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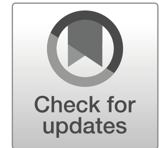

A Three-microRNA Panel in Serum: Serving as a Potential Diagnostic Biomarker for Renal Cell Carcinoma

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Abstract

Purpose Renal cell carcinoma (RCC) accounts for about 120,000 death each year. Although surgery is a routine treatment, RCC could be fatal if not diagnosed at an early stage. This study aims to search for suitable serum biomarkers and construct a miRNA panel with high diagnostic sensitivity or specificity.

Methods Totally 146 RCC patients and 150 normal control were involved in this three-stage study. Serum expression levels of 30 miRNAs selected from literature were tested by reverse transcription quantitative PCR (RT-qPCR) in the screening stage, the testing stage, and the validation stage. The diagnostic efficiency of miRNAs was evaluated by receiver operating characteristic (ROC) curve and area under curve (AUC) analysis. A panel with the highest diagnostic efficiency was constructed by backward stepwise logistic regression analysis. Additionally, bioinformatics analysis was used to investigate potential biological functions and mechanisms of candidate miRNAs.

Results MiR-224-5p, miR-34b-3p, miR-129-2-3p and miR-182-5p with low to moderate diagnostic ability (AUC = 0.692, 0.778, 0.687 and 0.745, respectively) were selected as candidate miRNAs after the three-stage study. The final diagnostic panel was consisted by miR-224-5p, miR-34b-3p and miR-182-5p with AUC = 0.855. No significance has been found between these four miRNAs and tumor location, Fuhrman Grade and AJCC clinical stages of RCC. Bioinformatic analysis suggested that the three-miRNAs panel may participate in tumorigenesis of RCC by targeting CORO1C.

Conclusions The three-miRNA panel in serum could serve as a non-invasive diagnostic biomarker of RCC.

Keywords Renal cell carcinoma · Serum miRNA · Biomarker · Non-invasive

Introduction

Every year, there are approximately 270,000 individuals puzzled by renal cell carcinoma (RCC) and nearly 120,000 people died because of it [1]. RCC, a radically metabolic disease, is linked to the acknowledged gene like the VHL, MET, FLCN and so on [2]. The most universal form of kidney cancer is clear-cell renal cell carcinoma which derives from the epithelium of renal tubules. Other types of kidney cancer including papillary, chromophobe, oncocytoma and collecting duct just accounts for about 25% [3]. Although numerous genes have been found to involve in the pathogenesis of RCC, the diagnosis and prognosis of RCC still at a preliminary stage of development. Despite surgery as a generally conventional treatment, kidney cancer could be fatal if it could not be diagnosed at an early stage or operable [4].

MicroRNAs(miRNAs) belong to one kind of RNA family, which is small and noncoding. They regulate the gene expression by involving in the post-transcriptional level. For instance, von Hippel–Lindau (VHL) is a well-known tumor suppressor gene. Mutant VHL gene is usually at high risk for the development of tumors, especially RCC [1, 5, 6]. Certain miRNAs have been found closely related to the

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dysregulation of the VHL gene [7]. According to other studies, there are many miRNA having been proven to be involved in cell invasion, proliferation, migration, invasion, apoptosis and neoplasms progression [8, 9]. Accordingly, miRNAs extracting from tumor tissue or serum have been deemed to be an available instrument for screening diagnosis, prognosis of tumor [10, 11].

Some studies have confirmed that specific one kind of miRNA could be used for detecting the progress of RCC like diagnosis or prognosis. However, those miRNAs lacked high sensitivity or specificity if detecting alone. Thus, our study aims to search for probable serum biomarkers according to the high variation of miRNA expression profiles and then constructing a miRNA assembling panel with high sensitivity or specificity. Our experiment was based on reverse transcription quantitative PCR (RT-qPCR) and receiver operating characteristic (ROC) curve analysis for the value of detecting capability. Bioinformatics analysis of candidate miRNAs was also applied in our study for exploring relative biological functions.

Materials and Methods

Collection of Human Serum Specimens

The study was exerted under the examination and approval of the Ethics Committee of Shenzhen Hospital, Peking University. All the participators understood the purpose of specimens completely and also signed the informed consent form. 126 serum samples gathered by patients that were diagnosed renal cell carcinoma (RCC) histologically and 130 normal control (NC) from January 2016 to May 2019 at Peking University Shenzhen Hospital (Shenzhen, China). For the patients, we differentiated their pathological feature based on the World Health Organization criteria. Additionally, all the patients have never received any treatment before diagnosing RCC. For NC, we extracted serum from volunteers who never have cancer history or any interferential disease. Table 1 displays the clinical manifestations of all participants.

All the serum samples were centrifuged at 3000 g for 10 min at 4 within 2 h shortly and then were stockpiled in fresh tubes at − 80 °C for preparation of the study.

Study design

Figure 1 showed an overview of the study design, including three screening miRNAs procedures, diagnostic miRNA panel construction as well as bioinformatics analysis.

Firstly, we picked up 30 miRNAs by searching literature. All selected miRNAs were found associated with RCC closely in renal cell or tissue but not enough evidence in serum. RCC pools and 4 NC pools were set up, which contained serum samples of 20 RCC patients and 20 healthy control, respectively. The selection criteria were single-peaked melting curve, fold change (FC) > 1 or < – 1, and cycle threshold (Ct) < 35. Secondly, at the testing stage, in order to check on the expression level of the selected miRNAs, we used another 30 RCC patients and 30 NC serum. The selection criterion was p-value < 0.05. Finally, to further confirm that the selected miRNAs could diagnose RCC effectively, we adopted the receiver operating characteristic (ROC) curve to calculate the area under the curve (AUC) with the rest 76 RCC patients and 80 NC. Additionally, the expression level of each candidate miRNAs was verified by p-value once again.

To reinforce the diagnostic efficacy, diagnostic miRNA panel construction would be established based on backward stepwise logistic regression. Moreover, we investigated whether there was a significant correlation between the expression level of selected miRNAs and clinical parameters.

For bioinformatic analysis, we applied miRWalk3 (http://mirwalk.umm.uni-heidelberg.de/), GEPIA (http://gepia.cancer-pku.cn/index.html) for genes prediction and utilized Enrichr database (http://amp.pharm.mssm.edu/Enrichr/) for KEGG pathway enrichment analysis and GO functional annotation.

RNA Extraction and RT-qPCR

During RNA extraction and RT-qPCR processes, each serum sample were added with 2 μl synthetic C. elegans miR-39 (cel-miR-39) (10 nM/L, RiboBio, Guangzhou, China) as a reference to normalize efficiency variation. All procedures were strictly in accordance with protocols of manufacturer. Through a NanoDrop 2000c system (Thermo Scientific, USA), we could know total RNA concentration which was extracted from serum specimens through TRIzolTM LS Reagent (Invitrogen, USA). Reverse transcription reaction was carried out under different temperatures sequentially with a general PCR machine (BIO-RAD, USA) by using the PrimeScript RT reagent Kit (TaKaRa, Dalian, China). With the help of an SYBR Green qPCR kit (SYBR Pre-mix Ex Taq II, TaKaRa) on LightCycler 480 Real-Time PCR System (Roche Diagnostics, Mannheim, Germany), we executed quantitative real-time polymerase chain reaction (qPCR) to detect the expression level of miRNAs. Similarly, the process of qPCR was applied in order, 95 for 20 s, forty cycles 95 for 10 s, 60 for 20 s, and then 70 for 10 s.

Statistical Analysis

Expression levels of microRNAs were calculated with the $2^{-\Delta\Delta Cq}$ method [12]. We utilized the ROC curve and AUC value to assess diagnostic ability: AUC is 0.5 to 0.7 (low), 0.7 to 0.85 (medium) and 0.85 to 1.0 (high). Backward stepwise logistic regression was wielded to examine the efficiency of diagnosis panel including the finally selected miRNAs. The software we used to statistical analyses was GraphPad 5.0 and

Table 1 Clinical manifestations of RCC patients and NCs

Variables	Testing stage (n = 60)			Validation stage (n= 156)		
	RCC patients (n = 30)	NCs (n = 30)		RCC patients (n = 76)	NCs (n = 80)	
Age	49.4 ± 11.3	50.2 ± 13.6	p = 0.81	50.5 ± 11.1	49.8 ± 9.4	p = 0.67
Gender			p = 0.43			p = 0.25
Male	20 (66.7%)	17 (56.7%)		55 (72.3%)	51 (63.8%)	
Female	10 (33.3%)	13 (43.3%)		21 (27.6%)	29 (36.3%)	
Location						
Left kidney	14 (46.7%)			35 (46.1%)		
Right kidney	16 (53.3%)			41 (53.9%)		
Fuhrman grade						
Grade 1	6 (20%)			12 (15.8%)		
Grade 2	10 (33.3%)			33 (43.4%)		
Grade 3	13 (43.3%)			28 (36.8%)		
Grade 4	1 (3.3%)			3 (4.0%)		
Clinical stage						
Stage 1	17 (56.7%)			47 (61.8%)		
Stage 2	3 (10%)			17 (22.4%)		
Stage 3	7 (23.3%)			7 (9.2%)		
Stage 4	3 (10%)			5 (6.8%)		

There was no significant difference in age or gender between RCC patients and NCs in the testing and validation stages. Data were presented as means ± SD or number (percentage). Statistical comparison was performed by using two-sided Student's t-test or Chi-square test.

SPSS. The method of verifying significance included student's-t test and one-way analysis of variance. Data were presented as means ± SD or number (percentage). $p < 0.05$ was considered statistically significant.

Results

Characteristics of Study Subjects

126 RCC patients and 130 NCs participated in the study. As shown in Fig. 1, this study was divided into three separate stages: the screening stage, the testing stage, and the validation stage. At each stage, there were no significant differences in gender and age distribution between RCC patients and NC. Table 1 lists the participants' statistics and clinical characteristics of the testing stage and the validation stage.

Selection of miRNAs from the Screening Stage

We first screened the 30 miRNAs selected from literature in 4 RCC pools and 4 NC pools (Fig. 2). 7 miRNAs were culled because of undetectable or the cycle threshold (Ct) value were greater than 35, while 23 miRNAs were detected with a single peak melting curve and the Ct values were less than 35. In these 23 miRNAs, 8 miRNAs with FC > 1 or < -1 were selected for further validation in the testing stage. Among them, 2 miRNAs (miR-149-5p, miR-224-5p) were expressed higher in RCC patients than NCs. In contrast, 6 miRNA (miR-34b-3p, miR-129-2-5p, miR-142-3p, miR-182-5p, miR-671-5p, miR-625-3p) indicates a lower level in RCC patients than NC.

Identification of Candidate miRNAs in the Testing Stage

We selected another 30 RCC patients and 30 NCs for further testing of the 8 selected miRNAs. The expression level of these miRNAs was mainly the same as the screening stage (Fig. 3). Five miRNAs were expressed significantly differently between CRC patients and NC and were identified as candidate miRNA. Of these, miR-149-5p and miR-224-5p were significantly more expressive in RCC patients than NCs ($p < 0.05$), while the other 3 miRNA expressions (miR-34b-3p, miR-129-2-3p and miR-182-5p) were significantly lower than NC ($p < 0.05$) in RCC patients. However, no significant differences were found in 3miRNA (miR-142-3p, miR-625-3p, and miR-671-5p)

Evaluation of Candidate miRNAs in the Validation Stage

The five previously discovered candidate miRNAs were further tested with 76 RCC patients and 80 NCs by using RT-

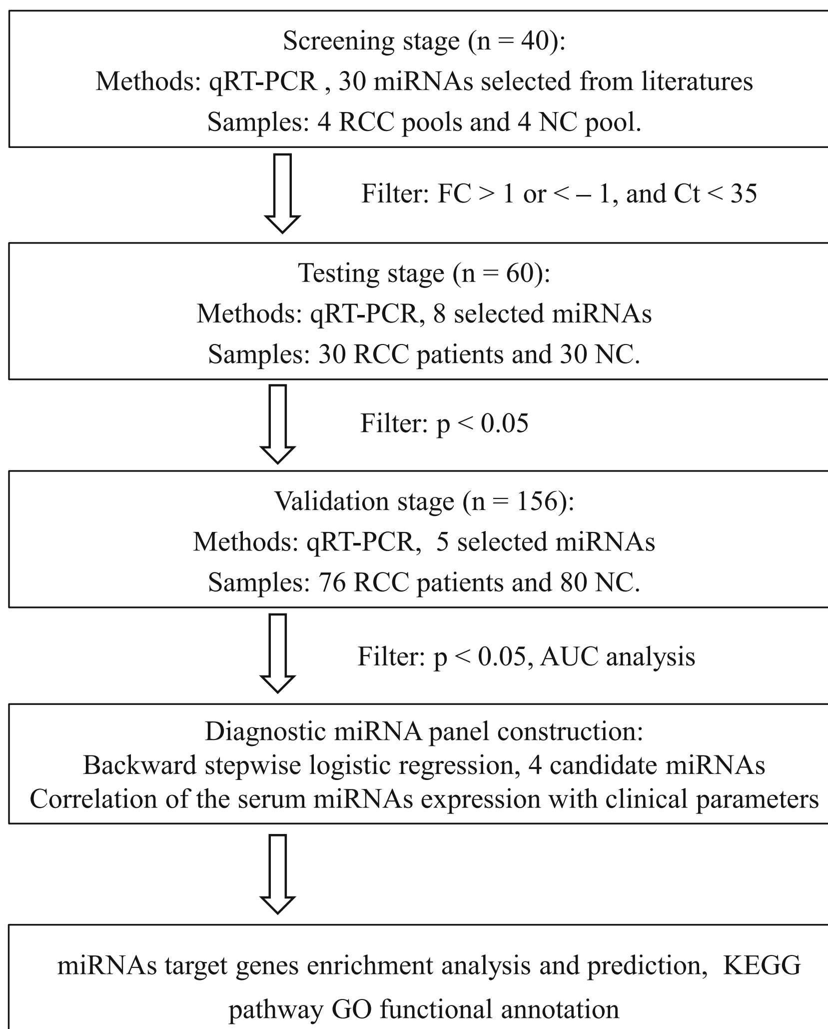

Fig. 1 Overview of the study design. A three-stage study was conducted to select candidate miRNAs for construction of serum diagnostic panel. RCC, renal cell carcinoma; NC, normal control; FC, fold change; Ct, cycle threshold; AUC, area under the curve

qPCR and ROC curve analysis. Except miR-149-5p, Significant difference was confirmed in miR-224-5p, miR-34b-3p, miR-129-2-3p and miR-182-5p between CRC patients and NCs (Fig. 4). Thereinto, miR-224-5p was up-regulated in the serum of RCC patients. In contrast, serum expression of miR-34b-3p, miR-129-2-3p and miR-182-5p were down-regulated in RCC patients than NCs. AUCs for miR-224-5p, miR-34b-3p, miR-129-2-3p and miR-182-5p were 0.692 (95% confidence interval (CI): 0.610–0.775), 0.778 (95% CI: 0.704–0.852), 0.687 (95% CI: 0.601–0.772) and 0.745 (95% CI: 0.670–0.820), respectively (Fig. 3). According to AUC analysis, miR-34b-3p and miR-182-5p have moderate diagnostic ability while miR-224-5p and miR-129-2-3p have low diagnostic ability.

To accurately diagnose RCC patients, a diagnostic panel was constructed with these 4 miRNAs by using backward reverse logic regression. In this process, miR-129-2-3p was culled and the three-miRNA panel was consisted of miR-224-5p, miR-34b-3p and miR-182-5p. ROC curve of the panel was drawn, and the AUC was 0.855 (95% CI: 0.797 to 0.912; sensitivity = 80.3%, specificity = 66.3%; Fig. 3f). The final logistic regression model was calculated with the equation:

$$\mathrm{logit}(P) = 1.783 + 0.542 \times \mathrm{miR} - 224 - 5\mathrm{p} - 1.882 \times \mathrm{miR} - 34\mathrm{b} - 3\mathrm{p} - 1.574 \times \mathrm{miR} - 182 - 5\mathrm{p}$$

The logistic regression model was validated in testing stage and the AUC was 0.879 (95% CI: 0.795 to 0.963; sensitivity = 80.0%, specificity = 69.0%; Fig. 3f).

Relationship Between Serum miRNAs Expression and Clinical Manifestations

Clinical significance of miR-224-5p, miR-34b-3p, miR-129-2-3p and miR-182-5p were evaluated by using Student's t-test or one-way ANOVA. RCC patients in the testing stage and the validation stage were grouped by location, Fuhrman Grade and AJCC clinical stages. As shown in Table 2, no significant relationship was found between the expression levels of these

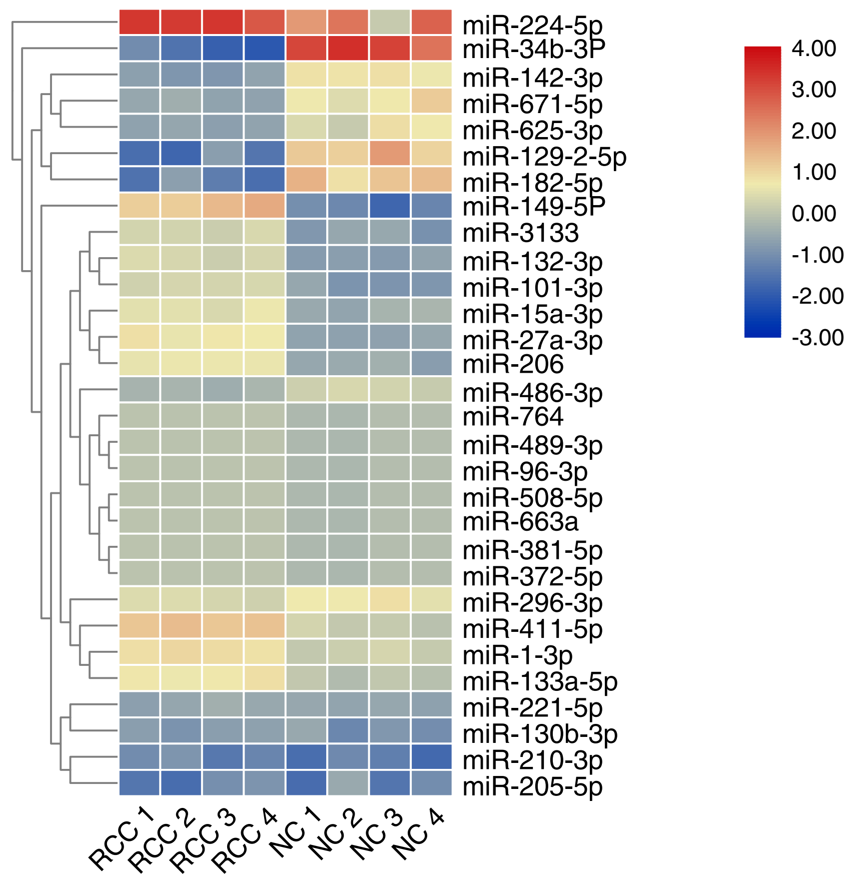

Fig. 2 Heatmap of 30 miRNAs in screening stage. Five serum sample were mixed up into one pool. 4 RCC pools and 4 NC pools were used in this stage, which contained serum samples of 20 RCC patients and 20 normal control, respectively. MiRNAs with single-peaked melting curve, fold change (FC) > 1 or < – 1, and cycle threshold (Ct) < 35 were selected for further testing

4 miRNAs and clinical parameters such as location, Fuhrman Grade and AJCC clinical stages.

Bioinformatic Analysis of miR-224-5p, miR-34b-3p and miR-182-5p

Possible target genes of the three-miRNA panel were predicted by using the TargetScan7.2. Venn diagram was drawn (Fig. 5a), and genes predicted in more than two miRNAs (736 totally) were used for KEGG pathway enrichment analysis and GO functional annotation by using the Enrichr database. The 39 genes predicted by all three miRNAs were validated in the GEPIA database. Among these 39 genes, CORO1C was the only gene that significantly upregulated in RCC patients (Fig. 5b). These three miRNAs may participate in tumorigenesis of RCC by mainly targeting CORO1C.

Top 10 of the biological process (BP), cellular component (CC), molecular function (MF) and KEGG pathways were shown in Fig. 5c, d, e, f. GO functional annotation include positive regulation of transcription, DNA-templated (GO:0045893); negative regulation of cellular macromolecule biosynthetic process (GO:2000113); RISC complex (GO:0016442); cytoplasmic stress granule (GO:0010494); translation factor activity, RNA binding (GO:0008135); ubiquitin-protein transferase activity (GO:0004842). Additionally, KEGG Pathways included the mTOR signaling pathway and longevity regulating pathway.

Discussion

Over the past few years, miRNAs have obtained more and more attention in RCC and other cancer like gastric cancer, lung cancer and so on [13, 14]. The dysregulation of miRNA can be found in the tissues, extracellular vesicles, serum as well as other fluid as a biomarker [15, 16]. miRNA as a novel biomarker can serve as a dependable guidance of the management of patients with RCC in terms of diagnosis, treatment and prognosis increasingly. RCC is the most common pathological pattern of kidney cancer [6]. Thus, the aim of our study is to search for a convenient and noninvasive measure for RCC diagnosis. After three-stage selection, finally, 3 miRNAs (miR-34b-3p, miR-182-5p and miR-34b-3p) with higher potency in screening RCC were found. According to Table 2, our miRNA panel was aberrant expression during the whole stage of RCC in serum constantly. However, Estelle Chanudet. et al. elaborated that there were significant relationships between the stage of 1–2 and 3–4 for some miRNA [17]. The 3 miRNAs we picked up were not included in their research result. What's more, according to the paper of Cheng

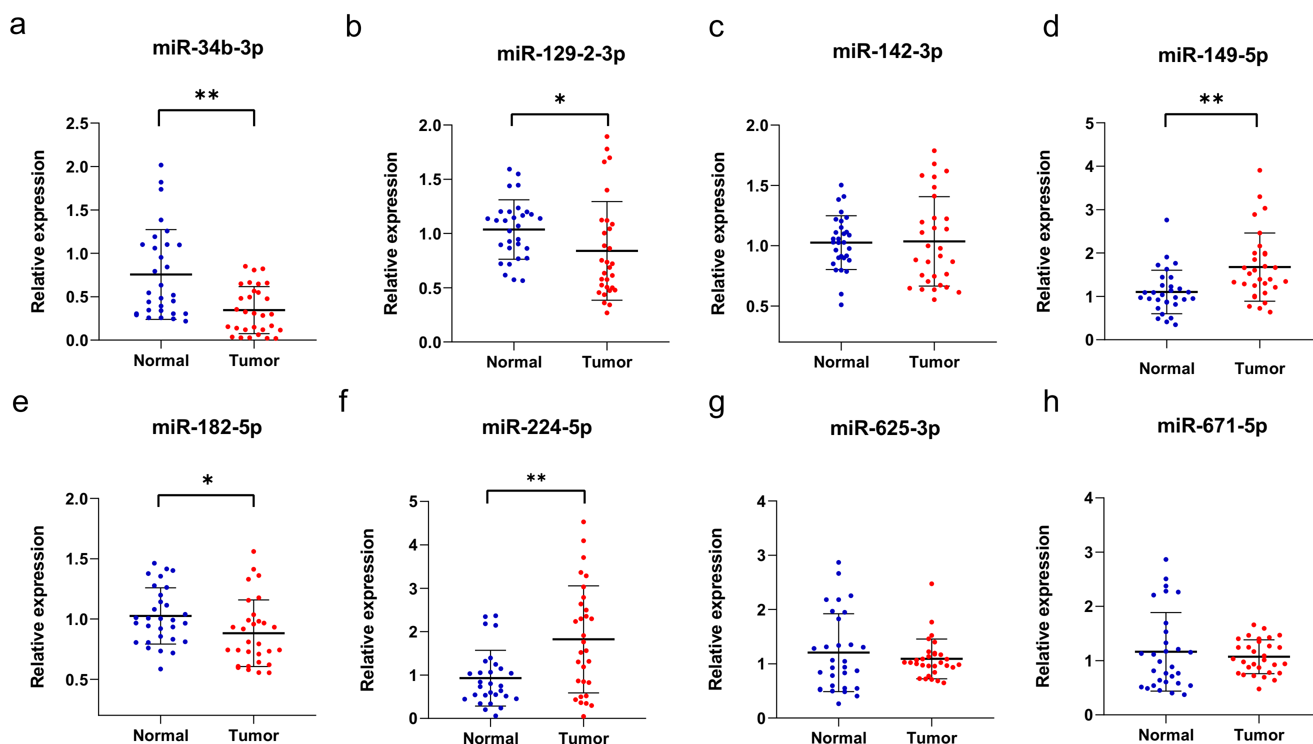

Fig. 3 Serum expression profiles of the 8 selected miRNAs in the testing stage. 30 RCC patients and 30 normal control were involved in this stage. Serum expression levels of miR-34b-3p (a), miR-129-2-3p (b), and miR-182-5p (e) were significantly lower in RCC patients than NC. On the contrary, serum miR-149-5p (d) and miR-224-5p (f) were significantly upregulated in RCC patients. The serum expression levels of There was no significant difference between RCC and NC in the serum expression levels of miR-142-3p (c), miR-625-3p (g), and miR-671-5p (h). * $p < 0.05$; ** $p < 0.01$

Wang. et al., their research found 5 serum miRNAs dysregulated particularly at early-stage renal cell carcinoma [18]. The reason why our study differs from 2 paper is likely to the different mechanism of miRNA in RCC. As stated in the study of Yijun Qi. et al., the kinds of miRNAs varied in different stages of RCC tissue[19]. Which raise a viewpoint: the variation in miRNAs expression depends upon their extremely complicated and multiple pathways or target genes, which induces the dysregulation of miRNAs to play distinct roles in the different stage of tumorigenesis. For the 3 serum miRNAs we select, it is necessary to discuss their pathways or targeted genes during the growth of RCC.

MiR-34b-3p, one member of the family of miR-34 which is located at human chromosome 11q23, participates in the network of p53(a tumor suppressor gene). miR-34b-3p contains CpG islands on its promotor region. When CpG methylation, miRNA-34b-3p destroy the p53-DAPK axis thorough dysregulation of important protein like Bcl2, cMYC, CDK4/6, and c-MET [20, 21]. In our study, the expression of miRNA-34b-3p was down-regulated in RCC serum but in the RCC tissue, oppositely, miR-34b-3p is up-regulated [22]. The reason why the distinction of expression of miR-34b-34p between serum and tissue needs further exploration.

For miR-182-5p, it belongs to the family of miR-183 which is located at human chromosome 7q32.2. In the study of Xin Xu. et al., they illustrated that the mechanism of miR-182-5p was activating the AKT/FOXO3a signaling pathway by the downregulation of the miRNA. Moreover, FLOT1 was certified as a target gene of miR-182-5p [23]. The same mechanism also could be discovered in hepatocellular carcinoma [24]. Additionally, MALT1 /NEAT2 (metastasis-associated lung adenocarcinoma transcript 1) was correlated with miR-182-5p. With the low level of miR-182-5p, the MALT1 was downregulated and led to the upregulation of p53, downregulation of CDC20, AURKA [25]. Thus, miR-182-5p could likely participate in different pathways.

There were only a few studies for the pathway of miR-224-5p in RCC. Then, for breast cancer, miR-224-5p suppress autophagy via inhibiting the expression of the Smad4 gene [26]. For the RCC, based on the study of Yufeng Jiang et al., LINC01094, a long non-coding RNA (lncRNA), played a crucial role in the development of RCC. It was activated by FOXM1 (a protein) at the transcriptional level, and then, the high-expressed LINC01094 served as a sponge to induce the gather of miR-224-5p/CHSY1 (the miR-224-5p-targeted mRNA) [27]. Thus, it can be seen the intricacy and unpredictability of the miRNA.

The pathway or target gene of our miRNA panel provide the available information for the development of therapeutic targets and diagnostic or prognostic biomarker for RCC.

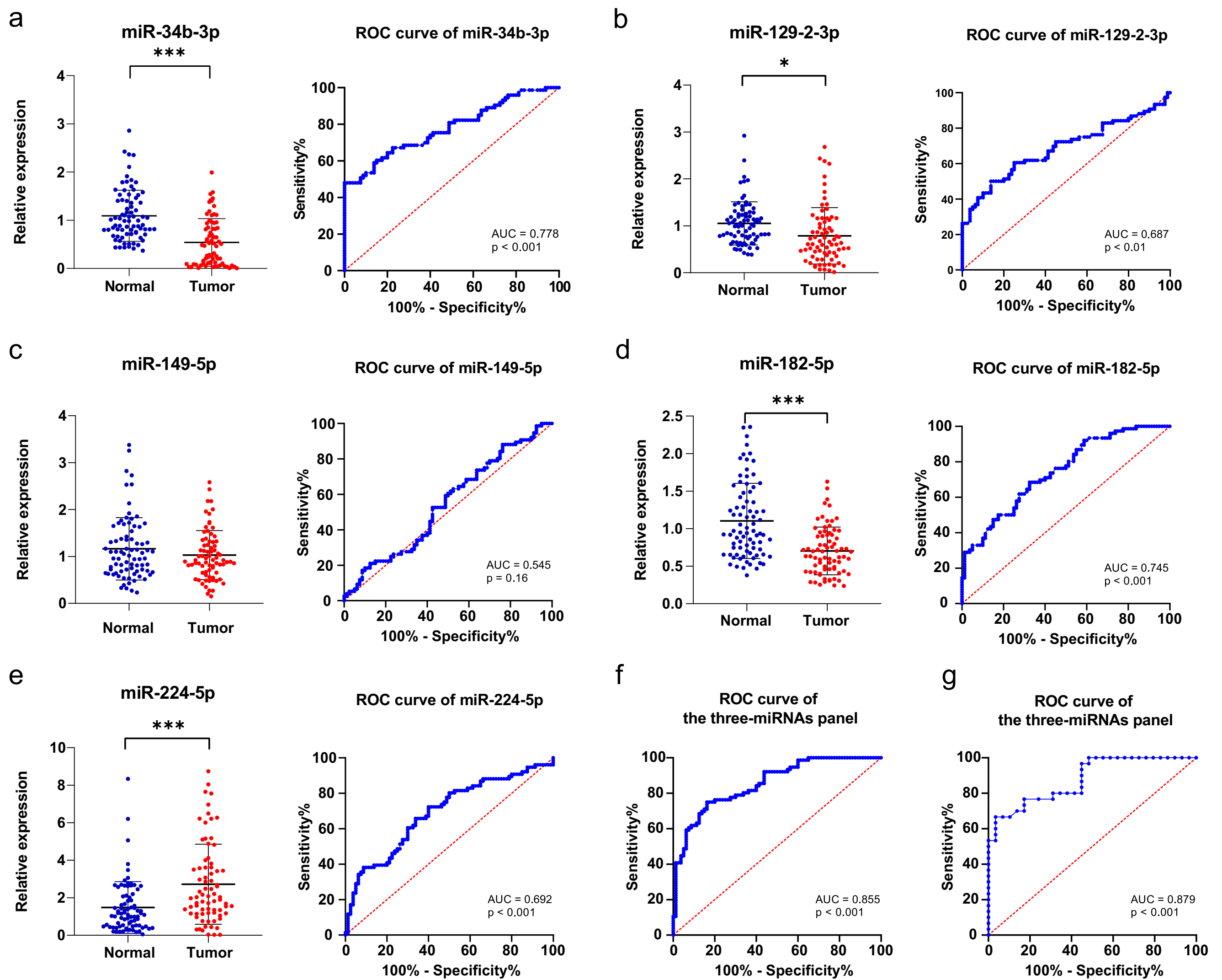

Fig. 4 Serum expression profiles of the 5 selected miRNAs and ROC curve analysis in the validation stage. 76 RCC patients and 80 normal control were involved in this stage. Results were mainly consisted with the testing stage, while miR-149-5p was excluded in this stage (c). AUC curve analysis showed that miR-34b-3p (a), miR-129-2-3p (b) and miR-182-5p (d) and miR-224-5p (e) had low to moderate diagnostic ability (AUC = 0.778, 0.687, 0.745 and 0.692, respectively). Construction of a three-miRNA (miR-224-5p, miR-34b-3p, and miR-182-5p) performed well both in the validation stage (f) and the testing stage (g). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Table 2 Correlations between miRNAs expression levels and clinical parameters of RCC patients

Variables	miR-34b3p		miR-129-2-3p		miR-182-5p		miR-224-5p	
Location		p = 0.27		p = 0.09		p = 0.41		p = 0.36
Left	0.37 ± 0.28		0.68 ± 0.41		0.79 ± 0.33		2.28 ± 2.01	
Right	0.30 ± 0.32		0.81 ± 0.67		0.72 ± 0.30		2.64 ± 1.91	
Fuhrman Grade		p = 0.22		p = 0.21		p = 0.96		p = 0.07
I - II	0.30 ± 0.34		0.74 ± 0.56		0.75 ± 0.33		2.76 ± 2.03	
III - IV	0.37 ± 0.25		0.88 ± 0.55		0.75 ± 0.30		2.06 ± 1.80	
AJCC clinical stages		p = 0.29		p = 0.07		p = 0.58		p = 0.98
I - II	0.93 ± 1.36		0.73 ± 0.51		0.74 ± 0.31		2.47 ± 1.87	
III - IV	1.28 ± 1.94		0.98 ± 0.66		0.78 ± 0.32		2.46 ± 2.21	

Data were presented as means ± SD or number (percentage). Statistical comparison wasperformed by using two-sided Student's t-test or Chi-square test.

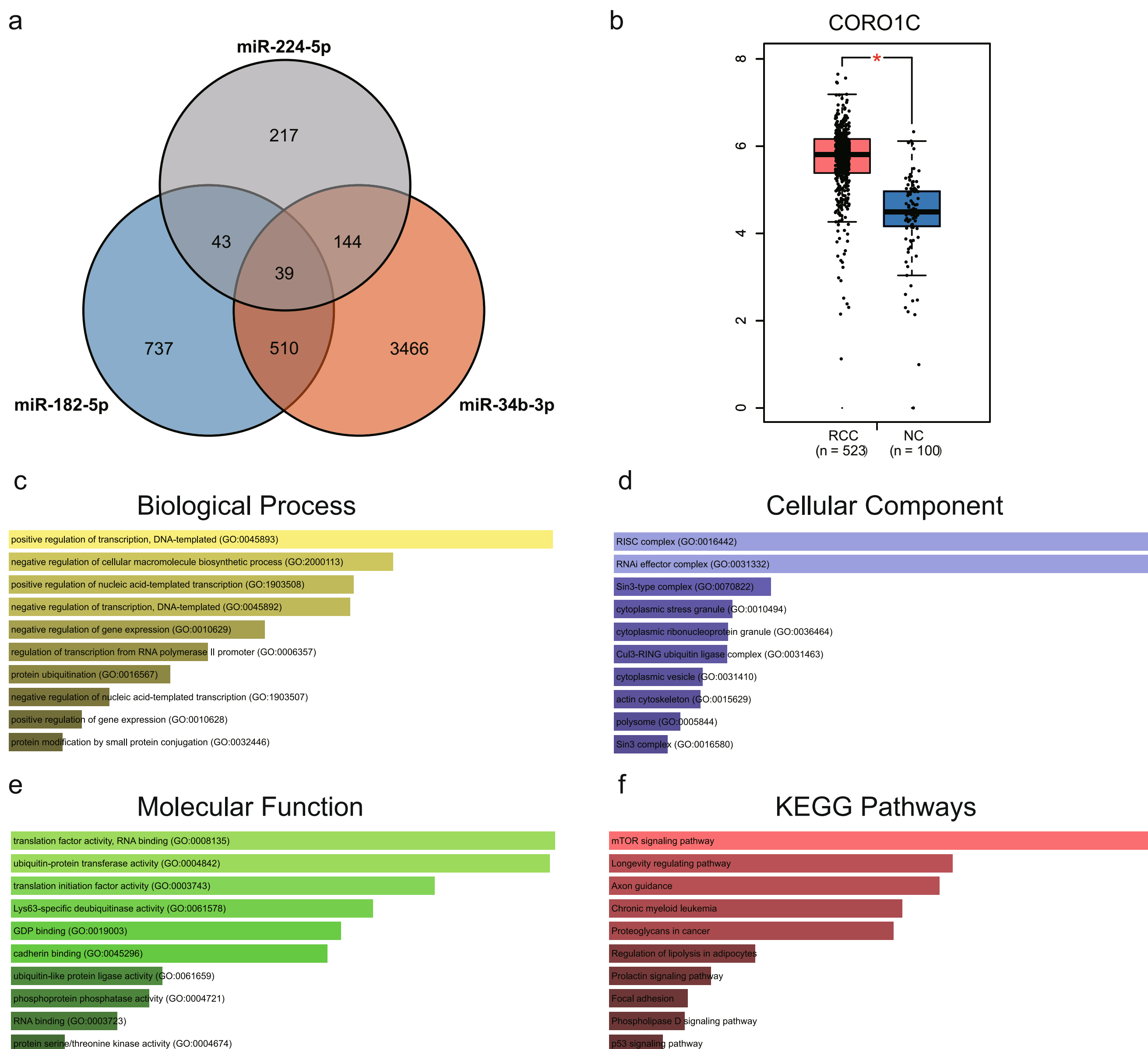

Fig. 5 Bioinformatic analysis of the three-miRNA panel. 736 target genes were predicted by miRWalk3 (**a**) and expression level of CORO1C in RCC was accessed from GEPIA (**b**). The Enrichr database was used for GO functional annotation (**c**, **d**, **e**) and KEGG pathway enrichment analysis (**f**)

Besides, the tumorigenesis of RCC may be affected by the complicated and synergetic miRNAs [17, 19, 28]. According to our bioinformatics analysis, important pathways include the mTOR signaling pathway and longevity regulating pathway were regulated by the miRNA panel. Additionally, CORO1C (Coronin 1C) was found significantly upregulated in RCC patients. Previously, Fan, L. et al. revealed that CORO1C can mediated cell migration and invasion by regulation of epithelial-to-mesenchymal transition (EMT) in nasopharyngeal carcinoma [29]. Our result suggested that the three-miRNA panel participated in RCC by targeting CORO1C.

Finally, we assessed clinical significance of the miRNA panel. No significant difference has been found between the miRNA panel with tumor location, Fuhrman Grade and AJCC clinical stages. However, based on the ROC curve, the AUC of miRNA panel was 0.855 (95% CI: 0.797 to 0.912) in the validation set and 0.879 (95% CI: 0.795 to 0.963) in the testing stage. The diagnostic ability of the miRNAs panel was obviously higher than individual miRNA. Therefore, we suggested that the serum three-miRNA panel consisted of miR-224-5p, miR-34b-3p, and miR-182-5p could serve as a non-invasive biomarker of RCC.

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Compliance with Ethical Standards

Conflict of interest There are no conflicts of interest.

Ethical approval The Ethics approval and consent to participate in this study was approved by the Ethics Committee of Shenzhen Hospital, Peking University.

Informed consent All the participators understood the purpose of specimens and signed the informed consent form.

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