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ARTICLE

MicroRNA-200 family expression analysis in metastatic clear cell renal cell carcinoma patients

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Abstract

The aim of this study is to analyse the of expression levels of microRNA-200 family members in patients with metastatic clear cell renal cell carcinoma (ccRCC). Analysis of microRNA expression was performed on 23 paired DNA samples extracted from kidney tumour tissue and the surrounding normal renal parenchyma. MicroRna-200c was found to have significantly lower expression (in kidney tumour tissue compared to normal renal parenchyma. No other microRna-200 family members showed statistically significant differences in expression levels between tumour and normal kidney tissue. Recent data suggest that the role of microRNA-200c in tumour pathogenesis is rather contradictory, and the underlying mechanisms by which microRNA-200c affects the carcinogenic potential of malignant cells remains unclear and requires further investigation at the molecular level.

Introduction

Clear cell renal cell carcinoma (ccRCC) is the most common carcinoma of the renal parenchyma, that accounts for ~70% kidney cancer cases [1]. ccRCC is the third leading cause of morbidity among genitourinary system tumours. Moreover, ccRCC is responsible for approximately 3% of cancer cases in adults, and the mortality rate is rising [2]. Although main forms of assigned treatment are surgical treatment combined with chemotherapy and radiotherapy, average ccRCC survival rate remains low. Tumour metastasis is one of the leading causes of kidney cancer mortality, thus, molecular mechanisms of epithelial-mesenchymal transition (EMT) are of particular interest, as invasive tumour growth becomes possible as a result of the detachment of malignant cells from the tumour mass due to a decrease or a complete loss of intercellular adhesion molecules, resulting in cells acquiring anomalously high motility, which enables them to penetrate stiff structural components of the surrounding stroma [3]. Invasion process heavily involves various molecular and cellular mechanisms, which, according to published data, depend directly on EMT. EMT is currently known to underlie embryogenesis, inflammation and tissue regeneration processes, and, undoubtedly, plays a pivotal role in carcinogenesis mechanisms [4]. Understanding the EMT process and its contributing factors will allow to create a theoretical framework for the development of new approaches to rational cancer therapy, particularly for prevention of tumour invasion and metastasis—the primary specific features of malignant growth. Metastasis involves general-purpose biochemical mechanisms, therefore microRNA that participate in systemic regulation mechanisms play an important part in regulation of corresponding genes [5]. Several metastasis and EMT-inducing transcription factors have been identified to date (Snail, Slug, Twist, ZEB1, ZEB2) [6]. Recent findings show that microRNA-205 and microRNA-200 family (microRNA-200a, microRNA-200b, microRNA-200c, microRNA-141 and microRNA-429)

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serve as epithelial markers and repressors of EMT [7]. MicroRNA-200 family members function to promote mesenchymal-epithelial transition (reverse process of EMT) and inhibit induction of EMT by influencing mRNA that code for ZEB1 and ZEB2. And conversely, ZEB1 repress microRNA-200 gene transcription by directly binding to their promotor region [8].

Aim

The role of microRNA that influence EMT and human cancer progression and metastasis is becoming more prominent [9, 10]. A significant number of studies analysed miRNA expression in kidney cancer, however, the results of different studies are contradictory, and there is no consensus on which miRNA are dysregulated in this disease.

Taking into account the direct involvement of miRNA-200 family in regulation of EMT-associated genes, we suggested that expression levels of the members of this family may correlate with kidney tumour development in patients with metastatic renal cell cancer. Thus, the aim of this study was an analysis of expression levels of miRNA-200 family members in patients with metastatic clear cell renal cell carcinoma.

Materials and methods

Analysis of microRNA expression was performed on 23 paired DNA samples extracted from kidney tumour tissue and the surrounding normal renal parenchyma. Tissue samples were obtained from unrelated patients with metastatic ccRCC, residents of Bashkortostan. The study included all available metastatic renal cancer patients operated during 5 years at Bashkir State Medical University hospital. None of the patients had received chemotherapy or radiation therapy prior to the surgery. Tissue samples and venous blood were collected by urology department faculty members. Ethical approval for this study was obtained from Institute of Biochemistry and Genetics Bioethics Committee. All samples investigated in this study were obtained with written informed consents of the participants.

Total RNA and microRNA extraction was performed using Direct-zol™ RNA MiniPrep (Zymo Research) kit with Zymo-Spin™ II C Column. Expression levels were measured using quantitative (real-time) PCR TaqMan MicroRNA Assays (Applied Biosystems) kit and CFX96™ real-time PCR detection system (BioRad). All reactions were performed in triplicates for each sample. $2^{-\Delta Ct}$ method was used for quantitative gene expression assessment. The $2^{-\Delta Ct}$ method is based on the assumption that the cycle threshold difference (ΔCt) between target gene and reference gene is proportional to relative target gene expression. Small nuclear RNA U6, characterized by stable expression in various tissues and cells, was used as endogenous control. The study personnel performing laboratory analyses were blinded to the sample groups.

Statistical analysis

Data were analysed using MS Office Excel 2003 [Microsoft], GraphPad Prism 6 software (GraphPad Software, Inc, La Jolla, CA). Two-sided $P < 0.05$ was considered to indicate statistical significance in all analysis methods. Independent variables significance hypothesis was tested using *t*-statistic coefficient and *p*-value for *t* coefficient. The heat map was built using d3heatmap package for R.

Results

MicroRNA expression profiling was performed on 23 paired tumour and normal kidney tissue samples from patients with metastatic clear cell kidney cancer using quantitative real-time PCR. Percentage of malignant cells in tumour tissue samples was estimated to be at least 75%. Clinicopathologic features of patients enrolled in the study are presented in Table 1. It was shown miRNA-200c family expression patterns were significantly different between ccRCC samples and normal kidney tissues just for one miRNA (Table 2). Results are shown as mean ± SEM. The raw data information of miRNA-200c family expression levels are presented in Supplementary Table 1S. Statistical analysis of acquired data demonstrated significantly decreased microRNA-200c expression levels (Fold change $= 0.034$, $p = 0.001$) in kidney tumour tissue relative to normal renal parenchyma (Fig. 1c). Expression levels of other microRNA-200c family members did not yield statistically significant differences between kidney tumour and normal kidney tissue (Fig. 1a, b, d, e). Figure 2 shows a heat map representing individual expression levels for each of the tested samples. The analysis of correlation between miRNA-200c expression and clinicopathologic factors did not show statistically significant differences.

Discussion

Current studies demonstrate that microRNA play an important role in pathophysiology of renal cell cancer. MicroRNA can promote oncogenesis and can be useful for molecular diagnostics, prognosis, and targeted therapy. MicroRNA-200 family is known to play an important part in cancer genesis, development and progression. For instance, microRNA-200b prevents malignant transformation of cells exposed to carcinogenic substances [11]. MicroRNA-200c inhibits matrix invasion of breast cancer

Table 1 Clinico-pathologic features of patients with metastatic ccRCC

Age (years): range; the average	Gender, *n* (%)		Tumour size, cm	Furman gradation, *n* (%)		Metastases, *n* (%)	Pathological status of lymph nodes, *n* (%)	
	Male	Female		1–2	3–4	M1	N0	N1
41–71; 56	13 (56.5)	10 (43.5)	4.0–20.2	12 (52.17)	11 (47.83)	23 (100.0)	2 (8.7)	21 (91.3)

Table 2 Relative expression levels in the tumour and normal tissue of patients with metastatic ccRCC

miRNA	ccRCC tissue ($n = 23$)	Normal kidney tissue ($n = 23$)	*p*-value*
microRNA-200a	9.373 ± 1.382	8.516 ± 1.048	0.6240
microRNA-200b	1.428 ± 0.133	1.301 ± 0.138	0.5109
microRNA-200c	0.110 ± 0.009	0.8087 ± 0.056	<0.0001
microRNA-141	0.293 ± 0.035	0.3565 ± 0.052	0.3250
microRNA-429	0.676 ± 0.057	0.6096 ± 0.075	0.4889

*Significance level (*p*-value) was calculated using Student's *t*-test

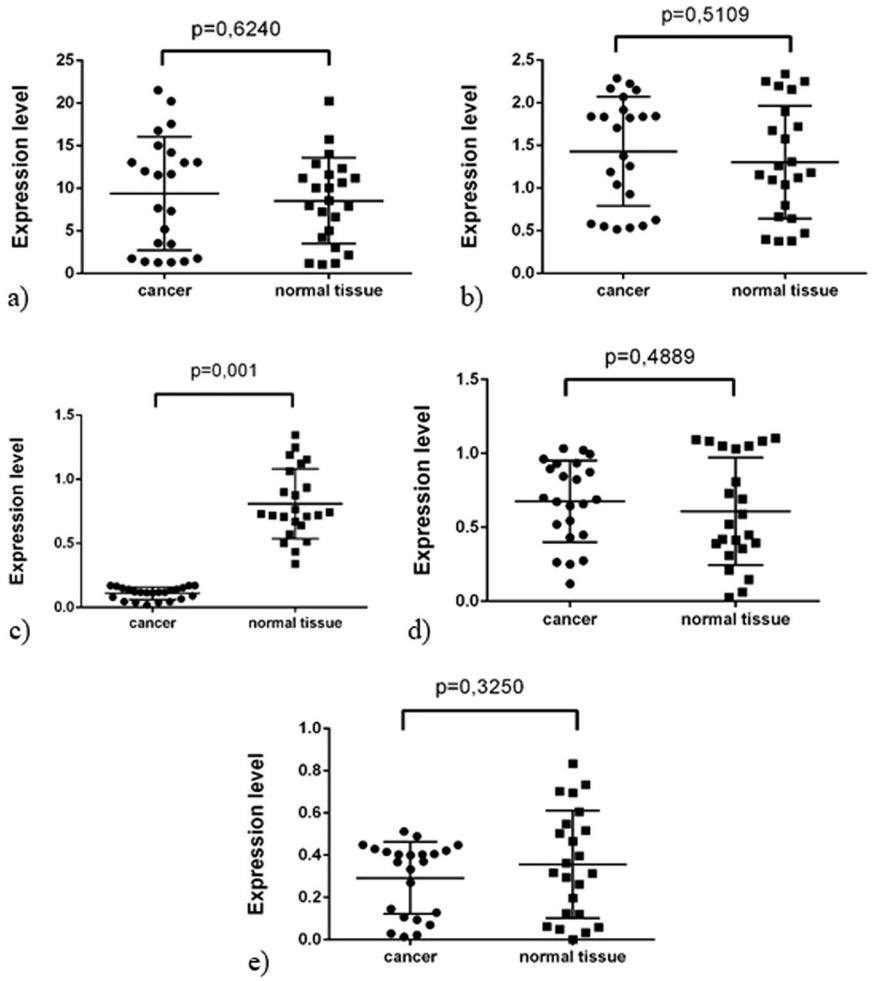

Fig. 1 Analysis of microRNA expression in kidney tumour and normal kidney tissue samples from patients with metastatic clear cell kidney cancer. MicroRNA-200a (**a**), microRNA-200b (**b**), microRNA-429 (**d**), and microRNA-141 (**e**) did not show significant differences in expression levels between tumour and normal kidney tissue. MicroRNA-200c (**c**) demonstrated statistically significant decrease in expression levels in kidney tumour tissue relative to normal renal parenchyma. Significance level (*p*-value) was calculated using Student's *t*-test

cells [12] MDA-MB-231, while miR-200b/c/429 cluster significantly decreases cell growth and promotes apoptosis [13]. Meanwhile, microRNA-200 family also inhibits EMT, repressing *ZEB1* [8] and *ZEB2* [14]. All these studies suggest antioncogenic function of microRNA-200 family.

The present study demonstrated a significant decrease in microRNA-200c expression levels in kidney tumour tissue relative to normal renal parenchyma in patients with metastatic kidney cancer. The link between microRNA-200c level and carcinogenesis has been explored in different

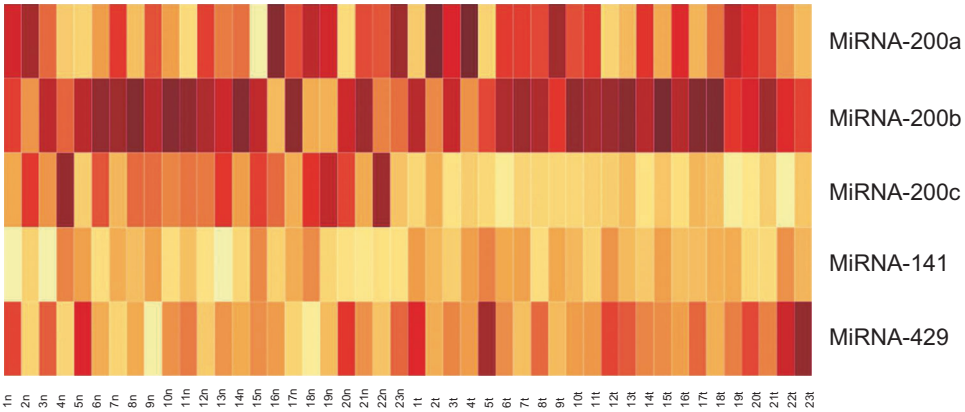

Fig. 2 Heat map illustrating differentially expressed miRNAs in tumour (t) and normal (n) kidney tissue. Colour intensity represents the magnitude of increase in expression levels

types of tumours. Lee et al. have found strong correlation between *ZEB1* expression [15] and miR-200c. *ZEB1* and *ZEB2*, confirmed targets of microRNA-141 and microRNA-200c, promote epithelial-mesenchymal transition and tumour cell migration [16]. Observation of patients with bladder cancer shows that decreased microRNA-200c expression is associated with disease progression to muscle-invasive bladder cancer and poor prognosis. Therefore, microRNA-200c expression can be useful for prediction of bladder cancer progression and treatment decision making [17]. Koo et al. have shown microRNA-200c to be an important modulator of EGFR signalling pathway cell survival, which is involved in radiation response of human tumour cells [18]. Additional regulatory functions of microRNA-200c in major signalling pathways have been described. MicroRNA-200c suppresses ubiquilin-1 expression in breast cancer cells and facilitates radiation-induced autophagy [19]. TANK-binding kinase-1 (TBK1, proposed as a target of microRNA-200c) inhibits radiation-induced apoptosis in breast cancer [20] and non-small-cell lung cancer [21]. MicroRNA-200c has been reported to regulate *Notch* signalling pathway activation in human tumour cell lines [22]. *Notch* gene family encodes transmembrane receptors that mediate cell-cell contact communications. *Notch* signalling pathway is associated with radiation resistance and is considered a novel therapeutic target for treatment of malignancies [23, 24]. Nakada et al. investigated microRNA expression profiles in renal cell cancer, including clear cell and chromophobe types, and found that microRNA-200c levels were significantly reduced in ccRCC [25]. Moreover, methylation has been shown to play a role in microRNA-200c levels in ccRCC. ccRCC cells treated with Aza (methyltransferase inhibitor) have shown a significantly decreased migration and invasion ability, accompanied by a significant increase in microRNA-200c expression, whereas microRNA-200c knockdown facilitated ccRCC cell migration and invasion [26]. It has also been noted that Aza treatment leads to decreased N-cadherin expression (cytoskeletal linker protein) and increased expression of E-cadherin (cell adhesion molecule) in ccRCC cells, which indicates EMT suppression. In contrast, microRNA-200c knockdown resulted in increased expression of N-cadherin and decreased expression of E-cadherin in ccRCC cells, which indicates EMT enhancement. As treatment with Aza also upregulated microRNA-200c expression in ccRCC cells, inhibitory effect of microRNA-200c on ccRCC cell migration and invasion may be partly attributed to inhibition of EMT [26]. Oncogenic microRNA-200c functions have been identified along with suppressor functions. Increased expression levels of this microRNA have also been seen in nasopharyngeal cancer, and in vivo suppression of microRNA-200c levels reduced tumour growth in mice. *PTEN* has been identified as a direct target of microRNA-200c [27]. Furthermore, elevated microRNA-200c expression has been found in non-small-cell lung cancer and reliably associated with lymph node metastasis. Kaplan-Meier analysis for patients with lymph node metastases, late stage TNM and high microRNA-200c expression showed poor 5-year relapse-free survival and overall survival prognosis [28].

Conclusion

There have been no studies done on the expression of miRNA-200 family in metastatic kidney cancer in Russia. We demonstrated a significant decrease in expression level of miRNA-200c (Fold change = 0.034, $p = 0.001$) in tumour tissue compared to normal renal parenchyma. Expression levels of other miRNA-200 family members did not show significant differences between tumour and normal kidney tissue. However, the study was carried out on a limited sample of patients with metastatic kidney cancer, and further research on broader populations of patients is needed to elucidate the obtained results. Furthermore, more research is needed to clarify the mechanisms of miRNA-200c effects on the malignant potential of the cell.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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