MASARYK UNIVERSITY

FACULTY OF MEDICINE

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HABILITATION THESIS

Brno 2022 **Michal Fedorko, MUDr., Ph.D.**

MASARYK UNIVERSITY

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DEPARTMENT OF UROLOGY

Habilitation thesis

Possibilities of diagnosis and prognosis of renal cell carcinoma using novel biomarkers

(Collection of previously published scholarly works)

MUDr. Michal Fedorko, Ph.D.

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MUDr. Michal Fedorko, Ph.D.

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1. Objectives of the habilitation thesis

The submitted habilitation thesis is a summary of published scientific works of which the applicant is the author or co-author. These works represent the results of long-term scientific research activities of the applicant related to renal cell carcinoma, especially in the search for potential non-invasive biomarkers for diagnosis and follow-up. Outputs presented by the applicant at international professional conferences are also included. The individual publications are connected by an accompanying commentary, which puts them in context with the current state of knowledge in the given issue. The work is divided into three thematically related parts with the following goals:

1. Introduce the issue of renal cell carcinoma in terms of its occurrence and prognosis of individual stages and reveal the complex molecular genetic mechanisms involved in its pathogenesis (chapters Introduction and Molecular genetic mechanisms in the pathogenesis of renal cell carcinoma).

2. To justify the need to search for biomarkers of this disease, to present the types and individual groups of biomarkers and possible areas in which these biomarkers are useful in clinical practice (chapter Biomarkers of renal cell carcinoma).

3. Describe in detail the individual groups of non-coding RNAs and their possible use in the diagnosis and prognosis of renal cell carcinoma, critically evaluate their benefits and potential direction of further research in this area (chapters Non-coding RNAs and their use in clinical practice, Short non-coding RNAs, Long non-coding RNAs).

2. Summary

Despite intensive research, there are currently no reliable biomarkers available for routine practice for early detection, prognosis, or follow-up of renal cancer including the treatment response, although it is a cancer with a significant proportion of advanced stages at the time of diagnosis and thus with a considerable lethality. We therefore rely on the detection of the disease by imaging examinations (very often incidentally) and we also use imaging practically uniformly when monitoring patients after surgical or systemic treatment. Non-coding RNAs whose levels can be determined in tissues and body fluids and which are able to differentiate tumour tissue from non-tumour tissue and tumour patients from or healthy individuals represent a significant shift forwards. Presented papers confirm the diagnostic and prognostic value of many groups of these RNAs. A detailed analysis of the tumour tissue may thus reveal biomarkers of potentially aggressive renal carcinomas, recurrent tumours or tumours poorly responsive to treatment. The determination of circulating non-coding RNA levels as a minimally invasive method of diagnosis fits into the attractive concept of so-called liquid biopsy of cancer. The results are encouraging. The use of urine as a completely non-invasive source of biomarkers is also attractive. Here, the data is still premature and insufficient. Last but not least, in vitro and in vivo tests reveal the therapeutic potential of many of the RNAs described, which, in combination with novel therapies for metastatic renal cancer, could lead to prolonged overall and cancer-specific survival in this group of patients.

3. Introduction

According to the latest data, renal cell carcinoma (RCC) accounts for 4.2% of all cancers in men and 2.6% in women (1). Worldwide, the age standardized incidence (ASR) of RCC is 4.6 cases per 100 thousand in both sexes (16th most common cancer), 6.1 per 100 thousand in males (10th most common cancer) and 3.2 per 100 thousand in females (14th most common cancer). Its current mortality is 1.8 per 100 thousand (2). In a long term, the highest incidence has been reported in Northern America and European countries (Fig. 1). The Czech Republic has had one of the highest incidences of RCC in world for several decades. The current ASR (14.4 per 100000) is the second highest after Lithuania (14.5 per 100000). In the Czech Republic, age standardized incidence is 22.1 per 100 thousand for men and 9.9 per 100 thousand for women, so it is 6th and 10th most common cancer, respectively (Fig. 2).

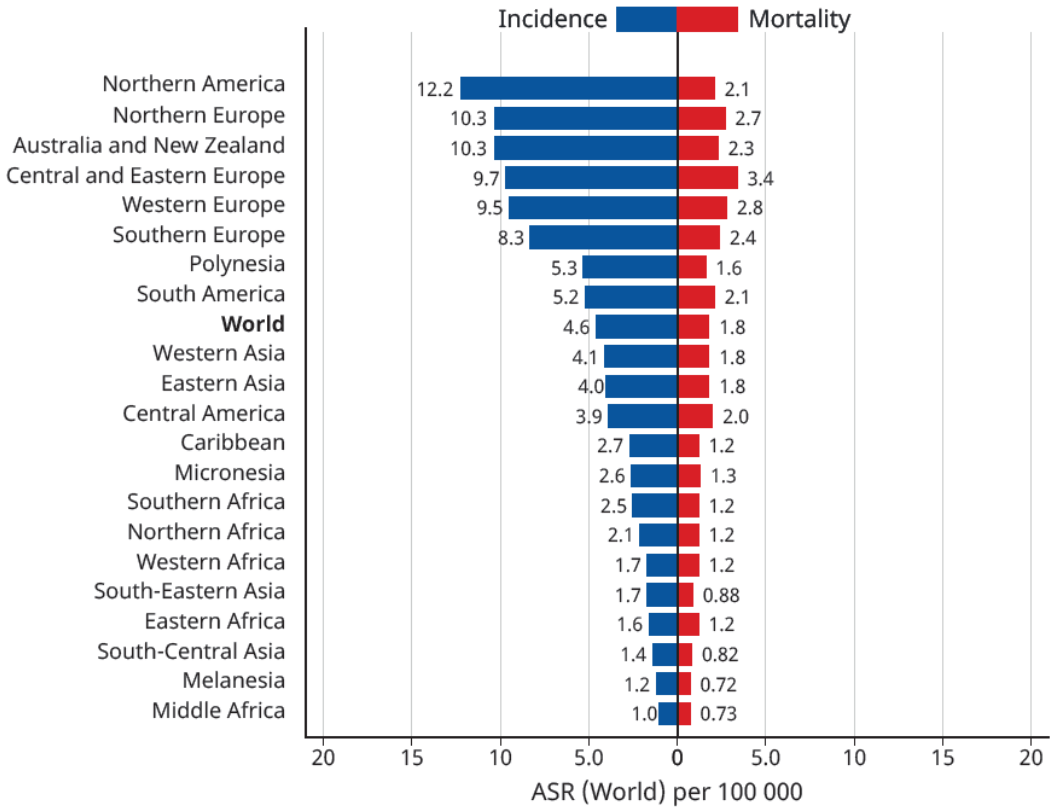


Fig. 1. Incidence and mortality (ASR) of RCC in different regions (2).

10 MAJOR CANCERS, ASR (WORLD) PER 100 000

Male		Female	
Non-melanoma skin cancer	78.4	Breast	64.6
Trachea, bronchus and lung	58.7	Non-melanoma skin cancer	58.4
Prostate	56.5	Corpus uteri	18.2
Colon	31.1	Colon	17.2
Rectum	25.9	Trachea, bronchus and lung	15.7
Kidney	22.1	Cervix uteri	13.7
Bladder	19.8	Ovary	12.5
Stomach	12.2	Rectum	11.3
Melanoma of skin	11.7	Melanoma of skin	10.5
Pancreas	11.6	Kidney	9.9
All sites	420.1	All sites	307.8

Fig. 2. Age-standardized incidence (ASR) of ten major cancers in both sexes in the Czech Republic (1).

According to the data of the National Cancer Registry (NOR), the age standardized incidence and mortality of RCC in 2018 was 14.37 / 100 thousand and 4.01 / 100 thousand, respectively (3). The development of incidence and mortality in the Czech Republic over time correlates with the development in other industrialized countries, the highest incidence was reached in 2005, mortality has been slowly declining (or remains at a stationary level) since 2002 (Fig. 3, 4).

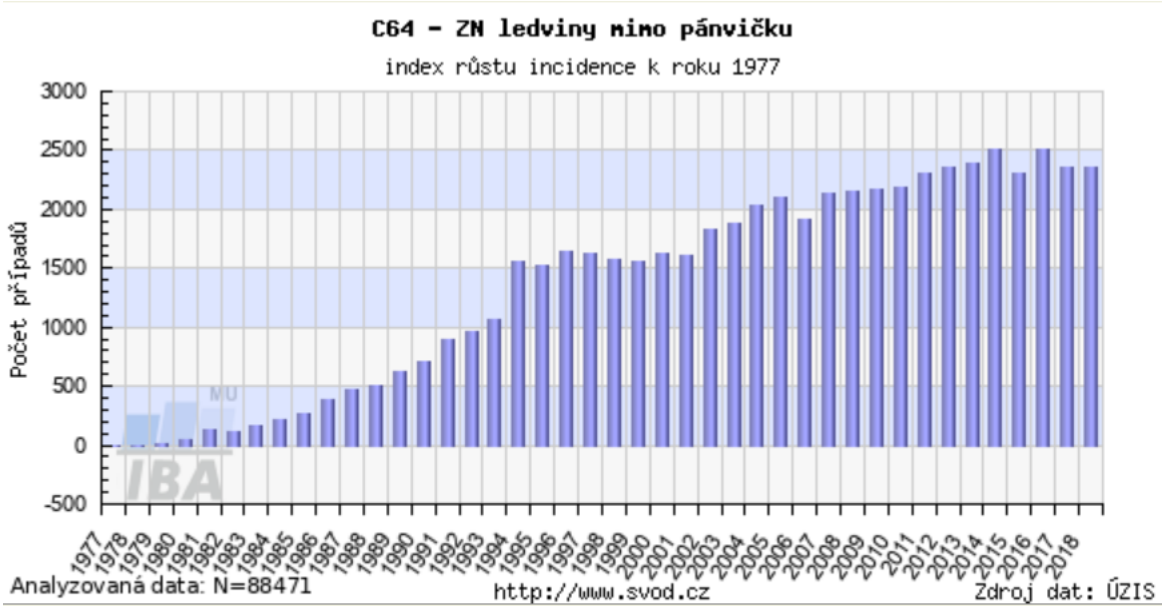


Fig. 3. RCC incidence growth index (1977 – 2018) (3)

C64 - ZN ledviny mimo pánvičku
Časový vývoj, ASR(W)

Rok	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997
Incidence	5.36	5.46	5.36	5.68	6.44	6.12	6.53	7.05	7.15	8.27	8.61	8.84	9.83	10.02	11.24	11.73	12.09	15.13	14.65	15.29	15.07
Mortalita	1.99	2.66	3.21	3.44	3.55	3.87	4.04	4.28	4.48	4.81	4.85	4.81	5.46	5.73	5.81	5.95	5.86	6.86	7.01	7.37	6.73

Rok	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018
Incidence	14.55	14.21	14.47	14.1	15.34	15.47	15.92	16.11	15.07	15.63	15.38	15.41	15.14	15.72	15.37	15.45	15.9	14.57	15.43	14.4	14.37
Mortalita	6.76	6.62	6.85	6.57	6.83	6.5	6.22	6.15	5.76	6.11	5.66	5.43	5.52	5.34	4.92	4.75	4.59	4.73	4.37	4.37	4.01

Fig. 4. Time evolution of incidence and mortality of RCC in the Czech Republic (3).

RCC is still one of the most lethal urological malignancies. Its five-year relative survival, i.e. a statistical comparison of the survival of cancer patients and other populations of the same age, race and sex without tumour (expressed as a percentage of patients who survive 5 years) is 74.8% (4). Of the urological malignancies, only malignant tumours of the penis and ureter reach lower values, but their incidence is less than 1 case per 100 thousand (Table 1).

Malignancy	**5-year relative survival**
Prostate cancer	98%
Testicular cancer	95,2%
Bladder cancer	77,1%
Kidney cancer	74,8%
Penile cancer	66,7%
Ureteral cancer	46,5%

Tab. 1. Comparison of five-year relative survival of patients with tumours of the urogenital tract (4).

The five-year relative survival of patients with RCC is gradually increasing (Fig. 5), but still a relatively significant proportion of patients are diagnosed at an advanced stage of the disease, i.e. with regional lymph node involvement or distant metastases (Fig. 6), when the percentage of surviving patients decreases dramatically (Fig. 7). The increasing proportion of localized tumours is mainly due to the use of imaging methods from another indication, which incidentally detect a kidney tumour, which would otherwise manifest itself in the advanced stage due to localization in the retroperitoneum (5).

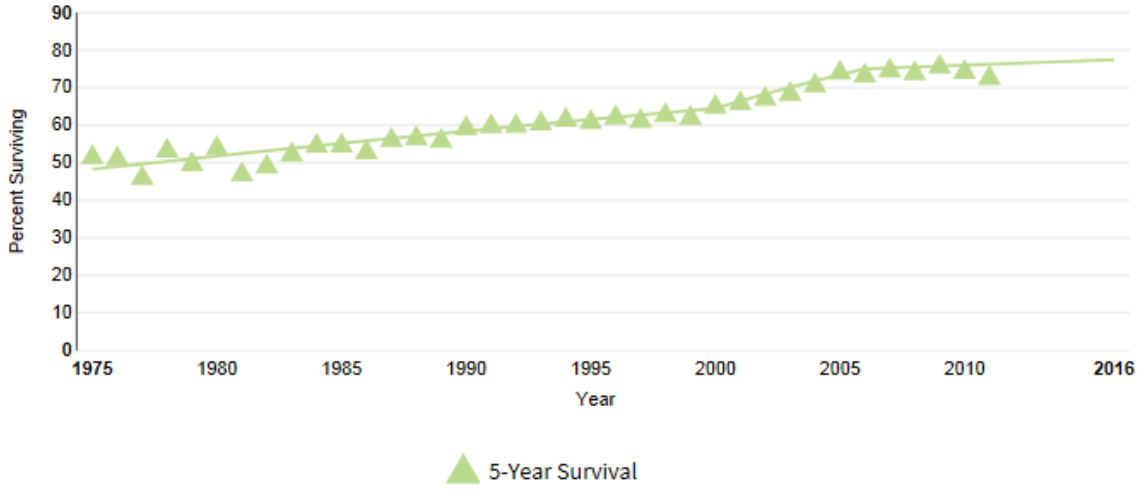


Fig. 5. Time evolution of 5-year relative survival of RCC patients (4).

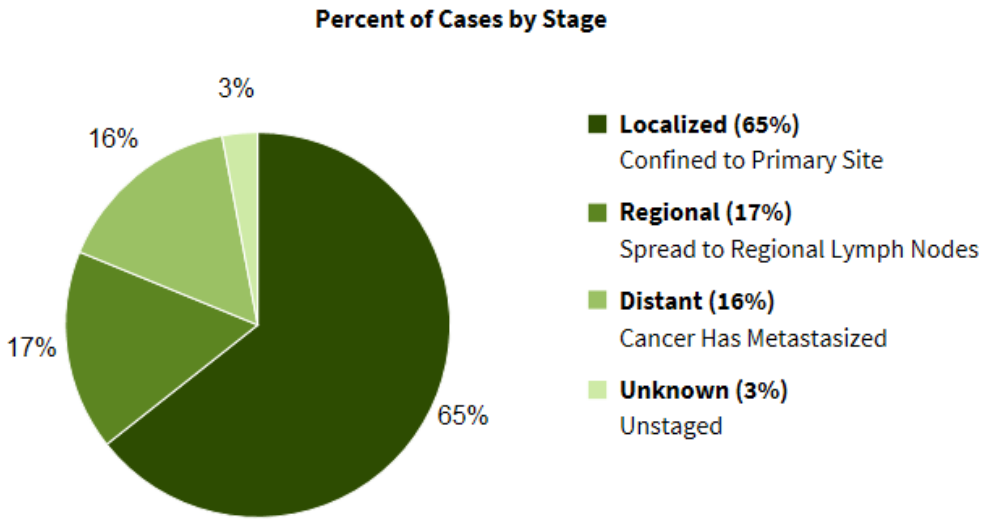


Fig. 6. Percentage of RCC stages at the time of diagnosis (4).

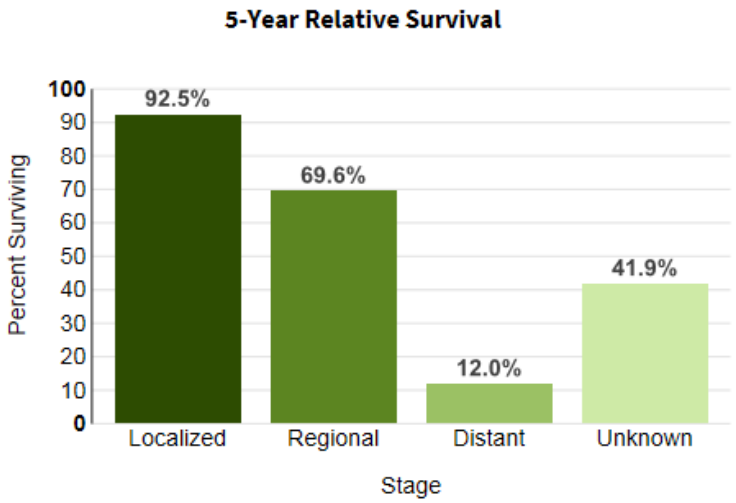


Fig. 7. Comparison of five-year relative survival according to the stage of RCC at the time of diagnosis (4).

The proportion of clinical stages of RCC in the Czech Republic at the time of diagnosis is similar, stage IV tumours make up to 20% (Fig. 8).

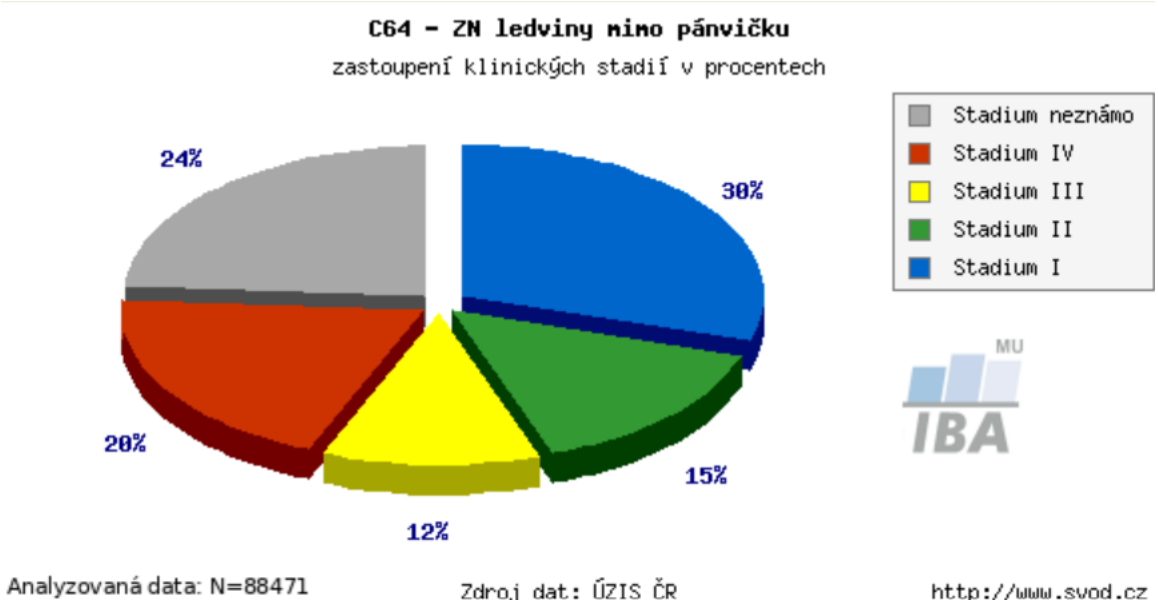


Fig .8. The proportion of clinical stages of RCC in the Czech Republic (3).

In addition to these advanced stages, tumours that progress or recur after primary treatment generally have a poor prognosis. Thus, it is clear that a prerequisite for effective treatment is the early diagnosis of RCC and the identification of patients at risk of disease relapse or the need for further treatment to achieve a better clinical effect.

Published work related to the topic:

Fedorko M, Kiss I, Pacík D. Nádory ledvin. In: Matějovská Kubešová H, Kiss I, eds. Geriatrická onkologie. Praha, Česká republika: Mladá fronta; 2015: 120-124.

The chapter in the monography provides summary information on kidney tumours in terms of epidemiology, diagnosis and treatment of localized and metastatic disease. It specifically targets a group of patients older than 65 years, describes the benefits, risks and results of RCC treatment in the geriatric population.

8.1 Nádory ledvin

Michal Fedorko, Igor Kiss, Dalibor Pacík

Karcinom z renálních buněk (renal cell carcinoma – RCC) je nejčastější solidní lézí ledviny a tvoří přibližně 90 % všech maligních nádorů ledvin. Zahrnuje různé podtypy se specifickými histopatologickými a genetickými charakteristikami, ze kterých nejčastější je konvenční, neboli světlobuněčný RCC, tvořící 70–80 % z renálních karcinomů. I když za posledních 30 let došlo k výraznému zvýšení relativního přežívání pacientů s RCC, stále se řadí mezi nejletálnější urologické malignity s pětiletým relativním přežitím 71,8 %. RCC reprezentuje 2–3 % všech maligních nádorů, jeho celosvětová standardizovaná incidence (ASR) představuje 4,0 případu na 100 tisíc a mortalita 1,6 případu na 100 tisíc. Výskyt RCC se v závislosti na geografické poloze mění více než desetinásobně. Česká republika je celosvětově na prvním místě z hlediska incidence RCC, hodnota ASR je až čtyřnásobně vyšší, než je celosvětový průměr, přičemž RCC tvoří v ČR až 5,6 % všech malignit a je 6. nejčastějším maligním nádorem celkově, 4. u mužů. Incidence nádorů ledvin má vzestupnou tendenci, mortalita se udržuje přibližně na stejné úrovni, respektive je zaznamenán její mírný pokles. Podle údajů Národního onkologického registru byla v roce 2011 ASR 15,34 na 100 tisíc, mortalita 5,12 na 100 tisíc, hrubá incidence dokonce více než 28 případů na 100 tisíc obyvatel. Nejvyšší hodnoty standardizované incidence bylo dosaženo v roce 2005. Z hlediska věkové struktury pacientů s RCC v české populaci je nejvyšší incidence ve věkové skupině 65–69 let.

Diagnostika

Variace v hodnotách incidence se připisují rozdílné diagnostické intenzitě. Více než 50 % nádorů ledvin je diagnostikováno náhodně neinvazivními zobrazovacími metodami (sonografie nebo CT břicha) indikovanými pro různé nespecifické příznaky a jiná abdominální onemocnění. Narůstá tak detekce malých nádorů, přesto je v současné době stále patrný poměrně vysoký záchyt také objemných nádorů, které udržují dlouhodobě stabilní úroveň mortality. Incidentální diagnostikou se zvyšuje počet nádorů zachycených ve stadiu lokalizovaného onemocnění (tedy nádoru ohraničeného na ledvinu) a v současné době toto stadium představuje více než 60 %. Incidentálně diagnostikované nádory mají navíc ve srovnání se symptomatickými nádory výrazně lepší prognózu. Klasická trias příznaků (bolesti v boku, makroskopická hematurie, hmatná rezistence) je známkou pokročilého onemocnění a v dnešní době se vyskytuje pouze v 6–10 %, paraneoplastické syndromy (hypertenze, kachexie, váhový úbytek, horečky, neuromyopatie, amyloidóza, zvýšená sedimentace erytrocytů, anémie, polycytemie, hyperkalcemie, porucha jaterní funkce) se objevují asi u 30 % pacientů.

Základem diagnostiky je zobrazovací vyšetření ledvin. Jakékoliv ložisko na ledvině, které nemá při ultrazvukovém vyšetření (UZ) charakter prosté cysty, by mělo být došetřeno CT vyšetřením po aplikaci kontrastní látky, které je nejdůle-

žitějším vyšetřením pro stanovení povahy expanze na ledvině. Vzhledem k vyššímu výskytu chronické renální insuficience (CHRI) v geriatrické populaci je potřeba zvážit riziko kontrastní nefropatie spojené s podáním kontrastní látky při CT proti riziku nefrogenní systémové sklerózy při magnetické rezonanci (MR) s gadoliniovou kontrastní látkou. Ačkoliv byla MR v minulosti považována za zobrazení volby u pacientů s CHRI, v dnešní době se preferuje kontrastní CT s přípravou (dostatečná hydratace před a po vyšetření, nefroprotektivní N-acetyl cystein). Pokud je nutné provedení MR u pacientů s CHRI, doporučuje se po vyšetření hemodialýza. MR je indikována u pacientů alergických na CT kontrastní látku. Další možností pro pacienty s CHRI je provedení UZ ledvin s kontrastní látkou, které je kromě CHRI využitelné i k posouzení komplexních cystických formací a odlišení infarktu ledviny nebo kortikální nekrózy.

Perkutánní biopsie může významně pomoci při rozhodování o léčebné strategii pacienta s RCC. Jedná se o bezpečnou techniku s nízkou morbiditou a zanedbatelným rizikem vzniku implantačních metastáz, zájem o ni proto narůstá. Doporučení stran indikací k biopsii na základě mezinárodního konsenzu je uvedeno v tabulce 8.1. Vzhledem k častější volbě aktivního sledování a ablačních technik u starších pacientů má v přístupu ke geriatrickému pacientovi s RCC biopsie z tumoru důležité místo. Diagnostická výtěžnost biopsií prováděných v centrech s dostatkem zkušeností dosahuje 78–97 %, s vysokou specificitou (98–100 %) i senzitivitou (86–100 %). Naproti tomu až 22 % biopsií může být nevýtěžných, stanovení gradingu podle Fuhrmanové je v bioptickém vzorku nepřesné a biopsie se nehodí pro cystické léze.

Strategie léčby

Volba vhodné strategie léčby u geriatrického pacienta s RCC vyžaduje důkladné zhodnocení stavu onemocnění (stadium, prognóza) a stavu pacienta (očekávaná doba života, performance status, komorbidity, předchozí léčba, spolupráce pacienta).

Chirurgická léčba a její alternativy

Základem léčby RCC je léčba chirurgická, která spočívá v kompletním odstranění ledviny s nádorem (radikální nefrektomie) nebo nefron šetřící operaci (nefron

Tab. 8.1 Doporučení pro biopsii z tumoru ledviny

Klinická situace	Biopsie ANO/NE/kdy
ablační metody	ANO – vždy
půred vs. po kryoablaci	před ablací
rozhodování o strategii léčby	ANO – s výjimkou jasné diagnózy (symptomy, vzhled při zobrazovacím vyšetření)
charakteristický vhled při zobrazovacím vyšetření	NE – není indikovaná
konzervativní léčba nezvažována	NE – není indikovaná
jiný primární tumor	ANO – doporučuje se
předchozí léze na ledvině	ANO – doporučuje se
vícečetné synchronní tumory	ANO – ze všech tumorů, pokud je to možné
aktivní sledování	ANO – doporučuje se
watchful waiting	NE – nedoporučuje se

sparing surgery – NSS), tedy odstranění části ledviny s nádorem (resekce ledviny neboli parciální nefrektomie). Oba tyto způsoby léčby mají u malých nádorů (do 4 cm) srovnatelné dlouhodobé onkologické výsledky, lze je provést otevřenou cestou nebo laparoskopicky. Výhodou NSS u starších pacientů je menší poškození renální funkce a s tím spojený menší výskyt proteinurie a CHRI. Tumory nevhodné pro NSS (větší nádory, nádory lokalizované v blízkosti renálního hilu) můžou být bezpečně léčeny radikální nefrektomií i ve vyšším věku. Operační léčba ve věkových kategoriích nad 75 let a nad 80 let je zatížená nízkou mírou komplikací, a samotný věk by proto neměl být kontraindikací chirurgické léčby.

Aktivní sledování (AS) je možností pro pacienty ve špatném celkovém stavu (četné komorbidity, vysoké riziko operace) nebo pro pacienty, kteří odmítají jiné způsoby aktivní léčby. Tato strategie se opírá o údaje z několika metaanalýz, které prokázaly pomalý růst malých nádorů ledvin (0,28–0,4 cm/rok) a nízké riziko vzniku metastáz (1–2 %). Ve skupině letitých pacientů (průměrný věk 76,6 roku) ve špatném celkovém stavu (ECOG 2–3), s ASA kategorií 3 nebo četnými komorbiditami bylo při AS prokázáno celkové pětileté přežití 43 %, nádorově specifické přežití až 93 % (dokonce 100 % pro nádory menší než 4 cm). Dalším argumentem pro tuto strategii je to, že z radiologicky podezřelých malých nádorů ledvin jsou v 20–25 % případů benigní léze. Pacient, kterému je nabídnuto AS, by měl být poučen na jedné straně o tom, že má potenciálně letální nádor a ani nulový růst neznamená s jistotou benigní charakter, na druhé straně musí být ubezpečen, že v případě progrese je možná aktivní léčba se stejnými výsledky jako v případě časné intervence. Pokud s ohledem na stav a věk pacienta neuvažujeme o případné aktivní léčbě, tuto strategii označujeme pojmem pozorné vyčkávání (watchful waiting).

Alternativou k chirurgické léčbě (pokud se jedná o malé nádory ledvin) jsou metody, které používají různé fyzikální principy k destrukci nádoru. Označujeme je pojmem ablační metody. Nejčastěji používanými způsoby ablačních metod jsou kryoablace a radiofrekvenční ablace tumoru ledviny (řadí se mezi tzv. termální ablace). Lze je provést perkutánně, laparoskopicky nebo otevřenou cestou. Cílem je snaha o zachování renální funkce s dodržením zásad onkologické bezpečnosti. Z hlediska starších pacientů je nevýhodou absence dat z dlouhodobého sledování, krátkodobé výsledky jsou však slibné a srovnatelné s chirurgickou léčbou. Riziko lokální recidivy je sice vyšší než v případě chirurgické léčby, tuto léčbu je však možno v případě potřeby opakovat. Na druhou stranu je potřeba pacienta poučit, že případná chirurgická léčba v případě progrese nálezu může být v terénu fibrózy velmi obtížná. Ačkoliv neexistují cílené studie na starší populaci, obvykle jsou tyto metody nabízeny pacientům ve vyšším věku, s komorbiditami a vyšším rizikem anestezie, v dostupných studiích je až polovina pacientů ve věku 70 let a více.

Léčba metastatického onemocnění

Zatímco v léčbě primárního nádoru či solitární metastázy je základní léčebnou modalitou chirurgická resekce či její alternativy (ablativní metody), v léčbě metastatického, neresekabilního onemocnění je to systémová léčba multikinázovými inhibitory (sunitinib, pazopanib, sorafenib, axitinib), bevacizumabem v kombinaci s interferonem alfa nebo mTOR inhibitory (temsirolimus a everolimus). Algoritmus léčby a sekvence jednotlivých linií léčby jsou shodné s pacienty mladšími a vychází ze skórovacího systému MSKCC kritérií, na jejichž základě mají pacienti prognózu dobrou, střední či špatnou. Se zohledněním histologického typu nádoru a prognostické klasifikace jsou doporučeny jednotlivé léčebné sekvence.

MSKCC kritéria a hodnocení prognózy onemocnění: LDH > 1,5násobek horní hranice normy, hemoglobin < dolní hranicí normy, korigované sérové kalcium > 2,5 mmol/l, Karnovsky index ≤ 70 %, interval < 1 rok od diagnózy do zahájení systémové léčby.

Prognóza dobrá: žádný faktor; prognóza střední: 1 nebo 2 faktory; prognóza špatná: 3 a více faktorů.

Výsledky systémové léčby a její tolerance u starších pacientů byly hodnoceny v řadě klinických studií a retrospektivních hodnocení. Nejvíce informací je publikovaných u starších pacientů léčených sunitinibem, kde výsledky ukazují srovnatelný efekt a toleranci léčby u pacientů pod a nad 65 let. Tyto výsledky byly statisticky srovnatelné v parametrech dosažené léčebné odpovědi RR 17 : 17 %, mediánu doby do relapsu onemocnění mPFS 11,3 : 10,9 měsíce a mediánu přežití mOS 18,2 : 18,4 měsíce. Tyto výsledky byly srovnatelné také při srovnání pacientů nad a pod 70 let, a to u pacientů v kategorii předléčených cytokiny či dosud neléčených (tab. 8.2).

Obdobně vyšly výsledky retrospektivní analýzy studie TARGET v případě sorafenibu. Vyšší věk není negativním rizikovým faktorem u pacientů léčených VEGF inhibitory. Obecně špatným prognostickým faktorem jsou komorbidity a horší celkový stav pacientů. Vyšší věk pacientů s nádory ledvin je provázen četnějšími komorbiditami a horším stavem, což se v praxi projevuje raritnější indikací k léčbě ve II. linii, 23 % : 39 % v neprospěch pacientů nad 75 let.

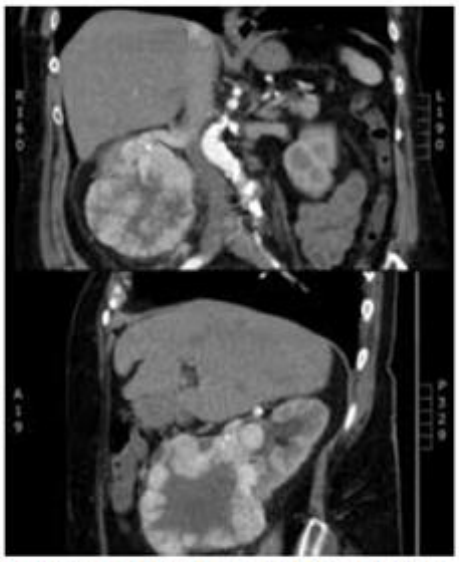
Tab. 8.2 Efektivita léčby metastatického renálního karcinomu u starších pacientů ve srovnání s pacienty mladšími

Studie	Počet pacientů	mPFS (měsíce)	mOS (měsíce)
Khambati (2014)	1 381	5,5	16,8
≥ 75 let sunitinib, sorafenib	144	7,5	19,8
< 75 let	1 237	p = 0,1388	p = 0,3321
Hutson (2014)			
sunitinib			
≥ 70 let	202	11,0	25,6
< 70 let	857	9,9	23,6
INF-α			
≥ 70 let	61	7,9	17,5
< 70 let	299	5,0	22,7
Gore (2009)			
sunitinib			
≥ 65 let	1 418	11,3	18,2
všichni	4 371	10,9	18,4
Jäger (2015)			
sorafenib II. linie			
63%			
≥ 70 let	532	6,1	–
< 70 let	1 765	7,8	–

Kazuistika

V roce 2013 jsme diagnostikovali tehdy 81leté pacientce objemný nádor pravé ledviny, který se manifestoval makroskopickou hematurií a renální kolikou vpravo. Již vstupní vyšetření prokázalo expanzi na pravé ledvině, cílené CT vyšetření potvrdilo roz-

sáhlý tumor velikosti 10 cm na pravé ledvině s podezřením na infiltraci okolních orgánů. Vstupní hodnota kreatininu byla 93 µmol/l, podíl funkce pravé a levé ledviny byl 53 % : 47 %. Pacientka prodělala před mnoha lety infarkt myokardu, léčila se s hypertenzí. Po anesteziologické stránce byla hodnocena jako kategorie ASA III–IV, schopna operace s vysokým rizikem. Vzdálené metastázy nebyly prokázány. U pacientky byla provedena radikální nefrektomie vpravo (podezření na infiltraci okolních orgánů se nepotvrdilo), histologické vyšetření prokázalo světlobuněčný karcinom z renálních buněk, infiltrující perirenální tuk (stadium pT3a). Samotná operace i pooperační průběh byly bez komplikací, nyní je pacientka 2 roky od operace bez známek lokální recidivy či metastáz, v dobrém celkovém stavu, pravidelně sledována, hodnota kreatininu je 119 µmol/l. Tento konkrétní případ demonstruje přínos a dobrý výsledek operační léčby i ve vysokém věku, navíc u nádoru, který byl symptomatický a svým rozsahem lokálně pokročilý.



CT ledvin s kontrastní látkou zobrazující objemný nádor v koronárním i sagitálním řezu pravou ledvinou

Shrnutí pro klinickou praxi

Česká republika je zemí s nejvyšší incidencí renálního karcinomu ve světě. Chirurgická léčba RCC je bezpečná i pro pacienty ve vyšších věkových kategoriích, aktivní sledování nebo ablační metody představují v geriatrické populaci vhodnou alternativu chirurgické léčby RCC. V případě metastatického onemocnění je léčba multikinázovými inhibitory dobře tolerovaná i populací starších pacientů a její efektivita je srovnatelná s výsledky léčby pacientů mladších věkových kategorií. Vyšší věk tedy není limitujícím faktorem pro chirurgickou léčbu ani léčbu systémovou, rozhodující je celkový stav pacienta, jeho komorbidity a jeho reálná očekávání z výsledku léčby.

4. Molecular genetic mechanisms in the pathogenesis of renal cell carcinoma

The vast majority of renal cell carcinomas are sporadic tumours (96%), the remainder being familial tumours manifested in known hereditary syndromes characterized by specific gene mutations, the histological subtype of RCC and comorbidities. Research into hereditary forms of RCC has led to the elucidation of the basic molecular genetic pathways of the pathogenesis of RCC with a significant impact on new therapeutic approaches, especially the so-called targeted treatment. These are the VHL / HIF cascade, PI3K / AKT / mTOR, Wnt / β-catenin, HGF / MET, epithelial-mesenchymal transition and other genetic and epigenetic alterations (6). In this chapter, these mechanisms are briefly described mainly for their significant influence by short and long non-coding RNAs, which can be used as diagnostic, prognostic and therapeutic biomarkers on the basis of these interactions (7).

VHL/HIF pathway

Molecular genetic studies in patients with von Hippel-Lindau (VHL) disease led in 1993 to the identification of the VHL tumour suppressor gene, which is located on the short arm of chromosome 3 (3p25-26). As with other solid tumours, RCCs are characterized by a state of hypoxia due to an imbalance between oxygen supply and consumption. A critical regulator of the hypoxic response is a mutation in the VHL gene. The VHL protein, together with other factors, forms a complex that is responsible for the degradation of so-called hypoxia inducible factors (HIF). These factors further regulate the expression of more than 200 genes whose target proteins significantly affect angiogenesis, glucose metabolism, invasion, mitogenesis, cell proliferation and survival and development of metastasis (VEGF, PDGF, EGFR, TGFα, Glut1, MUC1 and others). Under hypoxic conditions in the absence of VHL, HIF-1α and HIF-2α are stably expressed and induce the production of VEGF and other mentioned factors (Fig. 9). VHL inactivation is responsible for virtually all familial clear cell RCC (ccRCC) and at least 2/3 of sporadic ccRCC. Inactivation of both VHL gene alleles is necessary for RCC - in case of VHL disease one allele is inherited mutated and mutation of the other leads to RCC, in case of sporadic ccRCC both alleles are inactivated after birth, which leads to late RCC and unifocal occurrence (8).

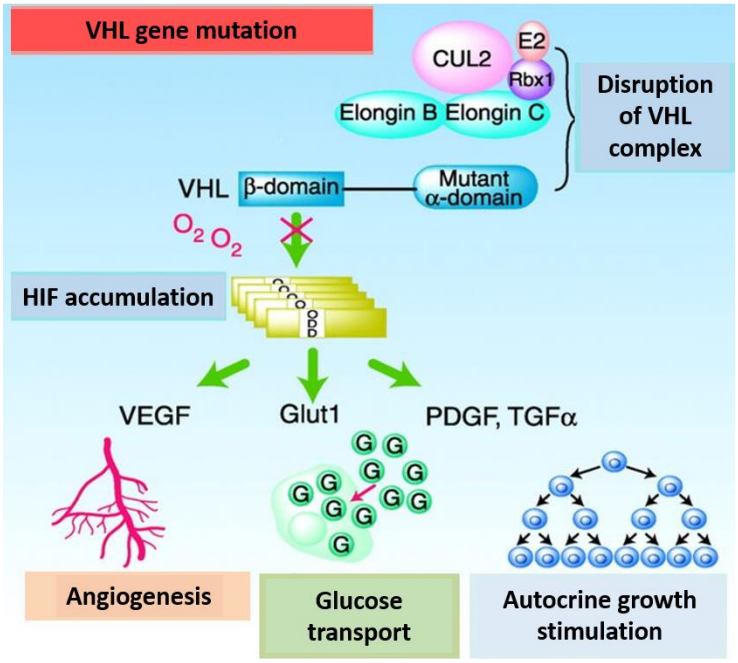


Fig. 9. VHL/HIF pathway. Adapted from Bratslavsky et al. (9).

PI3K/AKT/mTOR

Protein kinase 3 (AKT) and mammalian target of rapamycin (mTOR) significantly affect cell proliferation, cell survival, and angiogenesis. Binding of VEGF and PDGF to their receptors on tumour cells activates PI3K and leads to overproduction of PIP3, thereby transferring cytoplasmic AKT to the cell membrane, where it is activated. Activation of AKT results in inhibition of apoptosis by both inactivation of proapoptotic proteins (such as procaspase 9) and impaired degradation of proteins that promote cell cycle (e.g., cyclin D1) or proliferation (c-myc, β-catenin). Stimulation of mTOR production positively regulates cell cycle protein synthesis. The mechanism of AKT activation in tumours is probably through the decreased expression of the tumour suppressor gene PTEN (10). In addition, mTOR activation further stimulates HIF production and is therefore involved in the VHL / HIF cascade, too.

Wnt/β-catenin

Wnt is a family of glycoproteins that regulate cell proliferation, differentiation and migration. The final effector of Wnt is β-catenin, a transcriptional co-activator that enters the nucleus and activates the transcription of oncogenes, such as MYC. Increased β-catenin production is also described in the case of VHL inactivation, which may be another way in which VHL is involved in the pathogenesis of RCC. Wnt also stimulates the mTOR cascade by inhibiting GSK3.

Epithelial – mesenchymal transition

The kidney is of mesenchymal origin and the process of mesenchymal-epithelial transition results in the formation of epithelial structures, which further develop into nephrons. In the case of ccRCC, this process is reversed, i.e. epithelial-mesenchymal transition (EMT), which is a necessary prerequisite for the development of metastases. EMT requires activation of several transcriptional regulators (ZEB1, SIP1, Snail, Slug), whose target protein is E-cadherin (crucial for the epithelial phenotype). Loss of E-cadherin leads to dissociation of intercellular junctional complexes. Furthermore, the repression of E-cadherin releases β-catenin, which activates the transcription of mesenchymal markers (vimentin, fibroblast-specific protein 1). And, as with the aforementioned signalling pathways, the absence of VHL with overproduction of HIF-1α may be one of the factors leading to downregulation of E-cadherin. Interesting is the action of TGF-β, a multifunctional cytokine involved in EMT, which in normal cells acts as a tumour suppressor, but in the microenvironment of tumour cells acts as a promoter (11).

HGF/MET

Changes in the expression of hepatocyte growth factor (HGF) and its c-MET receptor are associated with the development of hereditary papillary RCC (pRCC). Mutations in the MET gene are also observed in 5-13% of sporadic pRCCs. MET phosphorylation stimulates the growth and metastasis of RCC by activating the PI3K / AKT and Ras / MAPK cascades. In addition, it induces the phosphorylation of β-catenin and subsequent transcription activation. However, HGF is also related to VHL expression in RCC tumour cells: the higher VHL expression, the more suppressed β-catenin production by HGF.

Genetic and epigenetic alterations in RCC

Genetic alterations are mainly gene mutations of various tumour suppressor genes, while epigenetic changes (i.e. without changing the nucleotide sequence of DNA) are changes in chromatin, the remodelling of which can activate regulatory factors, such as transcription factors in chromatin DNA. These are usually changes in histones (methylation, demethylation, acetylation) or ATP-dependent remodelling complexes. The most important chromatin remodelling genes include PBRM1 (mutated in up to 41% of ccRCC), SETD2 or BAP1 (12).

5. Biomarkers of renal cell carcinoma

RCC is considered a primary surgical disease because it can only be cured if it is localized and thus surgically removable. The diagnosis is based on imaging, either specifically due to the suspicion of a focal process (but the clinical suspicion usually indicates a locally advanced or metastatic tumour), or the kidney lesion is diagnosed incidentally, by imaging from another indication (more than half of all cases). Several studies have argued in favour of early diagnosis, showing a lower stage of the disease and a better prognosis of accidentally detected tumours compared to symptomatic tumours (13). In contrast, there is a group of small tumours (up to 4 cm) in which up to 20% are benign and clinically insignificant tumours for which early detection would lead to unnecessary diagnosis and treatment. Imaging examinations may therefore not reliably distinguish between benign and malignant tumours, tumour biopsy has its exact indications and is not routinely recommended (14). The exclusive role of imaging methods in the diagnosis and follow-up of RCC patients (as well as in the screening of selected group of patients such as end-stage renal disease or hereditary forms of RCC) could be changed by the identification of sufficiently accurate biomarkers.

In general, biomarkers of renal cell carcinoma can be divided into (i) biomarkers for early detection of RCC, (ii) diagnostic biomarkers for individual RCC subtypes, (iii) prognostic biomarkers, and (iv) biomarkers for prediction of treatment response (15).

In terms of **early detection**, there is currently no biomarker available in routine clinical practice and this topic is therefore a relatively urgent challenge, as there is still a relatively significant proportion of advanced forms of RCC at the time of diagnosis compared to other malignancies. The detection of aggressive tumours with highly malignant potential among so-called small tumours is also a challenge. Non-invasive detection assumes the presence of this biomarker ideally in the blood (e.g. M2 pyruvate kinase, circulating VEGF, carbonic anhydrase 9, TATI, M65) or urine of the patient (NMP22, AQP1, KIM-1, proteomic or metabolomics analysis).

Proteomics and metabolomics as an alternative way of identifying RCC have not yet found wider application in clinical practice. Research on the urine proteome / metabolome is particularly attractive. The main goal of proteomics is to detect quantitative and qualitative differences between normal and pathological samples and to identify differentially expressed proteins (16). Metabolomics examines endogenously produced metabolites in urine. In the case of RCC, metabolic activity is characterized by disorders of energy metabolism (glycolysis,

metabolism of amino acids and fatty acids), which are essential for cell growth and proliferation (17).

The term **"liquid biopsy"** is generally used for the attractive non-invasive alternative to a standard biopsy in order to obtain diagnostic tumour material (18). In relation to the early detection of RCC, its purpose could be to detect a micro-cancer that cannot be revealed by imaging. Although peripheral blood is usually mentioned as a fluid in this context, it can also be urine or cerebrospinal fluid. In this way, circulating tumour cells (CTC), tumor-derived cell-free DNA (ctDNA), tumor-derived cell-free miRNAs or extracellular vesicles (exosomes) can be detected. However, in the case of localized RCC, the evaluation of ctDNA and CTCs is very difficult compared to other solid tumors (19). Therefore, CTC detection tends to focus on metastatic RCC, where baseline positivity may be a prognostic marker of progression-free survival (20).

Diagnostic biomarkers to differentiate histological subtypes of RCC are used in immunohistochemical examination of tumour tissue. One specific biomarker for a particular subtype is not available, so the expression profiles of several proteins are determined. These can be enzymes (carbonic anhydrase 9), cytoskeletal proteins (vimentin, keratins), adhesion proteins (E-cadherin), CD proteins, transcription factors, glycoproteins or immunoglobulins. **Prognostic biomarkers** are expected to correlate well with the clinicopathological characteristics of the tumour and to be used in monitoring disease progression and patient survival (Bcl-2, survivin, Ki-67). At the time of targeted biologic therapy with tyrosine kinase inhibitors, mTOR inhibitors, and anti-growth factor antibodies, the use of **predictive biomarkers** of treatment response (e.g. high expression of carbonic anhydrase 9 in ccRCC associated with a favourable response to sorafenib and sunitinib, decreased VEGF levels during treatment, blood neutrophil-to-lymphocyte ratio) is very appropriate. Anyway, in the case of metastatic RCC, only MSKCC and IMDC risk scores have been validated as prognostic tools and included in relevant international guidelines (21). That is why research in the field of renal cancer biomarkers is increasingly focused on metastatic RCC.

One of the most important changes in the treatment of advanced stages of RCC in recent years has been the introduction of immune checkpoint inhibitors, i.e. drugs that block the inhibitory checkpoint molecules CTLA-4 and PD-1. The main well-studied biomarkers of immune checkpoint blockade, PD-L1 expression and tumour mutational burden did not demonstrate sufficient predictive ability (22, 23). Limitations on the use of PD-L1 expression include its

intra-tumoral heterogeneity, heterogeneous expression between primary tumor and metastases, unclear cut-off value defining positivity and variability of expression depending on sample age, previous treatment, analysed cell population or assay choice (24). Therefore, the search for other biomarkers is also fully justified here.

Published work related to the topic:

Fedorko M. Diagnostické možnosti časného zjištění nádorů ledvin, role nádorových markerů při stanovení dalšího postupu. Urol List 2011; 9(4): 7-11.

The review describes the issue of screening and early detection of RCC, known molecular biomarkers of renal cell carcinoma and their potential clinical applications based on the knowledge of the time.

Diagnostické možnosti časného zjištění nádorů ledvin, role nádorových markerů při stanovení dalšího postupu

M. Fedorko

SOUHRN

Incidence karcinomu ledviny celosvětově stoupá. Z urologických nádorů má po karcinomu prostaty a karcinomu močového měchýře třetí nejvyšší mortalitu. Prognózu významně ovlivňuje patologické stadium a biologická povaha nádoru. V souvislosti s pokroky v molekulární genetice a tzv. cílené léčbě karcinomu ledviny dochází k vyvíjení nových biologických markerů, které zejména při stanovení prognózy onemocnění doplňují tradiční klinickopatologické charakteristiky tumoru. Spolehlivé biomarkery by mohly mít důležitý dopad nejen na stanovení prognózy onemocnění a předpověď efektu léčby, ale i na časnou detekci karcinomu ledviny. Článek přináší přehled těchto potenciálních biomarkerů a možností jejich využití.

KLÍČOVÁ SLOVA

karcinom ledviny
časná detekce
nádorové markery
prognóza

SUMMARY

RENAL CANCER EARLY DETECTION, THE ROLE OF TUMOUR MARKERS IN FURTHER DIAGNOSTIC, THERAPEUTIC AND PROGNOSTIC STRATEGY

Incidence of renal cell carcinoma increases worldwide. It has the third highest mortality among urological malignancies, following prostate and bladder cancer. Its prognosis is highly related to pathological stage and biological behaviour. With the recent advances in molecular genetics and targeted therapies, novel biomarkers in renal cell carcinoma continue to be developed. These biomarkers have been used together with traditional clinicopathologic variables in estimating prognosis of renal cell carcinoma. As well as prediction of prognosis and therapeutic benefit, reliable biomarkers could have an important impact on early diagnosis, too. The article reviews these potential biomarkers.

KEY WORDS

renal cell carcinoma
early detection
tumour markers
prognosis

Karcinom z renálních buněk (RCC – renal cell carcinoma) představuje 2–3 % všech zhoubných nádorů dospělého věku [1]. Je nejčastější solidní lézí ledviny a tvoří až 90 % všech zhoubných nádorů ledvin. Zahrnuje několik subtypů se specifickými histopatologickými a genetickými charakteristikami [2] – konvenční renální karcinom (označovaný i jako světlebuněčný) (70–80 %), papilární renální karcinom (15 %), chromofobní renální karcinom (5 %), karcinom ze sběrných kanálků a medulární karcinom (< 3 %) a neklasifikovaný renální karcinom. Již v čase diagnózy má 25–30 % pacientů metastázy a až u 30 % pacientů dochází ke vzniku metastáz po chirurgické léčbě, tradičně 30–40 % pacientů s RCC umírá kvůli samotnému nádoru [3]. Pro prognózu pacienta má tedy zjištění nádoru v časném stadiu klíčový význam. Celosvětová standardizovaná incidence (ASR-W) RCC je 4,0/100 000, mortalita 1,6/100 000 [1]. Až na výjimky v podobě některých států lze od 70. let pozorovat postupné zvyšování incidence RCC ročně o 2–3 % jak v rámci Evropy, tak celosvětově [4,5], což lze vysvětlit častějším prováděním ultrazvukového a CT vyšetření břicha pro různé břišní potíže a jiná onemocnění. Tento trend koreluje s vyšším počtem incidentálně zjištěných a lokalizovaných nádorů a se zlepšením pětiletého

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přežívání pro pacienty v tomto stadiu onemocnění. Od konce 80. a začátku 90. let lze v Evropě pozorovat obecně stabilizaci a snižování mortality na RCC, i když jsou nadále některé státy, kde mortalita stoupá (Chorvatsko, Estonsko, Řecko, Irsko, Slovensko) [6]. ČR je celosvětově na prvním místě z hlediska incidence RCC – v roce 2008 byla standardizovaná incidence 14,89/100 000, standardizovaná mortalita představovala 5,4/100 000 [7].

Studium familiárních syndromů spojených s karcinomem ledviny vedlo k významným pokrokům v pochopení molekulární genetiky RCC, byly rozpoznány a charakterizovány tumor supresorové geny a onkogeny podílející se na vzniku jak familiárních, tak sporadických nádorů s přímým a příznivým dopadem na léčbu pacientů v pokročilém stadiu RCC [3]. Neustále přibývající nové molekulární markery, i ty již tradiční, jsou nadále zkoumány s cílem jejich využití v detekci nádorů, při odhadu odpovědi na léčbu, progrese a stanovení další prognózy onemocnění.

SCREENING A ČASNÁ DETEKCE NÁDORŮ LEDVIN

Vzhledem k lokalizaci ledviny v retroperitoneu jsou ve většině případů nádory ledvin asymptomatické a nehmatné až do pokročilého stadia onemocnění. Díky extenzivní zobrazovací diagnostice je více než 50 % tumorů detekováno náhodně [8], tyto tumory jsou obvykle ohraničené na ledvinu a mají lepší prognózu – pozitivní efekt časné a incidentální diagnostiky RCC byl prokázán v mnoha studiích [3]. Karcinom ledviny jako primárně „chirurgická" diagnóza je tedy atraktivním cílem pro screening, jeho implementace má však několik omezení [9], a to hlavně nízkou incidenci RCC (ASR 12/100 000/rok – uváděn údaj z USA), při které by screeningový test musel mít prakticky 100% specificitu. I kdyby měl daný test 100% specificitu a senzitivitu, výtěžnost by byla tak nízká, že poměr ceny a efektivity by byl nepříznivý. Dokonce i při selekci pacientů s rizikovými faktory, jako jsou mužské pohlaví, vyšší věk nebo kouření, je výtěžnost malá, protože relativní riziko spojené s těmito faktory je maximálně dvou- až trojnásobné [10]. Další limitací jsou klinicky insignifikantní nádory, které spolu s benigními nádory tvoří 10–20 % všech lézí a jejichž detekce by vedla k nadbytečné diagnostice a léčbě [11]. Hodnocení výsledků screeningu s chemickým vyšetřením moči k detekci hematurie, sonografickým vyšetřením nebo CT vyšetřením břicha potvrzuje kontroverzi populačního screeningu RCC. CT screening zachytí větší množství nádorů ohraničených na ledvinu a jeho výtěžnost je vyšší (v různých studiích udáváno široké rozmezí 23–300 nádorů na 100 tis. vyšetřovaných [3]), je však stále považována za relativně nízkou a vzhledem k ceně vyšetření nelze považovat tento screening za efektivní [12].

V současnosti má screening RCC význam u přesně definovaných cílových skupin – pacienti v konečném stadiu onemocnění ledvin, se získanou polycystózou ledvin, tuberózní sklerózou a familiárními formami RCC (tab. 1).

Kromě incidentálního radiologického vyšetřování není v současné době k dispozici žádná spolehlivá diagnostická metoda pro časnou detekci RCC. Pohled na časnou detekci a screening karcinomu ledviny by mohla změnit právě molekulární diagnostika a detekce biomarkerů RCC v moči a séru.

Tab. 1. Screening karcinomu ledviny – cílové skupiny [3].

Pacienti v konečném stadiu onemocnění ledvin
pouze pacienti s delším očekávaným přežíváním a bez významných komorbidit
periodické sonografické a CT vyšetření od třetího roku dialýzy
Pacienti s von Hippel-Lindauovou nemocí
CT vyšetření nebo sonografie každé dva roky počínaje 15.–20. rokem života
periodický klinický a radiologický screening non-renálních projevů
Příbuzní pacientů s von Hippel-Lindauovou nemocí
genetické vyšetření
při pozitivním nálezu screening jako u pacientů s von Hippel-Lindauovou nemocí
při negativním nálezu bez zvláštního doporučení, sledování vhodné
Příbuzní pacientů s jinými formami familiárního karcinomu ledviny
periodicky sonografie nebo CT vyšetření
zvážení genetického vyšetření
Pacienti s tuberózní sklerózou
periodické sonografické a CT vyšetření

MOLEKULÁRNÍ MARKERY KARCINOMU LEDVINY

Pochopení molekulárních základů von Hippelovy-Lindauovy nemoci (VHL) snad nejlépe ilustruje velký terapeutický potenciál odhalení molekulárních mechanizmů onkogeneze [13]. VHL jako familiární forma konvenčního karcinomu ledviny (clear cell renal cell carcinoma – CCRCC), který reprezentuje 70–80 % všech renálních karcinomů, je spojena se ztrátou funkce VHL genu, tedy mutací či inaktivací obou jeho alel. Defekt VHL genu byl však prokázán i u přibližně 60 % sporadických CCRCC [14]. Inaktivace VHL genu zatím nebyla prokázána u jiných histologických subtypů RCC, je tedy specifická pro CCRCC. Defekt VHL proteinu vede k akumulaci HIF 1A (hypoxia-inducible factor) a nadměrné expresi proteinů typických pro stav hypoxie, tyto proteiny však současně indukují angiogenezi (VEGF – vascular endothelial growth factor, PDGF – platelet-derived growth factor), buněčný růst (TGF-α, TGF-β – transforming growth factor alfa, beta), ovlivňují vychytávání glukózy (GLUT1), acidobazickou rovnováhu (CA IX – karboanhydráza IX) a podporují růst nádorových buněk. Působení TGF-α na EGFR (epidermal growth factor receptor) dále stimuluje proliferaci nádorových buněk [15]. Cílená inhibice HIF kaskády se stala doporučenou léčbou metastatického kar-

cinomu ledviny [16]. Jednotlivé součásti HIF kaskády začaly být zkoumány i jako možné prognostické faktory RCC. Postupně se spektrum možných prognostických a prediktivních biomarkerů rozšířilo o další skupiny (tab. 2).

Biomarkery HIF kaskády

Hladiny exprese VEGF jsou u různých subtypů RCC rozdílné a korelují s velikostí nádoru a pTNM stadiem [17]. Vysoké hladiny v tumorózní tkáni jsou negativním prognostickým znakem. Nadměrná exprese VEGF, izoforma A, je popisovaná u CCRCC s agresivním biologickým chováním a horším přežíváním pacientů [18]. Zhoršení prognózy pacientů s vysokými hladinami exprese VEGF a VEGF-R bylo potvrzeno i v dalších studiích [19]. Nezávislým negativním prognostickým faktorem CCRCC je i samotná zvýšená exprese HIF1A [20].

Karboanhydráza IX ovlivňuje za normálních okolností regulaci pH na buněčné úrovni v případě hypoxie. Je exprimována téměř výlučně u světlebuněčného karcinomu ledviny, velmi vzácně u ostatních subtypů RCC. V normálních tkáních je exprese prokazatelná v žaludeční sliznici, žlučovodech a pankreatu – expresi v normálních epiteliálních renálních buňkách blokuje právě VHL protein [3]. Studie analyzující imunohistochemickými metodami preparáty po nefrektomii prokázaly zvýšenou agresivitu tumorů a horší přežívání pacientů s CCRCC v případě nízké exprese CA IX [21,22]. Navíc byl prokázán vztah mezi expresí CA IX a odpovědí na imunoterapii RCC interleukinem 2 – vysoká exprese CA IX vede k lepšímu efektu léčby [23]. Role CA IX jako nezávislého prognostického faktoru je některými autory zpochybňována [24], jiní prokazují prediktivní význam spíše polymorfizmu jednotlivých nukleotidů CA IX genu [25]. V současné době probíhá 3. fáze mezinárodní klinické studie hodnotící monoklonální protilátku k CA IX jako adjuvantní léčbu po nefrektomii a její výsledky by mohly roli CA IX u RCC více objasnit [13].

Biomarkery proliferace

Ki-67 je akcepovaným markerem buněčné proliferace. Jeho zvýšená exprese v nádorové tkáni CCRCC koreluje s vyšším gradingem dle Fuhrmanové a horší prognózou [26]. Dle novějších studií je tento marker použitelný i v predikci rekurence RCC po radikální nefrektomii u pacientů s lokalizovaným nádorem [27]. Spolu s patologickým stadiem (pT1 vs ≥ pT2) a přítomností mikrovaskulární invaze je zvýšená exprese Ki-67 považována za nezávislý prognostický faktor – v případě pozitivity jednoho, dvou nebo všech tří těchto faktorů je riziko rekurence 7,1 %, 24 %, resp. 77 % [28]. Ve stejné studii nebyl prokázán nezávislý prognostický význam žádného z dalších studovaných biomarkerů včetně clusterinu, HSP27, MMP-2 a MMP-9.

Biomarkery ovlivňující buněčný cyklus a apoptózu

Dysregulace buněčného cyklu je kritickým mechanizmem, který rozhoduje o tom, zda nádorové buňky budou proliferovat, nebo dojde k jejich apoptóze. Nejvíce studovanými proteiny regulujícími buněčný cyklus s možným vlivem na prognózu RCC jsou cyklin D1 a p27 (označován i jako Kip1). p27 je inhibitor cyklinů a cyklin-dependentní kináza. Ztráta exprese p27 je nezávislým prediktorem horšího nádorově specifického přežívání i přežívání bez recidivy [29].

Zajímavý je vztah mezi expresí p53 a prognózou RCC. Jelikož je p53 známým tumor supresorovým genem, nabízí se předpoklad, že jeho zvýšená exprese bude spojena s lepší prognózou. Překvapivě je však zvýšená exprese spojena s rychlejší progresí onemocnění a kratším přežíváním [30]. Vztah mezi p53 a patogenezí RCC je tedy zřejmě komplexní a zahrnuje různé mechanizmy – důkazem toho je i nález korelace mezi zvýšenou hladinou p21 (proapoptotický cílový protein p53) a lepší prognózou u pacientů s lokalizovaným RCC, ale naopak horším přežíváním u pacientů s metastatickým RCC [31].

Intracelulární molekuly, které aktivují programovanou buněčnou smrt, jsou rovněž použitelné při stanovení prognózy a hodnocení rizika RCC. Absence Bcl-2 a Fas v preparátech po nefrektomii v případě metastatického RCC predikuje lepší odpověď na pooperační imunoterapii [32]. Vztah mezi expresí a metastatickým RCC ve skupině tzv. inhibitorů apoptózy (IAP1, IAP vázaný na X-chromozom, survivin) zatím není uspokojivě objasněn [33].

DNA markery

Profilování genové exprese DNA nádorů ledviny je slibnou metodou. Mikroanalýzou DNA a srovnáním tkáně RCC a benigní tkáně kůry ledviny bylo odhaleno množství genů, které by mohly být použity pro predikci prognózy RCC nezávisle na stadiu tumoru, grade a celkovém stavu – jedná se o stovky genů, ze kterých většina má neznámou funkci [34]. Prognostický význam při hodnocení rizika RCC má tedy spíše globální genetická informace než jednotlivé geny. Další výzkum se orientuje na posttranslační modifikace a vyšetřování stavu metylace různých genových promotorů ovlivňujících tumor supresorové geny [33].

Tab. 2. Vybrané potenciální molekulární prognostické a prediktivní parametry u světlebuněčného karcinomu ledviny [13].

HIF kaskáda	Buněčná adheze	Proliferace	Regulace buněčného cyklu
HIF1	EpCAM	Ki-67	Cyclin
CA IX	E-cadherin	MCM2	p27
CA XII	α-Catenin		
CXCR4	Catenin 6		
VEGF/VEGF-R			
ILGF1			
Regulace apoptózy	**m-TOR kaskáda**		
p53	PTEN		
Bcl2	Akt		
Smac	Phos S6k an		

Imunologické biomarkery

RCC je imunogenní tumor a imunoterapie přestavuje standardní systémovou léčbu metastatického RCC. Molekulární faktory, které modulují imunologickou odpověď u RCC, jsou logicky zkoumány jako možné prognostické biomarkery. Jedná se zejména o skupinu glykoproteinů B7. B7-H1 je molekula za normálních okolností exprimována makrofágy nebo aktivovanými T-lymfocyty. Zvýšená exprese u karcinomu ledviny blokuje antigen-specifickou funkci T-lymfocytů a je spojená s rychlejší progresí RCC a vyšší nádorově specifickou mortalitou [35]. Blokáda B7-H1 tedy může vést k stimulaci protinádorové imunitní odpovědi [36].

Sérové biomarkery

Na rozdíl od obvykle používané detekce molekulárních markerů v nádorové tkáni pomocí imunohistochemických metod má stanovení biomarkerů v séru výhodu možného využití v diagnostice karcinomu ledviny a jako neinvazivního způsobu jak stanovení prognózy, tak dalšího sledování u pacientů s diagnostikovaným RCC.

Z výše zmíněných biomarkerů je slibné stanovení antigenu CA IX a CA IX mRNA v séru pomocí ELISA, resp. PCR, které by mohlo být užitečné při stanovení prognózy a sledování pacientů s lokalizovaným CCRCC [37]. Autoři navíc navrhují i možné diagnostické využití ELISA stanovení CA IX při vyšetření aspirátu atypických renálních cyst.

Sérum amyloid A (SAA) je hlavním apolipoproteinem HDL. Jedná se o protein akutní fáze produkovaný v játrech, ale i extrahepatálně různými nádory. Při srovnání sérových hladin u pacientů s lokalizovaným RCC (N0M0), s postižením lymfatických uzlin (N1M0), metastatickým RCC (M1) a u zdravých jedinců byla zjištěna signifikantní elevace u M1 pacientů, zatímco u pacientů s lokalizovaným nádorem a kontrolní skupinou nebyl zjištěn rozdíl [38]. Hladina SAA signifikantně korelovala s T stadiem a stupněm diferenciace. Dle autorů je tedy SAA vhodným nástrojem pro detekci vzdálených metastáz, ale nikoli pro časnou diagnostiku karcinomu ledviny. Zároveň je SAA signifikantním nezávislým faktorem pro přežívání pacientů s RCC. Prognostický, nikoli diagnostický význam SAA byl potvrzen i další studií [39].

C-reaktivní protein (CRP) je reaktant akutní fáze produkovaný primárně v játrech jako reakce na elevaci cytokinů, zejména IL-6. I když je v praxi nejvíce využíván jako zánětlivý parametr, je prokázána i jeho prognostická hodnota (předoperační i pooperační elevace) u RCC z hlediska rizika rekurence a vzniku metastáz po nefrektomii [40]. Lepším prediktorem je pooperační hodnota – pacienti s přetrvávající elevací CRP, s rizikem proporcionálě zvýšeným podle absolutní hodnoty, by tedy měli být sledováni intenzivněji než ti, u kterých dojde po operaci k normalizaci [41]. Autoři navrhují nové využití CRP jako senzitivního biomarkeru rekurence onemocnění a metastáz.

KLINICKÉ APLIKACE

Pochopení jednotlivých kroků v karcinogenezi CCRCC vedlo k vývoji cílené léčby. V současné době schválenou léčbu metastatického RCC reprezentují [16]: sorafenib a sunitinib (inhibitory tyrozinkináz s aktivitou proti VEGFR2, PDGFR, c-KIT, FLT-3), bevacizumab (protilátka proti VEGF), pazopanib (inhibitor VEGFR, PDGFR, c-KIT), temsirolimus a everolimus (m-TOR inhibitory). Jsou zkoumány látky s aktivitou proti EGFR (panitumumab – protilátka proti EGFR, gefitinib a erlotinib – inhibitory EGRF tyrozinkinázové aktivity) a látky cíleně působící na HIF1A a CA IX [13].

Proteomická analýza umožňuje definování biomarkerů vhodných pro využití v diagnostice a stanovení prognózy RCC.

Co nejpřesnější stanovení prognózy u pacientů podstupujících operační odstranění maligního nádoru na základě obecně akceptovaného konsenzu je důležité pro schéma pooperačního sledování těchto pacientů a rozhodování o případné adjuvantní léčbě. Obzvláště to platí pro karcinom ledviny s fenotypem vysoce rezistentním ke konvenční nechirurgické léčbě [28]. Lze dohledat vícero nabízených kombinovaných molekulárně-klinicko-patologických prognostických modelů – kombinace CA IX, vimentin, p53, pTNM, ECOG performance status [42], BioScore – panel 3 biomarkerů (B7-H1, survivin, Ki-67) v kombinaci s klinickopatologickými charakteristikami [43] nebo kombinace patologického stadia, mikrovaskulární invaze a Ki-67 [28]. Jejich možné použití a případná revize prediktivního systému RCC vyžaduje další prospektivní studie zmíněných biomarkerů a externí validaci navrhovaných prognostických modelů.

Možnost diagnostiky RCC na základě stanovení exkrece definovaných produktů do moči se opírá o předpoklad eliminace látek, které jsou ve zvýšené míře exprimovány v nádorové tkáni. Vyšetřování moči jako potenciálního zdroje biomarkerů RCC není rozšířené – proteomika (analýza proteinů) v moči zatím dostatečně specifický biomarker neodhalila, mnohé studované proteiny nalezené u pacientů s RCC jsou vylučovány i u pacientů s jinými nádory nebo ledvinnými onemocněními [44]. Jako slibná se jeví analýza proteinů aquaporin-1 (AQP-1) a adipophylin (ADFP) [45]. Jejich koncentrace je signifikantně zvýšená u pacientů se světlebuněčným a papilárním RCC ve srovnání s kontrolními skupinami pacientů podstupujících operaci jinou než nefrektomii, zdravými dobrovolníky či pacienty s onkocytomem. Hladiny po nefrektomii (na rozdíl od jiných operací) výrazně klesly, což svědčí pro tumorózní původ těchto proteinů a stejné pre- i pooperační hodnoty u pacientů podstupujících jinou operaci vylučují vliv anestezie či samotného chirurgického zákroku. Tyto výsledky je potřeba potvrdit v dalších nezávislých studiích.

Dalším zdrojem potenciálních biomarkerů RCC je tzv. metabolomika, tedy kvalitativní a kvantitativní stanovení metabolitů v moči. Výsledky jsou však zatím spíše zklamáním – stav metabolitů ovlivňuje dieta pacienta, čas odběru vzorku, jsou pozorovány geografické rozdíly a není možné spolehlivě odlišit metabolity u stejného pacienta před odstraněním tumoru a po něm [46] – a zpochybňují metabolomiku jako screeningový nástroj dokonce i pro populaci s rizikem vzniku RCC.

ZÁVĚR

Nové informace o komplexních molekulárních alteracích, které vedou ke vzniku

a progresi karcinomu ledviny, umožňují identifikaci nových diagnostických a prognostických molekulárních markerů a otevírají dveře pro experimentální cílenou léčbu tohoto často letálního karcinomu. K tomu, aby bylo možné implementovat zmíněné biomarkery do běžné klinické praxe, je nutný další výzkum, který by potvrdil přesnější predikci prognózy RCC, než je tomu u běžně používaných klinickopatologických prediktivních modelů a dostatečnou diagnostickou hodnotu markerů vhodných pro časnou detekci RCC.

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6. Non-coding RNAs and their use in clinical practice

The central dogma of molecular biology is that RNA functions as an information intermediate between DNA (gene) and the protein it encodes. Thus, the vast majority of genetic information responsible for biological form and phenotype was thought to be expressed in proteins - surprisingly, the human genome encodes only about 20,000 proteins, which is less than 2% of its total content. In the human transcriptome (it is important to note that up to 90% of the genome is actively transcribed), in addition to the coding mRNA (which is actively translated), there are a number of antisense transcripts and non-coding RNAs thought to be transcriptional noise or evolutionary waste. However, current knowledge indicates that non-coding RNAs may play a crucial role in cell development, physiology and pathology (25). These non-coding RNAs can be divided into short and long (Fig. 10), the boundary between these groups being approximately 200 nucleotides.

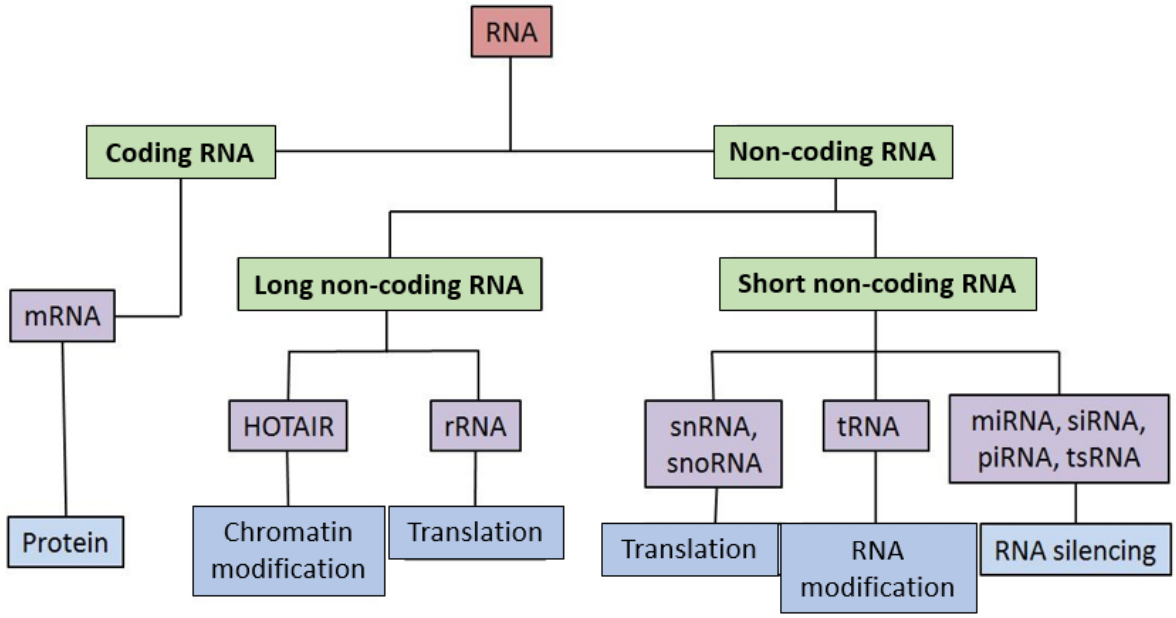

Fig. 10. Eukaryotic RNA - nomenclature and function. mRNA - mediator RNA, HOTAIR - HOX transcript antisense intergenic RNA, rRNA - ribosomal RNA, snRNA - small nuclear RNA, snoRNA - small nucleolar RNA, tRNA - transfer RNA, miRNA - microRNA, siRNA - small interfering RNA, piRNA - PIWI-interacting RNA, tsRNA - tRNA-derived small RNA. Adapted from Green et al (26).

6.1 Short non-coding RNAs

6.1.1 MicroRNAs

MicroRNAs (miRNAs) are short single-stranded RNAs (usually 21-25 nucleotides in length) that arise from a long primary transcript and from a precursor hairpin structure. The genes encoding the miRNA are transcribed into the primary miRNA, from which the precursor miRNA is formed in the nucleus by the action of the Drosha RNase. After transport to the cytoplasm, a mature miRNA is formed, which by the action of another RNase Dicer and binding to Argonaut protein forms an effector complex, the so-called RNA-induced silencing complex – RISC (27). The mature miRNA binds to the 3 'untranslated end of the target mRNA, causing its degradation or inhibition of translation (Fig. 11), thus regulating gene expression at the post-transcriptional level (28).

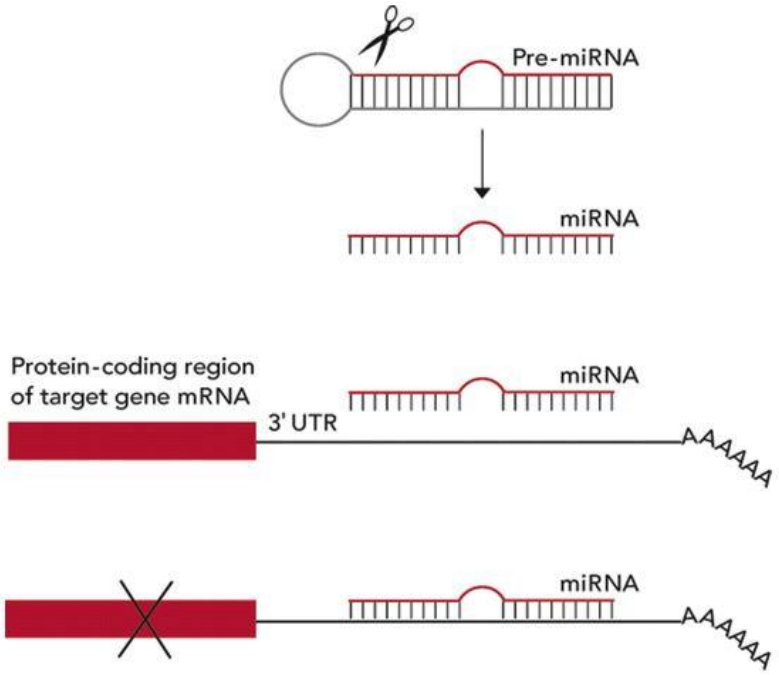

Fig. 11. Schematic representation of miRNA function.

The fact that miRNA sequences are highly conserved across various organisms suggests their role in basic biological processes such as development, differentiation, proliferation, apoptosis, regulation of cell stem properties, the immune system, or tumour transformation. Abnormal expression of miRNAs in tumours can have two basic pathogenetic consequences: miRNAs can function as an oncogene or as a tumour suppressor. In the pathogenesis of RCC, miRNAs affect fundamental mechanisms of carcinogenesis, such as hypoxia / VHL / HIF cascade, EMT,

cell proliferation, invasion, apoptosis or angiogenesis. Aberrant expression in cancer and different expression in tissues and body fluids make microRNAs suitable candidates for use as biomarkers.

Published works related to the topic:

Fedorko M, Pacík D, Varga G, Wasserbauer R, Ghazal M, Nussir M. MikroRNA v patogenezi renálního karcinomu a jejich využití pro stanovení diagnózy a prognózy RCC. Urol List 2015; 13(1): 27-31

A review describing the relationship of miRNAs to the pathogenesis of RCC and summarizing findings on miRNA deregulation in the comparison of tumour tissue, serum and urine of patients with RCC.

Fedorko M, Pacik D, Wasserbauer R, Juracek J, Varga G, Ghazal M, Nussir MI. MicroRNAs in the pathogenesis of renal cell carcinoma and their diagnostic and prognostic utility as cancer biomarkers. Int J Biol Markers 2016; 31(1): e26-37.

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An extended review describing in detail the pathogenetic mechanisms in which miRNAs are involved, supplemented by the latest knowledge regarding specific miRNAs as potential diagnostic and prognostic biomarkers of RCC.

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MikroRNA v patogenezi renálního karcinomu a jejich využití pro stanovení diagnózy a prognózy RCC

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KLÍČOVÁ SLOVA
mikroRNA, karcinom z renálních buněk, biomarker, patogeneze, diagnostika, prognóza

SOUHRN
MikroRNA (miRNA) jsou krátké nekódující RNA, které regulují genovou expresi na posttranskripční úrovni. Zúčastňují se řady kritických biologických procesů včetně onkogeneze. V patogenezi karcinomu z renálních buněk (renal cell carcinoma – RCC) mohou působit jako onkogeny i jako tumor supresory. Prostřednictvím cílových proteinů různých signálních drah ovlivňují apoptózu, buněčný růst, migraci, invazi, proliferaci, formaci buněčných kolonií či angiogenezi a jsou přímo zapojeny do procesů hypoxie a epiteliálně-mezenchymální proměny. Odlišně exprimované hladiny miRNA umožňují rozlišení zdravé a nádorové tkáně ledviny a dokonce jednotlivých podtypů RCC. Cirkulující a močové miRNA jsou potenciálními biomarkery pro neinvazivní diagnostiku RCC a sledování relapsu onemocnění. Navíc je prokázána prognostická hodnota některých miRNA, a to jak z hlediska identifikace vysoce rizikových primárních tumorů, tak z hlediska odpovědi na biologickou léčbu u metastatických nádorů. Cílem článku je poskytnout přehlednou a co nejaktuálnější informaci o roli miRNA v patogenezi RCC a miRNA využitelných pro diagnostiku a stanovení prognózy RCC.

KEY WORDS
microRNA, renal cell carcinoma, biomarker, pathogenesis, diagnosis, prognosis

SUMMARY
MicroRNAs IN THE PATHOGENESIS OF RENAL CELL CARCINOMA AND THEIR DIAGNOSTIC AND PROGNOSTIC VALUE
MicroRNAs (miRNA/miRNAs) are short non-coding RNAs that regulate gene expression at post-transcriptional level. They are involved in a number of critical biological processes including carcinogenesis. In the pathogenesis of renal cell carcinoma (RCC) they can act both as oncogenes and tumor suppressors. They regulate apoptosis, cell growth, migration, invasion, proliferation, colony formation or angiogenesis through target proteins involved in several signaling pathways and they are directly involved in mechanisms of hypoxia and epithelial-to-mesenchymal transition. Differentially expressed miRNAs can differentiate tumor tissue from healthy renal tissue and even different RCC subtypes. Circulating and urinary miRNAs could become biomarkers for non-invasive diagnosis or detection of the relapse of RCC. Moreover, prognostic value of several miRNAs has been shown. They may help identify high-risk primary tumors and estimate treatment response to biological treatment in metastatic tumors. The aim of the article is to provide comprehensive and the most up-to-date information about the role of miRNAs in the pathogenesis of RCC and their diagnostic/prognostic value.

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ÚVOD

Karcinom z renálních buněk tvoří 2–3 % všech maligních nádorů [1]. Navzdory kontinuálnímu zvyšování relativního přežívání během posledních 30 let zůstává jedním z nejletálnějších urologických nádorů s pětiletým relativním přežíváním 71,8 % [2]. V době diagnózy je 17 % nádorů lokálně pokročilých a 17 % metastatických, s pětiletým relativním přežíváním 64,2 %, resp. 12,3 % [2]. Základní metodou léčby je léčba chirurgická, s narůstajícím trendem k záchovným výkonům, zejména u malých nádorů (T1a) [3]. U starších pacientů nebo pacientů s vážnými komorbiditami lze zvolit observaci [4].

Vzhledem k tomu, že kromě zobrazovacích vyšetření není k dispozici jiná spolehlivá modalita pro diagnostiku a sledování onemocnění, pokračuje intenzivní výzkum s cílem identifikace biomarkerů pro časnou detekci, stanovení prognózy a sledování relapsu RCC.

MikroRNA jsou krátké nekódující RNA, které regulují genovou expresi na post-

transkripční úrovni prostřednictvím vazby na svou cílovou mRNA, čímž dochází k její inhibici a/anebo degradaci [5]. Zúčastňují se řady kritických biologických procesů, jako je buněčný růst, diferenciace nebo apoptóza. Inhibicí tvorby proteinů svých cílových genů (onkogenů nebo tumor supresorových genů) mohou ve výsledku působit jako onkogeny nebo jako tumor supresory [6]. Odlišná exprese různých miRNA je prokázána u mnoha nádorových onemocnění včetně RCC [7].

MikroRNA V PATOGENEZI RCC

Typickým znakem světlobuněčného RCC (clear-cell RCC – ccRCC) je inaktivace VHL genu, která je odpovědná za vznik prakticky všech familiárních a dvou třetin sporadických ccRCC [8]. Mutace VHL genu a zablokování tvorby jeho produktu, VHL proteinu, vede k neregulované expresi HIF-1α a HIF-2α s následnou produkcí tumorigenních cílových produktů, jako jsou VEGF, PDGF, TGFα a Glut1 [9]. VHL dependentní regulace byla pozorována u několika miRNA (miR-210, miR-155, let-7i, miR-8a) [10]. MiR-210 je HIF-1 dependentní (při hypoxii dochází k její zvýšené expresi), ale některé nádory bez známek VHL inaktivace vykazují rovněž zvýšené hladiny miR-210, což svědčí pro alternativní mechanizmy aktivace HIF, navíc v případě absence HIF-1 je miR-210 regulovaná prostřednictvím HIF-2 [11]. Asociace mezi expresí miR-210 a přežíváním pacientů podporuje předpoklad, že miR-210 není pouze markerem hypoxie, ale alterací genové exprese ovlivňuje samotnou povahu nádoru [10]. Cílovými proteiny miR-210 jsou proteiny ICSU 1, 2, které regulují mitochondriální transportní dráhy a vysvětlují rysy anaerobního chování nádorových buněk RCC. Útlum exprese miR-210 vede k stimulaci buněčné migrace a invazivního potenciálu RCC [12]. VHL a HIF-1 geny jsou cílovými geny i jiných miRNA, např. miR-17-5p a miR-224 [13]. Silná negativní korelace mezi expresí rodiny miR-200 (miR-200a/200b/200c/429/141) a hladinami VEGF svědčí pro regulaci HIF kaskády i těmito miRNA – ztráta regulace je pak odpovědná za aktivaci HIF [14]. U rodiny miR-200 je navíc popsána indukce epiteliálně-mezenchymální proměny (tedy proměny buněk epitelu v buňky mezenchymální, což zvyšuje tendenci k jejich invazivitě a metastazování) či regulace buněčného cyklu a EphA2/p-FAK/p-Akt/ /MMP2/9 kaskády, jak je tomu např. u miR-141, asi nejznámější z uvedené skupiny tumor supresorových miRNA [15,16].

Jiné hypoxií indikované miRNA jsou miR-155 a miR-21. Mechanizmů působení miR-21 je několik – jejími cílovými geny jsou tzv. apoptosis-related geny (TIMP3, FASL, PDCD4), které aberantně eprimovaná miR-21 inhibuje, dále reguluje invazi buněk prostřednictvím KISS1 proteinu, zvyšuje buněčnou proliferaci a migraci aktivací Akt kinázy/TORC a nakonec řídí expresi cyklinu D prostřednictvím NF-kappa B-dependentní transkripce [17–20].

Potenciálními cíli miR-155 jsou tumor supresorové geny SOCS-1 a BACH1. Inhibice miR-155 vede k potlačení buněčné proliferace a indukci apoptózy nádorových buněk [21].

Další onkogenní miRNA představují cluster miR-17-5p a miR-20a, miR-122 a miR-183.

Skupina tumor supresorových miRNA je velmi rozsáhlá. Tyto miRNA jsou ve tkáni RCC down-regulovány a transfekce nádorových buněk těmito miRNA nebo obnovení jejich hladin vede ke snížení nádorového potenciálu nádorových buněk.

MiR-584 narušuje mobilitu a viabilitu buněk inhibicí ROCK-1 onkogenu [22]. MiR-215 je jednou z nejvíce down-regulovaných miRNA u metastatického RCC ve srovnání s primárním nádorem. Její cílové geny (SIP1/ZEB2) ovlivňují hlavně epiteliálně-mezenchymální proměnu [23]. MiR-205 inhibuje protoonkogenní protein kinázy ze skupiny Src, obnovení hladin tumor supresorové miR-138 snižuje hladinu onkogenního vimentinu v buňkách RCC a výrazně alteruje schopnost jejich invaze [24]. Jiné tumor supresorové miRNA (miR-34a, miR-101, miR-199a, miR-1285, miR-1826, miR-187, miR-135a, miR-218, cluster miR-143/145, miR-133b, miR-199a-3b) působí cestou down-regulace různých onkogenů, jako jsou c-MYC, Notch1, EZH2, GSK3 beta, TGM2, MEK1, B7-H3, caveolin 2 nebo c-Met [25–30]. Transfekce buněčných linií RCC miR-99a indukuje zastavení buněčného cyklu ve fázi G1. Vzhledem k tomu, že jako přímý cílový protein miR-99a byl identifikován mTOR (mammalian target of rapamycin), tumor supresorový efekt miR-99a je zřejmě zprostředkován inhibicí mTOR kaskády [31]. Byl prokázán vztah mezi down-regulací miR-30a a zvýšenou nádorovou angiogenezí – cílovým proteinem je endoteliální ligand DLL4 [32].

Zajímavou a dosud neobjasněnou vlastností mikroRNA je schopnost konkrétní miRNA působit i jako onkogen i jako tumor supresor v závislosti na typu nádoru. Například miR-7, která je popsána u několika zhoubných nádorů u lidí jako tumor supresorová miRNA, působí u RCC jako onkogenní miRNA [33].

DIAGNOSTICKÉ MikroRNA V NÁDOROVÉ TKÁNI

U mnoha mikroRNA byl prokázán statisticky významný rozdíl v expresi mezi nádorovou tkání a mezi normálním renálním parechymem. Ze 470 analyzovaných miRNA identifikovali Nakada et al 43 odlišně exprimovaných miRNA mezi ccRCC a zdravým renálním parenchymem a 57 miRNA odlišně exprimovaných mezi chromofobním RCC a zdravým parenchymem. Nejvíce down-regulovanými miRNA byly miR-141 a miR-200c [34]. V jiné analýze s platformou 847 miRNA byla ve srovnání se zdravými kontrolami prokázaná v nádorové tkáni pacientů s ccRCC zvýšená exprese 38 a snížená exprese 48 miRNA – miR-16 a miR-451 byly mezi nejvíce up-regulovanými, zatímco miR-141 byla nejvíce down-regulovanou miRNA [35]. Juan et al popsali u pacientů s ccRCC 26 down-regulovaných a 9 up-regulovaných miRNA (včetně miR-21, miR-210 a miR-155, které jsou běžně popsané u jiných nádorů) [36]. Práce Chowa popisuje 80 odlišně exprimovaných miRNA u ccRCC, přičemž miR-122 a miR-200c jsou nejvíce dysregulované [37]. V Chengově studii vybraných osm miRNA byla prokázána zvýšená exprese miR-34a, miR-21 a miR-224; miR-141, miR-149 a miR-429 byly signifikantně down-regulovány [38]. Jako slibný biomarker pro odlišení nádorové

a nenádorové tkáně se jeví miR-129-3p s diagnostickou přesností 73,5 %. Nízké hladiny jsou navíc spojeny s kratším celkovým přežíváním (overal survival – OS) i přežíváním bez nádoru (cancer-specific survival – CSS) [39].

V několika studiích byla prokázána schopnost odlišit na základě exprese různých miRNA v nádorové tkáni jednotlivé histologické podtypy RCC a onkocytom. Youssef et al vyvinuli čtyřstupňový algoritmus na základě několika spárovaných miRNA, podle kterého lze vzorek zařadit do jednoho ze dvou možných histologických typů. V každém dalším kroku se možnosti zužují (normální tkáň vs tumor; ccRCC vs ostatní podtypy; papilární RCC vs chromofobní RCC a onkocytom; chromofobní RCC vs onkocytom). Senzitivita pro odlišení normální tkáně od RCC je 97 %, pro určení ccRCC 100 %, papilárního RCC 97 % a pro odlišení chromofobního RCC od onkocytomu 100 % [40]. Petillova studie byla zaměřená na nejvíce genomicky příbuzné podtypy RCC (ccRCC a papilární RCC; chromofobní RCC a onkocytom), odlišně exprimované miRNA jsou uvedeny v tab. 1 [41]. Onkogenní miR-21 vykazuje nejvyšší hladiny u ccRCC a papilárního RCC. Senzitivita a specificita pro odlišení těchto dvou podtypů od chromofobního RCC a onkocytomu je 83 %, resp. 90 % [42]. Ve Wachově studii s 9 miRNA odlišuje kombinace expresních profilů miR--145, miR-210, miR-200c a miR-502-3p nádor od zdravé tkáně s 92,9% přesností, kombinace miR-145 a miR-502-3p má 95% přesnost pro odlišení ccRCC a papilárního RCC, kombinace miR-210 a let-7c správně zařadí subtypy papilárního RCC (typ 1 vs typ 2) v 92,3 % (tab. 1) [43].

Tab. 1. Odlišně exprimované miRNA při srovnání ccRCC a papilárního RCC a při srovnání chromofobního RCC a onkocytomu [41].

Světlobuněčný RCC vs papilární RCC	Průměrný násobek hodnoty	Chromofobní RCC vs oncocytom	Průměrný násobek hodnoty
miR-203	4,74	miR-203	4,49
miR-424	4,34	miR-200b	2,89
miR-450	4,16	miR-197	1,99
miR-139	3,55	miR-320	1,50
miR-143	3,00	miR-186	–2,88
miR-503	2,97		
miR-224	2,94		
miR-145	2,59		
miR-126	2,27		
miR-31	–4,91		
miR-504	–2,62		
miR-371	–2,55		
miR-328	–1,68		
miR-425	–1,50		
miR-423	–1,36		

CIRKULUJÍCÍ MikroRNA JAKO POTENCIÁLNÍ BIOMARKERY RCC

MiR-210 je studována jako sérový biomarker pro stanovení diagnózy a progrese ccRCC. Studie na 34 pacientech s ccRCC a 23 zdravých subjektech prokázala signifikantně vyšší hladiny miR-210 u pacientů s ccRCC (senzitivita 65 %, specificita 83 %, AUC 0,77) [44]. Podobné výsledky byly publikovány i v další studii, které navíc prokázaly u pacientů s ccRCC signifikantní snížení hladiny miR-210 po odstranění nádoru [45]. Při srovnání exprese miRNA v séru a nádorové tkáni pacientů s RCC identifikovali Wulfken et al 36 miRNA se zvýšenými sérovými hladinami, které byly současně up-regulovány v nádorové tkáni. Sedm z nich bylo vybráno jako potenciální diagnostické biomarkery (miR-106b, miR-1233, miR-1290, miR-210, miR-7-1, miR-320b, miR-93) [46]. Práce českých autorů popisuje jako slibné diagnostické biomarkery RCC miR-378 a miR-451. Hladiny miR-378 jsou u pacientů s RCC zvýšené, hladiny miR-451 snížené. Kombinace obou miRNA umožnila odlišení séra pacientů s RCC se senzitivitou 81 %, specificitou 83 %, AUC 0,86 [47]. Signifikantně snížené plazmatické a tkáňové hladiny byly prokázány u tumor supresorových miR-508-3p a miR-509-5p [48,49]. Cheng et al prokázali ve srovnání s benigními nálezy na ledvině signifikantně vyšší sérové hladiny miR-34a, miR-21, miR-224 a nižní hladiny miR-141 u pacientů s ccRCC, hladina miR-21 navíc korelovala se stadiem onemocnění [38]. Dále jsou popsány signifikantně vyšší plazmatické hladiny miR-221 a miR-222 u pacientů s RCC, přičemž u pacientů s metastatickými nádory byla hladina miR-221 vyšší a negativně korelovala s OS [50].

MikroRNA V MOČI JAKO POTENCIÁLNÍ BIOMARKERY RCC

O expresi miRNA v moči pacientů s RCC je zatím velmi málo informací. Zatím v jediné studii je popsaná miR-15a, která je ve vzájemném vztahu s protein kinázou C alfa (PKC alfa), jejíž hladina je zvýšená u benigního onkocytomu, ale snížená u RCC. PKC alfa se jako součást transkripčního komplexu váže na primární transkript miR-15a a blokuje tvorbu miR-15a. Proto jsou hladiny miR-15a u pacientů s RCC zvýšené, zatímco u onkocytomu jsou snížené – vzhledem ke zvýšeným hodnotám v moči pacientů s RCC a naopak nedetekovatelným hladinám u onkocytomu a infekcí močových cest se miR-15a jeví jako slibný močový biomarker [51].

MikroRNA JAKO BIOMARKERY PROGNÓZY RCC

Lepší klinicko-patologické charakteristiky jsou popisovány u RCC s vyšší hladinou miR-210 (nižší stadium i grade) – na základě hladin v nádorové tkáni byly stanoveny tři skupiny, přičemž nejlepší celkové přežívání bylo zaznamenáno ve skupině s vysokými hladinami miR-210 [11]. Jasná korelace byla nalezena mezi expresí miR-21 a klinickými charakteristikami RCC – všichni pacienti s nízkou hladinou

přežili déle než pět let, zatímco u pacientů s vysokými hladinami bylo pětileté přežití pouze 50 %. Hladina miR-21 dále korelovala s T stadiem nádoru [42]. Jiná práce prokázala korelaci zvýšené hladiny miR-21 a snížené hladiny miR-126 s výskytem metastáz a horším CSS [52]. Jako nezávislý prognostický faktor u M0 pacientů s RCC je uváděn poměr miR(21/10b). Vysoký poměr je spojen s horší prognózou, což opodstatňuje striktnější sledování u high-risk pacientů podle poměru miR(21/10b) [53]. MiR-106b je ve tkáni ccRCC výrazně up-regulována. Její hladiny jsou však signifikantně nižší u tumorů těch pacientů, u kterých došlo ke vzniku metastáz. Čeští autoři proto navrhují miR-106b jako prediktivní biomarker relapsu po nefrektomii u pacientů s ccRCC [54].

Z miRNA, které odliší metastatický a nemetastatický ccRCC, byly prokázány nejvýraznější odchylky u miR-451, miR-221, miR-30a, miR-10b a miR-29a. Skupina 4 miRNA (let-7a, let-7c, miR-26a a miR-30a) navíc umožňuje rozlišení nádorů podle klinického průběhu (primárně metastatický, nemetastatický, metastazující s odstupem). Stejná studie prokázala i korelaci několika miRNA s přežíváním bez progrese (progression-free survival – PFS) a CSS [55]. V podobné studii srovnávající primárně metastatický ccRCC, vzdálené metastázy z plic a nemetastatický ccRCC bylo prokázáno 14 odlišně exprimovaných miRNA. Down-regulace miR-30c a up-regulace miR-451 a miR-126 signifikantně korelovaly s PFS a CSS [56]. Na základě odlišné exprese miRNA v lokalizovaném RCC a metastatickém RCC lze pomocí vybraných miRNA identifikovat se senzitivitou 76 % a specificitou 100 % primárně vysoce rizikové a nízko rizikové nádory pro vznik metastáz [57].

Z hlediska detekce časného relapsu po nefrektomii byla prokázána down-regulace miR-143, miR-26a, miR-145, miR-10b, miR-195 a miR-126 u pacientů s relapsem [58]. Dalším možným prediktorem relapsu je miR-514, která je výrazně down-regulována u primárně metastatických nádorů a u těch, u kterých vznikla recidiva [59].

Expresní profil miRNA v periferní krvi umožňuje stratifikaci pacientů s pokročilým RCC podle odpovědi na léčbu první linie sunitinibem. Dvacet osm a 23 z 287 analyzovaných miRNA bylo spojeno s krátkodobou (progrese do šesti měsíců), resp. dlouhodobou (progrese po 18 měsících) odpovědí [60]. Se špatnou odpovědí na léčbu sunitinibem je spojena i down-regulace miR-141 (projevující se hlavně epiteliálně-mezenchmální proměnou epiteliálních buněk). Reintrodukce miR-141 in vitro je schopna tento proces zvrátit [61]. Recentní studie srovnávala expresi miRNA u pacientů s výraznou senzitivitou a naopak výraznou rezistencí na sunitinib. MiR-942, miR-628-5p, miR-133a a miR-484 byly ve zvýšené míře exprimovány u pacinetů rezistentních na sunitinib. MiR-942 byla sama schopna odlišit tyto dvě skupiny pacientů. Čas do progrese a OS byly signifikantně redukovány u pacientů s hladinou miR-942 nad stanovenou hraniční hodnotu [62].

ZÁVĚR

MikroRNA se při vzniku RCC uplatňují v mnoha patogenetických mechanizmech. Některé z nich jsou zapojeny do regulace konkrétních signálních drah, u mnoha odlišně exprimovaných miRNA však cílové geny zatím nejsou objasněny. Odlišné hladiny miRNA v tkáni, séru/plazmě a moči pacientů s RCC jsou atraktivním cílem pro hledání biomarkerů použitelných v diagnostice a sledování. MiRNA analýza primárního tumoru může pomoci ve stanovení individuální prognózy pacientů. V blízké budoucnosti by se navíc některé ze zmíněných mikroRNA mohly stát novými terapeutickými cíli RCC.

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REVIEW

MicroRNAs in the pathogenesis of renal cell carcinoma and their diagnostic and prognostic utility as cancer biomarkers

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ABSTRACT

Purpose: To provide information about the role of microRNAs in the pathogenesis of renal cell carcinoma (RCC) and their diagnostic and prognostic utility as cancer biomarkers.

Methods: A literature search was performed in the PubMed and Web of Science databases using the keywords "renal cancer/renal cell carcinoma/kidney cancer" and "miR*/miRNA*/microRNA*". Articles dealing with the role of miRNAs in the pathogenesis of RCC, diagnostic miRNAs and prognostic miRNAs were separated.

Results: MiRNAs act both as oncogenes and tumor suppressors. They regulate apoptosis, cell growth, migration, invasion, proliferation, colony formation and angiogenesis through target proteins involved in several signaling pathways, and they are involved in key pathogenetic mechanisms such as hypoxia (HIF/VHL dependent) and epithelial-to-mesenchymal transition. Differentially expressed miRNAs can discriminate either tumor tissue from healthy renal tissue or different RCC subtypes. Circulating miRNAs are promissing as diagnostic biomarkers of RCC. Information about urinary miRNAs associated with RCC is sparse. Detection of a relapse is another implication of diagnostic miRNAs. The expression profiles of several miRNAs correlate with the prognosis of RCC patients. Comparison between primary tumor tissue and metastasis may help identify high-risk primary tumors. Finally, response to target therapy can be estimated thanks to differences in miRNA expression in tissue and serum of therapy-resistant versus therapy-sensitive patients.

Conclusions: Our understanding of the role of microRNAs in RCC pathogenesis has been increasing dramatically. Identification and validation of their gene targets may have direct impact on developing microRNA-based anticancer therapy. Several microRNAs can serve as diagnostic and prognostic biomarkers.

Keywords: Biomarker, Diagnosis, MicroRNA, Prognosis, Renal cell carcinoma

Introduction

Renal cell carcinoma (RCC) accounts for 2%-3% of all malignant tumors (1). Despite a steady increase in relative survival, it is still one of the most lethal urological malignancies, with 5-year relative survival 71.8% (2). At the time of diagnosis, 17% of tumors are locally advanced and 17% are metastatic, with 5-year relative survival rates of 64.2% and 12.3%, respectively (2). Biomarkers for early detection, prognosis and follow-up of RCC are needed as there is no reliable diagnostic modality other than radiological imaging.

MicroRNAs are short noncoding single-strand RNAs that regulate gene expression at the posttranscriptional level by binding to their target messenger RNA (mRNA) and causing mRNA inhibition and/or degradation (3). They have been shown to be involved in a number of critical biological processes including cell development, differentiation and apoptosis. They can have oncogenic or tumor suppressor effects by inhibiting the protein production of their tumor suppressor or oncogenic targets, respectively (4). Different miRNA expression has been associated with many human cancers including RCC (5). Every miRNA has numerous potential targets and multiple miRNAs may target the same mRNA. Because of the increasing interest in miRNAs as potential cancer biomarkers, we aimed to provide an up-to-date review on the

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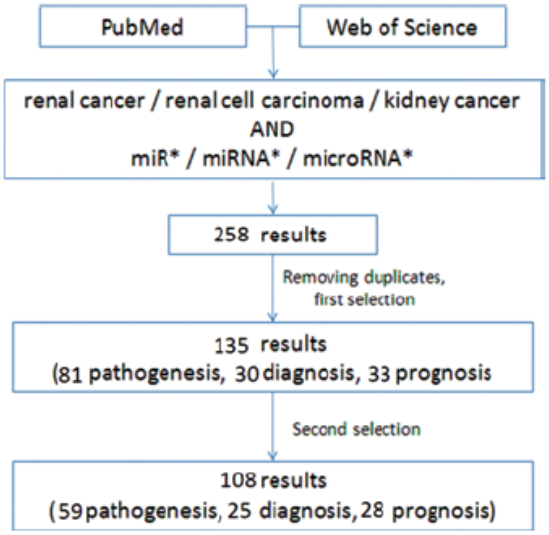

Fig. 1 - Workflow of evidence acquisition.

role of miRNAs in the pathogenesis of RCC with emphasis to their diagnostic and prognostic attributes.

Methodology

A thorough literature search was performed in the PubMed and Web of Science databases using the keywords "renal cancer/renal cell carcinoma/kidney cancer" and "miR*/miRNA*/microRNA*"; the search was restricted to articles published between 2004 and 2015. A total of 258 articles were found dating from 2008 (first relevant paper) to 2015 (April). During the first selection, all abstracts were reviewed and articles were excluded according to previously selected exclusion criteria: animal models, diagnostic/prognostic significance not proven, no abstract available, meeting abstracts, review articles, editorials or comments, articles ahead of print. After removing duplicates and performing the selection, 135 articles were analyzed (81, 30 and 33 discussing pathogenesis, diagnostic and prognostic miRNAs, respectively – some of them overlapping). The final selection omitted 27 articles that were considered irrelevant by the first author (did not provide additional information or could be confusing to the reader). The remaining 108 articles were summarized for the review (Fig. 1).

Results

MiRNAs in the pathogenesis of RCC

MiRNAs related to hypoxia and VHL/HIF pathway

Inactivation of the VHL gene is a typical sign of clear-cell RCC (ccRCC); it is responsible for virtually all familial ccRCC and two-thirds of sporadic ccRCC (6). VHL mutation and loss of its product, VHL protein, leads to unregulated expression of HIF-1α and HIF-2α, with subsequent induction of tumorigenic target proteins including VEGF, PDGF, TGFα and Glut 1 (7). VHL-dependent regulation has been observed in several miRNAs (miR-210, miR-155, let-7i, miR-8a) (8). MiR-210 is HIF-1 dependent (hypoxia induced), but some tumors without evidence of VHL inactivation also have elevated miR-210 levels, suggesting other mechanisms of HIF activation. In addition to HIF-1, HIF-2 can regulate miR-210 in the absence of HIF-1 (9). The association between miR-210 expression and patient survival indicates its influence on tumor behavior through alteration of gene expression rather than its role as a marker of tumor hypoxia only (8). MiR-210 targets iron-sulfur cluster protein (ISCU1,2) which is involved in the mitochondrial electron transport chain as a potential mechanism for regulating the anaerobic pattern of respiration seen in tumors. MiR-210 also stimulates cell migration and the invasive potential of RCC cells, as shown by silencing of miR-210 expression (10). A strong inverse correlation between miR-92a and VHL mRNA levels was found, indicating an alternative mechanism of VHL gene inactivation (11). MiR-138 inhibits the expression of HIF-1α in ccRCC cells, leading to increased apoptosis and reduced migration of tumor cells (12). The VHL and HIF-1α genes are likely direct targets of miR-17-5p and miR-224 (13).

There is a strong anti-correlation between the miR-200 family (miR-200a/200b/200c/429/141) and VEGF, suggesting regulation of HIF and its downstream targets by these miRNAs. Loss of this regulation may be responsible for activation of the HIF pathway (14). Proline oxidase (a mitochondrial tumor suppressor) acts through generation of reactive oxygen species and decreased HIF signaling. Overexpression of miR-23b downregulates the suppressor, and miR-23b can therefore function as an oncogene in renal cancer (15).

MiRNAs and epithelial-to-mesenchymal transition (EMT)

EMT increases the invasive and metastatic potential of epithelial cells. Decreased E-cadherin (through its repressors ZEB1 and ZEB2) and increased expression of vimentin lead to loss of adhesion and increased motility of tumor cells. Modulating EMT by the miR-200 family has been consistently described both in primary RCC and metastasis of RCC (16, 17). The possible target is ZEB1, which promotes tumor invasion and migration through E-cadherin silencing. Additionally, miR-141 targets cell division cycle (CDC) 25 phosphatases, important regulators of the cell cycle. Its loss facilitates genomic instability at an early stage of RCC development (18). However, modulating the EphA2/p-FAK/p-Akt/MMP2/9 signaling cascade seems to be crucial for miR-141 tumor-suppressive activity (19). Upregulation of miR-141 with subsequent EMT reversal could explain the anticancer effect of honokiol (isolated from Magnolia spp. bark) on RCC cell metastasis, suggesting a possible therapeutic strategy (20).

MiR-215 is one of the most downregulated miRNAs in metastatic RCC when compared to primary RCC. Its target (SIP1/ZEB2) has been shown to be involved in EMT. Transfection of kidney cancer cells with miR-215 decreases cell migration and affects cell proliferation (21). The E-cadherin regulators ZEB2 and BMI1 (together with the inhibitor of apoptosis survivin) are possible targets of the suppressor miR-708. Its restoration in RCC cell lines decreases cell

growth, clonability, invasion and migration and increases apoptosis primarily through survivin regulation (22). EMT is promoted by hypoxia-induced downregulation of miR30-c, too (23). Restoration of miR-138 in RCC cell lines leads to decreased vimentin expression at both the mRNA and protein levels, with significant inhibition of cell migration and invasion activities (24). EZH2 is another target oncogene of miR-138, inducing cell senescence of renal cancer cells (25).

MiRNAs and proliferation, invasion, apoptosis and angiogenesis

MiR-21 directly targets genes involved in the cell adhesion pathway, leading to degradation of intra- and extracellular matrix and abnormal cell growth patterns (14). Its key role in regulating cell apoptosis results from targeting multiple apoptosis-related genes including TIMP3, FASL and PDCD4 (26). Knockdown of miR-21 activates the caspase pathway and induces cell apoptosis in RCC (27). Aberrantly expressed miR-21 also regulates cell invasion through the TCF-21-KISS1 pathway. Anti-miR-21 upregulates KISS1 protein, with a subsequent decrease in cell invasion ability (28). Another pathogenetic mechanism of miR-21 function is presented by Akt kinase/TORC1 activation through posttranscriptional regulation of PTEN, resulting in increased cancer cell proliferation and migration (29). Finally, miR-21 controls the expression of cyclin D1 through NF kappa B-dependent transcription (30).

Expression of miR-155 is upregulated in RCC and miR-155 may function as an oncogene. Its suppression inhibits cell proliferation and migratory activity and induces apoptosis in renal cancer cells. Potential target genes are the suppressor genes SOCS-1 and BACH1 (31).

Oncogenic miR-23b-3p directly targets PTEN. Inhibition of miR-23b-3p induces PTEN gene expression with a concomitant reduction of PI3-kinase, total Akt and IL-32, and lower miR-23b-3p may be associated with better prognosis (32). The effect of overexpressed miR-17-92 cluster (miR-17-5p and miR-20a) on tumor cell proliferation was studied in primary and metastatic RCC cell lines. Its oncogenic effect may be explained by collaboration with the MYC oncogene, inhibition of PTEN or driving a proliferative signal by ectopic expression of the single miR-17-5p (33). Growth, invasion and migration of RCC cells are significantly increased after miR-122 transfection (34). Inhibition of endogenous miR-183 (upregulated in RCC tissues) suppresses cell proliferation, colony formation, migration and invasion of RCC cell lines *in vitro*. MiR-183 directly targets a tumor suppressor, phosphatase 2A. Upregulated miR-183 increases cell growth and metastasis and suppresses caspase activity (35).

MiR-584 acts as a tumor suppressor and is downregulated in RCC cell lines. It decreases cell mobility and viability through inhibition of the ROCK-1 oncogene. ROCK-1 is activated by Rhoa A and is associated with cell invasion in different cancers. In ccRCC, higher ROCK-1 mRNA expression is associated with shorter survival (36).

MiR-205 inhibits the proto-oncogenic Src family of protein kinases. Overexpression of miR-205 in cancer cells leads to inhibition of proliferation and cell motility factor and activation of STAT3 (37). Another target of miR-205, ZEB2, indicates its role also in EMT (38). Other tumor suppressor miRNAs (miR-34a, miR-101, miR-199a, miR-1285, miR-1826, miR-187, miR-135a, miR-218, miR-143/145 cluster, miR-133b, miR-199a-3b) act through downregulation of different oncogenes including c-MYC, Notch1, EZH2, protein kinase GSK3 beta, TGM2, beta-catenin, MEK1, B7 homolog 3, caveolin-2, hexokinase-2, matrix metallopeptidase 9 and c-Met (39-50). Downregulation of tumor-suppressive miR-1 and miR-133a leads to upregulation of oncogenic transgelin-2, which enhances cell proliferation and invasion (51). Similarly, downregulation of miR-145 leads to upregulation of ADAM17, a metalloprotease that is overexpressed in many cancer types including RCC (52). Matrix metallopeptidase 11 and the oncogenes ANGPT2 and NEDD9 are other targets of miR-145 (53, 54). Transfection of RCC cell lines with miR-99a induced G1-phase arrest. Since the mammalian target of rapamycin (mTOR) was identified as a direct target of miR-99a, the tumor suppressive role of miR-99a may be mediated primarily through mTOR regulation (55). Glut1 production is directly regulated by miR-1291 through SLC2A1 (56). A relationship between miR-30a downregulation and increased tumor angiogenesis by targeting the DLL4 endothelial ligand has been observed (57). WEE1 protein kinase (a mitosis inhibitor) is a common target of miR-424 and miR-381. Its downregulation (caused by the synergistic effect of both miRNAs) leads to Cdc2 activation and subsequent inhibition of cell proliferation and mitosis and abrogated G2/M arrest (58). MiR-381 also increases the sensitivity of RCC cells to 5-FU chemotherapy (59).

The association of miRNAs with possible novel regulatory pathways (e.g., Yin Yang 1 activation by miR-34a downregulation) has been suggested recently (60).

An interesting (and yet not fully understood) feature of miRNAs is the capability of a single miRNA to serve either as a tumor suppressor or oncogene depending on the type of cancer. MiR-7, which has been described as a tumor suppressor in several human cancers, has been characterized as an oncogene in RCC (61).

Recently, an anticancer effect of the quercetin and hyperoside combination (QH) on RCC cells has been shown. Besides a decrease in transcription factor levels, inhibition of oncogenic miR-27a was observed. Transfection of cells with miR-27a partially reversed the effects of QH (62). The anticancer activity of metformin may be explained by increased expression of miR-26a, which inhibits cell proliferation through downregulation of Bcl-2 and cyclin-D and upregulation of PTEN (63). The most recent papers describe the tumor suppressor effect of miR-506, miR-377, miR-184 and miR-125a-5p (64-67).

Diagnostic miRNAs

Differential expression of miRNAs in RCC and nontumorous renal tissue

Many microRNAs are differentially expressed between RCC subtypes and normal kidney tissue (Tab. I). Using a platform of 470 miRNAs, Nakada et al (68) identified 43 miRNAs that were differentially expressed between ccRCC and normal kidney and 57 miRNAs that were differentially expressed between chromophobe RCC (chRCC) and normal kidney. MiR-141 and miR-200c were the most significantly downregulated miRNAs in ccRCC (68). Thirty-eight upregulated and 48 downregulated miRNAs in cancer tissue were detected in another study

TABLE I - Differential expression of miRNAs between ccRCC and nontumor tissue

Author	Year	Differentially expressed miRNAs	Most upregulated	Most downregulated	Ref.
Nakada et al	2008	43	miR-155, miR-224, miR-210	miR-141, miR-200c, miR-138, miR-514	68
Jung et al	2009	76	miR-122, miR-18a, miR-452, miR-224, miR-210, miR-34b, miR-155, miR-21, miR-34a	miR-184, miR-514, miR-200c, miR-141, miR-510, miR-138, miR-429, miR-200b, miR-200a	71
Yi et al	2010	86	miR-451, miR-144, miR-491-3p, miR-193a-3p, miR-18b, miR-1280, miR-142-3p, miR-302f, miR-378, miR-15b, miR-16	miR-141, miR-1248, miR-200a, miR-664, miR-30a, miR-135a, miR-485-5p, miR-571, miR-10b, miR-200c	69
Juan et al	2010	35	miR-210, miR-155, miR-142-3p, miR-21, miR-592, miR-224, miR-34b, miR-34a	miR-141, miR-200c, miR-514, miR-429, miR-377, miR-135a, miR-154, miR-200a, miR-200b, miR-204, miR-211, miR-411, miR-10b	70
Chow et al	2010	80	miR-122, miR-210, miR-101, miR-19b, miR-489, miR-20b, miR-15a, miR-424, miR-17, miR-21,	miR-200c, miR-720, miR-150, miR-214, miR-1826, miR-182, miR-200b, miR-191	72
Osanto et al	2012	100	miR-21-5p, miR-451-3p, miR-210-3p, miR-34a-5p, miR27a-3p, miR-342-3p,	miR-125a-5p, miR-204-5p, miR-10a-5p, miR-10b-5p, miR-107-3p, miR-660-5p, miR-200b-3p	102
Zaravinos et al	2014	434	miR-25-5p, miR-489, miR-711, miR-498, miR-3687, miR-122-5p, miR-3656, miR-21-5p	miR-106b-3p, miR-141-3p, miR-656, miR-155-3p, miR-140-5p, miR-520	73
Wu et al	2012	56	miR-122, miR-210, miR-224, miR-885-5p, miR-7, miR-155, miR-144	miR-200c, miR-141, miR-514, miR-204, miR-138, miR-30a, miR-429	103

using a platform of 847 miRNAs in patients with ccRCC and healthy controls (69). Juan et al (70) found 26 downregulated and 9 upregulated miRNAs in ccRCC (including miR-21, miR-210 and miR-155, as commonly found in other cancers). Jung et al (71) identified 33 miRNAs with at least 2-fold change between ccRCC and nonmaligmant tissue. Chow et al (72) identified 80 differentially expressed miRNAs in ccRCC compared to normal tissue, 33 of them classified as a high signal group. MiR-122 and miR-200c were the most dysregulated. Zaravinos et al (73) examined miRNA expression profiles in ccRCC, a smaller cohort of papillary RCC (pRCC) and chRCC, and upper tract urothelial carcinoma versus normal kidney tissue. In ccRCC, miR-3648, miR-143-3p, miR-25-5p, miR-628-3p, miR-921 and miR-210 exhibited the best discriminatory ability versus normal kidney (73). In a study by Cheng et al (74), 8 miRNAs were selected for tissue and serum analysis. MiR-34a, miR-21 and miR-224 were significantly upregulated whereas miR-141, miR-149 and miR-429 were significantly downregulated. MiR-129-3p has been described as a promising diagnostic biomarker discriminating ccRCC tissues from normal tissues with 73.5% accuracy (75). Low miR-129-3p levels have been associated with short disease-free survival (DFS) and overall survival (OS). Analysis of dysregulated miRNAs and miRNA-mRNA dysregulation pairs may help identify disease-specific miRNAs. In a study by Hao et al (76), 5 potential biomarkers were identified (miR-425, miR-136, miR-340, miR-335 and miR-320d) (76). Table II summarizes up- and downregulated miRNAs that have been consistently reported among various studies.

MiRNA expression in different RCC subtypes

MiRNA signatures can distinguish RCC subtypes (and also oncocytoma). In the study by Zaravinos et al (73), despite the small cohorts, very good discriminatory miRNAs (AUC >0.8, $p<0.05$) were also found in pRCC (miR-3687, miR-25-5p) and chRCC (miR-3687, miR-4284, miR-141-3p) versus controls. Youssef et al (77) developed a 4-step decision tree based on a group of miRNA pairs that – based on their different expression – can classify a sample as 1 of 2 possible outcomes. Table III shows the miRNA pairs in consecutive steps. The system has a sensitivity of 97% in distinguishing normal kidney from RCC, 100% for ccRCC, 97% for pRCC, and 100% accuracy in distinguishing oncocytoma from chRCC.

Petillo et al (78) focused on the comparison between the subtypes with the greatest genomic similarity (chRCC/oncocytoma, pRCC/ccRCC). Table IV summarizes both comparisons. Oncogenic miR-21 has different expression among RCC subtypes, with the highest levels being observed in ccRCC and pRCC. Its expression can distinguish ccRCC and pRCC from chRCC and oncocytoma with 90% specificity and 83% sensitivity (79). In a study by Wach et al (80) examining 9 selected miRNAs, the combination of miR-145, miR-210, miR-200c and miR-502-3p correctly discriminated between tumor and normal tissue (accuracy of 92.9%), the combination of miR-145 and miR-502-3p predicted the RCC entity correctly in 95% of patients (ccRCC vs. pRCC), and the combination of miR-210 and let-7c was able to correctly predict the pRCC subtype (pRCC1 vs. pRCC2) in 92.3%.

TABLE II - MiRNAs consistently reported as upregulated or downregulated in RCC

Upregulated miRNAs	Author, year (reference)
miR-210	Nakada et al, 2008 (68); Jung et al, 2009 (71); Chow et al, 2010 (72); Juan et al, 2010 (70); Slaby et al, 2010 (97); Wu et al, 2012 (103); Osanto et al, 2012 (102); Redova et al, 2013 (10); Zaravinos et al, 2014 (73)
miR-21	Jung et al, 2009 (71); Chow et al, 2010 (72); Juan et al, 2010 (70); Zhang et al, 2012 (28); Faragalla et al, 2012 (79); Osanto et al, 2012 (102); Wu et al, 2012 (103); Cheng et al, 2013 (74); Wotschofsky et al, 2013 (109)
miR-155	Nakada et al,2008 (68); Jung et al, 2009 (71); Juan et al, 2010 (70); Slaby et al, 2010 (97); Wu et al, 2012 (103); Osanto et al, 2012 (102); Redova et al, 2013 (10)
miR-122	Nakada et al, 2008 (68); Jung et al, 2009 (71); Chow et al, 2010 (72); Wu et al, 2012 (103); Osanto et al, 2012 (102); Redova et al, 2013 (10)
miR-224	Nakada et al, 2008 (68); Jung et al, 2009 (71); Juan et al, 2010 (70); Wu et al, 2012 (103); Osanto et al, 2012 (102); Cheng et al, 2013 (74)
Downregulated miRNAs	
miR-141	Nakada et al, 2008 (68); Jung et al, 2009 (71); Juan et al, 2010 (70); Slaby et al, 2010 (97); Yi et al, 2010 (69); Osanto et al, 2012 (102); Hidaka et al, 2012 (42); Wu et al, 2012 (103); Redova et al, 2013 (10); Cheng et al, 2013 (74); Wang et al, 2013 (17); Zaravinos et al, 2014 (73)
miR-200c	Jung et al, 2009 (71); Chow et al, 2010 (72); Juan et al, 2010 (70); Slaby et al, 2010 (97); Yi et al, 2010 (69); Hidaka et al, 2012 (42); Wu et al, 2012 (103); Redova et al, 2013 (10); Wang et al, 2013 (17)
miR-200b	Jung et al, 2009 (71); Chow et al, 2010 (72); Juan et al, 2010 (70); Slaby et al, 2010 (97); Osanto et al, 2012 (102); Wu et al, 2012 (103); Redova et al, 2013 (10)
miR-138	Nakada et al, 2008 (68); Jung et al, 2009 (71); Wu et al, 2012 (103); Yamasaki et al, 2012 (24); Redova et al, 2013 (10); Wang et al, 2013 (17)
miR-429	Jung et al, 2009 (71); Juan et al, 2010 (70), Osanto et al, 2012 (102); Wu et al, 2012 (103); Cheng et al, 2013 (74); Redova et al, 2013 (10)
miR-204	Jung et al, 2009 (71); Juan et al, 2010 (70); Hidaka et al, 2012 (42); Wu et al, 2012 (103); Redova et al, 2013 (10)
miR-363	Jung et al, 2009 (71); Juan et al, 2010 (70); Hidaka et al, 2012 (42); Wu et al, 2012 (103); Wang et al, 2013 (17)
miR-200a	Jung et al, 2009 (71); Juan et al, 2010 (70); Yi et al, 2010 (69); Osanto et al, 2012 (102); Wu et al, 2012 (103); Redova et al, 2013 (10)
miR-218	Juan et al, 2010 (70); Hidaka et al, 2012 (42); Wang et al, 2013 (17)
miR-532	Jung et al, 2009 (71); Chow et al, 2010 (72); Osanto et al, 2012 (102); Wu et al, 2012 (103); Redova et al, 2013 (10)

TABLE III - Four-step decision tree discriminating normal tissue, RCC subtypes and oncocytoma (77)

Step 1	Step 2	Step 3	Step 4
Normal vs. kidney tumor	**ccRCC vs. other subtypes (pRCC, chRCC, oncocytoma)**	**pRCC vs. chRCC, oncocytoma**	**chRCC vs. oncocytoma**
miR-200c>miR-222	miR-194>miR-548m	miR-331-3p>miR-139-5p	miR-99a>miR-200b
miR-194>miR-15b	miR-192>miR-221	miR-191>miR-221	miR-22>miR183
miR-324-5p>miR-34a	miR-424>miR-183	miR-106a>miR-663	miR-625>miR-1300
miR-500>miR-425	miR-181b>miR-663		
miR-10b>miR-28-3p	miR-100>miR-182		
miR-532-5p>miR-93	miR-15a>miR-222		
	miR-195>miR-10a		
	miR-26b>let-7g		

ccRCC = clear-cell renal cell carcinoma; pRCC = papillary renal cell carcinoma; chRCC = chromophobe renal cell carcinoma.
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TABLE IV - miRNAs discriminating ccRCC vs. pRCC and chRCC vs. oncocytoma (78)

ccRCC vs. pRCC	Average fold-change	chRCC vs. oncocytoma	Average fold-change
miR-203	4.74	miR-203	4.49
miR-424	4.34	miR-200b	2.89
miR-450	4.16	miR-197	1.99
miR-139	3.55	miR-320	1.5
miR-143	3	miR-186	-2.88
miR-503	2.97		
miR-224	2.94		
miR-145	2.59		
miR-126	2.27		
miR-31	-4.91		
miR-504	-2.62		
miR-371	-2.55		
miR-328	-1.68		
miR-425	-1.5		
miR-423	-1.36		

ccRCC = clear-cell renal cell carcinoma; pRCC = papillary renal cell carcinoma; chRCC = chromophobe renal cell carcinoma.
Adapted from International Journal of Oncology, vol. 35, D. Petillo, E.J. Kort, J. Anema, K.A. Furge, X.J. Yang, B.T.Teh, MicroRNA profiling of human kidney cancer subtypes, p. 109-114, 2009, with permission from Spandidos Publications.

Circulating miRNAs as potential biomarkers of RCC

Serum miR-210 has been investigated as a biomarker for the diagnosis and the detection of progression of ccRCC. A study involving 34 ccRCC patients and 23 healthy controls found significantly higher serum levels of miR-210 in the patient group, with an AUC of 0.77 and a sensitivity and specificity of 65% and 83%, respectively (81). No association between miR-210 levels and age, sex, tumor size or existence of metastasis was found in the study. In another study, serum miR-210 levels yielded an AUC of 0.874 with a sensitivity of 81% and a specificity of 79.4%. In 10 samples 1 week after surgery, the average serum level of miR-210 was significantly decreased (82). Wulfken et al (83) compared miRNA profiles in serum and tissue of RCC patients and identified 36 increased circulating miRNAs that were overexpressed in corresponding tumor tissues. Seven miRNAs were selected as diagnostic biomarkers (miR-106b, miR-1233, miR-1290, miR-210, miR-7-1, miR-320b and miR-93). The level of miR-1233 was significantly increased in patients with RCC, although the diagnostic information was below expectations (sensitivity 77%, specificity 37.6%, AUC 0.588) and benign tumors did not show different miR-1233 levels (83).

MiR-378 and miR-451 have been put forward as promising serum biomarkers in RCC by Redova et al (84). Combination of miR-378 and miR-451 in serum enabled the identification of RCC with a sensitivity of 81%, a specificity of 83%, and an AUC of 0.86. However, Hauser et al (85) did not confirm a different level of miR-378. Also, miR-378 was not correlated with pT-stage.

A significant difference in plasma levels of miR-508-3p was found in RCC patients compared with healthy controls (86). MiR-509-5p was downregulated both in RCC tissue and plasma of RCC patients (87). In a study by Cheng et al (74), miR-34a, miR-21 and miR-224 were significantly upregulated in the sera of patients with RCC (compared with patients having benign lesions), whereas miR-141 was downregulated (74). The serum levels of miR-21 significantly correlated with the stage of ccRCC. Teixeira et al (88) observed higher plasma levels of miR-221 and miR-222 in RCC patients. Patients having metastases presented higher circulating levels of miR-221 than patients with no metastases. A significantly shorter OS in patients with higher expression levels of miR-221 was observed (88).

Recently, Wang et al (89) introduced a panel of 5 serum miRNAs for the early detection of RCC, including miR-193a-3p, miR-362, miR-572, miR-28-5p and miR-378. The AUCs for the combination were 0.807 (training set) and 0.796 (validation set).

Urinary miRNAs as potential biomarkers of RCC

There is limited information about urinary miRNAs in RCC patients. Von Brandenstein et al (90) described miR-15a, which is related to protein kinase C alpha (PKCα), as being upregulated in benign oncocytoma but downregulated in RCC. PKCα is a component of a transcription complex in tumors; it directly binds to the primary transcript of miR-15a in the nucleus and suppresses miR-15a. Therefore, miR-15a is upregulated in RCC but downregulated in oncocytoma. The miR-15a

TABLE V - Suggested prognostic miRNAs in renal cell carcinoma

Author	Year	miRNA	Expression (higher vs. lower)	Prognostic significance	Ref.
McCormick et al	2013	miR-210	H	Lower stage and grade, better OS	9
Zaman et al	2012	miR-21	H	Higher stage, worse OS	92
Faragalla et al	2012	miR-21	H	Shorter DFS and OS	79
Vergho et al	2014	miR-21/miR-126	H/L	Shorter CSS	93
Fritz et al	2014	miR 21/10b	H	Poor prognosis in M0	94
Wang et al	2013	miR-100	H	Worse OS and CSS	95
Shinmei et al	2013	miR-155	L	Poor prognosis in stage III and IV	96
Slaby et al	2010	miR-106b	L	Higher risk of metastasis	97
Zhao et al	2013	miR-187	L	Higher stage and grade, worse OS	44
Li et al	2013	miR-217	L	Higher stage and grade, worse OS	98
Zhao et al	2015	miR-497	L	Shorter OS	99
Fu et al	2014	miR-125b	H	Worse CSS, early recurrence	108
Wotschofsky et al	2013	miR-514	L	Primary M1 and recurrent	109
Heinzelmann et al	2011	miR-451, miR-221, miR-30a, miR-10b, miR-29a	L	M1 vs. M0	100
		miR-30a-d, miR-27b, let-7a-c, miR-26a, miR-125b, miR-130a	L	Primary M1 vs. late M1 and M0	
		miR-10a, let-7c, miR-26a, miR-143, miR-19b, miR-126a, miR-130a	L	Shorter PFS and CSS	
Heinzelmann et al	2014	miR-204, miR-30c, miR-30a-3p, miR-30a-5p, miR-30e-3p, miR-30e-5p, miR-30c-2-3p	L	M1 and distant metastasis vs. M0	101
		miR-30c	L	Shorter PFS and CSS	
		miR-126, miR-145	H		
Osanto et al	2012	miR-222-3p, miR-221-3p, miR-193a-3p, miR-130b-3p, miR-181a2-3p, miR-188-5p, miR-22-3p, miR-146a-5p	H	M1 vs. nonrecurrent	102
		miR204-5p, miR-139-5p, miR-26-5p, miR-27b-3p	L		
Wu et al	2012	miR-199-5p, miR-130b	H	Worse CSS	103
		miR-10b, miR-139-5p	L		
Khella et al	2012	miRNA-10b, miR-126, miR-196a, miR-204, miR-215, miR-192, miR-194	L	Metastasis vs. primary tumor	104
Khella et al	2013	miR-215	L	Reduced DFS	105
Slaby et al	2012	miR-143, miR-26a, miR-145, miR-10b, miR-195, miR-126	L	Tumor relapse	106
		miR-145, miR-126, miR-127-3p	L	Shorter relapse-free survival in M0	

OS = overall survival; DFS = disease-free survival; CSS = cancer-specific survival; PFS = progression-free survival; H = higher; L = lower; M0 = nonmetastatic; M1 = metastatic.

urinary levels are high in patients with RCC but undetectable in oncocytoma or urinary tract infections (90).

MicroRNAs as biomarkers of RCC prognosis (Tab. V)

Better clinicopathological features have been reported with high miR-210 expression (lower stage and grade) (9). Based on miR-210 ccRCC tissue levels, patients were separated into 3 groups, and a better OS was observed in the high miR-210 group. Samaan et al (91) reported shorter OS and a greater chance of disease recurrence in patients with miR-210 overexpression; however, after adjusting for tumor size and TNM stage the statistically significant association was lost (91).

A correlation was found between miR-21 expression and survival of RCC patients: all patients with low miR-21 expression survived 5 years, while only 50% of patients with high miR-21 expression survived. Higher expression of miR-21 was associated with more advanced stage of RCC (92). Significantly shorter DFS and OS in patients with high miR-21 expression was confirmed by another study involving 121 patients with different RCC subtypes (highest miR-21 in ccRCC and pRCC)

(79). In a study by Vergho et al (93), a significant correlation of miR-21 upregulation and miR-126 downregulation with metastasis and cancer-specific survival (CSS) was found (93). The miR(21/10b) ratio has been shown to be an independent prognostic factor for M0 ccRCC. A high miR(21/10b) ratio was associated with poor prognosis, suggesting the need for stricter surveillance in high-risk M0 patients according to the miR(21/10b) ratio (94). High miR-100 expression in RCC tissue was identified as an independent poor prognostic marker of both OS and CSS (95). In patients with stage III and IV ccRCC, low expression levels of miR-155 correlated with poor prognosis (96). MiR-106b is significantly overexpressed in ccRCC tissue; however, its expression levels are significantly lower in tumors of patients who develop metastasis and miR-106b may be a potential predictive marker of early metastasis after nephrectomy (97). Lower expression of several tumor suppressive miRNAs is also associated with a worse prognosis (44, 98, 99).

Of 33 miRNAs discriminating metastatic and nonmetastatic ccRCC, miR-451, miR-221, miR-30a, miR-10b and miR-29a showed the most significant downregulation. Additionally, a group of 4 miRNAs (let-7a, let-7c, miR-26a, miR-30c) distinguished the course of the disease (primary metastatic, late metastatic, nonmetastatic). Low expression levels of miR-26a, miR-10a, miR-143, miR-19b and let-7c showed a strong correlation with poor progression-free survival (PFS) and CSS, (100). A similar study comparing primary metastatic ccRCC and distant ccRCC metastases versus nonmetastatic ccRCC showed 14 differently expressed miRNAs. Downregulated miR-30c and upregulated miR-126 and miR-451 showed a significant correlation with PFS and CSS (101). Osanto et al (102) found 12 miRNAs to be uniquely discriminating between a metastatic and a nonrecurrent subgroup of patients. According to the difference between the miRNA expression in metastatic and localized renal tumors, 4 miRNAs were chosen to build a metastatic tumor signature: miR-10b, miR-139-5p (downregulated), miR-130b and miR-199b-5p (upregulated) (103). The validation test showed that the signature appeared to be more powerful in identifying concurrent metastases (81%) than subsequent/future metastasis of the primary tumors (69%); however, 22 of 29 patients who had metastatic disease had high-risk primary tumors while 6 of 6 with no metastasis had low-risk tumors predicted by the signature (sensitivity 76%, specificity 100%). Another study of an independent set of 20 pairs of metastatic ccRCC and matched primary tumors from the same patients showed as the most significantly differentially expressed miRNAs miR-10b, miR-126, miR-196a, miR-204, miR-215, miR-192 and miR-194 (104). A further study of miR-215, miR-192 and miR-215 by the same authors found both a convergent effect (the same molecule can be targeted by several miRNAs) and a divergent effect (the same miRNA can control multiple targets). Lower expression of miR-215 was associated with significantly reduced DFS time (105).

For the detection of early relapse after nephrectomy, miR-143, miR-26a, miR-145, miR-10b, miR-195 and miR-126 were confirmed as being downregulated in RCC patients who developed tumor relapse. MiR-127-3p, miR-145 and miR-126 significantly correlated with relapse-free survival of nonmetastatic patients (106). Prolonged DFS for higher miR-126 levels and lower levels in metastatic tumors were confirmed in a recent study by Khella et al (107). High tumor miR-125b indicated poor survival and early recurrence after nephrectomy for patients with ccRCC, especially with advanced-stage disease (108).

MiR-122 (upregulated) and miR-514 (downregulated) were differently expressed between samples of nonmetastatic and metastatic ccRCC. The expression of miR-514 was particularly downregulated in primary metastatic tumors and those that recurred (109).

MiR-21, miR-126 and miR-221 can predict CSS in ccRCC patients after nephrectomy with thrombectomy in case of inferior vena cava thrombus (110).

MiRNAs as biomarkers of treatment response

The microRNA signature in peripheral blood may stratify patients with advanced RCC according to their response to first-line treatment with sunitinib. Twenty-eight and 23 of 287 analyzed miRNAs were associated with poor response (progression before 6 months) or prolonged response (progression after 18 months), respectively (111). In the poor response model pre- and posttreatment levels of miR-192, miR-193a-3p and miR-501-3p were compared; the prolonged response model included pretreatment miR-410b and miR-1181c and miR-424c fold change.

In a study by Berkers et al (112), miR-141 downregulation-driven EMT in ccRCC was linked to an unfavorable response to sunitinib therapy. Reintroduction of miR-141 *in vitro* led to EMT reversal and increased sensitivity to a hypoxic environment.

Two groups of patients with metastatic RCC treated with sunitinib (marked sensitivity vs. resistance to sunitinib) were compared in a recent study by Prior et al (113). MiR-942, miR-628-5p, miR-133a and miR-484 were overexpressed in sunitinib-resistant patients. MiR-942 significantly discriminated between the 2 patient groups. Time to progression and OS were significantly reduced in patients with miR-942 expression above the mean. MiR-30a is a potent inhibitor of autophagy (which contributes to cancer resistance to treatment). Exogenous expression of miR-30a in RCC cells enhanced sorafenib-induced cytotoxicity, causing substantial cell apoptosis (114). MiR-200c could sensitize ccRCC cells to sorafenib and imatinib by targeting antiapoptotic heme oxygenase 1 (HO-1). A correlation was found between miR-200c and HO-1 levels and drug resistance (115).

Conclusion

MiRNAs are involved in various pathogenetic mechanisms of RCC development. Although some of them directly affect particular signaling pathways, the exact roles and gene targets of many differentially expressed miRNAs still have to be elucidated. The reverse effect of either knockdown or restoration of dysregulated miRNAs *in vitro* may herald the era of new therapeutic targets. Different expression of miRNAs in tumor tissue, serum/plasma and urine make miRNAs attractive biomarkers for diagnosis and follow-up; several studies have confirmed their consistent up- or downregulation in RCC. The

use of a combination of miRNAs rather than a single miRNA is desirable to identify suitable biomarkers for clinical practice. MiRNA analysis of the primary tumor might be an important tool for individual prognosis prediction. Specific miRNA signatures in nonmetastatic, primary metastatic and recurrent RCC may predict the course of the disease. In the management of advanced RCC, the identification of treatment-sensitive versus resistant tumors could lead to tailored therapy.

Disclosures

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Circulating miRNAs as diagnostic biomarkers

Several serum miRNAs have been shown to have relatively high accuracy in distinguishing between tumour patients and healthy individuals. In a group of 68 patients with RCC, serum miR-210 levels were significantly higher than in a control group of 42 healthy individuals (AUC 0.874 at a sensitivity of 81% and a specificity of 79%). One week after surgery, serum miR-210 levels were significantly lower than before surgery (29). In a study of Czech authors, a significantly higher level of miR-378 and a lower level of miR-451 were demonstrated. The combination of both miRNAs identifies the serum of RCC patients with a sensitivity of 81% and a specificity of 83%, AUC being 0.86 (30). Diagnostic accuracy can be increased by using a panel of several miRNAs, e.g. a combination of miR-193a-3p, miR-362, miR-572, miR-28-5p, miR-378 (31). Sensitivity of 100% and specificity of 73.3% for the diagnosis of ccRCC is described for the combination of miR-141 and miR-1233 (32).

Published work related to the topic:

Fedorko M, Stanik M, Iliev R, Redova-Lojova M, Machackova T, Svoboda M, Pacik D, Dolezel J, Slaby O. Combination of MiR-378 and MiR-210 Serum Levels Enables Sensitive Detection of Renal Cell Carcinoma. Int J Mol Sci 2015; 16(10): 23382-23389.
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In this original work, we demonstrated significantly higher expression of miR-210 and miR-378 in patients with ccRCC (serum sensitivity and specificity 80% and 76%, respectively, AUC 0.848) in a serum analysis of 195 patients with ccRCC and 100 healthy controls. At the same time, a significant decrease in plasma levels of both miRNAs after surgical removal of the tumour was demonstrated. A positive correlation between increased miR-378 levels and both disease-free survival and the stage of the disease was confirmed.

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Article

Combination of MiR-378 and MiR-210 Serum Levels Enables Sensitive Detection of Renal Cell Carcinoma

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Abstract: Serum microRNAs are emerging as a clinically useful tool for early and non-invasive detection of various cancer types including renal cell carcinoma (RCC). Based on our previous results, we performed the study to analyze circulating serum miR-378 and miR-210 in patients with various histological subtypes of RCC. RNA was purified from blood serum samples of 195 RCC patients and 100 healthy controls. The levels of miR-378 and miR-210 in serum were determined absolutely using quantitative real-time PCR. Pre- and postoperative levels of both microRNAs were compared in 20 RCC patients. Significantly increased serum levels of both miR-378 and miR-210 enabled to clearly distinguish RCC patients and healthy controls with 80% sensitivity and 78% specificity if analyzed in combination ($p < 0.0001$), and their levels significantly decreased in the time period of three months after radical nephrectomy ($p < 0.0001$). Increased level of miR-378

positively correlates with disease-free survival ($p = 0.036$) and clinical stage ($p = 0.0476$). The analysis of serum miR-378 and miR-210 proved their potential to serve as powerful non-invasive diagnostic and prognostic biomarkers in RCC.

Keywords: renal cell carcinoma; microRNA; blood serum; biomarker

1. Introduction

Renal cell carcinoma (RCC) is the most common neoplasm of adult kidney accounting for about 3% of adult malignancies with the mortality rate of over 40% [1]. There are several RCC subtypes, which can be further differentiated based on histological features including (i) clear cell RCC (conventional, 70%–80%); (ii) papillary RCC (10%–15%); and (iii) chromophobe RCC (5%) as the most common variants [2]. Demographically, the incidence rates are the highest in Europe, North America, and Australia, with Czech Republic having the highest incidence rate worldwide. Although there is a decreasing incidence of advanced stages of RCC (stage migration) in European cohorts in the last 25 years [3,4], the numbers of RCC patients diagnosed with advanced disease is still significant. As the survival rates for patients with localized disease compared with patients with regional and distant metastasis are significantly better, it is of great interest to provide early detection and treatment. Unfortunately, there is no standard serum biomarker enabling early and non-invasive diagnosis or monitoring of the disease. Recent reports highlighted the potential of serum microRNAs (miRNAs) to serve as a suitable tool for improving the RCC management.

MiRNAs are non-protein-coding, 18–25 nt in length, small RNAs involved in essential biological processes by their ability to regulate gene expression in a post-transcriptional manner. There is a growing number of evidence busting the myth of miRNAs being strictly intracellular molecules, confirming miRNAs in various body fluids, *i.e.*, serum/plasma, urine, and other body fluids in a highly stable form with similar signatures in men and women, as well as individuals of different age [5]. Although the existence of miRNAs in circulation and their originating from (i) microvesicles (released by exocytosis); (ii) exosomes (released upon fusion of late endosome with plasma membrane); or (iii) apoptotic vesicles and/or senescent bodies still remains to be clearly described and elucidated [6], circulating miRNAs constitute an elegant tool for early detection of RCC.

In our study, we analyzed circulating serum miRNAs (miR-378 and miR-210, formerly studied in both tissue and serum) [7–9] in patients with various histological subtypes of RCC by absolute quantification with respect to relevant clinical-pathological features confirming their potential to serve as a powerful non-invasive diagnostic and prognostic biomarker in RCC.

2. Results

The serum expression levels of both miR-378 and miR-210 were significantly increased in RCC patients ($n = 195$) compared to healthy donors ($n = 100$) ($p < 0.0001$ for both) (Figure 1A,B), confirming our previous results with miR-378 on an independent cohort of RCC patients. Receiver operating characteristic (ROC) curve analysis revealed that the serum levels of both analyzed miRNAs could serve

as appropriate biomarkers for differentiating serum of RCC patients from healthy controls with the area under curve (AUC) of 0.82 (95% CI, 0.77 to 0.86) for miR-378, and 0.74 (95% CI, 0.69 to 0.80) for miR-210, respectively (Table 1), even in the case when RCC patients with early clinical stages (stage I/II) were evaluated separately ($p < 0.0001$). Moreover, the combination of miR-378 and miR-210 could further improve the diagnostic accuracy with AUC of 0.85 (95% CI, 0.81 to 0.89, $p < 0.0001$) reaching the 80% sensitivity and 78% specificity (Figure 1F).

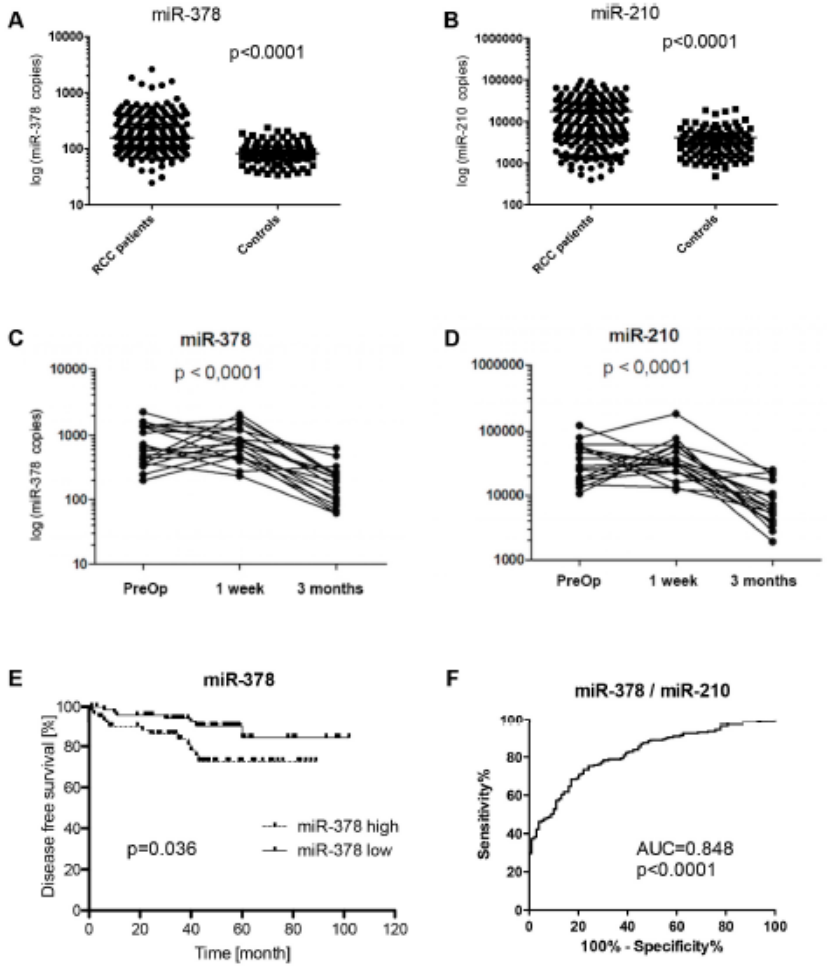

Figure 1. MiR-378 and miR-210 as biomarkers in renal cell carcinoma. Differences in serum levels of miR-378 (**A**) and miR-210 (**B**) in RCC patients and healthy controls; dynamics of miR-378 (**C**) and miR-210 (**D**) levels in blood serum one week and three months after radical nephrectomy; and positive correlation of miR-378 with disease free survival (**E**) and ROC analysis of miR-378/miR-210 combination for discrimination of renal cell carcinoma patients and healthy controls (**F**).

When miR-378 and miR-210 serum levels were analyzed one week and three months after radical nephrectomy, expression levels of both miRNAs were significantly decreased in the time period of three months ($p < 0.0001$) (Figure 1C,D).

Table 1. Summary of expression levels of miR-378 and miR-210 detected in serum of RCC patients and healthy controls expressed as median and interquartile range of miRNA copies.

Clinical Characteristic	*n*	MiR-378	MiR-210
RCC patients *vs.* healthy controls			
RCC	195	159 (109–278)	8544 (3344–27,269)
HC	100	83 (66–110)	2921 (1527–4953)
p-value		$p < 0.0001$	$p < 0.0001$
AUC		0.82	0.74
Histological subtype			
clear cell RCC	157	155 (109–270)	8111 (3288–26,929)
chromophobe RCC	12	201 (142–364)	9731 (3253–30,338)
papillary RCC	26	194 (88–281)	7760 (4551–23,223)
p-value		$p = 0.4200$	$p = 0.9999$
Clinical stage			
I	106	138 (104–238)	8775 (4385–25,151)
II	27	167 (113–230)	5000 (1904–17,732)
III	26	200 (152–268)	9078 (1561–27,439)
IV	36	226 (124–485)	14,909 (2683–36,245)
p-value		$p = 0.0476$	$p = 0.3985$
Fuhrman grade			
G1	34	141 (119–217)	8706 (3790–24,693)
G2	81	154 (102–287)	9999 (4151–32,338)
G3	55	159 (109–273)	7136 (2727–25,998)
G4	17	221 (167–501)	7286 (2357–15,398)
not available	8		
p-value		$p = 0.1925$	$p = 0.7516$

We also analyzed whether miR-378 and miR-210 serum expression levels were correlated to common clinical-pathological features of RCC. We observed a correlation between elevated serum miR-378 expression level and clinical stage ($p = 0.0476$), and miR-378 expression level and disease free survival ($p = 0.036$) (Figure 1E). We performed also the multivariate analysis of miR-378 together with common prognostic factors in RCC like T-, N-stage and Fuhrmann grade. Unfortunately, only T3 ($p = 0.001$) and N2 ($p = 0.0006$) stages were identified as independent prognostic factors. Circulating miR-378 has not reached statistical significance as independent prognostic factor in RCC ($p = 0.1672$).

However, neither serum miR-378 nor miR-210 levels were correlated with Fuhrman grade ($p = 0.1925$ for miR-378, $p = 0.7516$ for miR-210), and overall survival. We have not observed any difference in miRNA levels among RCC histological subtypes ($p = 0.4200$ for miR-378, $p = 0.9999$ for miR-210).

3. Discussion

Regarding the emerging evidence of circulating miRNAs to serve as relevant non-invasive biomarkers in cancer patients (e.g., miR-141 and miR-26a in prostate cancer [10,11], miR-29a and miR-92 in colorectal cancer [12], miR-195 in breast cancer [13], and based on our previous studies regarding the miR-378 and miR-210 potential to serve as an accurate biomarker both in circulating and tissue manner, we further evaluated these miRNAs in serum in the independent cohort of RCC patients undergoing radical nephrectomy (n = 195) and healthy donors (n = 100) using qRT-PCR. The present study showed that both serum miR-378 and miR-210 expression levels were significantly higher in RCC patients enabling clear distinguishing between RCC patients and healthy controls, even in early stages, and that their combination could serve as a powerful diagnostic biomarker with high accuracy (AUC 0.8480, 80% sensitivity, 78% specificity). The increased serum levels of miR-378 and miR-210 were observed also in the studies of Hauser *et al.* (2012), who described elevated miR-378 level in 25 ccRCC patients (p = 0.006), but were not able to distinguish between larger cohort of RCC with various histology and healthy controls [14], and Zhao *et al.* (2013) who observed significantly higher levels of miR-210 in the serum of 68 ccRCC patients ($p < 0.001$) [15]. Moreover, one of the typical and most prominent features of RCC tumors is hypoxia, and miR-210 is one of the well described so-called hypoxi-miRs [8]. We observed no changes in miR-378 and miR-210 serum levels one week after radical nephrectomy, probably due to pro-longed half-lives of both miR-378 and miR-210. However, in the time period of three months after surgery expression levels of both miRNAs significantly decreased ($p < 0.0001$), which was observed also by Zanutto *et al.* (2014) in colorectal cancer [16]. For the first time, we have described association of elevated serum levels of miR-378 with clinical stage ($p = 0.0476$) and disease-free survival ($p = 0.036$), extending also the results of our previous study [7]. Unfortunately, we were not able to prove circulating miR-378 as an independent prognostic factor in RCC by multivariate analysis.

Our study has several limitations, which should be discussed. There was a study published showing superiority of plasma over serum for circulating miRNAs analysis based on the release of platelets or WBC miRNA contents to the serum during the coagulation process [17]. Our measurement should not be biased from this perspective, because miR-378 and miR-210 were not shown to be associated with platelet or WBCs. Another limitation is definitely absolute quantification approach, which we have used for determination of studied miRNAs disabling to eliminate methodical inaccuracies, which could occur in processing of every sample and, finally, could bias comparisons of different groups of samples. However, we believe that the studied groups of patients and controls are large enough to overcome this bias and, mainly, there is no conclusively-defined reference gene, which should be used for normalization of circulating miRNAs expression.

In conclusion, serum miR-378 and miR-210 (or their combination) could serve as a powerful diagnostic and even prognostic biomarker in management of RCC patients.

4. Methods

4.1. Study Population

Serum samples from RCC patients were collected at the Masaryk Memorial Cancer Institute (MMCI; Brno, Czech Republic) and University Hospital Brno (UHB; Brno, Czech Republic). The study group included patients diagnosed for RCC and undergoing radical nephrectomy at MMCI (n = 195; male: 133, female: 62, median age: 64), cancer-free blood donor volunteers (n = 100; male: 65, female: 35, median age: 52) recruited from Department of Transfusion and Tissue Medicine of UHB, with no history of any type of cancer, and twenty RCC patients from UHB with serum samples additionally collected one week and three months after nephrectomy. Both cancer patients and healthy controls were of the same ethnicity (Caucasian). Clinical and pathological characteristics including stage and grade are summarized in Table 1. RCC serum samples were collected after signing an informed consent prior to surgery and stored at MMCI Bank of Biological Material. The study (MZ09-MOU-SlabyOndrej-B) has been approved by ethical committee of MMCI (3 September 2008).

4.2. RNA Isolation

Blood serum samples were collected just before surgery and initiation of any oncological treatment. Blood was processed for serum within one hour after extraction. Serum was stored in liquid nitrogen and the median of storage time to endpoint analysis was 20 months. Serum was obtained by centrifugation at 1200× g for 10 min at 4 °C. To complete the removal of residual cellular components, serum samples were re-centrifuged at 12,000× g for a further 10 min at 4 °C. Total RNA enriched for small RNAs was isolated using Qiagen miRNeasy Mini Kit (Qiagen, GmbH, Hilden, Germany) from 250 µL of blood serum according to modified manufacturers' protocol (we added 1.25 µL of MS2 RNA (0.8 µg/µL) to QIAzol (Qiagen, GmbH, Hilden, Germany). Concentration and purity of RNA were determined by measuring its optical density (A260/280 > 2.0; A260/230 > 1.8) using NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The samples were either stored at −80 °C or further processed.

4.3. qRT-PCR Quantification of MiRNA Expression in Serum

MiR-378 and miR-210 were quantified by TaqMan MicroRNA Assays (Applied Biosystems, Carlsbad, CA, USA) following reverse transcription (TaqMan MicroRNA Reverse Transcription Kit, Applied Biosystems) of 3 µL of RNA on Applied Biosystems 7500 instrument following the manufacturers' protocols. To reduce the possible high intra-assay variance introduced by low abundant miRNA, a pre-amplification step using TaqMan PreAmp Master Mix (Applied Biosystems) was performed for serum RNA samples prior to miR-210 analysis according to the manufacturer's instructions. Absolute quantification of miRNAs was performed in triplicate.

4.4. Statistics

Statistical analyses were performed using GraphPad Prism version 6 (GraphPad software, La Jolla, CA, USA). Sensitivity, specificity and area under curve (AUC) for miRNA levels were determined using

Receiver Operator Characteristic (ROC) analysis. Clinical-pathological parameters and miRNA levels were correlated using the Mann–Whitney-U or Kruskal–Wallis-test, as appropriate. Kaplan–Meier survival curves and long-rank test were used for survival analysis.

5. Conclusions

There are significantly increased levels of both miR-378 and miR-210 in blood serum of RCC patients when compared to age and gender-matched healthy donors. These miRNAs enable to clearly distinguish RCC patients and healthy controls with 80% sensitivity and 78% specificity if analyzed in combination, and their levels significantly decrease in the time period of three months after radical nephrectomy. Moreover, increased levels of miR-378 correlates with disease-free survival and clinical stage in RCC patients. In conclusion, analysis of miR-378 and miR-210 serum levels proved their potential to serve as powerful non-invasive diagnostic and prognostic biomarkers in RCC.

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Author Contributions

Michal Fedorko, Michal Stanik and Ondrej Slaby carried out the analysis and interpretation of data, and drafted the original manuscript; Dalibor Pacik and Jan Dolezel assisted in the organization of the survey and collection of the data; Robert Iliev and Tana Machackova assisted in the detailed methods of this study; Marek Svoboda and Martina Redova-Lojova reviewed the statistical analysis and revised the manuscript; and Michal Fedorko, Michal Stanik and Ondrej Slaby conceived the study. All authors approved the final version of the article, including the authorship list.

Conflicts of Interest

The authors declare no conflict of interest.

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The results of the work were presented by the applicant as a commented poster at the 30th Annual Conference of the European Urological Association (EAU) in Madrid (March 20-24, 2015) and published as a conference abstract in European Urology Supplements (IF 3,370).

For details see: **Fedorko M,** Staník M, Iliev R, Mlčochová H, Macháčková T, Pacík D, Doležel J, Slabý O. Circulating miRNA-378 and miRNA-210 in patients with renal cell carcinoma and their levels after surgical removal of the tumour. Eur Urol Suppl 2015; 14(2): e861. https://doi.org/10.1016/S1569-9056(15)60849-1

Diagnostic miRNAs in urine

A great advantage for the use of miRNAs in urine as biomarkers is their stability under different conditions. So far, however, data on their use in the diagnosis of RCC are insufficient. Recent work describes miR-30c-5p as a potential biomarker of early stage RCC. Its increased expression inhibits the growth of tumour cells by depleting the target heat-shock protein 5 (33). MiR-15a expression is significantly increased in the urine of RCC patients compared to healthy subjects, with no difference between ccRCC, papillary RCC, and chromophobic RCC. On the eighth day after nephrectomy, a 99.53% reduction in miR-15a expression was noted (34). The expression of miR-210, which was mentioned as a potential serum biomarker, was significantly increased in the urine of patients with ccRCC in one study. Sensitivity and specificity for differentiation of patients and healthy subjects were 57.8%, resp. 80%, AUC 0.76 (35).

Published work related to the topic:

Fedorko M, Juracek J, Stanik M, Svoboda M, Poprach A, Buchler T, Pacik D, Dolezel J, Slaby O. Detection of let-7 miRNAs in urine supernatant as potential diagnostic approach in non-metastatic clear-cell renal cell carcinoma. Biochem Med 2017; 27(2): 411-417.
IF 3,051

This original work can be considered as one of the pilot studies on the use of urinary miRNAs as a tool for RCC detection and the first study demonstrating the diagnostic potential of let-7a, which was the best of the six miRNAs studied to distinguish ccRCC patients from healthy controls.

Number of times cited (WoS, as of February 17th 2022): 26.

Short communication

Detection of let-7 miRNAs in urine supernatant as potential diagnostic approach in non-metastatic clear-cell renal cell carcinoma

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Abstract

Introduction: Urinary microRNAs (miRNAs) are emerging as a clinically useful tool for early and non-invasive detection of various types of cancer. The aim of this study was to evaluate whether let-7 family miRNAs differ in their urinary concentrations between renal cell carcinoma (RCC) cases and healthy controls.

Materials and methods: In the case-control study, 69 non-metastatic clear-cell RCC patients and 36 gender/age-matched healthy controls were prospectively enrolled. Total RNA was purified from cell-free supernatant of the 105 first morning urine specimens. Let-7 family miRNAs were determined in cell-free supernatant using quantitative miRNA real-time reverse-transcription PCR and absolute quantification approach.

Results: Concentrations of all let-7 miRNAs (let-7a, let-7b, let-7c, let-7d, let-7e and let-7g) were significantly higher in urine samples obtained from RCC patients compared to healthy controls ($P < 0.001$; $P < 0.001$; $P = 0.005$; $P = 0.006$; $P = 0.015$ and $P = 0.002$, respectively). Subsequent ROC analysis has shown that let-7a concentration possesses good ability to differentiate between cases and controls with area under curve being 0.8307 (sensitivity 71%, specificity 81%).

Conclusions: We have shown that let-7 miRNAs are abundant in the urine samples of patients with clear-cell RCC, and out of six let-7 family members, let-7a outperforms the others and presents promising non-invasive biomarker for the detection of RCC.

Key words: renal cell carcinoma; urine microRNAs; let-7; diagnostic biomarker

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Introduction

Renal cell carcinoma (RCC) accounts for 2–3% of all malignant tumours. There are several histological subtypes of RCC, with clear-cell (or conventional) histological type being the most frequent, presenting 70% – 80% of RCC cases (1). Despite a steady decrease in mortality rates, RCC remains one of the most lethal urological malignancies, with 5-year relative survival 72% (1). For advanced and metastatic RCCs (32% of all diagnosed cases), 5-year relative survival descends to 66% and 12%, respectively. Biomarkers for early detection of RCC are therefore necessary as there is no reliable diagnostic modality other than radiological imaging.

MicroRNAs (miRNAs) are short noncoding RNAs that regulate gene expression at the posttranscriptional level. They are involved in the number of critical biological processes including carcinogenesis. Besides tumour tissues, they are also present in different body fluids (*e.g.* serum, plasma, urine) with a high degree of stability indicating their extensive biomarker potential (2). Although variety of circulating miRNAs has been proposed as bio-

markers of RCC, urinary miRNAs have been mostly studied in bladder and prostate cancer (3).

The miRNA let-7 family is widely accepted as a tumour suppressor miRNA with important role in the regulation of cell cycle, cell differentiation and apoptosis (4). Downregulation of the members of let-7 family has been observed in various types of tumour tissue including RCC (5). Less frequent, upregulation of certain let-7 family members has also been observed, suggesting that let-7 does not play a tumour suppressor function under all circumstances and in all tissues (6). Higher levels of let-7 miRNAs in urine has been reported in bladder cancer (7). There are no data about urinary let-7 miRNAs in RCC, but increased urinary levels of some of the let-7 family members were found in patients with autosomal dominant polycystic kidney disease suggesting their abundance in urine and therefore also potential utility in other renal diseases such as cancer (8).

Based on that, we hypothesize, that let-7 family miRNAs differ in their concentrations in urine samples of RCC patients and heathy individuals, and could be potentially useful as diagnostic biomarkers of RCC. To this end, the aim of our study was to determine concentration of let-7 miRNAs in cell-free supernatant in group of prospectively enrolled patients with non-metastatic clear cell RCC and group of healthy controls, statistically evaluate the differences in concentrations between the groups and their ability to distinguish between RCC cases and healthy controls.

Material and methods

Study design and subjects

Between May 2015 and December 2016, adults undergoing partial or radical nephrectomy for RCC at Department of Urology, University Hospital Brno (UHB) were prospectively screened for participation in this observational case-control study. Inclusion criteria included: histologically proven clear-cell RCC, no distant metastasis or nodal involvement. Exclusion criteria included: active malignancy other than RCC, history of any malignancy, urinary tract infection, foreign bodies in urinary tract and urolithiasis. Urine samples of the cases were collected prior to surgically treatment. In the same time period participants of the control group were enrolled. Healthy controls included patients surgically treated at UHB for benign urological conditions like urethral stricture, phimosis, undescended testicle, stress urinary incontinence, hydrocele, benign prostatic hyperplasia, urethral caruncula, vesical neck sclerosis, simple renal cyst. Patients with active malignancy or history of any cancer, urinary tract infection, and foreign bodies in urinary tract or urolithiasis were excluded from control group. Urine samples of the control group were collected during regular post-operative follow-up visits. Study was approved by the Ethic committee at UHB and all participants signed informed consent before entering the study.

Out of 76 RCC patients approached, 2 declined to participate in the study, 15 patients were not included due to papillary or chromophobe histological type of RCC proved after surgery. Since we were not focused on the follow-up of patients, there was no additional drop-out from our study. All 36 healthy controls approached, agreed to participate on our study. Finally, 69 patients diagnosed with non-metastatic clear-cell RCC and 36 healthy controls were enrolled. Epidemiological and clinical characteristics of the cases and controls groups are summarized in Table 1.

Urine sampling and RNA isolation

The first morning urine samples were collected in 15 mL tubes (Sarstedt AG & Co., Numbrecht, Germany) with EDTA used for nucleic acid preservation and kept at 4 °C till further processing. As the next step, urine samples were centrifuged at 4 °C at 2000g for 15 minutes, and the cell-free supernatant was then collected and stored at – 80 °C until analysis. Before RNA isolation another centrifugation of urine sample was performed at 4 °C at 12,000g for 15 minutes. Total RNA from 1 mL of cell-free supernatant was isolated using manual column-based method, Urine microRNA Purification Kit (Norgen Biotek, Thorold, Ontario, Canada) according to the manufacturer's instructions. RNA concentration and purity was evaluated using Na-

Table 1. Epidemiological and clinical characteristics of study subjects.

	ccRCC patients	Healthy controls
	N = 69	N = 36
Male (N, proportion)	50 (0.72)	24 (0.67)
Age (years)	66 (33-87)	65 (40-79)
pT stage*		
pT1	54	NA
pT2	4	NA
pT3	11	NA
pT4	0	NA
pN stage*		
pN0	69	NA
pN1	0	NA
pM stage*		
pM0	69	NA
pM1	0	NA
Fuhrman grade†		
G1	11	NA
G2	40	NA
G3	13	NA
G4	5	NA

*pT,N,M stages accordingly to American Joint Committee on Cancer Staging Manual. †The grading scheme used in RCC. RCC - renal cell carcinoma, ccRCC - clear-cell renal cell carcinoma, NA - not applicable.

nodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Only the samples with concentration higher than 5 ng/µL and A260/A280 higher than 1.3 were further analysed in the study.

Quantitative miRNA real-time reverse-transcription PCR

Concentrations of let-7a, let-7b, let-7c, let-7d, let-7e, and let-7g were determined by quantitative miRNA real-time reverse-transcription PCR (qRT-PCR) accordingly to TaqMan MicroRNA assay protocol (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA was synthesized from 10 ng of total RNA in 15-µL reverse transcription (RT) reaction using microRNA-specific stem-loop RT primer and the TaqMan® MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) accordingly to manufacturer's recommendations. Real-time PCR was performed in 20-µL PCR reaction with 1.33 µL of RT product using specific TaqMan® MicroRNA assays (let-7a: ID000377, let-7b: ID002619, let-7c: ID000379, let-7d: ID002283, let-7e: ID002406, let-7g: ID002282; Thermo Fisher Scientific) on Roche LightCycler 480 PCR system (Roche, Basel, Switzerland) accordingly to manufacturer's recommendations. The reactions were carried out in a 96-well optical plate at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. All reactions were run in duplicates. After the reaction, the treshold cycle (Ct) values were determined using the fixed threshold settings, and the mean Ct values were calculated from duplicates. For each miRNA assay, a dilution series of synthetic miRNA oligo (IDT, Coralville, Iowa, USA) were carried out in parallel with qRT-PCR of biological samples to generate an absolute standard curve for quantification of let-7 concentrations. We also included inter-plate calibrator on each plate for each assay enabling us to correct for inter-plate variability. Quantitatively all measurements were standardized by use of the same amount of total RNA (10 ng) entering the reverse transcription and PCR reaction. Ct values of biological samples were converted to absolute concentration of miRNAs in the cell-free supernatant of the urine (fmol/L) based on relevant calibration curve equation (Figure 1) based on the recently described approach (9).

Statistical analysis

Statistical analysis was performed with GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). To compare urinary concentrations of miRNAs between RCC cases and healthy controls non-parametric Mann-Whitney U test was used since our experimental data do not follow a normal distribution. A P-value < 0.01 was considered statistically significant. The ROC analysis was performed to evaluate the ability of studied miRNAs to distinguish between urine of RCC patients and healthy controls.

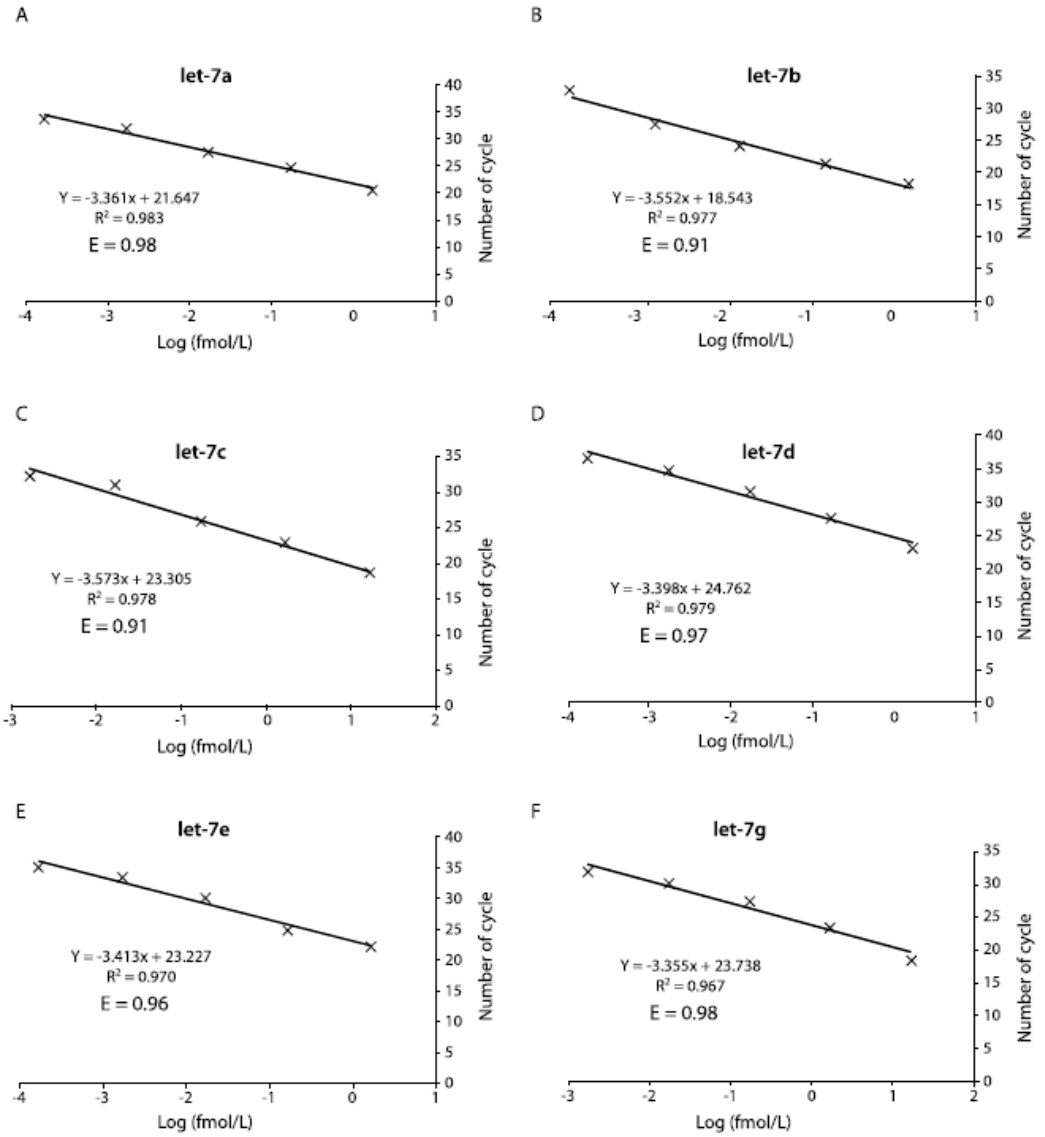

Figure 1. Absolute standard curves for let-7a (A), let-7b (B), let-7c (C), let-7d (D), let-7e (E) and let-7g (F) used for calculation of let-7 microRNAs concentrations (fmol/L) in the urine samples. E – qPCR reaction efficiency.

Results

We successfully purified RNA from urine samples of 105 subjects enrolled into our study. The concentration of RNA ranged from 5.1 to 17.3 with a median of 6.7 ng/μL and the purity (A260/A280) ranged from 1.3 to 2.0 with a median of 1.5.

The concentrations of all 6 miRNAs (let-7a, let-7b, let-7c, let-7d, let-7e, and let-7g) were significantly higher in urine samples obtained from RCC cases compared to healthy controls ($P < 0.001$; $P < 0.001$; $P = 0.005$; $P = 0.006$; $P = 0.015$ and $P = 0.002$, respectively; Table 2). Subsequent ROC analysis was performed to evaluate ability of urinary miRNAs to distinguish between RCC cases and controls. ROC curves indicated that urine concentration of let-7a possess satisfactory ability to differentiate between patients and controls with the AUC being 0.8307 (Figure 2A,B). The remaining let-7 miRNAs showed inferior analytical performance (AUC < 0.75; summarized in Table 2). We further evaluated analytical performance of combination of all let-7 miRNAs with AUC being 0.83.

Discussion

Noninvasive biomarker of RCC in urine presents a significant unmet medical need of urologic oncology. To prove our hypothesis that let-7 miRNAs concentrations in urine differ between RCC cases and healthy controls, we used the case-control design performed prospectively enabling us to control pre-analytical conditions, sample handling and processing. Common approaches to miRNA clinical testing include small RNA sequencing, qRT-PCR, miRNA microarray, multiplexed miRNA detection with color-coded probe pairs, and miRNA *in situ* hybridization. We decided to use qRT-PCR, since our approach is targeted and this method has several advantages in comparison to others:

Table 2. MiRNA concentrations in urine of RCC patients and healthy controls.

miRNA	RCC patients	Healthy controls	P-value	AUC	Sens. (%)	Spec. (%)
let-7a, fmol/L	7.510 (2.668–14.250)	1.525 (0.673–3.368)	< 0.001	0.83	71	81
let-7b, fmol/L	3.500 (1.835–6.160)	1.350 (0.1400–2.880)	< 0.001	0.75	73	67
let-7c, fmol/L	9.390 (4.090–18.82)	5.115 (2.180–9.253)	0.005	0.67	65	62
let-7d, fmol/L	5.540 (2.450–11.88)	3.505 (1.705–5.145)	0.006	0.66	66	61
let-7e, fmol/L	58.9 (25.03–106.6)	32.35 (15.33–63.60)	0.015	0.65	62	61
let-7g, fmol/L	22.48 (11.46–35.19)	12.89 (5.72–19.64)	0.002	0.69	70	60

Values of MiRNA concentrations are presented as median (interquartile range). AUC - Area under curve; Sens. – Sensitivity; Spec. – Specificity.

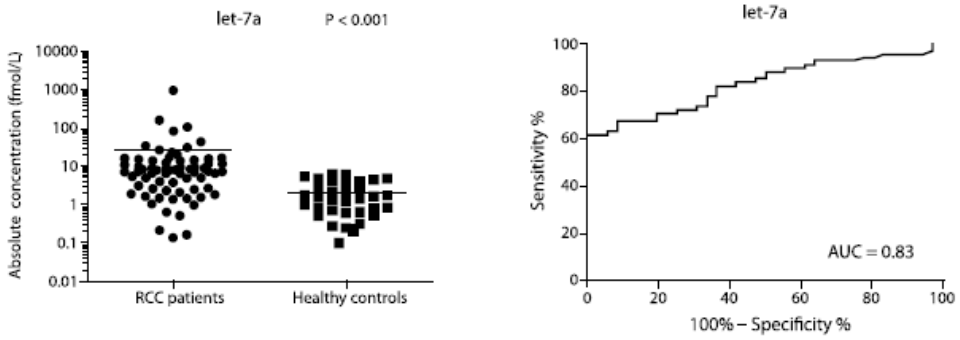

Figure 2. Differences of let-7a concentration between RCC patients and controls.
(A) Absolute concentrations of let-7a was determined by qRT-PCR in urine of RCC patients (N = 69) and healthy individuals (N = 36).
(B) ROC analysis of let-7a to evaluate the ability to distinguish RCC patients and healthy controls. AUC – area under curve.

high dynamic range, high sensitivity and specificity, small requests on RNA input, it is widely used in clinical diagnostics and comparatively inexpensive.

In our study, we confirmed our hypothesis, and found that urinary concentrations of let-7 miRNAs in RCC patients are significantly higher compared to healthy controls. Let-7a concentrations enabled to discriminate urine of the RCC patients and controls with a sensitivity of 71% and specificity of 81%, suggesting its diagnostic value for detection of RCC. We further evaluated analytical performance of combination of all let-7 miRNAs and there was no notable increase in AUC values observed in comparison to let-7a used as the only biomarker.

In contrast to bladder or prostate cancer, data about urinary miRNAs in RCC are sparse. In the pilot study of von Brandenstein *et al.* (23 RCC patients, 5 controls), higher levels of miR-15a were found in urine of RCC patients but was undetectable in oncocytoma, other tumours or urinary infection (10). In the recent study of Guorong *et al.*, urinary levels of miR-210 were found to be significantly higher in patients with clear-cell RCC (N = 75) compared to healthy controls (N = 45), with sensitivity, specificity and the area under ROC curve 57.8%, 80% and 0.76, respectively. In addition, the expression levels of urinary miR-210 significantly decreased one week after surgery (11). Based on our results, urinary let-7a indicates superior analytical performance to urinary miR-210 studied by Guorong *et al.* (AUC 0.83 vs. 0.76). Although analytical characteristics of urinary let-7a seem to be promising, there are not sufficient for clinical application of let-7a as the only biomarker. However, we believe, that urinary let-7a could add significant diagnostic value if combined with other emerging biomarkers in RCC or for monitoring of the RCC patients with initially increased levels of this biomarker. Analogically to other biomarkers in various cancers (*e.g.* carcinoembryonic antigen in colorectal cancer), we suppose, that in RCC, secretion of the let-7a presents biological feature of the subset and not all RCC cases.

Our study has several limitations, which should be discussed. The main limitation is the small group of RCC cases and controls and absence of the independent validation set. To this end, our study is a pilot study showing diagnostic potential of urinary let-7a concentrations in detection of RCC, but further independent studies are needed to confirm our results. Another limitation is absolute quantification approach, which we used for determination of studied urinary miRNAs disabling to eliminate methodical inaccuracies, which could occur in processing of every sample and, finally, could bias comparisons of different groups of samples. There were some transcripts used for normalization of urinary miRNAs (*e.g.* RNU6B or RNU48) (3), but to our knowledge there is no consensual reference gene. Therefore, we decided to use absolute quantification and to overcome this potential technological variability or bias, we implemented standardized protocols for urine samples collection, handling and storage.

In conclusion, we showed that let-7 miRNA family members are abundant in the urine cell-free supernatant of patients with clear-cell RCC, and confirmed our hypothesis, that let-7 miRNAs have different concentrations in the urine of RCC cases and healthy controls. Out of six let-7 members analysed, let-7a outperforms the others and may be considered as a promising noninvasive biomarker for the detection of clear-cell RCC.

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Potential conflict of interests

None declared.

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MiRNAs as prognostic biomarkers of RCC

Sufficient evidence can be found in the literature that aberrant miRNA expression affects the survival of patients with RCC, so the use of these miRNAs as biomarkers to identify patients at risk of disease relapse or progression can be considered. Most of the work concerns the determination of miRNA expression in tumour tissue. The works of Czech authors have a significant representation here. One of the pilot studies in this area was the study of miR-106b, the expression of which is significantly lower in patients who have metastasized and may therefore be a biomarker of early RCC metastasis after nephrectomy (36). Another of the Czech papers describes the reduced expression of miR-143, miR-26a, miR-145, miR-10b, miR-195 and miR-126 in patients with RCC relapse and primary metastatic tumour. In addition, it demonstrated a significant correlation with relapse-free survival for miR-127-3p and miR-126 (37). MiRNAs with increased expression and at the same time worse prognosis of RCC patients also include miR-21, miR-1260b, miR-210, miR-100, miR-125b, miR-221, miR-630, and miR-497, while lower expression and worse prognosis is described for miR-99a, miR-1826, miR-215, miR-217, miR-187, miR-129-3p, miR-23b and miR-27b (38). The role of decreased miR-126 expression as a biomarker of early relapse of ccRCC has been confirmed in recent research (39). At the time of targeted biologic therapy, the potential role of miRNAs as biomarkers for estimating therapeutic response in metastatic tumours appears. In patients treated with sunitinib, there was a significantly longer time to progression in patients with reduced expression of miR-155 and miR-484 in tumour tissue (40). Down-regulation of miR-141 is also associated with a poor response to sunitinib treatment (41). Sunitinib-resistant patients show higher expression of miR-942, miR-628-5p, miR-133a and miR-484 (42).

Published work related to the topic:

Machackova T, Mlcochova H, Stanik M, Dolezel J, Fedorko M, Pacik D, Poprach A, Svoboda M, Slaby O. MiR-429 in linked to metastasis and poor prognosis in renal cell carcinoma by affecting epithelial-mesenchymal transition. Tumour Biol 2016; 37(11): 14653-14658.

IF 2,926

This work demonstrated significantly reduced expression of tumour suppressor miR-429 in tumour tissue and ccRCC metastases with an effect on disease-free survival and overall patient survival. Transfection of miR-429 into TGF-β-treated tumour cell lines inhibited the loss of E-cadherin (caused by EMT) and thus reduced the migration capacity of tumour cells.

Number of times cited (WoS, as of February 17th 2022): 33.

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ORIGINAL ARTICLE

MiR-429 is linked to metastasis and poor prognosis in renal cell carcinoma by affecting epithelial-mesenchymal transition

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Abstract MicroRNAs (miRNAs) have been proven to be important oncogenes and tumor suppressors in wide range of cancers, including renal cell carcinoma (RCC). In our study, we evaluated miRNA-429 as potential diagnostic/prognostic biomarker in 172 clear cell RCC patients and as a potential regulator of epithelial-mesenchymal transition (EMT) in vitro. We demonstrated that miR-429 is down-regulated in tumor tissue samples ($P < 0.0001$) and is significantly associated with cancer metastasis ($P < 0.0001$), shorter disease-free ($P = 0.0105$), and overall survival ($P = 0.0020$). In addition, ectopic expression of miR-429 in 786-0 RCC cells followed by TGF-β treatment led to increase in the levels of E-cadherin expression ($P < 0.0001$) and suppression of cellular migration ($P < 0.0001$) in comparison to TGF-β-treated controls. Taken together, our findings suggest that miR-429 may serve as promising diagnostic and prognostic biomarker in RCC patients. We further suggest that miR-429 has a capacity to inhibit loss of E-cadherin in RCC cells undergoing EMT and consequently attenuate their motility.

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Keywords Renal cell carcinoma · Epithelial-mesenchymal transition · microRNA · miR-429 · E-cadherin

Introduction

Renal cell carcinoma (RCC) comprises of various cancer subtypes characterized by different genetic drivers, histological patterns, and clinical outcome resulting in different responses to the therapy [1]. The most common subtype of RCC is clear cell renal cell carcinoma (ccRCC) accounting for approximately 70 % of cases [2]. Due to the lack of sensitive diagnostic markers, high percentage of RCC patients is still diagnosed with metastatic disease [3]. Five years overall survival is reached by 55 % of patients, whereas in metastatic RCC, this percentage decreases rapidly to 10 %. In recent years, short non-coding RNA molecules called microRNAs (miRNAs) have emerged as critical modulators of broad spectrum of cellular biological processes through post-transcriptional regulation of mRNA expression levels mainly by binding to 3′ end of the untranslated mRNA region [4–6]. MiRNAs were also proven to be involved in the epithelial-mesenchymal transition (EMT), the process primarily responsible for metastatic development [7–9]. Members of the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, miR-429) and miR-205 are among the first described miRNAs participating in the EMT process [10]. Inactivation of these tumor suppressor molecules is considered to be the EMT's initial step. Loss of miR-200 family members causes up-regulation of expression levels of EMT inducers, ZEB1 and ZEB2, which further regulate expression of EMT-associated genes, such as E-cadherin through double negative feedback loop [11, 12]. Under physiological conditions, E-cadherin plays an important role in adherent junctions between epithelial cells and suppresses tumor cell invasion and metastasis. One of the most prominent features of the EMT process is

loss of E-cadherin [13, 14]. Tumor cells lose contact with each other and undergo multiple molecular changes, which can lead to tumor progression and finally development of metastatic disease. In the present study, we sought to determine the expression profiles of miR-429 in ccRCC tumor tissue, adjacent renal parenchyma, and metastases; its association with ccRCC clinicopathological features; and also the role of miR-429 in tumor cell migration and EMT in renal cell carcinoma lines in vitro.

Methods

Patient samples

One hundred eighty-seven patients diagnosed with ccRCC and surgically treated at the Masaryk Memorial Cancer Institute in Brno (Department of Urologic Oncology) and University Hospital Brno (Department of Urology), Czech Republic, were included in this study. In addition to primary tumor tissue, 45 samples which correspond to non-malignant kidney cortex were obtained during surgery from the same kidney as corresponding tumor tissue and 12 tissue samples were taken from ccRCC metastasis. All samples were frozen immediately after surgical resection in liquid nitrogen and further stored at −80 °C until RNA extraction. Patients did not receive any neo-adjuvant treatment before surgery. All ccRCC patients were of Czech origin and clinically and histologically verified as clear cell type carcinoma. Clinical stages were determined according to the 2011 Union for International Cancer Control TNM classification. All patients included in the study signed informed consent forms and the study was approved by the local Ethical Board at the Masaryk Memorial Cancer Institute and University Hospital Brno. Patient clinical characteristics are summarized in Table 1.

Table 1 Patients characteristics

Characteristics	Number of patients
Sample type	
Tumor tissue sample	187
Renal parenchyma	45
Metastasis	12
Gender	
Male	126
Female	61
Age	
Median (range)	65 (31–86)
TNM stage	
I	96
II	18
III	27
IV	46
Fuhrman grade	
1	37
2	81
3	52
4	17
Disease-free survival	
Without relapse	53 (3–121) months
Developed relapse	14 (2–60) months
Overall survival	
Alive patients	53 (3–126) months
Dead patients	15 (1–89) months

RNA isolation, reverse transcription, and real-time PCR

Total RNA was isolated from frozen tumor tissue samples, adjacent renal parenchyma, and metastases using mirVanaTM miRNA isolation kit (Ambion, TX, USA) and from cell line samples using TRIzol (Life Technologies, CA, USA). The RNA concentrations and purity were measured spectrophotometrically at 260/280 nm using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, DE, USA). Total RNA was reverse-transcribed using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and High-Capacity Reverse Transcription Kit (Applied Biosystems). The qPCR was performed using TaqMan Universal Master Mix II, no UNG (Applied Biosystems) and TaqMan Gene Expression MasterMix (Applied Biosystems) and QuantStudio 12 K Flex Real-Time PCR System according to manufacturer's recommendations. The quantification cycle (Cq) data were calculated using the default threshold settings set up at level 0.2. All miRNA and gene expression values were calculated according to the following formula: 2^{-dCt}, and normalized to RNU48 for miRNA analysis and to PPIA for E-cadherin expression analysis. All samples were run in duplicates.

Cell culture and in vitro studies

Renal cell carcinoma cell lines ACHN and 786-0 were obtained from ATCC (CA, USA) and maintained in recommended media supplemented with 10 % fetal bovine serum, 2 mM glutamax, 100 U/ml penicillin G, and 0.1 μg/ml streptomycin. For transfection experiments, Lipofectamine® RNAiMAX reagent (Invitrogen, CA, USA) and 33.3 nM pre-miR-429/negative control #1 (Ambion) were used according to manufacturer's protocol. To induce EMT, cells were treated with 10 ng/ml of recombinant human transforming growth factor-β (TGF-β) (Applied Biosystems). To study the role of miR-429 in EMT process in vitro, RCC cell lines were plated in 24-well plates 24 h prior to transfection. EMT was induced by TGF-β 1 day after transfection in ACHN (25×10^4 cells/well) and 786-0 (20×10^4 cells/well) cells. To study

expression profiles, cells were harvested with Qiazol (Qiagen, Germany) for total RNA isolation 4 days after treatment and levels of miR-429 and E-cadherin were evaluated by RT-qPCR. Values are presented as means of three independent experiments. For scratch assay experiments, cells were transfected with pre-miR-429/negative control #1 (MOCK) and treated with 10 ng/ml of TGF-β 1 day after seeding. Using TS scratch software, differences in cell-free area was evaluated 24 h (ACHN) and 12 h (786-0) after scratch was made.

Data analysis

Differences between subgroups were tested by non-parametric Mann–Whitney *U* test. Differences between in vitro experiments were evaluated by *t* test. ROC analysis was performed to identify the optimal miR-429 cutoff value enabling discrimination of patients accordingly to their DFS and OS. Survival analyses were calculated by Kaplan–Meier method using log-rank test. *P* values less than 0.05 were considered to be statistically significant.

Results

We determined expression levels of miR-429 in 172 tissue samples of ccRCC primary tumors, 45 renal parenchyma tissues, and 12 metastases. We observed significantly lower levels of miR-429 in tumor tissue and metastasis compared to renal parenchyma ($P < 0.0001$) (Fig. 1a, b). Lower expression of miR-429 was identified also in metastases when compared to primary tumor tissue ($P = 0.0391$). MiR-429 expression levels negatively correlated with TNM stage (stage I + II versus stage III + IV; $P < 0.0001$) (Fig. 1b) and Fuhrman grade (grade 1 + 2, grade 3 + 4; $P < 0.0001$) in ccRCC. Further, we evaluated the association of miR-429 expression and disease-free survival (DFS) and overall survival (OS) in ccRCC patients. Patients with higher expression levels of miR-429 showed significantly longer DFS after radical nephrectomy than patients with lower expression levels of miR-429 ($P = 0.0105$) (Fig. 1c). Survival analysis also showed that lower levels of miR-429 are associated with shorter OS of ccRCC patients ($P = 0.0020$) (Fig. 1d).

To determine the role of miR-429 in EMT in vitro, E-cadherin expression together with cell migratory capacity was studied in ACHN and 786-0 RCC cell lines by the use of TGF-β treatment to induce EMT and transfection of pre-miR-429 to increase the levels of miR-429. Expression levels of E-cadherin were measured 4 days after transfection and/or treatment. We observed a decrease in E-cadherin levels after TGF-β treatment, which was more prominent in 786-0 cell line (Fig. 2a, b). There was no effect of TGF-β on RCC cells transfected with pre-miR-429 prior to TGF-β treatment indicating

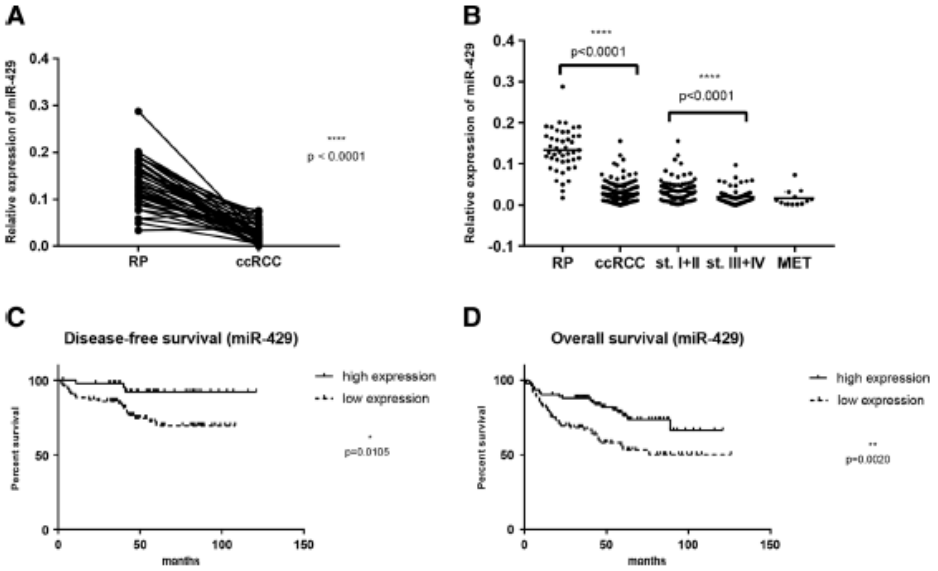

Fig. 1 Analysis of miR-429 expression in tumor and renal parenchyma tissue samples. MiR-429 **a** Normalized expression levels of miR-429 in paired tumor tissue and renal parenchyma samples. **b** Comparison of miR-429 expression in renal parenchyma, tumor tissue of the whole cohort, tumor tissue of patients with localized disease (stage I + II), metastatic disease (stage III + IV) and metastasis. Association of miR-429 expression levels with disease-free survival (**c**) and overall survival (**d**)

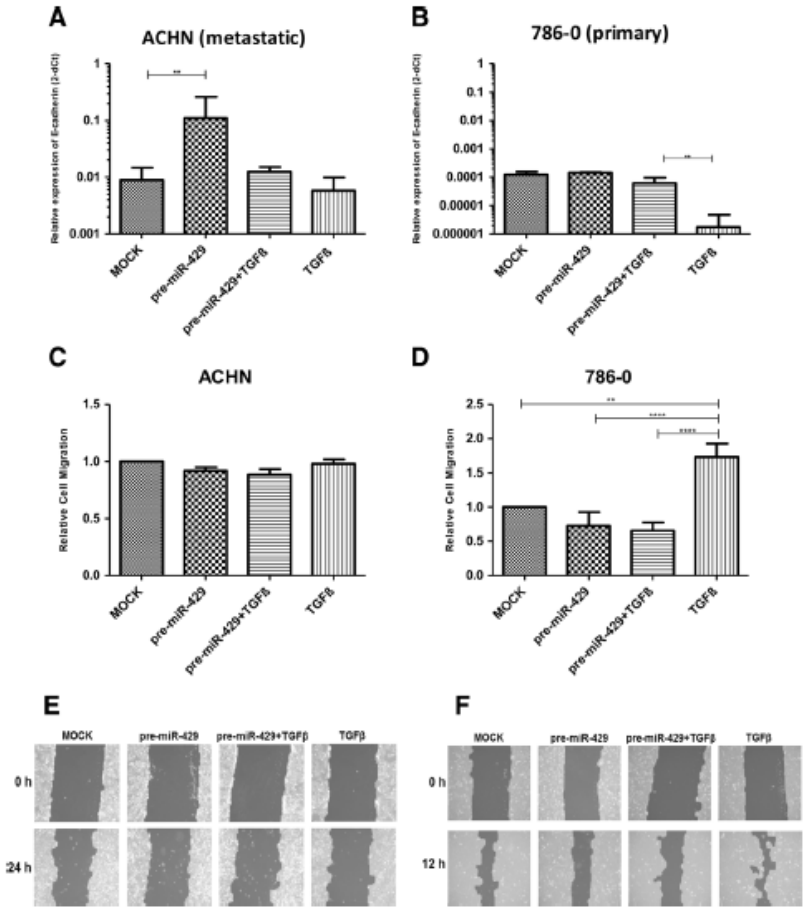

Fig. 2 MiR-429 involvement in the regulation of E-cadherin and cell motility. Effects of ectopic expression of miR-429 on the levels of E-cadherin in TGF-β treated ACHN (**a**) and 786–0 (**b**) RCC cell lines. Scratch migration assay for ACHN (**c**, **e**) and 786-O (**d**, **f**) was conducted 12 h/24 h after transfection with pre-miR-429. Images of RCC cells are presented as migration under three different conditions: negative control (MOCK), TGF-β treatment, pre-miR-429 + TGF-β treatment (magnification ×40). Data are presented as the mean of three experiments and the *bars* present the standard deviation (SD) of the mean. ** $P < 0.01$, **** $P < 0.0001$

capacity of miR-429 to inhibit E-cadherin loss induced by EMT. There was no effect of TGF-β treatment on E-cadherin expression levels in ACHN cells. We observed significant increase of E-cadherin levels in control ACHN cells after transfection with pre-miR-429 ($P < 0.01$); however, this effect was lost after TGF-β treatment (Fig. 2a). Further, migratory capacity of RCC cells was assessed by scratch wound assay. One day after transfection and treatment, the scratch wound assay was performed and after 12 h (786-0) and 24 h (ACHN), relative migration was evaluated. 786-0 cell line showed significantly increased relative cell migration after treatment with TGF-β in comparison to control cells ($P = 0.0011$). This potentiation of cell migration induced by TGF-β treatment in 786-0 was not observed in cells transfected with pre-miR-429 prior to treatment ($P < 0.0001$) (Fig. 2d, f). In ACHN cell line, we did not show any differences in relative cell migration regardless of transfection and/or treatment (Fig. 2c, e). These results indicate the ability of miR-429 to inhibit effects of TGF-β on cell migration in the 786-0 cell line.

Discussion

Approximately 40 % of all RCC patients with localized disease develops relapse of the disease after radical surgical removal of the tumor. This high rate of recurrence for clinically localized disease after nephrectomy underscores the importance of post-surgical surveillance [15, 16]. In recent years, miRNAs have been described as important regulators of EMT playing roles in metastatic development of RCC, which can also serve as potential biomarkers or therapeutic targets in RCC [17].

In our study, we observed significant decrease in miR-429 expression levels in primary tumors and metastasis of ccRCC patients. In addition, down-regulation of miR-429 was

associated with shorter DFS and OS. Our results are in accordance with the number of studies describing down-regulation of miR-429 in tumor tissue of renal cell carcinoma [18] and wide range of other cancers: colorectal carcinoma [19], hepatocellular carcinoma [20], cervical carcinoma [21], nasopharyngeal carcinoma [22], and in non-small lung carcinoma (NSCLC) [23]. Lower serum levels of miR-429 were also associated with poor overall survival of NSCLC patients suggesting its prognostic value in NSCLC [23]. In another study, elevation of miR-429 serum levels in NSCLC patients compared to controls was reported [24].

Recently, miR-429 was described as a potential regulator of E-cadherin restoration during EMT in bladder cancer [25]; however, its effects in EMT in ccRCC are not fully understood. Based on our results, we suggest that miR-429 has a capacity to inhibit loss of E-cadherin expression induced by TGF-β treatment in RCC cells followed by decreased cell migration capacity. Using scratch wound assay, an ability of miR-429 to reverse effects of TGF-β on cell migration in 786-0 cell line derived from non-metastatic RCC was proved.

ACHN is a metastatic RCC cell line, which already underwent EMT, therefore TGF-β treatment had no effect on their phenotype and also transient over-expression of miR-429 in these cells followed with TGF-β treatment does not result in any changes of their migration capacity. Effects of miR-429 and other members of miR-200 family on regulation of E-cadherin expression level during TGF-β-induced EMT were studied in several studies with various results in regard to the cell line used [26, 27]. For instance, down-regulation of E-cadherin was previously reported in ACHN cells after TGF-β treatment, but no changes were observed in cell migration [28]. Contrary to these results, Caki-2 cell line does not prove any E-cadherin expression before/after TGF-β treatment, but TGF-β treatment was followed by significantly higher motility of cells compared to controls. In addition, these changes were followed by significant cell shape alternation after TGF-β treatment [28]. In agreement with our observations, elevated invasiveness and metastatic ability of human 786-0 renal carcinoma cell line after TGF-β treatment were previously studied by Huang et al. [29]. It was suggested that TGF-β induces the expression of Fascin1 and thus improves metastatic potential in 786-0. These observations were reported also in the gastric cancer cells MKN45 [30].

In conclusion, we found out that miR-429 is significantly down-regulated in tumor and metastatic tissue of ccRCC patients compared to adjacent renal parenchyma. In addition, decreased levels of miR-429 were linked to shorter DFS and OS. In vitro experiments showed ability of miR-429 to suppress the cell motility through inhibition of E-cadherin loss induced by TGF-β treatment in non-metastatic 786-0 carcinoma cell line. Our data suggest that miR-429 acts as a tumor suppressor with role in metastatic development, especially through modulation of E-cadherin expression.

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Compliance with ethical standards All patients included in the study signed informed consent forms and the study was approved by the local Ethical Board at the Masaryk Memorial Cancer Institute and University Hospital Brno.

Conflicts of interest None.

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6.1.2. PIWI-interacting RNAs

As already mentioned in the miRNA characteristics, interaction at the RNA level, i.e. so-called RNA silencing, is one of the key regulatory processes within eukaryotic organisms. PiRNAs (PIWI-interacting RNAs) are short RNAs (24-32 nucleotides) that form RNA-induced quenching complexes with PIWI proteins, belonging to the family of so-called Argonaut proteins. PIWI proteins (P-element-induced wimpy testes) are expressed mainly in germ cells in the gonads and pi-RNA regulation is important for maintaining normal gametogenesis and reproduction (43). In addition to germ cells, piRNAs also perform regulatory functions in somatic cells (chromatin modification, attenuation of transposons to maintain genomic stability). In addition, however, they play an important role in the pathogenesis of various solid tumours by influencing apoptosis, proliferation, gene stability, invasion and metastasis of tumour cells (44).

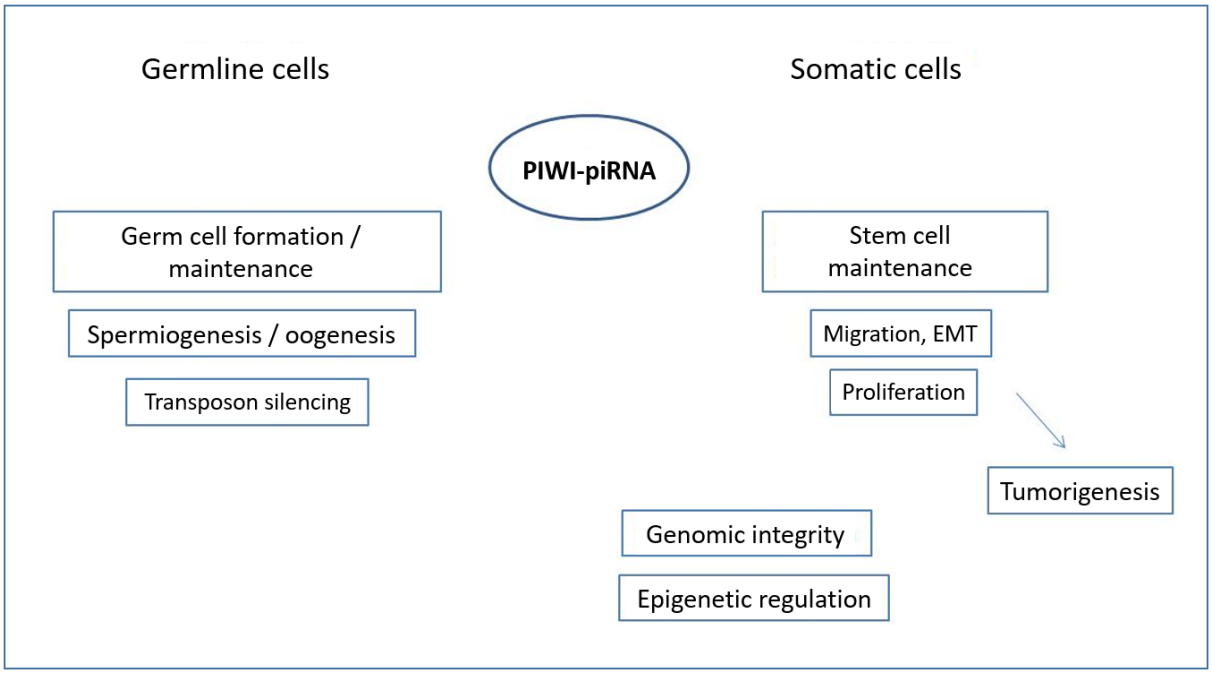

Fig. 12. Schematic representation of the cellular functions of PIWI-piRNA. Adapted from Litwin et al. (45).

PiRNAs as tumour biomarkers

Compared to non-tumour tissue, different expression of many piRNAs was demonstrated in tumour cell lines. Different levels of some piRNAs in peripheral blood (piR-651, piR-823 in gastric cancer, piR-5937, piR-28876 in colorectal cancer) were confirmed in studies on other

solid tumours (46). Thus, they may be potential biomarkers for the diagnosis and prognosis of cancer.

Published work related to the topic:

Iliev R, Fedorko M, Machackova T, Mlcochova H, Svoboda M, Pacik D, Dolezel J, Stanik M, Slaby O. Expression Levels of PIWI-interacting RNA, piR-823, are Deregulated in Tumor Tissue, Blood Serum and Urine of Patients with Renal Cell Carcinoma. Anticancer Res 2016; 36: 6419-6424.
IF 1,895

The original work showed significantly lower expression of piR-823 in RCC tissue compared to a healthy parenchyma and correlated with a worse prognosis of RCC patients. In contrast, in the serum and urine of patients, the expression of piR-823 was significantly increased, which can be explained by the active secretion of piR-823 by tumour cells.

Number of times cited (WoS, as of February 17th 2022): 47.

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Expression Levels of PIWI-interacting RNA, piR-823, Are Deregulated in Tumor Tissue, Blood Serum and Urine of Patients with Renal Cell Carcinoma

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Abstract. *Background: Renal cell carcinoma (RCC) is the most common neoplasm of adult kidney accounting for about 3% of adult malignancies. P-Element induced wimpy testis (PIWI)-interacting RNAs (piRNAs) are a new class of naturally occurring, short non-coding RNAs involved in silencing of transposable elements and in sequence-specific chromatin modifications. There were preliminary data published indicating that piR-823 expression is deregulated in circulating tumor cells and tumor tissue in gastric and kidney cancer, respectively. Patients and Methods: In our study, we analyzed piR-823 levels in 588 biological specimens: tumor tissue (N=153), adjacent renal parenchyma (N=121), blood serum (N=178) and urine (N=20) of patients undergoing nephrectomy for RCC; and in blood serum (N=101) and urine (N=15) of matched healthy controls. Expression levels of piR-823 were determined in all biological specimens by quantitative real-time polymerase chain reaction, compared in patients and controls, and correlated with clinicopathological features of RCC. Results: We identified a significant down-regulation of piR-823 in tumor tissue [p<0.0001, area under the curve (AUC)=0.7945]. On the contrary in blood serum and urine, the expression of piR-823 was significantly higher in patients with RCC compared to healthy individuals (p=0.0005, AUC=0.6264 and p=0.0157, AUC=0.7433, respectively). We further observed higher levels of piR-823 in tumor tissue to be associated with shorter disease-free survival of patients (p=0.0186) and a trend for higher piR-823 levels in serum to be associated with advanced clinical stages of RCC (p=0.0691). There were no other significant associations of piR-823 levels in any type of biological specimen with clinicopathological features of RCC. Conclusion: piR-823 is down-regulated in tumor tissue, but positively correlated with worse outcome, indicating its complex role in RCC pathogenesis. In blood serum, piR-823 is up-regulated, but with unsatisfactory analytical performance. Preliminary data indicate the promising diagnostic utility of urinary piR-823 in patients with RCC.*

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Key Words: PIWI-interacting RNA, piR-823, renal cell carcinoma; tumor tissue, blood serum, urine.

Renal cell carcinoma (RCC) is the most common neoplasm of the kidney in adults, accounting for 3% of adult malignancies and having the highest mortality rate at over 40% (1). P-Element induced wimpy testis (PIWI)-interacting RNAs (piRNAs) are a newly discovered class of short non-coding RNAs. They are 26-31 nucleotides long, which clearly differentiate them from other short non-coding RNAs such as microRNAs and siRNAs with 20-23 nucleotides in length (2). Initially, they were reported in mice testes, where they have a major role in the germ cell in maintaining genomic stability through binding to PIWI proteins and then silencing transposable elements (3). piRNA-based silencing is mediated by CpG methylation, chromatin remodeling and degradation of complementary transcripts (4). Recent studies suggest that the deregulated expression of PIWI proteins is common in tumor tissue, and PIWI expression levels are correlated to clinicopathological features and survival in patients with cancer [reviewed in (5)]. Therefore, they present promising cancer biomarkers. Recently, it was repeatedly reported that not only PIWI proteins, but also

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Table I. *Patient characteristics and piR-823 expression levels.*

	Tissue		Serum		Urine	
	N	*piR-823* copies*	N	*piR-823* copies*	N	*piR-823* copies*
Parenchyma, HC	121	465528±391450	101	2427±4230	15	93200±80495
Tumor, RCC patients	153	161841±191214	178	3828±6433	20	552264±773929
Fold-change in patients		0.3477		1.5772		4.663
AUC		0.7945		0.6264		0.7433
p-Value		**0.0001**		**0.0005**		**0.0157**
TNM stage						
I-II	82	201075±314067	117	2422±2823		NA
III-IV	48	151862±129437	60	3634±6378		NA
p-Value		0.7852		0.0691		NA
Fuhrman grade						
G1-2	81	213445±316014	106	2391±2601		NA
G3-4	49	135291±115428	66	3621±6326		NA
p-Value		0.1416		0.2096		NA
Association with DFS						
Cut-off		223038		2046		NA
p-Value		**0.0186**		0.9180		NA
Association with OS						
Cut-off		147727		2046		NA
p-Value		0.6042		0.9035		NA

RCC, Renal cell carcinoma; TU, tumor; RP, renal parenchyma; HC, healthy controls; AUC, area under curve; DFS, disease-free survival; OS, overall survival; NA: not available. *Data are the mean±SD.

piRNAs can play an important role in carcinogenesis. Over- or underexpression of piRNAs that target mRNA transcripts (*e.g.* those containing transposon-derived sequences in their 3' untranslated regions) could also play a driver role through degradation or inhibition of tumor-suppressor genes or oncogenes, respectively. Deregulated levels of many piRNAs were described in RCC (6, 7), gastric cancer (8, 9), hepatocellular carcinoma (10), multiple myeloma (11), lung cancer (12), bladder cancer (13), breast cancer (14) and pancreatic ductal adenocarcinoma (15). One of the first piRNAs identified as being linked to cancer is piR-823 (8). piR-823 was detected in cancer cell lines, white blood cells (16) and blood plasma (17, 18). This piRNA was described to be deregulated in gastric tumor tissue and myeloma cells and to be involved in regulation of tumor cell growth (8, 11). We published preliminary reports indicating its down-regulation in RCC tissue (19). Here, we decided to perform large-scale evaluation of piR-823 deregulation in tumor tissue, blood serum and urine of patients with RCC.

Patients and Methods

Samples of tumor tissue (N=153), renal parenchyma (N=121), blood serum (N=178) and urine (only for the last 20 patients included) used in this study were obtained from patients diagnosed with RCC who underwent radical nephrectomy at Masaryk Memorial Cancer Institute (MMCI) in Brno or The University Hospital Brno (UHB) between 2012 and 2015. The median age of the patients at the time of diagnosis was 64 years (range=21-84 years). Blood serum (N=101) and urine samples (N=15) were also obtained from healthy individuals who underwent preventative medical examination in MMCI. Patient's biological samples were frozen and stored at −80˚C until processing. Written informed consent was obtained from all participants (patients, healthy individuals), and the study was approved by the local Ethics Boards at MMCI and UHB. Clinical and pathological characteristics are summarized in Table I.

Total RNA from tissue samples was isolated by use of mirVana miRNA Isolation Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. For RNA isolation from serum, miRNeasy Serum/Plasma Kit (Qiagen, Valencia, CA, USA) was used, and for isolation from urine Urine microRNA Purification Kit (Norgen, Thorold, ON, Canada) was used. RNA concentration and purity were measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Using 10 ng of total RNA, cDNA was synthesized with TaqMan® MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's recommendations. Quantitative polymerase chain reaction was performed in a total volume of 15 μl using specific probe for *piR-823*. The primers and probe were designed and synthesized (Thermo Fisher Scientific) to be used for determination of *piR-823* sequence 5' AGCGUUGGUGGUAUAGUGGUGAGC AUAGCUGC-3'. The expression levels were quantified absolutely by use of a calibration curve using *piR-823* plasmids synthesized by Generi Biotech (Hradec Kralove, Czech Republic).

Differences in expression levels of *piR-823* between tumor tissue and renal parenchyma were evaluated by use of Wilcoxon test, and differences in serum and urine samples of patients and healthy controls were evaluated by use of Mann–Whitney test. For determining the differences according to clinical stage and grade, we used non-

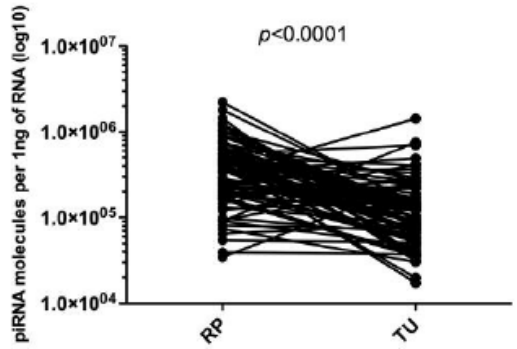

Figure 1. *Statistical significant difference in piR-823 expression (p<0.0001) between 121 paired samples of renal cell carcinoma tumor tissue (TU) and paired healthy renal parenchyma (RP).*

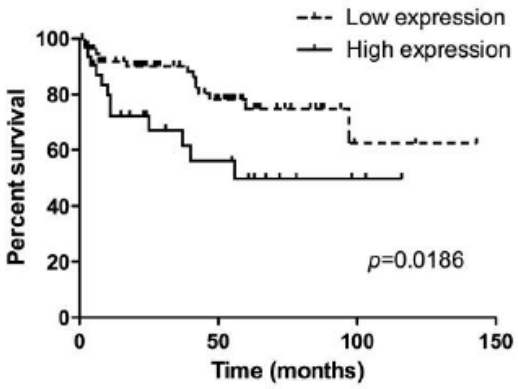

Figure 2. *piR-823 levels and disease-free survival of patients with renal cell carcinoma. There was a proven statistically significant (p=0.0186) association of longer disease-free survival in patients with low expression of piR-823 in tumor tissue compared to those with high expression.*

parametric Kruskal–Wallis test. Receiver operating curve (ROC) analysis was performed to identify cut-offs to distinguish patients with different prognoses. Kaplan–Meier survival curves and log-rank test were used for survival analysis. Calculations were performed in GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

In our study, we successfully determined *piR-823* expression levels in 588 biological specimens obtained from patients with RCC and healthy controls. We observed significant differences in *piR-823* expression levels between tumor and non-tumor tissue samples. *piR-823* expression was significantly decreased in tumor tissue compared to paired non-tumorous renal parenchyma (Figure 1, $p<0.0001$). ROC analysis showed that tissue levels of *piR-823* can differentiate tumor tissue from renal parenchyma with an area under the curve (AUC) of 0.795. However, we observed no association of *piR-823* levels in tumor with clinical stage or Fuhrmann grade. Interestingly, although the *piR823* levels in tumor tissue were decreased, a low level was associated with a longer disease-free survival (DFS) ($p=0.0186$; Figure 2). The median DFS in patients with RCC with high expression of *piR-823* was 56 months, whereas in the low-expression group median was not reached. Although we observed the same trend in OS, the association of *piR-823* level and OS did not reach statistical significance ($p>0.05$).

Furthermore, we recorded significantly higher levels of *piR-823* in serum of patients with RCC when compared to healthy controls ($p=0.0005$; Figure 3A), but ROC analysis showed this to have unsatisfactory analytical performance in distinguishing patients with RCC from controls (AUC=0.626). There was no correlation of *piR-823* in serum with Furhmann grade, DFS or OS. We did observe a trend for higher *piR-823* levels in serum to be associated with advanced clinical stages of RCC ($p=0.0691$). There was also no correlation between the piR-823 level in serum and that in paired tumor tissues samples ($p>0.05$).

Finally, we determined level of *piR-823* in urinary samples of small groups of patients with RCC and healthy controls. Preliminary results showed *piR-823* levels were significantly higher in urinary samples from patients with RCC in comparison to healthy controls ($p=0.0157$, Figure 3B).

Discussion

As the first aim, we determined expression of *piR-823* in paired samples of tumor tissue and adjacent renal parenchyma. In agreement with our previous pilot study (19), we observed significantly reduced expression of piR-823 in tumor tissue compared to adjacent non-tumorous renal parenchyma. Martinez *et al*.'s observed RCC tumors to be characterized by general down-regulation of *piRNA* (9). We did not find any significant association of tumor *piR-823* level with clinical stage, Fuhrmann grade or OS. However, we observed significant association of tumor *piR-823* and DFS, with the median DFS in patients with low expression being significantly longer.

Since piRNAs predominantly control the expression of transposable elements (20), piR-823 could be involved in maintenance of genomic stability and its frequently observed loss in RCC tumor tissues may be linked to genomic instability, which is a general hallmark of all malignant tumors. However, the link we found here to survival, where a higher levels of piR-823 is associated with worse DFS, indicates a more oncogenic than tumor-suppressive role for

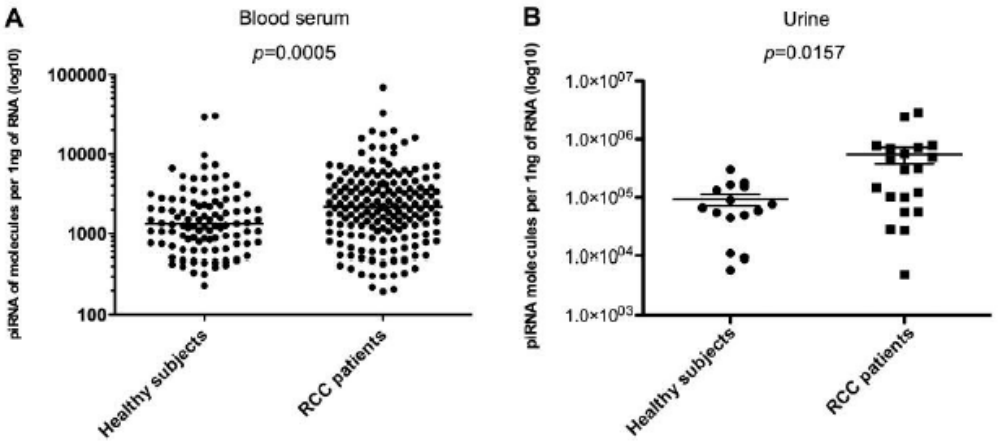

Figure 3. *piR-823 levels measured in blood serum (A) and urine (B). In patients with renal cell carcinoma (RCC), the piR-823 levels were increased compared to those in healthy individuals. Levels of piR-823 in urine of patients with RCC were higher in comparison with those in urine of healthy individuals. Lines represent medians, and bars are 25th and 75th percentiles.*

piR-823 in RCC. It seems that *piR823* might play a complex role in RCC pathogenesis characterized by different phenotypic effects in different stages of the disease.

In addition to transposable elements, piR-823 can potentially also target protein-coding genes. On the basis of base pair complementarity, cell-division cycle 5-like protein (CDC5L) represents a potential target of piR-823, since CDC5L contains a region which is fully complementary to piR-823. CDC5L is a cell-cycle regulator (21) whose its up-regulation in tumor tissue of osteosarcoma and cervical tumors led to aberrant cell-cycle control and contributed to the malignant phenotype (22). CDC5L is a part of the E3 ubiquitin ligase complex and is required for the correct functioning of the S-phase cell-cycle checkpoint (23). It is also involved in RNA processing, where it plays an important role as a pre-mRNA splicing factor and its down-regulation reduces cell viability (22). Unfortunately, the role of CDC5L in RCC is not known.

We also analyzed *piR-823* levels in blood serum and urine. We determined *piR-823* expression levels in a large group of patients with RCC and gender- and age-matched healthy controls. We observed significantly higher serum levels of piR-823 in the group of patients compared to healthy individuals (p=0.0005), which is in contrast to differences we found in tissue samples. This phenomenon might be explained by active release of *piR-823* by tumor cells leading to its decreased levels in tumors and increased levels in the circulation. The AUC for the ability of serum *piR823* to distinguish patients with RCC from healthy controls was 0.626, which is not high enough to enable diagnostic utility. Although differences in expression does not allow usage of piR-823 as a new independent biomarker of RCC, it could potentially be useful in combination with other available biomarkers, *e.g.* newly described microRNA biomarkers. We did not observe any significant association of *piR-823* with any clinical stage, Furhmann grade or survival. Therefore, we suggest that *piR-823* is more likely linked to some of the general hallmarks of RCC pathogenesis than to the tumor features behind tumor invasion and progression of the disease.

Finally, we evaluated *piR-823* levels in urine samples of pilot cohort of 20 RCC patients and 15 healthy controls. By this analysis, we identified significantly higher levels of *piR-823* in urine from patients and significantly higher levels of piR-823 in urine in comparison to serum, indicating urine to be more suitable for *piR-823* analysis than serum. In order to evaluate potential associations of urinary *piR-823* and clinicopathological data, study on the larger cohort of patients with RCC is needed.

There are certain limitations to our study. We performed absolute quantification of *piR-823* as we were not able to identify an appropriate endogenous control to be used for relative quantification. Therefore, our data might be biased due to the presence of inhibitors in the complex biological specimens. Moreover, *in vitro* experiments are needed to demonstrate the ability of RCC cells to release *piR-823* and to evaluate whether *piR-823* is exosomal, protein-bound or free. We have not performed an *in vitro* and *in vivo* study to describe functioning of piR-823 in RCC pathogenesis.

In conclusion, *piR-823* is down-regulated in tumor tissue, but in tumor positively correlated with worse outcome, indicating its complex role in RCC pathogenesis. In blood serum, *piR-823* is up-regulated, but with unsatisfactory

analytical performance. Preliminary data indicate the promising diagnostic utility of urinary piR-823 in patients with RCC

Disclosure Statement

The Authors have no conflict of interest.

Acknowledgements

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PIWI proteins as tumour biomarkers

Four PIWI proteins are currently known - PIWI 1 to PIWI 4 (also referred to as PIWIL1 - 4). Their aberrant expression is described in various types of cancer (Tab. 2).

PIWI	Tumour	Expression
PIWIL1	Lung cancer	↑
	Gastric cancer	↑
	Colorectal cancer	↑
	Renal cancer	↓
	Endometrial cancer	↑
	Invasive ductal carcinoma	↑
PIWIL2	Glioma	↑
	Cervical cancer	↑
	Non-small cell lung cancer	↑
	Renal cancer	↓
PIWIL3	Glioma	↓
	Gastric cancer	↑
	Multiple myeloma	↑
PIWIL4	Breast cancer	↑

Tab. 2. Deregulated PIWI proteins in tumour tissue. Adapted from Liu et al. (46).

Published work related to the topic:

Iliev R, Stanik M, Fedorko M, Poprach A, Vychytilova-Faltejskova P, Slaba K, Svoboda M, Fabian P, Pacik D, Dolezel J, Slaby O. Decreased expression levels of PIWIL1, PIWIL2, and PIWIL4 are associated with worse survival in renal cell carcinoma patients. Onco Targets Ther 2016; 9: 217-222.
IF 2,311

In this original work, a significantly reduced expression of PIWIL1 in RCC tissue and a correlation between decreasing expression of PIWIL1, PIWIL2 and PIWIL4 and a higher clinical stage of the tumour and between the expression of PIWIL2 and PIWIL4 and a higher nuclear grade was demonstrated. In the case of PIWIL1, PIWIL2 and PIWIL 4, low expression in tumour tissue is significantly associated with poorer overall survival.

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ORIGINAL RESEARCH

Decreased expression levels of PIWIL1, PIWIL2, and PIWIL4 are associated with worse survival in renal cell carcinoma patients

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Abstract: Piwi-interacting RNAs (piRNAs) are a newly discovered class of small non-coding RNAs involved in silencing of transposable elements and in sequence-specific chromatin modifications. PIWI proteins (PIWIL), which belong to the family of Argonaute genes/proteins, bind to piRNAs and function mainly in germ line cells, but more recently were described to be functional also in stem cells and cancer cells. To date, there have been four PIWI proteins discovered in humans: PIWIL1, PIWIL2, PIWIL3, and PIWIL4. Recent studies suggested that deregulated expression of PIWI proteins and selected piRNAs is common to many types of cancers. We found significantly lower expression of PIWIL1 ($P<0.0001$) and piR-823 ($P=0.0001$) in tumor tissue in comparison to paired renal parenchyma. Further, we observed a progressive decrease in PIWIL1 ($P=0.0228$), PIWIL2 ($P=0.0015$), and PIWIL4 ($P=0.0028$) expression levels together with increasing clinical stage. PIWIL2 ($P=0.0073$) and PIWIL4 ($P=0.0001$) expression also progressively decreased with increasing Fuhrman grade. Most importantly, low-expression levels of PIWIL1 ($P=0.009$), PIWIL2 ($P<0.0001$), and PIWIL4 ($P=0.0065$) were significantly associated with worse overall survival in renal cell carcinoma (RCC) patients. Our results suggest the involvement of PIWIL genes and piR-823 in RCC pathogenesis, and indicate PIWIL1, PIWIL2, and PIWIL4 as potential prognostic biomarkers in patients with RCC.

Keywords: kidney cancer, PIWIL, piRNA

Introduction

Renal cell carcinoma (RCC) represents approximately 75% of all kidney tumors and 3% of all cancers in the adult population.[1] Piwi-interacting RNAs (piRNAs) are a newly discovered class of small non-coding RNAs with a length of 26–31 nucleotides. piRNAs were first identified in the germ cells of various animal species.[2–4] In the human genome, there were more than 24,000 distinct piRNA sequences identified till now,[5] and their deregulated expression was observed in breast,[6] bladder[7] and gastric cancer,[8] and multiple myeloma.[9] piRNAs bind to a specific subfamily of Argonaute proteins called PIWI proteins. Argonaute proteins are a highly conserved group of proteins divided into two main classes: AGO and PIWI. In humans, four PIWI genes were identified. PIWI proteins in humans include PIWIL1/HIWI, PIWIL2/HILI, PIWIL3, and PIWIL4/HIWI2.[10] They bind piRNAs and amplify them in the so-called ping-pong cycle, which is endoribonuclease Dicer independent. Their physiological role is transposon repression through base-pairing and direct degradation.[11] They are also involved in DNA methylation but the exact mechanism of action remains unknown.[12] These days, there is increasing evidence to show that PIWIL proteins are linked to the hallmarks of cancer defined by Weinberg and Hanahan, such as deregulated cell proliferation, altered apoptosis, genomic instability, invasion,

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and metastasis.[13] Recent studies also suggested that the deregulated expression of PIWIL proteins is common in tumor tissue, and PIWIL expression levels are correlated to clinicopathological features and survival in patients with cancer and PIWIL proteins. Therefore, they present promising cancer biomarkers.[13] In our study, we determined the expression levels of PIWIL genes in clear cell RCC tumors and paired renal parenchyma, and evaluated their association with clinicopathological features in patients with RCC. Further, we investigated whether the piR-823 expression, observed to be downregulated in gastric cancer,[8] is similarly deregulated in RCC tumors, and if there is a correlation between piR-823 and PIWIL gene expression due to their involvement in piRNA biogenesis.

Materials and methods

Subjects

The patients with RCC who underwent radical nephrectomy at the Masaryk Memorial Cancer Institute (Brno, Czech Republic) and University Hospital Brno (Brno, Czech Republic) were included. Tissue samples were collected after signing an informed consent and stored at the Bank of Biological Material (Masaryk Memorial Cancer Institute). The samples were obtained from 57 patients (36 men and 21 women) with clear cell RCC with median age 64 (range 35–80) years at the time of diagnosis. In 38 cases, tissue samples of adjacent non-tumor renal parenchyma was available for the expression analysis. Clinical and pathological characteristics including clinical stage and Fuhrman grade are summarized in Table 1. The study has been approved by the local Ethical Committee of Masaryk Memorial Cancer Institute.

RNA extraction and qPCR

RNA isolation was performed using mirVana miRNA Isolation Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA yield and purity were measured using a spectrophotometer NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies) according to the manufacturer's recommendations. Quantitative polymerase chain reaction (qPCR) was performed in a total volume of 20 µL using specific probes for PIWIL1 (Hs01041737_m1), PIWIL2 (Hs00216263_m1), PIWIL3 (Hs00908825_m1), PIWIL4 (Hs00381509_m1; Life Technologies), and peptidylprolyl isomerase A (PPIA, 99999904_m1 Hs) as an endogenous control. cDNA synthesis of piR-823 was carried out using TaqMan MicroRNA Reverse Transcription Kit (Life Technologies). The primers and probe were designed and synthesized (Life Technologies) accordingly to the piR-823 sequence 5′-AGCGUUGGUGGUAUAGUGGUGAGCAUAGCUGC-3′. The expression level of piR-823 was computed relatively to endogenous control RNU44. qPCR was performed on an ABI 7500 (Life Technologies). Relative expression was calculated using a ΔCt method.

Table 1 Summary of PIWIL genes and piR-823 expression levels detected in tissue of RCC patients presented as median of normalized expression

	N=57	PIWIL1	PIWIL2	PIWIL3	PIWIL4	piR-823
Expression levels in tumor tissue and renal parenchyma (RP)						
RP		0.000131	0.000791	0.000004	0.001244	8.174421
Tumor		0.000016	0.000472	0.000003	0.001273	0.980179
Fold-change (tumor/RP)		0.12	0.60	0.59	1.02	0.12
P-value		**<0.0001**	0.3268	0.1325	0.4022	**0.0001**
Clinical stage						
I	11	0.0001023	0.001225	0.000003	0.001734	1.303027
II	4	0.000110	0.001636	0.000003	0.001745	1.080401
III	13	0.000028	0.001057	0.000002	0.001801	0.496938
IV	29	0.000013	0.000317	0.000003	0.000804	0.980179
P-value		**0.0228**	**0.0015**	0.3830	**0.0028**	0.4033
Fuhrman grade						
G1	6	0.000102	0.001356	0.000003	0.002421	0.462293
G2	24	0.000042	0.000711	0.000002	0.001597	1.388136
G3	17	0.000013	0.000459	0.000003	0.000760	0.980178
G4	10	0.000015	0.000253	0.000003	0.000804	0.671232
P-value		0.1099	**0.0073**	0.4665	**0.0001**	0.2898
Association with overall survival						
P-value		**0.009**	**<0.0001**	0.8548	**0.0065**	0.1418

Notes: Bold values indicate *P*-values lower than 0.05.
Abbreviation: RCC, renal cell carcinoma.

Statistic analysis

To calculate the significance of differences in PIWIL and piR-823 expression levels in cases of different clinical stages or Fuhrman grades, we used non-parametric Kruskal–Wallis test. To calculate the differences in expression between paired tumor tissues and adjacent non-tumor renal parenchyma, a nonparametric Wilcoxon matched pair test was used. For the sensitive discrimination of RCC patients with short and long overall survival cut-off values were identified for PIWIL genes and piR-823 by receiver operating curve analysis. Kaplan–Meier survival curves and long-rank test were used for survival analysis. Calculations were performed in GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

We determined the expression levels of PIWIL genes and piR-823 in tumor tissue of 57 patients with RCC and 38 samples of matched non-tumor renal parenchyma. First, we compared the expression levels of PIWIL genes and piR-823 in paired samples of RCC tumor tissue and non-tumor renal parenchyma. We found significant downregulation in the expression levels of PIWIL1 ($P<0.0001$) and piR-823 ($P=0.0001$; Figure 1A and B). Receiver operating curve

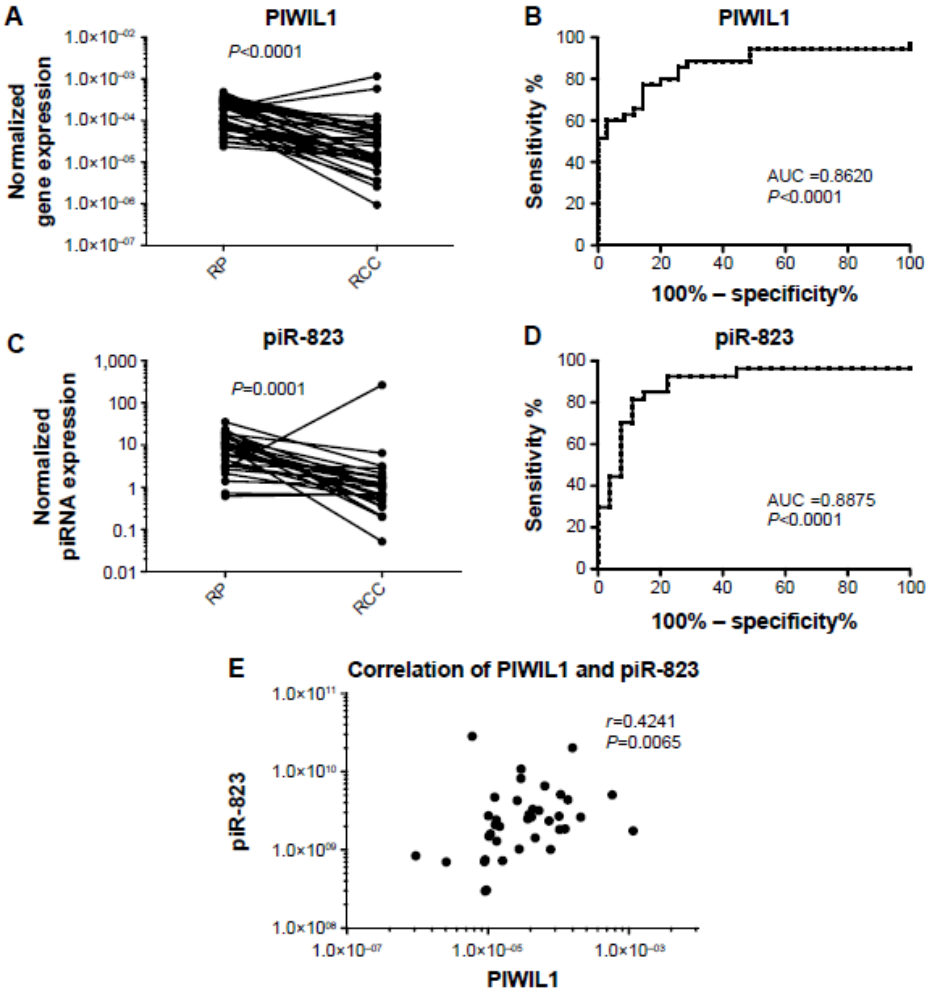

Figure 1 PIWIL1 and piR-823 are significantly downregulated in RCC tumors when compared to paired non-tumor renal parenchyma (**A** and **B**) and reached good ability to discriminate tumor tissue and non-tumor renal parenchyma (**C** and **D**). Expression of piR-823 is significantly correlated with PIWIL1 (**E**).
Abbreviations: AUC, area under curve; RCC, renal cell carcinoma; RP, renal parenchyma.

analysis revealed that the tissue levels of both PIWIL1 and piR-823 could serve as appropriate biomarkers for differentiating tumor tissue from non-tumor renal parenchyma with the area under the curve of 0.8620 (95% confidence interval: 0.7690–0.9543, $P<0.0001$) reaching the 86% sensitivity and 75% specificity for PIWIL1, and 0.9975 (95% confidence interval: 0.7908–0.9842, $P<0.0001$) reaching the 89% sensitivity and 78% specificity for piR-823, respectively (Figure 1C and D). Expression levels of piR-823 were significantly correlated with PIWIL1 expression levels ($r=0.4242$, $P=0.0065$; Figure 1E) supporting its involvement in piR-823 biogenesis. We have observed no significant differences in the expression levels of PIWIL2, PIWIL3, and PIWIL4 between tumors and paired renal parenchyma (Table 1). Further, we evaluated correlations of PIWIL genes and piR-823 with clinicopathological features and survival in patients with RCC (summarized in Table 1). We observed progressive decrease in PIWIL1 ($P=0.0007$, Figure 2A), PIWIL2 ($P=0.0174$, Figure 2B) and PIWIL4 ($P=0.00403$, Figure 2C) expression levels together with increasing clinical

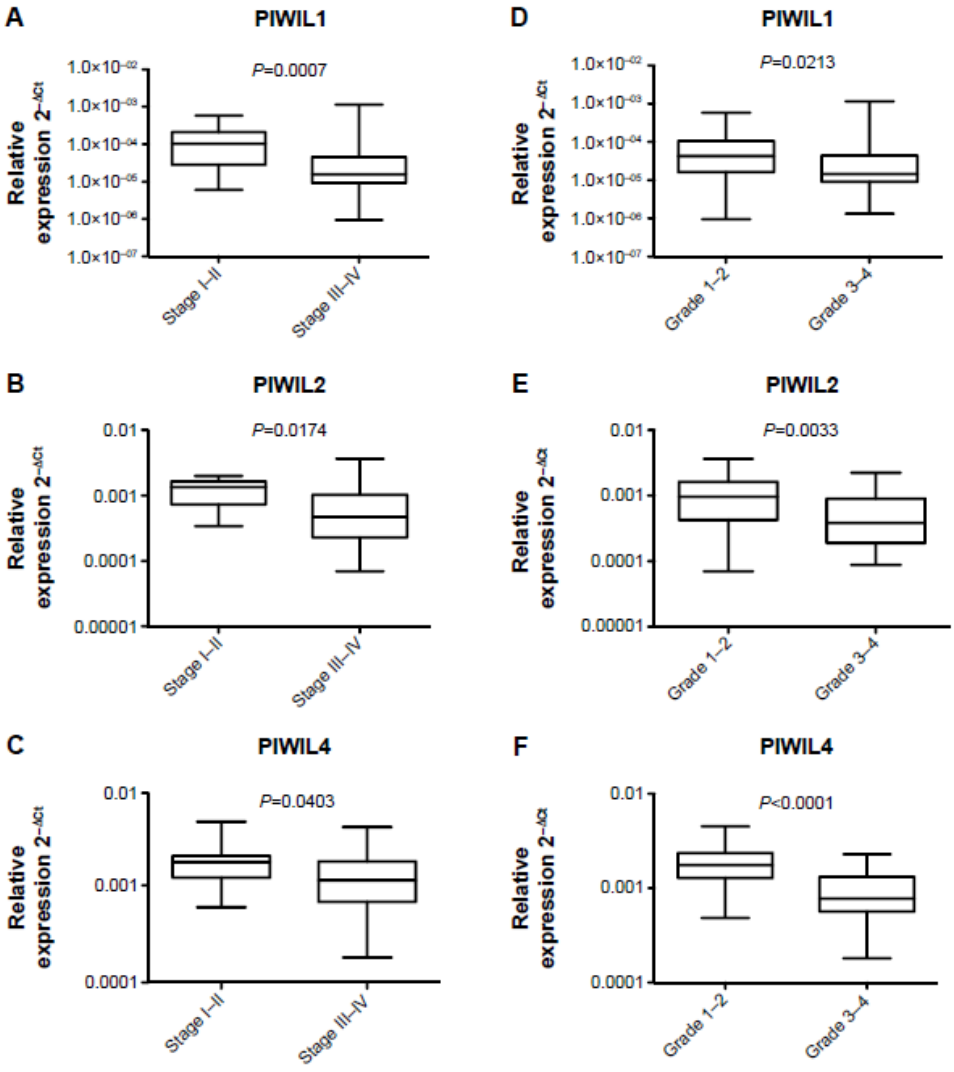

Figure 2 Associations of PIWIL genes expression and clinicopathological features of renal cell carcinoma.
Note: PIWIL1, PIWIL2, and PIWIL4 are significantly associated with clinical stage (**A–C**) and Fuhrman grade (**D–F**) in renal cell carcinoma patients.

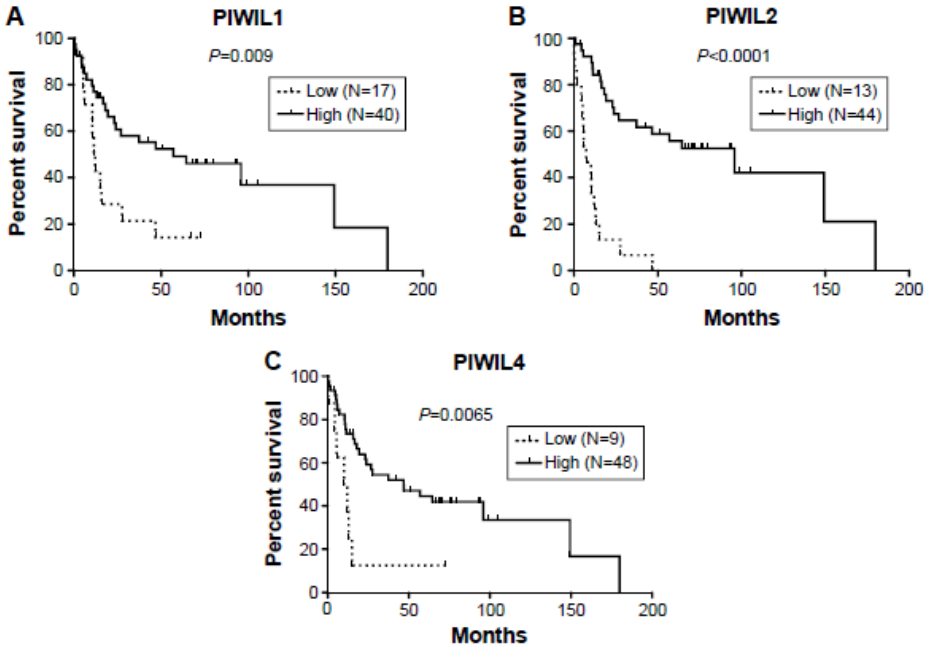

Figure 3 Expression of PIWIL1, PIWIL2, and PIWIL4 gene is associated with overall survival of renal cell carcinoma patients (**A–C**).

stage. PIWIL1 (P=0.0213, Figure 2D), PIWIL2 (P=0.0033, Figure 2E) and PIWIL4 (P<0.0001, Figure 2F) expression progressively decreased also with increasing Fuhrman grade. Most importantly, low-expression levels of PIWIL1 (P=0.009, Figure 3A), PIWIL2 (P<0.0001, Figure 3B) and PIWIL4 (P=0.0065, Figure 3C) were significantly associated with worse overall survival in patients with RCC. PIWIL3 and piR-823 were not correlated with any evaluated clinicopathological feature of RCC.

Discussion

In our study, we quantified the expression levels of PIWIL1, PIWIL2, PIWIL3, and PIWIL4 genes and piR-823 in paired samples of RCC tumor tissue and non-tumor renal parenchyma. Significant differences were observed only in the case of PIWIL1 and piR-823, whose levels were significantly decreased in tumor tissue. Accordingly, decreased levels of piR-823 were also observed in gastric cancer.[8] The strong correlation between PIWIL1 and piR-823 expression levels observed in our study suggest involvement of PIWIL1 in piR-823 biogenesis. In agreement with our results, Al-Janabi et al described the trend of PIWIL1 expression to be decreased in RCC tumors (P=0.089).[14] In addition, they observed a significantly higher level of PIWIL4 in tumors (P<0.001), which was not shown in tumor tissues in our cohort of patients with RCC. Analogically, the expression of PIWIL genes is downregulated in seminoma and non-seminoma testicular tumors, probably due to the existence of promoter CpG island hypermethylation-associated silencing.[15] Contrary to this observation and our results, upregulation of PIWIL1 (HIWI) in tumor tissue was shown in glioma,[16] cervical cancer,[17] breast cancer,[18] and non-small cell lung cancer, while PIWIL2 and PIWIL4 genes were downregulated.[19]

Further, we observed expression levels of PIWIL1, PIWIL2, and PIWIL4 to be progressively decreased with increasing clinical stage, and Fuhrman grade and lower levels of those genes to be significantly associated with the worse overall survival in patients with RCC. In agreement with our observations, Taubert et al described decreased levels of PIWIL2 protein to be significantly associated with poor prognosis in cohort of 202 bladder cancer patients (P=0.005).[20] A higher level of PIWIL4 mRNA was found to be associated with longer overall survival also in non-small cell lung cancer patients.[19] In patients with soft tissue sarcoma, Greither et al identified significant association between the low-expression levels of PIWIL2, PIWIL3, and PIWIL4 mRNAs and shorter tumor-specific survival.[21] Contrary to these observations and

also our results, the increased expression levels of PIWIL1 were correlated with tumor grade in gliomas[22] and associated with worse survival in colorectal cancer,[23] hepatocellular carcinoma,[24] and gastric cancer patients.[25] In gastric cancer it was also reported that PIWIL2 expression levels are associated with worse overall survival.[25]

To conclude, we observed deregulation of PIWIL1 and piR-823 in tumor tissue of patients with RCC and significant association of PIWIL1, PIWIL2, and PIWIL4 expression with the survival of patients with RCC. It seems that PIWIL mRNA levels are commonly deregulated in tumor tissue and correlated with clincopathological features of tumors, however, the tendency of changes varies according to the tissue of origin. In urological tumors (RCC and bladder cancer), the expression of PIWIL genes is more likely to be downregulated and the loss of their expression is associated with a more aggressive phenotype of tumors and worse survival.

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Disclosure

The authors report no conflicts of interest in this work.

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6.2. Long non-coding RNAs

Long non-coding RNAs (lncRNAs) are a group of non-coding RNAs longer than 200 nucleotides in length. They are actively involved in various cellular processes, such as differentiation, proliferation, response to DNA damage or chromosomal imprinting (47). With increasing knowledge of their biological functions, several of their basic properties crucial for the pathogenesis of cancer can be characterized: maintenance of proliferation signals, circumvention of growth suppressors, stimulation of cell replication, activation of invasion and metastasis, induction of angiogenesis and resistance to cell death (48). Unlike miRNAs, lncRNAs regulate gene expression at all levels. Nuclear lncRNAs are involved in epigenetic regulation, such as various chromatin modifications (association with histone modifying complexes, DNA methylation or chromatin remodeling), transcriptional regulation (in terms of activation or repression) and at further level of gene expression in post-transcriptional splicing of pre-mRNA (49-51). Cytoplasmic lncRNAs act at post-transcriptional (mRNA stability, miRNA binding - "sponging"), translational (translation activation or suppression) and post-translational (e.g. influencing protein stability) level of gene expression (52).

Differential expression of lncRNA in the tissue (or serum) of RCC compared to non-tumour samples and the relationship to the clinicopathological characteristics of the tumour suggest the potential use of lncRNA as diagnostic and prognostic biomarkers of RCC, or as therapeutic targets (53, 54). In addition to renal cell carcinoma, their use as biomarkers of other urological malignancies, such as prostate or bladder cancer, can be expected (55).

Published work related to the topic:

Fedorko M, Bohošová J, Poprach A, Pacík D. Dlouhé nekódující RNA a karcinom z renálních buněk. Klin Onkol 2020; 33(5): 340-349.

The review describes in detail the importance of long non-coding RNAs in the pathogenesis of renal cell carcinoma and the possibilities of their use in the diagnosis, prognosis and prediction of treatment response in patients with renal cell carcinoma.

Number of times cited (Scopus, as of February 17th 2022): 2.

Dlouhé nekódující RNA a karcinom z renálních buněk

Long non-coding RNAs and renal cell carcinoma

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Souhrn

Východiska: Poskytnout přehlednou informaci o významu dlouhých nekódujících RNA (lncRNA) v patogenezi karcinomu z renálních buněk (renal cell carcinoma – RCC) a možnostech jejich využití v diagnostice, stanovení prognózy onemocnění a predikci léčebné odpovědi. ***Materiál a metody:*** Vyhledávání v databázích PubMed a Web of Science s využitím variant klíčových slov „dlouhé nekódující RNA" („lncRNA", „long noncoding RNA", „long non-coding RNA") a „karcinom z renálních buněk" („renal cancer", „renal cell carcinoma", „kidney cancer"). Separace výsledků týkajících se patogeneze, diagnózy, prognózy a využití jako terapeutických cílů. ***Výsledky:*** Dlouhé nekódující RNA regulují genovou expresi na všech úrovních. Uplatňují se jako onkogeny i jako nádorové supresory. Mechanizmus jejich působení je objasněn pouze částečně, v patogenezi renálního karcinomu však aktivně regulují kaskádu faktorů indukovaných hypoxií, epiteliálně-mezenchymální tranzici, buněčnou proliferaci, buněčný cyklus, apoptózu, lokální invazi a vznik metastáz. Aberantní exprese ve tkáni nádoru ve srovnání se zdravým renálním parenchymem a korelace expresních hladin s klinicko-patologickými charakteristikami tumoru umožňují potenciální využití mnoha lncRNA jako biomarkerů pro časnou detekci a stanovení prognózy onemocnění vč. odpovědi na cílenou léčbu. Testy *in vitro* naznačují potenciální využití lncRNA jako terapeutických cílů. ***Závěr:*** Poznatků o dlouhých nekódujících RNA ve vztahu ke karcinomu z renálních buněk rychlým tempem přibývá. V současné době lze některé z nich považovat za slibné biomarkery. Před uvedením do rutinní klinické praxe je potřeba dalšího výzkumu.

Klíčová slova
biomarker – diagnostika – dlouhé nekódující RNA – karcinom z renálních buněk – prognóza

Summary

Background: To provide an overview of the importance of long non-coding RNAs (lncRNAs) in the pathogenesis of renal cell carcinoma and their utility as biomarkers for diagnosis, prognosis and prediction of treatment response. ***Materials and methods:*** A literature search in the Pubmed and Web of Science databases using the keywords variations of "long non-coding RNA" ("lncRNA", "long noncoding RNA", "long non-coding RNA") and "renal cell carcinoma" ("renal cancer", "renal cell carcinoma", "kidney cancer") was performed. The results related to the pathogenesis, diagnosis, prognosis and use as therapeutic targets were separated. ***Results:*** Long non-coding RNAs regulate gene expression at different levels. They act both as oncogenes and tumor suppressors. The mechanism of their action has not been fully elucidated, but they are actively involved in the regulation of hypoxia inducible factors pathway, epithelial-mesenchymal transition, cell proliferation, cell cycle regulation, apoptosis, local invasion and development of metastases. Aberrant expression in tumor tissue compared to healthy parenchyma and the correlation of expression levels with clinical-pathological features allow the potential use of many lncRNAs as biomarkers for early detection and prognosis of the disease, including the response to targeted therapy. *In vitro* assays indicate the potential use of lncRNAs as therapeutic targets. ***Conclusion:*** Our knowledge of long non-coding RNAs in relation to renal cell carcinoma is increasing rapidly. At present, some of them can be considered as promising biomarkers. Further research is needed before they can be introduced into routine clinical practice.

Key words
biomarker – diagnosis – long non-coding RNA – renal cell carcinoma – prognosis

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The Editorial Board declares that the manuscript met the ICMJE recommendation for biomedical papers.

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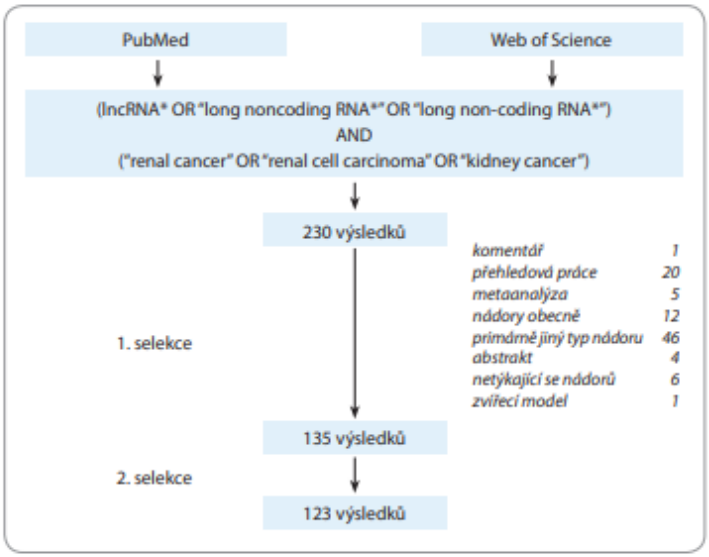
Úvod

Karcinom z renálních buněk (renal cell carcinoma – RCC) tvoří 4,2 % ze všech zhoubných nádorů u mužů a 2,6 % u žen [1]. Jeho incidence má stoupající tendenci. Nejvyšších hodnot dosahuje celosvětově v ČR, kde byla v roce 2016 standardizovaná incidence 15,17 případů na 100 000 obyvatel [2]. Pětiletým relativním přežitím na úrovni přibližně 75 % se řadí mezi nejletálnější urologické malignity [3]. Poměrně významná část pacientů je stále diagnostikována v pokročilém stadiu onemocnění, v ČR je až 20 % případů diagnostikováno v klinickém stadiu IV [2]. Kromě zobrazovacích vyšetření není k dispozici spolehlivý biomarker, který by byl použitelný v rutinní praxi pro časnou detekci či stanovení prognózy RCC.

Dlouhé nekódující RNA (long non-coding RNAs – lncRNA) patří do skupiny tzv. nekódujících RNA, což jsou transkripty genomu, které nejsou překládány, tedy nekódují funkční proteiny. Hranice mezi krátkými a dlouhými nekódujícími RNA je přibližně 200 nukleotidů. LncRNA regulují genovou expresi na více úrovních – v jádře buňky se uplatňují při modifikacích chromatinu, transkripční regulaci (aktivace i represe) i posttranskripčních úpravách mRNA (sestřih, transport, translace), v cytoplasmě regulují genovou expresi na posttranskripční (stabilita mRNA, vazba s miRNA), translační i posttranslační úrovni [4]. Uplatňují se v patogenezi různých onemocnění vč. zhoubných nádorů [5]. Regulují totiž zásadní projevy malignity, jako jsou buněčný růst, proliferace, invaze, angiogeneze či metastazování [6]. Cílem předkládané práce je přehled deregulovaných lncRNA u renálního karcinomu, popis jejich role v patogenezi RCC a možností využití v diagnostice, stanovení prognózy, příp. jako potenciálních terapeutických cílů.

Materiál a metody

Bylo provedeno systematické vyhledávání v databázích Web of Science a Pubmed k datu 3. 10. 2019, zahrnující časové období 2010–2019 a klíčová slova „lncRNA", „long noncoding RNA", „long non-coding RNA", „renal cancer", „renal cell carcinoma" a „kidney cancer". Zadáním („lncRNA*" OR „long noncoding RNA*" OR „long non-coding RNA*") AND („renal cancer" OR „renal cell carcinoma" OR „kidney cancer") v předmětu vyhledávání bylo nalezeno 230 prací, přičemž první relevantní výsledek byl z roku 2011. V první selekci bylo na základě abstrakt vyřazeno 95 prací odpovídajících předem stanoveným vylučovacím kritériím: duplikáty, nedostupný abstrakt, konferenční abstrakta, editorialy, komentáře, přehledové práce, metaanalýzy, zvířecí modely a práce týkající se primárně jiných malignit, zhoubných nádorů obecně nebo nenádorových onemocnění. Následně byla prvním autorem revidována abstrakta zbylých výsledků a ve druhé selekci bylo vyřazeno 12 výsledků, které nepřinášely novou informaci nebo by mohly být pro čtenáře matoucí. Přehled sumarizuje 123 výsledků, u kterých byly revidovány plné texty (schéma 1). Ačkoli se v mnoha pracích překrývá popis biologických funkcí konkrétních lncRNA, jejich prognostický význam i testy *in vitro*, jsou kvůli přehlednosti uvedeny samostatně. Nejvíce studovaným lncRNA je věnován samostatný prostor.



Obr. 1. Diagram vyhledávání.

LncRNA v patogenezi karcinomu z renálních buněk

Tumor supresorové lncRNA

Přehled tumor supresorových lncRNA uvádí tab. 1. Jejich funkce spočívá zejména ve snížení exprese onkogenů nebo útlumu patogenetických drah a regulátorů epiteliálně mezenchymální tranzice (EMT), dále mohou vázat mikroRNA (miRNA, tzv. miRNA „sponging"), příp. destabilizují androgenní receptor (AR). Hladiny těchto lncRNA jsou ve tkáních RCC snížené, což se ve výsledku projevuje stimulací buněčného cyklu, EMT, nadprodukcí onkogenních proteinů, a tím pádem zvýšenou proliferací nádorových buněk, jejich migrací a útlumem apoptózy [7–28].

MEG3

Exprese „maternally expressed gene 3" je u RCC signifikantně snížená. Indukuje apoptózu buněk RCC aktivací vnitřní mitochondriální dráhy, což vede ke snížení hladiny Bcl-2 a prokaspázy 9 a naopak zvýšení hladin kaspázy 9 a uvolnění cytochromu c do cytoplasmy [17]. Dále tlumí buněčný cyklus („G0/G1 arrest") prostřednictvím snížení exprese miR-7, která vede k nadprodukci RASL11B (Ras-like protein family member 11B), čímž navíc inhibuje proliferaci, invazi a migraci buněk ccRCC [16].

SARCC

Tato nádorově supresorová lncRNA se váže na androgenní receptor, čímž do-

Tab. 1. Přehled nádorově supresorových lncRNA v patogenezi karcinomu z renálních buněk a jejich biologických funkcí.

lncRNA	Mechanizmus působení	Poznámka	Odkaz
NR_023387	↓ EMT	↓ onkogen MGP	[7]
ADAMTS9-AS2	miR-27a-3p sponging	↓ FOXO1	[8]
ENST00000434223	↓ Wnt/katenin		[9]
SANT1	↓ SLC47A2	↓ supresorový komplex SFPQ/E2F1/HDAC1	[10]
GASL1	↓ proliferace		[11]
LINC00961	↓ EMT	↓ Slug, N-kadherin	[12]
XIST	miR-106b-5p sponging	↓ p21	[13]
OTUD6B-AS1	↓ Wnt/katenin, ↓ EMT	↓ E-kadherin, N-kadherin, Snail	[14]
KCNQ1DN	↓ c-Myc	↑ cyklin D1	[15]
MEG3	↓ miR-7	↑ zastavení buněčného cyklu ve fázi G0/G1	[16]
	↓ Bcl-2, ↓ prokaspáza 9		[17]
DHRS4-AS1		↑ zastavení buněčného cyklu ve fázi G0/G1	[18]
EGOT			[19]
DANCR			[20]
SARCC	destabilizace AR	↓ Akt, MMP-13, K-RAS, P-ERK	[21, 22]
	destabilizace AR	↓ HIF-2α, c-MYC	[23]
BX357664	↓ TGF-β1/p38/HSP27		[24]
CASC2	cíl miR-21		[25]
TRIM52-AS1			[26]
IRAIN	↑ cyklin D1, ↓ Bax		[27]
GAS5			[28]

ADAMTS9-AS2 – antisense RNA 2 ADAM metalopeptidáza s trombospondinem typu 1 motivu 9, CASC2 – kandidát na náchylnost k nádorům 2, DANCR – protein nekódující RNA antagonizující diferenciaci, DHRS4-AS1 – antisense RNA 4 z rodiny SDR dehydrogenázy/reduktázy, EGOT – transkript onkogeneze eozinofilních granulí, EMT – epiteliálně-mezenchymální tranzice, FOXO1 – vidličkový protein O1, GAS5 – transkript 5 specifický pro zástavu růstu, HDAC1 – histonová deacetyláza 1, IRAIN – intragenní antisense lncRNA na lokusu IGF1R, MGP – matrixový Gla protein, HSP27 – protein tepelného šoku 27, KCNQ1DN – následný soused KCNQ genu, MEG3 – maternálně exprimovaný gen 3, MMP-13 – matricová metaloproteináza 3, OTUD6B-AS1 – antisense RNA 1 obsahující OTU doménu 6B, SARCC – lncRNA potlačující androgenní receptor u renálního karcinomu, SFPQ – sestřihový faktor bohatý na prolin a glutamin, SLC47A2 – protein 2 vytlačující více léků a toxinů, TGF – transformující růstový faktor, TRIM52-AS1 – antisense RNA 1 z rodiny tripartitních motivů 52, XIST – X-neaktivní specifický transkript
↑ – zvýšení exprese, ↓ – utlumení exprese

chází k jeho destabilizaci a inhibici jeho funkce. Následné potlačení exprese miR-143-3p inhibuje další signály, jako jsou *Akt, MMP13, K-RAS* a *P-ERK*, tedy známé onkogeny [21,22]. Destabilizace AR tlumí i další onkogenní dráhu, *HIF-2α/c-Myc*, SARCC se tedy uplatňuje i v regulaci kaskády HIF (hypoxia-inducible factor) [23].

Onkogenní lncRNA

Přehled onkogenních lncRNA je uveden v tab. 2. Tyto lncRNA přímo stimulují produkci jiných onkogenů nebo tlumí expresi nádorových supresorů. V mnoha případech vážou miRNA, čímž přímo blokují jejich působení na cílové mRNA. Stimulují známé patogenetické dráhy RCC – HIF kaskádu, *Wnt/katenin, PI3K/Akt*, EMT. Ve tkáni nádorů jsou exprimovány ve zvýšené míře [29–88].

HOTAIR

„HOX transcript antisense RNA" je jednou z nejznámějších onkogenních lncRNA. V patogenezi RCC se typicky uplatňuje miRNa „sponging". Tímto mechanizmem inhibuje funkci nádorově supresorové miR-124, což vede k nadprodukci 2,8-sialyltransferázy 4, která stimuluje proliferaci, migraci a invazi RCC [42]. Vazba s miR-138, miR-200c, miR-204 nebo miR-217 stimuluje produkci onkogenů, jako jsou *ADAM9, EZH2, ZEB1 či ZEB2*, přičemž zvýšená exprese HOTAIR je stimulována působením estrogenového receptoru β, který se kromě RCC uplatňuje i u jiných nádorových onemocnění [43]. Kompetitivní inhibice miR-217 stimuluje produkci HIF-1α a následně AXL [44].

Tab. 2. Přehled onkogenních lncRNA v patogenezi karcinomu z renálních buněk a jejich biologických funkcí.

lncRNA	Mechanizmus působení	Poznámka	Odkaz
ATB	↓ p53	vazba p53 na DNMT1	[29]
	↑ EMT	cíl TGF-β	[30]
MALAT1	miR-203 sponging	↑ BIRC5 (survivin)	[31]
	miR-182-5p sponging		[32]
	miR-429 sponging		[33]
	↑ Livin		[34]
	↑ EMT	↑ EZH2, β-katenin	[35]
URRCC	↑ p-Akt	↓ FOXO3	[36]
ITGB1	↓ Mcl-1		[37]
GHET1	↑ EMT		[38]
HOTTIP	↑ PI3K/Akt		[39]
	miR-615-3p sponging	↑ IGF-2	[40]
	↓ LATS2		[41]
HOTAIR	miR-124 sponging		[42]
	miR-138, miR-200c, miR-204, miR-217 sponging	↑ estrogen receptor β	[43]
	miR-217 sponging	↑ HIF1α, AXL	[44]
ZFAS1	miR-10a sponging	↑ SKA1	[45]
EGFR-AS1	↓ degradace EGFR mRNA	↑ EGFR	[46]
LINC-PINT	↑ EZH2	↓ p53	[47]
AFAP1-AS1	↓ PTEN		[48]
PVT1	miR-16-5p sponging		[49]
	↑ EGFR		[50]
	↑ Mcl-1		[51]
	miR-200c sponging	↑ ZEB1, ZEB2	[52]
TUG1	miR-9 sponging	↑ YAP	[53]
	miR-196a sponging		[54]
CRNDE	↑ EMT		[55]
	↑ Wnt/katenin		[56]
HOXA11-AS	miR-146b-5p sponging	↑ MMP16	[57]
DUXAP8	miR-126 sponging		[58]
LUCAT1	miR-495-3p sponging	↑ SATB1	[59]
	↑ Akt		[60]
	↓ p57		[61]
SNHG14	miR-203 sponging	↑ N-WASP	[62]
SNHG15	↑ EMT		[63]
THOR	↑ IGF2BP1	↑ IGF, Myc, GLI1	[64]
SNHG1	↓ miR-137		[65]
MIAT	miR-29c sponging		[66]

MALAT1

„Metastasis-associated lung adenocarcinoma transcript 1", označovaný i jako NEAT2 (nuclear-enriched abundant transcript 2), stimuluje EMT a tlumí expresi miR-205 [35]. Inhibice miR-203 zvyšuje expresi onkogenu *BIRS5 (survivinu)*, což vede ke zvýšení proliferace buněk RCC [31]. MALAT1 váže i další tumor supresorové miRNA, jako jsou miR-182-5p a miR-429 [32,33]. Zvýšená exprese MALAT1 u RCC vede k nadprodukci proteinu Livin, který stimuluje nádorový růst zejména blokováním apoptózy [34].

LUCAT1

„Lung cancer associated transcript 1" je důležitým regulátorem proliferace. Stimuluje Akt – signální dráhu, dále se váže na polycomb represivní komplex 2 (PRC2), a tlumí tím exprese nádorového supresoru *p57* [60,61]. Inhibice miR-495-3p stimuluje proliferaci a invazi nadprodukcí genu *SATB1* [59].

HOTTIP

„HOX A transcript at the distal tip" stimuluje signální dránu PI3K/akt [39]. Kompetitivní inhibice miR-615-3p odblokuje produkci jejího cílového proteinu IGF-2, který má stimulační efekt na růst nádorových buněk [40]. Vazba HOTTIP na EZH2 a specifickou lyzinovou demetylázu 1 tlumí expresi nádorově supresorové kinázy LATS2 [41].

PVT1

„Plasmacytoma variant translocation 1" se aktivně zapojuje do procesu EMT. Vazba s miR-200c stimuluje expresi *ZEB1* a *ZEB2*, které snižují hladinu E-kadherinu. Ve tkáni RCC byla prokázána i vyšší exprese sestřihové varianty bez exonu 4 [52]. Ke stimulaci proliferace, invaze a EMT dochází interakcí PVT1 s miR-16-5p [49]. Zvýšená hladina PVT1 dále stimuluje expresi onkogenu Mcl-1, který je významným inhibitorem apoptózy [51]. Dalším popsaným mechanizmem působení PVT1 je aktivace signální dráhy EGFR [50].

UCA1

„Urothelial cancer associated 1", původně spojovaný s uroteliálními nádory močového měchýře, se uplatňuje jako

Tab. 2 – pokračování. Přehled onkogenních lncRNA v patogenezi karcinomu z renálních buněk a jejich biologických funkcí.

lncRNA	Mechanizmus působení	Poznámka	Odkaz
TP73-AS1	↑ PI3K/Akt/mTOR		[67]
CRPAT4	↑ AVL9		[68]
UCA1	miR-129 sponging	↑ SOX4	[69]
	↑ EZH2, ↓ miR-495	↓ p21	[70]
			[71]
GIHCG			[72]
H19	miR-29a-3p sponging	↑ E2F1	[73]
ROR	↓ p53	↑ c-Myc	[74]
RP11--436H11.5	miR-335-5p sponging	↑ BCL-W	[75]
Z38	↑ EMT		[76]
CCAT1	↑ Livin		[77]
NEAT1	miR-34a sponging	↑ c-MET	[78]
	↑ EMT		[79]
MRCCAT1	↓ NPR3	↑ p38-MAPK	[80]
SRLR	↑ IL-6/STAT3		[81]
HEIRCC	↑ EMT		[82]
ANRIL	↑ β-katenin, Ki-67, EMT		[83]
XIST	↓ miR-302c	↑ SDC1	[84]
LINC00152	↓ miR-205	↓ p16	[85]
UC009YBY.1			[86]
lncARSR	miR-34/miR-449 sponging	↑ AXL, c-MET	[87]
5'aHIF-1α	↑ HIF-1α		[88]
3'aHIF-1α			

↓ – snížení, ↑ – zvýšení

AFAP1-AS1 – antisense RNA 1 asociovaná s proteinem spojeným s aktinovými vlákny 1, Akt – protein kináza 2, ATB – lncRNA aktivovaná transformujícím růstovým faktorem β, ANRIL – antisense nekódující RNA na locusu INK4, AVL9 – AVL9 protein spojený s migrací buněk, AXL – tyrozin-protein kinázový receptor UFO, BCL-W – protein 2 podobný Bcl-2, BIRC5 – bakulovirový inhibitor 5 obsahující opakování apoptózy, CCAT1 – transkript 1 spojený s karcinomem tlustého střeva, c-MET – receptor hepatocytového růstového faktoru, CRNDE – protein nekódující gen odlišně exprimovaný u kolorektální neoplázie, CRPAT4 – transkript 4 spojený s prognózou světlobuněčného RCC, DNMT1 – DNA metyltransferáza 1, DUXAP8 – pseudogen 8 dvojitého homeoboxu A, EGFR – receptor epidermálního růstového faktoru, EZH2 – zesilovač zeste homologu 2, EMT – epiteliálně-mezenchymální tranzice, E2F1 – transkripční faktor E2F1, FOXO3 – vidličkový protein O3, GHET1 – transkript 1 vysoce exprimovaný u karcinomu žaludku, GIHCG – lncRNA postupně zvýšená během hepatokarcinogeneze, GLI1 – onkogen spojený s gliomem, HEIRCC – vysoce exprimovaný u renálního karcinomu, HIF1α – hypoxií indukovaný faktor 1 alfa, HOTAIR – antisense RNA transkript HOX genu, HOTTIP – transkript HOX A na distální špičce, HOX – homeobox, IGF – růstový faktor podobný inzulinu, IGF2BP1 – protein 1 vázající mRNA růstového faktoru podobného inzulinu 2, IL-6 – interleukin 6, ITGB1 – integrin beta-1, LATS2 – velká nádorově supresorová kináza 2, LINC-PINT – dlouhý intergenní protein nekódující transkript RNA indukovaný p53, lncARSR – lncRNA aktivovaná u RCC rezistentního na sunitinib, LUCAT1 – transkript 1 spojený s karcinomem plic, MALAT1 – transkript 1 spojený s metastazujícím karcinomem plic, MAPK – mitogenem aktivovaná protein kináza, Mcl-1 – protein diferenciace buněk myeloidní leukémie 1, MIAT – transkript spojený s infarktem myokardu, MMP16 – matrixová metaloproteináza 16, MRCCAT1 – transkript 1 spojený s metastatickým renálním karcinomem, Myc – myelocytomatóza, NEAT1 – jaderně obohacený abundantní transkript 1, NPR3 – C receptor natriuretického peptidu, N-WASP – nervový protein Wiskott-Aldrichova syndromu, PI3K – fosfatydilinositol-3-kináza, PTEN – homolog fosfatázy a tenzinu, PVT1 – translokace varianty plazmocytomu 1, RCC – karcinom z renálních buněk, ROR – regulátor přeprogramování, SATB1 – spaciální protein vázající AT bohatou sekvenci, SDC1 – syndekan 1, SKA1 – protein 1 spojený s vřeténkem a kinetochorem, SNHG1, SNHG14, SNHG15 – hostitelský gen pro malou nukleolární RNA 1, 14, 15, SOX4 – transkripční faktor SOX-4, SRLR – lncRNA spojená s rezistencí RCC na sorafenib, STAT3 – signální měnič a aktivátor transkripce 3, TGF-β – transformující růstový faktor beta, THOR – vysoce konzervovaná onkogenní lncRNA spojená s varletem, TP73-AS1 – antisense RNA 1 spojená s nádorovým proteinem 73, TUG1 – gen regulovaný taurinem 1, UCA1 – spojená s uroteliálním karcinomem, URRCC – lncRNA BX649059, XIST – X-neaktivní specifický transkript, YAP – yes-asociovaný protein 1, ZEB1, ZEB2 – homeobox vázající E-box zinkových prstů 1, 2, ZFAS1 – antisense RNA 1 zinkového prstu NFX

onkogen i v patogenezi RCC [71]. Inhibicí exprese miR-129 stimuluje expresi cílového genu *SOX4* s antiapoptotickým efektem [69]. Ke stimulaci buněčné proliferace buněk RCC dochází zvýšením exprese *EZH2* a interakcí s miR-495. *EZH2* dále tlumí expresi nádorového supresoru *p21* [70].

lncRNA jako diagnostické biomarkery RCC

Ve srovnání s nenádorovou tkání jsou ve tkáni RCC aberantně exprimovány

Tab. 3. Vybrané studie stanovující expresní profily lncRNA u karcinomu z renálních buněk.

Rok	Velikost souboru	Subtyp RCC	Odlišně exprimované lncRNA	Up-regulované	Down-regulované	Odkaz
2012	6	ccRCC	726	146	480	[89]
2013	11	ccRCC	40	14	26	[90]
2015	475	ccRCC	1 943			[91]
2015	15	ccRCC	1 308	568	740	[92,93]
2016	59	chRCC	143	41	102	[94]
2017	530	ccRCC	5 200	2 445		[95]
2018	519	ccRCC	1 518	1 059	459	[96]
2018	5	ccRCC	1 554	943	611	[60]

RCC – karcinom z renálních buněk

Tab. 4. Panely prognostických lncRNA u karcinomu z renálních buněk.

Počet lncRNA	Konkrétní lncRNA	Odkaz
6	CTA-384D8.35, CTD-2263F21.1, LINC01510, RP11-352G9.1, RP11-395B7.2, RP11-426C22.4	[102]
9	RP13-463N16.6, CTD-2201E18.5, RP11-430G17.3, AC005785.2, RP11-2E11.9, TFAP2A-AS1, RP11-133F8.2, RP11-297L17.2, RP11-348J24.2	[103]
6	AC003092.1, AC079160.1, COL18A1-AS1, LINC00520, LINC02154, SLC7A11-AS1	[104]
11	AC016773.1, HOTTIP, LINC00460, NALCN-AS1, PVT1, TRIM36-IT1, WT1-AS, COL18A1-AS1, LINC00443, LINC00472, TCL6	[105]
4	ENSG00000255774, ENSG00000248323, ENSG00000260911, ENSG00000231666	[106]
6	LINC00520, PIK3CD-AS1, LINC01559, CEACAM22P, MSL3P1, TREML3P	[107]
9	SLC25A5-AS1, COL18A1-AS1, WT1-AS1, AC016773.1, LINC00460, LINC00313, HOTTIP, FGF14-AS1, AS10502.1	[96]
19	LOC606724, SCART1, SNORA8, LOC728024, HAVCR1P1, FCGR1CP, LINC00240, LINC00894, GK3P, SNHG3, KIAA0125, URB1-AS1, ZNF542P, TINCR, LINC00926, PDXDC2P, COL18A1-AS1, LINC00202-1, LINC00937	[108]
4	RAB31, ACTN4	[109]
2	ENSG00000241684, NEAT1	[110]
6	COL18A1-AS1, WT1-AS, LINC00443, TCL6, AL356356.1, SLC25A5.AS1	[111]
7	AFAP1-AS1, GAS6-AS1, RP11-1C8.7, RP11-21L19.1, RP11-503C24.1, RP11-536I6.2, RP11-63A11.1	[112]
2	PVT1, DUXAP8	[95]
2	CRNDE, ENSG00000244020	[113]
5	AC069513.4, AC003092.1, CTC-205M6.2, RP11-507K2.3, U91328.21	[114]

stovky lncRNA (tab. 3). Výrazně deregulované lncRNA v nádorové tkáni ve srovnání s tkání nenádorovou tedy mohou představovat potenciální diagnostické biomarkery pro odlišení pacientů s RCC od pacientů bez nádoru. Expresní profily lncRNA jsou stanoveny vysokokapacitními metodami jako microarray assay, sekvenování nové generace nebo sekvenování na čipu. Novější práce obvykle využívají dostupné informace z TCGA (The Cancer Genome Atlas), kdy jsou vybrané lncRNA poté validovány na kohortě pacientů s RCC [60,89–96].

Ačkoli množství prací udává signifikantní rozdíly v expresi jednotlivých lncRNA mezi nádorovou a nenádorovou tkání, pouze v několika z nich se objevují údaje o diagnostické přesnosti umožňující odlišení pacientů od zdravých kontrol, jako je ROC analýza či stanovení senzitivity a specificity [14,18,97]. Výsledky u PVT1 (AUC 0,8567, senzitivita 86,67 %, specificita 76,67 %), LUCAT1 (AUC

0,7756, senzitivita a specificita 90 %) a LINC00982 (AUC 0,9578, senzitivita 76,67 %, specificita 66,67 %) se jeví z hlediska diagnostiky jako velmi slibné [98].

Stanovení hladin cirkulujících lncRNA jako minimálně invazivního způsobu diagnostiky RCC zapadá do konceptu tzv. tekuté biopsie (liquid biopsy). Kromě cirkulujících nádorových buněk a cirkulující nádorové DNA jsou již dostupné práce i o využití lncRNA v detekci různých nádorových onemocnění [99]. Informace o možném využití cirkulujících lncRNA jako biomarkerů RCC jsou zatím nedostatečné. Ojedinělé studie popisují dobrou diskriminační schopnost pro odlišení pacientů s RCC od zdravých kontrol (AUC 0,920, senzitivita 87 %, specificita 84,8 %) a dokonce odlišení pacientů v časném stadiu RCC od zdravých kontrol (AUC 0,886, senzitivita 80,7 %, specificita 84,8 a více) u onkogenní lncRNA GIHCG [72]. Panel pěti cirkulujících lncRNA (LET – low expression in tumor, PVT1 – plasmacytoma variant translocation, PANDAR – promoter of CDKN1A antisense DNA damage activated RNA, PTENP1 – phosphatase and tensin homolog pseudogene 1, linc00963) dokáže spolehlivě odlišit ccRCC od zdravých kontrol v tréninkové (AUC 0,90, senzitivita 79,2 %, specificita 88,9 %) i testovací kohortě (AUC 0,823, senzitivita 67,6 %, specificita 91,4 %). Pro odlišení ccRCC stadia I byla dosažena AUC 0,85 [100]. Sérové hladiny ACTB a MALAT1 nejsou u pacientů s urologickými nádory (vč. RCC) signifikantně vyšší než u nenádorových onemocnění [101].

LncRNA jako prognostické biomarkery

Aberantní exprese lncRNA koreluje u mnoha lncRNA s klinickopatologickými charakteristikami tumoru a ovlivňuje prognózu pacienta. Nejčastěji sledovanými parametry jsou celkové přežití a nádorově specifické přežití. Dostupná data se opírají buď o výzkum jednotlivých lncRNA, nebo se jedná o analýzu dat z TGCA databáze, kdy je obvykle jako prognostický biomarker hodnocen panel několika lncRNA, které jsou použity ke kalkulaci rizikového skóre. V případě onkogenních lncRNA je vyšší exprese vesměs spojena s horší prognózou,

Tab. 5. Vztah expresních hladin jednotlivých lncRNA s prokazatelným vztahem k prognóze karcinomu z renálních buněk.

lncRNA	Exprese	Prognóza	Odkaz
ADAMTS9-AS2	↓	↓	[8]
ENST00000434223	↓	↓	[9]
DHRS4-AS1	↓	↓	[18]
LOC389332	↓	↓	[115]
TCL6	↓	↓	[116]
SDPR-AS	↓	↓	[117]
NBAT-1	↓	↓	[118]
HOTTIP	↑	↓	[39]
ZFAS1	↑	↓	[45]
EGFR-AS1	↑	↓	[46]
OTUD6B-AS1	↑	↓	[14]
LINC-PINT	↑	↓	[47]
AFAP-AS1	↑	↓	[48]
PVT1	↑	↓	[49,50,52,119]
SNHG6	↑	↓	[120]
CRNDE	↑	↓	[55]
HOTTIP	↑	↓	[40]
LUCAT1	↑	↓	[59–61]
MIAT	↑	↓	[66]
TP73-AS1	↑	↓	[67]
GIHCG	↑	↓	[72]
ROR	↑	↓	[74]
RP11-436H11.5	↑	↓	[75]
NEAT1	↑	↓	[78,79]
MFI2-AS1	↑	↓	[121]
MRCCAT1	↑	↓	[80]
ATB	↑	↓	[122]
PANDAR	↑	↓	[123]
UCA1	↑	↓	[70]
SLINKY	↑	↓	[124]
TUG1	↑	↓	[125]
LINC00152	↑	↓	[85,126]
MALAT1	↑	↓	[35,127]
ZNF180-2	↑	↓	[97]
H19	↑	↓	[128]
CADM1-AS1	↓	↓	[129]
SPRY-IT1	↑	↓	[130]

↑ – zvýšená exprese, ↓ – snížená exprese / horší prognóza

Tab. 6. lncRNA jako potenciální terapeutické cíle léčby karcinomu z renálních buněk.

lncRNA	*In vitro* test	Efekt	Odkaz
ADAMTS9-AS2	↑	POS	[8]
ATB	↓	POS	[29,30]
GASL1	↑	POS	[11]
LINC00961	↑	POS	[132]
ITGB1	↑	NEG	[37]
GHET1	↓	POS	[38]
HOTTIP	↓	POS	[39,40]
	↑	NEG	[39]
ZFAS1	↓	POS	[45]
OTUD6B-AS1	↑	POS	[14]
KCNQ1DN	↑	POS	[15]
LINC-PINT	↓	POS	[47]
AFAP1-AS1	↓	POS	[48]
PVT1	↓	POS	[49,51,98]
MALAT1	↓	POS	[32,33,35,127]
	↑	NEG	[34]
SNHG15	↓	POS	[63]
THOR	↑	NEG	[64]
	↓	POS	[64]
SNHG1	↓	POS	[65]
LUCAT1	↓	POS	[60]
DHRS4-AS1	↑	POS	[18]
TP73-AS1	↑	NEG	[67]
CRPAT4	↓	POS	[68]
UCA1	↓	POS	[69–71]
GIHCG	↓	POS	[72]
H19	↓	POS	[73,128]
ROR	↓	POS	[74]
EGOT	↑	POS	[19]
RP11-436H11.6	↓	POS	[75]
DANCR	↑	POS	[20]
Z38	↓	POS	[76]
CCAT1	↓	POS	[77]
MRCCAT1	↓	POS	[80]
PANDAR	↓	POS	[123]
HOTAIR	↓	POS	[44]
SLINKY	↓	POS	[124]
HEIRCC	↓	POS	[82]
ANRIL	↓	POS	[83]

u tumor supresorových lncRNA je vztah opačný. Studie zkoumající prognostický význam panelů o různém počtu lncRNA jsou prezentovány v tab. 4 [95,96,102–114]. Přehled jednotlivých lncRNA ve vztahu k prognóze RCC je uveden v tab. 5 [8,9,14,18,35,39,40,45–50,52,55,59–61,66,67,70,72,74,75,78–80,85,97,115–130].

LncRNA jako prediktivní biomarkery odpovědi na cílenou léčbu

Byla identifikována ARSR (lncRNA activated in RCC with sunitinib resistance), jejíž vysoká hladina exprese koreluje se špatnou odpovědí na sunitinib. Mechanizmus tohoto působení spočívá v kompetitivní inhibici miR-34 a miR-449, čímž dochází ke zvýšení exprese *AXL* a *c-MET* v nádorových buňkách. Navíc se ARSR může inkorporovat do exozomů a přenášet i na senzitivní buňky, což dále zvyšuje rezistenci na sunitinib [87]. Některé genetické varianty ARSR, např. rs7859384ARSR, jsou však spojeny s lepší citlivostí na léčbu [131]. Hladina nádorově supresorové lncRNA SARCC (supressing androgen receptor in RCC) se během léčby sunitinibem zvyšuje, což potencuje efekt této léčby a citlivost na ni [21]. Ve vztahu k léčbě sorafenibem je popsána lncRNA SRLR (sorafenib resistence-associated lncRNA in RCC), která je ve zvýšené míře exprimována u RCC rezistentních na sorafenib. Její utlumení senzitizuje původně neodpovídající buňky k léčbě sorafenibem [81]. *In vitro* testování prokázalo zvýšení senzitivity buněk RCC na sorafenib i po utlumení exprese onkogenní NEAT1 [78].

LncRNA jako potenciální terapeutické cíle

Úvahy o využití lncRNA jako terapeutických cílů vychází z *in vitro* (příp. *in vivo* na zvířecích modelech) experimentů, při kterých je exprese studované lncRNA utlumena nebo zvýšena, případně je provedena transfekce buněčných linií konkrétní lncRNA. Následně je pozorován vliv na biologické vlastnosti buněčných linií, jako je proliferace, migrace či apoptóza. Tab. 6 prezentuje výsledky *in vitro* experimentů u konkrétních lncRNA, které autoři po-

Tab. 6. lncRNA jako potenciální terapeutické cíle léčby karcinomu z renálních buněk.

lncRNA	*In vitro* test	Efekt	Odkaz
XIST	↓	POS	[84]
SDPR-AS	↑	POS	[117]
NEAT1	↓	POS	[79]
FTX	↓	POS	[133]
BX357664	↑	POS	[24]
CRNDE	↑	NEG	[56]
UC009YBY.1	↓	POS	[86]
CASC2	↑	POS	[25]
TRIM52-AS1	↑	POS	[26]
IRAIN	↑	POS	[27]
LINC00152	↑	NEG	[126]
	↓	POS	[126]
MEG3	↑	POS	[17]
NBAT-1	↓	NEG	[117]
CADM-AS1	↑	POS	[129]
SPRY-IT1	↓	POS	[130]
GAS5	↑	POS	[28]

↑ – zvýšení exprese, ↓ – utlumení exprese, POS – příznivý efekt (potlačení nádorových vlastností v buněčné linii), NEG – nepříznivý efekt (stimulace nádorových vlastností v buněčné linii)

važují za potenciální terapeutické cíle u pacientů s RCC [8,11,14,15,17–20,24–30,32–35,37–40,44,45,47–49,51,56,60,63–65,67–77,79,80,82–84,86,98,117,118,123,124,126–130,132,133].

Závěr

U RCC je popsána aberantní exprese mnoha lncRNA. Uplatňují se jako onkogeny i jako nádorové supresory a v patogenezi RCC sehrávají důležitou roli při regulaci buněčné proliferace, buněčného cyklu, apoptózy, migrace, invaze a metastazování. Mechanizmus jejich působení zahrnuje známé dráhy jako VHL/HIF kaskáda, Wnt-katenin, PI3K/Akt, dále EMT či přímou regulaci známých onkogenů nebo represi tumor supresorů. V mnoha případech vážou miRNA („sponging"), a brání tak jejich vazbě na mRNA. Odlišné expresní profily, vztah k prognóze onemocnění a reakce na cílenou léčbu naznačují potenciální využití lncRNA jako biomarkerů pro časnou detekci RCC a stanovení jeho prognózy. Výsledky testů na buněčných liniích *in vitro* („knockdown" onkogenních lncRNA nebo stimulace nádorově supresorových lncRNA) jsou příslibem pro využití lncRNA jako terapeutických cílů. V době nastupující imunoterapie RCC lze očekávat další intenzivní výzkum lncRNA ve vztahu k receptoru PD-1, resp. PD-L1.

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7. Future perspectives

Short and long non-coding RNAs have been the target of intensive clinical research in the last two decades in terms of potential biomarkers and therapeutic targets for cancer treatment. Given the amount of preclinical evidence that have indicated the role of miRNAs in cancer resistance, another goal of research is to utilize miRNAs to increase the sensitivity of tumour cells to systemic treatment (56). Despite clinical evidence, miRNAs have not yet been used in routine clinical practice. There are several possible explanations (57):

1. The amount of miRNA biomarkers have been studied in groups with limited number of subjects and without validation cohorts.

2. It is difficult to overcome side effects of miRNA-based treatment, which is due to the simultaneous regulation of multiple targets. An example is MRX34 (liposomal formulation of miR-34a) halted due to severe immune-mediated adverse effects.

3. The need for a better delivery system and ensuring the highest possible stability of oligonucleotides in vivo.

Further research of other groups of small non-coding RNAs (piRNA, tsRNA) may be the key in terms of new therapeutic targets for RCC.

Similarly, there are several unresolved issues regarding lncRNAs, such as the exact mechanism of abnormal lncRNA expression in the tumour or lack of information on the various upstream regulatory mechanisms of lncRNA, such as histone status, DNA methylation patterns, transcription factors or post-transcriptional mechanisms (58).

Recent research on genomic enhancers and non-coding RNAs generated from enhancers, enhancer RNAs (eRNAs), has demonstrated their functional role in both normal and tumour cells, especially gene activation and interactions with transcriptional activators and co-activators (59). Due to their length, eRNAs are sometimes considered a subset of lncRNAs, although their properties are more variable than lncRNAs. Thus, eRNAs can be expected to become another target for the diagnosis and treatment of cancer.

An interesting area of research is exosomal lncRNA. Exosomes represent a medium for lncRNA transmission among tumour cells and their content (including lncRNAs) is another possible target of so-called liquid biopsy in tumour diagnosis (60). Engineered exosomes,

capable of carrying anti-tumour drugs, proteins or therapeutic miRNAs are promising in terms of treatment (61).

8. Conclusion

The habilitation thesis presents the author's systematic work in the field of renal cell carcinoma research in terms of suitable biomarkers for the diagnosis and monitoring of the disease. It documents close and productive multidisciplinary cooperation of the clinical field (urology) and molecular medicine, which results in the presented publication outputs in important professional periodicals supported by active participation in national and international forums. In the introductory part of the thesis presents the issue of renal cell carcinoma mainly in relation to the clinical stage and prognosis of the disease, thus building a rational basis for efforts to detect the disease early, while describing the main molecular genetic mechanisms involved in its pathogenesis. The next part justifies the need to find biomarkers of this disease and presents individual groups of biomarkers. The main part of the work is a detailed description of non-coding RNAs, where the presented results clearly indicate their possible use in the diagnosis and prognosis of renal cell carcinoma. It can be stated that the presented work fulfilled the goals set by the author and in addition to a detailed description of the issue can also serve as an impetus for further scientific work with the aim of implementing the acquired knowledge into clinical practice and finding other modern diagnostic and therapeutic methods in clinical oncology.

9. List of figures and tables

10. List of abbreviations

AKT	Protein kinase B
ASR	Age standardized rate
AQP1	Aquaporin 1
ATP	Adenosine triphosphate
AUC	Area under the ROC curve
BAP1	BRCA1 associated protein 1
Bcl-2	B-cell lymphoma 2
ccRCC	Clear-cell renal cell carcinoma
CD	Cluster of differentiation
CEITEC	Central European Institute of Technology
CTC	Circulating tumour cell
ctDNA	Circulating tumour DNA
CTLA4	Cytotoxic T-lymphocyte antigen 4
ČR	Czech Republic
DNA	Deoxyribonucleic acid
EAU	European Association of Urology
EGFR	Epidermal growth factor receptor
EMT	Epithelial – mesenchymal transition
eRNA	Enhancer RNA
FN	University Hospital
Glut1	Glucose transporter 1
GSK3	Glycogen synthase kinase 3
HGF	Hepatocyte growth factor
HIF	Hypoxia inducible factor
HOTAIR	HOX transcript antisense intergenic RNA
KIM-1	Kidney injury molecule 1

LF MU	Faculty of Medicine of Masaryk University
MAPK	Mitogen-activated protein kinase
MET	Hepatocyte growth factor receptor
miRNA	MicroRNA
mRNA	Mediator RNA
mTOR	Mammalian target of rapamycin
MUC1	Mucin 1
MYC	Myelocytomatosis viral oncogene homolog
NMP22	Nuclear matrix protein 22
NOR	National Cancer Registry
PBRM1	Protein polybromo-1
PDGF	Platelet-derived growth factor
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PFS	Progression-free survival
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
piRNA	PIWI-interacting RNA
PIWI	P-element induced wimpy testis
PIWIL	Piwi-like
PI3K	Phosphoinositide 3-kinase
pRCC	Papillary renal cell carcinoma
Pre-miRNA	Precursor microRNA
PTEN	Phosphatase and tensin homolog
RCC	Renal cell carcinoma
RISC	RNA-induced silencing complex
ROC	Receiver operating characteristic
RNA	Ribonucleic acid

rRNA	Ribosomal RNA
SETD2	SET domain containing 2
siRNA	Small interfering RNA
snoRNA	Small nucleolar RNA
snRNA	Small nuclear RNA
TATI	Tumour-associated trypsin inhibitor
TGFα	Transforming growth factor alpha
TGFβ	Transforming growth factor beta
tRNA	Transfer RNA
tsRNA	tRNA-derived small RNA
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-Lindau
Wnt	Wingless Int-1
WoS	Web of Science
ZEB1	Zinc Finger E-Box Binding Homeobox 1
SIP1	Zinc finger E-box-binding Homeobox 2

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