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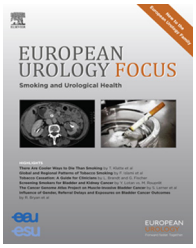

Platinum Priority – Kidney Cancer

Exosomal MicroRNAs Are Diagnostic Biomarkers and Can Mediate Cell–Cell Communication in Renal Cell Carcinoma

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Abstract

Background: Apart from an invasive biopsy, currently no tools are available to confirm the diagnosis of clear cell renal cell carcinoma (ccRCC); this resulted in approximately 30% of patients being diagnosed with metastatic disease.
Objective: To determine whether urinary microRNAs (miRNAs) can serve as biomarkers to confirm the diagnosis of ccRCC.
Design, setting, and participants: Global miRNA expression was assessed in 28 preoperative urine samples from patients with ccRCC and 18 healthy participants. The independent validation set consisted of 81 ccRCC patients, 24 patients with benign lesions, and 33 healthy participants. We extracted both cell-free and exosomal RNA for miRNA expression analysis using miRNA-specific polymerase chain reaction assays. We also investigated exosomal miRNA secretion in cell line models and performed exosome transfer between RCC and endothelial cell types.
Outcome measurements and statistical analysis: Receiver operating characteristic analysis was applied to identify the discrimination power of miRNAs.
Results and limitations: Overall, miR-126-3p combined with miR-449a or with miR-34b-5p could significantly distinguish ccRCC patients from healthy participants (miR-126-3p–miR-449a: area under the curve [AUC]: 0.84; 95% confidence interval [CI], 0.7620–0.9151; $p < 0.001$; miR-126-3p–miR-34b-5p: AUC: 0.79; 95% CI, 0.7013–0.8815; $p < 0.001$). The combination of miR-126-3p and miR-34b-5p was also able to distinguish small renal masses (pT1a, ≤4 cm) from healthy controls (AUC: 0.79; 95% CI, 0.6848–0.8980; $p < 0.001$). Using miR-126-3p and miR-486-5p in combination, we were able to differentiate between benign lesions and ccRCC (AUC: 0.85; 95% CI, 0.7295–0.9615; $p < 0.01$). The expression of a number of miRNAs returned to a level comparable with health after surgery. Kidney cancer cell lines were found to secrete exosomal miR-126-3p, miR-17-5p, miR-21-3p, and miR-25-3p, and these miRNAs were found to be internalized by other cell types.
Conclusions: We identified exosomal miRNAs as potential noninvasive diagnostic urinary biomarkers for ccRCC and provided evidence that miRNAs are secreted by the tumor and can function as a tool for intercellular communication.
Patient summary: We identified urinary microRNAs that can serve as diagnostic biomarkers for clear cell renal cell carcinoma.

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1. Introduction

Renal cell carcinoma (RCC) has the highest mortality rate among genitourinary cancers. The last 40 yr have seen a fivefold increase in disease incidence and a twofold increase in mortality [1]. It is a heterogeneous entity, with 70–85% of cases classified as the clear cell subtype (ccRCC). In the absence of symptoms, approximately 30% of patients present at the metastatic stage at the time of diagnosis. Localized kidney cancer can be cured surgically, whereas patient survival drops sharply if the disease becomes metastatic. Increasing incidental detection by imaging procedures and an aging population have led to active surveillance as an option for patients with small renal masses (SRMs) [2].

Apart from invasive biopsy, which has a limited success rate and a number of complications, currently no diagnostic tool is available to confirm the identity of renal masses [3], and sometimes nephrectomy is performed based on radiologic evidence alone. There is an urgent need for noninvasive urine or serum-based molecular testing to replace biopsy. In this regard extracellular microRNAs (miRNAs) are promising candidates.

The miRNAs are small noncoding RNA molecules that post-transcriptionally regulate protein translation. The differential expression of miRNAs in ccRCC has been documented by independent reports, and evidence shows their involvement in ccRCC pathogenesis [4,5].

Extracellular miRNAs in serum, plasma, saliva, and urine were shown to be associated with various pathologic conditions including cancer [6]. Extracellular miRNAs can be packaged in membrane vesicles or in a complex with miRNA-binding carrier proteins (eg, the Argonaute family of proteins that are highly specialized binding modules that accommodate small RNAs, like miRNAs, and coordinate downstream gene silencing by interacting with other factors) or high-density lipoproteins, leading to their stability and resistance to degradation [7]. The miRNA levels in biofluids were demonstrated to be reproducible across participants because miRNAs are resistant to robust enzymatic cleavage, freeze-thaw cycles, or pH changes [8]. Extracellular miRNAs in urine may be derived from urologic tumors, normal urothelium, the glomerulus, or the renal tubules, and they can also be secreted by tumor-reactive immunocytes.

Exosomes are a specific subtype (30–100 nm in size) of secreted membrane vesicles [9] that are formed intracellularly in endosomal compartments (multivesicular endosomes). Exosomes can be released to the extracellular compartment by the fusion of multivesicular endosomes with the plasma membrane [9], and they contain various molecules specific to their cell of origin including messenger RNA, miRNA, proteins, cytokines, and different surface receptors [10]. The stability of exosomal miRNAs has also made them good biomarker candidates [8].

The aim of this study was to examine the ability of urinary cell-free and exosomal miRNA expression as a noninvasive biomarker to diagnose ccRCC. We also tested their utility as diagnostic biomarkers for SRMs. Finally, we demonstrated that exosomal miRNAs are secreted by ccRCC cells in vitro and that this secretion supports the communication between ccRCC and endothelial cells.

2. Methods

2.1. Clinical samples

The study was approved by the local research ethics committee. For the discovery set, we collected 28 preoperative urine samples from patients having histologically verified ccRCC (10 women and 18 men; mean age: 59 yr ± 12.76) and 18 healthy participants. For the validation set, we used an independent set of 81 urine samples from ccRCC patients, 24 from patients with benign kidney tumors, and 33 healthy controls. Patient data are summarized in Supplementary Table 1 and 2. The 30–50 ml of urine was collected from each individual and centrifuged at 2000 *g* for 10 min at 4 °C and stored at −80 °C until further use.

2.2. Cell-free and exosomal RNA preparation from urine and tissue culture media

We compared cell-free and exosomal RNA extraction techniques. The miRNeasy Serum/Plasma Kit (item 217184; Qiagen, Hilden, Germany) was used for cell-free miRNA isolation. Exosomal urinary and tissue culture media miRNA isolation was done using the Norgen kit (item 47200; Norgen Biotek, Thorold, Canada) with 1 ml starting material. The miRNA concentration of the isolated RNA was measured using 2100 Bioanalyzer Small RNA Chip (Agilent, Palo Alto, CA, USA).

2.3. MicroRNA expression screening and quantitative polymerase chain reaction for microRNA detection

A 500-pg miRNA per sample was reverse transcribed using Custom Made Megaplex RT primer Pool and TaqMan MicroRNA Reverse Transcription Kit (part no. 4366597; Life Technologies, Carlsbad, CA, USA). After pre-amplification as previously described [11], real-time polymerase chain reaction (PCR) amplifications were run on ViiA7 Real Time PCR System (Life Technologies). The geometric mean of miR-16-5p and miR-106a-5p was identified as the most stable endogenous controls by the NormFinder algorithm (Supplementary Fig. 1). In the validation set, the expression of 12 miRNAs was determined using individual TaqMan MicroRNA Assays, as in our 2011 publications [11] (Supplementary Table 3). Expression levels and fold changes were calculated using the formula 2^{-ddCt}.

Details of the pilot miRNA screening on pooled samples, exosome preparation from tissue culture media, exosome transfer, and bioinformatic and statistical analysis are presented in the Supplement.

3. Results

3.1. Cell-free and exosomal microRNAs can distinguish between those with and without clear cell renal cell carcinoma

As an initial screening step, we compared the expression of 754 miRNAs in preoperative urines of ccRCC and healthy samples. Based on the result of this screening, our previously published miRNA microarray data on ccRCC tissue samples [12] and literature data, we selected 48 miRNAs with potential utility as diagnostic markers.

For the discovery phase, preoperative urine from each of 28 patients with histologically verified ccRCC and 18 healthy participants were tested for the expression of these 48 miRNAs by a custom-made miRNA array. Cell-free and exosomal miRNAs were measured separately. Using cell-free miRNAs, only miR-150-5p was significantly overexpressed in

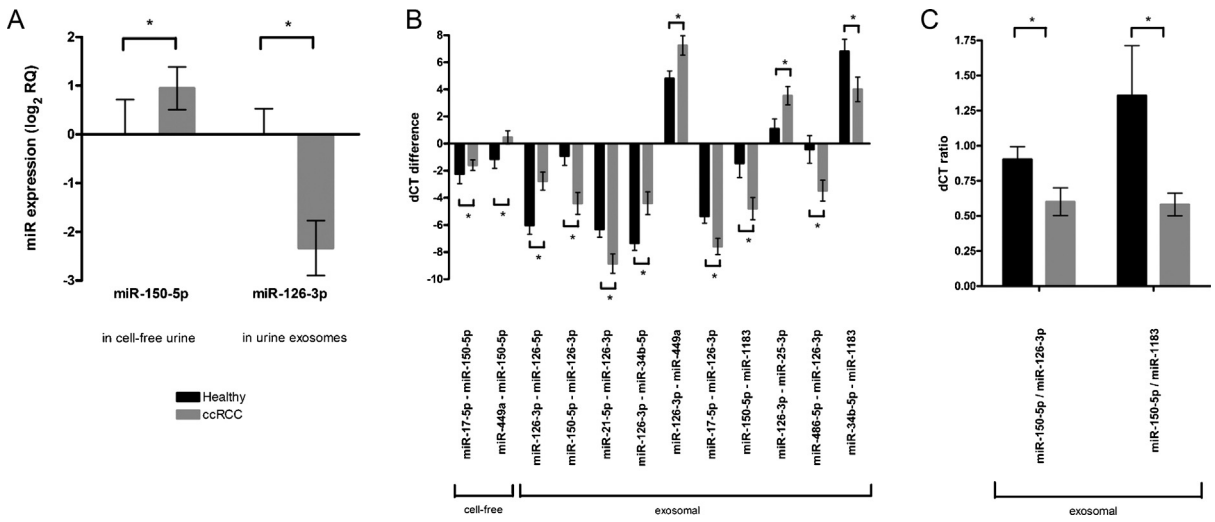

Fig. 1 – (A) miR-150-5p is significantly overexpressed in the urine of clear cell renal cell carcinoma (ccRCC) patients compared with healthy participants. (B) Differentially expressed microRNAs (miRNAs) and (C) miRNA dCt differences/ratios in cell-free urine RNA and exosomal RNA in ccRCC patients and healthy participants.
ccRCC = clear cell renal cell carcinoma; miR = microRNA.

ccRCC compared with healthy samples (fold change: 2.39; $p = 0.030$) (Fig. 1A); however, the receiver operating characteristic (ROC) did not reach statistical significance between the two groups (area under the curve [AUC]: 0.66; 95% confidence interval [CI], 0.4914–0.8260; $p = 0.058$). Measuring the expression of exosomal miRNAs, miR-126-3p was significantly downregulated (5.05-fold change) (Fig. 1A) and was able to discriminate between the ccRCC and control groups (AUC: 0.74; 95% CI, 0.5948–0.8880; $p = 0.004$).

Combinations of miRNAs were used to obtain better discrimination between healthy participants and those with cancer. Using exosomal miRNA data, we identified 12 miRNA combinations (10 dCt differences and 2 ratios) that can distinguish between the two groups. For cell-free miRNAs, two combinations were able to distinguish accurately between healthy participants and ccRCC patients (Fig. 1B). As shown in Table 1, the ROC analysis of exosomal miRNAs showed the ability of combinations of two miRNAs to obtain sensitivity of up to 86%, specificity of up to 85%, and AUCs reaching 0.77.

Because patients with early tumors (SRMs $\leq$4 cm) represent a diagnostic challenge, we tested the ability of miRNAs to detect SRMs. The miR-126-3p was found to be significantly downregulated in the urine of SRM ccRCC samples with a 5.2-fold change ($p = 0.012$), and it was able to distinguish patients with SRMs ($\leq$4 cm) from healthy participants (AUC: 0.74; 95% CI, 0.5948–0.8880; $p = 0.004$). Using different combinations of two miRNAs, we generated eight dCT differences and two dCT ratios for more significant discrimination as well (Table 1).

3.2. Biomarker validation

We validated our results in an independent set of 81 ccRCC patients, 24 patients with benign kidney tumors, and 33 healthy participants using individual reverse transcriptase-quantitative PCR reactions with miRNA-specific primers. Our validation results were very comparable with the initial discovery set (Table 1). For example, miR-126-3p showed a similar pattern of underexpression in ccRCC patients versus healthy participants with significant discriminatory power (AUC: 0.65; 95% CI, 0.5329–0.7645; $p = 0.018$).

Using a combination of two miRNAs, we were able to reach an AUC of up to 0.84. The sensitivity of the models reached 84%; specificity reached up to 83%.

Our miRNA combinations were still significantly useful in distinguishing healthy participants from those with SRMs ($\leq$4 cm) (Table 1 and Fig. 2).

When comparing the total population of tumors (both ccRCC and benign kidney tumors) with healthy participants, our model was able to distinguish between the two groups (Fig. 2 and Table 2). The miRNAs also displayed significant differential expression between healthy urine and that from patients with benign renal tumors (second section of Table 2). Combinations of miR-126-3p, miR-34b-5p, miR-150-5p, miR-449a, and miR-486-5p gave the best discrimination in most groups (Fig. 2 and Table 2). A miRNA combination (miR-17-5p and miR-25-3p) was significantly differentially expressed between benign renal tumors and ccRCC ($p = 0.026$; specificity: 87.5%). The ratio of miR-17-5p and miR-21-5p was also able to discriminate benign tumors from SRMs (Table 2).

To further examine if changes in miRNA expression are RCC related, we selected miR-126-3p, 449a, miR-17-5p, and miR-21-5p and performed a pilot analysis using three pairs of pre- and postoperative urine samples from the same patient. Our results show that miRNA levels returned to a level comparable with health after surgery (Fig. 3A).

3.3. Understanding the functional impact of microRNAs in clear cell renal cell carcinoma pathogenesis

We next explored the potential function of five miRNAs whose combination gave the best discrimination among

Table 1 – Receiver operating characteristic analysis of exosomal microRNA combinations that can distinguish between urine of clear cell renal cell carcinoma patients and healthy participants

	Discovery set						Validation set					
	Healthy urine vs all ccRCC samples											
miRNA combination	AUC	95% CI	*p* value	Cut-off	Sensitivity	Specificity	AUC	95% CI	*p* value	Cut-off	Sensitivity	Specificity
miR-126-3p–miR-126-5p	0.77	0.6376–0.9021	0.0010	>−5.5	72.4	70.0	0.77	0.6703–0.8633	<0.0001	>−2.9	70.0	75.9
miR-150-5p–miR-126-3p	0.77	0.6316–0.9064	0.0010	<−1.8	72.4	80.0	0.76	0.6639–0.8551	<0.0001	<0.2	67.5	72.4
miR-21-5p–miR-126-3p	0.77	0.6176–0.9031	0.0020	<−6.9	72.4	75.0	0.64	0.5363–0.7465	0.0245	<−6.9	70.0	55.2
miR-126-3p–miR-34b-5p	0.75	0.6141–0.8928	0.0028	>−7.7	82.8	65.0	0.79	0.7013–0.8815	<0.0001	>−3.4	77.5	72.4
miR-126-3p–miR-449a	0.75	0.6080–0.8989	0.0028	>5.1	82.8	70.0	0.84	0.7620–0.9151	<0.0001	>−3.5	83.8	62.5
miR-150-5p/miR-126-3p	0.75	0.6112–0.8853	0.0034	<0.6	65.5	80.0	0.76	0.6646–0.8613	<0.0001	<1.1	67.5	75.9
miR-150-5p/miR-1183	0.74	0.6009–0.8784	0.0047	<0.9	86.2	55.0	0.63	0.5157–0.7403	0.0417	<1.3	46.3	82.8
miR-17-5p–miR-126-3p	0.72	0.5689–0.8656	0.0104	<−5.5	72.4	55.0	0.71	0.6167–0.8117	0.0007	<−4.2	70.0	65.5
miR-150-5p–miR-1183	0.71	0.5551–0.8587	0.0147	<−0.1	86.2	55.0	0.63	0.5168–0.7366	0.0439	<1.1	51.3	79.3
miR-126-3p–miR-25-3p	0.71	0.5547–0.8556	0.0155	>2.4	62.1	85.0	0.66	0.5552–0.7612	0.0119	>2.6	52.5	86.2
miR-486-5p–miR-126-3p	0.69	0.5438–0.8424	0.0228	<−1.4	72.4	60.0	0.78	0.6739–0.8803	<0.0001	<−0.1	81.3	62.5
miR-34b-5p–miR-1183	0.67	0.5169–0.8245	0.0441	<7.7	69.0	65.0	0.72	0.6174–0.8136	0.0006	<4.1	67.5	69.0
	Healthy urine vs SRM ccRCC samples (<4 cm)											
miRNA combination	AUC	95% CI	*p* value	Cut-off	Sensitivity	Specificity	AUC	95% CI	*p* value	Cut-off	Sensitivity	Specificity
miR-150-5p–miR-1183	0.79	0.6431–0.9436	0.0034	<−1.5	86.7	70.0	0.60	0.4635–0.7313	0.1696	<0.9	45.0	82.8
miR-150-5p/miR-1183	0.79	0.6342–0.9391	0.0042	<0.8	86.7	65.0	0.64	0.5051–0.7673	0.0548	<1.4	60.0	62.1
miR-150-5p–miR-126-3p	0.78	0.6130–0.9404	0.0057	<−1.4	86.7	70.0	0.77	0.6616–0.8815	0.0001	<0.1	72.5	75.9
miR-34b-5p–miR-1183	0.75	0.5758–0.9175	0.0137	<7.6	80.0	65.0	0.70	0.5728–0.8186	0.0058	<4.6	70.0	62.1
miR-150-5p/miR-126-3p	0.74	0.5607–0.9127	0.0180	<0.6	66.7	80.0	0.77	0.6616–0.8815	0.0001	<0.1	72.5	75.9
miR-126-3p–miR-34b-5p	0.73	0.5413–0.9087	0.0245	>−7.6	80.0	65.0	0.79	0.6848–0.8980	<0.0001	>−3.4	77.5	72.4
miR-21-5p–miR-126-3p	0.71	0.5188–0.9012	0.0358	<−6.9	73.3	75.0	0.65	0.5162–0.7804	0.0366	<−7.4	65.0	65.5
miR-486-5p–miR-126-3p	0.70	0.5291–0.8776	0.0421	<−1.4	73.3	60.0	0.75	0.6246–0.8712	0.0010	<−0.1	77.5	62.5
miR-17-5p–miR-126-3p	0.70	0.5074–0.8860	0.0493	<−7.1	53.3	90.0	0.76	0.6461–0.8746	0.0002	<−4.8	72.5	72.4
miR-126-3p–miR-449a	0.68	0.4785–0.8748	0.0774	>5.1	73.3	70.0	0.89	0.8110–0.9682	<0.0001	>−2.2	77.5	91.7

AUC = area under the curve; ccRCC = clear cell renal cell carcinoma; CI = confidence interval; miRNA = micro RNA; SRM = small renal mass.

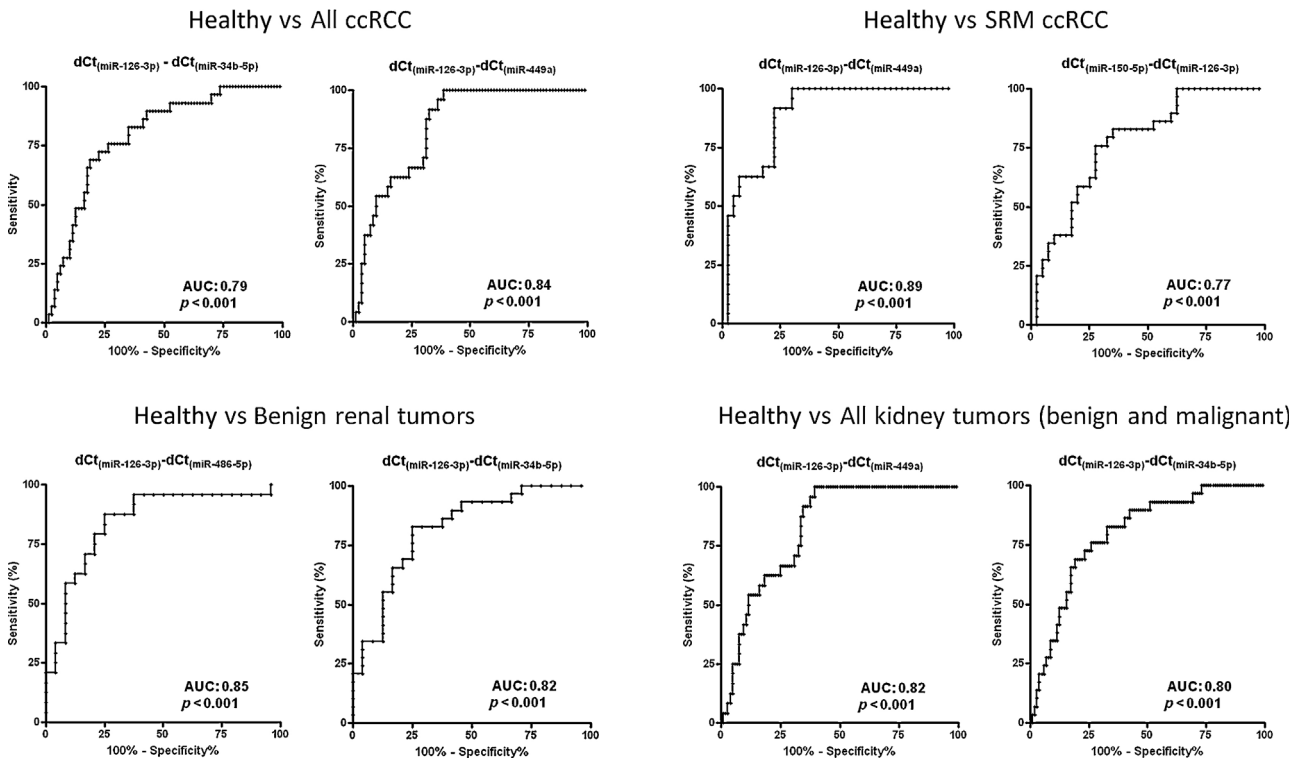

Fig. 2 – Receiver operating characteristic curves showing the discriminatory ability of different microRNA combinations of miR-126-3p, miR-34b-5p, miR-150-5p, miR-449a, and miR-486-5p to distinguish between healthy samples, clear cell renal cell carcinoma (ccRCC), and benign renal tumors. Sensitivity and specificity values are shown in Tables 1 and 2.
AUC = area under the curve; ccRCC = clear cell renal cell carcinoma; SRM = small renal mass.

Table 2 – MicroRNAs can distinguish between healthy participants and those with benign and malignant renal tumors

miRNA combination	AUC	95% CI	*p* value	Cut-off	Sensitivity	Specificity
Healthy urine vs all kidney tumors, benign and malignant						
miR-126-3p–miR-449a	0.82	0.7492–0.8974	<0.0001	>−1.8	60.6	100.0
miR-126-3p–miR-34b-5p	0.80	0.7132–0.8823	<0.0001	>−2.4	67.3	82.8
miR-126-3p–miR-486-5p	0.79	0.6958–0.8899	<0.0001	>0.8	52.9	95.8
miR-25-3p–miR-34b-5p	0.76	0.6656–0.8530	<0.0001	>−4.9	73.1	79.3
miR-21-5p–miR-34b-5p	0.76	0.6685–0.8494	<0.0001	>−10.5	74.0	72.4
miR-150-5p/miR-126-3p	0.76	0.6645–0.8528	<0.0001	<0.9	61.5	82.8
Healthy vs benign renal tumors						
miR-126-3p–miR-486-5p	0.85	0.7295–0.9615	<0.0001	>0.7	75.0	87.5
miR-126-3p–miR-34b-5p	0.82	0.7036–0.9343	<0.0001	>−2.2	75.0	82.8
miR-21-5p–miR-34b-5p	0.77	0.6421–0.9067	0.0006	>−10.5	79.2	72.4
miR-126-3p–miR-449a	0.77	0.6342–0.9110	0.0012	>−1.8	58.3	100.0
miR-17-5p–miR-34b-5p	0.77	0.6386–0.8988	0.0008	>−6.3	70.8	72.4
miR-25-3p–miR-34b-5p	0.76	0.6183–0.8961	0.0014	>−4.8	75.0	79.3
miR-34b-5p–miR-1183	0.75	0.6168–0.8889	0.0017	<4.7	75.0	62.1
Benign renal tumors vs all ccRCC						
miR-17-5p–miR-25-3p	0.65	0.5381–0.7609	0.0269	<−2.8	48.8	87.5
Benign renal tumors vs SRM ccRCC						
miR-17-5p/miR-21-5p	0.70	0.5658–0.8300	0.0084	>0.2	47.5	87.5
miR-17-5p–miR-25-3p	0.68	0.5456–0.8065	0.0191	<−2.8	52.5	87.5

AUC = area under the curve; ccRCC = clear cell renal cell carcinoma; CI = confidence interval; miRNA = microRNA; SRM = small renal mass.

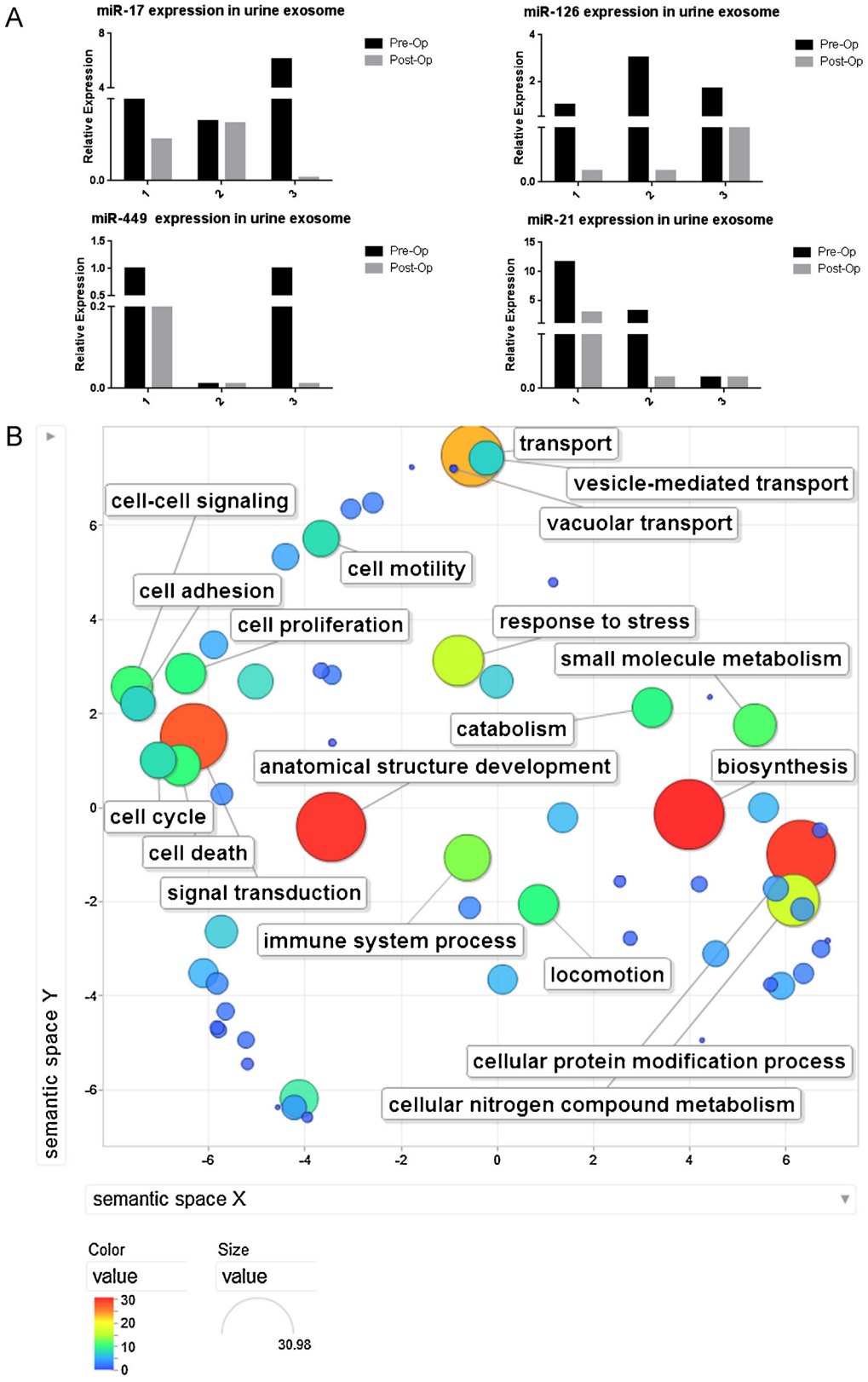

Fig. 3 – (A) Expression of miR-126-3p, miR-449a, miR-17-5p, and miR-21-5p in pre- and postoperative urinary exosomes in three patients. (B) Functional analysis of the predicted targets of the five most significant microRNAs (miRNAs) extracted from the urinary exosome of clear cell renal cell carcinoma patients. Terms represent "Biological Process" gene ontology (GO) categories. Colors and sizes indicate the percentage of GO term usage in the miRNA target gene list.
Pre-Op = preoperative; Post-Op = postoperative.

sample groups (miR-126-3p, miR-34b-5p, miR-150-5p, miR-449a, and miR-486-5p). Target prediction analysis followed by gene ontology (GO) was performed. GO classifies genes into controlled vocabularies (ontologies) that describe gene products in terms of their associated biologic processes, cellular components, and molecular functions. Our results showed that the most significant GO category was cell–cell signaling with terms including cell cycle, cell death, cell proliferation, adhesion, cell motility and transport, and vesicle-mediated transport (Supplementary Table 4 and Fig. 3B). Pathway analysis showed that "Cell Cycle: G1/S Check Point," "Pathways in Cancer," and "Angiogenesis" were the most significant pathways (Supplementary Table 5). This clearly indicates an active involvement of these miRNAs in ccRCC pathogenesis and shows that they are actively secreted.

3.4. Exosomal microRNA secretion into cell media and exosomal transfer

Next we tested whether ccRCC cell lines could secrete these exosomal RNAs. We cultured primary 786-O and metastatic ACHN and Caki-2 cell lines, and then we extracted exosomal RNA from the media and profiled the expression of the 10 selected miRNAs that we used for the validation set in patient samples. All three cell lines secreted miR-126-3p into the media (Fig. 4A). The miR-34b-5p was only secreted by metastatic Caki-2, and miR-17-5p, -21-5p, and -25-3p secretion into the media was only by the primary 786-O cells (Fig. 4B and 4C). These miRNAs were detectable in the exosome fraction compared with the exosome-depleted media, indicating active secretion through exosomes (Fig. 4D).

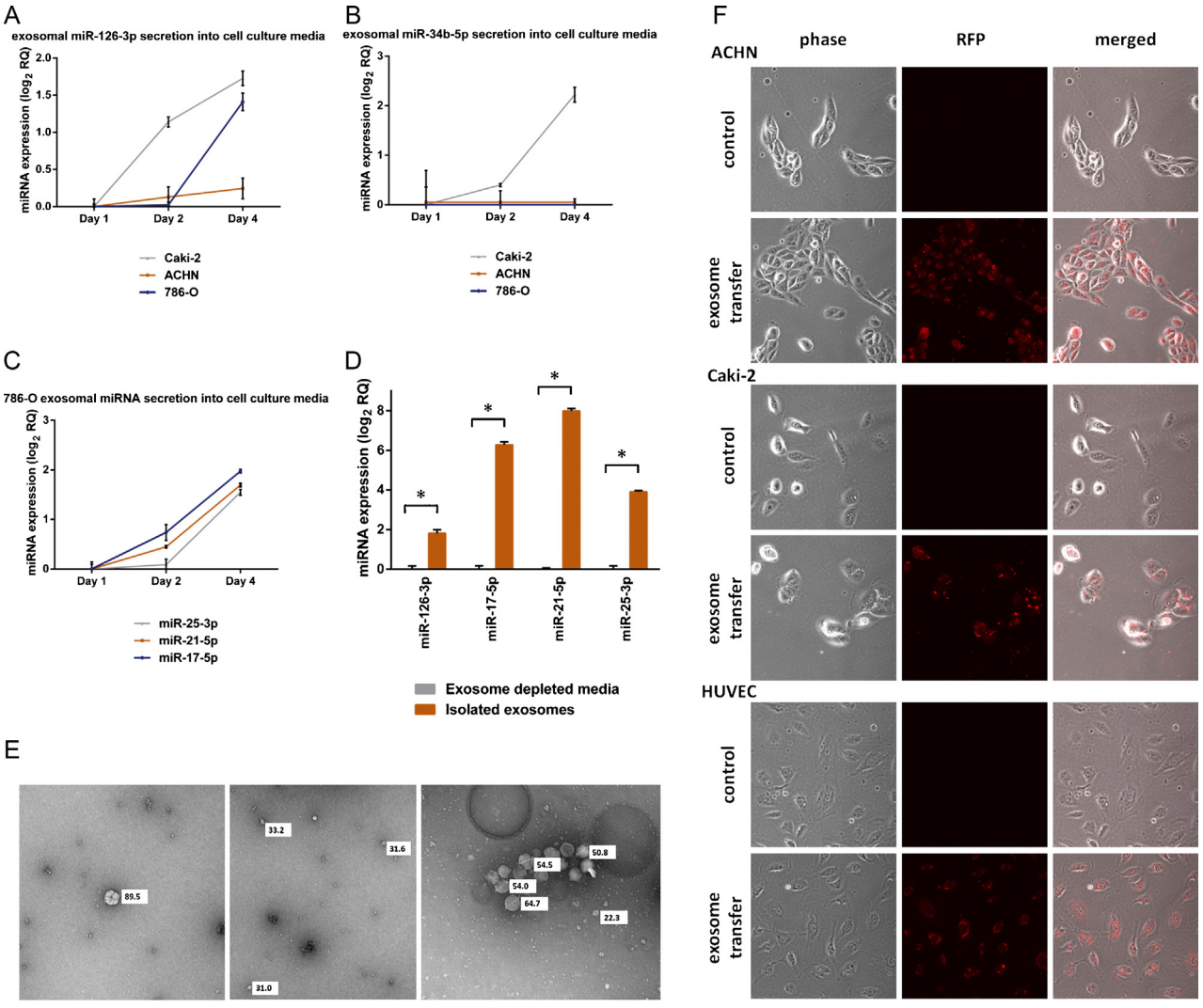

Fig. 4 – (A) miR-126-3p expression in exosomes secreted by 786-O cells. (B) Exosomal miR-34b-5p in cell culture media of three renal cell carcinoma (RCC) cell lines. (C) The miR-25-3p, miR-21-5p, and miR-17-5p are secreted by exosomes in the media of 786-O RCC cell lines. (D) All four microRNAs were detected in the exosomal but not the cell-free component of cell line media. *$p < 0.05$. (E) Electron micrographs of exosomes with the typical morphology and size (20–100 nm). Numbers indicate nanometers. (F) Exosomal RNA secreted by 786-O (stained by red fluorescent dye) was internalized into ACHN, Caki-2, and HUVEC endothelial cells after 24 h. All experiments were done in triplicate.
HUVEC = human umbilical vein endothelial cells; miRNA = microRNA; RFP = red fluorescent dye.

To examine if miRNAs can mediate cell–cell communication in RCC, we isolated exosomes secreted by 786-O cells as described in the Supplement. Exosomal structure and size was verified by electron microscopy as shown in Figure 4E. After labeling exosomal miRNAs, we transferred them to the culture media of ACHN and Caki-2 RCC cell lines and human umbilical vein endothelial cells (HUVEC). After 24 h, we detected exosomal RNA internalized by all the recipient cells (Fig. 4F). These results indicate the presence of miRNA-mediated communication between tumor cells and endothelial cells in RCC.

4. Discussion

Our results show that the combination of urinary miR-126-3p and miR-449a can distinguish between healthy participants and ccRCC patients with high sensitivity. This opens up the potential of using miRNAs as confirmatory biomarkers for ccRCC, especially for SRMs, before nephrectomy. Urinary miRNA testing can also replace invasive biopsies. Although it is impractical to screen the general population, screening high-risk groups can have a significant impact (eg, those with a family history). It can be especially useful for elderly patients with other comorbidities who are not fit for biopsy.

Our miRNA signature is useful to detect not only large renal masses but also SRMs ($\leq$4 cm). Most of these SRMs are currently diagnosed incidentally during imaging studies done for a different purpose. Early diagnosis can open the door to more treatment options including local ablation and active surveillance. It will be very practical to detect these tumors by a urine test. Our results also show that specific combinations can distinguish benign from malignant SRMs, although this awaits further validation.

Although our biomarkers did not reach the ideal combined sensitivity and specificity, they can still be clinically significant by avoiding the need for invasive biopsy in a substantial proportion of patients. Currently all patients must undergo an invasive biopsy to confirm ccRCC diagnosis. Our current sensitivity can save approximately 85% of patients the need for an invasive biopsy. Remaining patients, including both those who do not have a tumor and those with false-negative results, will still have a biopsy to confirm the nature of their lesions.

Our data are consistent with a study that reported miR-15a was significantly upregulated in the RCC urines of 10 patients [13]. This study differs from ours in that it combined different RCC subtypes, whereas ours focused on the clear cell subtype. Also, the potential utility of serum miRNAs as diagnostic markers was also recently suggested. One study showed that the combination of miR-378 and miR-451 in serum can distinguish healthy participants from RCC patients. This study also included multiple RCC subtypes in its analysis [14]. The miR-210 and miR-1233 were also reported to be promising serum biomarkers for ccRCC [15,16]. Analysis of urinary exosomal protein by liquid chromatography coupled with tandem mass spectrometry identified a 10-protein signature that was differentially expressed between healthy and RCC urine [17].

Although our results show that these miRNAs are cancer related, it will be important to validate these results in a larger patient cohort including those with other kidney disease and urinary tract infections.

Of our five miRNA candidates, only miR-34b was previously reported as overexpressed in ccRCC [13,18,19]; however, another study reported methylation of miR-34b in 100% of cases [20]. The miR-449a and miR-486-5p were reported as tumor suppressors in several malignancies [21,22]. We detected miR-126-3p to be downregulated in healthy urine compared with ccRCC. The miR-126-3p was also described to be underexpressed elsewhere. It was found to be a prognostic factor in metastatic versus primary tumors and in tumors that developed relapse [23,24]. In our previous work we also found that miR-126 showed negative correlation with its target, vascular endothelial growth factor A (VEGFA) [25].

Our study is the first to show the potential role of exosomal miRNAs in cell communication in RCC. It is suggested that genetic material exchange in the exosomes (including miRNAs) is part of cell–cell communications [26]. Our functional analysis revealed that the targets of our miRNAs can play a role in cell cycle, tumorigenesis, and angiogenesis. Cell–cell signaling was the most significant process through which these miRNAs can affect cancer. The literature suggests that secreted exosomes can have anti- and protumorigenic roles by inducing immunologic or immune-suppressive reactions, inducing apoptosis, facilitating tumor invasion and metastasis, or supporting survival and growth [26]. Our results, however, require further validation in vivo.

We presented that exosomal RNA can be delivered from primary ccRCC cells to metastatic kidney cancer and endothelial cells and that this very likely influences the behavior of the recipient cells and tumor progression. Interestingly, miR-150 was described to be delivered by microvesicles to tumor-associated macrophages and enhanced their VEGF secretion or promoted angiogenesis [27,28]. It was demonstrated that exosomes isolated from the ACHN metastatic RCC cell line inhibited Jurkat T lymphocyte proliferation and cytokine production, and induced apoptosis [29]. ACHN-derived exosomes also enhanced proliferative activity and suppressed apoptosis of the same cells. Similarly, autocrine exosome-driven proliferation was also demonstrated on OS-RC-2 RCC cells [30].

5. Conclusions

We provide strong evidence supporting the potential utility of urinary miRNAs as a potential diagnostic tool for ccRCC, especially SRMs. We also provide preliminary evidence that miRNAs can mediate cell–cell communication in kidney cancer.

Author contributions: George M. Yousef had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Butz, Yousef.
Acquisition of data: Butz, Nofech-Mozes.
Analysis and interpretation of data: Butz, Nofech-Mozes, Yousef.

Drafting of the manuscript: Butz, Yousef.
Critical revision of the manuscript for important intellectual content: Jewett, Finelli, Lee, Ordon, Stewart.
Statistical analysis: Butz, Szabo.
Obtaining funding: Yousef.
Administrative, technical, or material support: Butz, Yousef.
Supervision: Jewett, Finelli, Lee, Ordon, Stewart, Krylov.
Other (specify): None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.euf.2015.11.006.

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