Kidney Cancer

MicroRNAs in Serum Exosomes as Potential Biomarkers in Clear-cell Renal Cell Carcinoma

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\textbf{Abstract}

\textbf{Background:} Circulating microRNAs (miRNAs) in exosomes are emerging as clinically useful tools for cancer detection. However, little is known about their diagnostic impact in clear-cell renal cell carcinoma (ccRCC).

\textbf{Objective:} To investigate whether miRNAs in serum exosomes can serve as biomarkers in ccRCC.

\textbf{Design, setting, and participants:} Serum samples were obtained from 82 patients with ccRCC and 80 healthy volunteers. Exosomes were extracted and purified to selectively capture exosomes positive for tumor-associated epithelial cell adhesion molecule (EpCAM) via a magnetic bead technique. Total RNA was extracted and expression levels of miR-210, miR-1233, and miR-15a miRNAs were quantified and normalized to U6 levels.

\textbf{Outcome measurements and statistical analysis:} Expression levels were compared using a Mann-Whitney U-test, Friedman test, or Wilcoxon test. Receiver operating characteristic (ROC) curves were plotted to assess the diagnostic value of exosomal miRNAs for differentiation between ccRCC patients and controls.

\textbf{Results and limitations:} Expression levels of exosomal miR-210 and miR-1233 were significantly higher in ccRCC patients than in healthy individuals (both \(p<0.01\)). No significant difference was observed for exosomal miR-15a. Exosomal miR-210 and miR-1233 expression levels in different TNM stages were significantly higher than in the controls (all \(p<0.01\)). Exosomal miR-210 and miR-1233 expression levels were significantly lower in postoperative than in preoperative samples (both \(p<0.01\)). ROC analysis demonstrated that exosomal expression levels distinguished ccRCC patients from healthy individuals with 70% sensitivity and 62.2% specificity for miR-210, and 81% sensitivity and 76% specificity for miR-1233. The retrospective design and lack of other tumor subtypes are limitations of the study.

\textbf{Conclusions:} Serum exosomal miRNAs might represent potential diagnostic biomarkers in ccRCC in the future.

\textbf{Patient summary:} Circulating levels of exosomal microRNAs miR-210 and miR-1233 have potential as biomarkers for diagnostic and monitoring purposes in renal cancer in the future. These molecules can be measured in serum in so-called liquid biopsy.

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1. Introduction

Kidney cancer currently represents the third most common urological cancer in the USA, with an estimated 61,560 new cases and 14,080 deaths in 2015 [1]. Clear-cell or conventional renal cell carcinoma (ccRCC) is the most frequent RCC subtype, accounting for approximately 75–80% of these tumors, and is responsible for the majority of deaths caused by the disease [2]. Recent advances in diagnostic techniques have increased early ccRCC detection and decreased the mortality rate. However, up to 30% of patients with ccRCC present with metastatic disease at diagnosis, and nearly 20–30% of patients undergoing surgery will suffer recurrence [3]. Despite longer survival and improved response rates to targeted therapy for metastatic RCC, median overall survival is still <2 yr [4]. Therefore, it is highly desirable to have biomarkers suitable for ccRCC screening and monitoring.

Liquid biopsy testing of circulating tumor DNA or tumor cells has been considered the most promising tool for clinical application, although the majority of circulating tumor DNA is often not of cancerous origin and circulating tumor cells are detected incidentally [5,6]. RNAs transcribed from noncoding regions are arbitrarily classified as either short or long noncoding RNAs using a threshold of 200 nucleotides. Short noncoding RNA comprises micro-RNAs (miRNAs), which are regarded as small but highly influential molecules [7]. Several studies have demonstrated that selective loading of specific proteins and miRNAs in exosomes plays a role in cell-to-cell communication [8,9]. Exosomes can be secreted by cancer cells during tumor progression and metastasis [10]. Although the secretory mechanisms and biological functions of tumor-associated exosomes are still unclear, use of circulating exosomes as potential noninvasive biomarkers holds promise.

Several researchers, including our own group, recently reported differences in miRNA expression levels in blood samples between ccRCC patients and healthy controls [11–13]. It is still not known whether these miRNAs are also expressed in tumor-associated exosomes. To address this question, the present study had three aims: (1) to extract and purify tumor-associated epithelial cell adhesion molecule (EpCAM)-positive exosomes; (2) to confirm whether circulating exosomal miRNA is stable enough for amplification; and (3) to evaluate expression levels of three selected miRNAs in tumor-associated exosomes in serum from ccRCC patients and healthy controls.

2. Patients and methods

2.1. Patients and sample collection

This retrospective analysis included data for 82 ccRCC patients who underwent curative partial or radical nephrectomy and for whom a blood sample was collected 1 d before tumor resection at the Department of Urology at Zhejiang Cancer Hospital in Hang Zhou. Diagnoses were confirmed by two independent genitourinary pathologists. Pathologic T stages were uniformly adjusted according to the 7th edition of the TNM classification system [14]. ccRCC was graded according to the Fuhrman criteria [15]. The study was approved by the local ethics committee. Patients received necessary information concerning the study and a consent statement was signed by each patient. Control serum samples were collected from 80 healthy volunteers. In addition, serum samples were collected from ten patients 7 d after tumor resection. Details for the study population are presented in Supplementary Table 1.

2.2. Processing of serum samples

Serum samples were obtained via centrifugation of blood samples at 1200 × g (centrifuge model 5417R; Eppendorf, Hamburg, Germany) for 10 min at 4 °C to spin down the cells. The supernatant was collected and further centrifuged at 12,000 × g for 10 min at 4 °C (centrifuge model 5417R; Eppendorf) to completely remove cellular components. The cell-free serum samples were then stored at −80 °C until exosome isolation. Serum samples were processed and frozen within 4 h after the blood draw.

2.3. Isolation of total exosomes from serum

Exosomes were isolated using Total exosome isolation reagent (Invitrogen, Carlsbad, CA, USA). In brief, 500 μl of serum was centrifuged at 3000 × g for 30 min to remove cells and cell debris. The supernatant was mixed with 100 μl of exosome isolation reagent and incubated at 2–8 °C for 30 min. The sample was then centrifuged at 10,000 × g for 30 min at 4 °C. Total exosomal pellets were dissolved in SDS sample buffer.

2.4. Exosome purification with EpCAM beads

EpCAM positive exosomes were isolated using EpCAM isolation beads (Invitrogen) with 40 μl of beads per 100 μl of sample. After incubation for 3 h at 4 °C under slow rotation, the bead-bound exosomes were isolated on a magnetic separator and washed and eluted for downstream analysis.

2.5. Flow cytometry analysis and immunofluorescence of circulating exosomes

The beads were pelleted by centrifugation at 1000 × g for 3 min at room temperature (RT), washed twice with 1 ml of phosphate-buffered saline (PBS)/0.5% bovine serum albumin (BSA), and the exosome-bead complex was incubated with anti-EpCAM fluorescein isothiocyanate (FITC)-conjugated primary antibodies or corresponding isotype control (Invitrogen) at RT for 1 h. The labeled exosome-bead complexes were then pelleted and washed twice. The final complexes were resuspended in 150 μl of PBS/0.5% BSA for flow cytometry analysis. Flow cytometry was performed on a Beckman Coulter system (Brea, CA, USA). For immunofluorescence, exosome-bead complexes were incubated with anti-Cd9 antibodies conjugated to Alexa Fluor 488 (Abcam, Cambridge, UK) at RT for 1 h and washed twice as above. Exosome-latex bead-antibody complexes were spread onto a microscope slide and a cover slip was paled on top and sealed with nail polish. The slides were examined under an Olympus BX50 microscope (Tokyo, Japan) and images were taken with an Olympus DP70 camera using Leica imaging software.

2.6. Stability of circulating exosomes

2.6.1. Delayed processing

Exosome samples were divided into two groups (n = 6 per group) for experiments at RT and 4 °C. Each group was divided into three aliquots for delayed processing (0, 6, and 24 h). The exosome samples were then used for RNA extraction.

2.6.2. Freezing and thawing
Each exosome samples was divided into three aliquots. One aliquot was snap-frozen in liquid nitrogen and then immediately thawed at RT. Another aliquot was snap-frozen and thawed and the thawed sample was left at RT for 2 h. The exosome samples were then used for RNA extraction.

2.6.3. Lability of free RNA added to exosome samples
We added 1000 ng of commercially available human reference RNA (Stratagene, San Diego, CA, USA) to randomly selected exosome samples and incubated them for 0, 10, 20, or 30 s at RT. After incubation, QIAzol solution (Qiagen, Hilden, Germany) was immediately added to stop any ribonuclease activity before RNA extraction.

2.7. RNA extraction
Total RNA was extracted using a MicroMini kit (Qiagen) according to the manufacturer's protocol. In brief, 700 μl of QIAzol solution was added to each sample for lysis. RNA was eluted in 25 μl of RNase-free water. The RNA concentration and purity were monitored via UV spectrophotometry using a Nanodrop ND-1000 instrument (Thermo Scientific, Waltham, MA, USA). The RNA specimens were stored at −80 °C until reverse transcription (RT) was performed.

2.8. Reverse transcription
RT reactions were carried out with a miScript reverse transcription kit (Qiagen) in a total volume of 20 μl containing 10 μl of RNA extract, 4 μl of 5 × miScript RT buffer, and 1 μl of miScript reverse transcriptase mix. For synthesis of cDNA, the reaction mixtures were incubated at 37 °C for 60 min and 95 °C for 5 min, and then held at 4 °C. The cDNA specimens were stored at −20 °C until polymerase chain reaction (PCR).

2.9. miRNA quantification by real-time quantitative PCR
Amounts of miRNAs were quantified by qPCR using a miScript SYBR green PCR kit (Qiagen). A 2-μl aliquot of cDNA solution was amplified

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**Fig. 1** – (A) Flow cytometry analysis of tumor-associated exosomes. Appropriate gating reveal strong intensity for the marker EpCAM. (B) Immunofluorescence staining after separation of tumor-associated exosomes clearly showed the exosome marker CD9 on the surface of exosome-bead complexes.

using 12.5 μl of 2× QuantiTect SYBR green PCR master mix, 1 μl of 10× miScript universal primer, 1 μl of 10× miScript primer assay, and 8.5 μl of nuclease-free water in a final volume of 25 μl. Three miRNAs (miR-15, miR-210, and miR-1233; Qiagen) were selected on the basis of previous reports. Quantitative PCR was run on a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The reaction mixtures were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 56°C for 30 s, and 72°C for 35 s. Universal human reference RNA (Stratagene) was used as a positive control and RNase-free water (Qiagen) as a negative control in each PCR assay. Cycle threshold (Ct) values were calculated using SDS 1.4 software (Applied Biosystems). Each sample was run in duplicate for analysis. Expression levels of the miRNAs were normalized to U6, and were calculated using the $2^{-\Delta Ct}$ method.

### 2.10. Statistical analysis

Expression levels were compared using the Mann-Whitney U-test, Friedman test, or Wilcoxon test. Receiver operating characteristics (ROC) curves were plotted to assess the diagnostic value of exosomal miRNAs for differentiation between ccRCC patients and controls. A p value of <0.05 was considered statistically significant for multiple comparisons. All analyses were performed using SPSS version 13.0 and GraphPad prism 5.0 software.

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**Fig. 2** – Fig. 2. Scatter plots of expression levels of exosomal (A) miR-210, (B) miR-1233, and (C) miR-15 in serum from patients with clear-cell renal cell carcinoma (ccRCC; n = 82) and healthy controls (n = 80). The exosomal miR-210 level was significantly higher in ccRCC patients (mean expression $13.5E-3 \pm 4.6E-3$) than in the controls (mean expression $1.3E-3 \pm 2.2E-3$; p < 0.001). The exosomal miR-1233 level was significantly higher in ccRCC patients (mean expression $115.9E-3 \pm 30.7E-3$) than in the controls (mean expression $4.5E-3 \pm 6.0E-3$; p < 0.001). No significant difference was observed for the level of exosomal miR-15a (p = 0.619). miRNA expression levels (log10 scale on the y-axis) were normalized to U6. The line represents the median value. A Mann-Whitney U-test was used to determine statistical significance.

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3. Results

3.1. Flow cytometry analysis and immunostaining of circulating tumor-associated exosomes

To separate tumor-associated exosomes, total circulating exosomes were incubated with anti-EpCAM magnetic beads and the tumor-associated exosomes were isolated by magnetic separation. Tumor-associated exosomes were then visualized following incubation with fluorescence-conjugated antibodies to EpCAM and an isotype control (Fig. 1A). Immunostaining of the same beads clearly showed the exosome marker CD9 on the surface of the exosome-bead complexes (Fig. 1B).

3.2. Effect of different conditions on circulating exosomes

Circulating tumor-associated exosomal U6 RNA (as determined by real-time quantitative PCR) revealed no differences in exosomal miRNA expression for different exposure times and incubation temperatures (p > 0.05; Supplementary Fig. 1A). The quantity of RNA extracted (as determined by spectrometry after various processing steps, including freeze-thawing, 2-h storage after freeze-thawing, and no treatment) showed no significant differences in median RNA extracted (p > 0.05; Supplementary Fig. 1B). The quantity of RNA extracted at 10, 20, and 30 s after addition of exogenous RNA and without any RNA addition revealed an obvious decrease in exogenous free RNA over time, suggesting that free RNA added to the sample was immediately degraded by RNA enzyme in cell-free serum and became undetectable (p > 0.05; Supplementary Fig. 1C).

3.3. Expression levels of exosomal miR-210, miR-1233, and miR-15 in serum

Expression levels of exosomal miR-210, miR-1233 and miR-15 in serum were analyzed using scatter plots for comparison of ccRCC patients (n = 82) and healthy controls (n = 80). Both exosomal miR-210 (p < 0.001; Fig. 2A) and exosomal miR-1233 (p < 0.001; Fig. 2B) levels were significantly higher in ccRCC patients than in controls. There was no significant difference in exosomal miR-15a levels (p = 0.892; Fig. 2C). We examined the correlation between exosomal miRNA levels and clinical parameters. We found no significant association between exosomal miRNA levels and gender, age, or ccRCC grade (Table 1).

Next, we compared expression levels of exosomal miRNAs between the control subjects and ccRCC patients with different TNM stages. Exosomal miR-210 and miR-1233 expression levels in each stage were significantly higher than in the controls (all p < 0.01; Fig. 3A,B). No significant differences were observed among the different TNM stages.

3.4. Comparison of preoperative and postoperative serum exosomal microRNAs in ccRCC

Expression levels of serum exosomal miR-210 and miR-1233 were analyzed before and 7 d after surgical tumor removal in ccRCC patients. Expression levels of exosomal miR-210 (Fig. 4A) and exosomal miR-1233 (Fig. 4B) were significantly lower in postoperative than in preoperative samples.

3.5. Serum exosomal miR-210 and miR-1233 as a diagnostic tool in ccRCC

To assess the feasibility of serum exosomal miR-210 and miR-1233 as ccRCC diagnostic tools, we performed ROC curve analysis. The area under the ROC curve (AUC) for exosomal miR-210 was 0.69 (95% CI 0.61–0.77) with sensitivity of 70% and specificity of 62.2% in discriminating between ccRCC patients and controls (Fig. 5A). Exosomal miR-1233 yielded an AUC of 0.82 (95% CI 0.75–0.89) with sensitivity of 81% and specificity of 76% (Fig. 5B).

4. Discussion

Exosomes have emerged as a novel source of noninvasive cancer biomarkers as tumor-specific molecules can be
found in exosomes isolated from biological fluids. Several studies have examined exosomal miRNAs as potential tumor biomarkers in attempts to improve the accuracy of diagnostic methods [16]. Regarding urologic cancers, circulating exosomal miR-1290 and miR-375 might serve as sensitive biomarkers for prognostic purposes in castration-resistant prostate cancer [17]. Another study found that overexpression of exosomal miRNA in urine was associated with bladder cancer [18]. To the best of our knowledge, this is the first study to compare expression levels of serum exosomal miRNAs between ccRCC patients and healthy controls. Our results indicate that circulating exosomal miRNAs hold promise for so-called liquid biopsy for RCC diagnosis.
miRNA-15a levels from paraffin-embedded tissue samples and urine samples could differentiate malignant and benign samples. Taken together, these data suggest that not all overexpressed miRNAs can be loaded into secreted exosomes.

Wong et al [23] reported increased miR-1233 levels in patients with heart failure. Increased miR-210 levels have also been reported in the context of various cancers and other hypoxia-induced pathologic conditions [24,25]. miRNAs are expressed in all organisms, which could influence the diagnostic accuracy. Chevillet et al [26] quantified both the number of exosomes and the number of miRNA molecules in an exosome collection, and found that most exosomes did not harbor many copies of miRNA molecules. Exosomes can reflect their tissue or cell of origin by the presence of specific surface proteins [27]. We used magnetic beads bound to EpCAM antibodies to avoid secondary interference from nonepithelial tissues such as heart, which might improve the accuracy of ccRCC detection.

Exosomal miR-210 and miR1233 levels were significantly higher in patients with TNM stage 1 ccRCC and other stages compared to healthy controls (p < 0.01), but there were no differences among the different stages. We also compared preoperative and postoperative expression levels. Expression levels of miR-210 (p = 0.004) and miR-1233 (p = 0.008) were significantly lower postoperatively, suggesting that both miR-210 and miR-1233 might be released to some extent from the renal primary tumor into serum, and that miRNA changes might directly reflect cancer status. ROC analysis showed that serum exosomal miR-210 and miR-1233 levels can differentiate ccRCC patients from healthy controls.

Although this study was conducted according to the reporting recommendations for tumor marker prognostic studies [28], some limitations merit discussion. The sample size of our study cohort was relatively small, so further validation in larger patient cohorts is necessary. Owing to the enormous heterogeneity of renal neoplasms, it is highly unlikely that a single molecule would be ideal for evaluation and diagnostic purposes. The retrospective design is another limitation. We plan to validate our results in a prospective study in the future. In the present study, we did not evaluate exosomal miRNA expression levels in other renal neoplasm subtypes. Although ccRCC is considered the most common renal neoplasm, it will be interesting to investigate expression patterns for exosomal miRNAs in other renal neoplasm subtypes. Despite these limitations, our study clearly demonstrates that miRNA-containing exosomes in serum from ccRCC patients are stable enough to be amplified, and thus might serve as a novel source of RCC biomarkers in the future.

5. Conclusions

Serum exosomal miR-210 and miR-1233 were upregulated in ccRCC independently of clinical staging. Circulating exosomal miR-210 as well as miR-1233 might be potentially used in ‘liquid biopsies’ for diagnostic and monitoring purposes in RCC patients.
Author contributions: An Zhao 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: Zhao, Li.
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Analysis and interpretation of data: Wang, Ni, Zhao.
Drafting of the manuscript: Zhang, Zhu, Zhao, Li.
Critical revision of the manuscript for important intellectual content: Zhang, Zhao, Li.
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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.2016.09.007.

References