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UROLOGY - ORIGINAL PAPER

MicroRNA-15a expression measured in urine samples as a potential biomarker of renal cell carcinoma

Yulian Mytsyk[1] · Victor Dosenko[2] · Yuriy Borys[1] · Askold Kucher[3] · Katarina Gazdikova[4,5] · Dietrich Busselberg[6] · Martin Caprnda[7] · Peter Kruzliak[8,9,12] · Ammad Ahmad Farooqi[10] · Manyuk Lubov[11]

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Abstract

Introduction Currently, there is no accurate diagnostic molecular biomarker for renal cell carcinoma (RCC). The aim of this study was to assess the expression of microRNA-15a (miR-15a) in urine of patients with RCC and to evaluate its potential as a diagnostic molecular biomarker.

Materials and methods In total, 67 patients with solid renal tumors were enrolled: clear-cell RCC (ccRCC, $n=22$), papillary RCC (pRCC, $n=16$), chromophobe RCC (chRCC, $n=14$), oncocytoma ($n=8$), papillary adenoma ($n=2$) and angiomyolipoma ($n=5$). MiRNA-15a expression levels measurement in urine were performed using qPCR. Urine of 15 healthy volunteers without kidney pathology was used as control.

Results We observed a difference in mean miR-15a expression values in groups of pre-operative patients with RCC, benign renal tumors and healthy persons (2.50E−01 ± 2.72E−01 vs 1.32E−03 ± 3.90E−03 vs 3.36E−07 ± 1.04E−07 RFU, respectively, $p<0.01$). There was no difference in miR-15a expression between ccRCC, pRCC and chRCC ($p>0.05$). Direct association between RCC size and miR-15a expression values was obtained (Pearson correlation coefficient—0.873). On the 8th day after nephrectomy, mean expression value in patients with RCC decreased by 99.53% ($p<0.01$). MiR-15a expression differentiated RCC from benign renal tumors with 98.1% specificity, 100% sensitivity at a cut-off value of 5.00E−06 RFU, with AUC—0.955.

Conclusions MiR-15a expression measured in urine may be used as diagnostic molecular biomarker for RCC.

Keywords Renal cell carcinoma · MiRNA-15a · Biomarker · Diagnosis · Molecular marker

✉ Yulian Mytsyk
mytsyk.yulian@gmail.com

✉ Katarina Gazdikova
katarina.gazdikova@szu.sk

✉ Peter Kruzliak
kruzliakpeter@gmail.com

1 Department of Urology, Danylo Halytsky Lviv National Medical University, Pekarska Str. 69, Lviv, Ukraine

2 Department of General and Molecular Pathophysiology, Bogomoletz Institute of Physiology, National Academy of Sciences of Ukraine, Kiev, Ukraine

3 Department of Radiology, Danylo Halytsky Lviv National Medical University, Lviv, Ukraine

4 Department of Nutrition, Faculty of Nursing and Professional Health Studies, Slovak Medical University, Limbova 12, 833 03 Bratislava, Slovak Republic

5 Department of General Medicine, Faculty of Medicine, Slovak Medical University, Bratislava, Slovak Republic

6 Weill Cornell Medical College in Qatar, Qatar Foundation, Education City, Doha, Qatar

7 1st Department of Internal Medicine, Faculty of Medicine, Comenius University and University Hospital, Bratislava, Slovakia

8 Department of Internal Medicine, Brothers of Mercy Hospital, Brno, Czech Republic

9 Research and Development Services, Brno, Czech Republic

10 Laboratory for Translational Oncology and Personalized Medicine, Rashid Latif Medical College, Lahore, Pakistan

11 Department of Foreign Languages, Danylo Halytsky Lviv National Medical University, Lviv, Ukraine

12 2nd Department of Surgery, Faculty of Medicine, Masaryk University and St. Anne's University Hospital, Pekarska 664/53, 656 91 Brno, Czech Republic

Introduction

Renal cell carcinoma (RCC) is a multifaceted and therapeutically challenging disease. Most recent genetic and genomic studies have helped us to develop a better understanding of the mechanisms which underlie cancer development and progression.

RCC is a frequent form of malignant kidney lesion which represents 3% of total oncologic pathology, with a high index of relapse and a mortality rate of over 40%. RCC has increased significantly and rapidly, and developing resistance against therapeutics is a major hindrance in the application of therapy [1]. RCC is represented by characteristic histological subtypes, the most common is conventional or clear-cell RCC (ccRCC), which accounts for about 80–90% of cases. Other subtypes include papillary RCC (pRCC, 6–15%) and chromophobe RCC (chRCC, 2–5%). Knowing histologic subtype and grade of the RCC is fundamental for the prediction of oncologic outcomes. Fuhrman nuclear grade is the most widely accepted grading system of RCC; however, grade of differentiation can be used as an independent predictor of the disease behavior only in conventional and pRCCs, although tumor necrosis is an important prognostic factor in all RCC subtypes [2].

The clinical performance of percutaneous kidney biopsy (PKB) for differential diagnosis of RCC and small renal masses (SRM) is currently debated by urologists due to high index of uninformative biopsies, from 10 to 23% [3, 4]. Application of contemporary imaging techniques as contrast-enhanced CT/MRI/USG significantly improved early detection of RCC but is not reliable in differentiation between malignant and some benign renal tumors, such as oncocytoma (OC), fat-poor angiomyolipoma (AML) and papillary adenoma (PA). This results in performing of 7.5–33.6% of partial nephrectomies in RCC-suspected patients with SRM on benign renal tumors [5, 6].

Recently, numerous molecular markers such as carbonic anhydrase IX (CaIX), vascular endothelial growth factor (VEGF), hypoxia-inducible factor (HIF), Ki67, p53, p21, PTEN (phosphatase and tensin homolog), E-cadherin, C-reactive protein (CRP), osteopontin and CD44, CXCR4, and other cell cycle and proliferative markers have been investigated. None of these potential RCC biomarkers have clearly improved the predictive accuracy of current prognostic systems, none have been externally validated, and their use is not recommended in routine practice [7–10].

Molecular and clinical scientists are working on the identification of biomarkers for detection of malignant and benign tumors, characterization of RCC subtypes as well as their differentiation grades [11]. Based on the insights collected from a decade of research, it is probable that microRNAs play contributory role in cancer development, metastatic spread and development of resistance against an array of drugs [12, 13]. Abnormal miRNA expression in blood and in tumor tissues in patients with RCC has been published which provide wealth of information about contributory role of miR-27, miR-28, miR-30c, miR-106b, miR-135a, miR-141, miR-185, miR-199a, miR-200c, miR-210, miR-451, miR-508-3p, miR let-7f-2, but the methods used are invasive [14–16].

MiR-15a and miR-16-1 are encoded by adjacently located genes on chromosome 13q14.3. MiR-15a is downregulated in prostate cancer, chronic lymphocytic leukemia, nasopharyngeal carcinoma, malignant melanoma, glioma and breast cancer. It is a tumor suppressor, promoting apoptosis and inhibiting cell proliferation by targeting multiple oncogenes, including Bcl-2, Mcl1, CcnD1 and Wnt3A [17–19]. Nuclear binding of pri-microRNA-15a is a function of protein kinase Cα (PKCα), which plays an important role in endothelin-1 (ET-1)-mediated signaling—a system involved in a tumor growth [20, 21]. In addition, miR-15a takes part in non-canonical nuclear factor KappaB (NF-κB) pathway which regulates resistance to apoptosis, angiogenesis and multi-drug resistance. Moreover, the von Hippel Lindau gene is a negative regulator of NF-κB [22, 23].

Thus, to provide a non-invasive diagnostic biomarker of this disease, we investigated the correlation between urine miR-15a versus RCC type.

Materials and methods

Ethics statement

The study was approved by Ethical Committee of Danylo Halytsky Lviv National Medical University, Ukraine (protocol #5, dated 05/25/2015) and was followed in accordance with ethical standards formulated in the Declaration of Helsinki 1975. Our research was conducted at Urology Department of up-mentioned institution and at General and Molecular Pathophysiology Department of Bogomoletz Institute of Physiology of National Academy of Sciences of Ukraine during 2015–2017 years.

General data

In total, 67 adult patients with solid renal tumors according to clinical and imaging data were enrolled into study: suspected primary RCCs ($n = 58$), suspected OC ($n = 5$) and symptomatic fat-rich AMLs in association with persisting hematuria ($n = 4$). The gender was 41 men and 26 women. The age of the patients varied between 46 and 69 years (60.2 ± 6.4 years). The size of the tumors ranged from 1.26 to 12.7 cm with mean size of 6.23 ± 2.08 cm. In all patients, no PKB/previous RCC treatment was

performed. All patients underwent surgery with following pathologic analysis. Radical nephrectomy was performed in 42 (80.8%) patients and in 10 (19.2%) cases, nephron sparing surgery was performed. Diagnosis of RCC was pathologically confirmed in 52 (89.66%) of 58 RCC-suspected cases, and the other 6 (10.34%) diagnoses were benign renal tumors (OC, $n=3$), (PA, $n=2$), (fat-free AML, $n=1$). All RCCs were classified according to histologic subtypes—ccRCC ($n=22$), pRCC ($n=16$), chRCC ($n=14$). Simplified two-tiered Fuhrman grading system [24] was used in which grades I and II (low grade, $n=12$) and grades III and IV (high grade, $n=10$) were combined for the conventional RCC grading. RCC cases were classified concordantly to 7th edition of AJCC cancer staging manual: T1aN0M0 ($n=13$, 25.0%), T1bN0M0 ($n=15$, 28.85%), T2aN0M0 ($n=12$, 23.08%), T2bN0M0 ($n=5$, 9.62%), T3aN0M0 ($n=4$, 7.69%), T3aN1M0 ($n=3$, 5.77%). According to pathological reports in 9 (17.31%) patients with RCC area of tumor necrosis was present, at the same time no cases with sarcomatoid differentiation were observed. In all cases of suspected OCs and fat-rich AMLs, the diagnosis was postoperatively confirmed pathologically. More detailed characteristics of the final distribution by histologic type of the patients with solid renal tumors are presented in Table 1.

Control samples were obtained from 15 healthy individuals, according to clinical and imaging examinations (10 men and 5 women). The age ranged from 39 to 66 years (with median of 53.1 ± 8.2 years). With the aim to evaluate renal morpho-functional status, all healthy individuals underwent appropriate examinations, including general analysis of blood and urine, biochemical analysis of blood (creatinine, urea, ALT, AST), renal USG.

Table 1 Detailed characteristics of the patients' subgroups

Tumor histology	*N*	*M*	*F*	Mean age (years, mean ± SD)	Mean size of tumor (cm, mean ± SD)
RCC	52	32	20	60.77 ± 6.31	7.10 ± 2.96
ccRCC	22	13	9	60.59 ± 6.08	7.18 ± 3.10
pRCC	16	11	5	61.06 ± 5.58	6.79 ± 2.74
chRCC	14	8	6	60.71 ± 7.78	7.34 ± 3.16
Benign tumors	15	9	6	58.20 ± 6.33	5.39 ± 2.57
OC	8	5	3	58.38 ± 5.76	6.26 ± 2.07
PA	2	2	–	61.0 ± 8.49	1.48 ± 0.22
AML	5	2	3	56.8 ± 7.60	8.42 ± 2.84
Total	67	41	26	60.19 ± 6.36	6.23 ± 2.08

RCC renal cell carcinoma, *ccRCC* clear-cell renal cell carcinoma, *pRCC* papillary renal cell carcinoma; *chRCC* chromophobe renal cell carcinoma, *OC* oncocytoma, *PA* papillary adenoma, *AML* angiomyolipoma

Urinary samples

In all subjects, urine was collected in the morning (100–150 mL) into a sterile container, without use of any further stabilizing buffer, and stored at − 20 °C until further examination, then 1 day before a surgery and on the 8th day after nephrectomy.

miRNA isolation from urine

For miRNA isolation, 350 µL of urine was used for total RNA isolation by means of mirVana™ miRNA Isolation Kit (Applied Biosystems, USA) in accordance with manufacturers protocol. Concentration measurement of RNA was performed using spectrophotometry (NanoDrop ND-1000, NanoDrop Technologies Inc, USA).

qPCR data

MiR-15 expression in urine was measured using reverse transcription and real-time quantitative polymerase chain reaction (qPCR) analysis. Reverse transcription was conducted by means of TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, USA), and specific primer for MiR-15 and 10 ng of the total RNA was used for real-time qPCR using TaqMan MicroRNA Assays (Applied Biosystems, USA): U6 snRNA, ID 001973 (as endogenous control), hsa-miR-15a, ID 000389 (Applied Biosystems, USA). Temperature cycles were as follows: initial denaturation step at 95 °C/10 min; 50 cycles at 95 °C/15 s and at 60 °C for 60 s. U6 was used for data normalization of urine miRNA, and relative fluorescence was calculated using $2^{\Delta C_t * 100}$ presentation and presented in relative fluorescence units (RFU). Amplification was performed using 7500 Fast Real-time PCR (Applied Biosystems, USA).

Statistical analysis

Microsoft Excel 2016 and SPSS v.22 software packages were used for the statistical data analysis. In order to assess the difference in miR-15a expression levels in patients' subgroups, single-factor dispersion analysis was performed. The normality of data distribution was evaluated using Shapiro–Wilk test ($W=0.542$, $p=0.001$). When W statistics was significant ($p<0.05$), the null-hypothesis that data distribution is normal was rejected; therefore, difference was calculated by means of nonparametric Mann–Whitney U test. The results were considered statistically significant when p value was <0.05. The correlation was measured by means of Pearson method. The

diagnostic performance of expression levels for diagnostics of RCC was evaluated using receiver operating characteristics (ROC) analysis.

Results

The miR-15a expression levels were detected in urine samples of patients with RCC, benign renal tumors and controls. Strong statistical differences ($p < 0.01$) in miR-15a expression in groups were achieved (Fig. 1). In three cases of smallest RCC tumors sized < 3.4 cm (stage T1aN0M0), miR-15 expression values were within benign renal tumors range. In contrast, we observed two AMLs of biggest size (9.6 and 11 cm) with miR-15 expression levels within the range of RCCs. The relationship between grade of nuclear atypia in patients with high- and low-grade ccRCCs and miR-15a expression levels was detected: in low-grade ccRCCs, expression values were significantly lower ($p < 0.01$) in comparison with high-grade tumors (Fig. 2). Nevertheless, there was no significant difference ($p > 0.05$) in miR-15a expression levels between ccRCC, pRCC and chRCC subtypes (Fig. 3). The presence of pathologically proven necrosis had an impact on miR-15a regulation in patients with RCC resulting in significantly ($p < 0.01$) higher expression values in cases with necrosis in comparison with non-necrotic RCCs (Fig. 4). All descriptive statistics of our study is presented in Table 2.

In our study, direct association between RCC size and miR-15a expression value was noted: Pearson correlation coefficient (0.873) confers a strong positive association (Fig. 5). We observed direct relationship between T-stage of RCC and miR-15a expression levels (Fig. 6); however, there was no statistical significance ($p > 0.05$) between its values in subgroups with RCC stages T1aN0M0 versus T1bN0M0 and T2aN0M0 versus T2bN0M0, in the rest subgroups the difference was reliable ($p < 0.05$). The mean values of miR-15a expression in RCC subgroups according to TNM classification were as follows: T1aN0M0 = 5.77E−03 ± 3.93E−03 RFU, T1bN0M0 = 7.35E−02 ± 7.90E−02 RFU, T2aN0M0 = 3.57E−01 ± 1.63E−01 RFU, T2bN0M0 = 4.99E−01 ± 4.37E−02 RFU, T3aN0-1M0 = 7.18E−01 ± 1.56E−01 RFU.

We analyzed miR-15a concentration in urine samples of the patients with RCC after surgical treatment. On the 8th day after nephrectomy, the mean decreased by 99.53% from 2.50E−01 ± 2.72E−01 to 1.18E−03 ± 5.18E−03 RFU ($p < 0.01$). At the same time, the mean miR-15a level in post-op patients with RCC was significantly higher in comparison with benign tumors and healthy controls ($p < 0.01$). In these patients with small RCCs and false-negative results, miR-15a expression levels decreased insignificantly and remained within the range of benign renal tumors (Table 2, Fig. 1).

In order to determine the clinical reliability of miR-15a expression level to differentiate between RCCs, we calculated the specificity and sensitivity: at a cut-off value of 5.00E−06 RFU they constituted 98.1 and 100% (95% CI

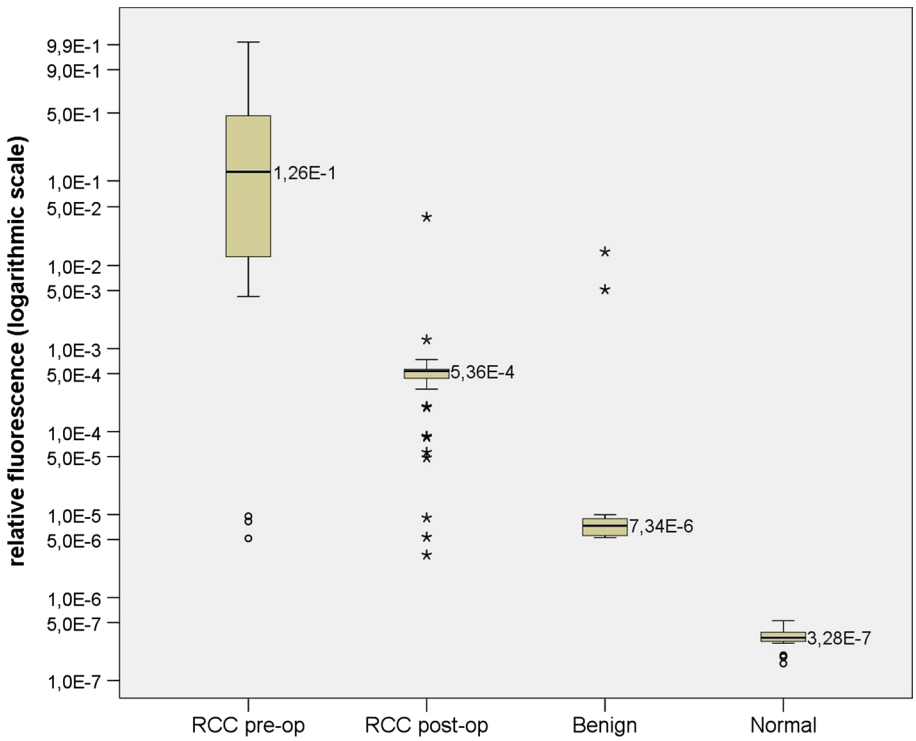

Fig. 1 MiR-15a expression in urine samples of patients with RCC (pre- and post-op), benign renal tumors and healthy controls. *RCC* renal cell carcinoma

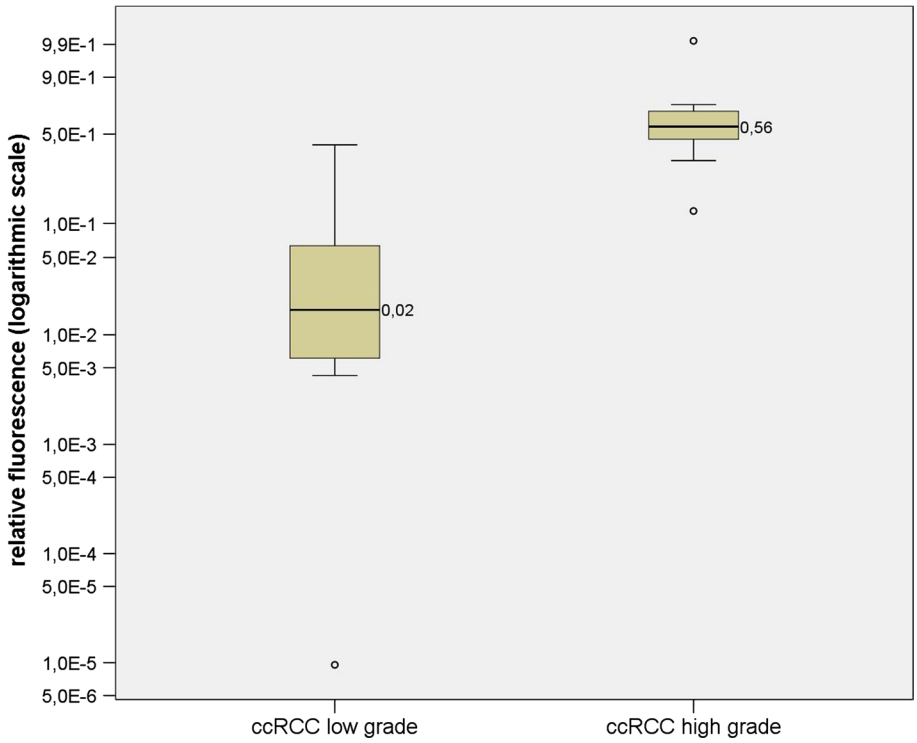

Fig. 2 MiR-15a expression of pre-op RCCs of low and high grade in urine. *ccRCC* clear-cell renal cell carcinoma

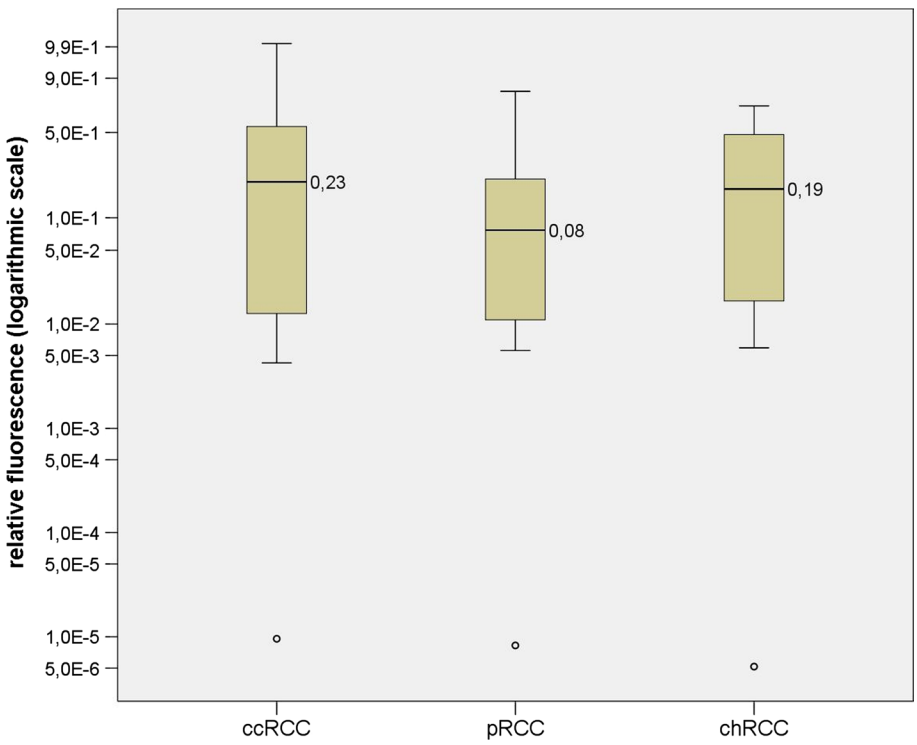

Fig. 3 Pre-op MiR-15a expression of clear-cell, papillary and chromophore RCC. *ccRCC* clear-cell renal cell carcinoma, *pRCC* papillary renal cell carcinoma, *chRCC* chromophobe renal cell carcinoma

0.9–1.0) accordingly while area under the curve (AUC) of the ROC curve was 0.955 (Fig. 7).

Discussion

The importance of miRNAs in diagnostic pathology, specifically non-oncologic kidney pathology has been

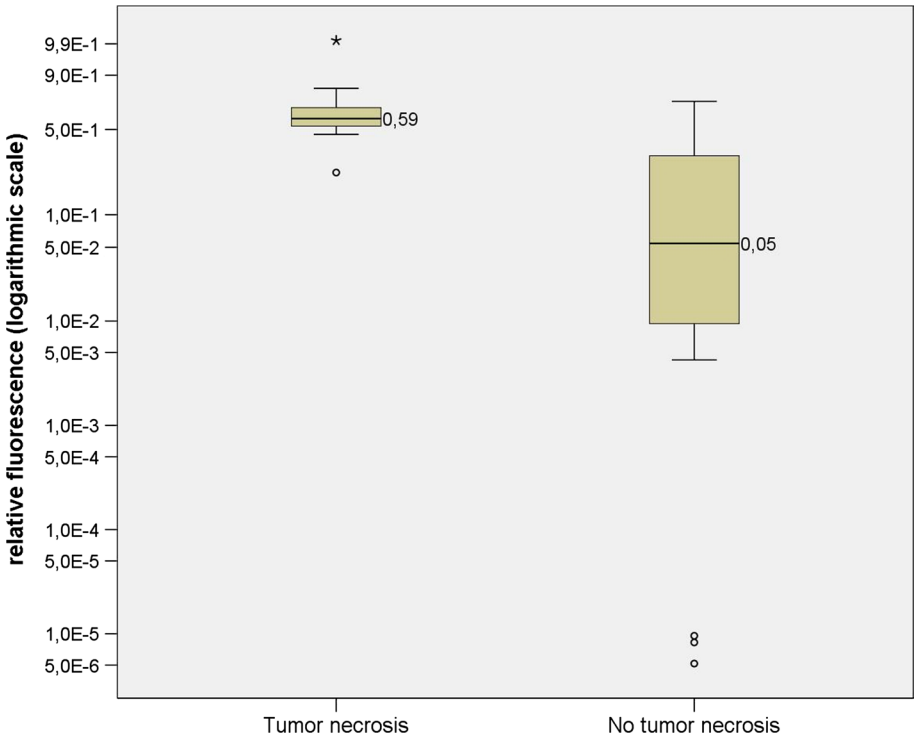

Fig. 4 MiR-15a expression levels in RCC in presence and absence of tumor necrosis

Table 2 MiR-15a mean and median expression levels in urine samples of patients with RCC, benign renal tumors and in healthy controls

Tumor type/patients subgroups	*N*	Mean	Median	SD	95% confidence interval for mean		Minimum	Maximum
					Lower bound	Upper bound		
RCC pre-op	52	2.50E−01	1.27E−01	2.72E−01	1.74E−01	3.25E−01	5.17E−06	9.93E−01
RCC post-op	52	1.18E−03	5.36E−04	5.18E−03	− 2.61E−04	2.63E−03	3.26E−06	3.78E−02
Benign	15	1.32E−03	7.34E−06	3.90E−03	− 8.40E−04	3.49E−03	5.28E−06	1.46E−02
HC	15	3.36E−07	3.28E−07	1.04E−07	2.79E−07	3.94E−07	1.60E−07	5.27E−07
ccRCC	22	3.00E−01	2.28E−01	3.06E−01	1.64E−01	4.36E−01	9.56E−06	9.93E−01
pRCC	16	1.77E−01	7.81E−02	2.33E−01	5.29E−02	3.02E−01	8.25E−06	8.21E−01
chRCC	14	2.53E−01	1.91E−01	2.54E−01	1.07E−01	4.00E−01	5.17E−06	7.13E−01
ccRCC low grade	12	8.28E−02	1.76E−02	1.44E−01	− 8.80E−03	1.74E−01	9.56E−06	4.25E−01
ccRCC high grade	10	5.61E−01	5.57E−01	2.33E−01	3.94E−01	7.27E−01	1.28E−01	9.93E−01
RCC with necrosis	9	6.13E−01	5.86E−01	2.15E−01	4.48E−01	7.79E−01	2.35E−01	9.93E−01
RCC without necrosis	43	1.74E−01	5.43E−02	2.16E−01	1.07E−01	2.40E−01	5.17E−06	7.25E−01

RCC renal cell carcinoma, *ccRCC* clear-cell renal cell carcinoma, *pRCC* papillary renal cell carcinoma, *chRCC* chromophobe renal cell carcinoma, *HC* healthy controls

described [25, 26]. As well, the evidence to use miRNAs in RCC pathogenesis as a diagnostic biomarkers is growing. Gowrishankar et al. [27] found a positive signature that included significant upregulation of miR-21-5p, 142-3p, let-7g-5p, let-7i-5p and 424-5p, as well as downregulation of miR-204-5p measured in tumor tissues, to be associated with ccRCC of high stage, high grade and quick progression. The encouraging results of other recent investigations supplemented existing database of miRNAs measured in tumor tissues and blood of patients that can be used as potential RCC biomarkers [11, 12, 14–16]. At the same, there is extremely small amount of data on miRs measured in urine samples of patients with RCC. In our study, we achieved valuable information about miR-15a expression levels in urine samples of patients with RCC of different histological subtypes and grades of differentiation. In pre-op period, miR-15a in RCC cases was significantly upregulated in comparison with benign renal

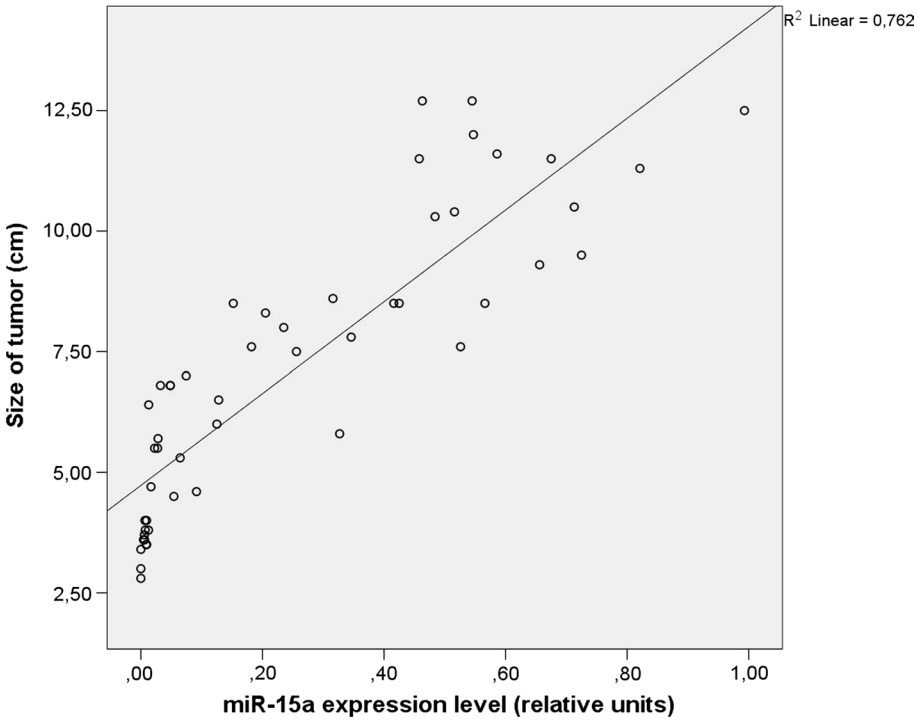

Fig. 5 Scatterplot of correlation between MiR-15a expression in urine and the tumor size in patients with RCC

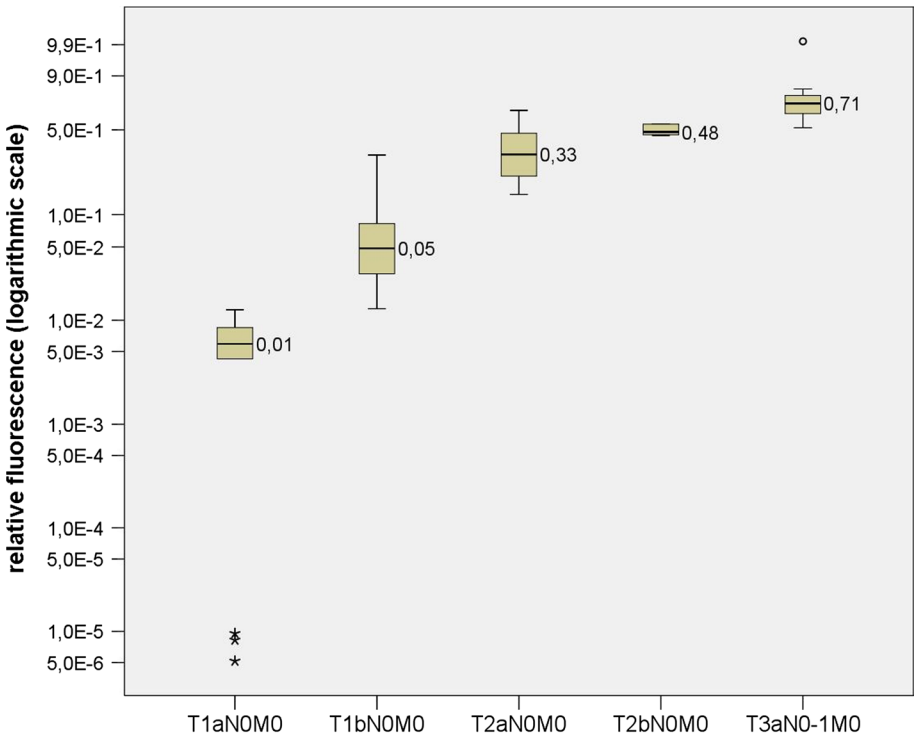

Fig. 6 MiR-15a expression in urine in patients with RCC in accordance with TNM classification

tumors and healthy controls ($p < 0.01$), while in post-op period the miR-15a expression in RCCs decreased significantly. Such association is achieved by Brandenstein data [28]; however, MiR-15a expression levels in pre-op RCC patients had significantly different range: 0.38–248.0 versus 0.01–0.99 RFU accordingly. We propose methodology differences to explain these discrepancies. RCC tumors with necrotic component were associated with highest miR-15a expression values that is opposed to Brandenstein results, albeit in latter cases of RCC necrosis were observed in "regressive tumors" characterized by > 50% of the tumor parenchyma destruction, massive hemorrhage

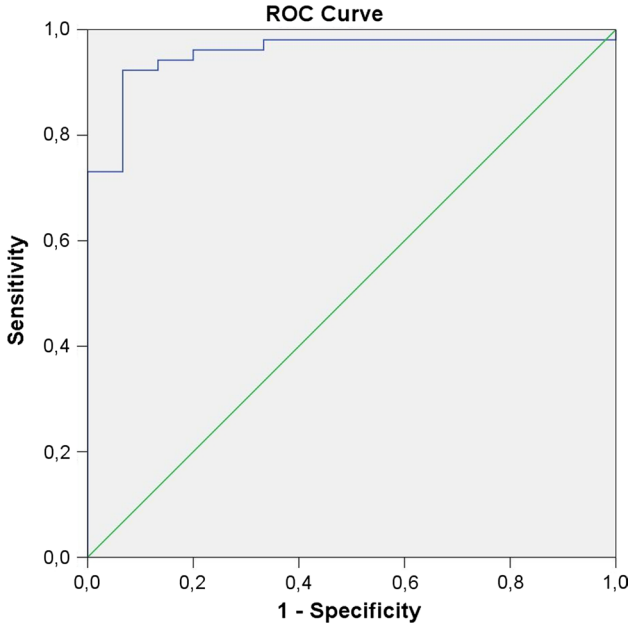

Fig. 7 Receiver operation characteristics (ROC) of MiR-15a expression in differentiation RCC from benign renal lesions using a cut-off value 5.00E−06 RFU

and inflammation, as well as subsequent restructuring by scaring that was not applicable to RCCs in our research.

An interesting finding of our study was that miR-15a was increased in patients with malignant tumors in comparison with cases of benign neoplasia and healthy controls: this exhibited an inverse correlation compare to the literature [17–19]. This discrepancy cannot be explained by a positive feed back loop initiated by PKCα that regulates DNA synthesis where PKCα activity induced by oncogenic stimuli activates mitogen-activated protein kinase (MAPK) and deregulates cyclin E expression by suppressing miR-15a, an inhibitor of cyclin E [29]. The evidence that miR-5a regulates signaling after ET-1 induction (the mediator induces decreasing PKCα levels, which can no longer suppress nuclear pri-miRNA release, resulting in cytoplasmic accumulation of mature miRNA-15a) could partially explain this issue, but requires further investigations [30].

The limitation of our study was relatively smaller sample size of patients with RCC of T3-stage and no cases of T4-stage, yet it is unlikely that a higher sample size of large RCC tumors will have a significant impact on our conclusions.

Conclusions

In conclusion, measuring expression of miR-15a in urine samples of patients with RCC can provide valuable information for differential diagnostics of this pathology and could be used as potential molecular biomarker with 98.1% specificity and 100% sensitivity.

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Compliance with ethical standards

Conflict of interest All authors declare no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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