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A serum three-microRNA panel: promising biomarkers for renal cell carcinoma screening

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Abstract

Aim: Renal cell carcinoma (RCC) screening is helpful to improve the prognosis of patients. However, the existing RCC detection methods are not suitable for large-scale screening. Serum microRNAs (miRNAs) is expected to be a convenient, economical, and non-invasive screening tool for RCC. This study aimed to identify relevant serum miRNAs as diagnostic markers for RCC.

Methods: This research included 112 patients with RCC and 112 healthy control individuals, carried out in three distinct phases. The objective was to identify serum miRNAs suitable for RCC diagnosis using quantitative reverse transcription polymerase chain reaction (RT-qPCR). Additionally, bioinformatics analyses were performed to predict target genes and provide functional annotations.

Results: Compared with healthy controls, patients with RCC highly expressed miR-221-3p and lowly expressed miR-124-3p, let-7b-5p, miR-30a-5p, and miR-302d-3p. After multiple rounds of combination screening, the



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combination of miR-124-3p, miR-221-3p, and let-7b-5p showed good diagnostic predictability. The diagnostic panel exhibited a 0.838 area under curve (AUC), achieving 75.00% sensitivity and 77.68% specificity.

Conclusion: Our analysis demonstrates that combining miR-124-3p, let-7b-5p, and miR-221-3p forms a non-invasive, economical, and remarkably effective diagnostic indicator for patients with renal cell carcinoma.

Keywords: microRNA, miR-221-3p, let-7b-5p, renal cell carcinoma, miR-124-3p, biomarker

INTRODUCTION

Among urological malignancies, renal cancer represents a significant health burden, accounting for approximately 5% of malignant neoplasms and standing as the sixth leading cancer type in males. It also represents 3% of malignancies, placing it tenth among women^[1]. According to Global Cancer Statistics 2020, there were 431,288 new cases of kidney cancer and 179,368 deaths attributed to this disease^[2]. Renal cell carcinoma (RCC), comprising approximately 90% of kidney cancers, originates from the renal tubular epithelial cells^[3]. RCC is a heterogeneous cancer that may originate from different cells throughout the nephrons. Examining histologic characteristics reveals that the predominant solid RCC varieties include clear cell RCC (comprising 70%-90% of cases), followed by papillary (types I and II) representing 10%-15%, and chromophobe accounting for approximately 3%-5% of cases^[4]. Surgical intervention remains the cornerstone of RCC treatment^[5]. In addition, adjuvant therapies post-surgery, such as hormone therapy, radiotherapy, immunotherapy^[6,7], vaccines, and target agents^[8], play crucial roles. Despite advances in modern medical treatment enhancing disease control and survival, many patients with RCC still develop locally advanced disease and metastatic stages^[9]. Clinical data show that metastases are present in 20%-30% of RCC patients at their first medical evaluation; 30%-50% develop metastases as the disease progresses, and nearly 40% of patients with localized disease eventually develop distant metastases post-surgery^[10]. According to the statistical results of patients diagnosed with RCC (or renal pelvis) from 2011 to 2017, the 5-year relative survival rate reaches 93% for patients with localized RCC, but drastically falls to 14% for those with distant metastases^[11]. Due to its severe health implications, early screening methods for renal cell carcinoma can significantly enhance patient survival rates.

Existing methods of RCC detection are not suitable for mass screening and preliminary screening. Traditional detection of kidney cancer depends on several imaging modalities - MRI, ultrasonography, and CT scanning; however, a definitive diagnosis still requires histopathological assessment and biopsy. These methods, though valuable, are inadequate for early screening due to their limited accuracy or high invasiveness. The diagnostic process of RCC primarily relies on contrast-enhanced CT scanning, which yields excellent results with 98.3% sensitivity and 92% specificity when applying a 100 HU threshold^[12]. However, its invasive nature and expensive and high cost render it impractical for widespread or early diagnosis. US, although promising for early detection, lacks specificity and involves a complex procedure, making it unsuitable for large-scale population screening. Although renal biopsy remains the definitive diagnostic approach for RCC, this invasive procedure's complications and complexity make it unsuitable for initial RCC screening. Given these challenges, there is a pressing need to develop new, more accurate, convenient, and cost-effective diagnostic tools for large-scale screening and preliminary screening of RCC. Although there are already many biomarkers for urinary tumors, there are currently no tissue or blood biomarkers for routine clinical practice of RCC^[13].

MicroRNAs (miRNAs) belong to endogenous non-coding small RNA species that regulate gene expression by binding to the 3' untranslated regions of messenger RNA, resulting in either translational inhibition or mRNA degradation. The role of miRNAs as diagnostic indicators has been well documented, with evidence

showing their significant involvement in multiple human cancer types' pathogenesis. Studies have shown that the highly stable miRNAs in circulation are mainly bound to lipid-protein complexes, including exosomes, macrovesicles, and apoptotic bodies^[14,15]. Various investigations have highlighted the diagnostic significance of exosomes across multiple cancer types, including bladder malignancies, tumors of the gastrointestinal tract, and neoplasms affecting the neurological system^[16-18]. Based on these advantages, blood-derived miRNA could serve as a minimally invasive and economical biomarker for RCC detection.

Our assumption was that expression levels of miRNAs in patient serum might be influenced by the presence of RCC. Consequently, this study aimed to identify crucial miRNAs as diagnostic biomarkers for RCC. Reverse transcription polymerase chain reaction (RT-qPCR) analysis was employed to quantify miRNA expression in serum specimens from RCC cases and healthy subjects. Subsequently, candidate miRNAs were screened, and stepwise logistic regression modeling was employed to identify a diagnostic panel for RCC. The potential biological roles of these miRNAs were subsequently investigated through bioinformatics analysis.

METHODS

Statement of ethical compliance

This research was conducted under the authorization of the Peking University Shenzhen Hospital Ethics Committee (reference: 2017-007-1). All procedures complied with the ethical principles outlined in the World Medical Association's Declaration of Helsinki. From September 2017 through May 2021, our study enrolled a total of 224 individuals at Peking University Shenzhen Hospital, comprising 112 patients diagnosed with RCC and an equal number of healthy controls (HCs). The serum collection procedure adhered to the ethical guidelines established by the committee.

The study enrolled RCC cases based on two key requirements: (1) All subjects provided written informed consent and had no previous therapeutic interventions at the time of blood collection; (2) The diagnosis of RCC must be validated through surgical specimens or pathological examination. Exclusion criteria for RCC patients: (1) Patients did not consent to participate in this study; (2) Biopsy pathology suggestive of RCC that has not been treated with surgery and other treatments, or the patient has not been treated at our hospital; (3) Patients who have had other benign or malignant tumor diseases; (4) This includes, but is not limited to, patients with combined cardiac insufficiency, acute and chronic infections, diabetes mellitus, and hepatic, renal, and cerebral impairment; (5) Patients assessed by the investigator as unsuitable for participation. Patients may not be included in the study if any of the above exclusion criteria are met.

Inclusion criteria for HCs: (1) All study participants completed and provided signed consent documentation; (2) The study subjects underwent comprehensive health screenings at Peking University Hospital (Shenzhen)'s Physical Examination Unit, with age and gender matching to RCC cases; (3) Participants were confirmed to be free of other diseases, including but not limited to cardiac insufficiency, acute and chronic infections, diabetes mellitus, hepatic, renal, and cerebral impairment, and various types of benign and malignant tumors after ultrasound, CT, and other examinations; (4) Participants had no history of other benign or malignant tumors and systemic diseases. Participants could not be included in HC if they did not meet any of the above criteria.

Research design

As depicted in [Figure 1](#), our research methodology encompassed four distinct phases. The first phase involved mining the Gene Expression Omnibus database through PubMed to identify differentially expressed miRNAs in renal RCC samples. Subsequently, we utilized the ENCORI platform to evaluate

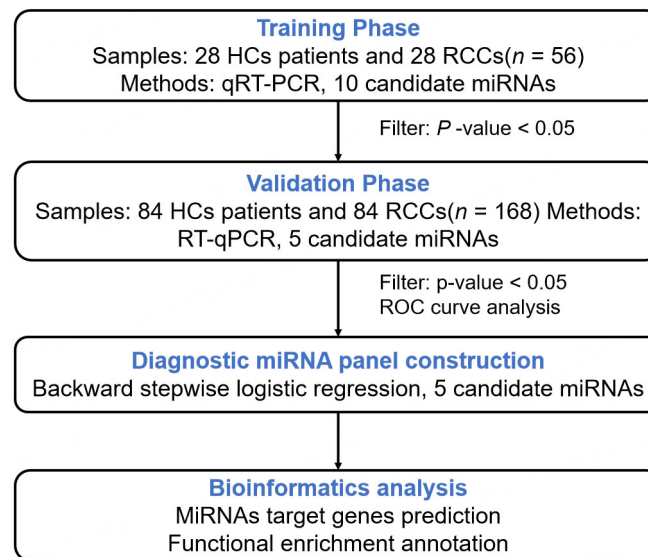


Figure 1. Study design overview. RCC: Renal cell carcinoma; RT-qPCR: reverse transcription polymerase chain reaction; miRNAs: microRNAs; ROC: receiver operating characteristic; HCs: healthy controls.

miRNA expression patterns during the screening stage. The selection criteria for these potential miRNAs were established based on statistical significance ($P < 0.01$) and expression magnitude (fold change exceeding 2 or below -2). To evaluate these potential biomarkers' performance, we implemented a two-stage study design. Initially, we performed RT-qPCR analysis to examine the differential expression of 10 miRNA candidates between RCC patient sera and healthy control samples. Those miRNAs demonstrating significant differences were subsequently validated using an enlarged cohort to confirm their expression patterns. Finally, we constructed a miRNA panel with the greatest diagnostic potential for RCC using reverse stepwise logistic regression analysis. Finally, we performed bioinformatics analysis of the miRNAs.

Serum samples collection

Blood specimens were obtained from RCC patients who were treatment-naïve, with no history of surgical procedures, radiation therapy, or anti-cancer drugs. All sample collection procedures were conducted in accordance with the ethical guidelines approved by the Peking University Shenzhen Hospital Ethics Committee. For each participant, peripheral blood (5-10 mL) underwent centrifugation (3,000 g, 4 °C, 10 min) within 2 h to obtain serum samples.

RNA extraction

The isolation of total RNA from serum was conducted with a TRIzol LS kit (manufactured by Thermo Fisher Scientific in USA, catalog #10296-028). To reduce variability in total RNA extraction process, each serum sample was treated with 2 µL of miR-54 (10 nM/L, RiboBio, China). The concentration and purity of the extracted RNA were subsequently measured using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Carlsbad, CA, USA).

RT-qPCR

For miRNA quantification, we employed the Bulge-Loop RT-qPCR primer sets supplied by RiboBio Corporation (Guangzhou) in the reverse transcription step. Expression analysis was performed on a Roche LightCycler 480 platform with Takara's Premix Ex Taq™ probe qPCR kit (catalog #RR390Q, Beijing). The thermal cycling protocol consisted of an initial 20-second step at 95 °C, with subsequent cycles comprising 10 s of DNA denaturation (95°C), primer annealing for 20 s (60 °C), and a final 10 s extension phase

(70 °C), repeated 40 times.

Bioinformatic analysis

For investigating the mechanistic implications of these miRNAs in RCC development, target prediction and miRNA-target interaction validation were performed through miRWalk 3.0 platform (<http://mirwalk.umm.uni-heidelberg.de/>). Subsequently, target gene sets underwent functional annotation and enrichment studies using the Enrichr online tool (<http://amp.pharm.mssm.edu/Enrichr/>)^[19].

Statistical analysis

The miRNA expression in serum specimens was quantified applying the $2^{-\Delta\Delta Cq}$ methodology during our validation and experimental testing^[20]. Furthermore, Student's t-test and chi-square analysis were employed to contrast miRNA expression patterns between separate cohorts. The comparison of clinicopathological variables across diverse cohorts utilized Kruskal-Wallis and Mann-Whitney methodologies. To assess miRNAs' diagnostic potential in RCC, we generated receiver operating characteristic (ROC) curves and determined their area under curve (AUC) values. The Youden index helped identify optimal diagnostic groupings. Statistical significance was established at $P < 0.05$. Analyses were performed with Medicare, SPSS Statistics 23.0, and GraphPad Prism 8.3.0 software. Results were expressed as numerical values or percentages, accompanied by means with standard deviations.

RESULTS

Clinical and demographic characteristics of participants

A total of 112 RCC patients and 112 HCs were recruited. All participants strictly met the exclusion and inclusion criteria for this study. For study design purposes, testing and validation groups were established using samples from RCC cases and healthy controls. Clinical profiles and demographic data of participants in both stages are detailed in Table 1. No notable age or gender disparities emerged between groups during either phase. Statistical analysis utilized the Wilcoxon-Mann Whitney methodology, with results presented as numerical values and their corresponding percentages.

Screening and validation of candidate miRNAs

A comprehensive PubMed search facilitated the preliminary miRNA screening. Expression patterns were evaluated through ENCORI, analyzing data from 517 cancerous and 71 normal kidney samples. Candidate miRNAs were selected based on stringent criteria: $P < 0.01$ and fold change (FC) beyond 2 or below -2. Based on the screening results, we identified and selected 10 RCC-related miRNAs as validation candidates for the subsequent training stage. The analysis involved a random selection of specimens from both RCC patients ($n = 28$) and healthy controls ($n = 28$). The results are shown in Figure 2 after RT-qPCR analysis. Among the 10 candidate miRNAs, five miRNAs (miR-30a-5p, miR-124-3p, miR-221-3p, let-7b-5p, miR-302d-3p) demonstrated significant differential expression between RCC patients and HCs. These five miRNAs were further investigated for subsequent studies.

Analysis of five microRNAs: expression patterns and their diagnostic potential in the validation stage

To evaluate these five miRNAs' potential as serum biomarkers in early RCC screening, we conducted a study involving 84 RCC patients and 84 healthy controls. As illustrated in Figure 3, serum analysis revealed that miR-221-3p displayed significant upregulation in RCC patients, whereas miR-30a-5p, miR-124-3p, let-7b-5p, and miR-302d-3p showed marked downregulation. The diagnostic capabilities of these miRNAs were assessed through ROC curve analysis. The analysis yielded AUC values of 0.651 for miR-30a-5p [95%confidence interval (CI): 0.584-0.713; Figure 3B], 0.703 for miR-124-3p (95%CI: 0.638-0.763; Figure 3D), 0.696 for miR-221-3p (95%CI: 0.631-0.756; Figure 3F), 0.708 for let-7b-5p (95%CI: 0.644-0.768;

Table 1. A total of 224 subjects, comprising RCC cases and healthy controls, were analyzed for their baseline features

	Testing phase (n = 56)			Validation phase (n = 168)		
	RCC	HCs		RCC	HCs	
Total number	28	28		84	84	
Age	66.8 ± 11.9	64.3 ± 10.2	P = 0.556	58.3 ± 14.1	60.3 ± 15.1	P = 0.382
Gender	24 (85.71%)	21 (75.00%)	P = 0.573	64 (76.19%)	62 (73.81%)	P = 0.379
Male						
Female	4 (14.29%)	7 (25.00%)		20 (23.81%)	22 (26.19%)	
Fuhrman grade						
I	5 (17.9%)			13 (15.5%)		
II	16 (57.1%)			48 (57.1%)		
III	6 (21.4%)			20 (23.81%)		
IV	1 (3.6%)			3 (3.6%)		
AJCC stage						
I	21 (75%)			60 (71.4%)		
II	4 (14.3%)			15 (17.9%)		
III	2 (7.1%)			6 (7.1%)		
IV	1 (3.6%)			3 (3.6%)		

RCC: Renal cell carcinoma; HCs: healthy controls.

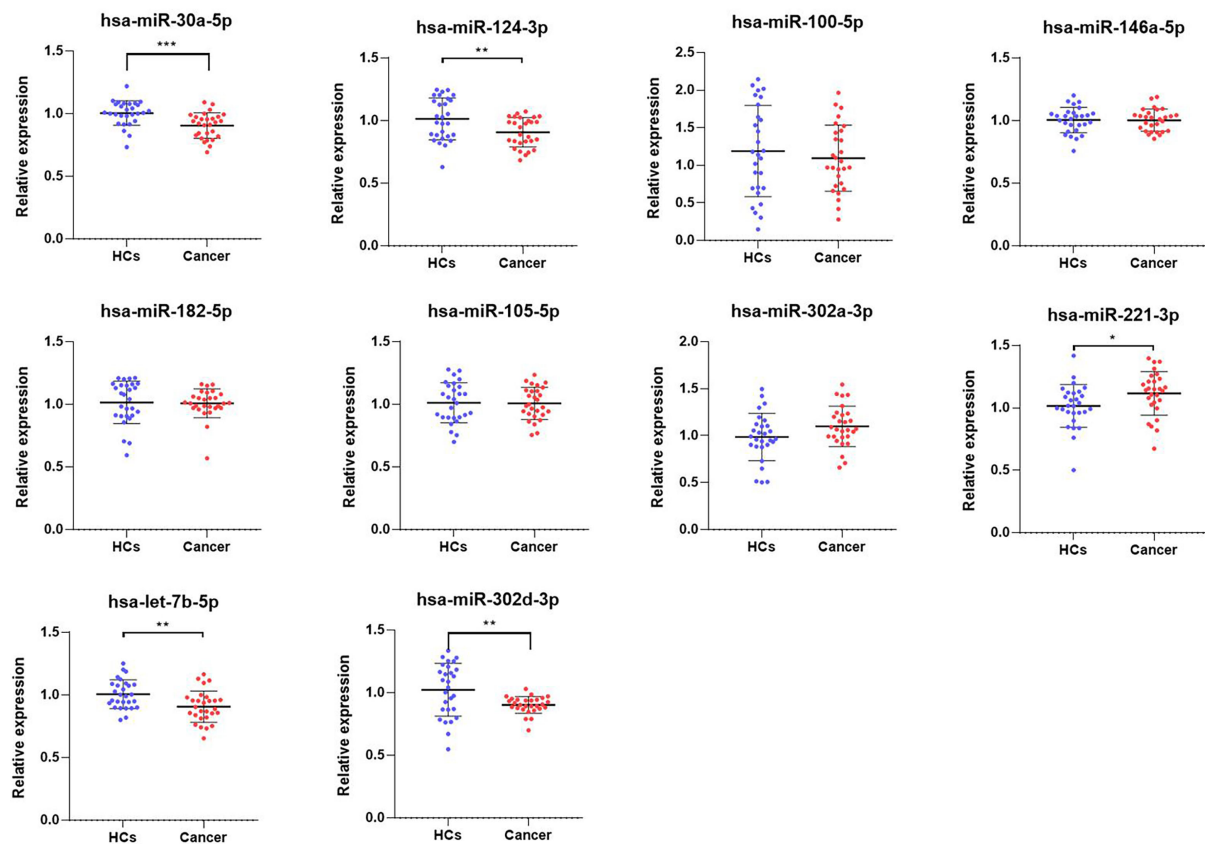


Figure 2. The expression analysis of ten microRNA candidates was conducted in the validation cohort. Statistical significance was established at three levels: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. The study encompassed matched specimens from equal numbers ($n = 28$) of RCC subjects and healthy controls. RCC: Renal cell carcinoma.

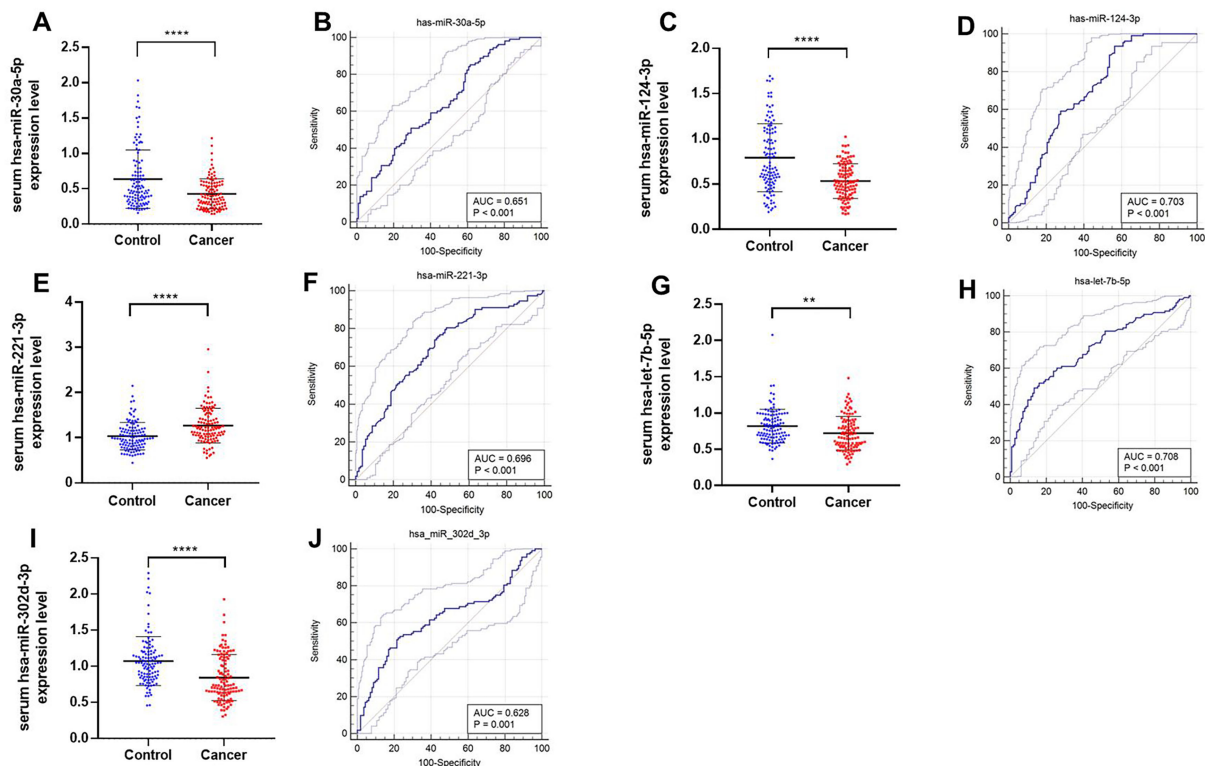


Figure 3. Expression profiles of five miRNAs and their respective ROC curves during the verification phase. The study examined serum samples from 84 individuals with RCC and 84 HCs. Analysis revealed significantly decreased expression of (A) miR-30a-5p, (C) miR-124-3p, (G) let-7b-5p, and (I) miR-302d-3p in RCC patients' serum. Conversely, (E) miR-221-3p showed significant upregulation; ROC analysis yielded the following AUC values: (B) 0.651 for miR-30a-5p [95%confidence interval (CI): 0.584-0.713], (D) 0.703 for miR-124-3p (95%CI: 0.638-0.763), (F) 0.696 for miR-221-3p (95%CI: 0.631-0.756), (H) 0.708 for let-7b-5p (95%CI: 0.644-0.768), and (J) 0.628 for miR-302d-3p (95%CI: 0.561-0.691). Asterisks indicate statistical significance levels: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. In the Figures, ROC curves are displayed in red, with green curves showing the 95% confidence intervals and blue lines representing the diagonal. RCC: Renal cell carcinoma; miRNAs: microRNAs; ROC: receiver operating characteristic; HCs: healthy controls.

Figure 3H), and 0.628 for miR-302d-3p (95%CI:0.561-0.691 Figure 3J). Following this, Table 2 presents the optimal cut-off values determined using the Youden index, along with the highest specificity and sensitivity for these five miRNAs in the diagnosis of RCC. The ROC analysis confirmed that miR-124-3p, miR-221-3p, and let-7b-5p (AUC = 0.703, 0.696, 0.708, respectively) possess robust diagnostic capabilities for RCC.

Discover the most suitable miRNA detection panel for RCC

During the last research stage, our findings confirmed miR-124-3p, miR-221-3p, and let-7b-5p as markers with superior diagnostic potential for RCC. Understanding that multiple miRNA combinations typically outperform single miRNA biomarkers in diagnostic accuracy, we proceeded to construct comprehensive diagnostic panels. By analyzing the expression profiles from the preceding phase and utilizing stepwise logistic regression modeling, we identified the optimal three-miRNA combination panel for RCC detection. The inclusion criterion for backward regression analysis was $P < 0.05$, and the exclusion criterion was $P > 0.1$. The regression equation we established is: $\text{Logit}(p) = -1.889 + (3.177 \times \text{miR-124-3p}) + (-2.447 \times \text{miR-221-3p}) + (3.548 \times \text{let-7b-5p})$. Figure 4 displays ROC curves for these three miRNA diagnostic combinations, demonstrating enhanced diagnostic performance with an AUC reaching 0.833, which surpassed individual miRNA effectiveness (95%CI 0.776-0.879; sensitivity: 75.00%, specificity: 77.68%; Table 2).

Table 2. Outcomes of receiver operating characteristic curves and Youden index for 5 candidate miRNAs and the three-miRNA panel

	AUC	P value	95%CI	Associated criterion	Youden index	Sensitivity (%)	Specificity (%)
miR-30a-5p	0.651	< 0.001	0.584-0.713	≤ 0.62	0.2358	85.19	38.39
miR-124-3p	0.703	< 0.001	0.638-0.763	≤ 0.81	0.3727	93.52	43.75
miR-221-3p	0.696	< 0.001	0.631-0.756	> 0.99	0.3142	80.36	51.79
let-7b-5p	0.708	< 0.001	0.644-0.768	≤ 0.61	0.3578	51.85	83.93
miR-302d-3p	0.628	= 0.0008	0.561-0.691	≤ 0.83	0.2864	50.89	78.57
three-miRNA panel	0.833	< 0.001	0.776-0.879	≤ 0.46	0.5268	75.00	77.68

AUC: Area under curve; CI: confidence interval; miRNA: microRNAs.

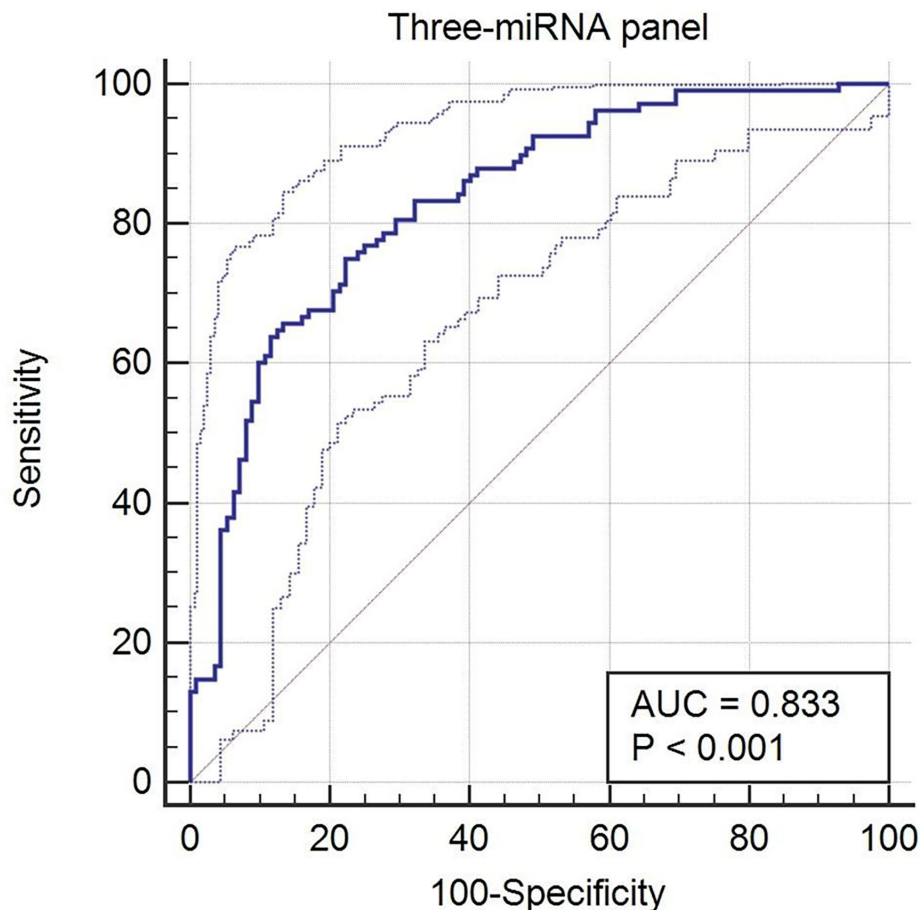


Figure 4. The receiver operating characteristic (ROC) analysis evaluated the diagnostic performance of the three-microRNA panel. When combining miR-124-3p, let-7b-5p, and miR-221-3p, the area under the curve reached 0.833 (with 95%CI: 0.776-0.879), demonstrating 75.00% sensitivity and 77.68% specificity. In the visualization, a deep blue line depicts the actual ROC curve, while the lighter blue shading indicates the 95% confidence region, and a diagonal red line serves as the reference baseline. AUC: Area under curve; CI: confidence interval; miRNA: microRNAs

Bioinformatics analysis of candidate miRNAs

To investigate the underlying molecular pathways of identified miRNAs in RCC, bioinformatic analyses were conducted on selected miRNAs displayed in Figure 5. Target gene prediction for miR-124-3p, let-7b-5p, and miR-221-3p was accomplished through MiRWalk2.0 platform. We focused on genes that were targeted by at least two miRNAs from our panel. The analysis revealed 331 potential target genes,

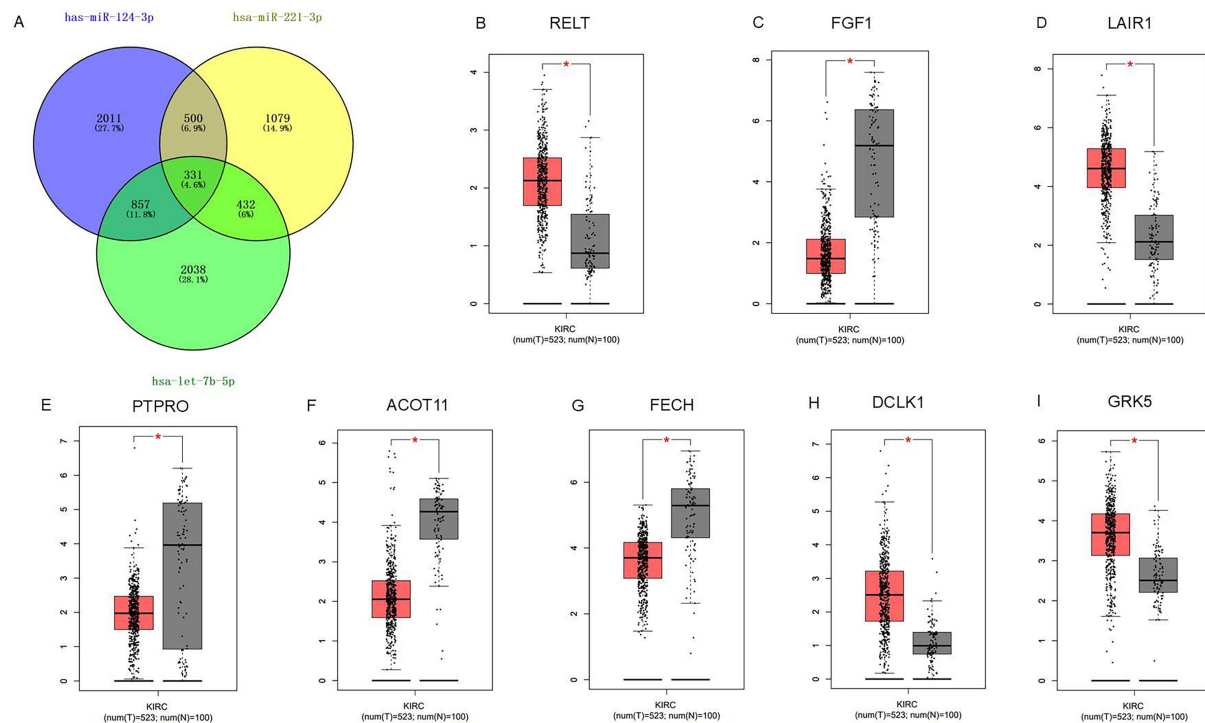


Figure 5. Prediction of Target Genes and Genes Differential Expression Analysis. (A) Target gene predictions and the number of genes predicted: 1789 (Genes predicted in more than two miRNAs were chosen as target genes); (B-E) Among the 331 predicted target genes, eight exhibited differential expression in KIRC ($|\log_2FC| > 1.5$, $P < 0.01$): RELT (B), FGF1 (C), LAIR1 (D), PTPRO (E), ACOT11 (F), FECH (G), DCLK1 (H), GRK5 (I). miRNAs: microRNAs.

which were subsequently visualized using a Venn diagram [Figure 5A]. Using the GEPIA database analysis, we discovered eight genes that display differential expression patterns in renal cancer, potentially serving as targets for the three-miRNA diagnostic panel. These include receptor expressed in lymphoid tissues (RELT) [Figure 5B], FGF1 [Figure 5C], LAIR1 [Figure 5D], protein tyrosine phosphatase receptor type O (PTPRO) [Figure 5E], ACOT11 [Figure 5F], ferrochelatase (FECH) [Figure 5G], DCLK1 [Figure 5H], and GRK5 [Figure 5I]. Each gene demonstrated significant differences with $\log_2 FC > 1.5$ and $P < 0.01$. Additional investigations utilizing the Enrichr database allowed for KEGG pathway enrichment and Gene Ontology (GO) functional annotation, as illustrated in Figure 6. Our findings indicated that the identified genes are expressed in various pathways including the Wnt and Hippo signaling pathways [Figure 6D]. The GO functional annotations were categorized into three aspects: biological processes (BP), cellular components (CC), and molecular functions (MF), as depicted in Figure 6A-C. Notable BP annotations comprised Endosomal Transport (GO:0016197), Vesicle-Mediated Transport (GO:0016192), and Positive Regulation Of Protein Localization To Membrane (GO:1905477). Significant CC annotations encompassed Cytoskeleton Of Presynaptic Active Zone (GO:0048788), Autolysosome (GO:0044754), and Cytoskeleton (GO:0005856). For MF, key annotations included DNA-binding Transcription Activator Activity specific to RNA Polymerase II (GO:0001228), Nuclear Receptor Binding (GO:0016922), and Protein Serine/Threonine/Tyrosine Kinase Activity (GO:0004712).

DISCUSSION

Various studies have documented that microRNAs significantly contribute to the regulation of metabolic pathways at the cellular level, enable communication between cells, and alter the microenvironment surrounding tumors. The identification of miRNAs within blood serum represents a cost-effective and

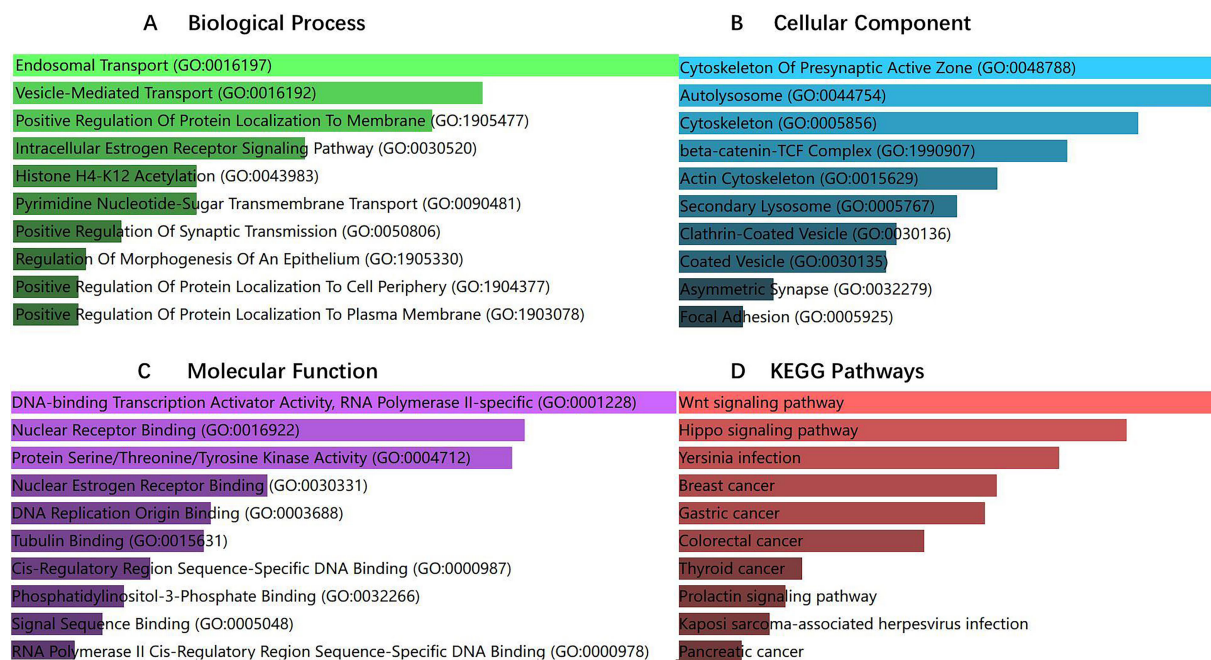


Figure 6. Target gene analysis: Functional annotation via GO and pathway enrichment through KEGG for let-7b-5p, miR-221-3p, and miR-124-3p. The figure presents: (A) biological process categorization; (B) cellular component distribution; (C) molecular function classification; and (D) enriched signaling pathways from the KEGG database. GO: Gene ontology; KEGG: kyoto encyclopedia of genes and genomes.

accessible detection method. Consequently, employing circulating miRNAs in serum as disease screening markers presents a convenient and non-invasive option compared to conventional diagnostic approaches, potentially facilitating broader implementation in everyday clinical practice^[21,22]. The goal of our research was to locate essential miRNAs that could serve as RCC diagnostic markers. Through our investigation, we found five miRNAs with notable differential expression in serum between HCs and RCC patients: let-7b-5p, miR-124-3p, miR-30a-5p, miR-221-3p, and miR-302d-3p. Subsequently, we developed a diagnostic signature incorporating three of these (let-7b-5p, miR-124-3p, and miR-221-3p), which exhibited enhanced accuracy and specificity for RCC detection compared to individual miRNA assessment.

In addition, we also explored the clinical significance and potential functions of each miRNA. In this panel, miR-124-3p was markedly downregulated in the serum of patients with RCC. Studies have shown that miR-124-3p plays a key role in RCC through the regulation of CAV1 and FLOT1 genes, suggesting its potential as a therapeutic target. Furthermore, studies have shown that miR-124-3p disrupts phospholipid metabolism to suppress cellular proliferation during acute kidney injury, establishing the scientific groundwork for both early detection and replacement therapy in renal conditions²⁴. Consequently, miR-124-3p exhibits potential as an effective diagnostic indicator for RCC.

In our investigation, we found that miR-221-3p exhibits notable upregulation in renal cell carcinoma tissues. Research conducted previously indicates that the axis of WDFY3-AS2 - miR-21-5p/miR-221-3p/miR-222-3p - TIMP3 serves as potential diagnostic and prognostic indicators for RCC development^[23]. Moreover, elevated levels of miR-221-3p are associated with adverse clinicopathological features of RCC^[24], underscoring its diagnostic value in RCC with the significance of ongoing research.

Research has shown that let-7b-5p is a key target for a variety of nephritis and kidney injury mechanisms^[25-27], and it also suppresses colon cancer and ovarian cancer progression^[28,29]. However, the mechanism of let-7b-5p in RCC remains unexplored, so it has the potential to be a direction for further research.

After target genes predictions, eight genes were identified that meet the criteria of $\log_2FC > 1.5$, $P < 0.01$, including RELT [Figure 5B], FGF1 [Figure 5C], LAIR1 [Figure 5D], PTPRO [Figure 5E], ACOT11 [Figure 5F], FECH [Figure 5G], DCLK1 [Figure 5H], GRK5 [Figure 5I], suggesting their potential targets for a three-miRNA diagnostic panel in RCC. Studies have shown that FGF1 expression is significantly reduced in RCC tissues and is significantly correlated with the overall survival of patients^[30]. In addition, research has suggested that patients with RCC exhibiting high expression of LAIR1 have diminished survival times compared to those with low expression of LAIR1. Therefore, LAIR1 has a role in promoting the pathogenesis of RCC^[31]. The expression of PTPRO is related to immune infiltration in patients with RCC, and both the RNA and DNA methylation levels of PTPRO may serve as biomarkers to predict the prognosis of patients with RCC^[32]. Additionally, ACOT11 also holds application value in the diagnosis and prognosis of RCC^[33]. The expression of FECH correlates with the immune microenvironment of RCC and likewise influences the prognosis of RCC^[34]. DCLK1 is identified as a dysregulated inhibitor within processes such as focal adhesion, epithelial-mesenchymal transition, and stemness in RCC^[35]. Moreover, the knockdown of GRK5 has demonstrated reductions in viability, invasion, migration, and proportion of RCC, suggesting GRK5 is a potential therapeutic target for RCC^[36]. Through comprehensive analysis, we identified that these eight genes exhibit significant enrichment in the Wnt signaling pathway and Hippo signaling pathway. They synergistically regulate critical biological processes including cellular transport and localization (specifically endosomal trafficking and vesicle transport), as well as protein serine/threonine/tyrosine kinase activity. Based on these findings, we propose the following hypothesis: The miRNA combination may disrupt Wnt/Hippo pathway homeostasis by suppressing FGF1 and GRK5 to respectively inhibit FGFR and GPCR signaling, thereby promoting cellular proliferation while suppressing apoptosis. Additionally, the downregulation of LAIR1 and PTPRO may alleviate immune checkpoint suppression, enhance CD8+ T cell infiltration, and improve the tumor immune microenvironment. However, further experimental validation is required to elucidate their specific roles in RCC.

Our research faces certain constraints. Firstly, the primary limitation stems from the relatively modest number of RCC patients included, as strict selection criteria hampered our capacity to gather a more extensive sample cohort across the study timeframe. While the results from the experimental data are promising, a larger sample size could lend more statistical power to these findings. Secondly, there are many potential confounding factors in the experiment. Although we controlled for variables such as gender, age, and other diseases, we did not evaluate the influence of tumor grade, stage, and subtypes of RCC, necessitating additional studies for comprehensive evaluation. Thirdly, the classification of RCC encompasses several subtypes, including clear cell renal carcinoma, granular cell renal carcinoma, renal carcinoma with mixed cellular components, and undifferentiated renal carcinoma variants. These subtypes were not distinguished in our present study, which requires more RCC samples and further experiments to verify the diagnostic value of miRNA across different subtypes. Fourthly, although numerous miRNAs are present in the serum of RCC patients and significantly differ from control groups, this study utilized only 10 miRNAs to minimize data processing errors. A more extensive and thorough bioinformatics analysis of miRNAs could enhance the study's depth. Additionally, the bioinformatics analysis has not been verified through basic experiments. The pathway enrichment analysis was conducted based on predicted targets and did not provide relevant evidence for the functional roles of these miRNAs. Lastly, this study has neither undergone external validation nor been compared to standard diagnostic tools. Our future investigations

will focus on assessing additional miRNAs and their potential utility in RCC detection, while also investigating their underlying biological mechanisms.

DECLARATIONS

Authors' contributions

Contributed to the concept and design of the study: Lai Y, Zhao, Z

Performed the experiments and wrote the manuscript: Ge Z, Lin S,

Collected the samples: Li H, Tao L

Performed the statistical analysis: Li R, Li X, Sun C, Wen Z, Chen W, Li Y

Read and approved the final manuscript: Ge Z, Lin S, Li X, Li R, Lu C, Sun C, Wen Z, Chen W, Li Y, Li H, Tao L, Zhao Z, Lai Y

Availability of data and materials

Data supporting this study can be obtained from the corresponding authors upon reasonable request.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

The Ethics Committee of Peking University Shenzhen Hospital has reviewed and granted approval ((Approval Number: 2017-007-1) for this study, which adheres to The Code of Ethics of the World Medical Association (Declaration of Helsinki). Informed consent was comprehensively explained to, understood by, and signed by all participants.

Consent for publication

Not applicable.

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