

Identification and validation of a four-miRNA (miRNA-21-5p, miRNA-9-5p, miR-149-5p, and miRNA-30b-5p) prognosis signature in clear cell renal cell carcinoma

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Purpose: Clear cell renal cell carcinoma (ccRCC) is one of the most common cancers with high mortality worldwide. However, biomarkers for predicting prognosis in ccRCC are limited. In this study, we attempted to identify potential prognostic biomarkers of ccRCC.

Methods: Clinical information and the preprocessed ccRCC mature miRNA expression profiles in The Cancer Genome Atlas database were downloaded from UCSC Xena. The miRNAs differentially expressed between ccRCCs and matched normal tissues were analyzed using the “limma” package. A miRNA-based signature was constructed using the multivariate Cox regression model with prognosis index (PI) formula. Patients with ccRCC were divided into low-risk and high-risk subgroups according to median PI. The survival times were compared between the two groups using Kaplan–Meier analysis with log-rank test. The training set was used to construct a miRNA-based signature for predicting prognosis. The test set was used to verify the signature. Target gene prediction and functional enrichment analysis of the four miRNAs were performed using miRNet.

Results: We identified four miRNAs, miRNA-21-5p, miRNA-9-5p, miR-149-5p, and miRNA-30b-5p, as independent prognostic indicators. Next, we used these four miRNAs to construct a four-miRNA PI for each patient. Results revealed that patients in the high-risk group (n=119) had significantly shorter survival time than those in the low-risk group (n=118) (high-risk/low-risk group log-rank $P=0.000$). This four-miRNA signature is an independent prognostic factor compared with routine clinicopathological features in the test set. These miRNAs targeted 1,634 genes, and a miRNA-target gene network was constructed using miRNet. The target genes of these four miRNAs were involved in various pathways related to cancer.

Conclusion: Our observations suggest that the four-miRNA signature correlated with the survival of patients with ccRCC and can be used as a prognostic biomarker of ccRCC.

Keywords: ccRCC, miRNA signature, overall survival, prognostic biomarkers

Introduction

Renal cell carcinoma (RCC) is a common malignant tumor of the urinary system, accounting for 2%–3% of adult malignancies,¹ and more than 100,000 people die of kidney cancer every year worldwide.² The most common subtype of RCC is clear cell renal cell carcinoma (ccRCC), which is associated with high morbidity and poor prognosis.³

miRNAs were first identified by Lee et al⁴ in *Caenorhabditis elegans* in 1993 as 19- to 24-nucleotide-long ncRNAs. It is estimated that miRNAs regulate the expression

of >60% protein-coding genes. miRNAs are involved in various biological processes, such as cell growth, proliferation, differentiation, and apoptosis.⁵ Owing to the tissue-specific expression of miRNAs, their expression profile has been associated with various diseases. Currently, abnormally expressed miRNAs have been detected in many human tumors, such as bladder cancer,⁶ lung cancer,⁷ prostate cancer,⁸ pancreatic cancer,⁹ gastric cancer,¹⁰ liver cancer,¹¹ and other malignancies. Several recent studies have suggested that miRNA expression profiling can be used to predict the clinical outcome of patients with malignant tumors.^{12,13} Specific miRNAs have been used as potential diagnostic tools to distinguish the four subtypes of RCC (clear cell RCC, papillary RCC, chromophobe RCC, and benign oncocytomas).¹⁴ However, studies on the association of miRNAs with ccRCC prognosis are limited. Currently, The Cancer Genome Atlas (TCGA) database (<https://cancergenome.nih.gov/>) can be used to analyze complicated clinical characteristics and cancer genomics. In this study, we screened the differentially expressed mature miRNAs between ccRCC tissues and matched normal tissues, and determined the association between these miRNAs and overall survival (OS). We constructed a four-miRNA signature that may be used as a potential prognostic biomarker of ccRCC.

Materials and methods

Data processing

The preprocessed ccRCC mature miRNA expression profiles in TCGA database, displayed as log₂ converted reads per million (log₂ (RPM + 1)), and clinical information, were downloaded from the UCSC Xena (<https://xenabrowser.net/datapages/>, version 09-08-2017). It contains miRNA expression data from two different platforms, including 311 samples (241 ccRCC tissues and 70 matched normal kidney tissues) based on the IlluminaHiSeq_miRNASeq platform (Illumina Inc., San Diego, CA, USA) and 259 ccRCC tissues based on the IlluminaGA_miRNASeq platform. The samples based on the IlluminaHiSeq_miRNASeq platform were used as the training set to identify differentially expressed miRNAs and to construct a miRNA-based signature for predicting prognosis. The samples based on IlluminaGA_miRNASeq platform were used as the test set to verify the signature. The mature miRNA sequencing data were processed using R language.

Screening of differentially expressed miRNAs

In the training set, miRNAs that were not expressed in >10% samples were removed. The differentially expressed miRNAs

between ccRCCs and matched normal tissues were analyzed using the “limma” package¹⁵ in R. The fold changes (FCs) in the expression of individual miRNAs were calculated, and differentially expressed miRNAs with |log₂FC|>1.0 and *P*<0.05 were considered to be significant. We applied bidirectional hierarchical clustering to the differentially expressed miRNAs based on Euclidean distance and displayed the results as a heat map.

Construction and validation of the miRNA-based prognostic signature for ccRCC

In the training set, the patients were separated into high- and low-level groups based on the median value of the differential expression of miRNAs, followed by univariate and multivariate Cox proportional hazards analyses. Finally, a miRNA signature-based prognosis index (PI) score was constructed on the basis of a linear combination of the expression level multiplied by a regression coefficient derived from the multivariate Cox regression model (β) using the following formula.

$$PI = M_1 * \beta_1 + M_2 * \beta_2 + M_3 * \beta_3 + \dots$$

The “ β ” value is the estimated regression coefficient of miRNAs and is derived from the multivariate Cox regression analysis, and “M” indicates the expression profiles of the miRNAs. Patients with ccRCC were divided into low- and high-risk groups based on median PI. The survival times were compared between the two groups using Kaplan–Meier analysis with log-rank test at *P*-value<0.05. The test set was used to confirm the robustness and transferability of the miRNA-based prognostic signature. We conducted the univariate and multivariate Cox proportional hazards analyses in the training and test sets to compare the relative prognostic value of this four-miRNA signature with that of routine clinicopathological features.

Target gene prediction and functional enrichment analysis

Target gene prediction and functional enrichment analysis of the four miRNAs were performed using miRNet (<http://www.mirnet.ca/>).¹⁶ miRNet is an easy-to-use web-based tool that offers statistical, visual, and network-based approaches to assist researchers understand miRNA function and regulatory mechanisms and construct a miRNA-target gene network. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was subsequently performed for the target genes. *P*-value <0.05 was set as the cutoff criteria.

Statistical analysis

The chi-squared test was used for categorical data, and the unpaired Student's *t*-test was used to screen differentially expressed miRNAs. Univariate/multivariate Cox proportional hazards analyses and Kaplan–Meier survival analysis were used to compare the two groups of patients. The chi-squared test and survival analysis were performed using IBM SPSS statistics software program version 22.0 (IBM, Armonk, NY, USA). All tests were two-sided, and $P < 0.05$ was considered statistically significant.

Results

Differential expression of miRNAs between ccRCC and matched normal kidney tissues

The detailed clinical characteristics of patients with ccRCC, including gender, age at diagnosis, histological grade, and TNM stage, are shown in Table 1. The training set contained more patients with metastasis than the test set (16.88% vs 14.67%, chi-squared test, $P = 0.000$). This also partially assisted us to test the prognostic value of this miRNA-based signature in different patients. According to the cutoff criteria

($P < 0.05$ and $|\log_2\text{FC}| > 1$), 138 miRNAs were differentially expressed between ccRCC and matched normal kidney tissues in the training set. These included 54 upregulated miRNAs and 84 downregulated miRNAs in ccRCC tissues. miRNA-21-5p was upregulated, whereas miRNA-9-5p, miR-149-5p, and miRNA-30b-5p were downregulated in ccRCC tissues. The results of the expression analysis are presented as a heat map (Figure 1), and the results of hierarchical clustering showed that the expression patterns of these differentially expressed miRNAs can correctly distinguish ccRCC from normal kidney tissues.

Construction of miRNA-based signature with differentially expressed miRNAs

Four samples were removed because of lack of survival record in the training set. For each of the 138 differentially expressed miRNAs, we used the median expression level as a cutoff to stratify the remaining 237 patients into high-level and a low-level groups. The univariate Cox proportional hazards regression analysis revealed that eight miRNAs possessed prognostic value (Table 2). Next, we performed a multivariate Cox proportional hazards regression analysis and identified four miRNAs, namely, miRNA-21-5p (Figure 2A), miRNA-9-5p (Figure 2B), miR-149-5p (Figure 2C), and miRNA-30b-5p (Figure 2D), as independent prognostic indicators. Thus, we used these four miRNAs to construct a four-miRNA PI as follows:

$$\text{PI} = \text{miRNA-21-5p} * 0.788 + \text{miR-9-5p} * 0.536 + \text{miR-149-5p} * 0.566 + \text{miR-30b-5p} * 0.683.$$

A PI was calculated for each patient in the training set. Then, 237 patients were separated into low- and high-risk groups according to median PI. Survival analysis was performed using the Kaplan–Meier method with log-rank test. Results revealed that patients in the high-risk group ($n = 119$) had significantly shorter survival time than those in the low-risk group ($n = 118$) (high-risk/low-risk group log-rank $P = 0.000$; Figure 3A). This four-miRNA signature is an independent prognostic factor compared with routine clinicopathological features (Table 3).

Verification of the four-miRNA signature in the test set

Similar to that observed in the training set, patients in the test set were divided into low- and high-risk groups according to median PI, and Kaplan–Meier analysis was used to compare the patient's OS. The results of survival analysis revealed

Table 1 Summary of patient cohort information

Factors	Training set	Test set	P
	N=237 (%)	N=259 (%)	
Gender			
Male	158 (66.67)	162 (62.55)	0.338
Female	79 (33.33)	97 (37.45)	
Age			
<65	153 (64.56)	162 (62.58)	0.711
≥65	84 (35.44)	97 (62.59)	
Histologic			
G1–2	99 (41.77)	119 (45.95)	0.227
G3–4	136 (57.38)	134 (51.74)	
Gx	2 (0.84)	6 (2.32)	
T			
T1–2	151 (63.71)	156 (60.23)	0.481
T3–4	86 (36.29)	103 (39.77)	
M			
M0	166 (70.04)	221 (85.33)	0.000
M1	40 (16.88)	38 (14.67)	
Mx	31 (13.08)	0 (0.00)	
N			
N0	97 (40.93)	123 (47.49)	0.323
N1	8 (3.38)	9 (3.47)	
Nx	132 (55.70)	127 (49.03)	
Stage			
I–II	143 (60.34)	146 (56.37)	0.507
III–IV	92 (38.82)	112 (43.24)	
Not reported	2 (0.84)	1 (0.39)	

Notes: Bold figure indicates statistically significant, $P < 0.05$.

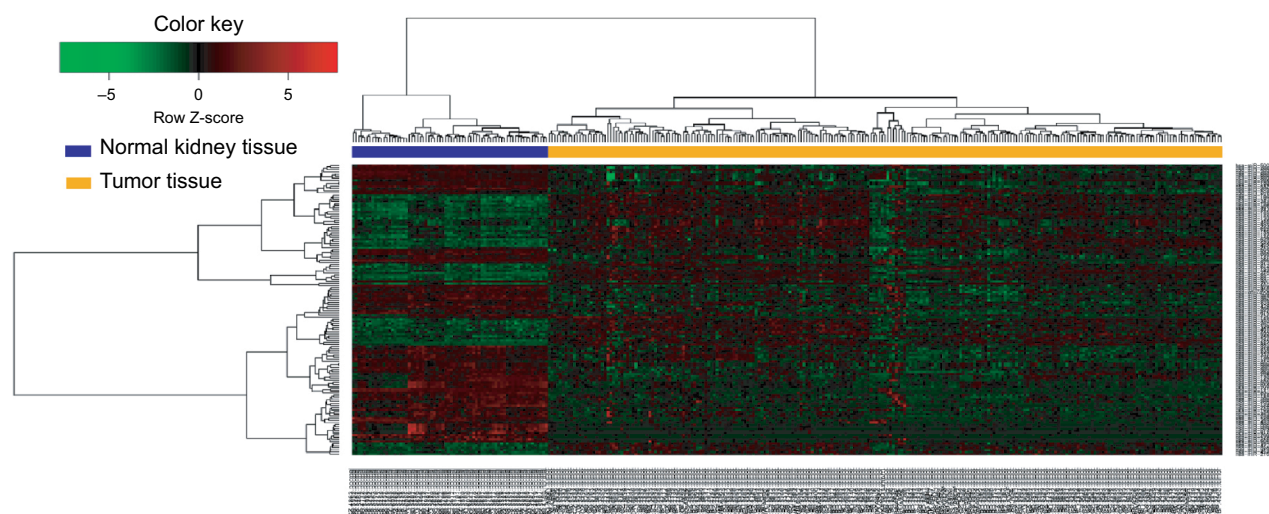


Figure 1 Hierarchical clustering dendrograms of expression patterns of differentially expressed miRNAs that can distinguish between normal kidney tissue and ccRCC tissue. **Abbreviation:** ccRCC, clear cell renal cell carcinoma.

Table 2 Univariate and multivariate analyses in ccRCC patients

miRNA	Univariate analysis		Multivariate analysis		
	P	95% CI	β	P	95% CI
miR-21-5p	0.000	1.884–5.149	0.788	0.031	1.073–4.502
miR-9-5p	0.004	1.270–3.465	0.536	0.044	1.014–2.883
miR-149-5p	0.003	1.303–3.473	0.566	0.027	1.066–2.908
miR-204-5p	0.003	0.293–0.775	−0.118	0.692	0.195–1.594
miR-146b-5p	0.001	1.436–3.800	−0.014	0.976	0.388–2.505
miR-223-3p	0.007	1.193–3.086	0.130	0.615	0.685–1.894
miR-30b-5p	0.009	1.173–3.069	0.683	0.006	1.211–3.236
miR-146b-3p	0.004	1.247–3.254	0.143	0.727	0.516–2.580

Notes: Bold figure indicates statistically significant, $P < 0.05$.

Abbreviation: ccRCC, clear cell renal cell carcinoma.

that patients in the high-risk group ($n=130$) had significantly shorter survival time than those in the low-risk group ($n=129$) (high-risk/low-risk group log-rank $P=0.000$; Figure 3B). The four-miRNA signature is an independent prognostic factor compared with routine clinicopathological features in the test set (Table 4). Overall, these results are consistent with that in the training set.

Target gene prediction and functional enrichment analysis

To investigate the potential biological functions of these four miRNAs, we predicted their target genes using miRNet. We observed that 1,634 genes were targeted by these four miRNAs and a miRNA-target gene network was constructed using miRNet (Figure 4). KEGG pathway enrichment analyses of the target genes revealed that they were involved in various pathways related to cancer, such as the MAPK, p53,

and Wnt signaling pathways, cell cycle, and RNA transport (Figure 5).

Discussion

ccRCC is one of the most common renal malignancies associated with high mortality and morbidity.¹⁷ However, clinical tools for predicting patient outcome utilize traditional clinical parameters. Therefore, accurate identification of predictive factors from data obtained from analysis of ccRCC specimens is clinically challenging. Identification and validation of novel biomarkers form an important part of practical studies on ccRCC. During tumorigenesis, miRNAs act as oncogenes or tumor suppressors; hence, the biological behavior of tumors can be inhibited by regulating miRNA levels for therapeutic purposes. Identification of RNA profiles and selective targets is the basis for individualized treatment of different tumors. Previous studies have shown that some specific miRNAs were aberrantly expressed in RCC and participate in its development.^{18–21} However, detailed analyses of the associations between miRNA expression and prognosis of patients with ccRCC remain limited.

In this study, we identified 138 miRNAs that were differentially expressed between ccRCC and normal kidney tissues. The univariate Cox proportional hazards regression analysis revealed that eight miRNAs possessed prognostic value. We confirmed that the four-miRNA (miRNA-21-5p, miRNA-9-5p, miRNA-149-5p, and miRNA-30b-5p) signature can be regarded as an independent predictor of prognostic OS after considering the various variables, including gender, age, histology, and stages. Previous studies have also identified

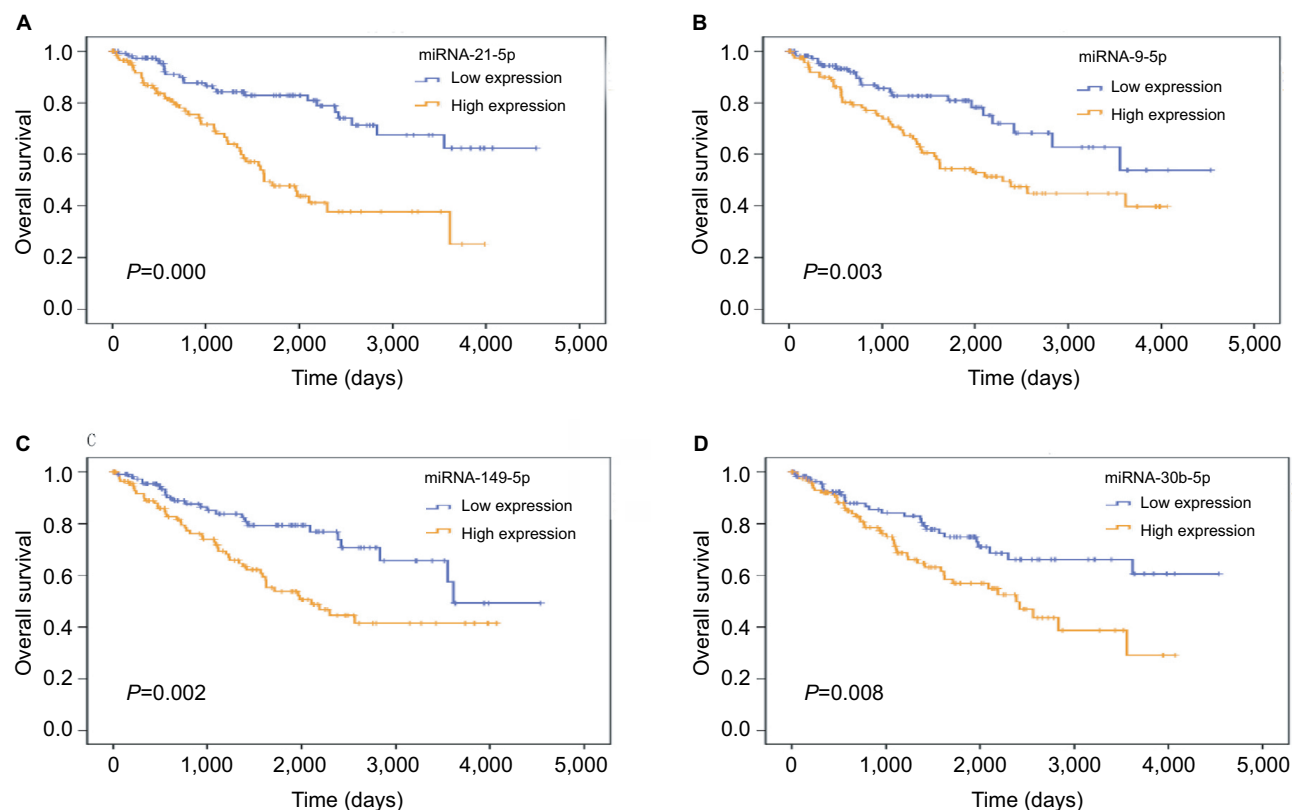


Figure 2 Four miRNAs were associated with overall survival in ccRCC patients using Kaplan–Meier curves and log-rank tests.

Note: (A) miRNA-21-5p; (B) miRNA-9-5p; (C) miRNA-149-5p; (D) miRNA-30b-5p.

Abbreviation: ccRCC, clear cell renal cell carcinoma.

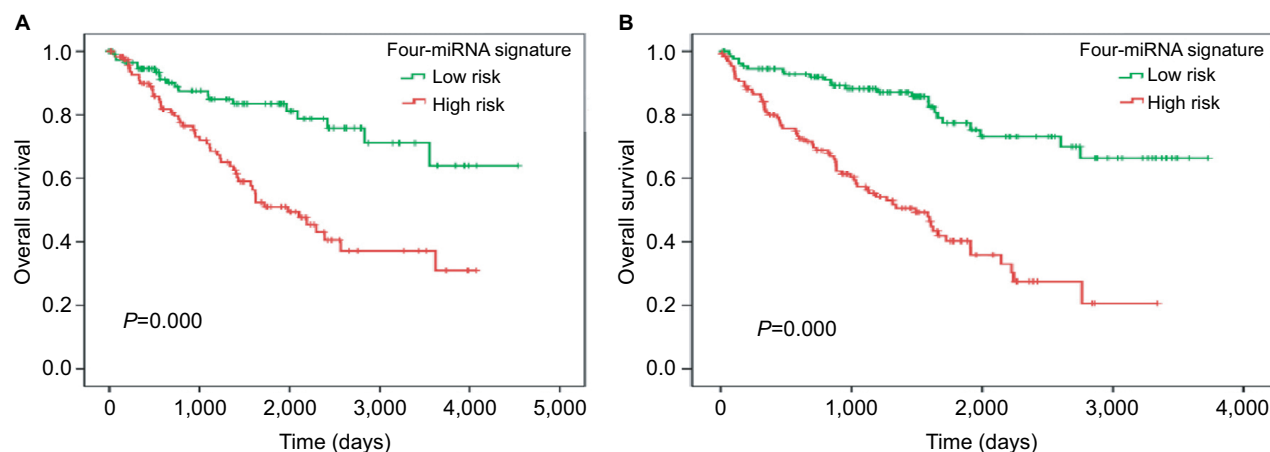


Figure 3 The Kaplan–Meier curves obtained using the four-miRNA signature to separate patients into high- and low-risk groups.

Notes: (A) Kaplan–Meier curve for training set; (B) Kaplan–Meier curve for testing set.

these four miRNAs, and several studies have investigated the relationship between miRNA expression patterns and cancer. Kowalczyk et al²² reported that special AT-rich sequence binding protein 1 (*SATB1*) may be a potential prognostic marker for ccRCC, as low *SATB1* expression in ccRCC may result from overexpression of miR-21-5p. *SATB1* downregulation and *miR-21-5p* upregulation were associated with shorter

patient survival. At present, the role of miRNA-9-5p in tumors has not been clarified. Certain studies show that downregulation of miR-9-5p expression can reverse the effect of miRNA-9-5p on proliferation, colony formation, cell cycle arrest, and apoptosis in osteosarcoma cells.²³ Okato et al²⁴ demonstrated that dual strands of pre-miR-149 (miRNA-149-5p and miRNA-149-3p) acted as antitumor miRNAs by

Table 3 Univariate and multivariate analyses of routine clinicopathological features and four-miRNA prognostic signature PI in the training set

Factors	Univariate analysis		Multivariate analysis	
	P	95% CI	P	95% CI
Gender (female/male)	0.862	0.579–1.580		
Age (<65 years/≥65 years)	0.149	0.885–2.245		
Histologic (G3–4/G1–2)	0.000	1.819–6.294	0.641	0.490–3.189
M (M0/M1)	0.000	2.933–7.587	0.702	0.467–3.103
N (N1/N0)	0.000	2.518–18.038	0.048	1.008–8.724
T (T1–2/T3–4)	0.000	2.018–5.283	0.408	0.146–2.187
Stage (I–II/III–IV)	0.000	2.677–7.515	0.116	0.729–17.584
PI (high/low risk)	0.000	1.692–4.759	0.008	1.363–7.740

Notes: Bold figure indicates statistically significant, $P < 0.05$.

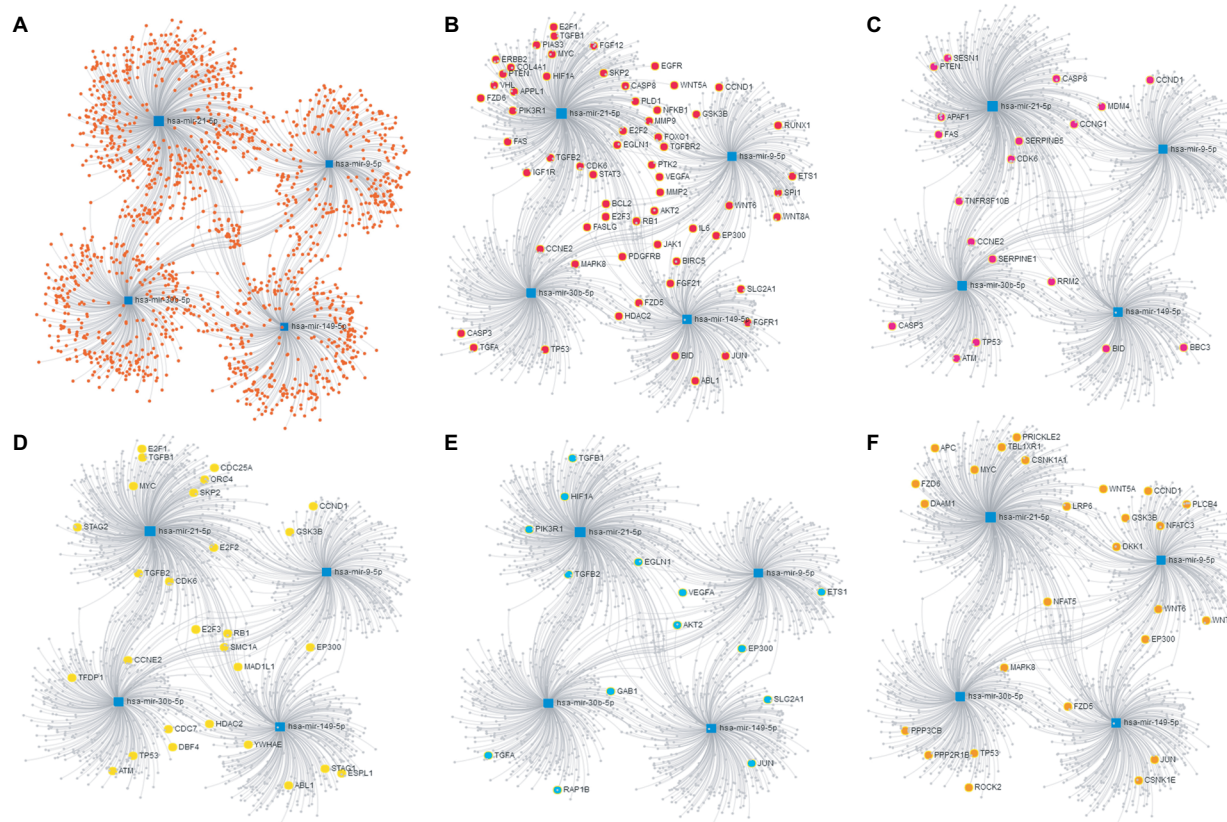
Abbreviation: PI, prognosis index.

Table 4 Univariate and multivariate analyses of routine clinicopathological features and four-miRNA prognostic signature PI in the test set

Factors	Univariate analysis		Multivariate analysis	
	P	95% CI	P	95% CI
Gender (female/male)	0.490	0.767–1.740		
Age (<65 years/≥65 years)	0.001	1.360–3.056	0.011	1.193–2.808
Histologic (G3–4/G1–2)	0.000	1.713–4.202	0.407	0.665–2.735
M (M1/M0)	0.000	2.057–4.719	0.108	0.820–7.363
N (N1/N0)	0.026	1.118–5.520	0.412	0.588–3.659
T (T3–4/T1–2)	0.000	2.688–6.388	0.000	2.189–9.396
Stage (I–II/III–IV)	0.000	2.314–5.529	0.349	0.155–1.931
PI (high/low risk)	0.000	2.481–6.265	0.000	1.847–8.377

Notes: Bold figure indicates statistically significant, $P < 0.05$.

Abbreviation: PI, prognosis index.

**Figure 4** The miRNA-target genes network constructed using the miRNet and the corresponding target genes involved in KEGG Pathways.

Note: (A) The total network of four miRNAs and their targets; (B) pathways in cancer; (C) p53 signaling pathway; (D) cell cycle pathway; (E) renal cell carcinoma; (F) Wnt signaling pathway.

Abbreviation: KEGG, Kyoto Encyclopedia of Genes and Genomes.

targeting *FOXM1*, which was shown to be associated with survival of patients with ccRCC. Liu et al²⁵ suggested that miR-30b-5p acts as a novel tumor suppressor to regulate RCC cell proliferation, metastasis, and epithelial to mesenchymal transition by downregulating GNA13 expression. In other words, miR-30b-5p may be considered a potential biomarker

for RCC diagnosis. However, studies demonstrating that the four differentially expressed miRNAs were predictors of ccRCC were lacking.

In this study, we constructed a four-miRNA signature, and the PI of this signature was calculated for each patient, which successfully separated patients into low- and high-risk groups.

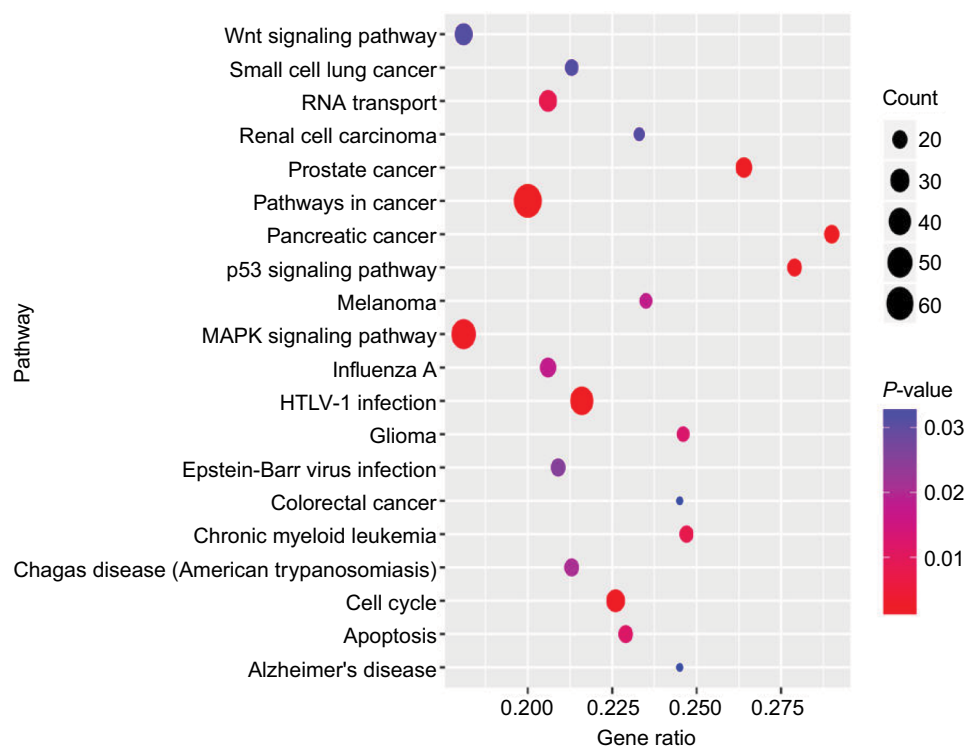


Figure 5 Kyoto Encyclopedia of Genes and Genomes pathway analysis of the predicted targets of the four miRNAs.

Abbreviation: HTLV-I, human T lymphotropic virus type I.

Specifically, patients considered high-risk by our four-miRNA signature had significantly poor prognosis than those in the low-risk group ($P < 0.001$). We confirmed that the four-miRNA signature is an independent predictor of OS in patients with ccRCC. Similar to that observed in the training set, patients in the test set were divided into low- and high-risk groups based on the risk score of individual patients, and Kaplan–Meier analysis was used to compare patient survival differences. Statistically significant differences ($P < 0.0001$) were observed between high- and low-risk groups. This confirmed that our four-miRNA signature is an independent and universal predictor of ccRCC.

It is well known that miRNAs modulate gene expression. Hence, we screened the target genes of these four miRNAs and used bioinformatics to predict the pathways and biological functions associated with their targets. The target genes were significantly enriched in multiple cancer-associated pathways, such as MAPK, p53, and Wnt signaling pathways. Abnormal regulation of these signaling pathways is involved in the development of various human cancers, such as breast cancer, hepatocellular carcinoma, hematological cancer, and lung cancer.^{26–28} Thus, abnormal regulation of signaling pathways may play a crucial role in the pathogenesis and progression of ccRCC. Further molecular investigations may provide new therapeutic targets for ccRCC.

Limitations

However, our study has few limitations. First, the ccRCC tissues were more than normal kidney tissues. Second, the miRNA expression profiles in the test set were not based on the same platform. Therefore, this four-miRNA signature has to be verified in a larger independent cohort of patients.

Conclusion

A comprehensive analysis of differentially expressed miRNA profiles and corresponding clinical information suggested that a four-miRNA signature was an independent and universal prognostic factor in patients with ccRCC. These miRNAs modulated genes associated with multiple cancer-associated pathways. However, further studies are required to verify our observations and establish the molecular mechanism underlying the interplay of miRNAs, their target genes, and ccRCC progression.

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Author contributions

All authors contributed toward data analysis, drafting, and critically revising the paper, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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