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

Received: March 12, 2017 *Accepted: May 08, 2017*

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.

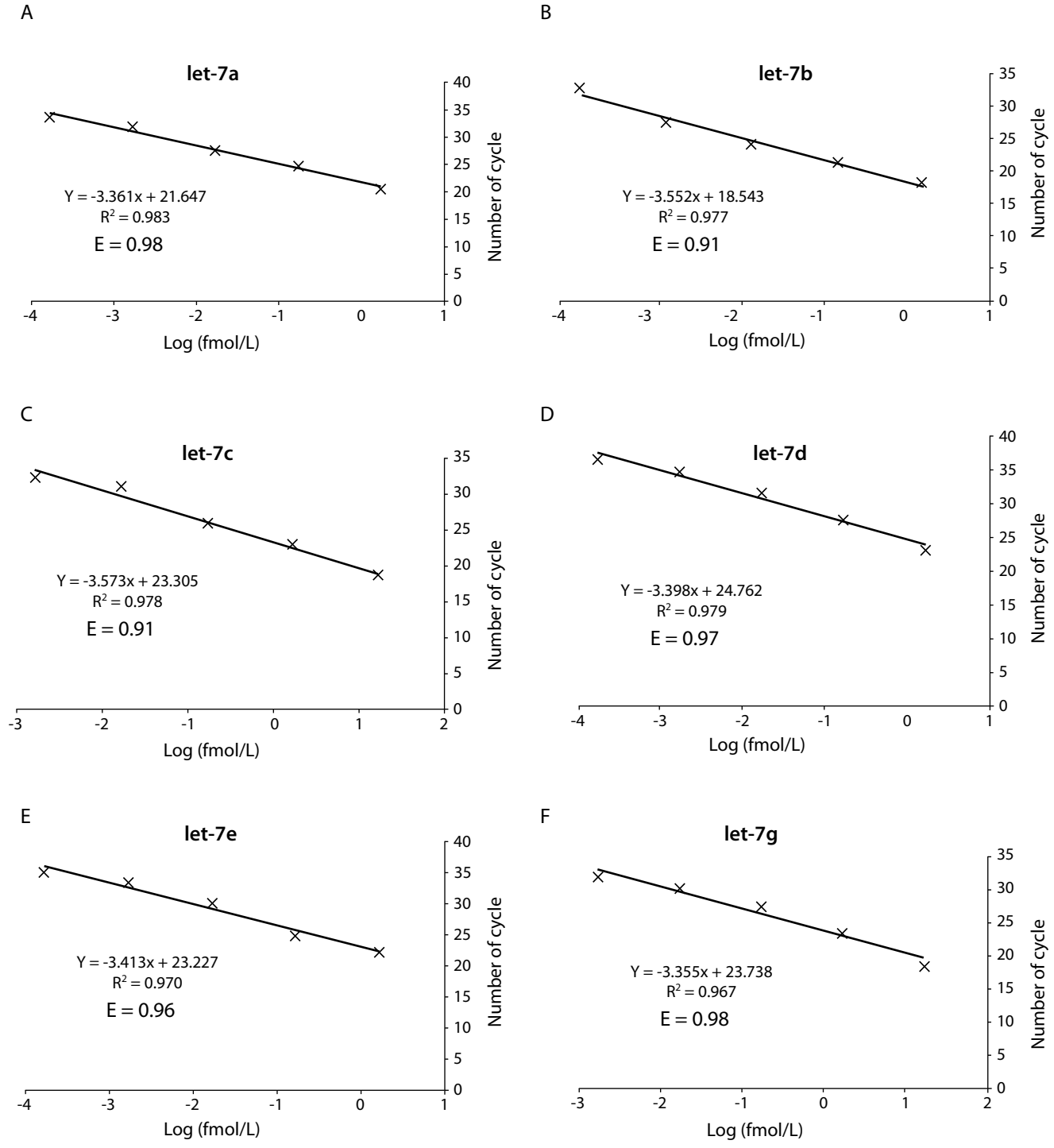

Figure 1. Absolute standard curves for let-7a (A), let-7b (B), let-7c (C), let-7d (D), let-7e (E) and let-7g (F) used for calculation of let-7 microRNAs concentrations (fmol/L) in the urine samples. E - qPCR reaction efficiency.

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.

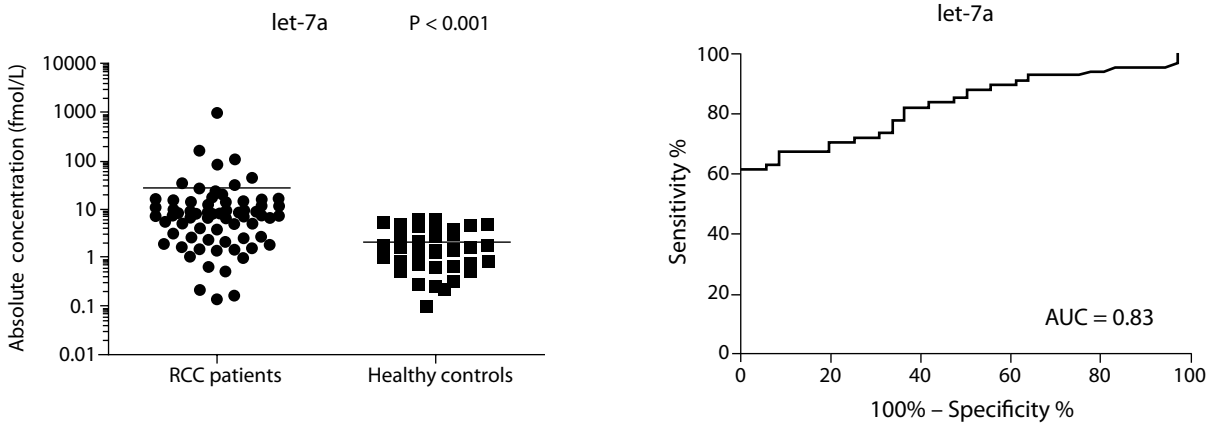

Figure 2. Differences of let-7a concentration between RCC patients and controls.
(A) Absolute concentrations of let-7a was determined by qRT-PCR in urine of RCC patients (N = 69) and healthy individuals (N = 36).
(B) ROC analysis of let-7a to evaluate the ability to distinguish RCC patients and healthy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

Acknowledgments

The project was supported by Ministry of Health of the Czech Republic, grant nrs. AZV 15-31071A, 15-34678A, and MZCR RVO (MOU, 00209805); the project CEITEC 2020 (LQ1601) provided by the Ministry of Education Youth and Sports of the Czech Republic.

Potential conflict of interests

None declared.

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