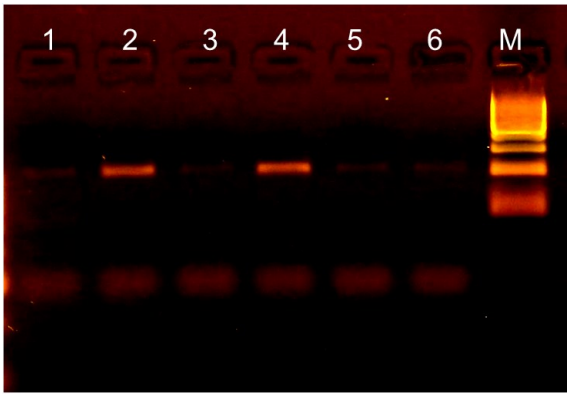
Histologie	N (%)
Epidermoidní karcinom	25 (50%)
Adenokarcinom	19 (38%)
Ostatní	6 (12%)

Exprese RECK mRNA byla stanovena pomocí real-time RT-qPCR na přístroji Rotor-Gene 2000 (Corbett Research, Australia) s použitím nespecifického interkalačního barviva SYBR Green I. Výsledky jsou udávány jako relativní normalizované k expresi glyceraldehyd-3-fosfátdehydrogenázy (GAPDH).



Obrázek 20 - Ukázka záznamu kvantifikace RECK mRNA (Rotor-Gene, SYBR Green I), ověření specificity analýzou křivky tání

Methylační statut byl analyzován methylačně specifickou PCR (Shames et al. 2007). Analyzovaným materiálem je v tomto případě DNA. Principem je konverze nemethylovaného cytosinu na uracil (bisulfitová modifikace), methylovaný cytosin zůstává nezměněn. Následně se provede PCR se specifickými primery pro nemethylovanou sekvenci (cytosin → uracil) a se specifickými primery pro methylovanou sekvenci (cytosin zůstává). Vyhodnocení jsme prováděli elektroforeticky.



Obrázek 21 - Ukázka elektroforetického vyhodnocení methylačně specifické PCR (primery pro methylovanou sekvenci, methylace promotoru je přítomna ve vzorcích 2 a 4)

Zaznamenali jsme nižší expresi mRNA genu RECK v nádorové tkáni ve srovnání s normální plicní tkání (p=0,0032). V histologické podskupině epidermoidního karcinomu jsme nalezli nižší expresi RECK ve srovnání s adenokarcinomem (p=0,0051). Dále jsme prokázali, že existují rozdíly v expresi podle stádia nemoci. Vyšší exprese byla nalezena u stádia IA ve srovnání se stádii IB-IIIA (p=0,0455).

Tabulka 4.9 - 2 - Rozdíly v expresi RECK mRNA mezi nádorovou a kontrolní tkání

N	tkáň	exprese RECK/GAPDH (medián)	Wilcoxon
50	NSCLC	0,0397797	**p=0,0032**
50	kontrolní tkáň	0,1102843	

Tabulka 4.9 - 3 - Rozdíly v expresi RECK mRNA mezi histologickými podtypy NSCLC

N	tkáň	exprese RECK/GAPDH (medián)	Median Two-Sample Test
19	epidermoidní karcinom	0,0297737	**p=0,0051**
25	adenokarcinom	0,1138185	

Tabulka 4.9 - 4 - Rozdíly v expresi RECK mRNA mezi histologickými podtypy NSCLC

N	stádium	exprese RECK/GAPDH (medián)	Median Two-Sample Test
11	IA	0,0543656	**p=0,0455**
39	IB-IIIA	0,0338051	

Zjistili jsme, že u vzorků s methylovaným promotorem genu pro RECK je signifikantně nižší exprese mRNA tohoto genu (p=0,0400). Nezjistili jsme žádný vztah methylace promotoru a exprese genu RECK ke klinickopatologickým hodnotám (DFI, OS).

Tabulka 4.9 - 5 - Rozdíly v expresi RECK mRNA dle methylace promotoru

N	methylace promotoru genu pro RECK	exprese RECK/GAPDH (medián)	Median Two-Sample Test
24	ANO	0,0283514	**p=0,0400**
24	NE	0,0560349	

Methylace promotoru genu RECK snižuje expresi mRNA tohoto genu a je tudíž jedním z jeho regulačních mechanismů. Naše výsledky potvrzují, že RECK může být pokládán za tumor supresorový gen a zajímavý cíl dalšího výzkumu inhibitorů matrixových metaloproteináz.

4.10 Prognostic significance of TMPRSS2-ERG fusion gene in prostate cancer (Příloha 10)

Kulda V, Topolcan O, Kucera R, Kripnerova M, Srbecka K, Hora M, Hes O, Klecka J, Babuska V, Rousarova M, Benson V, Pesta M: **Prognostic Significance of TMPRSS2-ERG Fusion Gene in Prostate Cancer.** *Anticancer Res* 2016, 36(9): 4787–4793.

Cílem této studie (Kulda et al. 2016) bylo stanovení exprese fúzního genu TMPRSS2-ERG a zhodnocení jeho prognostického potenciálu u karcinomu prostaty.

Karcinom prostaty je u mužů nejčastějším nádorem a třetí nejčastější příčinou úmrtí na maligní onemocnění. Naše pracoviště se tímto zhoubným onemocněním dlouhodobě zabývá, vznikla zde publikace zabývající se stanovením exprese prostate cancer gene 3 (PCA3) v moči za účelem časné diagnostiky (Klecka et al. 2010). Gen PCA3, dříve také nazývaný DD3 (diferential display 3) je dnes zařazen mezi geny pro long non-coding RNA (lncRNA) a je zapojen do regulací androgenní signalizace v buňkách prostaty (Lemos et al. 2016).

Karcinom prostaty je velmi heterogenní onemocnění. Ve většině případů roste pomalu, má malý potenciál k progresi a klinicky se někdy ani neprojevuje. Ale vyskytují se i formy s rychlým průběhem (Attard et al. 2016; Stangelberger et al. 2008). Rutinní stanovení sérových hodnot prostatického specifického antigenu (PSA) vede k odhalení časných stádií. Hlavní limitací PSA je však nízká pozitivní prediktivní hodnota. Agresivní terapie karcinomu prostaty přináší pro pacienta řadu komplikací (Storås et al. 2015; Dahl et al. 2015), proto jsou hledány nové markery schopné předpovědět progresi a generalizaci onemocnění. Současný výzkum ukazuje slibnou cestu, jak rozpoznat pacienty s nepříznivou prognózou (tj. pacienty, kteří by měli prospěch z radikální léčby). Jako klíčový v patogenezi agresivních forem karcinomu prostaty se ukazuje fúzní gen TMPRSS2-ERG (Tomlins et al. 2005; Hägglöf et al. 2014; Font-Tello et al. 2015). TMPRSS2 je transmembránová serinová proteáza specifická pro prostatu, jejíž exprese je řízena androgeny. ERG je gen z rodiny transkripčních faktorů ETS (erythroblastosis virus E26 transformující sekvence). Translokací vedoucí ke vzniku fúzního genu se dostane onkogen pod kontrolu promotoru závislého na androgenech. Ve způsobu, jakým translokace proběhne a jaký transkript je produkován, je velká diverzita (Clark et al. 2007).

Studovaná skupina zahrnovala 108 mužů, kteří podstoupili radikální prostatektomii pro karcinom prostaty. Předoperační hladina PSA proteinu jako rutinně používaného markeru byla měřena v periferní krvi. Přítomnost fúzního genu TMPRSS2-ERG byla stanovena v histologicky ověřené nádorové tkáni z FFPE bločků pomocí real-time RT-qPCR s LNA (locked nucleic acids) sondou na přístroji Stratagene Mx3005P. Zvolili jsme analýzu na úrovni transkriptu (RNA), tj. po izolaci celkové RNA byla provedena reverzní transkripce

s využitím náhodných hexanukleotidů jako primerů. Množství transkriptů pocházejících z fúzního genu TMPRSS2-ERG bylo následně stanoveno kvantitativní PCR metodou.

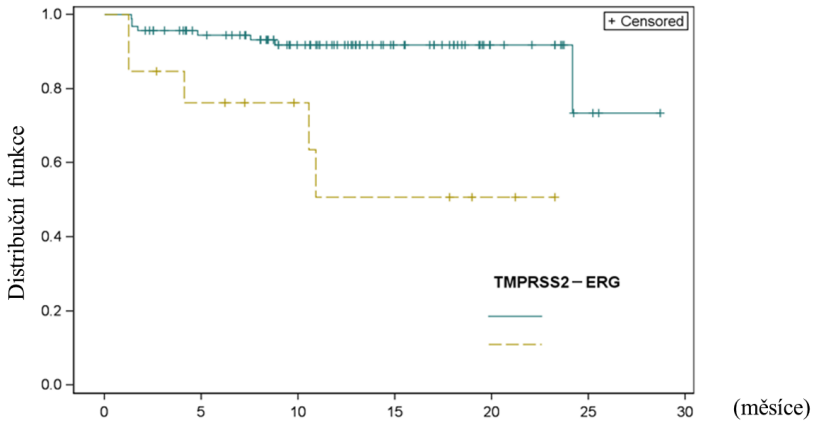
Obrázek 22 - Ukázka FFPE bločku a řezu obarveného H&E s označením nádorové tkáně

Fúzní gen TMPRSS2-ERG byl detegován u 13 vzorků (12 % pacientů). Přítomnost fúzního genu nezávisela na věku pacientů ani na Gleason skóre. U vyšších klinických stádií bylo vyšší procento výskytu fúze TMPRSS2-ERG.

Tabulka 4.10 - 1 - Přítomnost TMPRSS2-ERG dle klinických stádií

Stádium	N	TMPRSS2-ERG			
		negativní		pozitivní	
		N	%	N	%
I	16	15	94 %	1	6 %
II	63	57	90 %	6	10 %
III	29	23	79 %	6	21 %
celkem	108	95	88 %	13	12 %

Zaznamenali jsme statisticky významný vztah přítomnosti fúzního genu TMPRSS2-ERG v nádorové tkáni a délky bezpříznakového období (disease-free interval, DFI) (p=0,0020). Přítomnost fúzního genu znamenala horší prognózu.



Obrázek 23 - Kaplan-Meier křivky pro DFI

Vztah mezi přítomností fúzního genu TMPRSS2-ERG a bezpříznakového období (času do progrese) je klinicky zajímavý výsledek. Toto stanovení by mohlo identifikovat pacienty s vysokou pravděpodobností progrese onemocnění a pomoci zvolit pro takové pacienty nejvhodnější terapii.

Nedávno publikovaná práce ukazuje možnosti léčebných zásahů u nádorů, v jejichž patogenezi hraje roli fúzní gen (Gao et al. 2018). Další práce popisuje identifikaci nízkomolekulárního specifického inhibitoru ERG a naznačuje jeho možné použití v terapii karcinomů prostaty s přítomností fúzního genu TMPRSS2-ERG (Mohamed et al. 2018).

Ve stejných vzorcích byla stanovena hladina exprese miR-23b, miR-26a a miR-221, u kterých jsme na základě rešerše litetury očekávali, že by mohly mít také vztah k agresivitě nádoru a prognóze (Majid et al. 2012; Kato et al. 2015; Kneitz et al. 2014). U těchto mikroRNA jsme nenalezli žádný vztah k DFI ani k přítomnosti fúzního genu TMPRSS2-ERG. Zaznamenali jsme pouze jejich sníženou expresi v nádorové tkáni ve srovnání s okolní nenádorovou prostatickou tkání.

Rozeberu pobrobněji současný pohled na miR-221. Je to mikroRNA, která se prokazatelně podílí na karcinogenezi, ale u které nelze říci, zda působí jako onkogen nebo jako tumor supresor. Záleží na typu nádoru a dalších podmínkách působení (Abak et al. 2018). Např. u neuroblastomu působí onkogenně cílením na Nemo-like kinase (NLK) a tak zvyšuje expresi onkogenního transkripčního faktoru typického pro neuroblastom MYCN (He et al. 2017). U epiteliálních ovariálních nádorů naopak miR-221 cílením na ADP-ribosylation factor 4 (ARF4) působí jako tumor supresor (Wu et al. 2018). Velmi kontroverzní jsou poznatky o miR-221 v souvislosti s karcinomem prostaty. Mnoho prací ukazuje na její proliferaci a progresi nádoru podporující působení (Sun et al. 2014; Song et al. 2015; Shao et al. 2018), ale na druhé straně existuje řada prací včetně našeho zjištění, že tato mikroRNA je v tkáni karcinomu prostaty down-regulovaná (Spahn et al. 2010; Gordanpour et al. 2011; Xuan et al. 2015). Vysvětlení naznačuje zjištění, že exprese miR-221 je regulovaná androgeny tak, že androgeny snižují expresi miR-221. V léčbě karcinomu prostaty je běžná androgenní deprivační terapie (ADT), která vede k derepresi exprese miR-221. K další změně dojde v případě vývoje karcinomu prostaty rezistentního na kastraci (Gui et al. 2017). Tím jsem chtěl ukázat na obrovskou komplexitu sítí mikroRNA – cílové geny a ukázat, že i sama exprese regulačních molekul je pod vlivem dalších regulací.

5 Závěr

Naše výsledky založené na stanovení exprese mikroRNA v biologickém materiálu ukázaly, že tyto regulační molekuly mohou odrážet klinickopatologické vlastnosti nádoru a najít uplatnění jako biomarkery např. pro svoji prognostickou hodnotu nebo jako markery odpovědi na léčbu.

Naše zkušenosti potvrzují, že mikroRNA jsou stanovitelné nejen přímo v nativní nádorové tkáni, ale i ve formalínem fixované v parafínu zalité (FFPE) tkáni, která je rutinně připravována pro histopatologická vyšetření. Co je ale pro klinickou medicínu nejvýhodnější, vyšetřovaným materiálem může být i krev a její deriváty (krevní plazma). Odběr krve je minimálně invazivní a může být prováděn opakovaně za účelem monitorování průběhu onemocnění.

U pacientů s kolorektálním karcinomem jsme jako jedni z prvních ukázali na prognostický význam miR-21.

V tkáni karcinomu prostaty jsme popsali vyšší expresi miR-20a u hůře diferencovaných nádorů s vyšším Gleason skóre a ukázali, že přítomnost fúzního genu TMPRSS2-ERG znamená horší prognózu.

Novým poznatkem u nádorů hlavy a krku (HNSCC) bylo nalezení vztahu exprese miR-34a a p16-pozitivity jako markeru HPV infekce.

U pacientů s pokročilými stádii karcinomu žaludku jsme našli mikroRNA (miR-150, miR-224 a miR-342) s prognostickým významem, jejichž vysoká exprese v nádorové tkáni znamenala kratší celkové přežití.

Práce týkající se nemalobuněčného plicního karcinomu (NSCLC) ukázala, že methylace promotoru genu RECK snižuje expresi mRNA tohoto genu a je tudíž jedním z mechanismů potlačení jeho funkce v nádorové tkáni. Na souboru pacientů s pokročilým epidermoidním karcinomem léčených paliativní chemoterapií jsme našli vztah miR-34a, miR-224 a miR-342 k celkovému přežití. Zároveň jsme dospěli k závěru, že výsledek stanovení jedné jediné mikroRNA nemusí být postačující bez znalosti exprese dalších hráčů na poli mikroRNA – RNA interakcí. Vysoká hladina jedné konkrétní mikroRNA může být totiž za jistých okolností spojena s nepříznivou prognózou, za jiných okolností naopak s prognózou velmi dobrou.

Zatím nepublikované výsledky srovnání předoperačních a pooperačních plazmatických hladin cirkulujících mikroRNA ukázaly pokles miR-20a-5p, miR-23a-3p a miR-223a-3p po chirurgickém odstranění kolorektálního karcinomu.

Úplným závěrem shrnu, že deregulace genové exprese hraje roli v patogenezi nádorových onemocnění a regulační molekuly jako jsou mikroRNA představují slibné biomarkery.

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Seznam příloh

Práce vztahující se k tématu práce

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Příloha 8

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Příloha 11

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Příloha 12

Seznam prací autora

PŘÍLOHA 1

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CANCER GENETICS AND CYTOGENETICS

Relevance of miR-21 and miR-143 expression in tissue samples of colorectal carcinoma and its liver metastases

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Abstract MicroRNAs, which are endogenously expressed regulatory noncoding RNAs, have an altered expression in colorectal cancer. The aim of our study was to assess the relationship of miR-21 and miR-143 expression to the prognostic/clinicopathological features of colorectal carcinoma (CRC) and colorectal liver metastases (CLM). The estimation was performed in 46 paired (tumor and control) tissue samples of CRC. Further, we studied 30 tissue samples of CLM. MiR-21 and miR-143 expressions were quantified by using the quantitative reverse trascription polymerase chain reaction method. Relation of miR-21 and miR-143 expression to disease-free interval (DFI) (Wilcoxon; $P = 0.0026$ and $P = 0.0191$, respectively) was recorded. There was shorter DFI in patients with a higher expression of miR-21 and, surprisingly, also in patients with a higher expression of miR-143, which is a putative tumor suppressor. There was a higher expression of miR-21 and lower expression of miR-143 in CRC tissue in comparison with adjacent normal colon tissue ($P < 0.0001$; $P < 0.0001$, respectively). Similarly, we observed a higher expression of miR-21 and a lower expression of miR-143 in CLM in comparison with normal colon tissue ($P < 0.0001$; $P < 0.0001$, respectively). Our results support the hypothesis about oncogenic function of miR-21 and show its relation to DFI. The role of miR-143 in carcinogenesis seems to be more complex.

1. Introduction

Colorectal carcinoma (CRC) is the second most commonly diagnosed cancer in men and the third most common cancer in women in the Czech Republic, with an incidence of 91.3 men and 61.4 women out of 100,000 people in 2006 [1]. CRC ranks among the most frequent cancers worldwide [2].

Although the pathogenesis of CRC is one of the most characterized, new molecules that play a role in this process are still being discovered. Furthermore, CRC is not a homogeneous group of tumors. However, the mutation in the *KRAS* oncogene and the *PTEN* tumor suppressor gene are often present in all the CRC types [3–5]. We investigated miR-143 and miR-21 with a known relation to *KRAS* and *PTEN*, respectively [6,7].

MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression by the inhibition of the translation and/or decreasing of the stability of target mRNAs. It has been demonstrated that miRNAs play a significant role in tumorigenesis by downregulating tumor suppressor genes or oncogenes [8,9]. MiR-21 has a role in tumorigenesis, and in vitro experiments have shown that miR-21 is able to promote tumor cell growth and inhibit apoptosis [10]. It was identified that miR-21 targets the tumor suppressor genes tropomyosin 1 (*TPM1*), programmed cell death 4 (*PDCD4*), maspin, phosphatase and tensin homologue (*PTEN*), and reversion-inducing cysteine-rich protein with Kazal motifs (*RECK*). It shows that miR-21 has an oncogenic function [7,11–13]. Another miRNA that seems to

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be involved in CRC cancerogenesis is miR-143. Chen et al. demonstrated that miR-143 was suppressing colorectal cancer cell growth through inhibition of *KRAS* translation [6]. Although the experimentally verified targets mentioned above categorize miR-21 as oncogene and miR-143 as tumor suppressor gene, real behavior could be more complex. Single miRNA can regulate hundreds of different targets on which the overall effect is dependent [14]. It has been found that most miRNAs target both oncogenes and tumor suppressor genes, so they have dual nature [15].

The aim of our study was to assess the relationship of miR-21 and miR-143 expression to prognostic values (disease-free interval [DFI] and overall survival [OS]) as well as clinicopathological features of CRC and colorectal liver metastases (CLM). Control nontumorous tissue was also estimated.

2. Materials and Methods

2.1. Patients

The study included 46 patients with CRC (age 39.9–79.1 years, mean 62.8 years) who underwent surgery at the Department of Surgery, University Hospital Pilsen, between the years 2004 and 2005. The studied group reflects the natural distribution of clinicopathological characteristics of CRC patients. The location of the tumor was as follows: sigmoid colon (16 cases), rectum (15 cases), right colon (12 cases), and left colon (3 cases). Tumor samples were histologically verified. The diagnosis of adenocarcinoma was confirmed in all cases. The following histological subtypes were described: tubular (41 cases), mucinous (3 cases), and anaplastic (2 cases). Distribution according to tumor, node, metastasis system, grade, and stage (International Union Against Cancer [UICC]) classification is shown in Table 1. Postoperative adjuvant chemotherapy was indicated according to current guidelines of American Society of Clinical Oncology, 2004–2005. Eighteen patients were without adjuvant chemotherapy. The others received chemotherapy based on fluorouracil (5-FU) in different therapeutic schemes: FUFA (10 patients), FOLFIRI (3 patients), De Gramont (2 patients) or capecitabine (prodrug of 5-FU, 12 patients). One patient was treated with irinotecan. No radiotherapy was applied. Median duration of follow-up was 45.2 months.

The second studied group included 30 patients with CLM who underwent radical liver surgery at the Department of Surgery, University Hospital Pilsen, between the years 2002 and 2004. Patients' median age was 59.4 (range 39.6–82.1) years. The patients were treated by postoperative chemotherapy based on 5-FU (FUFA, 4 patients; FOLFIRI, 3; De Gramont, 3; FOLFOX, 2; XELOX, 2). Some patients received raltitrexed (3 patients) and oxaliplatin (2 patients). Eleven patients were treated by nonspecified chemotherapy. Informed consent was obtained from research participants.

Table 1
Distribution of CRC patients according to TNM, stage (UICC), and grade

	T				N		M	Stage (IUCC)				Grade				
Group	1	2	3	4	0	⩾1	0	1	I	II	III	IV	1	2	3	4	Unknown
Male ($n = 31$)	1	8	19	3	21	10	29	2	6	14	9	2	10	19	0	1	1
Female ($n = 15$)	1	3	11	0	12	3	15	0	4	7	4	0	3	7	2	0	3
All ($n = 46$)	2 (4%)	11 (24%)	30 (65%)	3 (7%)	33 (72%)	13 (28%)	44 (96%)	2 (4%)	10 (22%)	21 (46%)	13 (28%)	2 (4%)	13 (28%)	26 (57%)	2 (4%)	1 (2%)	4 (9%)

Abbreviations: CRC, colorectal carcinoma; TNM, tumor, node, metastasis system; UICC, International Union Against Cancer.

2.2. Tissue samples

Tissue samples were obtained from the patients during the surgical procedure. All samples were immediately frozen to −70°C until they were used. Tumor samples were taken directly from the CRC tissue, whereas the control tissues were taken from normal mucosa between the resection line and tumor itself. We obtained 30 CLM, and each of them was examined in two samples. The normal (control) liver tissue samples were taken from different patients who had undergone liver resection for traumatic liver rupture. All metastases samples were histologically verified.

2.3. Quantitative estimation of miRNA by reverse transcription real-time polymerase chain reaction

The total RNA was isolated from approximately 50 mg of tissue with the fastRNA Pro Green Kit (Q-BIOgene, Irvine, CA). A quantitative estimation was performed by a RT real-time polymerase chain reaction method by using TaqMan MicroRNA Assays (Applied Biosystems, manufactured by Roche, Branchburg, NJ). A two-step protocol requires reverse transcription with a miRNA-specific primer, followed by a real-time polymerase chain reaction with TaqMan probes. The assays target only mature miRNAs, not their precursors. We used total RNA as a normalizer. We also used the "normalization" genes RNU6B (U6snRNA) and miR-191, which are used in published studies [16–18]. Our approach of choice concerning normalization is described in detail in the Discussion.

Each sample was assessed twice in parallel. The Ct values were corrected by calibrators for the elimination of differences between runs of the iCycler apparatus (Bio-Rad, Prague, Czech Republic). The value of expression $i\Delta Ct = (Ct_{highest} + 1) - \text{measured Ct}$ ($Ct_{highest}$ is the highest Ct of all the measured samples of the real marker, e.g., miR-21) [19]. This approach allows for an easy solution to the problem of samples with an unmeasurable expression (value 0 is assigned to the iΔCt of these samples), and a further advantage is the logic that higher iΔCt means a higher expression (copy number). The iΔCt is the final result of the expression of miR-21 (miR-143) normalized to total RNA.

The results of the expression were also obtained as a relative copy number by using the Ct value of the measured miRNA and Ct value of normalizer (U6snRNA or miR-191) by the following approach: We calculated the value of the relative copy number R by the formula $R = 2^{(i\Delta Ct\ \text{miRNA} - i\Delta Ct\ \text{normalizer}) - \Delta Ct\ \text{highest}}$. Possible different $Ct_{highest}$ values of the measured miRNA and normalizer were corrected by the use of the $\Delta Ct_{highest}$ value, which was calculated as the difference between $Ct_{highest}$ of the measured miRNA and normalizer ($\Delta Ct_{highest}$ = miRNA $Ct_{highest}$ − normalizer $Ct_{highest}$).

Statistical analysis was performed by SAS software version 8.02 (SAS Institute, Cary, NC). The statistical results were calculated by a Wilcoxon nonparametric two-sample test. For the maximum hazard ratio (OS, DFI), the Cox regression hazard model was used. After finding this optimal cutoff for the examined markers, the Kaplan–Meier survival distribution functions of this optimal cutoff in given groups and subgroups were generated.

3. Results

There was a significantly higher expression of miR-21 and a significantly lower expression of miR-143 in CRC tissue in comparison with adjacent normal colon tissue ($P < 0.0001$; $P < 0.0001$, respectively). Similarly, we observed a significantly higher expression of miR-21 and a significantly lower expression of miR-143 in CLM tissue in comparison with normal colon tissue ($P < 0.0001$; $P < 0.0001$, respectively). We also compared CLM and normal liver tissue. We recorded a significantly higher expression of both estimated miRNAs, miR-21 and miR-143, in CLM tissue than in normal liver tissue ($P < 0.0001$; $P < 0.0001$, respectively; Table 2).

We did not find any differences in expression among CRC subgroups (T1–T3N0M0 vs. T1–T4N1M0–1, stage 1 + 2 + 3 vs. stage 4, stage 1 + 2 vs. stage 3 + 4 and T1–T4N0M0 vs. T1–T4N1M0–1); values are not presented here.

We also compared CRC and their subgroups (CRC T1–T3N0M0, CRC T1–T4N1M0–1, CRC stage 1 + 2 + 3, CRC stage 1 + 2, and CRC stage 3 + 4) with CLM tissue. We did not register differences in expression of miR-21. But

Table 2
Comparison of expression levels of miR-21 and miR-143 between studied groups

	miR-21					miR-143				
Group	*n*	25%	Median	75%	*P*-value	*n*	25%	Median	75%	*P*-value
CRC	46	6.650	7.165	8.030	<0.0001	46	10.250	11.350	12.750	<0.0001
Control colon tissue	45	5.650	6.200	6.770		44	11.700	14.250	16.000	
CLM	30	6.950	7.375	7.650	<0.0001	30	9.500	9.975	10.800	<0.0001
Control colon tissue	45	5.650	6.200	6.770		44	11.700	14.250	16.000	
CLM	30	6.950	7.375	7.650	<0.0001	30	9.500	9.975	10.800	<0.0001
Control liver tissue	29	3.130	3.730	4.730		29	5.450	7.030	9.280	
CRC	46	6.650	7.165	8.030	0.5749	46	10.250	11.350	12.750	0.0003
CLM	30	6.950	7.375	7.650		30	9.500	9.975	10.800	

Abbreviations: CRC, colorectal carcinoma; CLM, colorectal liver metastases.

Table 3
Comparison of expression levels of miR-21 and miR-143 between CLM and subgroups of CRC

	miR-21					miR-143				
Group	*n*	25%	Median	75%	*P*-value	*n*	25%	Median	75%	*P*-value
CLM	30	6.950	7.375	7.650		30	9.500	9.975	10.800	
X										
CRC T1-T3N0M0	31	6.700	7.150	8.130	0.7466	31	10.250	11.050	12.750	0.0025
CRC T1-T4N1M0–1	15	6.220	7.180	7.900	0.4738	15	10.250	12.000	12.600	0.0023
CRC stage 1 + 2 + 3	44	6.675	7.250	8.065	0.6929	44	10.300	11.350	12.750	0.0004
CRC stage 1 + 2	31	6.700	7.150	8.130	0.7466	31	10.250	11.050	12.750	0.0025
CRC stage 3 + 4	15	6.220	7.180	7.900	0.4738	15	10.250	12.000	12.600	0.0023

Abbreviations: CLM, colorectal liver metastases; CRC, colorectal carcinoma.

we found a significantly lower expression of miR-143 in CLM tissue compared to all individual CRC groups (Tables 2 and 3).

We recorded a statistically significant relation of miR-21 and miR-143 expression in CRC tissue to DFI (Wilcoxon; $P = 0.0026$ and $P = 0.0191$, respectively). Statistical values are summarized in Table 4. We recorded in patients with a higher expression of miR-21 shorter DFI (Fig. 1). In patients with higher miR-143 expression, we also registered shorter survival (Fig. 2). We found no relation of miR-21 and miR-143 expression to OS.

We also assessed the relationship of miR-21 and miR-143 expression to prognostic values (DFI and OS) in liver metastases. There was no relation of miR-21 and miR-143 expression in CLM tissue to DFI and OS; values are not presented here.

4. Discussion

CRC is not a homogeneous group of tumors. On the basis of clinical behavior and heredity, three subgroups can be distinguished: hereditary syndromes, sporadic CRC with a familial component, and sporadic CRC. The most common hereditary CRC syndromes are hereditary nonpolyposis colorectal cancer with an inherent mutation of one allele in mismatch repair genes and familial adenomatous polyposis with the inherent mutation of one allele in the *APC* gene. The loss of heterozygosity (mutation in the second allele) of these genes is at the beginning of the carcinogenesis of these syndromes. However, the initiation steps of hereditary nonpolyposis colorectal cancer and familial adenomatous polyposis are different; the mutations in many genes in the sequence of carcinogenesis overlap. Mutations in these genes are also observed in sporadic CRC with a familial component and in sporadic CRC, which is the most frequent CRC [20–22].

We obtained tumor samples from the Department of Surgery. The classification of patients to groups mentioned above was not possible to perform. Therefore, we chose to assess the miRNAs, regulating genes supposed to have a role in the carcinogenesis of both hereditary and nonhereditary CRC forms. We investigated miR-143 and miR-21—that is, miRNAs with a known relation to *KRAS* oncogene and *PTEN* tumor suppressor gene, respectively, as was described in the Introduction.

Our goal was to evaluate the relationship of the miR-143 and miR-21 expression in CRC and CLM tumor tissue to prognostic values—DFI and OS. We recorded the relationship of miR-21 and miR-143 expressions to DFI. There was shorter DFI in patients with a higher expression of miR-21. As described below, we and other investigators recorded a higher expression of miR-21 in tumor tissue. Taken together with verified targets of miR-21, which are tumor suppressor genes (*PTEN*, *TPM1*, *PDCD4*, maspin, and *RECK*), this result supports the oncogenic role of miR-21. There are only a few studies on miRNAs and prognosis. It was published that a high miR-21 expression is associated with a poor survival rate in colon adenocarcinoma [23]. The relation of miR-21 to prognosis was described also in non-small-cell lung cancer [24].

Surprisingly, there was also shorter DFI in patients with a higher expression of miR-143, which is a putative tumor suppressor. To our knowledge, this report is novel in its discussion about the relation of miR-143 expression and DFI.

The relation of miR-143 expression to DFI did not correspond to the logic of differences in miR-143 levels between tumor CRC tissue and control colon tissue. This could relate to that single miRNA can target hundreds of transcripts with different natures.

Table 4
Relation of miR-21 and miR-143 expression to DFI in CRC patients

	Patients above cutoff			Patients below cutoff			
miRNA	*n*	Median DFI (y)	Optimal cutoff	*n*	Median DFI (y)	Log rank *P*-value	Wilcoxon *P*-value
miR-21	11	0.84	8.1	33	3.16	0.0145	0.0026
miR-143	22	2.25	11.4	22	3.51	0.0151	0.0191

Abbreviations: DFI, disease-free interval; CRC, colorectal carcinoma.

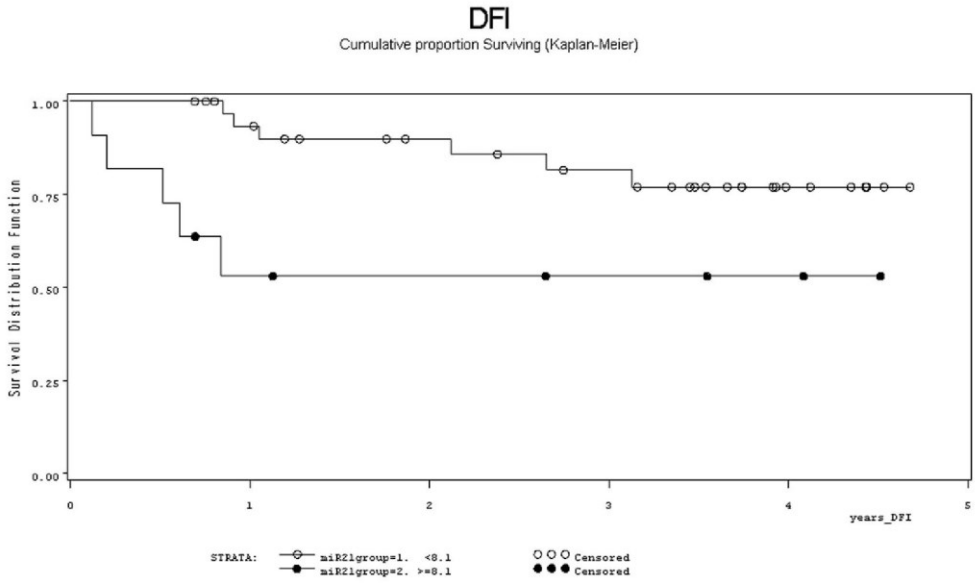

Fig. 1. Relation of miR-21 expression to disease-free interval (DFI) in colorectal carcinoma patients (Kaplan–Meier DFI curve).

We used commonly available predictive algorithms (microCosm, TargetScan, Pictar) [25] to identify the targets of miR-21 and miR-143. The predictive algorithms showed that besides the experimentally proven targets mentioned in the Introduction, there could be many other possible target mRNAs, including both oncogenes and tumor suppressor genes. For example, we found oncogenes *ABL2, ETV6, AFF1, TET1, ERBB3, MAF, RAB11, CBL,* and *MYBL2* and tumor suppressor genes *TMEM127, SMAD3,* and *DAPK1* among the predicted targets of miR-143 by the TargetScan human algorithm. On the basis of these data, the role of miR-143 in carcinogenesis could be more complex. We also used TargetScan prediction for identification of miR-21 targets. Among them, we found oncogenes *PLAG1, SKI, TET1,* and *CDK6* and tumor suppressor genes *PHF14, LIFR,* and *PDCD4*. Our results show that dominant is the oncogenic effect.

Naturally, we also compared the expression in tumor vs. control (normal adjacent) tissue in our cohort of patients. We recorded a higher expression of miR-21 and a lower

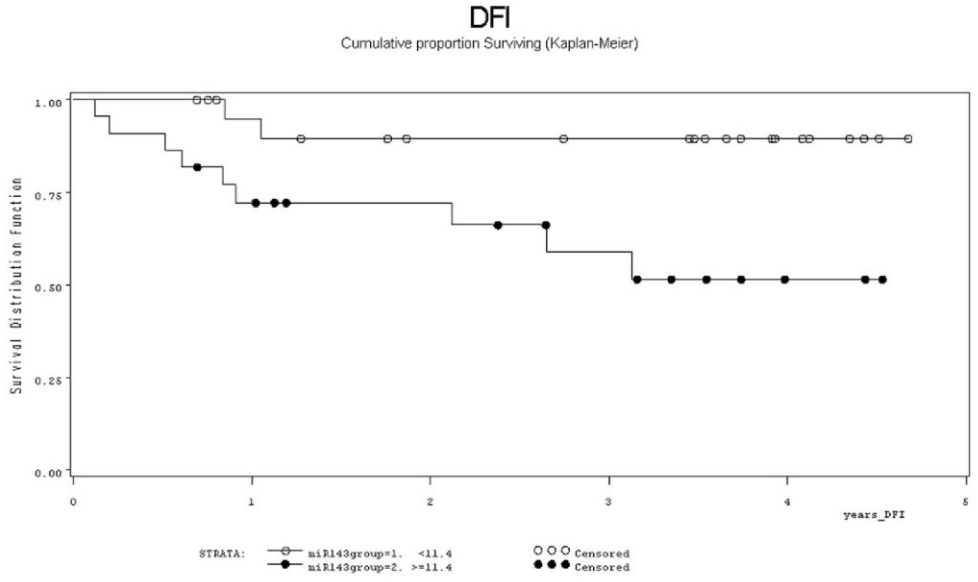

Fig. 2. Relation of miR-143 expression to disease-free interval (DFI) in colorectal carcinoma patients (Kaplan–Meier DFI curve).

Table 5
Comparison of different approaches to normalization and their results of expression

	Total RNA		U6RNA		miR-191	
Group	Median	*P*-value	Median	*P*-value	Median	*P*-value
miR-21						
CRC	7.165	<0.0001	1.105	0.2416	0.247	0.2814
Control colon tissue	6.200		1.189		0.187	
CLM	7.375	<0.0001	1.778	0.0047	0.284	0.1117
Control liver tissue	3.730		0.400		0.094	
CRC	7.165	0.5749	1.105	0.0004	0.247	0.4819
CLM	7.375		1.778		0.284	
miR-143						
CRC	11.350	<0.0001	8.787	<.0001	2.266	<.0001
Control colon tissue	14.250		163.144		22.627	
CLM	9.975	<0.0001	7.944	0.0401	1.125	0.9398
Control liver tissue	7.030		2.694		1.000	
CRC	11.350	0.0003	8.787	0.1696	2.266	0.0009
CLM	9.975		7.944		1.125	

Abbreviations: CLM, colorectal liver metastases; CRC, colorectal carcinoma.

expression of miR-143 in CRC tissue in comparison with adjacent normal colon tissue.

These results agree with the findings of Bandres et al. and Slaby et al. [26,27]. These authors used let-7a-1 as a normalizer. Recently, there has been evidence for the deregulated expression of let-7 in many tumors, including colorectal tumor [28–30]. The let-7 family is often present in multiple copies in the genome. Therefore, we chose a different approach to normalization, as is discussed below.

The comparison of miRNA expression between colorectal tumor tissue and CLM is also interesting. We did not register any differences in the expression of miR-21. But we found a lower expression of miR-143 in CLM tissue compared to CRC.

We also compared CLM and normal colon tissue. We found a higher expression of miR-21 and a lower expression of miR-143 in CLM tissue in comparison with normal colon tissue. This result corresponds with the comparison of CRC and normal adjacent tissue.

With respect to the process of development of CLM, the control tissue in comparison to CLM should be normal colon tissue. We also compared CLM and tissue adjacent to CLM (i.e., normal liver tissue). We observed both miR-21 and miR-143 to be significantly more expressed in CLM versus normal liver tissue. For interpretation of this result, it is necessary to compare expression of these miRNAs between normal colon and liver tissue. We found both miR-21 and miR-143 significantly more expressed in colon versus normal liver tissue, and therefore the comparison between CLM and normal liver tissue did not show new information about metastatic process.

As mentioned in Materials and Methods, we dealt with the choice concerning the normalizer. Normalization is a commonly known problem of quantitative estimation of miRNAs. There is no optimal generally recommended approach. We used normalization to total RNA, one of the accepted methods. We also used the "normalization" genes U6snRNA and miR-191 recommended by some authors who have published comparative analyses [31]. We recorded the conformity with recent accepted results (differences in miR-143 and miR-21 expression between tumor and control tissue of the colon, breast, lung cancer, and others) [29] by using total RNA as a normalizer. The results obtained by using "normalization" genes U6RNA and miR-191 were similar, but not exactly the same (Table 5).

In conclusion, our results support the oncogenic function of miR-21 and show its relation to DFI. The role of miR-143 in carcinogenesis seems to be more complex. The evaluation of the miRNAs expression could yield new information about CRC pathogenesis.

Acknowledgments

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PŘÍLOHA 2

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Importance of miR-20a Expression in Prostate Cancer Tissue

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Abstract. *Background: MicroRNAs (miRNAs), which are endogenously expressed regulatory noncoding RNAs, have an altered expression in tumor tissues. MiRNAs regulate cancer-related processes such as cell growth and tissue differentiation, and therefore, might function as oncogenes or tumor-suppressor genes. The aim of our study was to assess the expression of mir-20a, let-7a, miR-15a and miR-16 in prostate cancer (PCa) and benign prostatic hyperplasia (BPH) tissue and to investigate the relation between the expression of miRNAs and the clinico-pathological features of PCa. Patients and Methods: The study group comprised 138 patients: 85 patients with BPH and 53 patients with PCa. The total RNA was isolated from the tissue specimen core and miRNA expressions were quantified using a real-time RT-PCR method (TaqMan MicroRNA Assays). U6snRNA was used for the normalization of the miRNA expression. Results: miR-20a expression was significantly higher in the group of patients with a Gleason score of 7-10 in comparison with the group of patients with a Gleason score of 0-6 (p=0.0082). We found no statistical differences in the miRNA expressions (mir-20a, let-7a, miR-15a and miR-16) in the PCa tissue samples in comparison with the BPH tissue samples. Conclusion: Our result shows that the more dedifferentiated PCa cells have a higher expression of miR-20a and this supports the oncogenic role of miR-20a in PCa carcinogenesis. The evaluation of miRNA expression could yield new information about PCa pathogenesis.*

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Key Words: Prostate cancer, miRNA, miR-20a, let-7a, miR-15a, miR-16.

Prostate cancer (PCa) is the most commonly diagnosed cancer, and the second leading cause of cancer-related deaths of men in Western countries (1). Nevertheless, with regard to incidence, the molecular basis of PCa is insufficiently characterized. There is an increasing interest in the role of the new regulatory molecules, microRNAs (miRNAs), in cell processes, which could contribute to a better understanding of cancer pathology.

miRNAs are endogenously expressed, small non-coding RNAs, which regulate gene expression by the inhibition of the translation and/or decreasing of the stability of target mRNAs. Briefly, the miRNA expression in eukaryotic cells is realized in several steps. The miRNA gene is transcribed in the nucleus from the genomic DNA by RNA polymerase Pol II to produce a long transcript called a primary microRNA (pri-miRNA). This pri-miRNA is cleaved by a microprocessor complex containing the RNAse III enzyme Drosha and its cofactor Pasha (double-stranded RNA binding protein) into 70-100 nucleotide long precursor microRNA (pre-miRNA) with a typical hairpin structure. The pre-miRNA is exported by the transport protein exportin 5 into the cytoplasm, where the ~22 nucleotide miRNA duplex (miRNA/miRNA*) is excised from the pre-miRNA by RNAse III enzyme Dicer. Subsequently, one strand of the mature miRNA is incorporated into the RNA-induced silencing complex (RISC), which negatively regulates its target genes by preventing the production of their protein products.

Some miRNA genes are observed to be deregulated in cancer cells (2-6). The miRNAs can function both as oncogenes by down-regulating tumor-suppressor genes and as tumor-suppressor genes by down-regulating oncogenes (7). The reader is referred to reviews (2-5, 7, 8) and the public miRNA database available online at www.mirbase.org for more information on biogenesis, function and the role of miRNAs in carcinogenesis.

let-7 belongs to the first known miRNAs and is one of the most characterized. It was discovered in *Caenorhabditis*

Table I. *Comparison of expression levels of mirR-20a, let-7a, miR-15a and miR-16 between studied groups (BPH vs. PCa; organ-confined cancer (TNM 1c+2a+2b+2c) vs. advanced and metastatic (TNM 3a+3b+3c+1cM+2cM+3aM); Gleason score 0-6 vs. Gleason score 7-10).*

Group	N	miR-20a				let-7a			
		25%	Median	75%	p-Value	25%	Median	75%	p-Value
BPH	85	1.5476	3.8637	8.8766	0.3405	9.5137	17.2677	39.3966	0.5153
PCa	53	1.2746	3.5554	7.2100		8.0000	17.4550	30.9100	
Organ-confined cancer	41	1.2746	3.5554	.702100	0.9644	7.7275	12.8171	30.9100	0.0865
Advanced and metastatic	11	1.0353	3.6808	7.4643		15.4550	20.8215	83.2859	
Gleason score 0-6	27	1.1892	1.8661	3.8637	0.0082	6.9644	12.5533	27.8576	0.0707
Gleason score 7-10	20	3.5222	6.2467	15.0999		13.0431	18.0551	73.2024	
Group	N	miR-15a				miR-16			
		25%	Median	75%	p-Value	25%	Median	75%	p-Value
BPH	85	0.9013	1.6021	3.4343	0.8544	9.3827	19.6983	48.5029	0.3653
PCa	53	0.7738	1.6818	3.3636		6.1903	14.7230	42.2243	
Organ-confined cancer	41	0.7220	1.6586	3.3173	0.1033	4.6913	10.7779	42.2243	0.1708
Advanced and metastatic	11	1.6818	2.2974	3.4343		18.3792	20.8215	16.8507	
Gleason score 0-6	27	0.7738	1.6586	3.2043	0.0899	4.5315	12.3805	34.2968	0.1388
Gleason score 7-10	20	1.4315	2.9332	3.8025		8.7442	22.1075	90.5084	

elegans and its role in timing of stem cell division and differentiation was identified (9). let-7 is a member of the let-7 family and so far this family includes 10 mature sequences in humans (10). The let-7 family is often present in multiple copies in the genome. A letter is used to indicate isoforms with slightly different sequences. A subsequent number indicates the same sequences arising from different genomic locations (*e.g.* let-7a-1). In general, the function of let-7 is to promote the differentiation of cells.

The next most often studied group of miRNAs is the mir-17-92 cluster which contains miR-20a. Today there is evidence that the mir-17-92 cluster is involved in many types of human cancer. The amplification of the region containing this cluster was described in lymphoma (11, 12) and lung cancer (13). Loss of heterozygosity of these genes was recorded in breast cancer (14), hepatocellular carcinoma (15) and nasopharyngeal carcinoma (16-18). These results show that members of the mir-17-92 cluster could play a role both as oncogenes and tumor-suppressor genes.

A group of miRNAs with an antioncogenic role in cancerogenesis is the cluster miR-15 and miR-16. The role of these miRNAs was first identified in chronic lymphocytic leukemia (CLL). This cluster is located in the CLL frequently deleted or translocated region 13q14.3. Both miRNAs negatively regulate BCL2 at the post-transcriptional level (19).

Identification of deregulated miRNAs in cancer tissue can focus interest on these miRNAs and can help to determine their place in the cascade of carcinogenesis and finally they may become a promising targets for therapy.

The aim of our study was to asses the expression of let-7a, mir-20a, miR-15a and miR-16 in PCa tissue and benign prostatic hyperplasia (BPH) and investigate the relationship between expression of these miRNAs and clinicopathological features of PCa. We chose these miRNAs on the basis of previously published studies, based mainly on high throughput assays (20-23). This is the first direct study of these miRNAs in biopsied patient samples.

Patients and Methods

Patients. Our study group consisted of 138 patients who underwent a prostate biopsy between May 2006 and September 2008 in one of two Departments of Urology (the University Teaching Hospital in Pilsen and the University Teaching Hospital of the First Medical Faculty of Charles University in Prague). All the patients exhibited an elevated serum total prostate-specific antigen (tPSA) level and/or abnormal digital rectal examination. A previous biopsy was not an exclusion but had to have been performed at least three months prior to the study. The median age was 66.5 years (range 48-85 years). According to the histological verification, our group of patients was divided into 85 patients with BPH and 53 patients with PCa. The numbers of patients in compared groups are shown in Table I. The value of the Gleason score and TNM classification was not available for a small number of patients. Approval was obtained from the Institutional Ethics Committee and written informed consent from each patient. Patients who had had any transurethral manipulation, or radiotherapy, or who were on hormonal therapy, or had an indwelling catheter or acute urinary infection before the biopsy were excluded from the study.

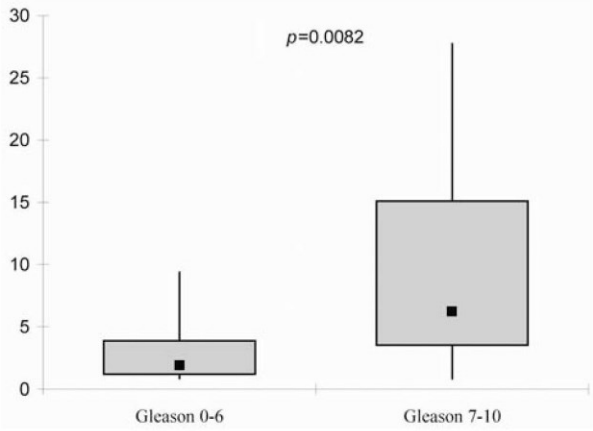

Figure 1. *Expression of miR-20a in PCa samples of Gleason score 0-6 and Gleason score 7-10. There was a significantly higher expression of miR-20a in Gleason score 7-10 group in comparison with Gleason score 0-6 group. The values shown in the figure are 10th and 90th percentiles (line), lower and upper quartiles (rectangle) and the median (small square) of miR-20a expression (relative copy number).*

Tissue samples. The tissue samples were obtained as a portion from a needle core bioptic sample, and were frozen at –70˚C until use.

Quantitative estimation of miRNA using RT real-time PCR. Total RNA was isolated from approximately 10 mg of tissue using a fastRNA Pro Green Kit (Q-BIOgene, Irvine, CA, USA). A quantitative estimation was performed by a RT real-time PCR method using TaqMan® MicroRNA Assays (Applied Biosystems, manufactured by Roche, Branchburg, NJ, USA). The two-step protocol requires reverse transcription with a miRNA-specific primer, followed by a real-time PCR with TaqMan® probes. The assays target only mature miRNAs, not their precursors. As a normalizer, RNU6B (U6snRNA), which is generally used in published studies (24-26) was used. Each sample and normalizer was assessed twice in parallel. The results of the expression were obtained as a relative copy number using the Ct value of the measured miRNA and Ct value of the normalizer (U6snRNA) by the following approach: The Ct values were corrected using calibrators for the elimination of differences between runs of the iCycler apparatus (Bio-Rad, Prague, Czech Republic) using the value of the expression: idCt=($Ct_{highest}$+1)–measured Ct, where $Ct_{highest}$ is the highest Ct of all the measured samples of the real marker *e.g.* miR-20a (27). This approach allows for an easy solution to the problem of samples with an unmeasurable expression (a value of 0 is assigned to the idCt of these samples) and a further advantage is the logic that a higher idCt means a higher expression (copy number). The final value of the relative copy number, R, was calculated using the following formula R=2(idCt miRNA–idCt normalizer)–dCt highest. Possible different $Ct_{highest}$ values of the measured miRNA and normalizer were corrected by the use of the $dCt_{highest}$ value which was calculated as the difference between $Ct_{highest}$ of the measured miRNA and the normalizer ($dCt_{highest}$=miRNA $Ct_{highest}$ –normalizer $Ct_{highest}$).

Statistical analysis was performed using the software SAS 8.02 (SAS Institute Inc., Cary, NC, USA). The statistical results were calculated by a Wilcoxon non-parametric two-sample test.

Results

We investigated differences in the expression of mirR-20a, let-7a, miR-15a and miR-16 in the BPH and PCa. All the measured values of expression and the P-values are summarized in Table I. We found no statistically significant differences in the expression of these miRNAs in BPH tissue in comparison with PCa tissue. Furthermore, we compared the expression in tumor tissue between different TNM stages of the disease. We compared the group of patients with organ-confined cancer (TNM 1c+2a+2b+2c) with those with a higher extent of the disease (advanced and metastatic; TNM 3a+3b+3c+1cM+2cM+3aM). We recorded no statistically significant differences in the expression of mirR-20a, let-7a, miR-15a and miR-16. Finally, we analyzed the differences in the expression of miR-20a, let-7a, miR-15a and miR-16 in two groups divided according to the degree of differentiation of PCa tissue, *i.e.* those with a Gleason score of 0-6 and those with a Gleason score of 7-10. We observed that the expression of miR20a was statistically significantly higher in the group with a Gleason score of 7-10 than in the group with a Gleason score of 0-6 (p=0.0082) (Figure 1). There were no differences in the expression of let-7a, miR-15a and miR-16 between these groups.

Discussion

In the past few years, research has revealed the role of miRNAs in the regulation of cell processes such as proliferation and apoptosis, as well as in the pathology of these processes. Despite the fact that the sequence of particular steps of the carcinogenesis of PCa is not known, it is evident that there is a deregulation of apoptosis and proliferation. We assessed the miRNAs which participate in these processes. We observed a higher expression of miR-20a in the group with a Gleason score of 7-10 than in the group with a Gleason score of 0-6. The Gleason score is an important variable describing the behavior of PCa and has been correlated with pathologic stage, metastasis and outcome (28). miR-20a is one of the members of the mir-17-92 cluster.

Sylvestre *et al*. described an overexpression of miR-20a in the human prostate cancer cell line PC3 using PCR (21). Volinia *et al*. recorded an up-regulation of miR-20a in PCa tissue using a microarray assay (22). The identified function of miR-20a is the modulation of the translation of the *E2F2* and *E2F3* mRNAs *via* binding sites in their 3'-untranslated region (21), this supports the oncogenic behavior of miR-20a. The same authors also observed antiapoptotic activity. miR-20a overexpression reduced apoptosis in the PC3 cell line (21). Our result supplements the previous findings and shows that the more dedifferentiated cancer cells (Gleason score 7-10) have a higher expression of oncogenic miR-20a.

It was reported that the other investigated miRNAs (let-7a, miR-15a and miR-16) have an antionco- or oncogenic function (3). The results of recent studies show that the *RAS* oncogene could be regulated at least by some members of the let-7 family (29). There is evidence that LIN28 plays an important role in the biogenesis of mature let-7 miRNA as a negative post-transcriptional regulator. High LIN28 levels prevent the processing of pri-let-7 by the microprocessor complex and also prevent pre-let-7 from turning into mature let-7 by dicer (10). This example also demonstrates that the assessment of pri-miRNA or pre-miRNA does not always corresponds with the level of mature miRNA. The miR-15a and miR-16 cluster is located in cancer frequently deleted or translocated region 13q14.3. Among the targets of miR-15a and miR-16, the antiapoptotic protein BCL2 was identified, which is overexpressed in many malignancies. Bonci *et al*. reported that in cancer cells of advanced prostate tumors, the miR-15a and miR-16 levels was significantly decreased (23). We did not observe differences in expression of miR-20a, let-7a, miR-15a and miR-16 between BPH and PCa. It should be noted that the behavior of BPH does not necessarily correspond with that of normal prostatic tissue; it was not possible for us to obtain normal prostate tissue.

In conclusion, our result extends the findings of previous studies and shows that the more dedifferentiated prostate cancer cells have a higher expression of miR-20a, supporting the oncogenic role of miR-20a in carcinogenesis of PCa.

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Cancer Epidemiology, Biomarkers & Prevention

Research Article

Diagnostic and Prognostic Value of microRNA-21 in Colorectal Cancer: An Original Study and Individual Participant Data Meta-analysis

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Abstract

Background: We aimed to systematically summarize the diagnostic and prognostic value of circulating/tissue miR21 in patients with colorectal cancer.

Methods: An original study was conducted to explore the potential value of circulating miR21 in colorectal cancer diagnosis and tissue miR21 in colorectal cancer prognosis. PUBMED and EMBASE were searched (to August, 2013) to identify eligible studies. To explore the diagnostic performance of circulating miR21, meta-analysis methods were used to pool sensitivity, specificity, positive and negative likelihood ratio, diagnostic OR and to construct a summary ROC curve. For prognostic meta-analysis, study-specific HRs of tissue miR21 for survival were summarized. Subgroup and sensitivity analyses were applied to explore heterogeneity.

Results: Finally, 14 studies (including our study) were included in the meta-analyses. The pooled sensitivity, specificity, and AUC of circulating miR21 were 0.76 [95% confidence interval (CI), 0.59–0.88], 0.81 (95% CI, 0.76–0.85), and 0.81 (95% CI, 0.78–0.85) in diagnosing colorectal cancer. Patients with higher expression of tissue miR21 had significant inferior overall survival (OS; pooled HR, 1.56; 95% CI, 1.16–2.11) and disease-free survival (DFS; pooled HR, 1.35; 95% CI, 1.08–1.69). The individual participant data (IPD) meta-analysis demonstrated that tissue miR21 level was independently associated with worse colorectal cancer OS (HR, 1.69; 95% CI, 1.07–2.67; $P = 0.023$), whereas this association seems to be confined to males ($P = 0.007$) but not for females ($P = 0.845$).

Conclusions: Circulating miR21 level has potential value for colorectal cancer early detection, whereas high tissue miR21 level is associated with adverse colorectal cancer prognosis.

Impact: miR21 is a promising biomarker for early detection and prognosis of colorectal cancer. *Cancer Epidemiol Biomarkers Prev; 23(12); 2783–92. ©2014 AACR.*

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Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

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Introduction

Colorectal cancer is one of the most common type of cancers and a leading cause of death worldwide (1). Screening tests for colorectal cancer, including colonoscopy and fecal occult-blood testing, have been frequently used in recent years (2). However, these screening tests are far from adequate because of their invasiveness, high cost, or low sensitivity (3). New biomarkers, especially noninvasive biomarkers are in urgent need for colorectal cancer early detection. In addition, new prognostic biomarkers for colorectal cancer are also in need to improve treatment strategies.

microRNAs (miRNA) are small noncoding RNAs that play important roles in the regulation of cell differentiation, cell-cycle progression, apoptosis, and tumorigenesis (4, 5). They regulate protein-coding mRNAs at the post-transcriptional level by binding to target mRNA, preventing its translation or targeting it for destruction, or by transcriptional gene silencing at the chromatin level (6, 7). About half of the human miRNAs are located at cancer-related regions of the genome, and miRNAs are reported

to act as oncogenes or tumor-suppressor genes (8, 9), implying their important roles in the progression and prognosis of cancers, including colorectal cancer (10, 11). miR21 is an oncogenic miRNA that can modulate the expression of cancer-related genes, including PTEN, TPM1, and PDCD (12–14). Interestingly, miR21 expression is elevated during tumor progression, for example, its level is upregulated in colorectal cancer tissues. Furthermore, it can influence patients survival and response to chemotherapy (10, 15, 16). Many studies have evaluated the application of miR21 in the diagnosis and prognosis of colorectal cancer; however, the results were contradictive (10, 17–19). As a consequence, the aim of this study was to comprehensively explore the potential value of miR21 in colorectal cancer diagnosis and prognosis.

Materials and Methods

Original study

We conducted an original study to explore the diagnostic and prognostic value of miR21 expression in colorectal cancer. Briefly, colorectal cancer tissues and corresponding normal tissues were obtained from 79 patients by surgical resection and blood samples were collected in EDTA tubes from 41 patients with colorectal cancer before surgical operation and from 30 healthy controls. Total RNA was extracted from tissue and plasma samples, followed with DNase I digestion to exclude genomic DNA contamination. Mature miR21 and internal control U6 were detected by stem-loop real-time RT-PCR methods. ROC analysis was performed and the AUC value was calculated to determine the diagnostic performance of serum miR21 level in colorectal cancer. Survival analyses were conducted using the Kaplan–Meier method. Univariate and multivariate Cox's proportional hazard regression analyses were applied to estimate HRs of death according to tissue miR21 expression levels. The detailed methods were described in the Supplementary File (Supplementary Methods for Original Study). The diagnostic and prognostic data calculated from the original study were pooled with studies identified from literature search in the meta-analysis process.

Meta-analysis

This meta-analysis was designed, conducted, and reported according to the PRISMA statement (20). The meta-analyses process, including the individual participant data (IPD) meta-analysis, was carried out in accordance with the Cochrane Handbook for Systematic Reviews of Intervention (21). The review has been registered in an international registry of systematic reviews PROSPERO (CRD42013005119).

Literature search and study selection

Comprehensive literature searches were conducted (to August, 2013) in PUBMED and EMBASE to identify eligible studies. The detailed selection process was presented in Supplementary File (Supplementary Methods for Literature search and Study selection).

Data extraction

Two reviewers (Drs. P. Li and H. Zhang), independently collected data using standardized forms and discrepancies were resolved by a third investigator. The following information from each study was extracted: first author, year of publication, origin of the study population, patient characteristics (age, sex, cancer type, and stage), source of samples, number of participants, miR21 assay method, follow-up time, and variables adjusted for in the analysis. For diagnostic studies, the numbers of true-positive (TP), false-positive (FP), true-negative (TN), and false-negative (FN) results were extracted. For prognostic studies, HR estimates with 95% confidence intervals (CI) for overall survival (OS), disease-free survival (DFS), recurrence-free survival, recurrence-free cancer–specific survival (RF-CSS), or progression-free survival were extracted. If the HRs and their 95% CIs were not provided, the numbers of deaths or recurrences and total samples in each study were extracted to calculate these numbers (22). An IPD meta-analysis approach was conducted to assess the prognostic performance of tissue miR21 expression in patients with colorectal cancer. The principal investigators of relevant studies were contacted and asked to provide the raw data. The quality of the diagnostic study was assessed using the quality assessment of diagnostic accuracy studies 2 (QUADAS-2; ref. 23). For prognostic studies, a modified version of the QUADAS-2 assessment tool (Supplementary Table S1) was applied to assess the risk of bias and the criteria for reporting observational studies proposed in the STROBE statement was used to complete the methodologic evaluation (24).

Statistical analysis

For the diagnostic meta-analysis, numbers of TP, FP, TN, and FN were analyzed and a bivariate model was constructed to summarize the sensitivity, specificity, positive and negative likelihood ratios (LR), diagnostic odds ratio (DOR), and generate the summary receiver operator characteristic curve (25). Confidence intervals were calculated, assuming asymptotic normality after a log transformation for variance parameters and for LRs and a logit transformation for proportions. The formula for a positive LR is sensitivity/(1-specificity), and the formula for a negative LR is (1-sensitivity)/specificity. The combined LRs provide the DOR (positive LR/negative LR). A clinically useful test was defined as having a positive LR greater than 5.0 and a negative LR less than 0.2 (26). Heterogeneity was assessed using the I^2 test (27), and the Deek funnel plot method was applied to test publication bias (28).

HR was adopted for prognostic evaluation in the current meta-analysis, because all the included studies used HR to measure the prognostic performance of miR21.

Study-specific HR estimates were pooled using a fixed-effects model, if there was no significant heterogeneity. Otherwise, a random-effects model was applied. The extent of heterogeneity across studies was checked using the χ^2 and I^2 tests; $P \leq 0.10$ and/or $I^2 > 50\%$ indicates significant heterogeneity. Subgroup analyses and sensitivity analysis were performed to dissect the heterogeneity. Begg funnel plots and Egger linear regression test were used to assess publication bias. In the Hong Kong Validation Cohort of Schetter and colleagues (10), tissue miR21 expression values were dichotomized with the highest tertile classified as high and the lower two tertiles defined as low, and this high–low cutoff was applied universally in the IPD analysis. Survival analyses were conducted using the Kaplan–Meier method. Then univariate Cox's proportional hazard regression analyses were applied to estimate HR of death according to tissue miR21 expression levels. And multivariate models were used to adjust potential confounding factors for death, including age, sex, TNM stage, pathologic differentiation, and side of the tumor (left or right).

All analyses were conducted using the Stata software (version 11.0; StataCorp.). A P value of <0.05 was considered statistically significant.

Results

The original study

A total of 71 individuals were included (41 patients with colorectal cancer and 30 normal controls) to evaluate the diagnostic value of miR21. Serum miR21 level was suggested to be a potential biomarker in discriminating patients with colorectal cancer from control subjects, with an AUC value of 0.657 (95% CI, 0.530–0.783; Supplementary Fig. S1), a sensitivity of 51.2% and specificity of 79.0%, respectively. To assess the prognostic value of tissue miR21 for patients with colorectal cancer, 79 patients with colorectal cancer were included. The mean follow-up time was 65.9 (95% CI, 61.5–70.3) months, and 22 patients died of colorectal cancer during the follow-up period. As Kaplan–Meier survival analysis indicated, patients with higher levels of miR21 in the tumor tissues had a nonsignificant worse OS (61.5 months vs. 68.4 months; $P = 0.280$;

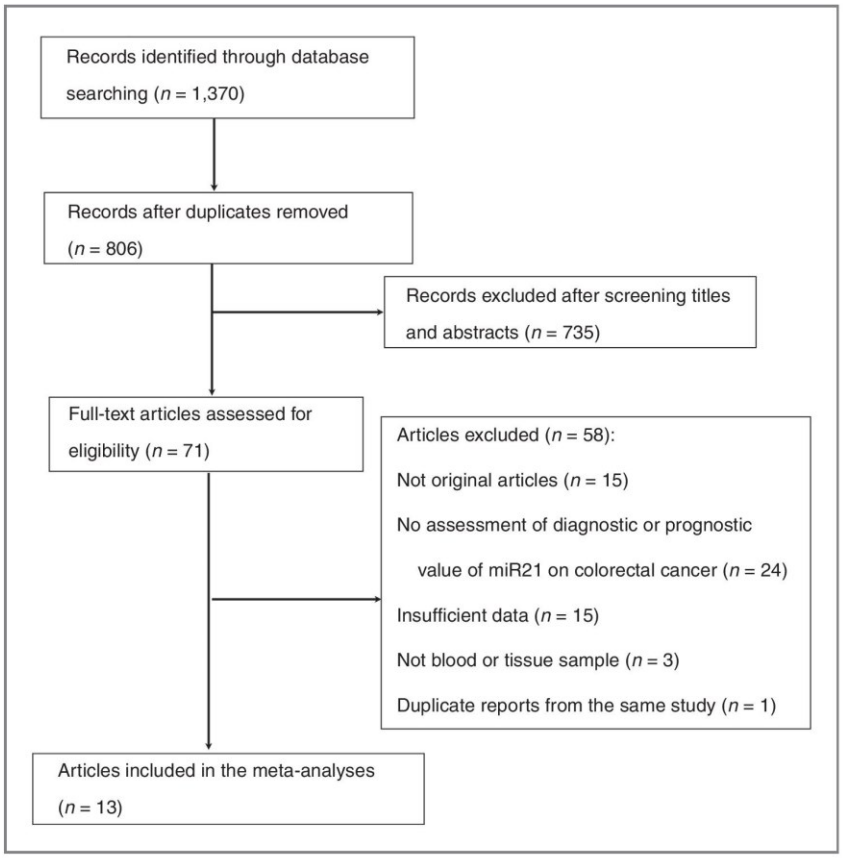

Figure 1. Flow diagram of the study selection process.

log-rank test; Supplementary Fig. S2). Furthermore, univariate Cox proportional hazard regression analysis revealed an HR of 1.58 (95% CI, 0.68–3.64; $P = 0.285$) for tissue miR21 in colorectal cancer prognosis. In the multivariable analysis, which included miR21 level, age, gender, side of the tumor, TNM stage, and differentiation, the HR for tissue miR21 in colorectal cancer prognosis was 1.92 (95% CI, 0.74–4.97; $P = 0.177$).

Study selection and characteristics

Searching PUBMED and EMBASE resulted in the inclusion of 1,307 articles. Finally, a total of 13 articles were identified as eligible studies (10, 16–19, 29–36). With our original study, 14 studies were finally included in the meta-analyses. The selection process was shown in Fig. 1, and the characteristics of the included studies were presented in Supplementary Tables S2 and S3. Among the included articles, 11 articles reported the prognostic value of miR21 (including our study; refs. 10, 16–19, 29–32, 36), whereas 6 examined diagnostic value of miR21 (including our study; refs. 17, 18, 33–35; 3 articles reported both prognostic and diagnostic value).

Diagnostic value of blood miR21 for colorectal cancer

Six studies with 1,071 patients assessed the diagnostic value of blood miR21 level for colorectal cancer. The included studies were conducted in Europe ($n = 1$), East Asian ($n = 4$), and the United States ($n = 1$). Sample size of each study ranged from 40 to 374. The types of specimen contain serum ($n = 3$) and plasma ($n = 3$). All the studies adopted the quantitative reverse transcription PCR (qRT-PCR) method to measure the expression of miR21. The quality assessments were shown in Supplementary Table S4.

The pooled sensitivity and specificity were 0.76 (95% CI, 0.59–0.88) and 0.81 (95% CI, 0.76–0.85), respectively (Fig. 2). And the area under the ROC curve was 0.81 (95% CI, 0.78–0.85; Fig. 3), indicating miR21 has a relatively high diagnostic performance in colorectal cancer. There was significant heterogeneity in sensitivity ($Q = 82.58$, df = 5, $I^2 = 93.94$, $P < 0.005$) but not in specificity ($Q = 6.57$, df = 5, $I^2 = 23.86$, $P = 0.25$). Sensitivity analyses indicated that country of origin, type of specimen, sample size, and study quality were not the source of heterogeneity. The pooled positive LR, negative LR, and DOR were 3.80 (95% CI, 3.00–4.81), 0.29 (95% CI, 0.17–0.50), and 13.68 (95% CI, 5.35–35.02), respectively.

The prognostic meta-analyses

A total of 11 studies were included in the prognostic analyses. All the studies were published in English and conducted in Europe ($n = 4$), East Asian ($n = 6$), and the United States ($n = 2$; one study included both American

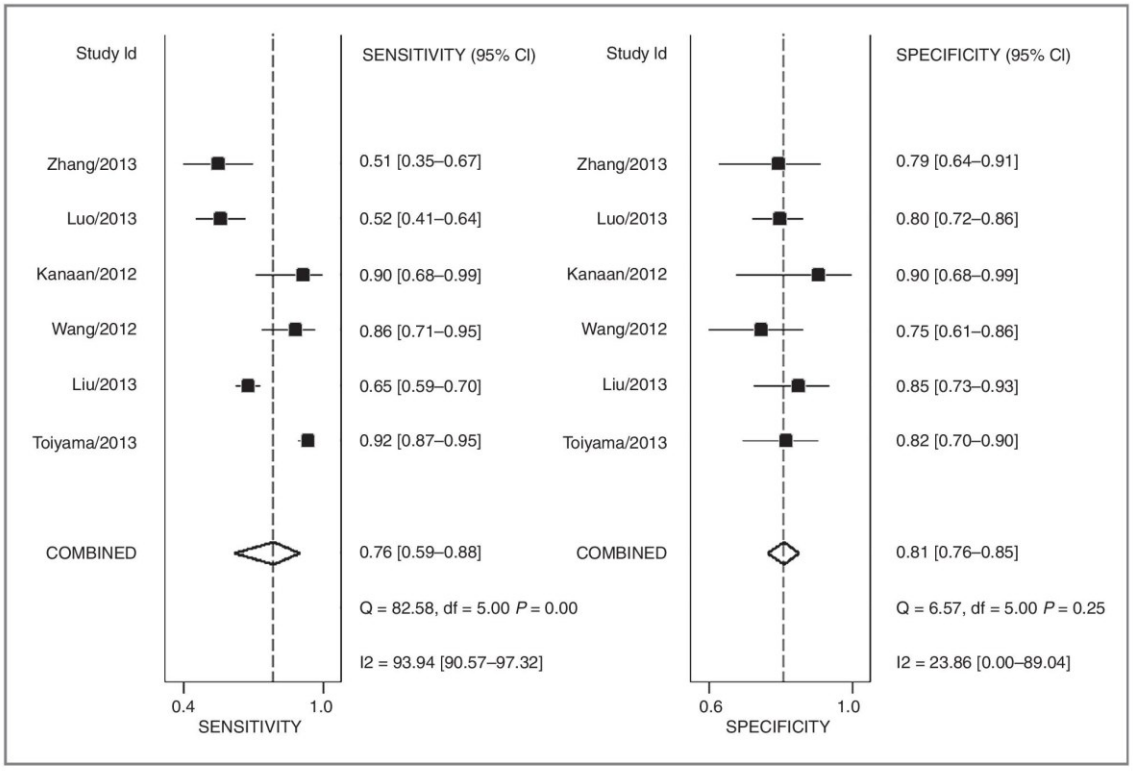

Figure 2. Forest plots of sensitivities and specificities of circulating miR21 in the diagnosis of colorectal cancer.

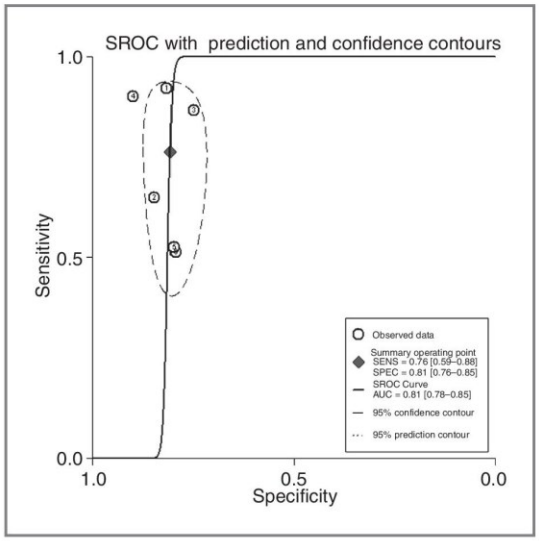

Figure 3. Summary ROC curves for miR21 in the diagnosis of colorectal cancer.

and Chinese population). Each study assessed 46 to 520 patients with colorectal cancer. The types of specimen contain tissue ($n = 9$), serum ($n = 2$), and tissue stroma ($n = 1$; one study evaluated both tissue and serum sample). Nine studies adopted the qRT-PCR method to measure the expression of miR21, whereas two studies applied the *in situ* hybridization (ISH) assay method. Three studies (including our study) presented IPD (10, 36). The quality assessments were shown in Supplementary Tables S5 and S6.

Tissue miR21 level and prognostic outcome

A total of nine studies (including our study) with 1,598 patients assessed the impact of tissue miR21 expression on colorectal cancer OS. The pooled HR was 1.56 (95% CI, 1.16–2.11) for all the studies, indicating that higher tissue miR21 expression level predicate poorer OS for patients with colorectal cancer (Fig. 4A). Significant heterogeneity across studies was observed ($I^2 = 85.1\%$, $P < 0.001$; Fig. 4A). Subgroup and sensitivity analyses suggested that methods to measure miR21 expression, country of origin might contribute to heterogeneity across studies (Table 1).

We then applied a meta-analysis of IPD to further explore the potential value of miR21 in colorectal cancer prognosis. Three studies (including our study) presented IPD data (10, 36), and Schetter and colleagues (10) only provided IPD of the Hong Kong Validation Cohort because the Maryland Test Cohort was not tested by the qRT-PCR method. A total of 236 patients with colorectal cancer were included. There was no significant association between miR21 expression and age, gender, tumor location, colorectal cancer stage, and differentiation (all $P > 0.05$). Kaplan–Meier survival analysis demonstrated that patients with higher miR21 levels in the tumor tissues had statistically worse OS (84.1 months vs. 110.6 months; $P = 0.001$; log-rank test; Fig. 5). As univariate analysis indicated, higher tissue miR21 level was associated with shorter OS (HR, 2.06; 95% CI, 1.35–3.15; $P = 0.001$). In the multivariable analysis, which included miR21 level, age, gender, side of the tumor, TNM stage, and differentiation, higher miR21 level served as an independent prognostic marker for indicating poorer OS in patients with colorectal cancer (HR, 1.69; 95% CI, 1.07–2.67; $P = 0.023$). Interestingly, the association between tissue miR21 expression and colorectal cancer survival appeared to be confined to male patients (multivariable HR, 2.47; 95% CI, 1.28–4.77; $P = 0.007$) but not for females (multivariable HR, 1.07; 95% CI, 0.53–2.16; $P = 0.845$).

Five studies comprising 953 patients evaluated colorectal cancer DFS for miR21. We found a significant association between higher miR21 expression level and poorer colorectal cancer DFS (pooled HR, 1.35; 95% CI, 1.08–1.69; Fig. 4B). There was significant heterogeneity in the analysis ($I^2 = 66.4\%$, $P = 0.018$; Fig. 4B). Through subgroup and sensitivity analyses, we found that methods to measure miR21 expression, country of origin, and sample size of the study might be the source of heterogeneity (Table 1).

Circulating miR21 level and prognostic outcome

Two studies explored the performance of circulating miR21 levels in the prognosis of colorectal cancer (17, 18). The HR of these two studies for colorectal cancer OS was 4.12 (95% CI, 1.10–15.4) and 1.58 (95% CI, 0.77–3.21), respectively.

Publication bias

The funnel plots of the diagnostic and prognostic meta-analyses were shown in Supplementary Fig. S3. Funnel plot tests (Begg and Egger tests) indicated no significant publication bias in this study. However, because of the limited number of the included studies, it is still difficult to ascertain whether publication bias exists or not.

Discussion

Although significant progress has been achieved in the diagnosis and prognosis of colorectal cancer over the years, development of better biomarkers is still important for colorectal cancer early detection and for predicting patients' outcome. The application of miRNAs as biomarkers for cancer diagnosis and prognosis has gained much attention in recent years. miR21 is one of the most studied miRNAs as a potential biomarker of colorectal cancer diagnosis and prognosis. To examine the reported diagnostic and prognostic accuracies and evaluate whether miR21 can be a useful biomarker for colorectal cancer, we performed this systematic review on 14 diagnostic or prognostic studies.

As the present meta-analysis showed, circulating miR21 achieved a pooled sensitivity of 0.76, specificity of 0.81, and AUC of 0.81. These results suggested that measuring blood miR21 level is a promising noninvasive method for colorectal cancer diagnosis. The DOR

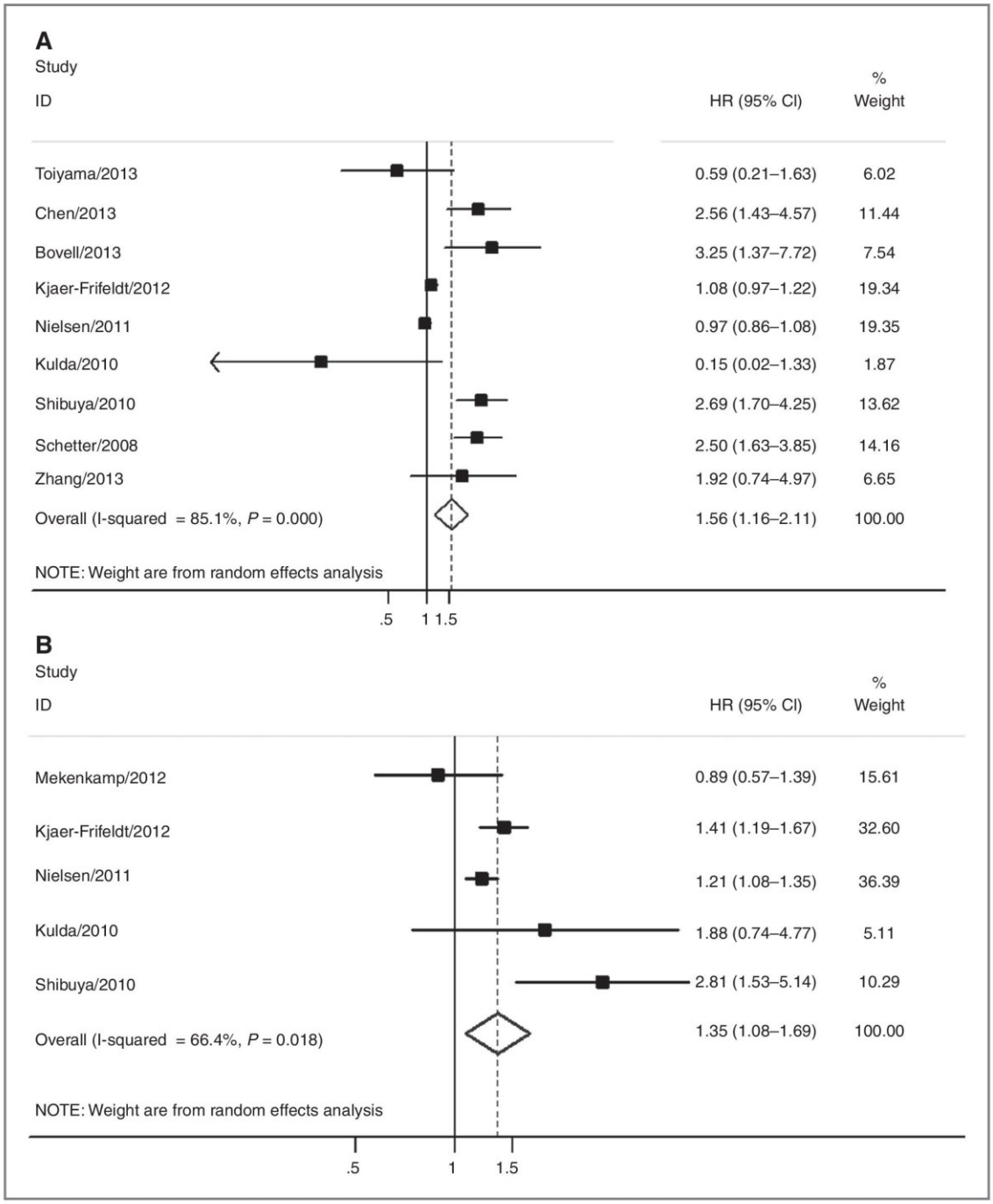

Figure 4. Forrest plots of studies evaluating tissue miR21 expression level and colorectal cancer prognosis. A, forrest plots of OS; B, forrest plots of DFS.

combines the strengths of both sensitivity and specificity, and was reported to be a useful indicator for evaluation of the diagnostic method (37). The DOR value of miR21 was 13.68, indicating a moderate diagnostic accuracy. However, the positive LR (3.8) and negative LR (0.29) suggested that miR21 may be not adequate enough to

Table 1. Subgroup analyses for association of tissue miR21 expression level with OS and DFS in colorectal cancer

	OS			DFS		
	No. of studies	Pooled HR	I^2 (%)	No. of studies	Pooled HR	I^2 (%)
Biochemical method						
qRT-PCR	7	1.98 (1.28–3.04)	59.3	3	1.62 (0.73–3.60)	78.8
ISH	2	1.02 (0.94–1.11)	41.1	2	1.27 (1.15–1.39)	54.2
Country of origin						
Asia	4	1.91 (1.10–3.31)	59.6	1	2.81 (1.53–5.14)	—
Europe	3	1.01 (0.86–1.19)	59.2	4	1.25 (1.14–1.37)	42.7
USA	2	2.63 (1.79–3.87)	0	—	—	—
Sample size						
Large (>100)	7	1.61 (1.18–2.20)	87.8	3	1.44 (1.12–1.85)	76.9
Small (< = 100)	2	0.64 (0.05–7.61)	78.7	2	1.15 (0.57–2.31)	50.3

discriminate and distinguish patients with colorectal cancer. We found a significant heterogeneity in sensitivity, whereas sensitivity analyses indicated that country of origin, type of specimen, sample size, and study quality were not the source. Different cutoff values of miR21 expression across studies may be one source of heterogeneity.

Measuring circulating miR21 might also be a useful screening method for colorectal advanced adenoma. In a study conducted by Toiyama and colleagues (17), in which 43 patients with Japanese advanced adenoma and 53 control subjects were enrolled, miR21 had a relatively high diagnostic performance for advanced adenoma (AUC value of 0.813, sensitivity of 0.811, and a specificity of 0.767). In another study, which included 50 Chinese advanced adenoma patients and 80 healthy controls, miR21 yielded an AUC value of 0.709 for discriminating advanced adenomas from controls (18). However, it should be noted that these two studies were both conducted in East Asia and the sample size was not large; thus, more studies are warranted to clarify this issue.

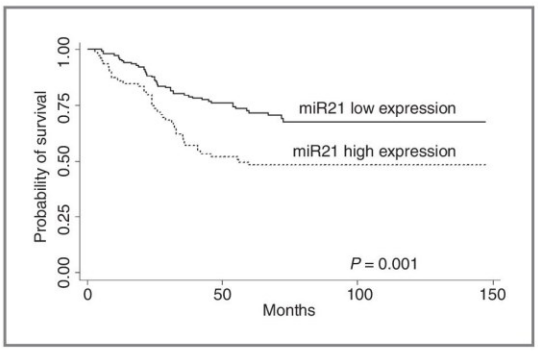

Figure 5. Kaplan–Meier curve of tissue miR21 expression in relation to OS of patients with colorectal cancer using IPD.

The results of the meta-analyses indicated that tissue miR21 expression level was a promising biomarker to predict survival in patients with colorectal cancer. Compared with patients with low tissue miR21 expression level, patients with an increased level of miR21 expression had a 1.56-fold higher risk of poor OS and 1.35-fold higher risk of poor DFS. There was significant heterogeneity in the meta-analyses of the data for OS and DFS. To decipher the reason for the heterogeneity, we applied subgroup and sensitivity analyses and found that methods used to measure miR21 expression, country of origin and sample size of the study could partially explain the heterogeneity. Besides, different cutoff values among the included studies may also be a potential source of heterogeneity. An IPD meta-analysis approach was conducted to further explore the prognostic potential of miR21 in patients with colorectal cancer. As the results demonstrated, higher miR21 level was an independent marker for predicting OS in patients with colorectal cancer. And our results showed that the effect of miR21 in predicting colorectal cancer survival was observed only in male participants, suggesting that gender may modify the observed effect. More well-designed studies with large sample size are warranted to clarify this issue and explore the relevant mechanisms. Besides, whether the prognostic value of other miRNAs is differed by gender may also need further study. Circulating miR21 was also developed as a noninvasive prognostic biomarker for colorectal cancer, and studies indicated that higher circulating miR21 level might be associated with poor OS for colorectal cancer (17, 18).

Though sensitivity and subgroup analyses were applied, heterogeneity in both diagnostic and prognostic meta-analyses was not fully explained. The heterogeneity across studies was probably due to the different methodology of evaluating miR21 expression. Different kinds of samples are used in assessing miR21 expression, including frozen tissues, formalin-fixed paraffin-embedded (FFPE) tissues, serum, and plasma. One previous study found that the results of frozen tissues differed from FFPE

tissues in evaluating the prognostic performance of miR21 expression for non–small cell lung cancer, suggesting that type of samples may influence the outcomes (38). Normalization is another problem for quantitative estimation of miRNAs. For the included studies, RNU6B, miR16, and total RNA were used by different studies. However, there is no conclusion about the performance of these normalization controls in the estimation of miRNAs and no optimal approach is generally recommended. To draw a convincing conclusion on the value of miR21 for the diagnosis and prognosis of colorectal cancer, an appropriate and unified method should be established and applied.

It is hypothesized that miRNAs enter the circulation directly secreted by cells, released by cells via exosomes, and via shedding of microvesicles (39). miRNAs have unusually high stability in tissues, and previous study has indicated that tumor-derived miRNAs are also present in plasma and serum in a remarkably stable form (40). Mostly, miRNAs demonstrate the same change in expression in blood (plasma or serum) and tumor tissues of patients with various types of cancer (39, 40). And thus, circulating miRNAs may serve as ideal biomarkers for cancer detection and prognosis. miR21 was reported to be one of the most relevant oncogene-like factors among various miRNA. Colon cancer cell lines with higher miR21 expression levels showed an enhanced ability of motility and invasion (41). Furthermore, suppression of miR21 inhibits cell growth *in vitro* and inhibits tumor growth in animal models by indirectly downregulating the antiapoptotic factor, B-cell lymphoma 2 (42). Studies in human cell lines further investigated the physiologic targets of miR21, and showed that miR21 could target tumor-suppressor genes, such as phosphatase, tensin homolog (PTEN), tropomyosin 1 (TPM1), and programmed cell death 4 (PDCD4; refs. 13, 43, 44). Besides, miR21 has been reported to play an important role in suppressing proapoptotic genes and modulating the vital components of the Ras/MEK/ERK pathway (45). Therefore, miR21 may be involved in the critical steps in carcinogenesis the genesis and progression of human cancer by promoting tumor growth, proliferation, antiapotosis, and migration (42, 43, 46, 47). It has been further demonstrated that tissue miR21 expression is associated with lymph node positivity and the development of distant metastases for colorectal cancer, and therefore miR21 expression serves as a marker clinicopathologic feature of the disease (15). These findings support a vital role for altered miR21 expression in tumorigenesis.

This systematic review had several important strengths. First, we conducted a relatively thorough systematic search and applied a comprehensive analytic approach to evaluate the diagnostic and prognostic value of miR21 in patients with colorectal cancer. Second, an original study was also conducted to explore the diagnostic and prognostic potential of miR21 in colorectal cancer, and an IPD meta-analysis was then used, which further supported the conclusions of the study. The methods of this study were rigorous and followed the guidelines for conducting and reporting systematic reviews.

There were also some limitations in our analysis. First, most of the diagnostic studies enrolled healthy people as controls and were not blind designed. This may affect the diagnostic performance. Second, there was considerable heterogeneity for both the diagnostic and prognostic meta-analyses. Subgroup and sensitivity analyses were applied, whereas the results could not fully explain the observed heterogeneity. Third, the different chemical assays used in the included studies might result in systematic errors among studies. Finally, only Asians and Caucasians were enrolled in this study, and thus the conclusions should be taken cautiously for other ethnic populations.

Taken together, in this study, it is concluded that circulating miR21 level is a useful biomarker for colorectal cancer detection, and tissue miR21 is a promising marker for colorectal cancer prognosis. Further research is needed to explore the combination of other variables associated with colorectal cancer diagnosis and prognosis, in an effort to develop better diagnostic and prognostic models with higher discriminative capacity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Disclaimer

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the article.

Authors' Contributions

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Development of methodology: H. Zhang, M. Pesta
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Zhang, H. Ju, M. Pesta, V. Kulda, M. Cai, Y. Wu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): W. Jin, C. Liu, H. Wu, J. Xu, Y. Ye, G. Zhang, J. Cai, Y. Wu
Writing, review, and/or revision of the manuscript: H. Zhang, P. Li, Y. Wu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Ju, M. Pesta, V. Kulda, G. Zhang, E. Xu, M. Lai
Study supervision: D. Xia

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MicroRNA Profile in Site-specific Head and Neck Squamous Cell Cancer

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Abstract. *Background/Aim: MicroRNAs (miRs) are non-coding RNA molecules regulating diverse cellular processes essential in carcinogenesis. Little is known regarding miRs in head and neck squamous cell cancer (HNSCC). The aim of the present study was to investigate miRs in relation to the clinicopathological features of site-specific HNSCC. Materials and Methods: The study comprised of 51 patients with HNSCC (23 oropharyngeal, 24 laryngeal and 4 hypopharyngeal carcinomas). Total RNA was extracted from tumor tissue and normal squamous epithelium using the miRNeasy FFPE Kit. A quantitative estimation of let-7a, miR-21, miR-200c, miR-34a, miR-375 was performed by a real-time polymerase chain reaction (PCR) method using the TagMan® MicroRNA assay. Additionally, p16 expression was detected by immunohistochemistry. Results: Significant differences of let-7a, miR-200c, miR-34a levels between oropharyngeal and laryngeal cancers were found ($p<0.05$). Compared to non-neoplastic tissues, miR-21, miR-200c, miR-34a were up-regulated and miR-375 was down-regulated in tumors of all sites. MiR-34a tumor levels significantly correlated with oropharyngeal origin ($p=0.0284$) and p16 positivity ($p=0.0218$). Conclusion: The microRNA profile seems to play a potential role in the pathobiology of oropharyngeal and laryngeal HNSCC. Up-regulation of miR34a in p16-positive oropharyngeal cancer has not been so far described and additional studies are warranted.*

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Key Words: MicroRNA, let-7a, miR-21, miR-200c, miR-34a, miR-375, head and neck squamous cell carcinoma, oropharyngeal, laryngeal, hypopharyngeal cancer.

The head and neck squamous cell cancer (HNSCC) represents a broad scale of tumors from the oral cavity to larynx (1). HNSCC of different anatomical sites seems to be associated with different etiopathogenesis, molecular characteristics and clinical outcomes, despite the same histological type. The majority of previous studies on HNSCC were performed irrespective of tumor location and published results were not site-specific. Detailed knowledge of the molecular basis of these tumors is, thus, required and new HNSCC biomarkers are warranted.

Nowadays, there is an increasing interest in the role of microRNAs (also known as miRNAs or miRs) in physiological and pathological cell processes, which bring new insights to cancer pathobiology. MicroRNAs are newly-recognized, non-coding, regulatory RNA molecules, 18-25 nucleotides in length. Their biogenesis is a multi-step process under the control of several enzymes and enzymatic complexes (Figure 1). Briefly, this process starts in the nucleus, where the primary-micro RNA (pri-micro RNA) with long nucleotide sequence is produced. During the next step, the hairpin-shaped pri-micro RNA enters a complex consisting of the enzyme Drosha and an essential cofactor Pasha to be processed into pre-microRNA and then transported to the cytoplasm. Enzymes Dicer and helicase are responsible for shortening of double stranded RNA and subsequently unwinding of this duplex into two mature microRNAs. They are incorporated into the RNA-induced silencing complex (RISC), which regulates the final effect of microRNA. For detailed description of micro RNA biogenesis, we recommend earlier reviews (2-4). Expression of

microRNA is tissue-specific and each alteration of tissue microRNA profile is associated with distinct disease-status. MicroRNAs participate in post-transcriptional regulation of gene expression to control development and maintain diverse cellular processes, including proliferation, apoptosis, senescence, differentiation, motility and morphogenesis. Each microRNA can regulate a considerable number of genes downstream by targeting many messenger RNA (mRNA) transcripts. One mRNA may be influenced by more types of microRNA. The final effect of this interaction is mRNA cleavage or translational repression (depending on the degree of complementarity with the target mRNA) with simultaneous quick degradation of microRNA. Thus, microRNA can control more than one-third of the protein-coding genes in the human genome inclusive of genes coding for transcription factors and RNA regulating proteins (5). Transregulation represents subsequent functional effects of microRNAs that may alter the levels of other RNAs (5).

MicroRNAs are involved in many diseases, both non-neoplastic and cancers. Their link to cancer is not surprising taking into consideration that microRNAs are involved in many processes essential in carcinogenesis and cancer progression (*e.g.* proliferation, migration, apoptosis). MicroRNA dysregulation is attributed to germ-line or somatic mutations of microRNA genes or epigenetic changes (6, 7). Two principal effects of microRNAs in human cancer have been described: oncogenic and tumor-suppressor. The former is linked to negative regulation of tumor suppressor genes, while the latter is mediated by translational repression of oncogenes (8, 9). However, a dual effect of some microRNAs, both oncogenic and tumor suppressing, has also been described (10).

More than 2,500 mature microRNAs have been identified in humans (see miRBase Sequence Database, Release 21, available online at www.miRbase.org) with different roles in cancerogenesis (11). In our study, we focused on 5 selected microRNAs (in Table I) with potentially crucial roles in the pathobiology of site-specific HNSCC: (i) Let-7a belongs to the large let-7 family. The let-7a precursor was the first microRNA identified from the study of developmental timing in *Caenorhabditis elegans*. The ubiquitously expressed let-7/miR-98 was one of the first mammalian microRNAs to be described. The let-7 family is often present in multiple copies in the genome and isoforms with slightly different sequences are indicated by a letter (12). Along with the general function to promote the differentiation of cells, its tumor suppressor role by targeting multiple oncogenes, namely *RAS*, in various human cancers, including head and neck cancer, was recently described (13). Moreover, its repressive role in cancer stem cells with stem-like properties ablation was also recently described (14). (ii) MicroRNA-21 is one of the first microRNAs identified in a number of cancers also having an important role as an oncogene (15). MiR-21 has been proven to be up-regulated in a wide variety of malignant tumors, namely in human glioblastomas, breast, colon, lung, pancreas, prostate cancer and in head and neck squamous cell carcinoma as well (16, 17). MiR-21 over-expression was significantly associated with worse outcome promoting cell proliferation (16, 18). Recently, miR-21 was introduced as a promising diagnostic and prognostic biomarker and therapeutic target as well (17, 19-21). (iii) MicroRNA-200c is one of the five members of microRNA-200 family that regulates the epithelial-mesenchymal transition (EMT) by targeting EMT-related gene expression. MiR-200c exhibits tumor suppressive properties. Its down-regulation promotes EMT-facilitating tumor cell invasion and progression of cancer, while up-regulation induces mesenchymal-epithelial transition (22, 23). (iv) MicroRNA-34a, like one of the three members of miR-34 family, is characterized by its tumor suppressor action that induces apoptosis, cell-cycle arrest, senescence and EMT. The p53 family has been described as one of the principal inducers of miR-34a gene expression, although alternative regulatory pathways also exist (24, 25). MiR-34a dysregulation plays an important role in many human cancers; this small molecule is, usually, down-regulated and associated with poor outcome (26). Common causes of miR-34a low expression are deletion, CpG promoter methylation of the *MIR-34A* gene (RNA gene) on chromosome 1 or the alteration of p53. This shows the important therapeutic potential of miR-34a as an anticancer drug (27). However, little is known regarding the role and expression status of miR-34a in head and neck squamous cell carcinomas. (v) MicroRNA-375 is typically expressed in the pancreas and pituitary gland. It has a great impact on development and endocrine function of the pancreas. MiR-375 has been implicated in a number of different cancers being down-regulated in cancer cells when compared to adjacent non-neoplastic tissues. Since there exist several molecular targets and different pathways of miR-375 regulation, it is necessary to investigate the expression pattern in specific cancers (28). Generally, miR-375 acts as tumor suppressor in human cancer, although an oncogenic function in breast cancer has been described (29).

Therefore, the aim of the present study was to investigate the profile of the above-mentioned microRNAs in HNSCC of different anatomical sites in relation to etiological factors and other clinicopathological features of these tumors.

Patients and Methods

Patients. The study patient population comprised of 51 patients with HNSCC treated from July 2009 to December 2012 at the Department of Otorhinolaryngology and Head and Neck Surgery, Faculty Hospital in Hradec Kralove, Czech Republic. The HNSCC groups included three sub-groups represented by histologically-verified squamous carcinomas of the oropharynx (n=23 patients), hypopharynx (n=4 patients) and larynx (n=24 patients). Patients included in the study were both smokers and non-smokers. Grading of tumors was obtained from pathological reports. Staging of tumors

Table I. *Selected microRNAs evaluated in the present study.*

MicroRNA (miR)	Principal effect of miR in cancerogenesis	Principal function mRNA of miR/target
let-7a	TS	Promotes differentiation of cells, represses CSC
miR-21	ONC	Promotes cell proliferation
miR-200c	TS	Regulates EMT/MET
miR-34a	TS	Induces apoptosis, cell cycle arrest, senescence
miR-375	TS/dual in distinct cancers	Regulates development of pancreas and glucose-induced insulin secretion

TS, Tumor suppressive; ONC, oncogenic; CSC, cancer stem cells; EMT, epithelial-mesenchymal transition; MET, mesenchymal-epithelial transition.

was evaluated according to pTNM Union for International Cancer Control (UICC) pathological staging criteria (30). All participants were informed on the research study and written inform consent was obtained from each patient. This work was approved by the Ethics Committee of the University Hospital in Hradec Kralove. Patients with other malignancies, inflammatory diseases or infections were excluded. Treatment decision-making was based on clinical status of patients and on grading and staging of tumors. All patients underwent surgery with resection of the tumor. The clinicopathological profile of the study populations is shown in Table II.

Tissue samples. Formalin-fixed, paraffin-embedded (FFPE) tissue samples from patients with HNSCC were prepared by standard laboratory technique and stored at room temperature until use. Samples, collected during a 4-year period (from 2009 to 2012), were processed at the Fingerland Institute of Pathology at the University Hospital in Hradec Kralove. Four-μm-thick paraffin sections were stained with hematoxylin and eosin and microscopically evaluated to ascertain regions suitable for macrodissection. Minimally, two FFPE tissue samples from each patient were histologically analyzed by a pathologist (M.L.) in order to find sites with cancer cells and sites of adjacent non-cancerous epithelial tissue. All selected areas destined for microRNA analysis were manually highlighted on hematoxylin and eosin stained slides. Thereafter, FFPE tissue samples were cut into 50 μm sections and parts of these sections, corresponding with previously highlighted areas, were separated. A total tissue area of approximately one cm^2 was used for RNA extraction. The estimation was performed in 51 paired (tumor and control) tissue samples of HNSCC.

RNA isolation. RNA was extracted from 50 μm FFPE sections following macrodissection of HNSCC tumor tissue and adjacent non-cancerous tissue using the miRNeasy FFPE Kit (Qiagen, Hilden, Germany).

Real-time quantitative PCR (qRT-PCR). A quantitative estimation of let-7a, miR-21, miR-200c, miR-34a, miR-375 and U6snRNA was performed by a qRT-PCR method using TaqMan® MicroRNA Assays (supplier, address). A two-step protocol requires reverse transcription with a miRNA-specific primer followed by a real-time PCR with TaqMan® probes (give more details and/or reference).

Table II. *The clinicopathological profile of the study population.*

Feature		Anatomic site					
	N	Oropharynx (n=23/45.1%)		Hypopharynx (n=4/7.8%)		Larynx (n=24/47.1%)	
		N	%	N	%	N	%
Sex							
Male	41	16	69.6	3	75.0	22	91.7
Female	10	7	30.4	1	25.0	2	8.3
Age							
≤60	28	15	65.2	3	75.0	10	41.7
>60	23	8	34.8	1	25.0	14	58.3
Mean	60.33		58.26		58.50		62.63
Median	60		57		60		61.5
Min-Max	45-85		45-70		53-61		46-85
Abuses							
Non-smoker	13	12	52.2	0	0	1	4.2
Smoker	38	11	47.8	4	100.0	23	95.8
TNM staging[a]							
I-II	14	4	17.4	0	0	10	41.7
III-IV	37	19	82.6	4	100.0	14	58.3
Histological grading							
G1-2	30	14	60.9	2	50.0	14	58.3
G3	21	9	39.1	2	50.0	10	41.7
Nodal status							
N0	24	6	26.1	0	0	18	75.0
N1-3	27	17	73.9	4	100.0	6	25.0
P16 status							
$P16^+$	17	17	73.9	0	0	0	0
$P16^-$	34	6	26.1	4	100.0	24	100.0
p-values				$p<0.0001$[b]			
Local recurrence/persistence							
No	43	18	78.3	3	75.0	20	83.3
Yes	8	3	21.7	1	25.0	4	16.7

LQ, Low quartile; UQ, upper quartile; SD, standard deviation. [a]UICC, 7th edition, [b]Chi-square test

The assays target only mature microRNAs, not their precursors. We used RNU6B (U6snRNA) as a normalizer. Each sample was assessed twice in parallel. The Ct values were corrected using calibrators for the elimination of differences between runs of the Stratagene Mx3000P Real-Time PCR apparatus (Agilent Technologies, Santa Clara, CA, USA). For the statistical analysis, we used the ddCt approach (2-Δ Δ CT algorithm; Applied Biosystems, Foster City, CA, USA).

Determination of p16 status. In our Hospital (Faculty Hospital in Hradec Kralove, Czech Republic), p16INK4a (hereafter denoted as p16) expression is routinely evaluated by immunohistochemistry within the field of activity of the histological examination of every HNSCC. Therefore, p16 status data are available as they were retrieved from hospital records. Immunohistochemistry was performed with the CINtec® Histology Kit (Roche mtm laboratories AG, Heidelberg, Germany). The p16 immunostaining was scored as follows: negative (0-50% tumor cells-nuclei and/or cytoplasmstained); positive (51-100% tumor cells-nuclei and/or cytoplasm-stained).

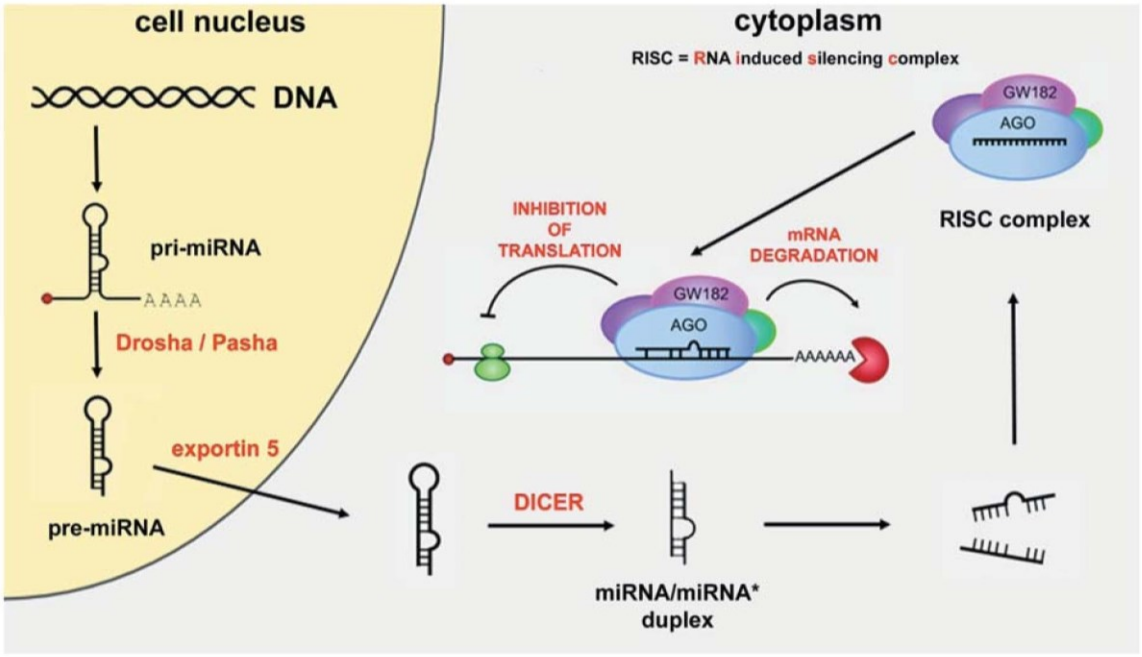

Figure 1. *Biogenesis of microRNA.*

Statistical analysis. Data analysis was performed using the SAS 8.02 software (SAS Institute Inc., Cary, NC, USA). The statistical results were calculated by the Wilcoxon non-parametric two sample test. Values of p lower than 0.05 were considered to be statistically significant in all analyses.

Results

The clinicopathological data of the HNSCC patients in the studied cohort are summarized in Table II. The expression levels of let-7a, miR-21, miR-200c, miR-34a and miR-375 were compared between site-specific sub-groups (oropharyngeal, hypopharyngeal and laryngeal) of HNSCC and between neoplastic and non-neoplastic tissues of the same patient. Comparison of the expression ratio of studied microRNAs with the clinicopathological and etiological characteristics of tumors was also performed.

Comparison of expression status of microRNAs between neoplastic and non-neoplastic tissues and between site-specific tumor subgroups. Comparing the expression values of the studied microRNAs between tumorous (irrespective to tumor site) and normal tissues of the same patient, we found statistically significant differences in miR-21 (p=0.0001) and miR-200 (p=0.0335), as well as in miR-375 (p=0.0020) expression values. The first two mentioned microRNAs were up-regulated, while the last one was down-regulated in the tumor tissue. All measured values of the expression of the studied microRNAs in tumorous and healthy tissues are summarized in Table III. MiR-34a (p=0.0155) was significantly up-regulated in oropharyngeal carcinomas when only comparing neoplastic and normal epithelial cells.

Statistically significant differences were recorded in the expression of let-7a (p=0.0242) and miR-200c (p=0.0378) between oropharyngeal and laryngeal cancers, in the expression value of miR-34a between oropharyngeal and laryngeal (Table IV), oropharyngeal and hypopharyngeal, as well as laryngeal and hypopharyngeal carcinomas (p=0.0178; p=0.0355; p=0.0317, respectively). In all cases, the highest expression levels of miR-34a were found in oropharyngeal cancers.

Expression tumor status of microRNAs in relation to clinico-pathological features of HNSCC patients. No correlation was observed between microRNA tumor expression and traditional clinicopathological factors, like sex and age. Furthermore, taking all HNSCC tumors together (irrespective to tumor site), statistically significant differences were found in tumor expression levels of let-7a and miR-200c between low stage (I-II) and high stage (III-IV) cancers (p=0.0204; p=0.0492, respectively), whereas higher tumor microRNA expression was associated with high HNSCC stage in both biomarkers. We also recorded significantly higher expression of miR-200c (p=0.0092) in less differentiated HNSCC (G3) and miR-375 (p=0.0231) in lymph node-positive (N1-3)

Table III. *Significant differences of microRNAs expression: tumor versus control non-neoplastic tissue.*

Group	N	miR-21				mir-200c				miR-375			
		25%	Med	75%	*p*-Value	25%	Med	75%	*p*-Value	25%	Med	75%	*p*-Value
HNSCC	51	6.62	20.1	52.5	0.0001	4.78	7.56	13.09	0.0335	0.02	0.06	0.29	0.0020
Control tissue	51	2.10	5.99	20.7		2.13	4.62	9.60		0.09	0.53	1.76	

Med, median.

laryngeal carcinomas. There were no significant differences in the miR-21, miR-34a, let-7a and miR-375 or miR-200c expression between low-grade and high-grade tumors, cancer lymph node status (N0 *versus* N1-3) and non-smoker and smoker patient groups. However, a significantly higher miR-21 (p=0.0005) and lower miR-375 (p=0.0010) expression in tumor than in healthy tissues was found in smokers but not in non-smokers.

HNSCC microRNAs expression in relation to p16 status. Comparing the p16 expression in site-specific head and neck tumors, p16 positivity has a statistically significant relationship to oropharyngeal cancer (p=0.0001) but not to hypopharyngeal and laryngeal tumors. Moreover, oropharyngeal carcinomas had a significant relationship between p16 expression and non-smoking (p=0.0428) and response to therapy (p=0.0018). Taking all HNSCC cases together, a statistically significant p16 tumor positivity was proven in patients with lymph node metastases (p=0.0173), in non-smokers (p=0.0001) and in tumors without recurrence/ persistence (p=0.0294). With respect to tumor site, the aforementioned significant relationship was confirmed in oropharyngeal tumors only (p=0.0018).

Down-regulation of miR-375 in HNSCC neoplastic cells was significantly associated with p16 tumor negativity (p=0.0038). Comparing all the site-specific p16 negative cancer sub-groups, the highest statistically significant miR-375 expression was found in oropharyngeal tumors (p=0.0441).

MiR-34a was significantly up-regulated in oropharyngeal tumors (p=0.0155) and in p16 positive carcinomas (p=0.0267). MiR-21, let-7a, miR-375 and miR-200c levels did not significantly correlate with p16 expression.

Discussion

HNSCCs represent about 6% of all cancer cases worldwide, with the majority being oropharyngeal and laryngeal squamous carcinomas (31). Smoking, alcohol abuse and human papillomavirus (HPV) infection have been acknowledged by the International Agency for Research against Cancer (IARC) as basic etiological factors of HNSCC (32). Generally, HNSCC is considered to be an aggressive neoplasm with unfavorable prognosis, despite the improvement of therapy in the last decades, and is often studied together as a single disease. However, behavior diversity of these tumors in different head and neck locations probably reflects miscellaneous pathways of cancerogenesis and specific intrinsic tumor properties, which are known from clinical practice (33). The locoregional lymph node metastasis is an important prognostic factor for these cancers. Moreover, many node-negative (N0) classified HNSCCs patients harbor occult neck node metastases (34). HNSCC remains difficult to be clinically managed. Therefore, a better understanding of the molecular pathobiology of head and neck squamous cell cancer is required. MicroRNAs represent new molecules regulating gene expression at the post-transcriptional level by the translational repression and/or degradation of target mRNA (35). MicroRNAs have distinct expression profiles in multiple pathophysiological conditions, including cancer. A number of studies on microRNA expression profiles of breast, prostate, colorectal, brain and other cancers have been so far published (18, 21, 35-37). However, little is known regarding the role of microRNAs in head and neck squamous carcinomas mainly with respect to their site of origin. Therefore, further studies of microRNA expression profile in site-specific HNSCC with potential clinical efficacy for diagnosis, prognosis and treatment seem to be fully substantiated (38). Certain studies have focused on microRNA expression profile of head and neck cancer (35, 39). The results seem to be inconclusive as various microRNAs were studied under different methods in different samples/cell lines and in different clinicopathological conditions. Although a vast number of de-regulated microRNAs have been reported, only a limited number of microRNAs share a common type of de-regulation pointing out to their important role in head and neck cancerogenesis and their potential prognostic function (35). In the study of Avissar *et al.* (2009), comparing neoplastic and normal tissues, it was shown that miR-21, miR-221, miR-18 were up-regulated in HNSCC tumors, whereas miR-375 was down-regulated (40). Moreover, the expression ratio of miR-221/miR-375 was introduced to be a promising diagnostic marker in distinguishing tumor from normal tissue. The same study revealed significant discrepancies in microRNA expression between cell lines and tumor tissues warning

Table IV. *Significant differences of microRNAs expression: oropharyngeal tumors versus laryngeal tumors.*

Group	N	let-7a				mir-200c				miR-34a			
		25%	Med	75%	*p*-Value	25%	Med	75%	*p*-Value	25%	Med	75%	*p*-Value
Oropharynx	23	1.96	3.40	5.37	0.0242	5.98	9.09	15.48	0.0378	0.64	1.01	1.64	0.0178
Larynx	24	0.84	1.72	3.05		4.35	6.75	8.75		0.19	0.43	0.90	

Med, median.

about limited utility of cell lines as a model system for the identification of clinically relevant microRNA biomarkers (40). MicroRNAs tested in the present study have been previously described to play crucial role(s) in carcinogenesis either in HNSCC or other cancer types.

In the present study, by comparing tumors to healthy tissues, we found significant differences in miR-21, miR-200c and miR-375 expression. Up-regulation of miR-21, a repeatedly confirmed finding, was first revealed in HNSCC (39-42). Down-regulation of miR-375 (being under-expressed by 9-fold compared to normal tissue in the present study) has also been reported in previous studies (40). Little is known about the deregulation of miR-200c in head and neck cancer, although its tumor-suppressive role in renal cell carcinoma, prostate, bladder, breast, pancreatic and gastric cancers has been described (35). Up-regulation of miR-200c was proven to inhibit the cancer stem cell-like properties and support MET by targeting the *ZEB1/ZEB2*, *BMI-1* and *SOX2* genes (23, 35). However, the role of miR200c has not been yet reported in the regulation of tumorigenicity and clinical behavior of HNSCC. MiR200c was significantly up-regulated and a considerable correlation of its over-expression with less differentiated cancers, but not with high-stage cancers, was also revealed in our recent study. However, this over-expression, with respect to its tumor-suppressive function, could be expected to be related to low-grade cancer. The small number of cases studied and incomplete elucidation of miR-200c interaction are shortcomings for better interpretation of these results.

Concerning the evaluation of microRNA expression profiles in different site-specific sub-groups of HNSCC and comparing them with each other, we found that let-7a, miR-200c and miR-34a revealed significant differences between oropharyngeal and laryngeal tumors. Surprisingly, miR-34a was significantly more up-regulated in oropharyngeal carcinomas. We are aware of only one study by Hui *et al.* that focused on microRNA profiling in subgroups of HNSCC (43). However, the authors did not find any differences in microRNAs expression of the oropharynx, the larynx and the hypopharynx. Besides miR-21 and miR-375, the other evaluated microRNAs were different from those explored in our study (43).

Taking all HNSCC cases together, we found significant differences between let-7a, as well as miR-200c expression, and stage of tumors. In laryngeal carcinomas, only miR-200c expression was higher and significantly associated with high stage of cancer, whereas miR-375 was associated with laryngeal lymph node-positive carcinoma. Generally used prognostic parameters, like disease free interval (DFI) and/or overall survival (OS), could not be evaluated in relation to microRNA expression levels due to relatively short follow-up in our study. Nevertheless, the monitoring of let-7a, miR-200c and miR-375 seems to have some potential prognostic significance. The prognostic role of selected microRNAs has been evaluated in HNSCC by several authors but the results seem to be heterogeneous and inconclusive for clinical practice (44).

Cancer pathobiology and the molecular differences between the anatomical locations and tumors of different etiology (viral, smoking or alcohol-induced) are important for developing new molecular targeted strategies. MicroRNA patterns are tissue-specific and may be influenced by the diversity of environmental factors.

Smoking-related cancers are the common object of microRNA studies, namely lung cancer. There exist different pathways of microRNA dysregulation: modification of microRNA gene expression followed by mutation or epigenetic regulation of distinct genes or by alteration of tumor suppressor or oncogenic microRNA function (45). Smoke-induced cancers show usually a bimodal microRNA expression profile with an initial down-regulation and a final up-regulation in advanced stages of cancer. In our study, we revealed significantly higher miR-21 (p=0.0005) and lower miR-375 (p=0.0010) expression in the tumor of smokers than in healthy tissues.

Alcohol represents one of the risk factors of HNSCC pathobiology. Alcohol has been described to contribute to dysregulation of microRNAs by several mechanisms, like the irritation of inflammation, interference with the absorption of folate and/or its degradation into carcinogenic acetaldehyde. Expression of miR-375 has been shown to increase with alcohol consumption in HNSCC (46). Our research group did not reveal any association between the studied microRNAs and alcohol use. However, it is necessary to take into consideration that clinical data in relation to drinking can be subject of variability.

The role of HPV/p16 in the regulation of microRNA expression in head and neck cancers is largely unknown (47). P16 positivity of HNSCC is considered to be a surrogate

marker of HPV etiology. However, other causes of p16 expression are also suggested. Clinical features and prognosis of HNSCC are greatly influenced by the HPV/p16 status, which is favorable in p16-positive tumors (48). The function of p16 seems to be more complex and differs between tumors of various locations. Therefore, the study of p16 role in tumors, in relation to its prognostic and therapeutic consequences, should be site-specific (49, 50). Oncogene-induced senescence represents a barrier against tumorigenesis. P16 is known as a key regulator of cellular senescence in cooperation with senescence-associated micro-RNAs. Namely, accumulation of miR-34a has been described during senescence in a range of cell types (25, 49, 51). The tumor-suppressive function of miR-34a has been reported in multiple cancers. Tumor suppressive effects consist of deregulation of oncogene expression and are mediated by changes in a number of target mRNAs, including *MYC*, cyclin B1 (*CCNB1*), *BCL-2*, *E2F3* and surivivin (*BIRC5*) (11, 25, 52, 53). In the majority of cancers, miR-34 is down-regulated and associated with tumor progression (11, 26). Ogawa *et al*. described miR-34 down-regulation to be associated with cis-diamminedichloroplatinum (CDDP) resistance of sinonasal squamous cell carcinoma (54). Contrary to the majority of cancers, miR-34 was over-expressed in our study, namely in p16-positive oropharyngeal carcinomas. This fact could be responsible for more favourable prognosis of these tumors described earlier (48). Unfortunately, the number of cases in our pilot study was limited. Therefore the studies of larger cohort of p16-positive oropharyngeal carcinomas are required to confirm miR-34a overexpression. The elucidation of its underlying pathobiological mechanisms and possible association miR-34a up-regulation with oncogene-induced senescence and clinical consequences are warranted.

Published articles on head and neck microRNA de-regulations are still scarce but expanding. However, since different conclusions have been formulated in reported studies, the use of microRNA profile of HNSCC in routine clinical practice cannot be currently asserted (42, 47, 55). Our study was performed using FFPE tissue samples. Although FFPE samples do not seem to be useful for mRNA, as well as DNA analysis due to their degradation during the fixation process and deterioration over time, the isolation of small-sized microRNA from these samples seems to be fully substantiated as micro-RNAs are known to be more stable (56). Moreover, the study of microRNAs in HNSCC should be more complex by taking many exogenous and endogenous aspects, including tumor location, into consideration. Nevertheless, based on our pilot study and the literary knowledge, we are convinced that microRNA profiling of HNSCC is behind the diagnostic, prognostic and therapeutic potential of these tumors. Therefore, microRNAs seem to potentially extend the family of tumor biomarkers in the future.

Conclusion

This study revealed that certain microRNA profiles are de-regulated in head and neck cancer (up-regulation of miR-21, miR-200c and miR-34a; down-regulation of miR-375) playing a potential role in the pathobiology of HNSCC. Significant differences of microRNA expression (let-7a, miR34a) between oropharyngeal and laryngeal cancers support the hypothesis of site-specific cancerogenesis in HNSCC. Up-regulation of miR34a expression in p16-positive oropharyngeal carcinomas has not been so far described. The role of miR-34a in carcinogenesis is more complex and remains to be elucidated. Additional microRNA expression studies in site-specific HNSCC in relation to prognosis, smoking and HPV infection/p16 status, respectively, are warranted.

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Conflicts of Interest

The Authors declare that there exist no conflicts of interest regarding this work.

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Tissue microRNAs as predictive markers for gastric cancer patients undergoing palliative chemotherapy

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Abstract. MicroRNAs have the potential to become valuable predictive markers for gastric cancer. Samples of biopsy tissue, routinely taken from gastric cancer patients undergoing palliative chemotherapy, constitute suitable material for microRNA profiling with the aim of predicting the effect of chemotherapy. Our study group consisted of 54 patients, all of whom underwent palliative chemotherapy based on 5-fluorouracil (5-FU) or 5-FU in combination with platinum derivatives between 2000 and 2013. The expression of 29 selected microRNAs and genes BRCA1, ERCC1, RRM1 and TS, in gastric cancer tissue macrodissected from FFPE tissue samples, was measured by quantitative RT-PCR. The relationship between gene expression levels and time to progression (TTP) and overall survival (OS) was analysed. From the set of the 29 microRNAs of interest, we found high expression of miR-150, miR-342-3p, miR-181b, miR-221, miR-224 and low levels of miR-520h relate to shorter TTP. High levels of miR-150, miR-192, miR-224, miR-375 and miR-342-3p related to shorter OS. In routinely available FFPE tissue samples, we found 6 miRNAs with a relation to TTP, which may serve as predictors of the effectiveness of palliative treatment in gastric cancer patients. These miRNAs could also help in deciding whether to indicate palliative chemotherapy.

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Introduction

Gastric cancer rates as the fourth most common malignancy worldwide and is the third most common cause of death from a malignant disease, after lung and liver cancer (1). Eastern Asia accounts for more than 50% of cases registered globally. A gradual decrease in the number of cases has been observed in regions such as the United States, Western and Central Europe (2,3).

In 2012, the incidence of gastric cancer in the Czech Republic was 14.6 cases per 100,000 inhabitants. Most cases are diagnosed in the late stages of the disease and only palliative treatment remains possible. Chemotherapy is often embarked upon as part of palliative treatment and most patients receive the same medications, overall survival varies greatly from patient to patient, however. One possible cause of this variability could be the gene expression changes occurring in cancer tissue, which may alter the effect of cytostatics. Evaluating the expression of specific genes including genes for microRNA (miRNA) could help single out chemotherapy efficacy predictors in gastric cancer patients undergoing palliative treatment (4,5). By identifying patients with chemoresistant tumors, we hope to spare them the strain of inefficient chemotherapy.

The tissue samples were the same as those used by the pathologists when making the diagnoses, to measure the levels of chemotherapy response predictors. The use of efficacy predictors makes up an important part of the development of new targeted therapy drugs and their introduction into clinical practice; this is no less the case for conventional chemotherapeutics.

The ability of cancer cells to overcome the effects of chemotherapy by changing the expression of repair genes, enzymes partaking in nucleic acid metabolism and genes involved in apoptosis limits the success of chemotherapy. Eukaryotic cells react to DNA damage by activating repair mechanisms with these key functions: DNA damage detection, stopping replication of damaged DNA, repair of damaged area if possible

Table I. Primer sequences for quantitative reverse transcription polymerase chain reaction (qRT-PCR) with the probes of Universal Probe Library.

Symbol	Gene name	Function of the product of this gene	Primer sequence 5'-3'	UPL probe
Reference genes				
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Enzyme of glycolysis.	AGCCACATCGCTCAGACAC GCCCAATACGACCAAATCC	60
HPRT	Hypoxanthine guanine phosphoribosyltransferase	Purine salvage pathway.	TGACCTTGATTTATTTTGCATACC CGAGCAAGACGTTCAGTCCT	73
Predictors of treatment response				
ERCC1	Excision repair cross-complementary group 1	Nucleotide excision repair pathway, catalyzes the 5' incision in the process of excising the DNA lesion. Studied as a predictor of platinum chemotherapy drugs.	GAAATTTGTGATACCCCTCGAC GATCGGAATAAGGGCTTGG	79
RRM1	Ribonucleotide reductase subunit M1	Subunit of an enzyme essential for the production of deoxyribonucleotides needed for DNA synthesis. Studied as a predictor of vinorelbine.	AAGCACCCTGACTATGCTATCC GTTATAGAGGTCTTCCATCACATCAC	71
BRCA1	Breast cancer 1	Protein involved in DNA repair of double-stranded breaks, and recombination. Studied as a predictor of platinum drugs.	TTGTTGATGTGGAGGAGCAA CAGATTCCAGGTAAGGGGTTC	11
TS	Thymidylate synthase	Methylation of deoxyuridylate to deoxythymidylate needed for DNA synthesis. Studied as a predictor of 5-fluorouracil and folate antimetabolites.	CCCAGTTTATGGCTTCCAGT GCAGTTGGTCAACTCCCTGT	43

or else the induction of apoptosis. Nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR) and homologous recombination repair (HRR) belong to the most important repair mechanisms of eukaryotic cells (6,7). Damage to these mechanisms means that mutations are passed on to the next cell generation. Impaired function of DNA repair mechanisms is thus linked to both ageing and cancerogenesis. However, increased activity of these mechanisms can hinder chemotherapy by forestalling further effective damage to DNA and thereby preventing the activation of apoptosis in proliferating tumor cells (8).

Cisplatin acts cytotoxically by creating adducts, which participate in crosslinking DNA, and in so doing activates programmed cell death. Decrease in the scale of damage to DNA, whether as a result of fewer adducts being created (in lower drug dosage, for instance) or because of their repair, can lead to a decrease of efficacy of this chemotherapeutic substance (9).

5-Fluorouracil (5-FU) is one of the most commonly used chemotherapeutics in gastric cancer treatment regimens (10). The primary site of action for 5-FU is thymidylate synthase (TS), an essential enzyme in *de novo* biosynthetic pathway of deoxythymidylate (dTMP). Thymidylate synthase catalyzes the reductive methylation of dUMP (deoxyuridine-5-prime monophosphate or deoxyuridylate) to dTMP (deoxythymidine-5-prime monophosphate or deoxythymidylate) using 5,10-methylenetetrahydrofolate as a cofactor. Maintaining a dTMP pool is crucial for DNA replication and repair.

We measured the levels of mRNA of the excision repair cross-complementary group 1 gene (ERCC1), ribonucleotide reductase subunit M1 gene (RRM1), breast cancer 1 gene (BRCA1) and TS, all of which participate in repair to damaged DNA. We determined their relationship to time to progression (TTP), using the definition of progression according to RECIST (response evaluation criteria in solid tumours) criteria (11), and overall survival (OS). Table I lists the basic characteristics of the genes of interest.

In addition to genes whose products are directly involved in DNA repair and nucleic acid metabolism, we also focused on gene products that seem to affect the expression of genes

involved in the above mentioned processes. MicroRNAs (also known as miRNAs or miRs), small non-coding RNA molecules 18-23 nucleotides in length, make up an immense group of regulatory molecules involved in carcinogenesis. The human genome may encode over 2,500 miRNAs, which may target ~60% of mammalian genes and are abundant in many human cell types (see miRNA database available online at www.mirbase.org). MicroRNAs participate in the post-transcriptional regulation of gene expression controlling development and maintain diverse cellular processes including proliferation, apoptosis, differentiation, motility and morphogenesis.

The effect of microRNA regulatory networks in cancer tissue can be oncogenic (by targeting tumor suppressor genes) or tumor-suppressive (by post-transcriptional repression of oncogenes) (12). However, the final effect of any particular miRNA is time and tissue dependent (13).

Many studies have described changes in expression of miRNAs and their involvement in carcinogenesis, tumor progression, invasion, metastasis and the effects of treatment in gastric cancer tissue. MicroRNAs may become valuable diagnostic markers and therapeutic targets for gastric cancer (14). Based on our research of published literature, we chose 29 miRNAs, which have a potential role in carcinogenesis or drug metabolism and therefore could be expected to influence the efficacy of treatment. A list of the main characteristics of miRNAs of interest is shown in Table II.

Materials and methods

Ethics statement. The present study was approved by the ethics committee of the University Hospital in Pilsen (decision from 11.7.2012 to the grant NT14227). Anonymised data were used to conduct this study.

Patients. This was a retrospective study. The patients were treated at the Complex Oncology Center of the University Hospital in Pilsen between 1st January 2000 and 30th June 2013. The inclusion criteria of this study were: patients with gastric cancer, with no gastric resection treatment, who underwent palliative chemotherapy only. We evaluated nearly 1,300 cases, all of which were treated at the Complex Oncology Center, but our inclusion criteria meant only 54 cases could be used in the present study. Stage of disease was determined using the TNM (tumor-node-metastasis) system of the International Union Against Cancer (IUCC, 7th edition) (15). All patients were in the fourth stage of the disease. Each diagnosis of gastric cancer was verified by a pathologist.

Tissue samples. Biopsy tissue samples, gathered, using endoscopy, from gastric cancer patients for diagnostic purposes prior to chemotherapy, were processed by standard laboratory techniques at the Institute of Pathology of the University Hospital in Pilsen, Czech Republic. FFPE tissue samples were stored at room temperature until use. Paraffin sections (4-μm thick) were stained with hematoxylin and eosin (H&E), microscopically verified by pathologists and examined in order to identify sites with cancer cells and sites of adjacent non-cancerous epithelial tissue suitable for macrodissection. Areas selected for expression analysis were highlighted manually.

RNA isolation. Total RNA (including microRNA) was extracted from 10-μm FFPE sections following macrodissection of tumor tissue and adjacent non-cancerous tissue using the miRNeasy FFPE kit (Qiagen, Hilden, Germany) as we previously described (16). The paired samples (tumor and adjacent non-cancerous tissue) were only available from 18 patients. The 10-μm sections corresponded to H&E representatives, on the areas highlighted for macrodissection.

Quantitative estimation of protein coding gene expression. Quantitative estimation of mRNA of selected genes (BRCA 1, ERCC1, RRM1 and TS) was performed by a real-time RT-PCR method with Universal Probe Library (UPL) probes (Roche, Mannheim, Germany). Reverse transcription was performed on 50 ng of total RNA with Superscript III reverse transcriptase (Life Technologies, Carlsbad, CA, USA) and random hexamers as primers. The sequences of primers and corresponding UPL probes generated by ProbeFinder software (Roche) are shown in Table I. The primers were synthesized by East Port Praha (Prague, Czech Republic). The quantitative estimation was performed in technical duplicates on Stratagene Mx3005P apparatus (Agilent Technologies, Santa Clara, CA, USA). The 20-μl PCR reactions included 1.0 μl of RT product, FastStart TaqMan Probe Master (Roche), 2.5 μl of each primer and 2.5 μl of UPL probe. The reactions were incubated in 96-well plates at 95˚C for 10 min and then followed by 48 cycles of 95˚C for 10 sec and 60˚C for 30 sec.

All the samples were also assessed for the expression of reference genes *GAPDH (*glyceraldehyde-3-phosphate dehydrogenase) and HPRT (hypoxanthine-guanine phosphoribosyltransferase). Due to generally low HPRT expression and small yield of RNA isolated from FFPE tissue (small tissue samples), we were unable to measure the expression of HPRT in all of our samples. Therefore, we did not use HPRT data for normalization along with GAPDH and total RNA.

Quantitative estimation of microRNA expression. A quantitative estimation of selected microRNAs (Table II) was performed by a RT real-time PCR method using TaqMan® MicroRNA assays (Applied Biosystems, Foster City, CA, USA) in technical duplicates on Stratagene Mx3005P apparatus (Agilent Technologies). A two-step protocol requires reverse transcription with a miRNA-specific primer, followed by a real-time PCR with TaqMan® probes. The assays target only mature miRNAs, not their precursors. We used RNU6B (U6snRNA) as a normalizer.

Processing of real-time PCR data. Samples were assessed in technical duplicates. The Ct values were corrected using calibrators to eliminate differences between the individual runs of the real-time PCR apparatus. In cases where there was a discrepancy between the results obtained from both technical duplicates, the sample assessment was repeated. The results are presented as normalized values as a ratio of the number of copies of the given gene to that of the reference gene. To obtain gene expression data we used the $\Delta\Delta$Ct approach ($2^{-\Delta\Delta Ct}$ algorithm).

Statistical analysis. The statistical analysis was performed using SAS 9.3 software (SAS, Institute Inc., Cary, NC, USA).

Table II. The analysed microRNAs and their involvement in the cancer process.

Symbol	miRBase accession no.	Role in gastric cancer/Prediction potential	Ref.
miR-15b	MIMAT0000417	Regulates cisplatin resistance and metastasis by targeting PEBP4 in lung adenocarcinoma cells	(40)
		Modulates multidrug resistance by targeting BCL2 in human gastric cancer cells	(41)
miR-16	MIMAT0000069	Associated with chemosensitivity in gastric cancer	(42)
		Modulates multidrug resistance by targeting BCL2 in human gastric cancer cells	(41)
miR-21	MIMAT0000076	Stimulates gastric cancer growth and invasion by inhibiting many tumor suppressors (PTEN, PDCD4)	(43)
		Confers cisplatin resistance in gastric cancer cells by regulating PTEN	(44)
miR-27a	MIMAT0000084	Functions as an oncogene in gastric adenocarcinoma by targeting prohibitin	(45)
		Potential biomarker for predicting resistance to fluoropyrimidine-based chemotherapy	(46)
miR-34a	MIMAT0000255	Inhibits the growth, invasion and metastasis of gastric cancer by targeting PDGFR and MET expression	(47)
		Regulates cisplatin-induced gastric cancer cell death by modulating PI3K/AKT/survivin pathway	(48)
miR-99a-3p	MIMAT0004511	Predicts fluoropyrimidine-based chemotherapy response in patients with advanced colorectal cancer	(49)
miR-101	MIMAT0000099	Downregulated in gastric cancer and involved in cell migration and invasion	(50)
		Enhances cisplatin sensitivity in bladder cancer cells	(51)
miR-106a	MIMAT0000103	Confers cisplatin resistance by regulating PTEN/Akt pathway in gastric cancer cells	(52)
		Induces multidrug resistance in gastric cancer by targeting RUNX3	(53)
miR-107	MIMAT0000104	Significantly dysregulated in gastric adenocarcinoma tissues	(54)
		Regulates cisplatin chemosensitivity of A549 non-small cell lung cancer cell line by targeting cyclin dependent kinase 8	(55)
miR-141	MIMAT0000432	Inhibits tumor growth and metastasis in gastric cancer	(56)
		Overexpression of miR-141 results in enhanced resistance to cisplatin in gastric cancer cells	(57)
miR-143	MIMAT0000435	Suppresses gastric cancer cell growth and induces apoptosis	(58)
		Involved in cisplatin resistance of gastric cancer cells via targeting IGF1R and BCL2	(59)
miR-145	MIMAT0000437	Suppress invasion-metastasis cascade in gastric cancer	(60)
		Reverses 5-FU resistance in tumor xenograft models	(61)
miR-150	MIMAT0000451	Promotes gastric cancer proliferation by negatively regulating the pro-apoptotic gene EGR2	(22)
		Reduces cisplatin chemosensitivity and promotes invasiveness of muscle-invasive bladder cancer cells	(62)
miR-181b	MIMAT0000257	Aberrantly overexpressed in gastric cancer cells and primary gastric cancer tissues	(26)
		Prognostic significance in gastric cancer patients treated with S-1/oxaliplatin or doxifluridine/oxaliplatin	(25)
miR-192	MIMAT0000222	miR-215/192 significantly upregulated in gastric cancer tissues from gastrectomy	(28)
		miR-192/miR-215 influence 5-FU resistance in colorectal cancer cell lines	(63)
miR-193a-3p	MIMAT0000459	Associated with precancerous lesions of gastric cancer	(64)
		Regulates the multi-drug resistance of bladder cancer	(65)
miR-202	MIMAT0002811	Inhibits the expression of γ-catenin and BCL-2; miR-202 has decreased expression in gastric cancer	(66)

Table II. Continued.

Symbol	miRBase accession no.	Role in gastric cancer/Prediction potential	Ref.
miR-206	MIMAT0000462	Suppresses gastric cancer cell growth and metastasis	(67)
		Inhibition of gastric cancer progression through the c-Met pathway	(68)
miR-211	MIMAT0000268	Associated with gastric cancers as potential biomarkers for gastric cancer diagnosis and treatment	(69)
		Downregulation of ribonucleotide reductase	(70)
miR-218	MIMAT0000275	Inhibits invasion and metastasis of gastric cancer	(71)
		Regulates cisplatin chemosensitivity in breast cancer by targeting BRCA1	(72)
miR-221	MIMAT0000278	miR-221/222 are encoded in tandem and they have the same seed sequence;	(33)
miR-222	MIMAT0000279	miR-221 and miR-222 regulate gastric carcinoma cell proliferation and radioresistance by targeting PTEN	
miR-224	MIMAT0000281	Promotes chemoresistance of lung adenocarcinoma cells to cisplatin	(73)
		5-FU chemosensitivity is significantly increased in miR-224 knockdown cells	(74)
miR-342-3p	MIMAT0000753	Upregulation is associated with chemosensitivity in gastric cancer	(42)
miR-372-3p	MIMAT0000724	Maintains oncogene characteristics by targeting TNFAIP1 and affects NF-κB signaling in human gastric carcinoma cells	(75)
miR-375	MIMAT0000728	Downregulated in gastric cancer, inhibits cell migration and invasion by targeting JAK2	(76)
		Predictive for response for non-small cell lung cancer treated with cisplatin-vinorelbine A	(77)
miR-509-3p	MIMAT0002881	Inhibits cell proliferation and migration by targeting CDK2, Rac1, and PIK3C2A	(78)
miR-575	MIMAT0003240	Significantly upregulated in gastric cancer	(79)
miR-520h	MIMAT0002867	Downregulates histone deacetylase 1 and so contributes to the chemotherapeutic effect of doxorubicin	(39)
		Controls ABCG2 level and thereby anticancer drug response	(80)

The statistical results were calculated by a Wilcoxon non-parametric two sample test. For the maximum hazard ratio (OS, TTP) the Cox regression hazard model was used. After finding an 'optimal cut off' given by the lowest P-value of log-rank test for the examined markers, the Kaplan-Meier survival distribution functions determined by the 'optimal cut off' in given groups and subgroups were generated.

Results

Prior to our analysis of the relationship between gene expression and TTP and OS, we compared gene expression in cancer and adjacent non-cancer epithelial gastric tissue. We found the expression of miR-221 in cancer tissue to be significantly higher, while the expression of miR-202 and miR-509 proved to be lower compared to healthy tissue (P-value 0.013, 0.011 and 0.018, respectively). However, when drawing conclusions from this analysis, we have to take into account the low number of paired samples, caused by the lack of non-cancerous tissue in some of the FFPE samples.

The Cox regression hazard model was used to determine the relation of marker level to OS or TTP. Results for all markers are summarized in Table III. From the set of the 29 microRNAs of interest, we found high expression levels of miR-150, miR 342-3p, miR-181b, miR-221, miR-224 and low levels of miR-520h related to shorter TTP. High levels of miR-150, miR-192, miR-224, miR-375 and miR-342-3p related to shorter OS. For markers with statistically significant results, optimal cut off values were chosen and Kaplan-Meier survival distribution functions for OS and TPP were generated.

The treatment regimen of all patients included 5-FU. We noted a definite correlation between high levels of miR-150 and miR-342-p in cancerous tissue and shorter TTP as well as OS. High expression of miR-224, however, only proved to have a relation to shorter OS (Table IV and Fig. 1).

In the subgroup of patients receiving 5-FU as the only treatment, we recorded a relation between high levels of miR-181b and shorter TTP and between high levels of miR-150, miR-192 and miR-342-p and shorter OS. In the subgroup treated with both 5-FU and cisplatin, we noted that high levels of miR-221, miR-224 and low levels of miR-520 related to shorter TTP and high levels of miR-221, miR-224 and miR-375 to shorter OS (Table V and Fig. 2).

Discussion

The present study focused on patients in advanced stages of gastric cancer. Therefore, these patients could not have the

Table III. Relation between level of given marker and TTP or OS (Cox regresion hazard model).

	All patients				5-FU alone				5-FU/cisplatin			
	OS		TTP		OS		TTP		OS		TTP	
Marker	P-value	HR	P-value	HR	P-value	HR	P-value	HR	P-value	HR	P-value	HR
ERCC1	0.9896	1.000	0.6452	0.547	0.2203	1.083	0.1464	1.103	0.2740	*	0.0679	*
RRM1	0.8615	*	0.8486	*	0.7395	*	0.7137	*	0.4886	*	0.1703	*
BRCA1	0.1658	17.340	0.4511	4.261	0.1881	*	0.2095	*	0.9996	*	0.4076	*
TS	0.3422	*	0.4118	*	**0.0524**	**0.985**	0.4693	*	0.5148	*	0.3000	*
miR-15b	0.3772	1.247	0.5953	1.199	0.2288	2.749	0.7050	1.380	0.2717	1.468	0.4768	1.297
miR-16	0.4178	1.003	0.7514	1.001	0.4816	1.003	0.6070	1.002	0.1216	1.053	0.1344	1.050
miR-21	0.5122	1.001	0.7040	1.001	0.5362	1.001	0.5916	1.001	0.4965	1.007	0.3586	1.011
miR-27a	0.1592	1.105	0.3201	1.072	0.1169	1.315	0.4536	1.141	0.1402	1.387	0.0716	1.568
miR-34a	0.5279	1.014	0.8185	1.005	0.5976	1.019	0.8735	1.006	0.0567	1.536	0.0806	1.626
miR-99a-3p	0.1951	*	0.7863	2.705	0.4441	*	0.7846	5.008	0.4712	*	0.7964	5.091
miR-101	0.3734	4.040	0.6222	2.142	0.3617	4.703	0.6637	2.110	0.9744	1.299	0.0966	*
miR-106a	0.4283	1.034	0.5075	1.028	0.4467	1.038	0.6843	1.022	0.4066	1.106	0.7731	1.039
miR-107	0.4980	1.829	0.6703	1.438	0.6056	1.713	0.9425	0.928	0.1013	*	0.1739	*
miR-141	0.2236	1.241	0.1042	1.295	0.0646	1.946	0.1968	1.609	0.7076	1.134	0.2205	1.460
miR-143	0.5144	1.002	0.8042	1.001	0.5847	1.002	0.6593	1.002	0.0619	1.395	0.0537	2.322
miR-145	0.4063	1.001	0.8176	1.000	0.5373	1.001	0.6376	1.001	0.0727	1.057	0.1059	1.121
miR-150	**0.0494**	**1.004**	**0.0056**	**1.006**	**0.0438**	**1.039**	0.0743	1.034	0.1311	1.004	0.2351	1.046
miR-181b	0.1406	1.061	0.0882	1.063	0.0564	1.130	**0.0333**	**1.777**	0.3327	1.185	0.2270	1.082
miR-192	0.7227	1.011	0.8569	1.006	**0.0233**	**1.200**	0.3684	1.080	0.8814	0.992	0.5269	0.959
miR-193a-3p	0.2323	4.798	0.7265	1.577	0.2401	7.129	0.5706	2.464	0.3158	*	0.0606	*
miR-202	0.3803	7.022	0.6739	0.320	0.4932	6.787	0.6010	4.384	0.1694	*	0.0946	*
miR-206	0.0594	*	0.4452	*	0.1810	*	0.2963	*	0.2441	*	0.1440	*
miR-211	0.1548	*	0.2151	*	0.1155	*	0.3240	*	0.5506	*	0.4309	*
miR-218	0.0639	*	0.6789	2.981	0.4176	*	0.0687	*	0.0961	*	0.0847	*
miR-221	0.1934	1.051	0.3627	1.034	0.6144	1.032	0.7147	1.023	**0.0160**	**2.438**	**0.0371**	**2.099**
miR-222	0.5086	1.001	0.6742	1.000	0.5679	1.001	0.6445	1.001	0.2627	1.012	0.2698	1.012
miR-224	**0.0175**	**7.609**	0.2724	2.620	0.1441	4.532	0.3603	2.602	**0.0283**	**322.120**	**0.0367**	**436.694**
miR-342-3p	**0.0286**	**1.261**	**0.0144**	**1.383**	**0.0443**	**2.516**	0.1531	2.077	0.1383	1.272	0.1641	1.692
miR-372-3p	0.6113	1.000	0.1651	7.159	0.5731	*	0.5128	*	0.2737	6.344	0.2863	6.080
miR-375	0.1968	*	0.9422	1.000	0.4974	1.001	0.9324	1.011	**0.0427**	**1.362**	0.8672	1.000
miR-509-3p	0.2446	3.507	0.6196	*	0.6909	*	0.5540	*	0.1246	*	0.1607	*
miR-575	0.1999	*	0.4849	*	0.4778	*	0.5664	*	0.6211	*	0.5531	*
miR-520h	0.1712	*	0.1190	*	0.3799	*	0.2106	*	0.0977	*	**0.0483**	**0.584**

*Clinically unrealistic hazard ratio (HR) values due to the coincidence of extreme marker level and extreme time to event in some cases.

tumors surgically removed and underwent palliative treatment only. The main clinical concern in such cases is deciding which chemotherapeutic regimen is indicated. The question was whether we could predict the effect of chemotherapy and thereby determine, if the aggressive chemotherapeutic treatment, which inevitably decreases quality of life, would prolong survival. If a low effect of treatment is predicted, it would be appropriate to offer to those patients inclusion in new ongoing studies.

In our laboratory assessment we used tissue samples taken gastroscopically for routine diagnostic purposes and macrodissected a sample of cancerous tissue, verified by a pathologist from FFPE sections, to analyse the expression of genes influencing the effects of therapy. This approach can be easily translated into clinical practice. We conducted expression analysis from a large number of tumor cells. Cancerous tissue is heterogeneous by nature, and originates in the process of clonal evolution, and therefore examining the collective

Table IV. Relation between level of given microRNA and TTP or OS (Kaplan-Meier estimation).

Marker	No. of patients	Cut-off	Patients below cut-off		Patients above cut-off		P-value
			N	Median (days)	N	Median (days)	
Time to progression (TTP)							
miR-150	40	45	37	113	3	26	0.0016
		6.7	23	138	17	90	0.0232
miR-342-3p	42	2.7	40	106.5	1	12	0.0006
		0.6	31	113	10	66	0.0997
Overall survival (OS)							
miR-150	41	45	38	215	3	69	0.0020
		6	21	424	20	172.5	0.0145
miR-342-3p	42	0.45	25	304	17	170	0.0319
miR-224	41	0.048	15	494	26	175	0.0090

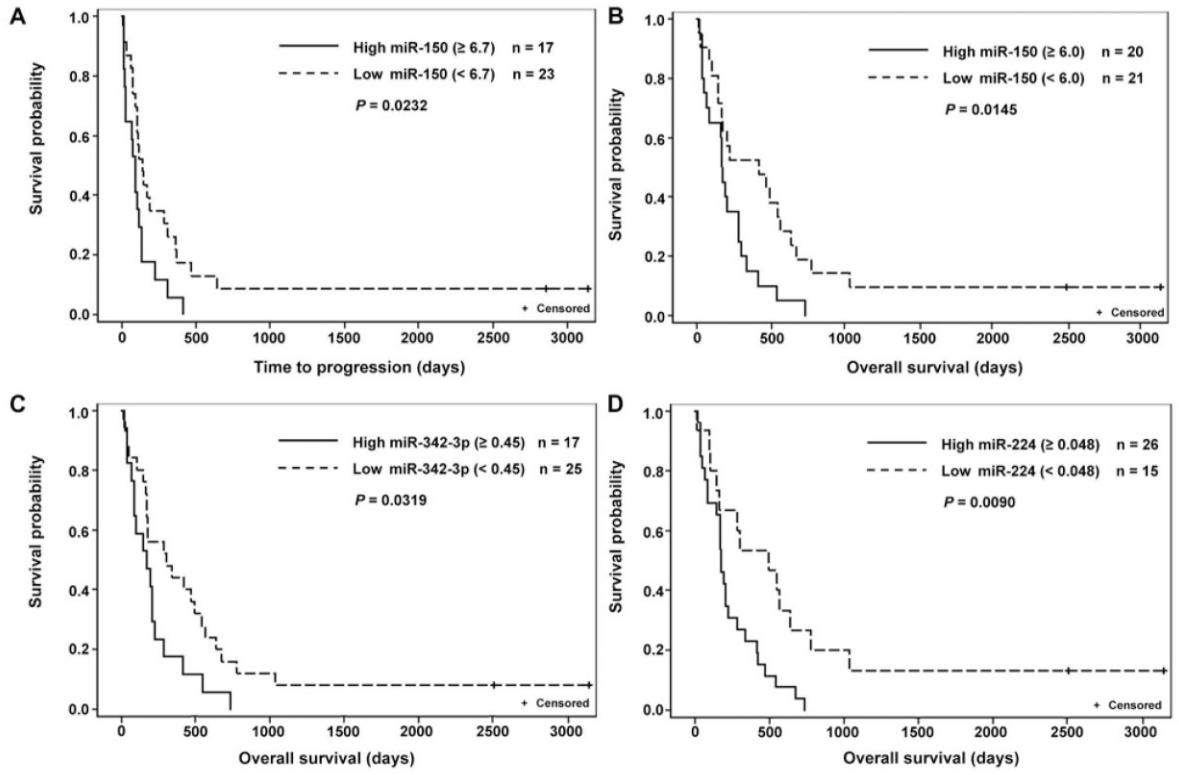

Figure 1. Relation of microRNA expression to time to progression (TTP)/overall survival (OS) in all gastric cancer patients (Kaplan-Meier curves).

changes in expression of a variety of cancer cell types (gathered by macrodissection) provides a more complex insight regarding prognosis. We set out to ascertain the prognostic potential of chosen miRNAs, which influence apoptosis and cell proliferation and in so doing interact with the mechanism of chemotherapy indicated in the cases we examined. However, we could not leave out monitoring protein coding genes frequently investigated as possible treatment outcome predictors.

Many studies have noted the prognostic value of low ERCC1 expression in gastric cancer patients undergoing chemotherapy; a meta-analysis published in 2015 concluded ERCC1 may be a useful prognostic factor for gastric cancer and furthermore that low mRNA levels of ERCC1 appear to be associated with a significant OS benefit to patients treated with platinum-based chemotherapy (17). However, the predictive value of the ERCC1 gene for survival and response to platinum-based chemotherapy in gastric cancer remains

Table V. Relation between level of given marker and TTP or OS based on the treatment (Kaplan-Meier estimation).

Marker	No. of patients	Cut-off	Patients below cut-off		Patients above cut-off		P-value	Relation to OS/TTP
			N	Median (days)	N	Median (days)		
Treatment								
5-fluorouracil								
TS	14	0.008	11	282	3	547	0.0226	OS
miR-150	23	6.300	12	424	11	170	0.0099	OS
miR-181b	21	0.260	2	13.5	19	147	0.0038	TTP
miR-192	24	2.300	16	339	8	39	0.0001	OS
miR-342-3p	24	0.600	18	282	6	62.5	0.0141	OS
5-fluorouracil/cisplatin								
miR-221	10	0.600	4	732	6	129.5	0.0038	OS
	10	1.500	4	262.5	6	67	0.0356	TTP
miR-224	9	0.150	5	684.5	4	84	0.0049	OS
	9	0.150	5	108	4	43	0.0027	TTP
miR-375	9	26.000	5	637	4	76.5	0.0027	OS
miR-520h	10	40.000	8	88	2	563.5	0.0265	TTP

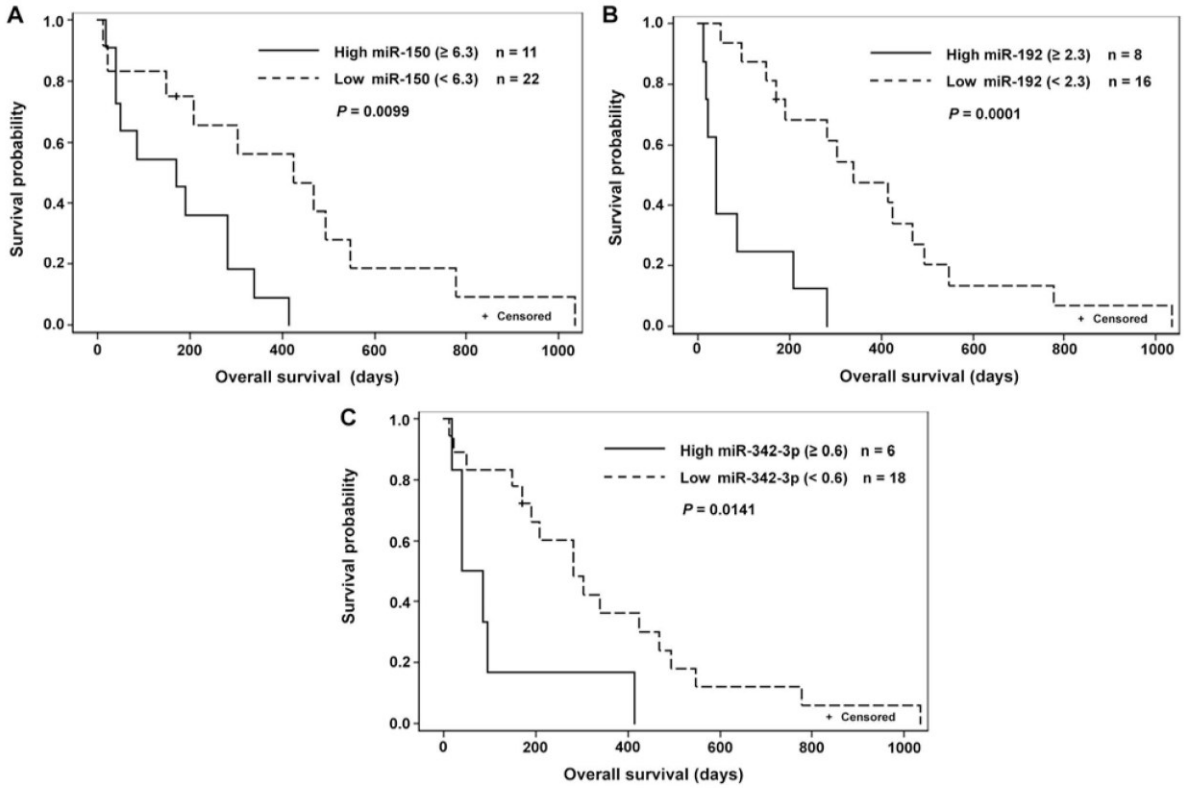

Figure 2. Relation of microRNA expression to overall survival (OS) in the subgroup of gastric cancer patients receiving 5-fluorouracil (5-FU) as the only treatment (Kaplan-Meier curves).

controversial (18). We found no statistically significant relation between the levels of ERCC1 and survival. It is possible our results reflect our test group, as in all cases we examined 5-FU was part of the treatment regimen but platinum-based

chemotherapy was only used in a subgroup, whose analysis was affected by the small number of patients.

The very nature of the TS enzyme makes its expression the most commonly examined in relation to 5-FU chemotherapy. We determined high levels of TS predict longer OS (Table V). Similar results were published by Wei *et al* (19), who conducted their analysis on a group of patients treated in the same way and also used RT-PCR to assess TS expression. On the other hand, some studies have shown high levels of TS to have the opposite effect (20).

From the set of the 29 microRNAs of interest, we found that high expression levels of miR-150, miR-181b, miR-192, miR-221, miR-224, miR 342-3p, miR-375 and low expression of miR-520h relate to unfavourable outcomes for patients (shorter TTP or OS) Table III.

MicroRNAs have the potential to become accurate, easily measurable biomarkers, with features fortuitous for diagnostic testing methods, such as stability in FFPE tissue, blood and perhaps other bodily fluids (21).

Wu *et al* (22) found miR-150 was overexpressed in gastric cancer cell lines and tissue samples and demonstrated overexpression of miR-150 could promote proliferation and growth of cancer cells by targeting the tumor-suppressor EGR2. In undifferentiated gastric cancer, higher miR-150 levels appeared to be associated with shorter postoperative patient survival, however, miR-150 was deemed to be an insufficiently independent prognostic factor in these cases (23). In the study of Chen *et al* (24), miR-150 showed decreased expression in gastric cancer patients compared to healthy test subjects. In the present study, higher levels of miR-150 showed a relation to shorter TTP and OS (Table IV; Figs. 1A and B and 2A).

Compared to normal gastric tissue samples, there is an overexpression of miR-181b in gastric tumors. Lower levels of miR-181b relate to longer OS of patients on regimens based on 5-FU and platinum derivatives (25). Furthermore, overexpression of miR-181b was found to downregulate the tissue inhibitor of metalloproteinases 3 (TIMP3) (26). Our results hint at the association of higher miR-181b levels to shorter TTP of patients treated with 5-FU (Table V). The results of another study show the ambiguous nature of the effects of certain miRNA; Chen *et al* (27) observed miR-181b was downregulated in human gastric adenocarcinoma tissue samples compared to adjacent normal gastric tissue and also described how miR-181b could suppress tumor cell proliferation by downregulating the expression of cAMP responsive element binding protein 1 (CREB1).

Our analysis of miR-192 levels showed its high expression related to shorter OS in the group of patients treated with 5-FU (Table V and Fig. 2B). To the best of our knowledge, no other study dealing with the predictive value of miR-192 in gastric cancer patients treated with 5-FU has been published. Xu *et al* (28) found miR-192 to be upregulated in gastric cancer tissue samples obtained by gastrectomy. The upregulation of both miR-192 and miR-215 was related to clinical characteristic such as lymph node metastases, while the inhibition of miR-215 or miR-192 significantly decreased gastric cancer cell invasion. The results reported by Chen *et al* (29) demonstrate that elevated circulating miR-192 has the potential to improve the early detection of distant metastases of GC.

Recently published studies show that miR-221 is an oncogenic microRNA involved in several malignancies (30,31). We found higher miR-221 expression in tumor samples in comparison to adjacent noncancerous tissue. This is in accordance with Liu *et al* (32) who found miR-221 was upregulated in 88% of gastric cancer tissue samples. Moreover, we observed a relation of high miR-221 expression to shorter TTP and OS in 5-FU monotherapy treated patients (Table V). The influence of miR-221 on the effect of chemotherapy is corroborated by published experiments conducted on the human gastric cancer cell line SGC7901 showing the knockdown of miR-221 inhibited cell growth and invasion and increased the radiosensitivity of the cells (33).

We found higher levels of miR-224 indicate shorter OS (Table IV and Fig. 1D). Mao *et al* (34) concluded that miR-224 is overexpressed in human gastric cancer cells. Reducing the expression of miR-224 can effectively inhibit growth and promote apoptosis of gastric cancer cells. These results are also supported by the study of Liu *et al* (35) who investigated the expression of miR-224 in the human gastric cancer cell line SGC-7901. In examining the effects of miR-224 mimics, they observed miR-224 could negatively regulate the expression of Raf-1 kinase inhibitor protein (RKIP). RKIP contributes to the suppression of proliferation and invasion of gastric cells.

We determined higher levels of miR-342-3p correlated to shorter TTP and OS in 5-FU monotherapy treated patients (Table V and Fig. 2C); similar results were described in colorectal cancer. High levels of miR-342-3p were associated with shorter survival time (36). Kim *et al* (37) screened miRNAs associated with response to chemotherapy using microarrays and found miR-342-p belongs to the miRNAs, whose upregulation is associated with chemosensitivity in gastric cancer.

Our observations of the relation of high miR-375 expression to shorter OS in 5-FU monotherapy treated patients (Table V) could be explained by the findings of Liu *et al* (38), who showed that miR-375 downregulated p53 expression through an interaction with the 3' UTR region of p53. In addition, they observed the expression of miR-375 desensitized cells to ionizing radiation and etoposide.

We demonstrated that higher levels of miR-520h correlated to longer TTP in 5-FU and cisplatin therapy treated patients (Table V). Shen *et al* (39) found that miR-520h downregulates histone deacetylase 1 and, thus, contributes to the chemotherapeutic effect of doxorubicin.

Summarizing the aforelisted results, amongst the miRNAs we examined, we found six miRNAs (miR-150, miR-181, miR-221, miR-224, miR-342-p and miR-520h) with a relation to TTP, which could serve as predictors of the effectiveness of treatment. These results merit multifactorial analysis, which we were, however, unable to perform due to the limited number of samples.

In our experience, microRNAs can generally be assessed with more precision and ease than mRNA of coding genes. This is essentially due to the fact that miRNA analysis is less demanding in terms of both quality and quantity of isolated RNA, features problematic in samples of RNA extracted from FFPE tissue. FFPE tissue samples are routinely taken and analysed during standard gastric cancer management and

that is why we believe microRNAs could become clinically applicable predictors of the effectiveness of palliative treatment in gastric cancer patients.

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Predictive relevance of miR-34a, miR-224 and miR-342 in patients with advanced squamous cell carcinoma of the lung undergoing palliative chemotherapy

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Abstract. Attributing to their pathophysiological role and stability in biological samples, microRNAs (miRNAs) have the potential to become valuable predictive markers for non-small cell lung cancer (NSCLC). Samples of biopsy tissue constitute suitable material for miRNA profiling with the aim of predicting the effect of palliative chemotherapy. The present study group included 81 patients (74 males, 7 females, all smokers or former smokers) with the squamous cell carcinoma (SCC) histological subtype of NSCLC at a late stage (3B or 4). All patients received palliative chemotherapy based on platinum derivatives in combination with paclitaxel or gemcitabine. The expression of 17 selected miRNAs was measured by reverse transcription-quantitative polymerase chain reaction in tumor tissue macrodissected from formalin-fixed paraffin-embedded (FFPE) tissue samples. To predict the effect of palliative chemotherapy, the association between gene expression levels and overall survival (OS) time was analyzed. From the 17 miRNAs of interest, low expression levels of miR-342 and high expression levels of miR-34a and miR-224 were associated with a reduced OS time in subgroups of patients based on smoking status and treatment modality. Using cluster analysis, associations between combinations of miR-34a, -224 and -342 expression levels with patient survival were identified. The present study revealed that patients with the simultaneous high expression of miR-224 and -342 had a similar prognostic outcome to those with the low expression of miR-224 and -342, which was significantly reduced, compared with patients exhibiting high expression of either miR-224 or miR-342 with low expression of the other. We hypothesize that the effect of a particular miRNA is dependent on the expression level of other members of the miRNA network. This finding appears to complicate survival analyses based on individual miRNAs as markers. In conclusion, the present study provides evidence that specific miRNAs were associated with OS time, which may be candidate predictors for the effectiveness of palliative treatment in SCC lung cancer patients. This objective can be better achieved by combining more markers together than by using individual miRNAs.

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Key words: microRNA, lung cancer, palliative treatment, biomarkers

Introduction

Lung cancer is the most common type of cancer, with high mortality rates worldwide (1); the incidence in the Czech Republic was 86.9 cases in men and 38.0 in women per 100,000 people in 2011 (2). Approximately 85% of all lung cancer cases are non-small cell lung cancer (NSCLC), which includes two major histological subtypes: Squamous cell carcinoma (SCC) and adenocarcinoma. SCC represents ~25-30% of cases of NSCLC (3). The prognosis for patients with advanced SCC is poorer than that of those with adenocarcinoma (4).

Chemotherapy is an essential modality of palliative treatment for inoperable SCC at advanced stages. The response rate to chemotherapy varies widely from patient to patient; therefore, it is of interest to find biomarkers that predict the effect of cytostatic therapeutics. The resistance of cancer cells to chemotherapy can be caused by the increased export of anticancer drugs out of the cells, improved DNA repair ability or apoptosis resistance (5). The expression of genes participating in these processes is regulated by the microRNA (miRNA/miR) network. miRNAs are small non-coding RNA molecules of ~22 nucleotides that participate in the post-transcriptional regulation of gene expression (6). The human genome encodes >2,500 miRNAs (7), which target ~60% of mammalian genes and are abundant in a number of human cell types (8) (see miRNA database available online at www.mirbase.org).

The aim of the present study was to evaluate the association of the expression of miRNAs involved in the processes resulting in chemotherapy resistance with the overall survival (OS) time of patients with advanced SCC receiving palliative

care. All patients in the cohort of the present study received palliative chemotherapy based on platinum derivatives (cisplatin or carboplatin) in combination with paclitaxel or gemcitabine.

On the basis of previously published literature, the present study focused on miRNAs whose effect on the processes involved in chemotherapy resistance may be expected (miR-15b, miR-21, miR-27a, miR-34a, miR-99a, miR-106a, miR-107, miR-143, miR-150, miR-192, miR-193, miR-211, miR-218, miR-221, miR-224, miR-342 and miR-375). A list of the main characteristics of miRNAs of interest, including references, is included in Table I.

Patients and methods

Ethics statement. This study was approved by the Ethics Committee of the University Hospital in Pilsen (Pilsen, Czech Republic). Written informed consent was obtained from all the subjects. Anonymized data were used to conduct the present study.

Patients. The present study was retrospective. The study group consisted of 81 patients with late-stage (3B or 4) and the SCC histological subtype with an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0-2 of NSCLC treated between January 2000 and June 2014 at the Department of Pneumology and Phthisiology of the University Hospital in Pilsen. Stage of disease was determined using the TNM (Tumor Nodus Metastasis) system of the International Union Against Cancer (IUCC; 7th edition) (9). The median patient age was 62.4 years (range, 32.7-79.3 years), and there were 74 males and 7 females. All patients underwent palliative chemotherapy using platinum derivatives in combination with paclitaxel or gemcitabine. The use of sequential radiotherapy was permitted for patients with stage 3B disease; patients with concurrent radiotherapy were excluded from the present study. In certain patients with stage 3B disease, radiotherapy was not indicated due to poor PS. The exclusion criteria for entering the study were >80 years of age, other malignancy and high cardiopulmonary risk. Clinicopathological data, including age at the time of diagnosis, smoking status, clinical disease stage, radiotherapy status and chemotherapy regimen, are listed in Table II.

Tissue samples and RNA isolation. Biopsy tissue samples were obtained using bronchoscopy for diagnostic purposes prior to chemotherapy and were processed by standard laboratory techniques at the Department of Pathology of the University Hospital in Pilsen. Formalin-fixed paraffin-embedded (FFPE) tissue samples were stored at room temperature until use. Paraffinized sections were stained with hematoxylin and eosin, microscopically verified by pathologists and examined to identify sites with cancer cells for macrodissection. Total RNA (including miRNA) was extracted from 15-μm thick FFPE sections following the macrodissection of tumor tissue using the miRNeasy FFPE kit (Qiagen, Hilden, Germany) as described previously (10).

Quantitative estimation of microRNA expression. A quantitative estimation of 17 selected miRNAs (Table I) was performed by the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) method using TaqMan® MicroRNA assays (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in technical duplicates on the Stratagene Mx3005P apparatus (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's protocol. The two-step protocol included reverse transcription with a miRNA-specific primer, followed by qPCR with TaqMan® probes. Briefly, 5 ng of RNA was reverse transcribed in a 20-μl reaction containing 2.5 μl of primers specific to particular miRNA (Applied Biosystems; Thermo Fisher Scientific, Inc.). The 20-μl PCR reactions included 2.5 μl of RT product. The reactions were incubated in 96-well plates at 95˚C for 15 min and then followed by 48 cycles of 95˚C for 15 sec and 60˚C for 60 sec. The $2^{-\Delta\Delta Cq}$ method was used for the quantification of qPCR data, as described previously (11); the expression was normalized to RNU6B (U6snRNA). Details of all kits for estimation of all miRNAs and RNU6B are included in Table I.

Statistical analysis. SAS version 9.3 statistical software (SAS Institute, Inc., Cary, NC, USA) was used to perform all statistical calculations. Although the group of patients in the present study was as homogenous as possible (SCC subtype of NSCLC, stages 3B and 4), certain patients underwent radiotherapy, which may have caused confounders. The potential effect of treatment inconsistency was mitigated by evaluating miRNA expression in the subgroups of patients.

The evaluation of prognostic significance (the association between markers and time to recurrence) was performed as a univariate analysis of maximum likelihood estimates using the Cox regression hazard model. For markers significant in the Cox model, an optimal cut off was identified. There is no standard method for biomarker cut-off determination to split continuous variables into two groups; the simplest approach used in exploratory studies is to set the median as a cut-off value. However, in the case of unequal distribution of events in the studied group, this approach is far from optimal. The present study used continuous search for the cut-off value by searching for the lowest P-value of the log-rank test, as described previously (12,13).

miRNAs identified as significant by univariate analysis were incorporated into a multivariate analysis and the Kaplan-Meier survival distribution functions were generated for combinations of miRNA expression levels (clustering) with the cut-off values from univariate analysis and later, the median. $P<0.05$ was considered to indicate a statistically significant difference. DIANA-TarBase v7.0 and DIANA-miRPath v3.0 bioinformatic tools were used to identify overlapped target genes of the miRNAs of interest (14,15).

Results

Effect of treatment modalities on OS time. Prior to the analysis of the association between gene expression and OS, the outcomes for subgroups of patients undergoing different treatment were compared. A significantly longer OS time was identified in the subgroup of patients who underwent chemotherapy combined with radiotherapy in comparison with patients who underwent chemotherapy alone (P=0.0498; Fig. 1A). There were no significant differences in OS between

Table I. Analyzed miRNAs and their involvement in pathogenesis and treatment of NSCLC.

Symbol	miRBase accession no.	Cat. no. 4427975 assay ID	Relation to NSCLC	(Refs.)
miR-15b	MIMAT0000417	000390	Regulates cisplatin resistance and metastasis by targeting PEBP4 in lung adenocarcinoma cells	(35)
miR-21	MIMAT0000076	000397	Regulates NSCLC cell invasion and chemo-sensitivity through SMAD7	(36)
miR-27a	MIMAT0000084	000408	Higher expression levels in advanced NSCLC patients resistant to EGFR-TKI	(37)
miR-34a	MIMAT0000255	000426	Sensitizes lung cancer cells to cisplatin via p53/miR-34a/MYCN axis	(38)
miR-99a-3p	MIMAT0004511	002141	Promotes proliferation, migration and invasion of NSCLC cell lines	(39)
miR-106a	MIMAT0000103	000578	Confers cisplatin resistance in non-small cell lung cancer A549 cells	(40)
miR-107	MIMAT0000104	000443	Regulates cisplatin chemosensitivity of A549 non small cell lung cancer cell lines by targeting cyclin dependent kinase 8	(41)
miR-143	MIMAT0000435	002249	Regulates cell apoptosis in lung cancer by targeting PKCε	(42)
miR-150	MIMAT0000451	000473	Downregulation induces cell proliferation inhibition and apoptosis in NSCLC by targeting BAK1	(43)
miR-192	MIMAT0000222	000491	Regulates chemo-resistance of lung adenocarcinoma for gemcitabine and cisplatin combined therapy by targeting Bcl-2	(44)
miR-193a-3p	MIMAT0000459	002250	Suppresses the metastasis of NSCLC by downregulating the ERBB4/PIK3R3/mTOR/S6K2 signaling pathway	(45)
miR-211	MIMAT0000268	000514	Promotes NSCLC proliferation by targeting SRCIN1	(46)
miR-218	MIMAT0000275	000521	Regulates cisplatin chemosensitivity in NSCLC by targeting RUNX2	(47)
miR-221	MIMAT0000278	000524	Overexpressed in aggressive NSCLC and regulates TRAIL resistance through PTEN and TIMP3	(48)
miR-224	MIMAT0000281	002099	Is implicated in lung cancer pathogenesis through targeting caspase-3 and caspase-7	(19)
miR-342-3p	MIMAT0000753	002260	Suppresses proliferation and invasion of NSCLC by targeting RAPB2	(21)
miR-375	MIMAT0000728	000564	Predictive for response for non-small cell lung cancer treated with cisplatin-vinorelbine A	(49)
RNU6B	-	001093	Reference gene	(50)

miR/miRNA, microRNA; NSCLC, non-small cell lung cancer.

subgroups of patients with different chemotherapy regimens (Fig. 1B).

Association of miRNA expression with OS time. The Cox regression hazard model was used to determine the association between the levels of miRNA expression with OS time. From the 17 miRNAs of interest, in the subgroup of smokers, the low expression of miR-342 (P=0.0500) and high expression level of miR-34a and miR-224 (P=0.0338 and P=0.0400, respectively) were associated with a shorter OS time. High expression levels of miR-34a were associated with shorter OS time in the subgroup of patients treated with platinum derivate-based chemotherapy in combination with gemcitabine (P=0.0364). High expression levels of miR-224 were associated with shorter OS time in the subgroup of patients who underwent chemotherapy combined with radiotherapy (P=0.0250).

For the statistically significant miRNA markers, optimal cut-off values were identified and Kaplan-Meier survival distribution functions for OS were generated. Statistically significant differences in OS time between subgroups with marker expression levels below and above the cut-off value were obtained for miR-342 in the subgroup of smokers (P=0.0243; Fig. 2A), miR-34a in a subgroup of patients that were treated with gemcitabine in chemotherapy regimen (P=0.0239; Fig. 2B) and miR-224 in the subgroup of patients that underwent chemotherapy combined with radiotherapy (P=0.0093; Fig. 2C). Statistical values obtained from the Kaplan-Meier analyses are summarized in Table III.

miRNAs associated with OS (miR-34a, -224 and -342) were the subject of the subsequent cluster analysis. Pairs of these miRNAs (miR-34a and -224, miR-34a and -342, and miR-224 and -342) were analyzed for their association with

Table II. Clinicopathological characteristics of patients with squamous cell carcinoma of the lung (n=81).

Characteristic	Patients, n (%)
Sex	
Male	74 (91.4)
Female	7 (8.6)
Age, years	
<55	11 (13.6)
55-65	41 (50.6)
>65	29 (35.8)
Smoking status	
Non-smoker	0 (0)
Ex-smoker	42 (51.9)
Smoker	39 (48.1)
Clinical stage	
3B	42 (51.9)
4	39 (48.1)
Eastern Cooperative Oncology Group performance status	
0	2 (2.5)
1	58 (71.6)
2	18 (22.2)
3	3 (3.7)
Radiotherapy	
Yes	25 (30.9)
No	56 (69.1)
Chemotherapy	
Paclitaxel and carboplatin	35 (43.2)
Gemcitabine and cisplatin	46 (56.8)

OS time. For each pair of miRNAs, patients were stratified into groups according to the miRNA expression being above (high) or below (low) the cut-off value: Group A (high miR-224 and high miR-342); group B (high miR-224 and low miR-342); group C (low miR-224 and high miR-342); and group D (low miR-224 and low miR-342). Initially, the cut-off value obtained from univariate analysis was used also for cluster analysis; however, this led to a highly disproportional distribution of patients among subgroups. Therefore, a median was used as a cut off value for cluster analysis.

Fig. 3 demonstrates the Kaplan-Meier survival distribution functions of patients stratified into groups according to the expression of miR-224 and miR-342. There are two pairs of groups with similar OS distributions; Fig. 4 includes a comparison of the OS of two groups of patients created by combining the groups from Fig. 3 with similar survival outcomes (group A/D vs. group B/C). There was a significant difference in survival between these groups (P=0.0018), as detailed in Table IV. The same approach was used to analyze the other pairs of miRNAs; however, no significance was identified. All three miRNAs were analyzed together in the same manner (miR-34a, -224 and -342). Patterns of expression associating patients with significantly shorter survival times were identified (Fig. 5; Table IV).

Identification of potential target genes for miR-34a, -224 and -342. Using DIANA-TarBase v7.0 and DIANA-miRPath v3.0 bioinformatic tools (11,12), 6 overlapping target genes with P<0.05 were identified between miR-34a, -224 and -342. These genes, including GNAS complex locus (GNAS), insulin like growth factor 1 receptor (IGF1R), cyclin D1 (CCND1), cyclin G2 (CCNG2), serpin family E member 1 (SERPINE1) and ribonucleotide reductase regulatory subunit M2 (RRM2), are associated with cell cycle regulation, p53 signaling and DNA repair.

Discussion

miRNAs may have the potential to become accurate, easily measurable biomarkers, with features convenient for diagnostic testing methods, including stability in FFPE tissue blocks, blood, and potentially, other bodily fluids (16). The present study focused on patients with the NSCLC SCC subtype with an advanced-stage SCC. The included patients were unable to undergo surgical resection and received palliative treatment only. For these patients, there were multiple treatment modalities. The main clinical concern in such cases is deciding which therapeutic regimen is indicated. However, in the group of patients in the present study, it was only possible to analyze the potential predictors for the treatment response to platinum base derivatives in combination with either paclitaxel or gemcitabine, with or without the application of radiotherapy.

Initially, the present study focused on a univariate analysis of the association between miRNA expression and OS time. Subsequently, a multivariate analysis was performed that included the miRNAs that had been identified to exhibit associations with OS. On the basis of the results of the present study, we hypothesize that the effect of a single miRNA may depend on the level of expression of other members of the miRNA network, to be further discussed.

Higher levels of miR-224 indicated shorter OS times for patients with chemotherapy combined with radiotherapy in the present study. Cui *et al* (17) reported that miR-224 expression was significantly upregulated in NSCLC tissues and suggested it performed its oncogenic role in lung cancer pathogenesis through targeting caspase-3 and -7. Wang *et al* (18) identified through microarray analysis that miR-224 expression was upregulated in cisplatin-resistant cell lines, and demonstrated that miR-224 could promote cisplatin resistance via regulating the G_1/S cell cycle transition and apoptosis by targeting p21. These findings indicated the association of miR-224 with the effect of chemotherapy based on DNA damage, and its potential as a predictor for the response to treatment. However, Zhu *et al* (19) reported that miR-224 expression levels were downregulated in NSCLC compared with non-cancerous lung tissue. These authors also observed that decreased miR-224 expression was significantly associated with lymph node metastasis, an advanced tumor-node-metastasis stage and a reduced OS time (19). Furthermore, Wang *et al* (20) recently identified that miR-224 was significantly upregulated in NSCLC tissues and hypothesized that miR-224 expression promotes NSCLC cell proliferation by downregulating Ras association domain

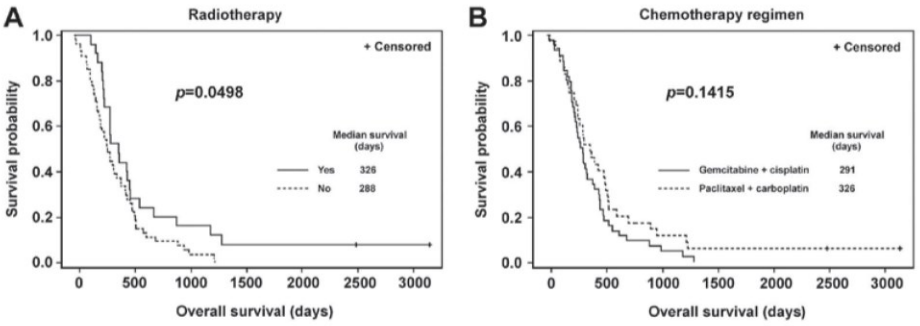

Figure 1. Association between the treatment modality and OS time for non-small cell lung cancer patients, as determined with the Kaplan-Meier method. All patients were treated with chemotherapy. (A) There were significantly longer OS times in the subgroup of patients who underwent chemotherapy combined with radiotherapy, compared with the patients who underwent chemotherapy without radiotherapy. (B) OS was independent of the chemotherapy regimen received. OS, overall survival.

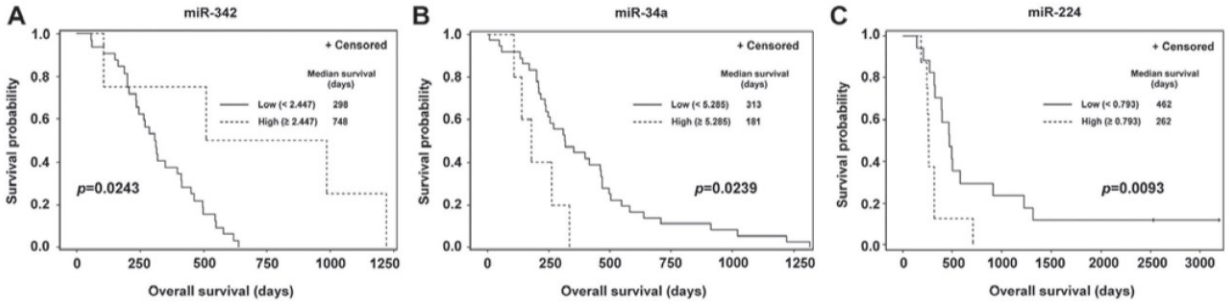

Figure 2. Association of miRNA expression with OS in the subgroups of non-small cell lung cancer patients (Kaplan-Meier curves). (A) Low expression of miR-342 was associated with shorter OS time in the subgroup of smokers. (B) High expression of miR-34a was associated with shorter OS time in the subgroup of patients treated with chemotherapy based on platinum derivatives in combination with gemcitabine. (C) High expression of miR-224 was associated with shorter OS time in a subgroup of patients who underwent chemotherapy combined with radiotherapy. OS, overall survival; miR, microRNA.

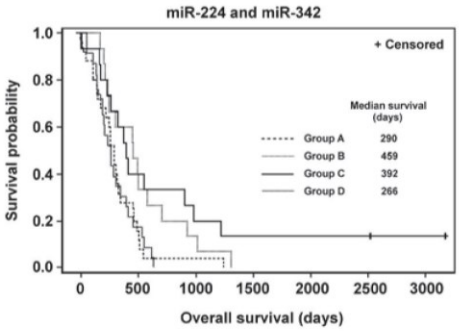

Figure 3. Kaplan-Meier curves for the overall survival of patients stratified into four groups according to the expression of miR-224 and -342: Group A, high miR-224 and -342; group B, high miR-224 and low miR-342; group C, low miR-224 and high miR-342; and group D, low miR-224 and -342. miR, microRNA.

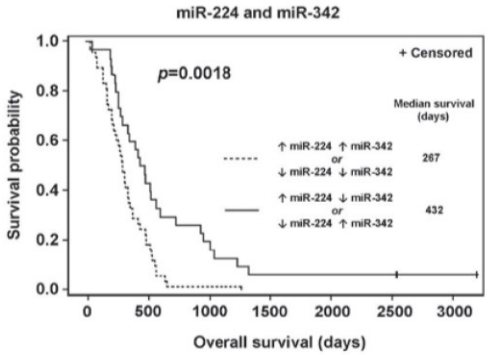

Figure 4. Comparison of survival of two groups of patients created by putting groups with similar survival from Fig. 3 together (group A and D vs. group B and C). Patients with the high expression of both miR-224 and -342 or low expression of both miR-224 and -342 have significantly shorter OS times than those with the high expression of either miR-224 and -342 and the low expression of the other. miR, microRNA.

family member 8 expression; the inconsistency of these studies will be discussed in the following paragraph.

In the present study, low levels of miR-342 indicated a poorer outcome in patients with a history of smoking, independent of treatment modality. Xie *et al* (21) demonstrated that miR-342 was downregulated in NSCLC and acted as a tumor suppressor through the repression of RAP2B, member of RAS oncogene family. Similarly, Tai *et al* (22) identified that miR-342 was capable of indirectly regulating MYC activity via the direct repression of E2F transcription factor 1. Takahashi *et al* (23) investigated how cigarette smoking altered plasma miRNA profiles; they identified that there was a decrease in plasma miR-342 in subjects who quit smoking, compared with smokers.

Table III. Association between the level of miRs and overall survival time as determined by Kaplan-Meier estimation.

					Below cut-off		Above cut-off		
Patient group	Treatment	Marker	Patients, n	Cut-off	n	Median, days	n	Median, days	P-value
Smokers only	Chemotherapy	miR-342	36	2.447	32	298	4	748	0.0243
Smokers and ex-smokers	Gemcitabine and cisplatin	miR-34a	41	5.285	36	313	5	181	0.0239
Smokers and ex-smokers	Chemotherapy and radiotherapy	miR-224	25	0.793	17	462	8	262	0.0093

miR, microRNA.

Table IV. Association between combinations of miRs and OS (Kaplan-Meier estimation).

Expression pattern	Patients, n	Median OS, days
miR-342 and -224		
High miR-224 and -342, or low miR-224 and -342	48	267
High miR-224 and low miR-342, or low miR-224 and high miR-342	30	432
miR-342, -224 and -34a		
High miR-224, -342 and -34a, or low miR-224, -342 and -34a	39	250
Other combinations	35	451

OS, overall survival; miR, microRNA.

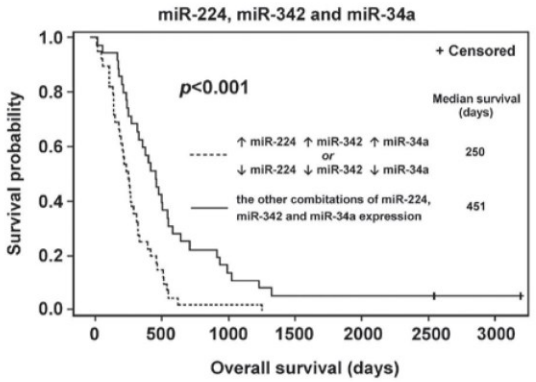

Figure 5. Comparison of the OS of two groups of patients based on patterns of miR-224, -342 and -34a expression. Patients with the high or low expression of all three miRs exhibit a significantly shorter OS time than those with other combinations of miR-224, -342 and -34a expression. OS, overall survival; miR, microRNA.

miR-34a is a member of the miR-34 family that is associated with the p53 pathway, and is implicated in cell death/survival signaling (24). The miR-34 family is transcriptionally activated by p53; in turn, p53 is a direct miR-34a target. However, the effect of miR-34a on p53 depends on the cellular context (25). miR-34a can have also a positive effect on p53 transcriptional activity and protein stability by targeting multiple p53 inhibitor genes (including MDM4, p53 regulator, sirtuin 1, metastasis associated 1 family member 2, histone deacetylase 1 and YY1 transcription factor) (26). A previous study identified that miR-34a inhibits cell proliferation (27). Expression of the miR-34 family was downregulated in tumor tissue compared with normal tissue, and low levels of miR-34a expression were associated with a higher probability of relapse in surgically resected NSCLC (28). However, higher levels of circulating miR-34a were observed in patients with NSCLC compared with healthy controls (29). Higher levels of miR-34a indicated a shorter OS time in patients receiving palliative platinum derivate-based chemotherapy in combination with gemcitabine in the present study.

Multivariate analysis was performed with the miRNAs (miR-34a, -224 and -342) that were identified as associated with OS. The most notable finding was that patients with the high expression of miR-224 and -342 exhibited similar outcomes to those with low expression of miR-224 and -342, which was significantly shorter than that of patients with high expression of either miR-224 or miR-342 and the low expression of the other (Figs. 3 and 4).

We hypothesize that the effect of a single miRNA is dependent on the level of expression of the other members of the miRNA network. It has been established that an miRNA can have a predominantly oncogenic role in one type of cancer and a tumor suppressive role in another; for instance, miR-224 was identified to be a tumor suppressor in prostate cancer (30), whereas in other types of malignancy, including gastric (11,31) and colorectal cancer (32), an oncogenic role for miR-224 was described. The ambiguous role of miR-224 was also observed within the SCC histological subtype of NSCLC in the present study. Tumor progression occurs as a result of the dysregulation of a number of protein-coding genes and epigenetic processes,

including the deregulation of a number of miRNAs. Therefore, to understand the role of one particular miRNA, it is necessary to determine the levels of the other 'co-players'. In the present study, OS time was influenced by the mutual association of miR-224 and -342. The high level of miR-224 can be associated with adverse or favorable outcomes, depending on the simultaneous level of miR-342. These findings could explain the inconsistent results of previously published studies on miR-224 expression in NSCLC. In 2014, Zhu *et al* (19) reported that miR-224 was significantly downregulated in NSCLC and that a decrease in miR-224 expression was significantly associated with shorter OS time (19). Also in NSCLC, Cui *et al* (33) identified that miR-224 was significantly upregulated, with the increased expression of miR-224 promoting cell migration, invasion, and proliferation. As aforementioned, the present study also identified that the high expression levels of miR-224 were associated with shorter OS time in one subgroup of patients, specifically those who underwent chemotherapy combined with radiotherapy.

Using bioinformatic tools, the present study identified overlapping experimentally validated target genes for miR-34a, -224 and -342. Notably, all overlapping target genes identified in the present study (GNAS, IGF1R, CCND1, CCNG2, SERPINE1, and RRM2) are involved in processes associated with carcinogenesis, including cell cycle regulation, p53 signaling and DNA repair. This may explain the complicated mutual dependency of those miRNAs in relation to tumor progression and the effectiveness of treatment. We hypothesize that these molecules could be involved in competing endogenous RNA crosstalk, where RNA transcripts co-regulate each other by competing for shared miRNAs, thereby titrating miRNA availability (34). However, one limitation of the present study is the absence of immunoprecipitation data and reporter assays, which are methods that may confirm the interactions among the set of 3 miRNAs and 6 target genes. Nevertheless, the results of the present study may provide a stimulus for further research in this area.

With cluster analysis, novel associations between miR-34a, -224 and -342 that affected patient survival time were identified in the present study. The result may demonstrate that the effort to find a particular miRNA as a perfect marker for a particular event may be fruitless due to the complex interactions between RNA transcripts. In order to understand all aspects of the effect of miRNAs on the regulation of gene expression in cancer and their associations with phenotype and treatment outcome, miRNA profiling and deep bioinformatic analysis will be necessary. Only this approach can facilitate the future application of miRNAs in clinical practice. miRNAs can generally be assessed with more precision and ease than the mRNAs of coding genes, as miRNA analysis is less demanding in terms of the quality and quantity of isolated RNA, features that may be problematic in RNA samples extracted from FFPE tissue (16). FFPE tissue samples are routinely taken and analyzed during standard lung cancer management, which is why miRNAs may become clinically applicable predictors of the effectiveness of palliative treatment in patients with lung cancer. Nevertheless, the findings of the present study demonstrated that, due to the complex network of interactions, this objective could be achieved by combining more markers together rather than by using individual miRNAs. On the basis of the results of the current study, miR-224, -342 and -34a could be members of this panel of predictors of treatment efficacy.

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PŘÍLOHA 7

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Vliv exprese vybraných protein kódujících genů a mikroRNA na riziko relapsu plicních adenokarcinomů stadia 1

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SUMMARY

Advanced lung cancer – underrated or overrated advances?

Introduction: Adjuvant chemotherapy has become an integral part of treatment of radically operated patients with lung cancer stages 2, 3 and 1B larger than 4 cm. For smaller tumors (stages 1B and 1A), the benefits of this treatment have not been demonstrated for the overall patient population. However, it is known that the risk of disease recurrence following surgical removal of the tumor is not negligible. The aims were to assess the relationship between expression of selected protein-coding genes and microRNAs and disease-free interval (DFI) and overall survival (OS) in patients with early-stage lung adenocarcinoma and to try to find a possible marker of risk of disease recurrence and thus identify the patient population that might benefit from adjuvant chemotherapy.

Patients and Methods: The study included 42 patients (31 males and 11 females; all but 4 patients were smokers or ex-smokers) with radically operated stage 1A and 1B lung adenocarcinoma not receiving adjuvant chemotherapy. Expression of selected mRNAs and miRNAs was measured by quantitative RT-PCR in tumor tissues obtained by macrodissection from formalin-fixed paraffin-embedded (FFPE) tissue biopsies. The relationships between gene expression levels of selected mRNAs and miRNAs and DFI and OS was analyzed.

Results: In the entire set of mRNAs and microRNAs of interest, no statistically significant relationship was found between their expression and DFI or OS. In the subgroup of smokers or ex-smokers only, a significant relationship was demonstrated between the mRNA level of BRCA1 and OS.

Conclusion: Using routinely prepared FFPE tumor samples, a relationship between shorter OS and the level of BRCA1 was demonstrated in the subgroup of smokers or ex-smokers. Given the small sample size, however, the results need to be confirmed by further studies.

Keywords: NSCLC, adenocarcinoma, stage 1, prognostic factor, mRNA, miRNA

SOUHRN

Úvod: Adjuvantní chemoterapie se stala nedílnou součástí radikálně operovaných pacientů s plicními karcinomy stadií 2, 3 a dále pak i 1B větších než 4 cm. Pro menší nálezy stadií 1B a stadium 1A nebyl pro celkovou populaci pacientů prokázán přínos této léčby. Nicméně je známo, že i zde není riziko recidivy onemocnění po chirurgickém odstranění nádoru zanedbatelné. Naším cílem bylo posoudit vztah exprese vybraných protein kódujících genů a mikroRNA k době do progrese onemocnění (DFI) a celkovému přežití (OS) pacientů s plicními adenokarcinomy nízkých stadií, a pokusit se tak najít možný marker rizika recidivy onemocnění a identifikovat pacienty, kteří by mohli mít prospěch z podání adjuvantní chemoterapie.

Pacienti a metody: Naše studie zahrnovala 42 pacientů (31 mužů a 11 žen, vyjma 4 nemocných byli všichni kuřáci či bývalí kuřáci) s radikálně operovaným plicním adenokarcinomem stadií 1A a 1B bez adjuvantně podávané chemoterapie. Exprese vybraných mRNA a miRNA byla měřena pomocí kvantitativní RT PCR v nádorové tkáni, získané makrodisekcí z formalinem fixovaných parafínových bločků (FFPE) bioptované tkáně. Byl analyzován vztah mezi hladinou genové exprese vybraných mRNA a miRNA a DFI a OS.

Výsledky: Z celkového setu mRNA a mikroRNA našeho zájmu jsme neprokázali žádný statisticky významný vztah mezi jejich expresí a DFI/OS. Pouze u podskupiny kuřáků/exkuřáků byl prokázán signifikantní vztah mezi hladinou mRNA BRCA1 a OS.

Závěr: V rutinně připravovaných FFPE nádorových vzorcích jsme prokázali vztah mezi kratším OS a hladinou BRCA1 u podskupiny kuřáků/exkuřáků. Tento výsledek je však vzhledem k malé skupině souboru nutné potvrdit dalšími studiemi.

Klíčová slova: NSCLC, adenokarcinom, stadium 1, prognostický faktor, mRNA, miRNA

ÚVOD

Plicní karcinom patří mezi nejčastější příčiny úmrtí na nádorová onemocnění na světě, kdy dominantní roli zaujímá nemalobuněčný plicní karcinom (NSCLC) [1]. Chirurgická resekce je zlatý standard léčby pacientů stadia 1A a 1B (dle 7. TNM klasifikace), kdy následné pětileté přežití je uváděno v rozmezí 60 až 90 %, část nemocných umírá v důsledku recidivy tumoru [1,2]. Adjuvantní léčba se v současné době řídí stadiem nemoci. Pro pacienty stadií 2–2A je doporučována adjuvantní chemoterapie (CHT) platinovým doubletem (obvykle 4 cykly cisplatiny a vinorelbinu), pro pacienty s pozitivními N2 uzlinami je možné též doplnění o postoperační radioterapii (PORT) s cílem snížit riziko lokální recidivy tumoru [3,4]. Adjuvantní CHT u těchto nemocných vedla dle dat z velkých randomizovaných studií (IALT, JBR10, ANITA, BLT) k prodloužení pětiletého přežití o 4–15 %, metaanalýza (LACE) uvádí prodloužení pětiletého přežití 5,4 % [5,6]. Ačkoliv existují práce, dokládající přínos adjuvantní CHT i u pacientů stadia 1B (někteří autoři doporučují navýšit léčbu na 6 cyklů) [7,8], v nejrespektovanější studii CALGB nebyl obecný benefit pro tyto nemocné prokázán [5]. Post-hoc analýza prokázala význam pouze u nemocných s tumorem větším než 4 cm [3]. Další snahy se proto logicky upíraly k nalezení dalších klinických (udáván vliv pohlaví, buněčné diferenciace, vaskularizace nádoru nebo výkonnostního stavu), či biologických (stanovení exprese samostatných genů nebo vybraného panelu genů) parametrů, které by pomohly lépe definovat prospěch adjuvantní chemoterapie, a případně ji tak nabídnout i některým nemocným stadia 1 [1,2,9,10]. Jiné práce posuzují použití cílené léčby u vybraných nemocných místo standardní CHT – např. nadějná studie ALCHEMIST, zkoumající cílené užití tyrozinkinázových inhibitorů u pacientů s prokázanými senzitivními mutacemi genu EGFR či ALK translokacemi [11,12]. Dosud však žádný z těchto postupů nevedl k užití v klinické praxi. Naším cílem bylo zjistit možný vztah exprese některých DNA opravných genů, ABC transportérů a vybraných mikroRNA k období bez recidivy onemocnění (DFI) a celkovému přežití (OS) u pacientů s radikálně resekovanými adenokarcinomy stadia 1 bez následně podávané adjuvantní chemoterapie, a tím najít kandidátní biomarker, který nám umožní určit skupinu pacientů, pro které může být podání adjuvantní CHT přínosné.

Tabulka 1: Soubor pacientů

Pohlaví	muži 31 (74 %)	ženy 11 (26 %)
Kuřácký statut	ex/kuřáci 38 (90 %)	nekuřáci 4 (10 %)
Stadium	1A 27 (64 %)	1B 15 (36 %)
Recidiva	ano 19 (45 %)	ne 23 (55 %)
Přežívání 2/2016	ano 20 (48 %)	ne 22 (52 %)

PACIENTI A METODY

Design studie

Retrospektivní studie zahrnovala 42 pacientů stadia 1A a 1B s histologicky ověřeným plicním adenokarcinomem s radikálním operačním řešením bez adjuvantně podávané chemoterapie, léčených na Klinice pneumologie a ftizeologie, FN Plzeň. Pacienti podstoupili operační výkon v letech 2003–2011, stadium bylo potvrzené histologickým vyšetřením plicního resekátu (včetně disekovaných lymfatických uzlin hilu a mediastina). Naším cílem bylo stanovení exprese vybraných mRNA a mikroRNA, zjištění jejich vztahu k DFI a OS, a tak se pokusit najít možný marker rizika recidivy onemocnění, který by umožnil identifikovat pacienty s horší prognózou nemoci, a ti by mohli mít prospěch z podání adjuvantní chemoterapie.

Pacienti byli pooperačně standardně sledováni, první rok byl prováděn kontrolní skiagram plic každé 3 měsíce, 2. rok každého půl roku, každý rok až do 5 let od výkonu pak standardně probíhalo CT plic+mediastina jedenkrát ročně, po 5. roce od doby resekce byl prováděn pouze skiagram plic1x ročně, v prvních 5 letech pak u většiny nemocných (pokud nevyjádřili svůj nesouhlas) probíhala bronchoskopická kontrola s cílem vyloučit recidivu v místě pahýlu operační rány. OS bylo stanoveno jako doba přežití od operačního výkonu do úmrtí/konce sledování. DFI bylo definováno jako doba od operačního výkonu do recidivy, resp. konce sledovaného období.

Soubor pacientů

Náš soubor zahrnoval celkem 42 pacientů s mediánem věku 65 let (rozmezí 48–77 let). 31 (74 %) pacientů představovali muži, 11 (26 %) pak ženy; kuřáků bylo 18 (42,9 %), exkuřáků 20 (47,6 %) a nekuřáci 4 (9,5 %); 27 nemocných mělo stadium 1A (64 %), 15 (36 %) stadium 1B (shrnuje tabulka 1). Recidiva se objevila u 19 nemocných, 23 pacientů v době sledování recidivu nemělo. V době vyhodnocování (únor 2016) přežívalo celkem 20 pacientů (5 s recidivou a 15 bez recidivy), přičemž 8 pacientů bez recidivy zemřelo z jiného důvodu než plicního karcinomu, ve skupině s recidivou zemřelo všech 14 nemocných v souvislosti s plicním karcinomem.

Vzorky tkáně a izolace RNA

Bioptické vzorky byly odebrány během chirurgické resekce tumoru (lobektomie) a zpracovány standardními laboratorními metodami na Šiklově ústavu patologie Fakultní nemocnice Plzeň, s cílem diagnostiky tumorů – barvení hematoxylinem a eozinem (HE) a imunohistochemické vyšetření (pomocí p63 a TTF1). FFPE (formalinem fixované parafínové bločky) tkáňových vzorků byly skladovány při pokojové teplotě do doby analýzy. Parafínové řezy užité pro další zpracová-

ní byly opět barveny HE s cílem mikroskopicky verifikovat nádorové buňky, a jejich okrsky byly patologem vyznačeny pro následnou makrodisekci pro získání RNA. Celková RNA (včetně mikroRNA) byla extrahována soupravou miRNeasy FFPE Kit (Qiagen, Hilden, Germany) z 15 µm širokých FFPE řezů, získaných makrodisekcí dle korespondujících HE barvených sklíček, na kterých patolog vyznačil nádorovou tkáň.

Stanovení exprese vybraných protein kódujících genů

Kvantitativní stanovení mRNA vybraných genů (ABCC1, ABCC10, ABCG2, ATP7B, SLC22A1, SLC29A1, ERCC1, BRCA1) byla uskutečněna pomocí real-time RT-PCR s užitím Universal Probe Library (UPL) sond (Roche, Mannheim, Germany) v technických duplikátech na přístroji Stratagene Mx3005P (Agilent Technologies, Santa Clara, CA, USA), jak bylo publikováno dříve [163].

Stanovení exprese vybraných mikroRNA

Kvantitativní stanovení vybraných 17 mikroRNA (miR-15b, miR-21, miR-27a, miR34a, miR-99a, miR-106a, miR-107, miR-143, miR-150, miR-192, miR-211, miR-218, miR-221, miR-224, miR-342 a miR-375) bylo provedeno pomocí real-time RT-PCR metody za užití TaqMan® MicroRNA Assays (Applied Biosystems, Foster City, CA, USA) v technických duplikátech na přístroji Stratagene Mx3005P (Agilent Technologies, Santa Clara, CA, USA) podle instrukcí výrobce sond. Exprese RNU6B (U6snRNA) byla užita jako normalizátor za užití tzv. ΔCt přístupu (2-ΔCt algoritmus).

Statistická analýza

Statistický software SAS verze 9.3 (SAS Institute Inc., Cary, NC, USA) byl užit pro všechny statistické výpočty. Zhodnocení prognostické signifikance (vztah markerů k DFI a OS) bylo provedeno analýzou maximální věrohodnosti (Coxův regresní model), Kaplan-Meierovy distribuční funkce byly generovány pro signifikantní markery z Coxova modelu. Výsledky s hodnotou $p < 0,05$ byly považovány za statisticky významné.

VÝSLEDKY

Z celkového panelu protein kódujících genů a mikroRNA, které jsme stanovovali, jsme neprokázali žádný statisticky významný vztah mezi jejich expresí a DFI/OS v celkovém souboru. Pouze u podskupiny kuřáků/exkuřáků byl prokázán signifikantní vztah mezi hladinou mRNA BRCA1 a OS ($p = 0,0415$, HR = 2,23).

DISKUZE

Znalost individuálního průběhu karcinogeneze, kterou můžeme hodnotit dle konkrétních de novo mutací, nebo dle změn expresního profilu vybraných genů, by měla pomoci nejen cílit moderní léčbu, ale dle již publikovaných výsledků je nadějná i pro nádorovou diagnostiku a výběr chemoterapie [1,9,13].

Naším cílem bylo posoudit vztah exprese některých DNA opravných genů, ABC transportérů a vybraných mikroRNA k DFI a OS a pomocí nich identifikovat nemocné, kteří by mohli mít na základě tohoto vyšetření prospěch z adjuvantní chemoterapie i ve stadiu 1.

Lidský genom obsahuje geny pro 48 ABC (ATB binding cassette) transportérů, které se dělí do 7 rodin (ABCA, ABCB, ABCC, ABCD, ABCE, ABCF, ABCG). Přestože se jedná o různorodou skupinu proteinů, jejich společnou vlastností je schopnost aktivně přenášet různé látky přes membránu za spotřeby ATP. Z hlediska protinádorové léčby je významné, že některé z těchto proteinů mají schopnost exkrece toxických látek z buňky [14,15]. Na základě rešerše literatury jsme vybrali 6 genů z této skupiny ve vztahu k nádorům plic (ABCC1, ABCC10, ABCG2, ATP7B, Slc22a1, Slc29a).

ABCC1 (190 kDa) je efluxní pumpou, která patří do skupiny C transmembránových genů ABC [16]. U NSCLC byl zkoumán její vliv na rezistenci na léčbu taxany či etoposidem [17,18]. Její zvýšená exprese byla popsána na hematoencefalické bariéře, ve střevech a sliznici úst [18]. Byla pozorována její zvýšená exprese v plicích, což zřejmě souvisí s její úlohou protektivního faktoru před znečištěním ovzduší a inhalačními toxiny [18]. Její zvýšená exprese je popisována některými autory jako negativní faktor pro OS i PFS u některých druhů CHT, a dokonce i u TKI [17,18]. Roli mohou hrát i „varianty" tohoto proteinu způsobené polymorfismy, např. SNP (single nucleotide polymorfisms) [17]. Její exprese se zdá být vyšší u dlaždicových karcinomů a stadia 1 [19]. V naší studii jsme žádný vztah k DFI či OS nezaznamenali.

ABCC10 (171 kDa) je dalším transmembránovým transportérem z rodiny C skupiny ABC efluxních pump [16,20]. Normálně je exprimována v řadě tkání, zejména v kůži, tlustém střevě a varlatech [21]. U NSCLC je pak popisována větší exprese ve srovnání s normální plicní tkání, což může mít vliv na rezistenci např. k taxanům, vinca alkaloidům či TKI [16,20,22]. Je tedy možným prognostickým/prediktivním faktorem u NSCLC. V naší studii jsme však toto neprokázali.

ABCG2 je další z rodiny ABC transportérů, který vytváří transmembránové homodimery či tetramery [23]. Tento gen, nalézající se na 4. chromozomu, je exprimován v řadě tkání, např. placentě, tenkém střevě, játrech, ledvinách, mozku či hematopoetických kmenových buňkách [24,25]. Jeho zvýšená exprese, popř. některé z polymorfismů, se dávají do souvislosti nejen s rezistencí na topotecan či platinu, ale též byl popsán vztah k EGFR-TKI i ALK inhibitorům [23,26,27,28,29,30,31]. Jinde je naopak udáván vliv gefitinibu na jeho inhibici [23,32,33]. Navíc je popisován i jeho vztah k některým nežádoucím účinkům CHT [34] či TKI [35,36]. ABCG2 protein je považován za marker kmenových buněk, které se podílejí na proliferační aktivitě tumoru [37,38]. Proto nepřekvapí, že řada pra-

cí udává jeho vztah k prognóze pacientů s NSCLC [38,39,40,41]. U raných stadií je jeho vztah k OS sporný [42], možná v důsledku spolupůsobení dalších faktorů – udáván např. vliv CD133 [43]. Naše práce žádný vliv mezi expresí mRNA tohoto genu a DFI či OS neprokázala.

ATP7B patří do rodiny ATPáz transportujících těžké kovy – mimo jiné i měď [44]. Gen je exprimovaný především v ledvinách a játrech, kde má za úkol odstraňovat přílišné množství mědi z buněk, v játrech tak jeho mutace může vést ke vzniku Wilsonovy choroby [44]. Jeho zvýšená exprese je dávána do souvislosti s rezistencí k cisplatině u řady nádorů, včetně NSCLC, tyto vztahy jsou ale možná komplexní a nezávislé na samotné expresi ATP7B [45,46]. U NSCLC byla popsána zvýšená exprese tohoto genu, která byla spojená se sníženou diferenciací nádorových buněk [47] a horší prognózou nemocných [45,47]. Naše práce tuto asociaci neprokázala, což mohlo být způsobeno i nízkým stadiem onemocnění (publikované práce se zaměřovaly na pokročilá stadia).

Slc22a1 je transmembránový transportér exprimovaný především v játrech, ledvinách a střevě. Je spojen s efluxem řady léků a toxinů [48]. Je exprimován u řady nádorů, včetně NSCLC, kdy může souviset s chemorezistencí na některé druhy CHT a nepříznivou prognózou [49,50]. Vliv tohoto genu je u NSCLC prozatím málo prozkoumán – v jedné práci byly dávány do souvislosti jeho SNP se stupněm vyrážky u pacientů léčených EGFR-TKI [51]. V naší práci jsme pak nezaznamenali jeho vztah k prognóze.

Slc29a1 je transmembránový transportér nukleosidů a nukleosidových analog, lokalizovaný na 6. chromozomu [52]. U NSCLC byla popsána jeho deregulovaná exprese, která se může měnit i v závislosti na některých SNP [52,53,54]. Jeho zvýšená exprese je spojována s horší léčebnou odpovědí na gemcitabin [55,56]. Nebyl prokázán rozdíl mezi expresí u adenokarcinomu a dlaždicového karcinomu [57]. U pacientů léčených gemcitabinem je popisován jeho vliv na OS, nikoliv na PFS (čas do progrese). U naší skupiny pacientů nebyl žádný vztah k prognóze prokázán.

Funkcí genů ERCC1 a BRCA1, stejně jako jejich možným prognostickým/prediktivním významem u pokročilých tumorů, se zabývá náš předchozí článek [58]. Řada autorů se pak zabývala i vztahem k možné predikci odpovědi na adjuvantní chemoterapii, resp. prognóze radikálně operovaných pacientů s NSCLC, kdy některé práce naznačují možný prognostický a prediktivní potenciál, ale definitivní závěr prozatím nebyl stanoven [5,9,10]. V naší práci jsme vztah ERCC1 a BRCA1 k DFI či OS u celkového souboru nezaznamenali, ovšem v podskupině kuřáků/exkuřáků jsme prokázali signifikantní vztah exprese BRCA1 k OS. Je tedy možné, že expresi BRCA1 v nádorové tkáni ovlivňuje i kuřácký statut. Toto tvrzení je ale nutné potvrdit dalšími studiemi. BRCA1 přispívá k opravě dvojitých zlomů DNA a dále funguje jako regulátor chemoterapií indukované apoptózy – především u antimikrotubulózních látek (taxany, vinca alkaloidy) [9,10]. Na toto téma vzniklo několik prací, kdy část publikací se zabývala pacienty s adjuvantní či neoadjuvantní léčbou a některé pak, stejně jako naše práce, i pacienty bez adjuvantní léčby. Taron et al. prokázali lepší efekt neoadjuvantní CHT (cisplatina + gemcitabin) u 55 pacientů s nízkou expresí BRCA1 [59]. V naší práci jsme obdobný vztah nezaznamenali, BRCA1 měla vztah pouze k OS u dlaždicových karcinomů [58]. Studie fáze III SCAT pak u 500 pacientů adjuvantně léčených v jednom rameni cisplatinou s docetaxelem a v druhém dle exprese BRCA1, buď cisplatinou + gemcitabinem (při nízké expresi BRCA1), nebo cisplatinou + docetaxelem (při střední expresi BRCA1), případně jen docetaxelem (při vysoké expresi BRCA1) nezaznamenala rozdíl v OS mezi oběma rameny [60]. Rossel et al. pak prokázali u 126 pacientů bez adjuvantní léčby (stadia 1–3) horší přežití u 40 pacientů s vyšší expresí BRCA1 (medián OS 29 měsíců) oproti 83 nemocným s nízkou expresí BRCA1 (medián OS v době vydání studie nedosažen) [61]. Další studie potvrzovala tyto závěry u pacientů se stadii 1B–2B [62]. Sanchis et al. se pak věnovali výlučně stadiu 1, kde u 64 pacientů též neprokázali vliv exprese BRCA1 na OS, avšak vyšší exprese BRCA1 měla vztak k nižšímu DFI [63].

MikroRNA (miRNA) jsou krátké (22–24 nukleotidů), jednovláknové, nekódující RNA molekuly, které negativně regulují genovou expresi na posttranskripční úrovni prostřednictvím inhibice translace a/nebo snížením stability cílové mRNA. Tento základní biologický proces byl objeven teprve nedávno a je intenzivně studován (Andrew Fire, Craig Mello – Nobelova cena za rok 2006). Odhaduje se, že exprese až 50 % genů je ovlivněna touto cestou. Bylo identifikováno už více než 2 500 lidských miRNA (databáze miRBase). Konkrétní mikroRNA se označuje předponou miR- následovanou číslem. Jedna konkrétní miRNA může cílit stovky různých RNA transkriptů, ale i obráceně, jedna konkrétní mRNA může být cílem pro mnoho různých miRNA. Z uvedeného je tedy zřejmé, o jak komplexní regulační síť se jedná. Řada miRNA se účastní karcinogeneze, a to ovlivněním exprese tumorsupresorových genů nebo onkogenů. Tzn. některé miRNA vykazují tumorsupresorový nebo onkogenní efekt a jsou typické pro nádorové buňky. V současnosti je pro většinu onkologických onemocnění, resp. typů nádorové tkáně, znám charakteristický profil exprese molekul miRNA a je vkládána naděje, že některé najdou využití jako nádorové markery. V našem případě jsme se zajímali o mikroRNA, které hrají roli v patogenezi nádorů [64].

miR-15b byla v souvislosti s NSCLC zkoumána především jako součást možného diagnostického molekulárního panelu [13,65]. U NSCLC je popsána její zvýšená exprese [65] a korelace se stupněm

diferenciace (grade) nádoru [66]. Proto by její exprese mohla mít souvislost i s prognózou nemocných [13]. To se však u našeho souboru pacientů nepotvrdilo.

Mezi mikroRNA s onkogenním efektem patří u řady nádorů (včetně NSCLC) intenzivně studovaná miR-21, jejíž gen se nachází na 17. chromozomu [67]. Podílí se na řízení proliferace, angiogeneze, invazivity a migrace nádorových buněk, kdy je mimo jiné dávána do souvislosti s regulací EGFR, PTEN, BCL-2 či KRAS [68,69,70,71,72,73,74]. Byl popsán vztah miR-21 k odpovědi na chemoterapii jak platinovými deriváty [69,70,75,76], tak i na radioterapii [77,78]. U NSCLC je popisována její zvýšená exprese v tkáni, plazmě i séru [67,79,80,81,82,83,84], a to zejména u adenokarcinomů [68], vyšších TNM stadií [86,87] a při nižším stupni diferenciace (vyšší grade) tumoru [67]. Uplatňuje se i jako součást diagnostických panelů NSCLC včetně raných stadií [72,85,87,88,89]. Rovněž je poukazováno na její prognostický význam v jednotlivých studiích [67, 75,76,81] i v metaanalýze [90]. Prognostický význam (signifikantní vztah k DFI i OS) byl popsán i u stadia 1 [91]. Naše práce pro tyto nemocné žádný vztah k prognóze nezaznamenala.

miR-27a se podílí na regulaci proliferace a apoptózy u řady nádorů [92]. U NSCLC je udávána její snížená koncentrace v séru a v plazmě [92,93]. V pre-miRNA i samotné miRNA bylo nalezeno několik polymorfismů, u kterých někteří autoři uvádějí vliv na OS, některé práce tento vztah neprokázaly [94,95]. V souvislosti s miR-27a je u NSCLC věnován zájem především možnému vlivu na rezistenci k EGFR-TKI cestou MET (snížená exprese miR vede ke zvýšené expresi MET) a případně SPROUTY7 (ovlivňujícího též MET) [92,96]. Ale byl zkoumán i možný vliv miR-27a na angiogenenezi a rezistenci k platinovým derivátům [94]. V naší práci jsme však žádný vliv na prognózu nemocných neprokázali.

miR-34a je tumor supresorová mikroRNA, lokalizovaná na 1. chromozomu [97]. Je deregulovaná u řady tumorů – karcinomu prsu, prostaty, osteosarkomu i NSCLC [98]. K jejímu snížení může docházet jednak vlivem některých mutací, ale zejména pak epigeneticky pomocí metylace jejího promotoru [99,100]. Ovlivněním procesu reparace DNA se miR-34a podílí na citlivosti nádorové tkáně k radioterapii [96,100], dále je uváděn její vliv na účinnost EGFR-TKI díky ovlivňování drah MET a KRAS onkogenů [101,102,103,104], ale vztah je popisován i k imunitě skrze působení na NK buňky či TGFβ [105]. Rovněž se podílí na řízení apoptózy – cestou bcl2 a p53 – a proliferace nádorových buněk – cestou Rb, TGF či PDGFR [97,106,107]. Jedna práce pak, v kontrastu k předchozím publikacím, uvádí zvýšenou expresi u NSCLC [108]. U tumorů nízkých stadií je popisováno snížení u dlaždicových forem (SCC) a naopak zvýšení u adenokarcinomů [101,109]. Odlišné složení souboru pacientů tak může být vysvětlením pro zvýšenou expresi miR-34a u výše zmíněné práce. U NSCLC nízkých stadií byl též zmiňován vliv na prognózu pacientů [109,110]. My jsme však, podobně jako Wang et al. [111], takovýto vztah neprokázali.

miR-99a je mikroRNA s tumor supresorovým efektem, jejíž gen je lokalizován na 22. chromozomu. Je uváděna její snížená exprese ve tkáni NSCLC (ACC i SCC) [112]. Díky cílení na mTOR a řadu genů podílejících se na epiteliálně mezenchymální přeměně (EMT) inhibuje proliferaci, migraci a invazivitu NSCLC[113,114]. Lze tedy předpokládat působení této miRNA na prognózu nemocných. Takovýto závěr jsme ale v naší práci nepotvrdili.

miR-106a byla původně považována za onkogenní se zvýšenou expresí u NSCLC s vlivem na proliferaci, invazivitu a migraci nádorových buněk [115,116]. Rovněž byla její zvýšená exprese dávána do souvislosti s rezistencí na cisplatinu díky vlivu na efluxní pumpu ABCA1 [114]. Novější práce pak ukazuje i na její možný tumorsupresorový potenciál díky vlivu na autofagii tumorózních buněk [117]. Dle našich znalostí vliv na prognózu u pacientů ve stadiu 1 nebyl dosud zkoumán, a naše práce tuto možnou korelaci nepotvrdila.

miR-107 má tumor supresorový efekt u řady nádorů (např. karcinom žaludku, nádory hlavy a krku), kdy se podílí na řízení buněčné proliferace a apoptózy [118]. U NSCLC byla popsána též snížená exprese této mikroRNA s vlivem na buněčný cyklus a možným navozením rezistence k cisplatině [119,120]. Snížená exprese byla v práci Zhonga et al. ve vztahu ke kratšímu OS [121]. V naší práci jsme tento vztah na našem souboru pacientů stadia 1 nepotvrdili, což může být způsobeno odlišným vlivem této mikroRNA u různých TNM stadií NSCLC [121].

miR-143 je mikroRNA s tumor supresorovým působením, jejíž gen je lokalizován na 5. chromozomu. Hraje významnou úlohu u řady karcinomů včetně NSCLC [122,123]. Díky působení na některé onkogeny (např. KRAS, MMP-13) se podílí na regulaci buněčné proliferace, apoptózy, migrace a metastázování u NSCLC [123,124,125]. Dále je dáván vztah miR-143 do souvislosti s řízením autofagie a glykolýzy [122]. miR-143 byla rovněž zkoumána jako potencionální diagnostický marker pro NSCLC [126]. Její snížená exprese u NSCLC byla spojena s kuřáckým statutem [127], což podobně jako u našeho výsledku u BRCA1 podporuje vliv kuřáckého návyku na některé molekuly spjaté s rozvojem NSCLC. I přes značný zásah miR-143 do onkogeneze NSCLC a převahu kuřáků v našem souboru jsme nezaznamenali vztah této mikroRNA k prognóze.

Gen pro onkogenní miR-150 se nalézá na chromozomu 19. Tato mikroRNA je zvýšeně exprimována u řady nádorů včetně NSCLC (ačkoliv lze najít i práci dokládající snížení její exprese u NSCLC) [128,129]. Toho lze potencionálně využít též při

diagnostice nízkých stadií NSCLC [130]. Nicméně exprese miR-150 se zvyšuje se stoupajícím stadiem [128,131]. Mezi funkce miR-150 patří regulace apoptózy (spjata s p53) a buněčné proliferace [132,133]. Rovněž je dávána do souvislosti s toxicitou radioterapie díky ovlivňování zánětlivých cest [134]. Ačkoliv byl popsán její vztah k prognóze NSCLC [128], v našem souboru pacientů jsme tento vztah neprokázali.

Onkogenní miR-192 je zvýšeně exprimována v NSCLC [135]. Podílí se na řízení proliferace (cestou PIK3-AKT-mTOR) a apoptózy (ovlivňuje bcl-2 a p53) [136,137]. Její zvýšená exprese je uváděna jako jedna z cest rezistence k chemoterapii (zkoumán režim cisplatina-gemcitabin) [135]. V našem souboru jsme neprokázali její vztah k prognóze pacientů.

Další onkogenní mikroRNA se zvýšenou expresí u NSCLC je miR-211. Tato mikroRNA ovlivňuje buněčnou proliferaci a invazivitu nádorových buněk [138]. Je uváděna jako jeden z možných biomarkerů dlaždicového karcinomu [139], obdobný vztah u adenokarcinomu nebyl dosud zkoumán. V našem souboru jsme neprokázali její vztah k prognóze pacientů.

Tumor supresorová miR-218 vykazuje sníženou expresi ve tkáni NSCLC [140]. Její snížení může být způsobeno jednak polymorfismy genu (SNP), nebo hypermetylací jejího promotoru. Tato hypermetylace může mít souvislost s kouřením [141, 142,143]. Funkčně se tato mikroRNA podílí na buněčné migraci a invazivitě [142,144]. Je udávaná souvislost mezi její sníženou expresí a rezistencí na léčbu EGFR-TKI [140,145], a díky regulaci apoptózy i platinovým derivátům [146,147]. Tento tumorsupresor je dáván do souvislosti s horší prognózou pacientů s NSCLC [141,144]. Tento vztah však naše studie u souboru pacientů stadia 1 nepotvrdila.

miR-221 byla původně považována za onkogen aktivovaný pomocí MET [148]. Její zvýšená exprese u NSCLC negativně ovlivňuje inhibitory proliferace a migrace (PTEN, TIMP-3, MMP, AKT) [149]. Následně byl popsán i tumorsupresorový efekt této mikroRNA [150], přesný vliv na NSCLC tedy není dostatečně znám a patrně závisí na místním kontextu. V naší studii jsme neprokázali vliv miR-221 na prognózu našeho souboru pacientů.

miR-224 byla popisována u různých tumorů jako tumorsupresor i onkogen [151]. U NSCLC je dávána do souvislosti spíše s tumorsupresorovým efektem, tzn. její snížená exprese je spojena s horší prognózou pacientů v pokročilých stadiích [152]. Jiná práce pak dokládá vliv na OS jen u nemocných s mutací p53/KRAS [151]. Funkčně se podílí na ovlivňování proliferace, apoptózy, invazivity a migrace nádorových buněk [152,153]. Naše práce vztah miR-224 k prognóze u časných stadií tumorů neprokázala.

miR-342 vykazuje sníženou expresi v tkáni NSCLC s vlivem na proliferaci a invazivitu tumoru patrně díky ovlivnění exprese RAP2B [154]. V naší studii jsme neprokázali vliv této mikroRNA na prognózu nemocných.

miR-375 se podílí na řízení proliferace, migrace a invazivity u řady karcinomů, vykazuje tumorsupresorový efekt [155]. Snížení exprese miR-375 může být navozeno metylací jejího promotoru [156]. U NSCLC je uváděn podíl miR-375 na rezistenci vůči CHT (cisplatina-vinorelbin), což může být způsobeno vlivem na DNA opravný gen ERCC1 [157,158]. U NSCLC je popisována snížená exprese miR-375 u ACC ve srovnání s SCC, což může být potencionálně i diagnosticky využitelné [158, 159,160]. Její snížená exprese je spojena s kratším OS v několika studiích i jedné metaanalýze [155, 156,161], jedna studie naopak uvádí opačnou souvislost [162]. Naše studie žádný vztah k prognóze neprokázala.

Rozdílné výsledky mezi různými studiemi mohou záviset jednak na rozdílné metodice stanovení a použitých cut-off pro dané markery a dále zejména na rozdílném zastoupení klinických skupin (stadium, pohlaví, věk, kuřácký status, PS, etnikum) pacientů v jednotlivých studiích [9]. Proto jsme se snažili v naší pilotní studii provést měření na homogenním souboru pacientů, odrážejícím reálné klinické podmínky. To však mělo za následek výrazné snížení velikosti souboru nemocných, kdy jsme nemuseli mít dostatečně velký soubor pro prokázání menších odchylek. Byla by tedy vhodná validace našich výsledků na větší multicentrické studii.

ZÁVĚR

Naším cílem bylo pokusit se najít možný marker rizika recidivy onemocnění u pacientů s plicními adenokarcinomy nízkých stadií, kteří podstoupili pouze radikální chirurgickou léčbu. Tento marker by mohl být následně využitelný pro identifikaci pacientů s nepříznivou prognózou, tzn. těch, kteří by mohli mít prospěch z podání adjuvantní chemoterapie.

V rutinních FFPE nádorových vzorcích jsme prokázali vztah mezi kratším OS a hladinou BRCA1 u podskupiny kuřáků/exkuřáků. Tento výsledek je však vzhledem k malé skupině souboru nutné potvrdit dalšími studiemi. V celkové skupině pacientů jsme vztah mezi expresí námi vybraných genů a DFI/OS neprokázali. V tomto kontextu tedy, dle výsledků naší studie, nelze žádný ze zkoumaných markerů považovat za vhodný indikátor podání či nepodání adjuvantní chemoterapie u resekovaných nemocných s adenokarcinomem stadia 1.

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PŘÍLOHA 8

10. MikroRNA u karcinomu prostaty

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Karcinom prostaty (CaP – cancer of prostate) patří celosvětově mezi nejčastější maligní nádory diagnostikované u mužů, incidence je velmi vysoká v rozvinutých zemích, což také souvisí s intenzivním rozvojem screeningových metod záchytu pomocí stanovení sérových hladin prostatického specifického antigenu (PSA) [1]. V České republice byla v roce 2009 incidence 119,5 na 100 000 mužů, od roku 2005 jsou zhoubné nádory prostaty na prvním místě malignit u mužů před nádory plic a kolorektálním karcinomem, nepočítáme-li nádory kůže [2,3].

Přestože patogeneze CaP není na molekulární úrovni tak přesně zmapována jako například patogeneze kolorektálního karcinomu, v posledních letech se naše znalosti v této oblasti značně prohloubily. Kromě mapování genetických změn (změn sekvence nukleotidů v DNA) jsou v posledních deseti letech intenzivně studovány i změny epigenetické, které také přispívají k iniciaci a progresi tohoto onemocnění. V současnosti jsou intenzivně zkoumány tři typy epigenetických změn, a to metylace DNA, remodelace chromatinu a regulace genové exprese molekulami mikroRNA (miRNA) [4], které se bude věnovat tato kapitola.

Nynější model patogeneze CaP předpokládá postupnou kumulaci změn ve tkáni prostaty zahrnující ztrátu exprese ribonukleázy L kódované genem HPC1, změny týkající se genu pro androgenní receptor (AR) a genu pro MSR1. Vliv mají také hereditární změny sekvence DNA, například mutace v genech BRCA1 a BRCA2. Předpokládá se vliv prostředí, ale i virové infekce. Výsledkem je změna normální tkáně prostaty ve tkáň označovanou jako prostatická intraepiteliální neoplazie (PIN) [5]. V závislosti na vzniku fúzního genu TMPRSS2-ETS v lézích typu PIN jsou popisovány dvě různé cesty patogeneze CaP [6]. Ke vzniku tohoto fúzního genu dochází u časné high-grade PIN (HGPIN), a to přibližně u 20 % karcinomů prostaty. Vzniká fúzí promotoru androgeny regulovaného genu specifického pro prostatu – transmembránové proteázy serinového typu 2 (TMPRSS2, lokus 21q22.2) a genu z rodiny transkripčních faktorů erytroblastosis virus E26 transformující sekvence ETS (ETV1, lokus 7q21.2 nebo ERG, lokus 21q22.3).

Ve tkáni HGPIN, ve které došlo ke vzniku fúzního genu TMPRSS2-ETS, dochází ke snížení exprese tumor supresorových genů PTEN a NKX3.1 a ke zvýšení exprese onkogenu c-MYC. Takto změněná tkáň pokročilého HGPIN se mění v CaP, zvyšuje se exprese AR, c-MYC a EZH3, snižuje exprese p53 a vzniká pokročilý CaP. Od objevení fúzního genu TMPRSS2-ETS v roce 2005 se intenzivně zkoumá jeho role v patogenezi CaP. Ukazuje se, že pokud dojde k jeho vzniku a následně k metastatickému rozsevu onemocnění, zachovávají si metastázy podobný typ

fúze TMPRSS2-ETS. Naopak, pokud před vznikem metastáz nedojde k translokaci a fúzní gen nevznikne, neobjevuje se ani u metastáz. Ve tkáni HGPIN, ve které nedojde k translokaci částí genů TMPRSS2 a ETS, je průběh patogeneze na molekulární úrovni podobný, navíc dochází ke zvýšení exprese například genu MUC1 a vznikají další aktivační mutace. Nepřítomnost fúzního genu se ukazuje být příznivým prognostickým faktorem CaP [7].

K výše uvedeným genetickým změnám přispívají v patogenezi CaP také změny epigenetické. Jako první z těchto změn byla u CaP popsána aberantní metylace DNA. U metastatického CaP dochází ke globální hypometylaci cytozinů, tato hypometylace je spojena s chromozomální instabilitou a progresí onemocnění [4]. Zvýšená exprese v důsledku hypometylace promotorů byla u CaP zjištěna například u genů IGF2, CAGE, CYPB1, HPSE, WNT5A a dalších. Na druhé straně dochází během patogeneze CaP, a to i v jejím časném stadiu, k hypermetylaci genů účastnících se kontroly buněčného cyklu, oprav DNA, hormonální odpovědi, apoptózy a také k hypermetylaci promotorů některých genů kódujících miRNA. Další mechanizmus přispívající k deregulaci exprese genů během patogeneze CaP je remodelace chromatinu a posttranslační modifikace histonů. Těchto procesů se účastní řada genů, nejlépe prostudovaná je úloha genu EZH2 kódujícího histon metyltransferázu, polycomb protein katalyzující trimetylaci histonu H3 na lyzinu 27 (H3K27) [8].

Ještě koncem 90. let si málokdo uvědomoval, jaký vliv na řízení genové exprese mají malé molekuly RNA (velikosti kolem 22 nukleotidů). Zjistilo se, že jev původně nazývaný postranskripční umlčování genů se podílí také na nádorové transformaci, a protože samotná deregulace exprese molekul miRNA nemění primární strukturu DNA (pořadí nukleotidů), je tento mechanizmus zařazen mezi epigenetické změny. MiRNA jsou krátké nekódující RNA molekuly, které regulují genovou expresi prostřednictvím inhibice translace a/nebo snížením stability cílové mRNA.

První práce systematicky analyzující expresi miRNA u CaP, a to pomocí microarray technik, byla publikována v roce 2007 [9]. Porovnáním exprese miRNA mezi maligními a benigními buňkami prostaty bylo nalezeno mnoho molekul miRNA se sníženou nebo zvýšenou expresí. Na základě dalších výsledků se pozornost u karcinomu prostaty soustřeďuje na roli miRNA ve vztahu ke schopnosti nádorových buněk zabránit apoptóze a dále na ovlivnění androgenní signalizace molekulami miRNA.

Nejprve se však podívejme, jaké mechanizmy mohou vést k aberantní expresi molekul miRNA. Stejně jako u protein kódujících genů může být i patologická změna exprese a funkce miRNA způsobena jak genetickými změnami, tak změnami chromatinu, které přímo nemění primární strukturu DNA (epigenetické změny). Aberantní expresi molekul miRNA způsobují mechanizmy zahrnující změnu počtu kopií genu pro danou miRNA a epigenetické modifikace jako například metylace promotoru genu kódujícího miRNA snižující expresi příslušné miRNA. Mutace jsou nalézány v oblastech DNA přepisovaných do prekurzorů miRNA

(pri-miRNA, pre-miRNA), tyto mutace mohou výrazně ovlivňovat zpracování prekurzorů miRNA a hladinu vlastní maturované miRNA. Kritické jsou mutace v oblastech dvouřetězců (stem) sekundární struktury pre-miRNA nebo v nespárovaných doprovodných oblastech, které jsou důležité pro interakci s proteinem DGCR8 a štěpení ribonukleázou Drosha. Mutace DNA projevující se v nespárované stem loop struktuře, smyčce, nemají většinou vliv na zpracování miRNA [10]. Změna exprese miRNA může být ovlivněna dalšími faktory, včetně chyb při zpracování miRNA. Výše uvedené mechanizmy mají bezprostřední vliv na výslednou hladinu miRNA ve tkáni karcinomu prostaty.

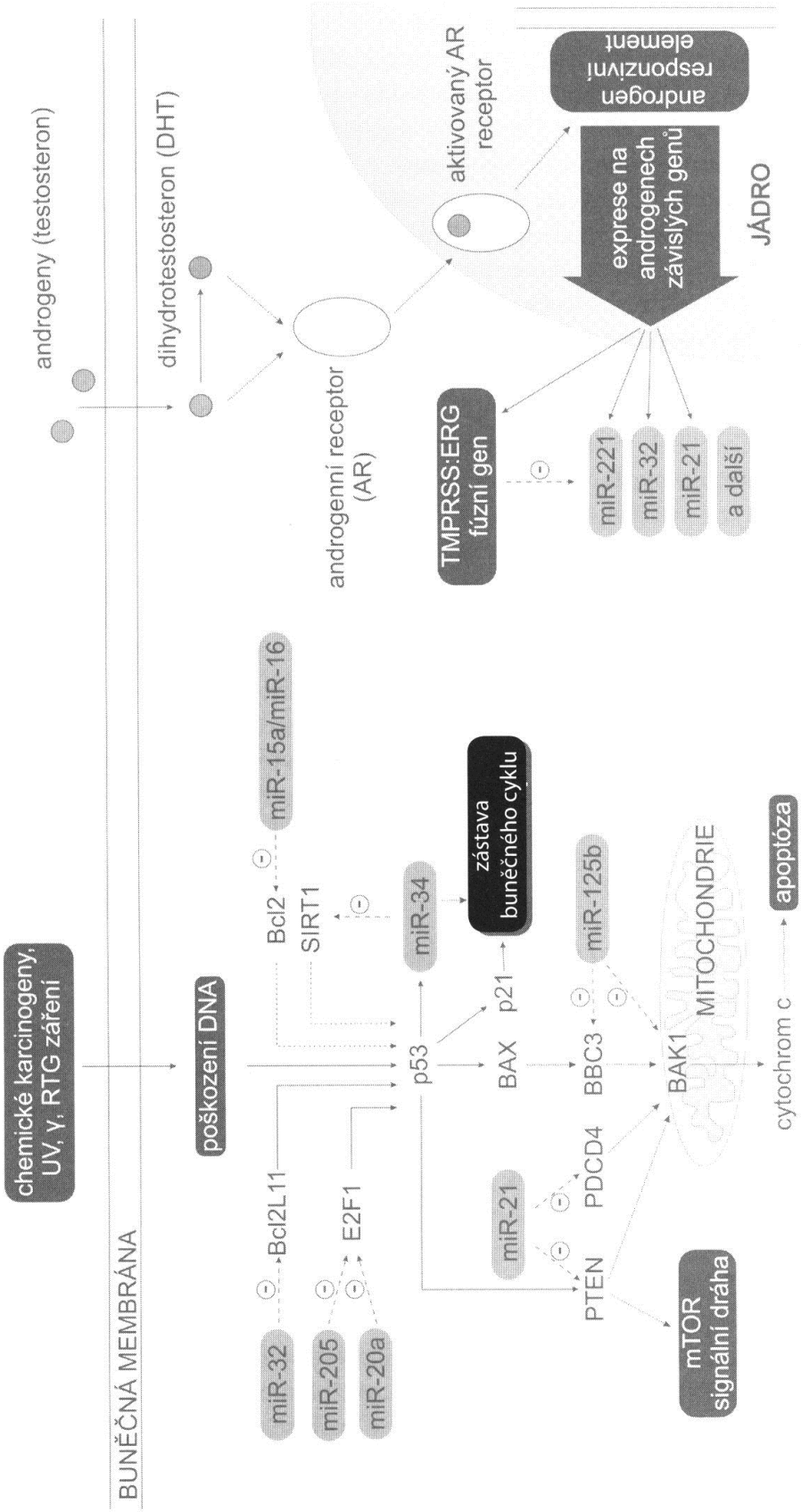
Konkrétně u karcinomu prostaty byla zjištěna zvýšená exprese ribonukleázy Dicer, což vede k deregulaci exprese jednotlivých miRNA. Tato zvýšená exprese Diceru je ve vztahu ke stadiu onemocnění a Gleason skóre, byla nalezena také u agresivnějších CaP [11]. V okolí genů kódujících některé miRNA se u karcinomu prostaty vyskytuje hypermetylace CpG ostrůvků, která negativně koreluje s expresí příslušných miRNA. Vrba et al. zjistili, že u nádorové linie karcinomu prostaty PC3 dochází v důsledku hypermetylace CpG ostrůvků v blízkosti genů miR-200c a miR-141 ke snížení jejich exprese v porovnání s nádorovými liniemi karcinomu prostaty LNCaP a DU145, u kterých je metylační status CpG ostrovků genů miR-200c a miR-141 nezměněn [12].

Pokud se genetické varianty (například jednonukleotidové polymorfizmy) nacházejí uvnitř genů miRNA v místech, která kódují sekvenci zralých miRNA nebo v oblastech vazebných míst na cílových mRNA, mohou tyto změny ovlivnit specificitu miRNA a vést k deregulaci exprese cílových mRNA. Tyto genetické varianty byly nalezeny u řady miRNA, například miR-423, miR-125b [13].

10.1. MikroRNA a apoptóza u karcinomu prostaty

Deregulace exprese miRNA u karcinomu prostaty ovlivňuje aktivaci apoptózy, a to inhibicí proapoptotických signálů (obr. 10.1.). Upregulace klastru miR-17-92 (na chromozomu 13) vedoucí k nadměrné tvorbě miR-20a inhibuje expresi transkripčních faktorů E2F1-3. V závislosti na fázi buněčného cyklu podporuje snížená hladina E2F1-3 proliferaci buněk a snižuje hladinu proteinu p53, a inhibuje tak apoptózu zprostředkovanou kaspázami. Zároveň transkripční faktory E2F1-3 řídí transkripci miR-20a, vzniká tak autoregulační smyčka [14]. Další miRNA inhibující apoptózu je miR-21. Její antiapoptotické mechanizmy jsou realizovány prostřednictvím sítě proteinu p53. U karcinomu prostaty bylo prokázáno, že miR-21 inhibuje expresi genu PDCD4 [15] a tumor supresorového genu PTEN [16].

Rodina miR-34 je exprimována částečně pod kontrolou p53. Ztráta p53 vede ke snížení hladiny miR-34a, a tím ke snížení inhibice exprese genu SIRT1. Následně zvýšená hladina SIRT1 vede k další snížené transkripci genu p53 [17]. Další výzkum ukázal, že expresi transkripčního faktoru E2F1 inhibují také miR-25



Obr. 10.1. Vztah molekul mikroRNA k apoptóze a androgenní signalizaci v patogenezi karcinomu prostaty. Upraveno podle [21].

Levá část obrázku

Zabránění apoptóze je klíčová událost v karcinogenezi. Řada mikroRNA působí zásahem do buněčných signálních drah, které přímo souvisejí s buněčným cyklem a apoptózou. MiR-125b inhibuje proapoptotické geny BAK1 a BBC3, miR-21 cílí tumorsupresorové geny se vztahem k apoptóze PTEN a PDCD4. Některé miRNA mají vztah k proteinu p53. Prostřednictvím miR-34 je vykonávána alespoň část efektů p53 vedoucích k zástavě buněčného cyklu. Klastr miR-15a/miR-16 indukuje apoptózu represí antiapoptotického proteinu BCL2. MiR-32 brání apoptóze inhibicí exprese proapoptoticky působícího BCL2L11, miR-20a a miR-205 jsou zapojeny do řízení hladiny transkripčních faktorů rodiny E2F.

Pravá část obrázku

Androgeny (testostron) prostupují buněčnou membránou dovnitř buňky. Účinkem enzymu 5-alfa-reduktázy vzniká z testosteronu mnohem účinnější dihydrotestosteron (DHT). Jak testosteron, tak DHT se vážou na androgenní receptor (AR), který vazbou androgenů mění konformaci a dimerizuje. Aktivovaný androgenní receptor působí jako specifický transkripční faktor, váže se na sekvenci DNA nazývanou androgen responzivní element, a podporuje tak transkripci androgeny řízených genů, mezi něž patří mimo jiné i některé geny pro miRNA (miR-21, miR-32, miR-221) a v patogenezi karcinomu prostaty důležitý fúzní gen TMPRSS2: ERG.

a miR-205 [18]. Do současnosti bylo identifikováno nejméně 10 miRNA inhibujících apoptózu, a to včetně těch, které se zapojují do zmíněné zpětnovazebné smyčky.

Kromě inhibice apoptózy je pro nádorové buňky klíčová deregulace proliferace. Zvýšená exprese miRs-221/222 (lokalizovány na chromozomu X) se vyskytuje v buňkách nádorové linie karcinomu prostaty PC3. Tyto miRNA přímo inhibují expresi P27KIP1, tím je buňce umožněn další průchod buněčným cyklem [19]. Přechod G1/S kontrolním bodem buněčného cyklu upregulací cyklinu D1 je podpořen u nádorových buněk snížením exprese miR-15a/16-1 (klastr na chromozomu 13q14). Snížená exprese miR-15a/16-1 byla zaznamenána u 80 % nádorů prostaty. Tyto miRNA také inhibují expresi WNT3a, což vede k aktivaci dráhy Wnt [20,21].

Tím, že každá molekula miRNA má potenciál regulovat desítky až stovky genů, několik málo deregulovaných miRNA může ovlivňovat většinu klíčových drah karcinogeneze. Například zvýšená exprese miR-21 může inhibovat apoptózu, zároveň indukovat proliferaci a migraci buněk.

10.2. MikroRNA a androgenní signalizace

Proces patogeneze karcinomu prostaty je spjat s androgenní signalizací, která je také ovlivňována molekulami miRNA. Vzniká tak vztah zpětné vazby, kdy androgen-responzivní molekuly regulují miRNA a další miRNA modulují androgenní signalizaci. Exprese miR-125b je regulována androgeny prostřednictvím androgen-responzivních elementů (ARE) nacházejících se v promotoru genu pro miR-125-b2. Upregulace miRNA-125b usnadňuje růst nezávislý na androgenech u LNCaP buněk a tlumí apoptózu inhibicí exprese BAK1, BBC3 a p53 genů [22, 23].

Ribas et al. [24] zjistili, že po navázání androgenu na AR se tento komplex váže na promotor genu miR-21 a indukuje expresi miR-21, tzn. že u CaP je podpora růstu buněk androgeny realizována také prostřednictvím miR-21. Kromě toho, že se v promotoru miR-21 nachází ARE sekvence, může být exprese miR-21 prostřednictvím různých drah spuštěna v nepřítomnosti androgenů.

Další miRNA řízená androgeny se ukazuje být miR-146a. Snížená exprese miR-146a byla pozorována v buňkách androgen-senzitivních v porovnání s buňkami androgen-nezávislými. Dále bylo zjištěno, že miR-146a inhibuje expresi genu ROCK1, který se podílí na vzniku karcinomu prostaty nezávislého na kastraci [25].

Exprese miR-141 je účinně indukována androgeny. Amplifikace androgenních receptorů zvýší expresi miR-141 8–10krát. Také u karcinomu prostaty byla pozorována zvýšená exprese této miRNA [26].

Dále bylo zjištěno, že exprese molekul miRNA řízená prostřednictvím androgenů je ovlivněna celistvostí dráhy androgenů. Zároveň je exprese miRNA aktivnější v buňkách androgen závislých než v buňkách androgen nezávislých [27].

Inhibicí exprese cílových genů molekuly miRNA také modulují androgenní signalizaci. Tyto poznatky byly získány zejména pokusy na buněčných liniích. Při zkoumání androgen senzitivních a rezistentních buněk byla zaznamenána zvýšená exprese miR-221/222 [28], zároveň tyto miRNA ovlivňují odpověď buněk na dihydrotestosteron.

MiRNA také regulují androgenní signalizaci prostřednictvím sdílených transkripčních faktorů jiných signálních drah. ErbB-2 (HER2/Neu) je receptorová tyrozin kináza se zvýšenou expresí u podskupiny CaP. Jeden z mechanizmů, jehož výsledkem je zvýšená exprese ErbB-2, je snížení exprese miR-331-3p, a tím i ztráta inhibice ErbB-2. *In vitro* exprese miR-331-3p sníží expresi ErbB-2 a rovněž zabrání androgenní signalizaci. Tento účinek je nezávislý na přítomnosti androgenních receptorů a může být zvýšen pomocí antiandrogenu bicalutamidu, což naznačuje sdílené dráhy. Také další výzkum ukázal důležitou roli miRs-145/331-3p v karcinogenezi prostaty [29,30].

10.3. Vybrané mikroRNA se vztahem ke karcinomu prostaty

10.3.1. MiR-21

Zvýšená exprese miR-21 byla nalezena u řady tumorů [31–33], deregulace miR-21 by snad mohla být pokládána i za obecnou vlastnost nádorových buněk. Ověřené cíle miR-21 zahrnují mnoho genů uplatňujících se v potlačování migrace a invazivity buněk, jako příklad uveďme dobře známé tumor supresorové geny PDCD4, TPM1 a PTEN. Jako další cílový gen byl identifikován MARCKS kódující protein s vlivem na buněčnou adhezi a motilitu. U CaP byla tato miRNA dlouho opomíjena, ale začíná se ukazovat, že i zde se tato miRNA podílí na patogenezi. Exprese miR-21 koreluje se stadiem onemocnění, přítomností metastáz v lymfatických uzlinách, Gleason skóre, obdobím do biochemické rekurence po radikální prostatektomii [34,35].

10.3.2. MiR-15a/miR-16

Oblast obsahující klastr dvou genů miR-15a a miR-16 bývá často postižena delecí u chronických lymfocytárních leukemií. U CaP byla zjištěna snížená exprese miR-15a/miR-16 u 80 % nádorových vzorků a také u nádorově asociovaných fibroblastů [36,37]. Bylo prokázáno, že tyto miRNA indukují apoptózu represí

antiapoptotického proteinu BCL2. Dalšími významnými cílovými geny těchto miRNA jsou geny zasahující do buněčné proliferace (například cyklin D1), geny aktivující onkogenní dráhy (WNT3A se vztahem k AKT a MAPK signální dráze). Prostřednictvím regulace hladiny VEGF ovlivňují tyto miRNA angiogenezi [36]. Ztráta funkce miR-15a/miR-16 může proto přispívat různými cestami k progresi karcinomu prostaty.

10.3.3. MiR-20a

Často studovaný je i klastr miR-17-92, ve kterém je obsažena miR-20a. Oblast klastru bývá amplifikována u řady tumorů, včetně CaP [31,14,38]. Předpokládá se, že miR-20a je spolu s dalšími miRNA obsaženými ve stejném klastru (například miR-17-5p) zapojena do autoregulační smyčky, která řídí hladinu transkripčních faktorů rodiny E2F podílejících se na kontrole buněčného cyklu a apoptózy. Princip autoregulační smyčky spočívá v tom, že exprese klastru miR-17-92 je regulována faktory E2F, produkované miRNA naopak inhibují translaci E2F faktorů. Experimenty na buněčných liniích CaP ukazují na antiapoptotické působení miR-20a [14].

10.3.4. MiR-32

Další miRNA bránící apoptóze je miR-32, která inhibuje expresi BCL2L11 (BIM) – proapoptoticky působícího zástupce BCL2 rodiny. U CaP byla popsána zvýšená exprese miR-32 spojená se zvýšenou expresí hostitelského genu C9orf5 (miR-32 je obsažena v intronu genu C9orf5), jehož role v onkogenezi není známa [27]. Tvorba miR-32 je regulována androgeny. Zvýšená exprese miR-32 je typická pro hormonálně rezistentní karcinom prostaty [39].

10.3.5. MiR-34

Velice zajímavé je zapojení miR-34 do sítě drah spojené s proteinem p53. Exprese miR-34 se zvyšuje při poškození DNA cestou závislou na p53. Alespoň část efektů p53 je zprostředkována skrze miR-34, tato miRNA inhibuje cyklin-dependentní kinázy (CDK4 a CDK6), cyklin D1 a E2, transkripční faktor E2F3, a tím vyvolá zástavu buněčného cyklu, případně indukuje apoptózu. Navíc se zde uplatňuje i pozitivní zpětná vazba, kdy miR-34 aktivovaná působením p53 inhibuje SIRT1, a tím dále posiluje aktivitu p53 (SIRT1 má schopnost bránit apoptóze závislé na p53). Tyto popsané jevy jsou v souladu i s nálezem ztráty exprese miR-34 u androgen refrakterních p53 defektních buněčných linií CaP [40–42].

10.3.6. Rodina miR-200

Členové rodiny miR-200 inhibicí exprese ZEB proteinů kontrolují epiteliálně mezenchymální tranzici, což je událost související s procesem tvorby metastáz [43].

10.3.7. MiR-221/miR-222

Obě tyto miRNA jsou pokládány za onkogenní a po boku miR-21 se řadí k nejčastěji deregulovaným u různých typů nádorů včetně CaP [44]. Negativně regulují hladinu inhibitoru cyklin dependentních kináz P27KIP1, což vysvětluje podporu proliferace nádorových buněk při jejich zvýšené expresi. Role miR-221 u CaP je ale komplikovanější, u CaP pozitivních na přítomnost fúzního genu TMPRSS2: ERG je prokazována snížená exprese miR-221 [45].

10.3.8. MiR-125b

O miR-125b je známo, že inhibuje proapoptotické geny BAK1 a BBC3 (Puma) [23], což by mohlo naznačovat onkogenní působení, ale závěry výzkumu jsou zatím rozporuplné. Existují práce, které ukazují jak na zvýšenou [46], tak na sníženou [9,47] expresi této miRNA u CaP. U karcinomu prsu bylo popsáno tumor supresorové působení miR-125b zprostředkované inhibicí ERBB2 (HER2) a ERBB3 (HER3) [48]. Musíme si uvědomit, že daná konkrétní miRNA může regulovat stovky genů, mezi kterými jsou jak onkogeny, tak tumor-supresory, a celkový efekt závisí na „čase a místě" působení dané miRNA.

10.4. MikroRNA a léčba

S přibývajícími poznatky na poli RNA interference a nekódujících RNA molekul je snaha přenést tyto znalosti do klinické praxe, nejen jako možných diagnostických nástrojů, ale i v podobě nových léčiv. Pro léčebné účely jsou syntetizovány jak molekuly pre-miRNA, tak anti-miRNA, které budou pomocí speciálních partikulí bezpečně dodávány do těla pacientů. Tyto molekuly mohou být podávány lokálně nebo systémově. Je očekáván léčebný efekt na nádorové buňky, při zasažení normálních buněk se nepředpokládají vedlejší účinky, případně je očekáváno protinádorové působení.

Deregulace exprese molekul miRNA, které ovlivňují výslednou hladinu produktů genů zapojených v reparaci DNA, může ovlivnit léčebnou odpověď. Poškození DNA, vyvolaná například ozářením, spustí v buňce mechanizmus, je-

hož výsledkem je opravení chyb nebo smrt buňky. U karcinomu prostaty snižuje zvýšená exprese miR-521 reakci na poškození DNA inhibicí exprese genu Cockayne syndrom protein A [49].

Lee et al. geneticky modifikovali herpes simplex virus-1 začleněním oblastí cílových sekvencí (tzv. seeds) pro miR-143 a miR-145 do jednoho ze základních genů tohoto viru. Zvolili miRNA, které jsou vysoce exprimovány v normálních tkáních, ale mají silně potlačenou expresi v buňkách CaP. Takto vytvořený miRNA-regulovaný onkolytický virus se dokáže replikovat (a tím způsobovat buněčnou lýzu) jen v buňkách CaP. V buňkách s normální expresí miR-143 a miR-145 tyto miRNA posttranskripčně inhibují expresi virového genu, a tak jsou před lytickým účinkem viru chráněny. U myšího modelu vedl takto modifikovaný virus k 80% snížení objemu nádoru [50].

10.5. Využití molekul mikroRNA jako biomarkerů u karcinomu prostaty

Stále více prací ukazuje, že molekuly miRNA mohou být zdrojem klinickopatologických informací [51]. Použití molekul miRNA pro získání informací o nádorové tkáni poprvé publikovali v roce 2005 Lu et al., kteří pomocí stanovení exprese miRNA rozlišili nádory různých typů (tj. epiteliální vs. hematopoetické) a tkáňového původu [52]. Další studie zdůraznily potenciál stanovení expresních profilů miRNA jako možnost přesně určit tkáňový původ nádoru [53–55]. Kromě výše uvedeného mají molekuly miRNA i další vhodné vlastnosti pro využití coby biomarkerů. MiRNA jsou mimořádně stabilní v různých typech klinických vzorků, dokonce i ve tkáních fixovaných ve formalínu a archivovaných v parafinových bločcích [56]. Molekuly miRNA lze snadno a specificky detekovat kvantitativní RT-PCR (qRT-PCR). Tyto testy lze provádět multiplexově, což je důležité pro efektivitu detekce ve vztahu k úvaze, že diagnostického nebo prognostického závěru bude dosaženo spíše kombinací biomarkerů než stanovením jednoho jediného analytu. Většina molekul miRNA je mezi druhy vysoce konzervovaná, což pro preklinické studie umožňuje použití modelů onemocnění u zvířat.

V roce 2008 řada autorů popisovala, jak výskyt nádorového onemocnění změnil expresi určitých miRNA v periferní krvi. MiRNA byly izolovány z plazmy nebo ze séra [57,58]. Tyto práce ukázaly na možnost použití miRNA v klinické praxi ze snadno dostupného biologického materiálu. Později byly publikovány i práce popisující kvantifikaci miRNA například v moči nebo ve spermatu. Pro využití molekul miRNA u karcinomu prostaty jako biomarkerů bylo důležité prokázat prostup molekul miRNA z prostaty do periferní krve, což v roce 2008 demonstrovali Mitchell et al. na myším modelu. Zjistili, že u myší s xenograftním CaP systémem vstupují lidské miRNA do myší plazmy [57].

Kromě studií porovnávajících hladiny jednotlivých miRNA mezi nádorovou tkání a kontrolní tkání prostaty, případně hledající vztah mezi hladinou miRNA v nádorové tkáni a klinicko-patologickými charakteristikami, se pozornost řady autorů upíná k stanovování hladin miRNA v periferní krvi, resp. plazmě nebo séru. Důvodem je případné diagnostické využití těchto stanovení v klinické praxi. Tyto studie jednak srovnávají expresi miRNA v plazmě/séru mezi zdravými probandy a pacienty s karcinomem prostaty v různém stadiu onemocnění a dále hledají vztah mezi hladinami miRNA a prognózou onemocnění či predikcí odpovědi na léčbu.

Studie Yaman Agaoglu et al. uvádí zvýšenou hladinu miR-21 a miR-221 v plazmě mužů s lokalizovaným karcinomem prostaty v porovnání se zdravými kontrolami [59]. Podobně Mahn et al. nalezli zvýšenou hladinu miR-26a, miR-195 a let-7i v séru mužů s lokalizovaným CaP v porovnání s muži s benigní hyperplazií prostaty (BPH – benign prostatic hyperplasia) [60]. Další studie zkoumající jiný set molekul miRNA nalezla signifikantně vyšší koncentrace miR-107 a miR-574-3p v plazmě mužů s CaP v porovnání se zdravými muži. V této studii byly koncentrace miR-107 a miR-574-3p stanovovány také v moči, a to s lepším diagnostickým výsledkem než při použití mRNA genu PCA3, nově zaváděného markeru CaP [61]. Tyto studie naznačují využitelnost exprese miRNA pro diagnostiku karcinomu prostaty.

Další studie zjišťují možnost předpovídat vývoj onemocnění, prognózu, dle stanovení hladin miRNA v plazmě/séru. Brase et al. se zabývali analýzou 69 miRNA v séru a nalezli tři miRNA (miR-141, miR200b a miR-375), jejichž hladina stoupala se vzrůstajícím stadiem onemocnění a Gleason skóre [62]. Studie Moltzahna et al. nalezla vztah řady miRNA, včetně miR-24, miR-106a, miR-451 a miR-93, s CAPRA skóre [63]

Bryant et al. nalezli 16 miRNA v plazmě, včetně miR-141, miR-20b a miR-375, umožňujících rozlišit mezi lokalizovaným a metastatickým karcinomem prostaty [61]. Tyto studie ukazují na možnost stanovení hladin miRNA v plazmě/séru pro prognózu onemocnění, resp. pro identifikaci nádorů s nepříznivou prognózou. Tato stanovení by mohla doplnit stávající laboratorní schémata.

Ukazuje se, že existuje silná asociace s metastatickým onemocněním u molekul miR-141, miR-200b a miR-375. Tyto markery by mohly být použity v době stanovení diagnózy pro identifikaci pacientů s agresivním onemocněním nebo pro předpověď recidivy po primární léčbě [61]. Provedené studie však nedosahují takových počtů pacientů jako ve studiích klasických markerů, a proto je třeba tyto výsledky ověřit ve velkých studiích včetně dlouhodobého klinického sledování.

Zároveň se objevují studie hledající vztah mezi hladinou miRNA a léčebnou odpovědí. Je však třeba zmínit, že se jedná o práce, ve kterých je vyšetřován malý počet pacientů, což komplikuje závěry jednotlivých studií. Zhang et al. publikovali práci, jejímž cílem bylo ověřit možnost rozlišit pacienty s BPH, lokalizovaným a pokročilým karcinomem prostaty pomocí stanovení exprese miR-21. Tuto možnost sice nepotvrdili, avšak při dalším vyhodnocování zjistili, že hladina miR-21 byla signifikantně zvýšena u čtyř pacientů s lokalizovaným CaP, kteří nereagovali

na chemoterapii. Pacientům byl podáván docetaxel [64]. Tato práce poprvé uváděla vztah mezi cirkulující miRNA a predikcí léčby. Další studie, Gonzalese et al., měla za cíl zjistit prediktivní význam plazmatické hladiny miR-141. Do studie vstoupilo 21 pacientů, a to s metastatickým karcinomem prostaty a léčených různým typem léčby (chemoterapie, hormonální terapie, nové léky jako součást klinických studií). Stanovení hladiny miR-141 ukázalo její prognostický význam, byl nalezen vztah mezi hladinou této miRNA a klinickou progresí. Avšak nebyl zaznamenán vztah k léčebné odpovědi [65].

Závěr

Jak bylo uvedeno v předchozích kapitolách, efekt jednotlivých molekul miRNA se neomezuje na interakci s několika dalšími molekulami, ale s desítkami až stovkami molekul, což komplikuje jejich využití jako léčebného cíle, ale i biomarkeru, zvláště vezmeme-li v úvahu heterogenitu nádorových onemocnění. Přesto lze předpokládat, a to i vzhledem k vhodným chemickým vlastnostem miRNA pro úlohu biomarkerů, že z velkého množství zkoumaných molekul se jich několik osvědčí a že budou v budoucnu využívány v klinické praxi.

Postupná identifikace onkogenů a tumorsupresorů, které jsou klíčové pro patogenezi karcinomu prostaty spolu s pochopením úlohy molekul miRNA, nám doufejme otevře nové možnosti diagnostiky a léčby tohoto onemocnění.

Poděkování

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Significance of Methylation Status and the Expression of *RECK* mRNA in Lung Tissue of Patients with NSCLC

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Abstract. *Objectives: RECK (reversion-inducing cysteine-rich protein with Kazal motifs) is a glycoprotein which negatively regulates the activity of matrix metalloproteinases (MMPs). We analyzed differences in RECK mRNA expression in histological types of non-small cell lung cancer (NSCLC) and the relationship between promoter methylation status of RECK gene, level of RECK mRNA expression and clinicopathological values of patients with NSCLC. Patients and Methods: Methylation status of the promoter and the expression of RECK mRNA were analyzed in paired tissue samples (tumor and control) of 50 patients with NSCLC. The methylation status of the RECK promoter was assessed using methylation-specific PCR. The level of RECK mRNA expression was measured using an RT real-time PCR method. Results: Lower expression of RECK mRNA in NSCLC tissue was recorded compared to normal tissue (p=0.0032). Significantly lower expression of RECK in squamous cell carcinoma (SCC) tissue was observed in comparison with adenocarcinoma tissue (p=0.0051). Significant differences in expression of RECK in stages IB-IIIA were found in comparison with stage IA (p=0.0455). There was a significantly lower expression of RECK mRNA in NSCLC tissue in samples with positive RECK promoter methylation status in comparison with samples with negative promoter methylation status (p=0.0400). Conclusion: We showed that there were differences in expression between histological types of NSCLC (SCC, adenocarcinoma). There was a higher expression of RECK in stage IA in comparison with stages IB-IIIA. Our results indicate that RECK could be classified as a tumor suppressor gene and is an interesting target for further investigation of MMP inhibitors.*

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Key Words: Non-small cell lung cancer, RECK, tissue samples, mRNA, RT-PCR, promoter methylation.

RECK (reversion-inducing cysteine-rich protein with Kazal motifs) is a membrane anchored glycoprotein which regulates matrix metalloproteinases (MMPs) and inhibits angiogenesis. Recent analyses indicate that RECK expression is frequently down-regulated in tumor tissues in comparison with the surrounding non-tumorous tissues in several common types of cancer including colon, mammary, and pancreatic carcinoma (1). We focused on RECK expression in lung cancer, which is the leading cause of cancer-related mortality, not only in the Czech Republic, but also around the world (2, 3).

RECK protein is able to inhibit MMP-2, MMP-9 and MMP-14. Reduction of active MMP-2 is probably due to direct inhibition of its processing enzymes, MMP-14 and also MMP-2, by RECK: purified recombinant RECK was found to inhibit the proteolytic activities of MMP-2 and MMP-14 *in vitro*. The mechanism for the reduction of pro-MMP-9 is less clear. Pro-MMP-9 production is probably halted at some point between transcription and secretion (1). The effect of RECK in tumorigenesis is realized mainly through the relationship with MMPs (1). MMPs not only degradate the extracellular matrix (ECM) components and basal membranes, but they also influence changes in the growth, apoptosis, and migration of healthy cells. Through remodelling or destruction of the ECM, MMPs contribute to processes of tumor cells migration (4).

The decreased inhibition of active MMPs is trigged by the down-regulation of RECK expression by K-ras (5). Two mechanisms involving K-ras were described. The first is mediated by the methylation of CpG islands in the *RECK* promoter. Oncogenic RAS increases the binding of DNA methyltransferase DNMT3b to the promoter of *RECK* and this binding induces promoter methylation, which could be reversed by DNMT3b small interfering RNA (siRNA) (6). The second mechanism of this RECK down-regulation is *via* the target SP1 site on the *RECK* promoter sequence and appears to be multifactorial and also tumor specific. This theory supposes that Ras facilitates the phosporylation or other modification of Sp1/Sp3 factors which increases binding to the SP1 site in the promoter region of *RECK* gene

and thus decreases the transcription activity of *RECK*. This theory was confirmed by the results of Chang *et al*. (7). The next possible regulatory mechanism of the expression of RECK was published by Zhang *et al*., which showed that RECK was a *bona fide* target of miR-21 (8).

We investigated differences in *RECK* mRNA expression in histological types of non-small cell lung cancer (NSCLC). We further analyzed the relationship between promoter methylation status of *RECK* gene, level of *RECK* mRNA expression and clinicopathological values of patients with NSCLC.

Patients and Methods

Patients. We studied a group of 50 patients with NSCLC (median age of 62.4 years, range 47.5-77.8, stage IA 12 (24%) and stages IB – IIIB 38 (76%)), who had undergone lung surgery at the Department of Surgery, University Hospital Pilsen, between 2005-2007.

Tissue samples. Fifty paired (tumor and control) lung tissue samples were taken directly from tumor tissue and from the adjacent, histologically cancer-free lung tissue (normal lung tissue) in the same patient during surgery. These resected tissue samples were immediately frozen to –70˚C and stored at this temperature until use. All the samples were histologically verified. The distribution according to histology is shown in the Figure 1.

Assesment of the methylation status of the RECK promoter. DNA was isolated from approximately 20 mg of 50 tumor lung tissue samples using the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany). Genomic DNA conversion was performed using EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA). DNA after conversion was used for analyses of the methylation status of the *RECK* promoter using methylation-specific PCR. The sequence of primers taken from the publication of Chang *et al*. was modified (9). The following primers were used for the methylated sequence: M-sense 5'-AATAAAGAGTTTTGGTACGGGGTAC-3'; M-antisense, 5'-AAAACCGCGAAATACTCGAA-3' and for the unmethylated sequence: U-sense 5'-TAAAGAGTTTTGGTAT GGGGTATGT-3'; U-antisense 5'-CTCCAAAACCACAAAATA CTCAAA-3', synthesized by GeneriBiotech (Hradec Kralove, Czech Republic).

Quantitative estimation of mRNA using RT real-time PCR. Total RNA was isolated from 100 mg of 50 paired control and tumor lung tissue. We used the fast RNA Pro Green Kit (Q-BIOgene, Irvine, CA, USA). Reverse transcription (RT) was performed from 3 μg of total RNA with Superscript III Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA) and oligo $d(T)_{21}$ as a primer. The sequence of primers used for *RECK* was as follows: forward primer 5'-ATCATTCCCGTCGATCACTATC-3'; reverse primer 5'-ATATGTCC AGAGCAAGTGCAAG-3' synthesized by GeneriBiotech (Hradec Kralove, Czech Republic).

In all the samples, we also assessed the expression of mRNA of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The real-time PCR procedure and the sequence of *GAPDH* primers were described in our previous publication (10). The results are presented as normalized values, the ratio of the number of copies of *RECK* to the housekeeping gene *GAPDH*. Statistical analysis was performed using software SAS 8.02 (SAS Institute Inc., Cary, NC, USA). The statistical results were calculated by a Wilcoxon two-sample test. For the maximum hazard ratio (disease-free interval, DFI; overall survival, OS) the Cox regression hazard model was used. After finding the optimal cut-off for the examined markers, the Kaplan-Meier survival distribution functions of this optimal cut-off in given groups were computed.

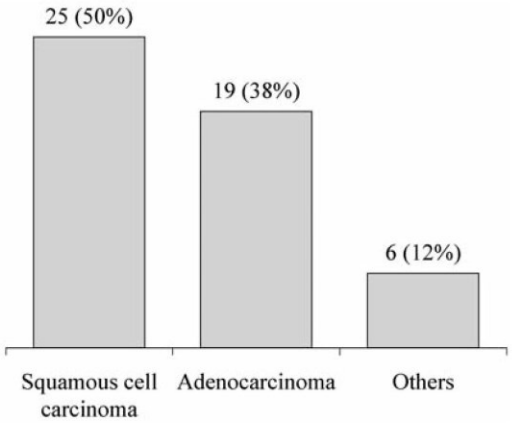

Figure 1. *The distribution of carcinoma tissues according to histological type*.

Results

We investigated differences in the expression of *RECK* mRNA in NSCLC and normal lung tissue. We found a statistically significant lower expression of *RECK* in tumor tissue in comparison with normal lung tissue (p=0.0032). We recorded a statistically significant lower expression of *RECK* in squamous cell carcinoma (SCC) tumor tissue in comparison with normal lung tissue (p=0.0003), but we did not observe differences between adenocarcinoma tissue and normal lung tissue (p=0.3208) (Table I). Futhermore we recorded a significantly lower expression of *RECK* in the SCC tumor tissue in comparison with the adenocarcinoma tissue (p=0.0051) (Figure 2).

We also observed a significantly higher expression of *RECK* in stage IA in comparison with IB-IIIA (p=0.0455) using the median two-sample test (Table II).

The samples of tumor tissue tested positively on promoter methylation status expressed a statistically significant lower level of *RECK* mRNA in comparison with unmethylated *RECK* promoter tumor tissue samples (p=0.0400) (Figure 3).

We found no statistically significant relation between the expression of *RECK* and clinicopathological values (DFI, OS) in all studied groups (NSCLC, SCC and adenocarcinoma), hence the p-values are not presented here. We recorded no statistical significance in the relation between RECK promoter methylation status and DFI and OS in all compared groups

Table I. *Differences in expression of RECK mRNA.*

N	Tissue	Median expression *RECK/GAPDH*	Wilcoxon *p*-Vaue
50	NSCLC	0.0397797	0.0032
49	Control	0.1102843	
25	SCC	0.0297737	0.0003
24	Control	0.1043990	
19	Adenocarcinoma	0.1138185	0.3208
19	Control	0.1359807	
25	SCC	0.0297737	0.0051
19	Adenocarcinoma	0.1138185	

Table II. *Difference between expression of RECK in stage IA and IB-IIIA.*

N	Stage	Median expression *RECK/GAPDH*	Two-sample test
11	IA	0.0543656	*p*=0.0455
39	IB-IIIA	0.0338051	

Table III. *Relation of DFI and OS to RECK promoter methylation status (only the patients with obtained DFI and OS data were included).*

Studied groups	DFI			OS		
	Methylation status		Log-rank *p*-value	Methylation status		Log-rank *p*-value
	–	+		–	+	
NSCLC	23	22	0.0869	24	24	0.4275
SCC	11	12	0.2878	11	13	0.7679
Adenocarcinoma	11	5	0.5682	12	6	0.6575

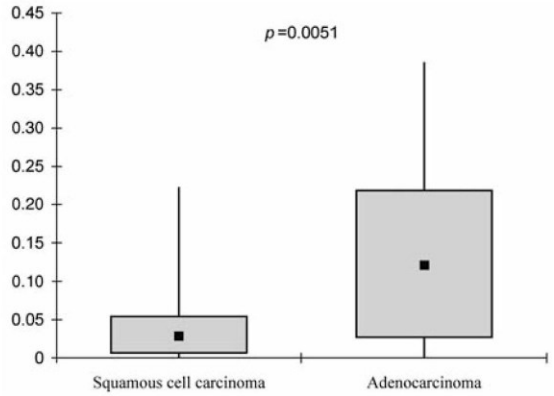

Figure 2. *Difference between expression of RECK in SCC and adenocarcinoma tumor tissue. There was a significantly lower expression of RECK in the SCC tumor tissue in comparison with the adenocarcinoma tissue. Minimum and maximum (line), lower and upper quartile (rectangle) and median (small square) values are shown.*

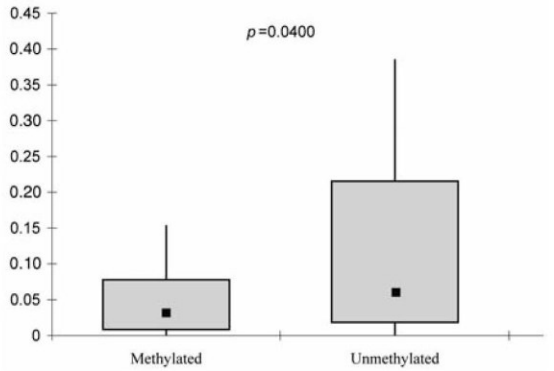

Figure 3. *Relation between methylation status and expression of RECK in NSCLC tissue. The samples with positive promoter methylation status (24 patients) expressed a statistically significant lower level of RECK mRNA (median of expression 0.0283) in comparison with tumor tissue samples unmethylated RECK promoter (24 patients; median of expression 0.0560). Minimum and maximum (line), lower and upper quartile (rectangle) and median (small square) values are shown.*

(NSCLC, SCC and adenocarcinoma) (Table III). It is interesting to note the *p*-value of 0.0869 for the relation between the *RECK* promoter methylation status and DFI in NSCLC group.

Discussion

The MMPs are enzymes which are involved in many processes associated with tumor growth and metastasis, *e.g.* angiogenesis, degradation of the extracelular matrix and basal membrane during tumor enlargment and invasion (11, 12). Therefore molecules regulating their expression and function attract the attention of investigators, for instance as a potential target of anticancer therapy (11, 13). Previous investigation of tissue inhibitors of MMPs has identified the molecule metallopeptidase inhibitor 1 (TIMP-1) as a promising prognostic marker and the study of other molecules with a strong effect on the function on MMPs continues (14-17).

Another molecule which inhibits MMPs (MMP-2, MMP-9, MMP-14) is RECK. We investigated expression of *RECK* mRNA and the methylation status of *RECK* gene promoter in NSCLC. The expression of *RECK* mRNA is decreased in NSCLC tissue in comparison with adjacent cancer-free lung tissue. In contrast, to results of Chang *et*

al. (9), we recorded a lower expression of *RECK* in SCC tissue in comparison with normal lung tissue, but we did not register differences between adenocarcinoma tissue and normal lung tissue. Futhermore, we observed lower expression of *RECK* in the SCC tumor tissue in comparison with the adenocarcinoma tissue. This shows that these histological types are different in *RECK* expression. Expression of RECK in NSCLC was also investigated by Takemoto *et al*., but these investigators did not find differences in expression in histological types (SCC, adenocarcinoma). The same authors did describe a much higher expression of MMP-9 in SCC than in adenocarcinoma (18). Taken together with the results of Takagi *et al*. that RECK negatively regulates MMP-9 transcription (19), this corresponds with our finding that *RECK* expression is lower in SCC tissue.

Next we investigated whether the expression of *RECK* in NSCLC depends on the stage of the disease. We compared stage IA (better prognosis) and stage IB-IIIA (worse prognosis). We observed higher expression of *RECK* in stage IA in comparison with stages IB-IIIA. Takemoto *et al*. observed similar results, but only in adenocarconoma of the lung (18). According to these results, we supposed that patients having higher expression of *RECK* in tumor tissue would achieve longer DFI and OS, but we did not confirm this idea, neither in NSCLC nor in subgroups (SCC, adenocarcinoma). Nevertheless Takemoto *et al*. recorded a relationship between lower *RECK* expression and shorter survival in patients with adenocarcinoma (18). Assessing the RECK protein (20, 21), Takenaka *et al*. observed a higher 5-year survival rate for patients with tumors with strong RECK expression.

We investigated the relation between the methylation status of *RECK* promoter and DFI and OS in NSCLC, but we did not observe any statistically significant differences (DFI, p=0.0869; OS, p=0.4275). We only found, that in solid tumor tissue of NSCLC, the expression of *RECK* is down-regulated by *RECK* promoter methylation. This result agrees with data published by Chang *et al*., who showed a correlation between *RECK* expression down-regulation and promoter methylation (9). More correctly, however, these observations do not exclude other mechanisms which could contribute to *RECK* down-regulation.

In conclusion, we show that the expression of *RECK* mRNA in SCC is lower in comparison with normal tissue and there are also differences in expression between histological types of NSCLC (SCC, adenocarcinoma). There is a higher expression of *RECK* in stage IA in comparison with stages IB-IIIA. These results show that *RECK* could be classified as a tumor suppressor gene with deregulated expression in the SCC tissue. *RECK* is an interesting target for further investigation of MMP inhibitors, in relation to tumors, at least as TIMPs.

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Prognostic Significance of *TMPRSS2-ERG* Fusion Gene in Prostate Cancer

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Abstract. *Background/Aim: Current research of prostate cancer (PCa) offers a promising way of identifying patients with adverse prognosis who do benefit from radical treatment that can affect quality of life as resections are associated with numerous side-effects. The aim of our study was to evaluate the relationship of TMPRSS2-ERG fusion gene status, tumor tissue prostate-specific antigen (PSA), prostate cancer antigen 3 (PCA3), miR-23b, miR-26a and miR-221 expression levels in combination with preoperative serum PSA level to the risk of PCa recurrence after radical prostatectomy. Patients and Methods: The study group consisted of 108 patients who underwent radical prostatectomy. PSA was measured in peripheral blood collected preoperativelly. The expression of TMPRSS2-ERG transcript and the expression of miR-23b, miR-26a and miR-221 in formalin-fixed, paraffin-embedded (FFPE) tumor tissues was analyzed by reverse transcription (RT) real-time polymerase chain reaction (PCR). Results: Significantly shorter time to recurrence was observed in patients with high expression of TMPRSS2-ERG (p=0.0020). High levels of preoperative PSA (>10.0 ng/ml) proved to be marker of shorter time to recurrence (p=0.0153). The most promising marker of the risk of recurrence after radical prostatectomy was a combination of high level of preoperative serum PSA and high expression of TMPRSS2-ERG fusion transcript in tumor tissue (p=0.0001). Conclusion: A combination of high preoperative serum PSA and high expression of TMPRSS2-ERG could be promising in distinguishing those tumors that are aggressive and life-threatening.*

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Prostate cancer (PCa) belongs to the most commonly diagnosed cancers worldwide having a higher incidence in Western countries, which might be due to environmental and lifestyle factors, as well as the greater scale of prostate-specific antigen (PSA) screening in the developed countries. PCa has been proven to be a very heterogenous disease, with individual cases differing in both the speed of progression and overall prognosis (1). The main issue of PCa management is to distinguish those tumors that are subsequently progressing. This would make it possible to limit the number of patients undergoing radical surgery to cases of life-threatening tumors as radical prostatectomy is associated with numerous side-effects (2, 3). To assure radical treatment in cases of aggressive prostate cancer a set of markers reliably indicating the nature of the individual case needs to be identified.

Current research offers a promising way of identifying patients with adverse prognosis. It is based on the knowledge of the pathogenesis of PCa and on the role of the fusion gene *TMPRSS2-ERG* described and linked to PCa by Tomlin *et al.* in 2005 (4). The origin of the *TMPRSS2-ERG* oncogene can be traced to a recurrent rearrangement (translocation, interstitial deletion) on long arm of chromosome 21 that fuses androgen-regulated prostate-specific gene promoter of the transmembrane protease serine 2 (*TMPRSS2*), locus 21q22.3, and the gene *ERG*, locus 21q22.2, a member of the transcription factor erytroblastosis virus E26 transforming sequence family (ETS) (5). Prostate tissue has a strong

androgen dependency and so does PCa, with the signal pathways being mediated by androgen receptors (6). As a result of a *TMPRSS2-ERG* fusion, overexpression of oncogenic transcription factor ERG is driven by androgens through androgen-responsive elements of *TMPRSS2* 5'-untranslated region. Nevertheless, as the disease evolves into castration-resistant PCa, *TMPRSS2-ERG* fusion gene overcomes androgen regulation (7). Fusion gene *TMPRSS2-ERG* appears in early high grade prostatic intraepithelial neoplasia (PIN) and it has been observed that its presence in PCa cells promotes the loss of the tumor supressor gene *PTEN* and other alterations, which in turn lead to the speed up of progression of the disease (8).

In addition to analysis of *TMPRSS2-ERG* fusion gene significance, we also focused on selected microRNAs. MicroRNAs are non-coding RNAs consisting of a small number of nucleotides (~22 nt) that can influence gene expression by interfering with translation or by destabilising target mRNAs. In this way, microRNAs can affect cancerogenesis in both directions; slow the progression by inbibiting oncogen expression or fasten it by down-regulating tumor supressors (9). Many studies have described changes in expression of microRNAs in PCa tissue and their involvement in initiation, progression and the effects of treatment (10). Based on our research of published literature, we chose three tumor-suppressive miRNAs (miR-23b, miR-26a and miR-221) whose levels could be of interest in making prediction of tumor aggressiveness.

The expression level of miR-23b has been observed to be down-regulated in PCa and restoration of the expression inhibited cancer cell proliferation, migration and invasion in PCa cell lines (11). In PCa, miR-23b supresses proto-oncogene Src kinase and its up-regulation shows a strong positive correlation to better prognosis (12). Both miR-26a and miR-221 function as cell proliferation inhibitors, miR-26a inhibits prostate cancer progression by repression of Wnt5a (13) and La-related protein 1 (LARP1) (14). Lower miR-221 expression was associated with a higher risk of recurrence after radical prostatectomy (15).

Furthermore, we included analysis of tumor tissue expression of PSA and prostate cancer antigen 3 (PCA3), formerly referred to as differential display code 3 (DD3). *PCA3* is a long non-coding RNA (lncRNA) beginning to be used as a diagnostic marker of PCa. Recent meta-analysis concluded that urine PCA3 test had acceptable sensitivity and specificity for the diagnosis of PCa (16). There are studies showing association of high PCA3 expression with pathological features of PCa, being predictive of high Gleason score, high-stage and high-volume disease (17, 18). However, less is known about prognostic significance of PCA3 on the basis of the follow-up of patients.

The aim of our study was to evaluate the relationship of *TMPRSS2-ERG* fusion gene status, tumor tissue PSA, PCA3, miR-23b, miR-26a and miR-221 expression levels in combination with routinely used preoperative serum PSA level to the risk of PCa recurrence after radical prostatectomy and, in this way, to predict the aggressivity of the tumor.

Patients and Methods

Patients. Our study group consisted of 108 patients who underwent radical prostatectomy between January 2011 and June 2012 at the Department of Urology of the University Hospital in Pilsen, Czech Republic. All the patients exhibited elevated serum PSA levels or abnormal digital rectal examination. Indication for surgery was confirmed by positive biopsy results, namely the detection of cancerous cells. The median age was 62.9 years (range=43.8-73.9). Clinicopathological data, such as age at the time of surgery, preoperative PSA level, stage of the disease according to the International Union against Cancer (IUCC) and Gleason score are listed in Table I. Approval was obtained from the Institutional Ethics Committee and written informed consent from each patient.

Tissue samples and RNA extraction. The FFPE (formalin-fixed, paraffin-embedded) tissue samples were used for RNA extraction as we described previously (19, 20). Briefly, areas selected for analysis highlighted by pathologist (either tumor tissue or adjacent normal prostate tissue) were manually macrodisected from 15-μm thick FFPE tissue sections prepared with microtome (Leica RM 2135; Leica Biosystems, Nussloch, Germany). Total RNA was isolated using the RNeasy FFPE Kit (Qiagen, GmbH, Hilden, Germany) according to the protocol of the manufacturer. The concentration of the isolated RNA was measured with NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA); in case of lower concentration than 15 ng/μl the isolation was repeated.

Quantitative estimation of TMPRSS2-ERG, PSA and PCA3 expression. Reverse transcription (RT) was performed from 250 ng of total RNA with Superscript III Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA) and random hexamers as primers. Quantitative estimation of *TMPRSS2-ERG*, *PSA* and *PCA3* transcripts was performed by real-time polymerase chain reaction (PCR) method with UPL probes (Universal ProbeLibrary; Roche, Mannheim, Germany) on Stratagene Mx3005P apparatus (Agilent Technologies, Santa Clara, CA, USA). The sequences of primers for *TMPRSS2-ERG* fusion gene (forward primer 5'-TAGGC GCGAGCTAAGCAG-3' targeting *TMPRSS2* exon 1 and reverse primer 5'-GTCCATAGTCGCTGGAGGAG-3' targeting ERG exon 4) were previously designed and validated by other researchers (21, 22). We found the appropriate UPL probe (probe #1) for this pair of primers by ProbeFinder Software (Roche, Mannheim, Germany). The sequences of primers and corresponding UPL probes for *PSA* mRNA (forward primer 5'-GTGCTTGTGG CCTCTCGT-3', reverse primer 5'-CAGCAAGATCACGCTTTTGT-3', probe #44) and *PCA3* lncRNA (forward primer 5'-TGGGAAGGACCTGAT GATACA-3', reverse primer 5'-TGTGTGGCCTCAGA TGGTAA-3', probe #66) were generated by ProbeFinder Software. The PCR reactions were carried out in 96-well plates in a volume of 20 μl containing 1.0 μl of RT product, 2.0 μl of each primer and 2.0 μl of UPL probe and FastStart TaqMan Probe Master reaction mix (Roche, Mannheim, Germany). The reaction conditions were initial

Table I. *Clinicopathological characteristics of patients with prostate cancer (n=108).*

Characteristics	Number of patients	%
Age (years)		
<55	13	12.0
55-60	48	44.5
>60	47	43.5
Preoperative PSA (ng/ml)		
<4.0	5	4.6
4.0-10.0	58	53.7
>10.0	45	41.7
Clinical stage		
I	16	14.8
II	63	58.3
III	29	26.9
Gleason score		
6	37	34.2
7	54	50.0
8	11	10.2
9	6	5.6

PSA, Prostate-specific antigen.

Table II. *TMPRSS2-ERG fusion gene status in relationship to the stage of the disease and Gleason score.*

TMPRSS2-ERG	Presence detected		High expression	
all (n=108)	34	31.5%	13	12.0%
Clinical stage				
I (n=16)	7	43.8%	1	6.3%
II (n=63)	13	20.6%	6	9.5%
III (n=29)	14	48.3%	6	20.7%
Gleason score				
6 (n=37)	8	21.6%	3	8.1%
7 (n=54)	17	31.5%	7	13.0%
8 (n=11)	4	36.4%	2	18.2%
9 (n=6)	5	83.3%	1	16.7%

Table III. *Relation between TMPRSS2-ERG fusion gene status and time to recurrence (Cox model).*

TMPRSS2-ERG status	Number of patients		HR	95% CI	*p*-Value
	+	–			
Mere presence	34	74	1.52	0.48-4.80	0.4739
High expression	13	95	6.14	1.95-19.39	0.0020*

HR, Hazard ratio; CI, confidence interval. *$p<0.05$.

denaturation at 95˚C for 10 min, followed by 50 cycles of 95˚C for 10 s and 60˚C for 30 s. The expression levels were normalized to total RNA and PSA (*PCA3/PSA* ratio). All samples were assessed in technical duplicates. If Ct values obtained from technical duplicates were in discrepancy, the sample assessment was repeated.

Quantitative estimation of microRNAs. A quantitative estimation of miR-23b, miR-26a and miR-221 was performed by a RT real-time PCR method using TaqMan® MicroRNA Assays (Applied Biosystems, manufactured by Roche, Branchburg, NJ, USA). We used *RNU6B* (U6snRNA) as a normalizer. The Ct values were corrected using calibrators to eliminate differences between individual runs of the Stratagene Mx3005P Real-Time PCR apparatus (Agilent Technologies, Santa Clara, CA, USA). The results are presented as normalized values as a ratio of the number of copies of the given gene to that of the reference gene. To obtain gene expression data we used the ΔΔCt approach ($2^{-\Delta\Delta Ct}$ algorithm).

Quantitative estimation of blood serum PSA protein. Preoperative blood samples were taken from the cubital vein before any procedures involving the prostate manipulation, collected in VACUETTE® blood collection tubes (Greiner Bio-One, Kremsmünster, Austria). The serum was separated by centrifugation at 1700 × *g* for 10 minutes and PSA determined using the UniCel DxI 800 chemiluminescent immunoassay system (Beckman Coulter, Brea, CA, USA) as described previously (23).

Statistical analysis. SAS version 9.3 statistical software (SAS Institute Inc., Cary, NC, USA) was used for all statistical calculations. The results with $p<0.05$ were considered statistically significant. Non-parametric two-sided Wilcoxon signed-rank test was used for comparing the two groups (tumor tissue and normal prostate tissue) and Pearson's Chi-squared test for evaluating categorical data. Evaluation of prognostic significance (the relation of markers to time to recurrence) was performed as analysis of maximum likelihood estimates (Cox regression hazard model); the Kaplan-Meier survival distribution functions were generated for markers significant in Cox model.

Results

We have previously analyzed the *TMPRSS2-ERG* fusion gene status by the RT-qPCR method with UPL probes quantifying mRNA transcripts, so we were able to classify cases not only into categories (fusion gene present/absent) but according to the expression level as well. We detected presence of the *TMPRSS2-ERG* fusion in 34 out of 108 samples (31.5%). Nevertheless, in about two thirds of positive cases it was a late amplification with cycle threshold (Ct) above 40. Therefore, we decided to distinguish a category of high level of *TMPRSS2-ERG* fusion transcript expression (Ct≤40.0) in which 13 out of 108 samples (12.0 % fell within). We did not record statistically significant differences of the presence or high expression of *TMPRSS2-ERG* fusion gene in relationship to the stage of the disease, but there were differences related to Gleason

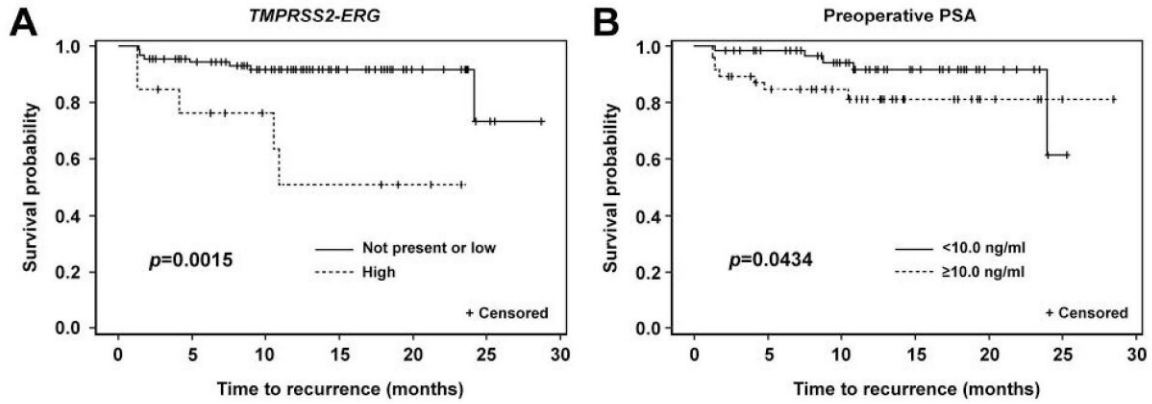

Figure 1. *Relation of TMPRSS2-ERG fusion transcript expression (A) and preoperative serum PSA level (B) to time to recurrence (Kaplan-Meier curves).*

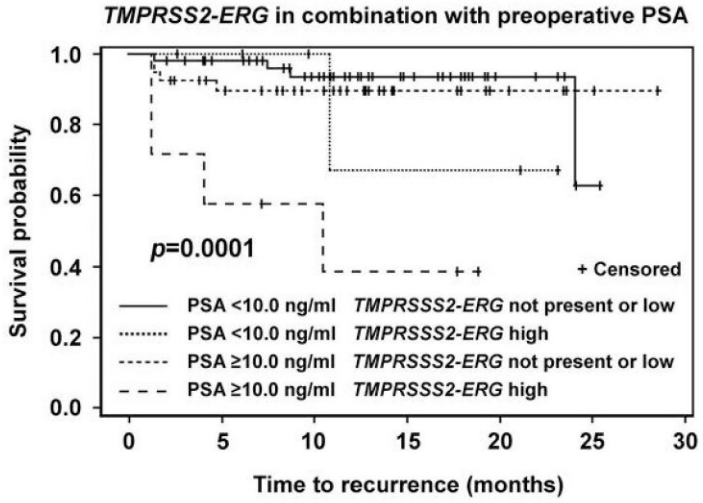

Figure 2. *Relation of TMPRSS2-ERG fusion transcript expression in combination with preoperative serum PSA level to time to recurrence (Kaplan-Meier curves). A combination of high PSA level and high TMPRSS2-ERG expression was associated with the shortest time to recurrence.*

score (Pearson's Chi-squared test, p=0.0259). Higher Gleason score was associated with higher percentage of *TMPRSS2-ERG* positivity (Table II).

The *TMPRSS2-ERG* fusion gene status was also evaluated in relation to time to recurrence (disease-free interval (DFI)). The mere presence of *TMPRSS2-ERG* had no relation to DFI; however, we found significantly shorter DFI in patients with high expression of *TMPRSS2-ERG* in the carcinoma tissue (p=0.0020, Cox model, analysis of maximum likelihood estimates); details in Table III. Preoperative PSA level, the currently available biomarker for PCa, was also evaluated by Cox model and its high levels proved to be marker of shorter DFI (p=0.0153). There was no correlation between preoperative PSA level and *TMPRSS2-ERG* fusion gene status. Kaplan-Meier survival curves were generated for both *TMPRSS2-ERG* fusion gene status (Figure 1A) and preoperative PSA (Figure 1B). Kaplan-Meier survival curves were also computed for the combinations of *TMPRSS2-ERG* status and preoperative serum PSA level (Figure 2). The category of patients with high preoperative PSA level splits according to the *TMPRSS2-ERG* status into groups with different prognosis; those with high expression of *TMPRSS2-ERG* have shorter DFI.

Furthermore, we evaluted the expression levels of microRNAs and found miR-23b, miR-26a and miR-221 to be down-regulated in tumor tissue compared to normal prostate

tissue (p=0.0031, p=0.0022, p<0.0001, respectively). We found no correlation of the expression of these microRNAs to clinical stage, Gleason score, *TMPRSS2-ERG* status or prognosis.

There was no significant difference in the expression of *PSA* mRNA in tumor tissue compared to normal prostate tissue. *PCA3* was significantly overexpressed in tumor tissue compared to normal prostate tissue for both ways of expression values normalization, to total RNA (p<0.0001) and as a *PCA3*/*PSA* ratio (p<0.0001). We found no correlation of the expression of either PSA or PCA3 to clinical stage, Gleason score, *TMPRSS2-ERG* status or prognosis.

The expression of miR-23b, miR-26a and miR-221 had no relation to *TMPRSS2-ERG* status and DFI. We recorded significantly lower expression of miR-221 in tumor tissue compared to normal prostate tissue (p=0.0005).

Discussion

It is known that some prostate carcinomas may not cause any trouble or only progress very slowly. Microscopic foci of prostate cancer are frequently randomly found in autopsies of men over the age of 50 who died from other reasons (24). Due to the progress in PCa screening, a rising number of men are diagnosed with PCa; nevertheless, not in all cases immediate radical treatment is necessary. There is an increasing need to distinguish tumors that are rapidly progressing to spare patients with slowly growing tumors the strain of overtreatment. To identify those patients with tumors that tend to progress, we used a panel of genes whose expression is significantly involved in PCa carcinogenesis (*TMPRSS2-ERG*, *PCA3*, miR-23b, miR-26a and miR-221) (12, 14, 25). We assessed their expression on RNA level that allowed their quantification using RT real time PCR. We combined these markers with serum PSA, as a routinely used marker of high senzitivity for detection of PCa, but with a limited prognostic value.

As the most promising indicator of worse outcome, we have revealed a combination of high level of preoperative serum PSA and a presence of high level expression of *TMPRSS2-ERG* fusion gene in tumor tissue. Despite of limitation in specificity, serum PSA is one of the most useful biomarkers in oncology and still has not lost its potential that is evident from currently developing concept of prostate health index (Phi) (23). Therefore, the identification of new biomarkers that can be used alone or in combination with PSA is welcome to improve distinguishing the aggressive from the indolent ones (26).

The *TMPRSS2-ERG* fusion was identified to be the most common gene rearrangement in PCa; published data report its prevalence approximately 50% in PSA screened localized PCa (27). In our cohort, we detected presence of the *TMPRSS2-ERG* fusion in 31.5% of PCa samples. The mere presence of the fusion gene in our cohort had no relation to prognosis. The meta-analysis of Pettersson *et al*. concluded that *TMPRSS2-ERG* or *ERG* overexpression did not strongly predict recurrence or mortality among men treated with radical prostatectomy; the cohort of this meta-analysis included patients from Europe, North America and Asia (28). It appears, however, that the levels of *TMPRSS2-ERG*, not merely its presence, have the dicisive effect on progression (29). Similarly, in our cohort, we found significantly shorter time to biochemical recurence in patients with high expression of *TMPRSS2-ERG* transcript.

TMPRSS2-ERG fusion gene presence can be detected non-invasivelly from samples of urine. Stephan *et al*. in their work focused on diagnostics of PCa and concluded that *PCA3* and Phi were superior to the other parameters and further stated that the advantage of *TMPRSS2-ERG* might be seen in subgroups of aggressive PCa (30).

MicroRNAs, whose expression was assessed (miR-23b, miR-26a and miR-221) in our study, were chosen on the base of described role in pathogenesis of PCa (12, 14, 25). Nevertheless, we did not record any prognostic significance. We observed lower expression of miR-221 in PCa tumor tissue. This finding supports tumor suppressor role of miR-221 in PCa, which was described in several previously published studies (15, 31, 32). However, some other studies (33, 34) indicate up-regulation of miR-221 in PCa.

In conclusion, a combination of high preoperative serum PSA and high expression of *TMPRSS2-ERG* could be promising in distinguishing those tumors that are aggressive and together with patient status make it possible to decide if radical resection is beneficial.

Conflicts of Interest

The Authors declared they have no conflicts of interest.

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PŘÍLOHA 11

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CIRKULUJÍCÍ MIKRORNA U PACIENTŮ S KOLOREKTÁLNÍM KARCINOMEM

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Východisko: MikroRNA (miRNA) jsou krátké jednovláknové RNA molekuly, které se významným způsobem podílejí na regulaci genové exprese, a tak zasahují do mnoha fyziologických i patologických procesů včetně patogeneze nádorových onemocnění. Ukázalo se, že molekuly miRNA jsou přítomny i v tělesných tekutinách (krev, moč) a jejich hladiny zde odrážejí děje uvnitř organismu. Tyto vlastnosti dávají cirkulujícím miRNA potenciál stát se v budoucnu klinicky použitelnými biomarkery. Kolorektální karcinom patří celosvětově k nejčastějším maligním nádorům. Chirurgická léčba (radikální resekce) je základní léčebnou modalitou s cílem vyléčení pacienta. U řady pacientů ale dochází v průběhu let k recidivě onemocnění. Rutinně stanovovaným nádorovým markerem za účelem stanovení prognózy a časného odhalení recidivy je karcinoembryonální antigen (CEA). Senzitivita a specificita ale není dostačující, hledání nových markerů má proto smysl.

Cíl: Cílem naší studie bylo porovnáním předoperačních a pooperačních plazmatických hladin vybraných miRNA u chirurgicky léčených pacientů s kolorektálním karcinomem najít ty miRNA, které vykazují statisticky signifikantní změnu po odstranění nádorové tkáně.

Metodika: Studovaný soubor zahrnoval 88 pacientů (58 mužů, 30 žen, medián věku v den operace 65 let) s kolorektálním karcinomem, kteří podstoupili resekci nádorové tkáně. Histologicky se jednalo o adenokarcinomy. U každého z pacientů byl analyzován předoperační a pooperační (odběr mezi 5. - 10. dnem po operaci) vzorek krve. Celková RNA byla izolována z 200 µl krevní plazmy po přidání cel-miR-39 jako exogenní referenční kontroly (tzv. spike-in). Stanovení hladiny deseti vybraných miRNA bylo provedeno kvantitativní RT real-time PCR metodou kitem TaqMan Advanced miRNA Assays (Thermo Fisher Scientific). Pro hodnocení rozdílu předoperačních a pooperačních hladin byl použit Wilcoxonův párový test.

Výsledky: Nalezli jsme statisticky signifikantní pokles hladiny cirkulující miR-20a-5p ($p=0,0069$), miR-23a-3p ($p=0,0010$) a miR-223a-3p ($p=0,0088$) v krevní plazmě po chirurgickém odstranění kolorektálního karcinomu. Jedná se o molekuly, jejichž funkce byla popsána jako onkogenní, kdy vysoké hladiny těchto miRNA jsou dávány do vztahu s progresí nádorového onemocnění.

Závěr: Nalezli jsme tři miRNA, které splňují základní podmínku, aby o nich bylo možné uvažovat jako o možných markerech recidivy onemocnění, a to změnu hladiny po odstranění nádorové tkáně. Dále budeme hodnotit prognostický potenciál těchto miRNA (vztah hladin exprese k celkovému přežití) a provedeme multivariantní analýzu spolu s klasickými markery (CEA).

PREOPERATIVE AND POSTOPERATIVE PLASMA LEVELS OF CIRCULATING MICRORNAS IN PATIENTS WITH COLORECTAL CANCER

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BACKGROUND

Colorectal cancer (CRC) ranks among the most common cancers worlwide. Surgical removal remains the best strategy of treatment for resectable tumours. One of the ways how to improve the estimation of prognosis of surgically treated patients with CRC is the use of a combination of already proven tumours markers (*e.g.* carcinoembryonic antigen, CEA) with new promising biomarkers.

MicroRNAs have the potential to become valuable biomarkers for this purpose. MicroRNA molecules exhibit high stability in body fluids and tissues and can be assessed from blood plasma samples.

The aim of this study was to identify microRNAs whose plasma levels reflect the course of the disease and have a relation to prognosis.

PATIENTS AND METHODS

This study involved 88 patients (58 men and 30 women, the median age = 65 years) with CRC who underwent surgical removal of the tumour tissue.

Paired (preoperative and postoperative) blood plasma samples were analyzed.

Ethics statement

This study was approved by the ethics committee of the University Hospital in Pilsen. Anonymised data were used to conduct the study.

RNA isolation

The total RNA was isolated from 200 µl of blood plasma with cel-miR-39 as spike-in control.

Quantitative estimation of microRNA expression

A quantative estimation of selected microRNAs (miR-20a-5p, miR-21-5p, miR-23-3p, miR-29-3p, miR-92a-3p, miR-155-5p, miR-199a-3p, miR-210a-3p, miR-223-3p) was performed by an RT real-time PCR method using TaqMan Advanced MicroRNA Assays (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in technical triplicates on Stratagene Mx3005P apparatus (Agilent Technologies, Santa Clara, CA, USA).

RESULTS I

A decrease in plasma levels after surgical removal of tumour tissue was revealed for three microRNAs:

miR-20a-5p, miR-23a-3p, and miR-223a-3p

sample	N	miR-20a-5p				miR-23a-3p				miR-223a-3p			
		25%	median	75%	P-value	25%	median	75%	P-value	25%	median	75%	P-value
preoperative	88	0,048	0,259	0,835	0,0069	0,149	0,660	3,555	0,0010	0,237	1,279	4,084	0,0088
postaoperative	88	0,024	0,200	0,467		0,104	0,366	1,266		0,163	0,547	2,282	

CONCLUSION

We have revealed statistically significant decrease in plasma levels for miR-20a-5p, miR-23a-3p, and miR-223a-3p after surgical removal of the tumour tissue. We will investigate these microRNAs as markers for detection of recurrence of the disease.

We have revealed the prognostic significance of miR-20a-5p. High level was associated with unfavourable outcome. This result shows the oncogenic features of miR-20a-5p in CRC.

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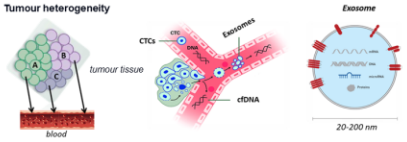
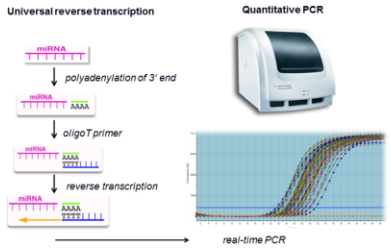
LIQUID BIOPSY

Liquid biopsy is based on the analysis of cells (circulating tumour cells, CTC), subcellular particles (exosomes), and molecules (DNA, RNA) released from tumour tissue into the blood stream.



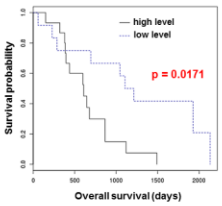
Liquid biopsy is a minimally invasive approach suitable for repeated monitoring of disease which overcomes tumour heterogeneity.

TAQMAN ADVANCED MICRORNA ASSAYS



RESULTS II

Prognostic significance of blood plasma miR-20a-5p level was tested on a subgroup of palliatively treated CRC patients (n=27).



Kaplan-Meier curves for the overall survival of patients stratified into two groups according to the expression of miR-20a-5p.

Patients with high blood plasma level of miR-20-5p had significantly shorter overall survival.

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