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# Expression of miRNA-106b in conventional renal cell carcinoma is a potential marker for prediction of early metastasis after nephrectomy

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## Abstract

**Background:** MicroRNAs are endogenously expressed regulatory noncoding RNAs. Previous studies have shown altered expression levels of several microRNAs in renal cell carcinoma.

**Methods:** We examined the expression levels of selected microRNAs in 38 samples of conventional renal cell carcinoma (RCC) and 10 samples of non-tumoral renal parenchyma using TaqMan real-time PCR method.

**Results:** The expression levels of miRNA-155 ( $p < 0.0001$ ), miRNA-210 ( $p < 0.0001$ ), miRNA-106a ( $p < 0.0001$ ) and miRNA-106b ( $p < 0.0001$ ) were significantly over-expressed in tumor tissue, whereas the expression of miRNA-141 ( $p < 0.0001$ ) and miRNA-200c ( $p < 0.0001$ ) were significantly decreased in RCC samples. There were no significant differences between expression levels of miRNA-182 and miRNA-200b in tumor samples and renal parenchyma. Our data suggest that expression levels of miRNA-106b are significantly lower in tumors of patients who developed metastasis ( $p = 0.030$ ) and miR-106b is a potential predictive marker of early metastasis after nephrectomy in RCC patients (long-rank  $p = 0.032$ ).

**Conclusions:** We have confirmed previous observations obtained by miRNA microarray analysis using standardized real-time PCR method. For the first time, we have identified a prognostic significance of miRNA-106b, which, after validation on a larger group of patients, maybe useful as a promising biomarker in patients with RCC.

## Background

Renal cell carcinoma (RCC) accounts for approximately 3% of cancers in adults as well as 85% of all primary malignant kidney tumors. It is the third most common urological cancer after prostate and bladder cancer but it has the highest mortality rate at over 40% [1,2]. Clear cell (conventional) carcinoma is the most common subtype of RCC and accounts for approximately 75-80% of these tumors [3]. Apart from surgery, it is both chemotherapy and radiotherapy resistant. The present absence of biomarkers for early detection and follow-up of the disease is responsible for late diagnosis and subsequent poor prognosis. It is necessary, therefore, to improve our understanding of RCC's pathogenesis, identify new biomarkers

enabling prediction of early metastasis after nephrectomy, and develop new targeted therapies.

One of the most modern and progressive approaches for molecular characterization of tumors today is based on microRNA expression profiles. MicroRNAs (miRNAs) are short noncoding RNAs, 18-25 nucleotides in length, that post-transcriptionally regulate gene expression. Depending upon the extent of their complementarity with target mRNA, miRNAs act by two mechanisms of post-transcriptional regulation of gene expression, which lead to target mRNA degradation or repression of its translation and consequent decrease of particular protein levels. Bioinformatics have predicted that miRNAs have the capacity to regulate one third of all mammalian genes, among which are included a significant number of important oncogenes and tumor suppressor genes [4,5]. MiRNAs have been studied most intensively in the field of oncological research, and emerging evidence suggests

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that altered miRNA regulation is involved in the pathogenesis of cancer [6-8]. Changes in the expression of miRNAs have been observed in a variety of human cancers [9-11].

Several studies have focused on miRNAs' significance in RCC [12]. These papers described the potential of miRNA profiles to distinguish tumor tissue from normal renal parenchyma [13-20], classify renal cell carcinomas according to histological subtypes [13-15], identify expression profiles to predict metastasis from primary tumors [13,16], and determine prognosis for particular renal cell carcinoma patients [13,16]. Most of these studies have been methodologically based on hybridization microarray platforms and mostly included only small numbers of patients. Nevertheless, there exists a certain intersection between groups of miRNAs identified in individual studies, and several interesting mechanistic studies have revealed the functions of some miRNAs in vitro [21].

The aim of our study was to validate expression changes of selected miRNAs identified in previous microarray studies (miR-155 [16], miR-106a [19], miR-106b [19], miR-200b [16,19], miR-200c [15,16,19], miR-141 [15,16], miR-182 [13] and miR-210 [16,19]) by the standardized and more quantitative method that is real-time polymerase chain reaction (PCR). For the first time, we have correlated miRNAs with the relapse-free survival of RCC patients in order to evaluate them as potential predictive biomarkers of early metastasis after nephrectomy.

## Patients and methods

### Study population

Thirty-eight patients (24 men, 14 women) diagnosed for clear cell renal cell carcinoma at Masaryk Memorial Cancer Institute (Brno, Czech Republic) between 2003 and 2009 were included in this study. The study has been approved by the local Ethical Committee. Patients' ages ranged between 41 and 89 years, with a median of 68. Histological diagnosis was established according to the guidelines of the World Health Organization. Cases were selected according to tissue availability and were not stratified for any known preoperative or pathological prognostic factor. Clinical follow-up data in the form of annually assessed survival time was available for all patients. The median follow-up time for all cases was 40 months and ranged from 3 to 105 months. Clinical characteristics of the patients are summarized in Table 1.

### Tissue sample preparation and miRNA purification

Under the supervision of an experienced pathologist, 48 tissue samples were collected (before any treatment was started) from surgically resected tissues - 38 samples from primary tumors and 10 from adjacent non-tumoral

**Table 1: Patient characteristics**

Factor	Number
<b>Age</b>	
mean	68
range	41-89
<b>Sex</b>	
male	24
female	14
<b>Stage</b>	
T1+T2	19
T3	19
<b>Fuhrman grade</b>	
G1	6
G2	25
G3	7
<b>Early recurrence</b>	
Yes*	15
No**	23

\* Recurrence-free survival = 11.5 (5-36) months

\*\* Follow-up = 50 (41-62) months

Medians and 25th and 75th percentiles in parentheses.

renal parenchyma. All samples were immediately stored in liquid nitrogen until RNA extraction. Samples were homogenized (Retch MM301) in sterile conditions before total RNA isolation. Total RNA isolation and small RNA enrichment procedures were performed using the mir-Vana miRNA Isolation Kit (Ambion, USA) according to the manufacturer's instructions. DNA was extracted using the Qiagen DNA Mini Kit (Qiagen, Germany), again following the manufacturer's instructions. Nucleic acid concentration and purity were controlled by UV spectrophotometry (A260:A280 > 2.0; A260:A230 > 1.8) using a Nanodrop ND-1000 (Thermo Scientific, USA).

### Real-time quantification of miRNAs by stem-loop RT-PCR

Complementary DNA (cDNA) was synthesized from total RNA using gene-specific primers according to the TaqMan MicroRNA Assay protocol (PE Applied Biosystems, Foster City, Calif., USA). Reverse transcriptase (RT) reactions utilized 10 ng of RNA sample, 50 nM of stem-loop RT primer, 1 × RT buffer and 0.25 mM each of dNTPs, 3.33 U/μl MultiScribe RT and 0.25 U/μl RNase inhibitor (all from the TaqMan MicroRNA Reverse Transcription kit of Applied Biosystems; 4366597). Reaction mixtures (15 μl) were incubated in a TGradient thermal cycler (Biometra) for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, and then held at 4°C. Real-time PCR was performed using the Applied Biosystems 7500 Sequence

Detection System. The 20- $\mu$ l PCR reaction mixture included 1.3  $\mu$ l of RT product, 1  $\times$  TaqMan (NoUmpErase UNG) Universal PCR Master Mix, and 1  $\mu$ l of primer and probe mix of the TaqMan MicroRNA Assay protocol (PE Applied Biosystems). Reactions were incubated in a 96-well optical plate at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 10 min. The threshold cycle data were determined using the default threshold settings. All real-time PCR reactions were run in triplicate and average threshold cycle ( $C_T$ ) and SD values were calculated.

#### Data normalization and statistical analysis

Expression data were normalized according to expression of the RNU6B reference DNA (Assay No. 4373381; Applied Biosystems). Statistical differences between miRNA levels in RCCs and RP and differences in therapy response in relation to miRNA levels were evaluated using the nonparametric Mann-Whitney U test between 2 groups. Survival analyses were performed using the long-rank test and Kaplan-Meier plots approach. All calculations were performed using Statistica software version 6.0 (StatSoft Inc., USA).

#### Results

We identified gene expression levels of the studied miRNAs in 38 RCCs and 10 non-tumoral renal parenchyma (RP). Differences between the two groups were evaluated using the Mann-Whitney test and also by the Wilcoxon test for ten paired samples. Both methods identified highly significant differences between RCC and RP in the expression levels of the most studied miRNAs. Significance levels and medians of the relative expression values with their ranges defined by the 25th and 75th percentiles are presented in Table 2. The real-time PCR analysis indicated no significant difference between RCC and the RP in expression levels of miR-200b and miR-182. By contrast, the expression levels of miR-155, miR-210, miR-106a and miR-106b were significantly upregulated in the tumor compared to the RP. The most significant difference was seen for miR-210, for which the expression levels were more than 60 times higher in RCC tissue. Conversely, miR-141 and miR-200 were significantly downregulated in RCCs (Table 2). The most significant difference was observed in miR-141, with levels in RCCs approximately 15 times lower than in the RP.

To evaluate the potential association of individual miRNAs with the prognosis of RCC patients after nephrectomy, the expression levels of each miRNA in the group of patients who developed metastasis were compared with their levels in the group of patients in remission. Metastatic patients tended to have lower levels of miR-155, miR-106a and miR-106b in RCCs, but only miR-106b reached statistical significance ( $p = 0.03$ ) (Table 3).

To determine differences for relapse-free survival on the basis of miR-106b expression, Kaplan-Meier plots were constructed and the long-rank test was performed. This analysis demonstrated significant difference ( $p = 0.032$ ). On the other hand, no association was found between the development of metastasis and miR-155, miR-210, miR-106a, miR-200b and miR-200c (Table 3).

#### Discussion

It has become evident that genomic information for transcribing miRNAs is implemented in the human genome, and miRNAs constitute a robust regulatory network with post-transcription regulatory efficiency for almost one third of human coding genes. A number of different approaches to quantifying miRNAs in tumors and non-tumoral tissue have been described, including microarrays, modified invader assay, bead-based flow cytometric assay, and real-time PCR [11]. The main advantage of real-time PCR is the fact that it is a more quantitative and more sensitive method compared with other high-throughput assays. In our study, we have analyzed expression levels of selected miRNAs previously identified by global miRNA profiling studies in RCC clinical specimens as suspected diagnostic biomarkers using a standardized TaqMan real-time PCR approach on a larger group of RCC patients. This validation is necessary if one is to draw conclusions from the findings derived from hybridization microarray analysis.

One of the most frequently studied miRNAs in cancer biology, miR-155, has repeatedly been identified through miRNA microarray profiling as upregulated also in RCC tissue [15,16]. We have confirmed observations from these studies, inasmuch as miR-155 levels were almost 30 times higher in RCCs compared to RP. The available experimental evidence indicates that miR-155 is overexpressed in a variety of malignant tumors (breast, lung, colon, head/neck), which allows us to include this miRNA into the list of oncogenic miRNAs with high importance in cancer diagnosis and prognosis [22].

Three miRNA microarray studies have revealed downregulation of miR-141 and miR-200c in RCC tissue [15,16,18]. In agreement with these results, we have observed 20 times higher levels of miR-141 and 10 times higher levels of miR-200c in RP compared to RCCs. Both miR-200c and miR-141 are members of the miR-200 family that is mechanistically associated with the process of epithelial-mesenchymal transition (EMT). EMT is characterized by a decrease of E-cadherin, loss of cell adhesion, and increased cell motility leading to promotion of metastatic behavior of cancer cells (including RCC) [23]. A molecular link between EMT and the miR-200 family is represented by zinc-finger E-box binding homeobox 1 (ZEB1), a crucial inducer of EMT in various human tumors directly suppressing transcription of miR-141 and

**Table 2: Expression levels of selected miRNAs in renal cell carcinoma (RCC) in comparison to renal parenchyma (RP)**

	non-paired samples		p-value	paired samples		p-value
	RCC n = 38	RP n = 10		RCC n = 10	RP n = 10	
<b>miRNA-155</b>	12.261*	0.430	<b>&lt; 0.0001</b>	17.882	0.430	<b>0.002</b>
	3.945-24.127**	0.180-0.811		9.234-30.451	0.179-0.811	
<b>miRNA-141</b>	0.107	1.937	<b>0.002</b>	0.296	1.937	<b>0.004</b>
	0.047-0.630	1.128-2.805		0.107-1.235	1.128-2.805	
<b>miRNA-210</b>	38.947	0.592	<b>&lt; 0.0001</b>	28.264	0.592	<b>0.002</b>
	25.153-74.817	0.488-0.701		7.750-73.600	0.488-0.701	
<b>miRNA-200c</b>	0.268	2.41	<b>&lt; 0.0001</b>	0.259	2.41	<b>0.002</b>
	0.171-0.508	1.910-3.408		0.171-0.565	1.910-3.408	
<b>miRNA-106a</b>	20.557	4.934	<b>&lt; 0.0001</b>	22.846	4934	<b>0.004</b>
	13.302-31.403	3.857-7.910		13.302-28.127	3.857-7.910	
<b>miRNA-106b</b>	14.102	2.937	<b>&lt; 0.0001</b>	16.356	2973	<b>0.002</b>
	8.807-20.746	1.941-3.963		12.101-30.239	1.942-3.963	
miRNA-200b	7.384	5.702	0.559	5.756	5702	0.846
	3.599-15.578	4.715-8.260		1.892-9.571	4.715-8.260	
miRNA-182	0.147	0.121	0.919	0.19	0.121	0.322
	0.047-0.515	0.086-0.154		0.038-0.491	0.086-0.154	

\* Medians of expression level related to RNU6B with \*\*25th and 75th percentiles.

miR-200c, which strongly activate epithelial differentiation in pancreatic, colorectal and breast cancer cells [24]. On the other hand, ZFH1B, also known as ZEB2 and Smad-interacting protein 1 (SIP1), was identified as the common target of miR-141 and miR-200c. It already has been reported that ZFH1B is upregulated in a variety of human carcinomas and that it may function as a transcriptional repressor for E-cadherin [23].

Huang et al. [20] have described induction of miR-210 expression under the hypoxic conditions dependent on HIF- $\alpha$  expression. Mutations in the *VHL* gene, one of the key events in RCC pathogenesis, is associated with accumulation of HIF- $\alpha$ . Consistent with these findings and with previous profiling studies [16,19,20], we have observed more than 60 times higher levels of miR-210 in tumors.

We have identified significant upregulation of miR-106a and miR-106b in RCCs with approximately 4 and 5 times higher levels, respectively. Our data are in agreement with the results of Chow et al. [21], who used an approach based on real-time PCR. Interestingly, miR-106a and miR-106b upregulation has not been detected by any other group focused on miRNA profiling in RCC [13-18], probably due to the lower sensitivity and lower dynamic range of hybridization-based microarrays. Overexpression of miR-106b, however, has been observed in a

variety of human tumors, including colorectal cancer [25], gastric cancer [26], hepatocellular carcinoma [27] and head and neck squamous cell carcinomas [28].

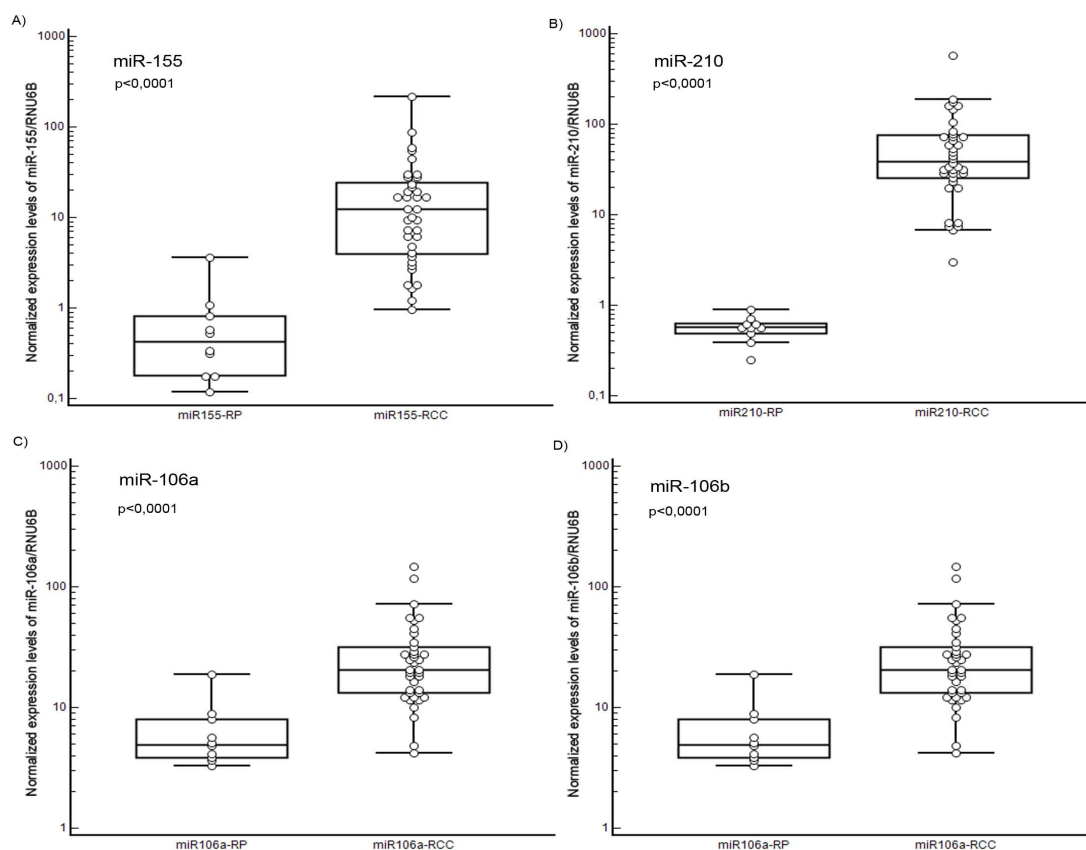
We have not confirmed significant differences in miR-182 and miR-200b levels between RCCs and RP as reported by Petillo et al. [13], Jung et al. [16] and Chow et al. [19].

To date, only one study was done focusing on miRNAs' significance in RCC prognosis, and that involved a group of 8 RCC patients (4 patients indicated good and 4 poor prognosis) [13]. Petillo et al. [13] identified a group of 20 miRNAs enabling classification of RCC patients according to their prognosis. We have tested only one (miR-182) of these 20 miRNAs and have not proven its prognostic significance. Moreover, other analyzed miRNAs were evaluated as possible prognostic factors enabling the prediction of early metastasis after nephrectomy, and, except for miR-106b, none of these indicated significant potential to predict prognosis. Surprisingly, miR-106b, considered to be oncogenic [29], has significantly higher expression levels in RCC of patients with better prognosis. A possible explanation for this contradiction lies in the involvement of the miR-106b family (miR-106b, miR-93, and miR-25) in TGF- $\beta$  signaling [30]. The role of TGF- $\beta$  signaling in cancer pathogenesis is characteristically ambiguous [31]. In the early events of carcinogene-

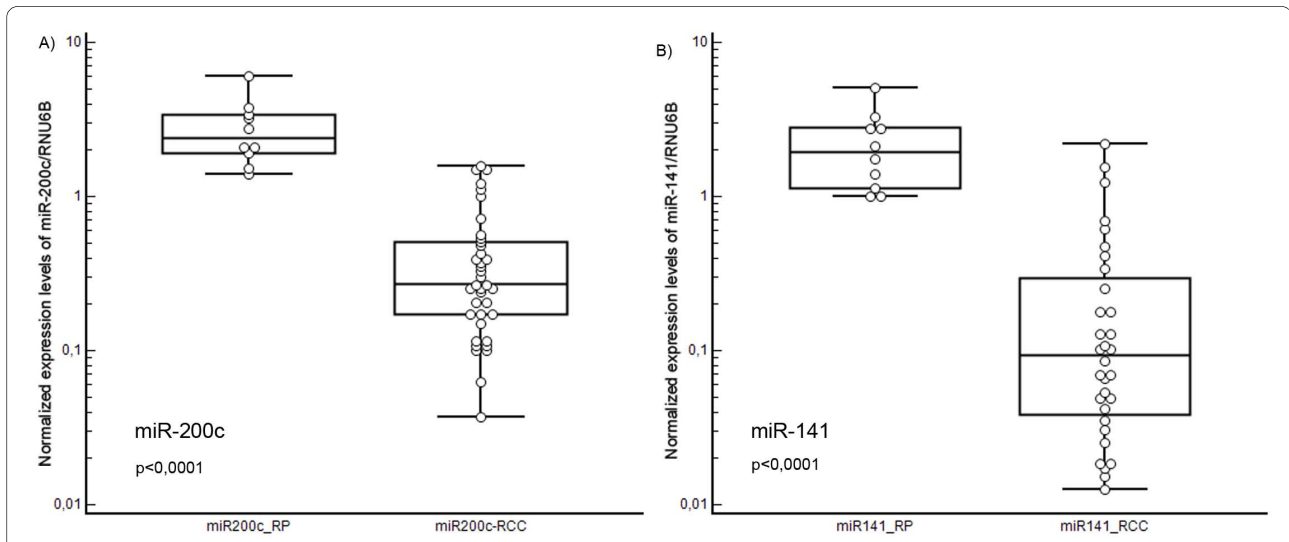
**Table 3: Expression levels in RCC of patients with/without development of metastatic disease**

	Metastatic disease n = 15	Relapse-free disease n = 23	p-value
miRNA-155	6.07* 1.80-21.03**	15.58 7.29-27.58	0.095
miRNA-141	0.072 0.033-0.671	0.102 0.046-0.181	0.682
miRNA-210	36.758 12.147-62.417	41.137 28.581-82.332	0.317
miRNA-200c	0.266 0.108-0.487	0.301 0.185-0.517	0.502
miRNA-106a	14.522 12.469-24.862	25.126 16.923-42.519	0.081
<b>miRNA-106b</b>	10.387 6.591-15.950	16.451 11.119-25.831	<b>0.030</b>
miRNA-200b	6.556 3.849-14.096	7.804 3.697-14.676	0.87
miRNA-182	0.302 0.051-0.728	0.081 0.036-0.321	0.194

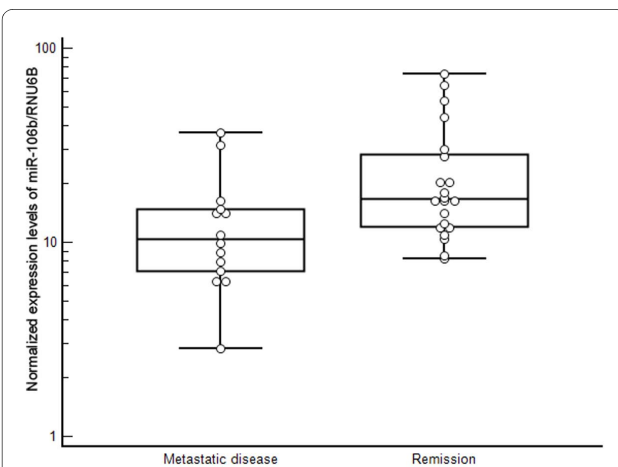
\* Medians of expression level related to RNU6B with \*\*25th and 75th percentiles.



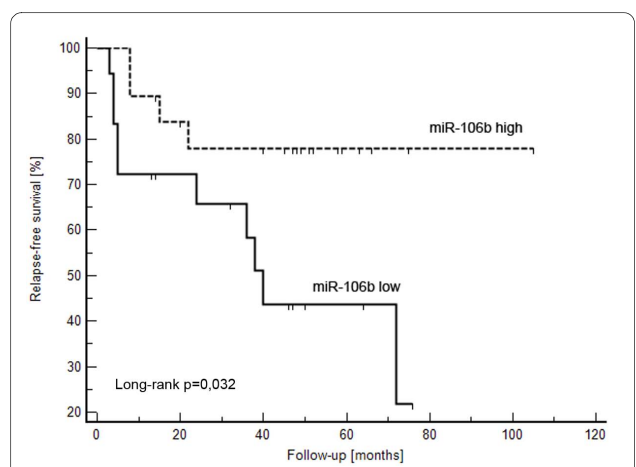
**Figure 1** Comparison of miR-155, miR-210, miR-106a and miR-106b expression levels in renal parenchyma (RP) and renal cell carcinomas (RCC).



**Figure 2** Comparison of miR-200c and miR-141 (tumor suppressive miR-200 family) expression levels in RP and RCC.



**Figure 3** Comparison of miR-106b expression levels in RCC stratified according to the development of metastatic disease after nephrectomy.



**Figure 4** Relapse-free survival of patients with RCC based on the miR-106b expression levels (cutoff = median of miR-106b expression).

sis, TGF- $\beta$  levels are lower and indicate features of a tumor suppressor, but in the late phase, within the development of metastatic disease, the degree of TGF- $\beta$  activation increases and leads to the promotion of immunosuppression, neoangiogenesis and progression of the disease. In relation to the TNM stage of RCCs, we have observed a general tendency for miR-106b levels to decrease from earlier stages towards advanced. Higher levels of miR-106b in selected RCCs may be connected with anti-neoplastic effects due to interference with TGF- $\beta$  signaling.

### Conclusions

To our knowledge, this is the first report observing that the expression of miR-106b has a correlation with the

development of metastasis and relapse-free survival in RCC patients after nephrectomy. Our findings also support the importance of miR-155, mR-210, miR-106a, miR-106b, miR-200c and miR-141 in RCC pathogenesis. While further studies and validations are needed, we suggest that miRNA-106b might be used for predicting early metastasis after nephrectomy in clinical practice. If validated, this would represent a next step to better treatment decisions and, ultimately, improvement in the survival rate of RCC patients.

### Abbreviations

RCC: renal cell carcinoma; miRNAs: microRNAs; RP: non-tumoral renal parenchyma; EMT: epithelial-mesenchymal transition; ZEB1: zinc-finger E-box binding homeobox 1; ZFHXB1: zinc finger homeobox 1B gene; SIP1: Smad-interacting protein 1; VHL: von Hippel-Lindau gene; HIF- $\alpha$ : hypoxia-induced factor alpha; TGF- $\beta$ : transforming growth factor beta.

### Competing interests

The authors declare that they have no competing interests.

### Authors' contributions

JJ carried out most of the experiments and organized data for the manuscript. PF and LK performed histopathological diagnosis of clear cell renal cell carcinoma and participated in manuscript drafting. MS, RL, AP, JM and RV participated in data organization and manuscript drafting. OS performed project design, coordinated the study and writing of the manuscript. All authors read and approved the final manuscript.

### Acknowledgements

This work was supported by grant IGA NS/10361-3/2009 from the Czech Ministry of Health and Project MZMOMOU2005.

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Received: 3 June 2010 Accepted: 7 July 2010

Published: 7 July 2010

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doi: 10.1186/1756-9966-29-90

**Cite this article as:** Slaby et al., Expression of miRNA-106b in conventional renal cell carcinoma is a potential marker for prediction of early metastasis after nephrectomy *Journal of Experimental & Clinical Cancer Research* 2010, **29**:90