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Identification of prognostic markers for hepatocellular carcinoma based on miRNA expression profiles

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ABSTRACT

Aims: The aim of the study was to identify key miRNAs related to hepatocellular carcinoma (HCC) and then to explore their potential function and clinical significance.

Materials and methods: The miRNA expression profiles of 387 HCC and 62 normal liver tissues were obtained from the Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) databases. GEO2R tool and edgeR package in R/Bioconductor were used to screen out HCC-related miRNAs. VennDiagram package was used to identify key miRNAs related to HCC. The miRWalk tool and multiple R packages, such as pROC and survival, were used to explore potential function and clinical significance of these key miRNAs.

Key findings: A total of 17 and 300 HCC-related human miRNAs were identified in GEO dataset and TCGA, respectively. Thereinto seven miRNAs including hsa-miR-199a-3p, hsa-miR-199b-3p, hsa-miR-139-5p, hsa-miR-139-3p, hsa-miR-424-3p, hsa-miR-1269b and hsa-miR-1269a were key miRNAs related to HCC. Functional enrichment analysis showed that these key miRNAs were involved in multiple biological processes, such as telomere maintenance via telomerase, protein sumoylation, histone mRNA metabolic process and angiotensin maturation. Cox regression analysis indicated that hsa-miR-139-5p expression was associated with the prognosis of HCC patients. ROC curve analysis suggested that survival prediction model developed based on tumor stage and hsa-miR-139-5p exhibited good performance in predicting 3-year overall survival of HCC patients.

Significance: The present study identified several HCC-related miRNAs, which might serve as new diagnostic markers and therapeutic targets for HCC. In addition, hsa-miR-139-5p might act as a promising prognostic indicator for HCC patients.

1. Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer in adults, and the leading cause of cancer-related mortality worldwide [1,2]. Although surgery, radiofrequency ablation and chemoembolization have been widely applied for HCC therapy, the survival rate of HCC patients is still low, partly due to high heterogeneity of HCC. Furthermore, due to the fact that biological processes involved in the occurrence and progression of HCC are very complicated, there have not been effective prognostic biomarkers until now. Therefore, it is necessary to explore new HCC-related molecules for the diagnosis, prognosis and treatment of HCC.

MicroRNAs (miRNAs) are a class of short, evolutionarily conserved, endogenous, single-stranded, non-coding RNA molecules which can regulate gene expression via the degradation of mRNAs or inhibition of translation [3]. A large number of studies have shown that miRNAs participate in various biological processes (BPs), such as cell cycle, differentiation, development and metabolism [4–8]. In addition, the dysregulation of miRNAs can affect the occurrence, development and prognosis of tumors including HCC [9–11]. In recent years, microarrays and sequencing technologies have been extensively used as efficient tools for identification of disease-related molecules [12–14]. Meanwhile, a large amount of data generated by these high-throughput detection technologies has been deposited in public databases, such as The Cancer Genome Atlas (TCGA) and the Gene Expression Omnibus (GEO), and is freely available via the web to researchers worldwide, which provides abundant resources for integrated studies to obtain more reliable information and more feasible measures in the field of medicine.

In the present study, we identified several key miRNAs related to

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HCC via mining miRNA expression profiles of HCC in GEO and TCGA databases, and further explore their potential function and clinical significance by multiple bioinformatics methods.

2. Materials and methods

2.1. miRNA expression profiles

miRNA expression profiles of 12 HCC and corresponding paracancerous tissues were obtained from the GSE115016 dataset in GEO (https://www.ncbi.nlm.nih.gov/geo/) [15]. These miRNA expression profiles were generated by Affymetrix Multispecies miRNA-4 Array (Santa Clara, CA, USA). The miRNA expression profiles of 375 HCC and 50 normal liver tissues and clinical information of corresponding patients were downloaded from TCGA (https://portal.gdc.cancer.gov/). These miRNA expression profiles were generated by miRNA-Seq.

2.2. Identification of HCC-related miRNAs

GEO2R, an interactive web tool (https://www.ncbi.nlm.nih.gov/geo/geo2r/), was used to identify differentially expressed human miRNAs between HCC and normal liver tissues in GSE115016 dataset. Multiple-testing correction was performed by Benjamini & Hochberg method. The miRNAs with the adjusted P value < 0.05 and $|\log FC| > 1$ were chosen as HCC-related miRNAs. The edgeR was a Bioconductor package for differential expression analysis of digital gene expression data, and was used to identify HCC-related miRNAs based on TCGA [16,17]. The miRNAs with $|\log FC| > 1$ and false discovery rate (FDR) < 0.05 were considered as HCC-related miRNAs. Subsequently, HCC-related miRNAs overlapped in the two datasets were screened out by VennDiagram package [18] and defined as key miRNAs.

2.3. Potential function analysis of key miRNAs related to HCC

The new version of miRWalk (http://mirwalk.umm.uni-heidelberg.de/) not only stored predicted data obtained with a maschine learning algorithm including experimentally verified miRNA-target interactions, but also offered a standard enrichment analysis of gene set such as Gene Ontology (GO, http://geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.kegg.jp/) pathway enrichment analysis based on the hyper geometric tests [19]. In the present study, miRWalk with default parameters was firstly used to identify potential target genes of key miRNAs related to HCC. Subsequently, enrichment analysis of miRNA target genes was performed to explore potential function of key miRNAs related to HCC. The adjusted P value < 0.05 was considered significant.

2.4. Clinical significance analysis of key miRNAs related to HCC

To evaluate performance of key HCC-related miRNAs in distinguishing between HCC tissues and normal liver tissues, pROC package (https://cran.r-project.org/web/packages/pROC/index.html) specifically dedicated to receiver operating characteristic (ROC) analysis was used to analyze normalized miRNA expression profiles of 375 HCC and 50 normal liver tissues [20]. Area under the ROC curves (AUC) > 0.7 was defined as a good performance.

The association between the expression of key HCC-related miRNAs and the prognosis of HCC patients with complete clinical data was analyzed by Cox regression model based on survival package. P value < 0.05 was considered significant. The rms package was used to develop a nomogram for predicting 3- and 5-year survival rate of HCC patients by combining independent prognostic factors. Subsequently, survivalROC package (https://cran.r-project.org/web/packages/survivalROC/index.html) was used to assess performance of the survival prediction model. AUC > 0.7 was defined as a good performance. According to risk score of each patient calculated by the prediction model, patients were classified into high-risk group ($>$ the median risk score) or low-risk group ($\leq$ the median risk score). Kaplan-Meier (K-M) survival curves with log-rank test was used to assess the prognostic difference of high-risk versus low-risk group.

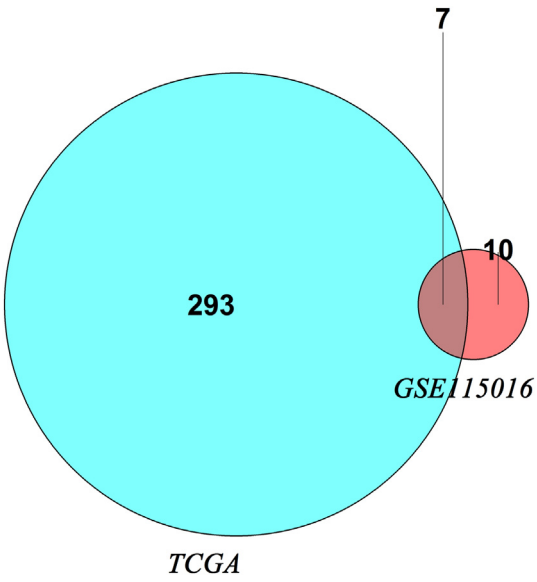

Fig. 1. Venn diagram of HCC-related miRNAs in GSE115016 and TCGA (Light blue and pink circles indicates HCC-related human miRNAs in GSE115016 and TCGA, respectively.). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Identification of HCC-related miRNAs

A total of 17 and 300 HCC-related human miRNAs were identified in GSE115016 and TCGA, respectively (Table S1 and S2). Concretely speaking, 2 up-regulated and 15 down-regulated human miRNAs in HCC were found in GSE115016. For TCGA dataset, there were 260 up-regulated and 40 down-regulated human miRNAs in HCC. Further intersection analysis showed 7 miRNAs (hsa-miR-199a-3p, hsa-miR-199b-3p, hsa-miR-139-5p, hsa-miR-139-3p, hsa-miR-424-3p, hsa-miR-1269b and hsa-miR-1269a) overlapped in GSE115016 and TCGA. The seven miRNAs were defined as key miRNAs related to HCC (Fig. 1 and Table 1).

3.2. Potential function of key miRNAs related to HCC

Functional enrichment analysis based on miRNA target genes showed that the seven key miRNAs were involved in 20 biological processes (BPs), such as pathway-restricted SMAD protein

Table 1
HCC-related miRNAs overlapped in GSE115016 and TCGA.

miRNA_ID	GSE115016		TCGA	
	logFC	adj.P.Val	logFC	FDR
hsa-miR-199a-3p	−3.80	1.95E−02	−1.31	6.51E−11
hsa-miR-199b-3p	−3.80	1.95E−02	−1.31	6.08E−11
hsa-miR-139-5p	−3.69	1.26E−03	−1.45	4.32E−20
hsa-miR-139-3p	−2.94	3.49E−03	−1.92	3.01E−39
hsa-miR-424-3p	−1.67	1.95E−02	−1.36	7.64E−25
hsa-miR-1269b	2.81	1.95E−02	6.53	3.30E−12
hsa-miR-1269a	3.65	2.18E−02	5.74	1.25E−17

Abbreviations: logFC, log2 Fold Change; adj.P.Val, adjusted P value; FDR, False discovery rate.

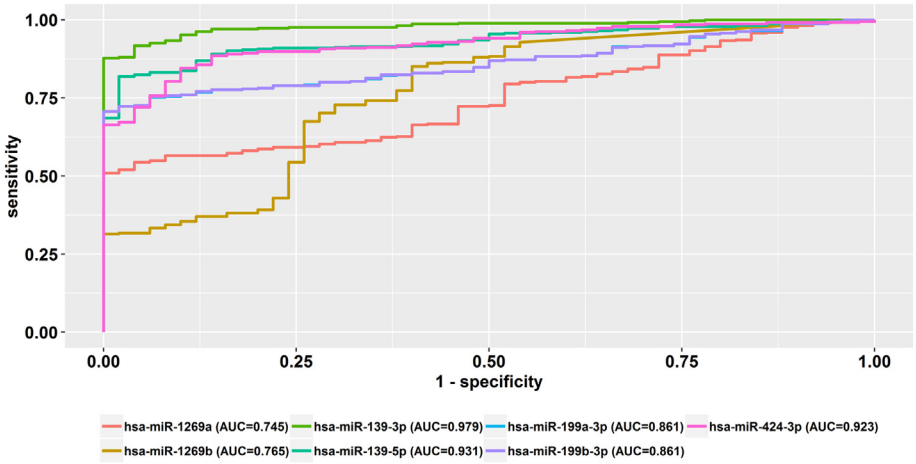

Fig. 2. ROC curves showing the diagnostic performance of seven HCC-related miRNAs (AUC indicates the area under ROC curve).

phosphorylation, BMP signaling pathway, protein sumoylation, telomere maintenance via telomerase, canonical Wnt signaling pathway, fibroblast growth factor receptor signaling pathway and histone mRNA metabolic process, and 32 molecular functions (MFs), such as GTPase activator activity, protein tyrosine phosphatase activity, MAP kinase activity, endoribonuclease activity and phosphoprotein binding (Table S3 and S4). Furthermore, they could also affect 13 cell components (CCs), such as mitotic spindle pole, ER to Golgi transport vesicle, endoplasmic reticulum quality control compartment and RISC-loading complex (Table S5).

3.3. Clinical significance of key miRNAs related to HCC

ROC curve analysis indicated that these key miRNAs related to HCC could effectively distinguish between HCC and normal liver tissues (Fig. 2)(AUC > 0.7). Thereinto hsa-miR-139-3p had the best distinction capability (AUC = 0.979). Univariate Cox regression analysis showed that tumor stage and the expression levels of hsa-miR-139-5p, hsa-miR-139-3p and hsa-miR-424-3p in HCC tissues were significantly associated with the prognosis of HCC patients ($P < 0.05$) (Table 2). High tumor stage was associated with poor prognosis in HCC patients (Hazard ratio: 1.7, $P < 0.001$). High expression levels of hsa-miR-139-5p (Hazard ratio: 0.72, $P < 0.001$), hsa-miR-139-3p (Hazard ratio:0.77, $P < 0.001$) and hsa-miR-424-3p (Hazard ratio:0.81, $P = 0.022$) were associated with good prognosis in HCC patients. Further multivariate Cox regression analysis indicated that tumor stage and hsa-miR-139-5p were independent prognostic factors ($P < 0.01$) (Table 2). Survival prediction model was developed based on the two independent predictors and displayed on a nomogram (Fig. 3). ROC curve analysis suggested that the prediction model exhibited good performance in predicting 3-year survival of HCC patients (AUC = 0.74). K-M analysis showed that the prognosis of HCC patients with high-risk score was significantly lower than that of HCC patients with low-risk score ($P < 10^{-5}$). (See Figs. 4 and 5.)

Table 2
Identification of factors associated with the prognosis of HCC patients.

Factors	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P*	HR	95%CI	*P*
Gender	0.81	0.55–1.19	0.285			
Age	1.01	0.99–1.02	0.242			
Tumor stage	1.7	1.38–2.1	< 0.001	1.52	1.21–1.9	< 0.001
hsa-miR-199a-3p	0.91	0.82–1.01	0.072			
hsa-miR-199b-3p	0.91	0.82–1.01	0.07			
hsa-miR-139-5p	0.72	0.63–0.82	< 0.001	0.69	0.53–0.9	0.007
hsa-miR-139-3p	0.77	0.67–0.88	< 0.001	1.19	0.9–1.57	0.22
hsa-miR-424-3p	0.81	0.68–0.97	0.022	0.89	0.73–1.07	0.216
hsa-miR-1269b	1.04	1–1.09	0.078			
hsa-miR-1269a	1.04	0.99–1.08	0.112			

Abbreviations: HR, Hazard ratio; CI, Confidence interval.

4. Discussion

To elucidate the molecular mechanism of the occurrence and progression of HCC and decrease mortality and improve the prognosis of HCC patients, new HCC-related molecules needed to be revealed. In the present study, we firstly identified and validated seven differentially expressed miRNAs (hsa-miR-199a-3p, hsa-miR-199b-3p, hsa-miR-139-5p, hsa-miR-139-3p, hsa-miR-424-3p, hsa-miR-1269b and hsa-miR-1269a) between HCC and normal liver tissues based on large miRNA expression profiles. Thereinto the expression of hsa-miR-199a-3p, hsa-miR-199b-3p, hsa-miR-139-5p, hsa-miR-139-3p, hsa-miR-424-3p were down-regulated in HCC tissues and might function as tumor suppressors in HCC. The expression of hsa-miR-1269b and hsa-miR-1269a were up-regulated in HCC tissues and might act as onco-miRNAs in HCC. By reviewing the published literature, we found that these miRNAs, except hsa-miR-139-3p, had been reported in HCC [21–25]. For instance, hsa-miR-199a-3p could inhibit tumorigenesis of HCC cells by targeting ZHX1/PUMA signal [21]. The hsa-miR-139-5p could inhibit the growth of HCC cells through targeting SPOCK1 [22]. The hsa-miR-139-3p could suppress tumor growth and metastasis in HCC by repressing ANXA2R [24]. Due to research limitations, these identified miRNAs might also have other functions waiting to be revealed. Thus, we continued to explore their potential function by enrichment analysis of miRNA target genes. The results showed that these miRNAs were involved in multiple cancer-related BPs, such as BMP signaling pathway, protein sumoylation, water transport, canonical Wnt signaling pathway and fibroblast growth factor receptor signaling pathway [26–30]. In addition, these miRNAs also participated in the regulation of many MFs and CCs, such as GTPase activator activity, protein tyrosine phosphatase activity, MAP kinase activity, endoribonuclease activity, phosphoprotein binding, mitotic spindle pole, ER to Golgi transport vesicle, endoplasmic reticulum quality control compartment and RISC-loading complex. The above findings contributed to pointing out a new direction for subsequent research on the role of the seven miRNAs in HCC. In order to obtain miRNAs with potential diagnostic value from these HCC-related miRNAs, we assessed their capability for distinguishing between HCC and normal liver tissues in large samples by ROC curves. The results revealed that hsa-miR-139-3p, hsa-miR-139-5p and hsa-miR-424-3p had high accuracy (AUC > 0.9), and hsa-miR-1269a, hsa-miR-1269b, hsa-miR-199a-3p and hsa-miR-199b-3p had moderate accuracy (0.9 < AUC < 0.7), which suggested that hsa-miR-139-3p,

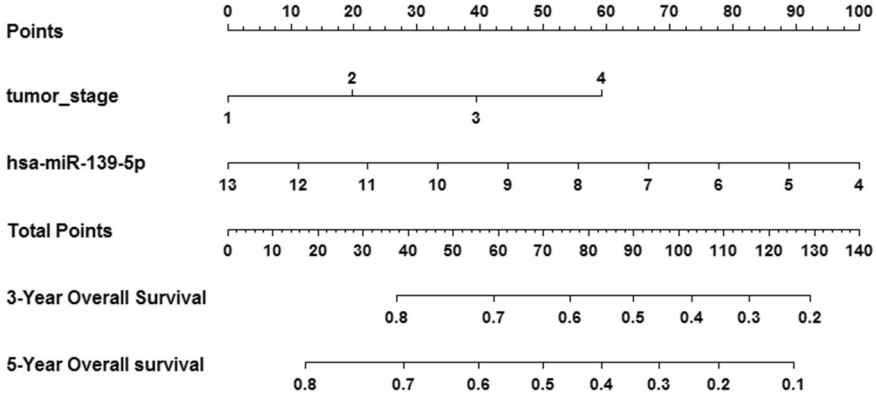

Fig. 3. A nomogram predicting overall survival rates of HCC patients based on tumor stage and hsa-miR-139-5p.

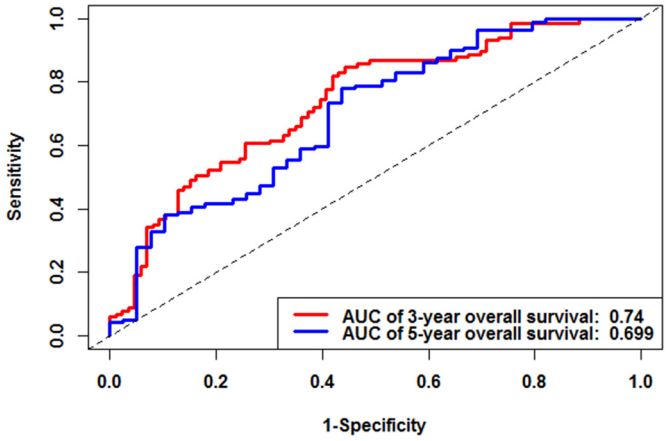

Fig. 4. ROC curves showing distinction capability of the survival prediction model for 3- and 5-year overall survival rates of HCC patients (AUC indicates the area under ROC curve).

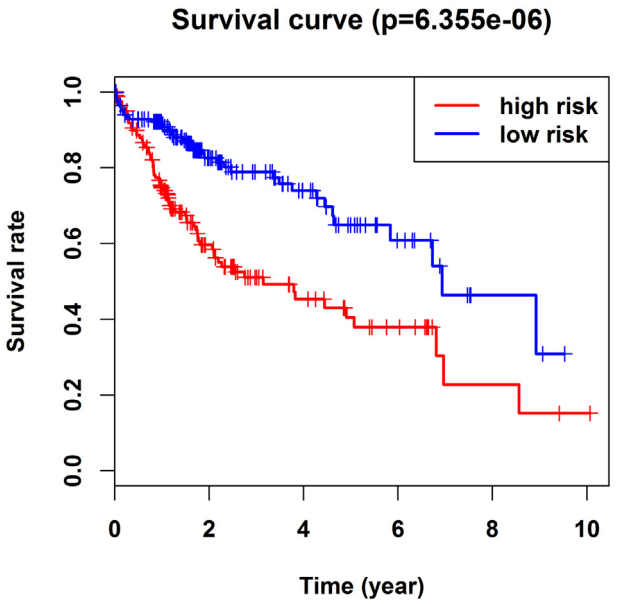

Fig. 5. Kaplan-Meier survival curves of HCC patients with high- or low- risk score (High-risk group indicates the risk score of each patient is greater than the median risk score. Low-risk group indicates the risk score of each patient is less than or equal to the median risk score.).

hsa-miR-139-5p and hsa-miR-424-3p had more effective distinction capability and might serve as promising diagnostic markers for HCC.

Nomograms have been recognized as reliable and pragmatic prediction tools to quantify individual risk by incorporating multiple significant prognostic factors [31,32]. Therefore, we developed a prognostic nomogram for predicting overall survival (OS) of HCC patients based on tumor stage and hsa-miR-139-5p, which were identified as two independent prognostic factors in the current study. The result of ROC analysis suggested that the nomogram had a good performance in predicting 3-year survival of HCC patients, and might serve as a useful guide in the management of HCC patients. In addition, we divided all HCC patients from TCGA into high- or low-risk group based on the median risk score calculated by the prognostic prediction model, and then observed that OS in the high-risk group was significantly worse than that in the low-risk group, suggesting that the model could help clinicians to identify patients with high risk before the treatment, and to make better clinical decisions and follow-up surveillance.

In conclusion, the present study identified several HCC-related miRNAs, which might serve as new diagnostic markers and therapeutic targets for HCC. In addition, hsa-miR-139-5p might act as a promising prognostic indicator for HCC patients.

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None.

Ethical approval

This article does not contain any studies with human participants performed by any of the authors.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116596.

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