

Lower expressed miR-198 and its potential targets in hepatocellular carcinoma: a clinicopathological and in silico study

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Purpose: To investigate the clinicopathological value and potential roles of microRNA-198 (miR-198) in hepatocellular carcinoma (HCC).

Methods: Ninety-five formalin-fixed paraffin-embedded HCC and the para-cancerous liver tissues were gathered. Real-time reverse transcription quantitative polymerase chain reaction was applied to determine the miR-198 expression. The association between the miR-198 expression and clinicopathological features was examined. Meanwhile, potential target messenger RNAs of miR-198 in HCC were obtained from 14 miRNA prediction databases and natural language processing method, in which we pooled the genes related to the tumorigenesis and progression of HCC and classified them by their frequency. The selected target genes were finally analyzed in the Gene Ontology analysis and Kyoto Encyclopedia of Genes and Genomes pathway.

Results: miR-198 expression was significantly lower in HCC than that in adjacent noncancerous liver tissues (1.30 ± 0.72 vs 2.01 ± 0.58 , $P < 0.001$). Low miR-198 expression was also correlated to hepatitis C virus infection ($r = -0.48$, $P < 0.001$), tumor capsular infiltration ($r = -0.43$, $P < 0.001$), metastasis ($r = -0.26$, $P < 0.010$), number of tumor nodes ($r = -0.25$, $P = 0.013$), vaso-invasion ($r = -0.24$, $P = 0.017$), and clinical tumor node metastasis stage ($r = -0.23$, $P = 0.024$). Altogether, 1,048 genes were achieved by the concurrent prediction from at least four databases and natural language processing indicated 1,800 genes for HCC. Further, 127 overlapping targets were further proceeded with for pathway analysis. The most enriched Gene Ontology terms in the potential target messenger RNAs of miR-198 were cell motion, cell migration, cell motility, and regulation of cell proliferation in biological process; organelle lumen, membrane-enclosed lumen, and nuclear lumen in cellular component; and enzyme binding, protein domain-specific binding, and protein kinase activity in molecular function. Kyoto Encyclopedia of Genes and Genomes analysis showed that these target genes were obviously involved in focal adhesion and pathways in cancer.

Conclusion: Lower expression of miR-198 was related to several clinicopathological parameters in HCC patients. miR-198 might play a regulatory role through its target genes in the development of HCC.

Keywords: miR-198, hepatocellular carcinoma, metastasis, RT-qPCR, in silico

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Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent malignancies in the world. The estimate incidence of liver cancer ranked fourth and its mortality ranked third in People's Republic of China, 2015.¹ Currently, several genes have been identified to be related with the tumorigenesis and progression of HCC, but the molecular mechanisms of their functions and interactions have not been clearly

studied. Therefore, it is urgent to find the potential function of these genes during the development of HCC.

MicroRNA (miRNA) is classified as endogenous noncoding RNA, repressing protein translation by binding to their target genes.² From nearly all biological processes, miRNA can exert the function as posttranscriptional regulation.³ Aberrant expression of microRNA-198 (miR-198) has been found to be correlated to the carcinogenesis and disease progression of several classes of cancers, including lung adenocarcinoma,⁴ esophageal cancer,⁵ squamous cell carcinoma of tongue,⁶ retinoblastoma,⁷ pancreatic adenocarcinoma,⁸ multiple myeloma,⁹ colorectal cancer,¹⁰ and prostate cancer.¹¹ However, there were only three research papers studying the expression of miR-198 in HCC. Varnholt et al first reported the lower expressed miR-198 in Caucasian patients with hepatitis C virus (HCV) related HCC.¹² Tan et al identified the suppressing role of miR-198 in HGF/c-MET pathway that influenced migration and invasion of HCC cells.¹³ Elfimova et al found another pathway that was repressed by miR-198 in vitro which could negatively regulate cell growth and migration.¹⁴ However, whether miR-198 is related to clinicopathological parameters has not been studied, there still remains a mystery regarding miR-198's role in the key processes of hepatocellular carcinogenesis.

Thus, in this study, we assessed the expression of miR-198 in tumor tissues of HCC patients with their paired noncancerous liver. In addition, the relationship of miR-198 with several clinicopathological parameters was also investigated. Moreover, we predicted the target genes of miR-198 via 14 miRNA databases and filtered in the natural language processing (NLP) to achieve the specific messenger RNAs (mRNA) profiles of miR-198 targets for HCC. Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation were further undertaken to explore the function of these mRNAs. We attempted to pioneer the use of real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR) and in silico method to clarify the potential molecular mechanism of miR-198, providing guidelines for the following research on miR-198 in HCC.

Materials and methods

Tissue samples

There were 95 cases of HCCs and their paired paraneoplastic liver formalin-fixed paraffin-embedded (FFPE) tissues included in this retrospective study. The age of the HCC patients ranged from 29 to 82 years old, with a mean age of 52 years. All the clinicopathological features are provided in Table 1. Adjacent noncancerous liver tissues were obtained

Table 1 Relationship between the expression of miR-198 and clinicopathological features in HCC

Clinicopathological feature	n	miR-198 relevant expression (2 ^{-ΔCq})		
		Mean ± SD	t	P-value
Tissue				
Adjacent noncancerous liver	95	2.01±0.58	7.53	<0.001***a
Noncirrhotic liver	50	1.96±0.57	-0.76	0.449 ^b
Cirrhotic liver	45	2.06±0.59	5.67	<0.001***c
HCC	95	1.30±0.72	6.25	<0.001***d
Age, years				
≥50	46	1.42±0.74	1.66	0.10
<50	49	1.18±0.68		
Sex				
Male	75	1.30±0.69	0.04	0.97
Female	20	1.29±0.83		
Differentiation				
High	6	1.87±0.52	10.20	<0.001***e
Moderate	60	1.45±0.74		
Low	29	0.87±0.45		
Size (cm)				
<5	18	1.48±0.72	0.99	0.33
≥5	77	1.26±0.72		
Tumor nodes				
Single	52	1.47±0.76	2.72	0.008**
Multi	43	1.08±0.61		
Metastasis				
Without metastasis	46	1.49±0.73	2.62	0.01*
With metastasis	49	1.12±0.66		
Clinical TNM stage				
I-II	22	1.60±0.72	2.34	0.021*
III-IV	73	1.21±0.69		
Portal vein tumor embolus				
-	63	1.35±0.71	1.03	0.31
+	32	1.19±0.72		
Vaso-invasion				
-	59	1.42±0.69	2.23	0.028*
+	36	1.09±0.71		
Tumor capsular infiltration				
With complete capsule	45	1.64±0.77	4.92	<0.001***
No capsule infiltration	50	0.99±0.50		
HCV				
-	63	1.53±0.71	5.89	<0.001***
+	32	0.83±0.44		
HBV				
-	17	1.42±0.78	0.77	0.44
+	78	1.27±0.70		
AFP				
-	41	1.40±0.79	1.36	0.18
+	38	1.17±0.71		
Cirrhosis				
-	50	1.21±0.71	-1.30	0.20
+	45	1.40±0.72		
nm23				
-	20	1.29±0.67	-0.07	0.95
+	75	1.30±0.73		

(Continued)

Table 1 (Continued)

Clinicopathological feature	n	miR-198 relevant expression (2 ^{-ΔCq})		
		Mean ± SD	t	P-value
P53				
–	40	1.29±0.75	–0.04	0.97
+	55	1.30±0.69		
P21				
–	62	1.34±0.78	0.81	0.42
+	33	1.22±0.58		
VEGF				
–	25	1.15±0.65	–1.21	0.23
+	70	1.35±0.73		
Ki-67LI				
Low	47	1.40±0.72	1.37	0.18
High	48	1.20±0.70		
MVD				
Low	47	1.24±0.67	–0.73	0.47
High	48	1.35±0.76		

Notes: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. *Adjacent noncancerous liver (including liver with and without cirrhosis) vs HCC. ^aNoncirrhotic liver vs cirrhotic liver. ^cNoncirrhotic liver vs HCC. ^dCirrhotic liver vs HCC. ^eDifferentiation group pairwise comparison: high vs low differentiation ($t = 4.851$, $P < 0.001$) and moderate vs low ($t = 4.610$, $P < 0.001$) have significant differences, high vs moderate ($t = 1.336$, $P = 0.186$) has no difference.

Abbreviations: AFP, alpha fetal protein; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; miR-198, microRNA-198; MVD, microvessel density; SD, standard deviation; TNM, tumor node metastasis; VEGF, vascular endothelial growth factor.

from the location at least 2 cm away from the tumor node. The adjacent tissue was from the same HCC patient. We designed these paired sample groups in order to eliminate the individual difference. Tumor sizes of HCC ranged from 1 to 11 cm (mean size, 6.4 cm). All cases were from patients who were under initial hepatectomies without any treatment and they were randomly chosen from hepatectomy surgeries in the First Affiliated Hospital of Guangxi Medical University, People's Republic of China between March 2010 and December 2011. The research protocol was approved by the Ethical Committee of the First Affiliated Hospital of Guangxi Medical University. Written informed consent was signed by the patients for the treatment and sample usage in this study. All samples were diagnosed by three pathologists independently (Dian-zhong Luo, Zhen-bo Feng, Gang Chen).

RT-qPCR

Total RNA including miRNA was extracted from tumor and nontumorous liver sections and the miRNeasy FFPE kit (QIAGEN, KJ Venlo, the Netherlands) was applied according to our previous reports.^{15–17} RNA concentrations were measured by NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA). The OD260/OD280 ratio of the total mRNA (including miRNA) isolated from the FFPE tissues ranged from 1.84 to 2.06, and OD260/OD230 from 1.90 to 2.04.

The PCR amplification efficiency of all the quantitative real-time PCR (RT-qPCR) reactions ranged from 91.0% to 95.2%. A combination of RUN6B and RUN48 was chosen as the house-keeping reference for detection of miR-198 expression. The primers of miR-198, as well as RNU6B and RNU48, were provided by TaqMan® MicroRNA Assays (4427975, Applied Biosystems, Life Technologies, Grand Island, NY, USA). Sequence of miRNA and references in the paper were as follows: miR-198 (Applied Biosystems Cat No 4427975-000468): UCCCAAUGUAGACAAAGCA; RNU6B (Applied Biosystems Cat No 4427975-001093): CGCAAGGAUGACACGCAAUUCGUGAAGCGUCCAUAUUUUU; RNU48 (Applied Biosystems Cat No 4427975-001006): GAUGACCCAGGUAACUCUGAGUGUGUCGUGCUGAUGCCAUCACCGCAGCGCUCUGACC. The reverse transcription was carried out with TaqMan® MicroRNA Reverse Transcription Kit (4366596, Applied Biosystems, Life Technologies) in a total volume of 10 μL. Real-time qPCR for miRNA trial was performed with Applied Biosystems PCR7900. The normalization of miR-198 quantity in each sample was compared with its internal reference. At last, the formula $2^{-\Delta Cq}$ was used to calculate the expression of miR-198 in this experiment.¹⁵

Potential target mRNAs of miR-198 in HCC

To achieve predicted target mRNAs of miR-198, 14 miRNA in silico databases were used in this study, including TargetScan, miRanda, RNAv22, miRWalk, miRTarBase, PicTar, TargetS, DIANA, miRDB, miRNAmap, PITA, TargetMiner, Human miRNA Targets, and PolymiRTS Database. More than 16,000 target mRNAs were predicted once in total. Only mRNAs that were simultaneously predicted by four or more than four databases were selected for subsequent study. Other validated genes targeted by miR-198 were also added to the study throughout literature review.

NLP analysis of liver cancer

To identify the specific genes that were abnormally expressed in HCC, we conducted a literature search in the PubMed and EMBASE with an attempt to cover all studies available in publication from January 1980 and May 2015 through the following strategy: (liver or hepat*) and (cancer or carcinoma or tumor or neoplasm or malignan* or carcinogenesis or tumorigenesis) and (resistance OR prognosis OR metastasis OR recurrence OR survival OR sorafenib OR bevacizumab) (“1980/01/01” [PDAT]: “2015/05/25” [PDAT]). We then identified all the genes and mRNAs that were associated with the key message, and used a biomedical tool – entity recognizer (ABNER)¹⁶ to add them to a list via gene mention tagging.¹⁷

Gene-enrichment and functional annotation analysis

To evaluate the function of the potential target mRNAs, we used the GO terms and KEGG pathway annotation for the miR-198 targets based on functional annotation summaries provided by DAVID (Database for Annotation, Visualization, and Integrated Discovery, <http://david.abcc.ncifcrf.gov/>).¹⁸ The GO terms with a modified Fisher exact P -value <0.01 and the pathways with P -value <0.05 were chosen for the next analysis. The enrichment map of annotation analysis was drawn by software Cytoscape 3.3.0.¹⁹

Validation of the expression of some potential target genes based on The Cancer Genome Atlas (TCGA) dataset

To further study the potential role of miR-198, additional analyses were performed on the expression of some predicted genes randomly selected from one top pathway generated by the signaling pathway analysis. All gene expressions of 369 patients of liver HCC and 50 adjacent liver tissues were extracted and analyzed from the TCGA RNAseq profiles (<http://cancergenome.nih.gov>).

Statistical analysis

SPSS 21.0 (IBM Corporation, Armonk, NY, USA) was employed for statistical analysis. Values were shown as the mean \pm standard deviation. Student's paired or unpaired t -test was adopted to analyze the significance between paired and unpaired groups respectively. One-way analysis of variance test was applied to analyze the significance between groups of various differentiations of HCC. Fold change (FC) was calculated to describe the degree of difference on a log 2 scale. Statistical significance was defined at a $P<0.05$ level.

Results

Expression of miR-198 in HCC

The relative level of miR-198 was 1.23 ± 0.72 in HCC tissues, remarkably lower than that in adjacent noncancerous liver tissues (2.01 ± 0.58 , $P<0.001$) (Table 1, Figure 1A). Furthermore, the area under curve (AUC) of miR-198 accessed by receiver operating characteristic (ROC) curve was 0.775 (95% confidence interval (CI): 0.708–0.841, $P<0.001$) (Figure 2A). The cutoff value for miR-198 was 1.78. The sensitivity was 66.3% and the specificity was 75.8%. However, there was no significant difference of the miR-198 level between the noncirrhotic liver (1.96 ± 0.57) and cirrhotic liver (2.06 ± 0.59 , $t=-0.76$, $P=0.449$) (Table 1). And miR-198 expression was markedly downregulated in HCC (1.30 ± 0.72) as compared

to noncirrhotic liver ($t=5.67$, $P<0.001$) and cirrhotic liver ($t=6.25$, $P<0.001$), respectively.

Expression of miR-198 with clinicopathological parameters in HCC

Considering correlation between miR-198 expression and clinical features, several clinicopathological parameters were related to miR-198. Remarkably higher expression of miR-198 was found in the tumor with complete capsular (1.64 ± 0.77) than in the tumor with no capsule or with tumor infiltration (0.99 ± 0.50 , $P<0.001$) (Figure 1B). MiR-198 differential expression could be found in tissue differentiation, and significant differences existed between high differentiation (1.87 ± 0.52) and low differentiation (0.87 ± 0.45 , $P<0.001$) (Figure 1C), and similar variation was also found between moderate (1.45 ± 0.74) and low differentiation (0.87 ± 0.45 , $P<0.001$) (Figure 1C). However, high differentiation and moderate one showed no significant difference. HCV positive patients had a noticeably lower miR-198 expression level (0.83 ± 0.44) than negative ones (1.53 ± 0.71 , $P<0.001$) (Figure 1D). In tumor without metastasis, higher miR-198 expression (1.49 ± 0.73) was observed compared with that with metastasis (1.12 ± 0.66 , $P=0.010$) (Figure 2E). Expression of miR-198 was higher in patients with single tumor node (1.47 ± 0.76) than in those with multitumor nodes (1.08 ± 0.61 , $P=0.008$) (Figure 1F). As for clinical tumor node metastasis (TNM) stage, miR-198 expression in early stages (I and II, 1.60 ± 0.72) was higher than that in advanced stages (III and IV, 1.21 ± 0.69 , $P=0.021$) (Figure 1G). Concerning the status of vaso-invasion, expression of miR-198 was lower in positive ones (1.09 ± 0.71) than in negative ones (1.42 ± 0.69 , $P=0.028$) (Figure 1H). Simultaneously, additional Spearman's correlation test confirmed the relationship between miR-198 expression and the following clinicopathological features: differentiation ($r=-0.43$, $P<0.001$) and clinical TNM stage ($r=-0.23$, $P=0.024$). However, no significant differences between miR-198 expression and other clinicopathological parameters were noted, such as age, sex, tumor size, portal vein tumor embolus, hepatitis B virus, alpha fetal protein, cirrhosis, expression of other markers: nm23, p53, p21, vascular endothelial growth factor, Ki-67, or microvessel density. The detection of the expression of nm23, p53, p21, vascular endothelial growth factor, Ki-67, and CD34 to present microvessel density was carried out by the immunohistochemistry method (Figure 3A–L). And hematoxylin and eosin staining images of HCC, cirrhosis, and noncirrhotic liver tissues are shown in Figure 4. We also searched for the data of the expression of miR-198 in Gene Expression

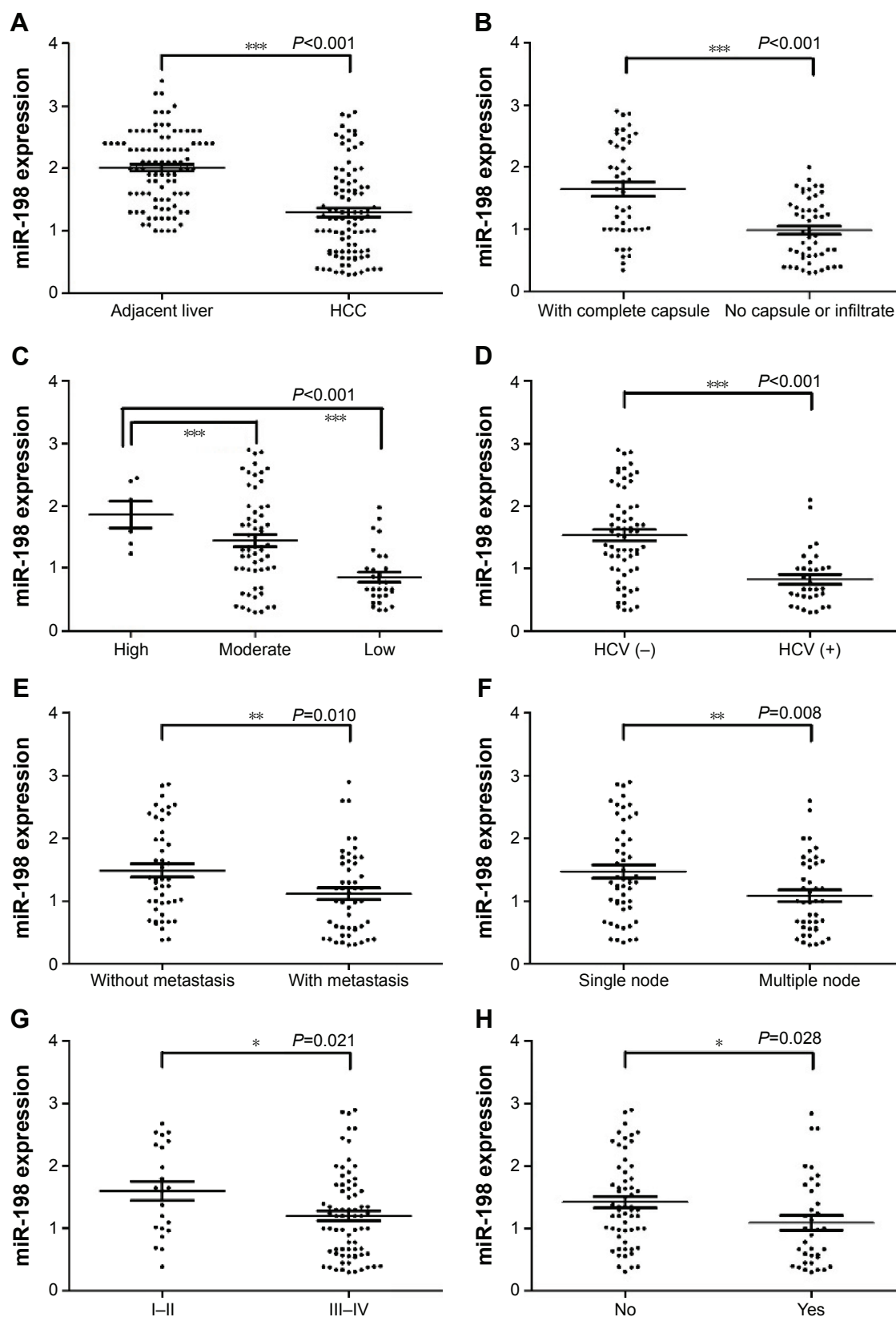


Figure 1 miR-198 expression in HCC tissue.

Notes: Quantitative real-time PCR was performed to detect the expression of miR-198 in HCC tissue and adjacent noncancerous hepatic tissue. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. (A) Tissue, (B) tumor capsular infiltration, (C) differentiation: high differentiation (1.8650 ± 0.5217) is higher expressed than low differentiation (0.8652 ± 0.4475 , $P < 0.001$), moderate differentiation (1.4495 ± 0.7414) is higher expressed than low differentiation (0.8652 ± 0.4475 , $P < 0.001$). (D) HCV infected, (E) metastasis, (F) tumor nodes, (G) clinical TNM stage, (H) vasculectomy.

Abbreviations: HCC, hepatocellular carcinoma; HCV, hepatitis C virus; miR-198, microRNA-198; PCR, polymerase chain reaction; TNM, tumor node metastasis.

Omnibus datasets and brought in all the HCC-related miR-198 expression chip data to perform a meta-analysis. Standardized mean difference with 95% CIs were calculated and pooled by using STATA 12.0 (StataCorp LP, College Station, TX, USA). Six microarray datasets with miR-198 expression level were employed. But the results obtained by a random-effects model showed no statistical significance ($P>0.05$) between the expression of miR-198 in HCC and noncancer tissues (data not shown). So, the more expanded sample size will be needed for the validation of miR-198 expression in the future.

ROC analysis for miR-198 expression in HCC

ROC curve was also performed to identify the predictive value of miR-198 level in HCC patients for clinicopathological

features. The AUC to discriminate HCC from adjacent noncancerous liver accessed by ROC curve was 0.775 (95% CI: 0.708–0.841, $P<0.001$) (Figure 2A). The cut-off value for miR-198 was 1.78. The sensitivity was 66.3% and the specificity was 75.8%. The AUC to judge the capsule and tumor infiltration was 0.748 (95% CI: 0.649–0.846, $P<0.001$) (Figure 2B). The cut-off value for miR-198 was 1.73. The sensitivity and specificity were 48.9% and 96%, respectively. The AUC to judge the HCV infected was 0.794 (95% CI: 0.702–0.886, $P<0.001$) (Figure 2C). The cut-off value for miRNA-198 was 1.12. The sensitivity and specificity were 73% and 81.2%, respectively. The AUC to evaluate without tumor metastasis was 0.652 (95% CI: 0.542–0.761, $P=0.011$) (Figure 2D). The cutoff value for miR-198 was 0.62. The sensitivity and specificity were 93.5% and 34.7%,

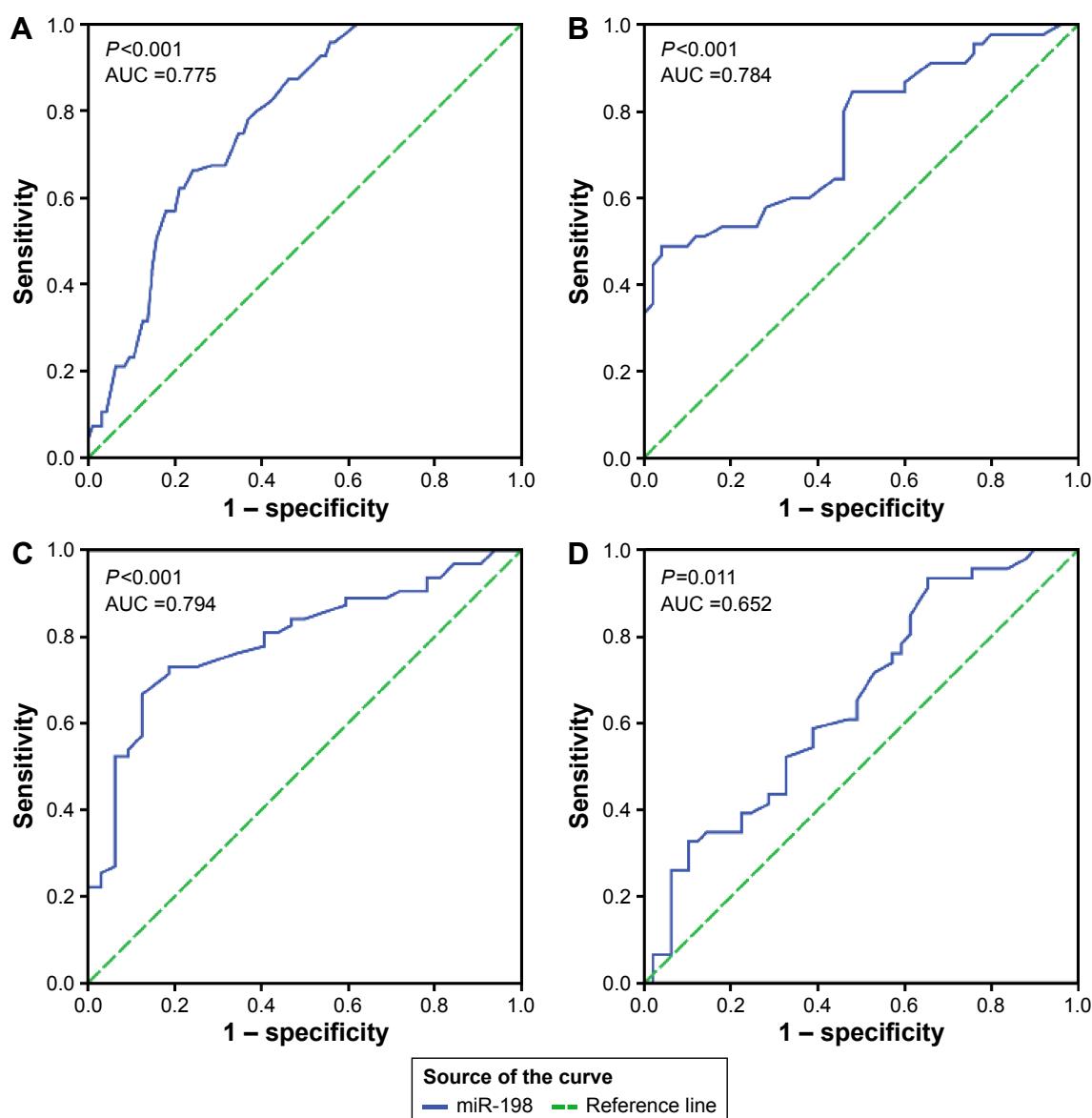


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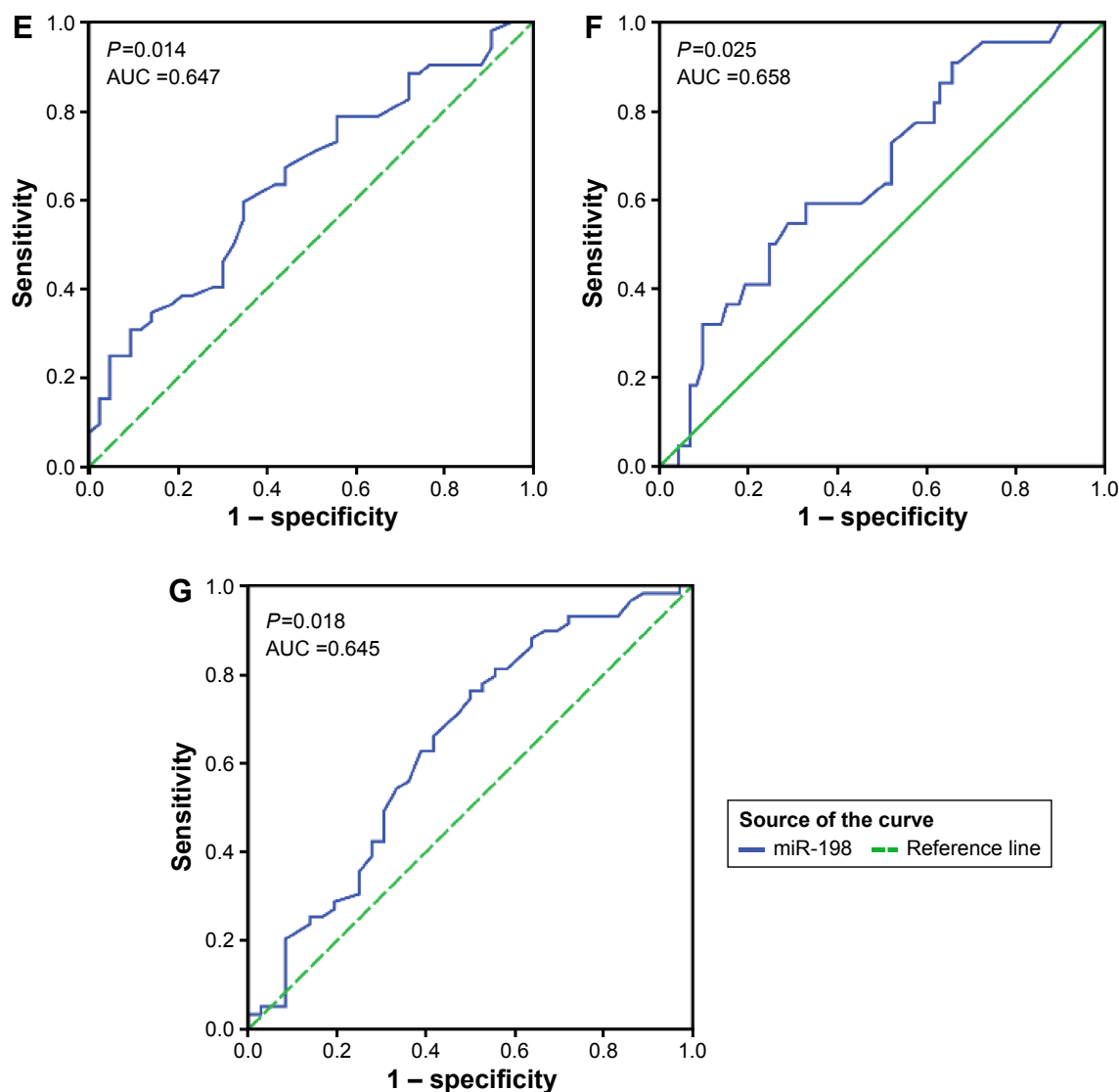


Figure 2 Significance of miR-198 with different parameters in HCC with the ROC curve analysis.

Notes: (A) Discrimination of HCC from adjacent noncancerous liver, (B) evaluating capsular infiltration, (C) HCV infected, (D) metastasis, (E) tumor nodes, (F) TNM stage, (G) vaso-invasion.

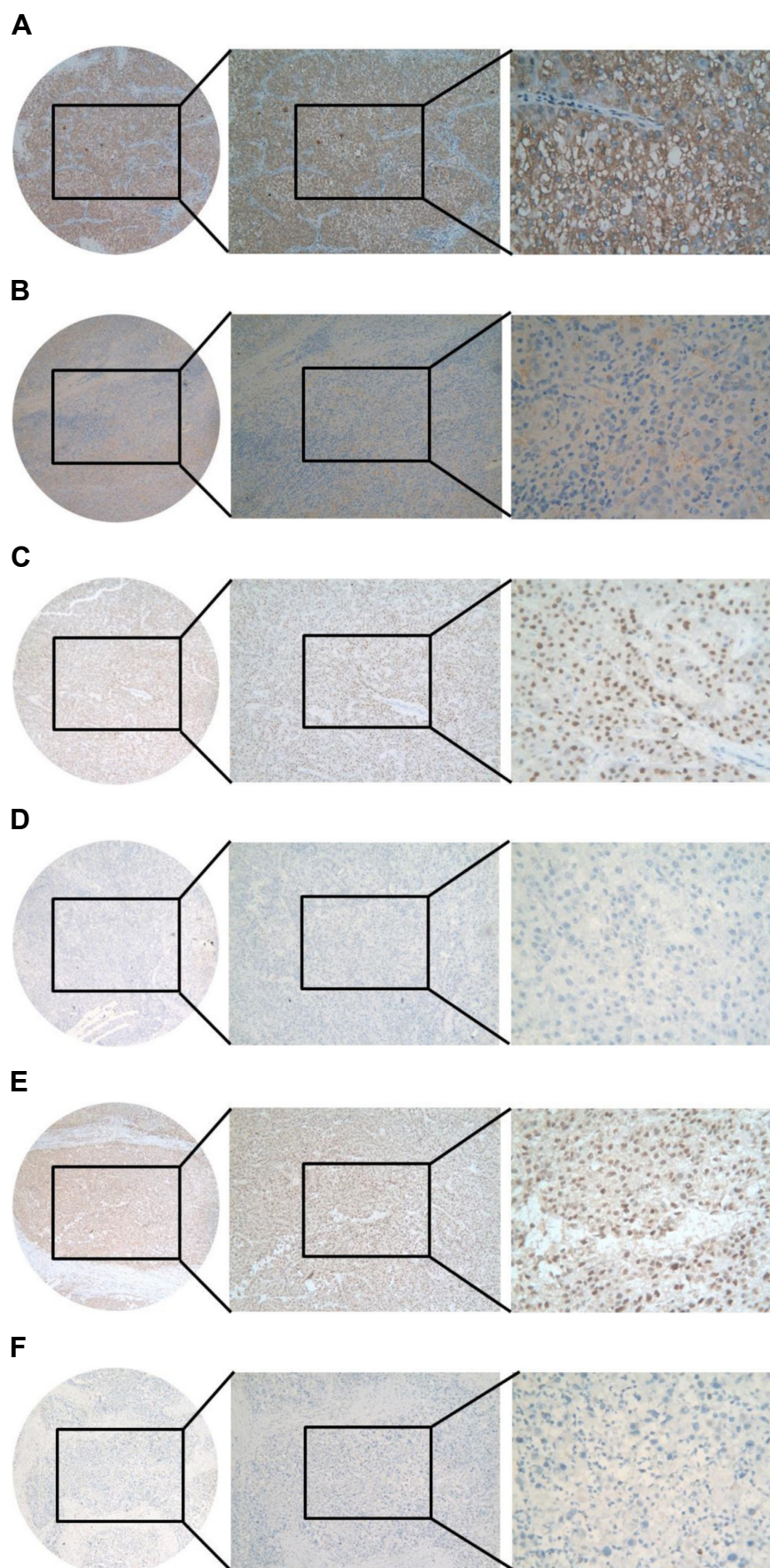
Abbreviations: AUC, area under the curve; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; miR-198, microRNA-198; ROC, receiver operating characteristic; TNM, tumor node metastasis.

respectively. The AUC to predict single tumor node was 0.647 (95% CI: 0.537–0.757, $P=0.014$) (Figure 2E). The cutoff value for miR-198 was 1.22. The sensitivity and specificity were 59.6% and 65.1%, respectively. The AUC to estimate TNM stage was 0.658 (95% CI: 0.533–0.784, $P=0.025$) (Figure 2F). The cutoff value for miR-198 was 1.365. The sensitivity and specificity were 59.1% and 67.1%, respectively. The AUC to predict vaso-invasion was 0.645 (95% CI: 0.526–0.763, $P=0.018$) (Figure 2G). The cutoff value for miR-198 was 0.935. The sensitivity and specificity were 76.3% and 50%, respectively. The sensitivity and specificity were 73% and 81.2%, respectively (Figure 2B). The cutoff value for miR-198 was 1.73. The sensitivity and specificity were 48.9% and 96%, respectively. The AUC in

HCV negative patients was 0.794 (95% CI: 0.702–0.886, $P<0.001$) (Figure 2C). The cutoff value for miR-198 was 1.12. The sensitivity and specificity were 73% and 81.2%, respectively.

Role of miR-198 on recurrence of HCC patients

We further studied the value of miR-198 on patient recurrence. Of the 95 patients studied, 70 had the complete follow-up data: 34 on low miR-198 level (lower than the median level of 1.2000) and 36 on high miR-198 level. There was no significant difference of recurrence between low miR-198 expression group and the high group ($P=0.542$).

**Figure 3** (Continued)

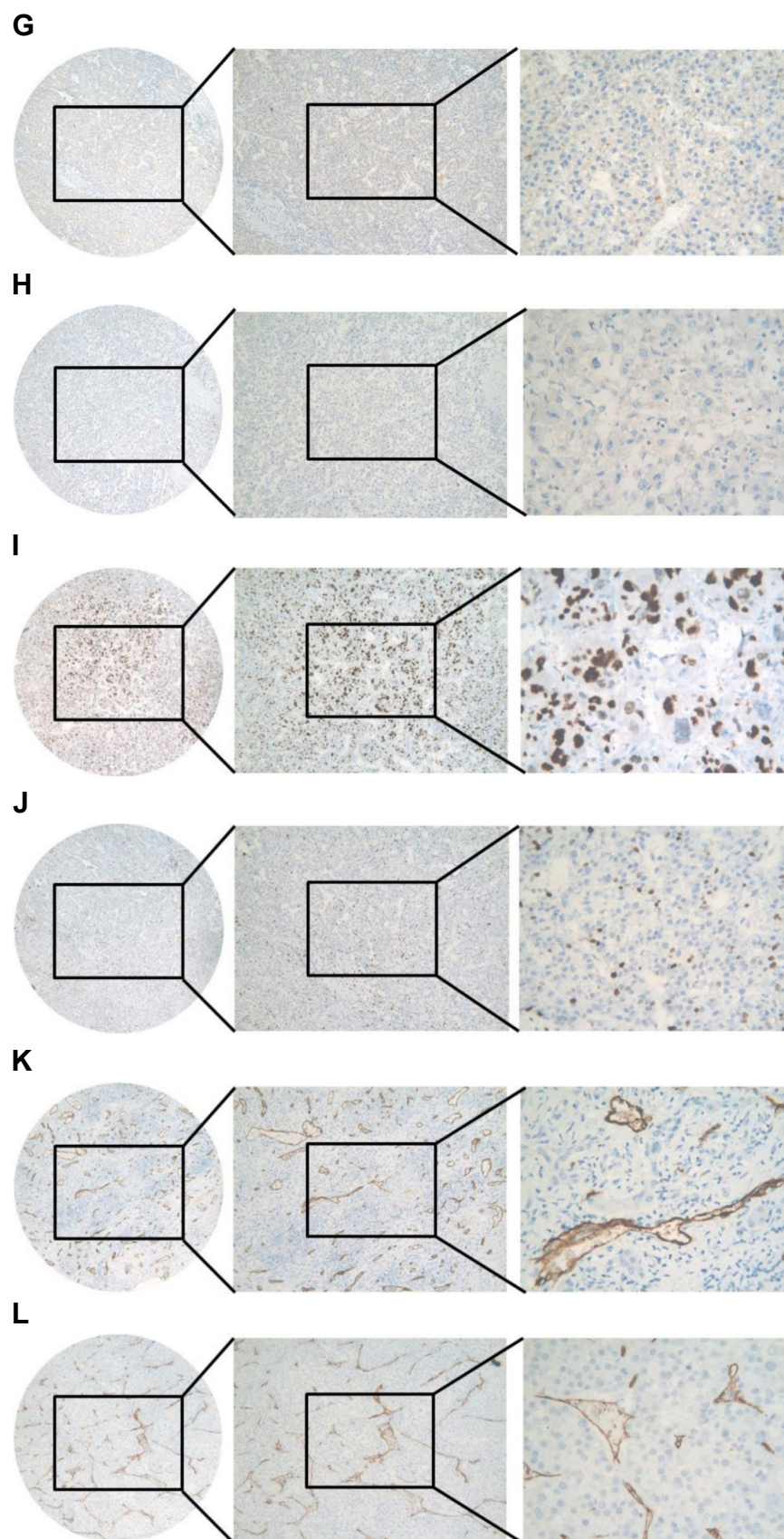


Figure 3 Immunohistochemistry images of HCC under microscope (×40, ×100, and ×400).

Notes: (A) nm23 positive; (B) nm23 negative; (C) p21 positive; (D) p21 negative; (E) p53 positive; (F) p53 negative; (G) VEGF positive; (H) VEGF negative; (I) Ki-67 LI high; (J) Ki-67 LI low; (K) MVD high; (L) MVD low.

Abbreviations: HCC, hepatocellular carcinoma; MVD, microvessel density; VEGF, vascular endothelial growth factor.

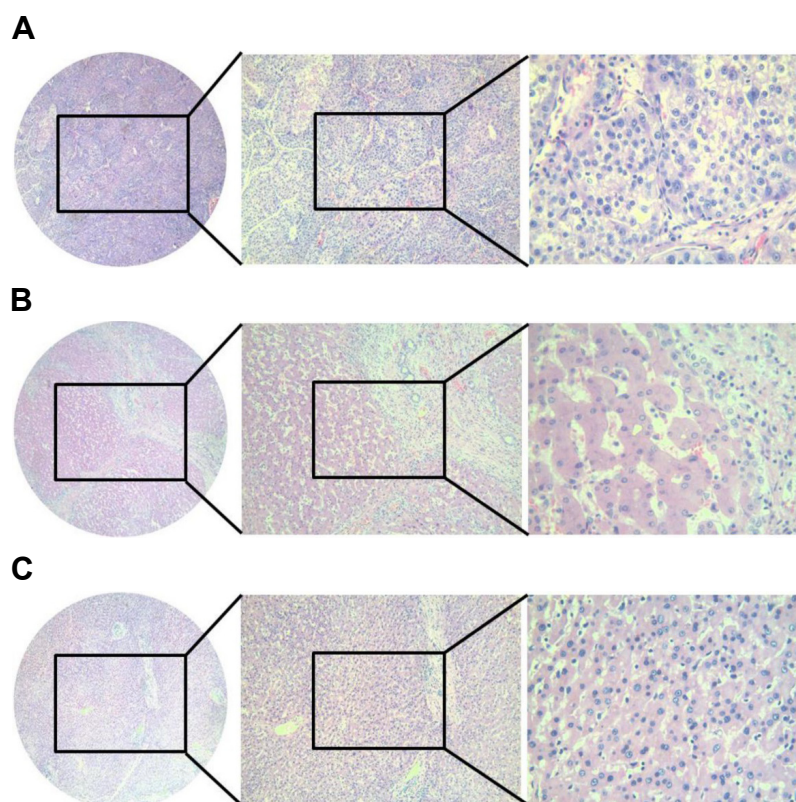


Figure 4 HE staining images of HCC (A), cirrhosis (B), noncirrhotic liver tissue (C), under microscope ($\times 40$, $\times 100$, $\times 400$).
Abbreviations: HCC, hepatocellular carcinoma; HE, hematoxylin and eosin.

Potential targets of miR-198 in HCC

To get predicted targets of miR-198, we conducted the prediction process online via 14 computational algorithms mentioned above, and attained a total number of 16,381 mRNAs after excluding duplications generated from the prediction process, and subsequently in order to increase reliability of our study we screened 1,048 mRNAs which were considered as more or less likely predictions. Another 37

genes validated to be the targets of miR-198 were added to our study through literature review. In addition, the abstracts or titles of 64,577 studies had been included through data and text mining tools in the process of NLP, and a total of 1,800 HCC-related genes were subsequently identified. Furthermore, we attained the potential target mRNAs of miR-198 in HCC by combining the predicted target list and genes specifically expressed in liver cancer, as shown in Table 2. Altogether,

Table 2 Potential target mRNAs of miR-198 in HCC

VCP	CDH11	CCND2	PAK6	ADARBI	DGCR8	MSI2	SERPINF2
HMGA1	DENND2D	CDK6	PAPSS1	APBA2	DDK3	MYB	SHOX2
PTEN	HOXA1	CSF1	PER2	ARHGEF9	ELAVL1	NLK	SMAD2
CDK4	LARPI	CTBP2	POLR2E	BIRC5	FANCD2	NOVA1	SP1
FUT8	MAPRE1	DDR1	PPP2R5E	BMF	FOXJ1	NTRK3	SPRY2
MET	NCOR2	EGF	SERPINA5	BTG2	GPM2	PAFAH1B1	SRC
SLC2A1	NEK8	FASN	SETD4	BVES	H2AFX	PCDH9	TLN1
CNR1	OGT	FRS2	SETDB1	CACNA1C	HDAC4	PEA15	TNFRSF18
MSR1	PDCD1LG2	FTCD	SPTBN1	CAPN2	HDGF	PEG10	TNK2
PPP1R9B	PTPN1	HOXB13	STK17B	CBL	HDLBP	PLAU	TNRC6B
ADAM12	RAD52	IDS	TFCP2	CD4	HFE	POLD1	TRIM3
ARHGAP1	SORT1	ITGA5	TLN2	CDKN2B	INPPL1	PPARA	YWHAG
ASPH	THBS1	MAP2K7	TNFRSF8	CNTN2	ISX	PSD3	ZEB2
BAG3	AMFR	MLL2	TPM3	CTGF	KRT14	PVR	SLC16A7
BCL2L1	BMPRIIA	MST1R	ULK1	CYCS	MPZL1	PVRL2	SPDYA
CBS	CAPN9	MTSSI	WHSC1	DACH1	MSI1	RPL41	

Abbreviations: HCC, hepatocellular carcinoma; miR-198, microRNA-198; mRNA, messenger RNA.

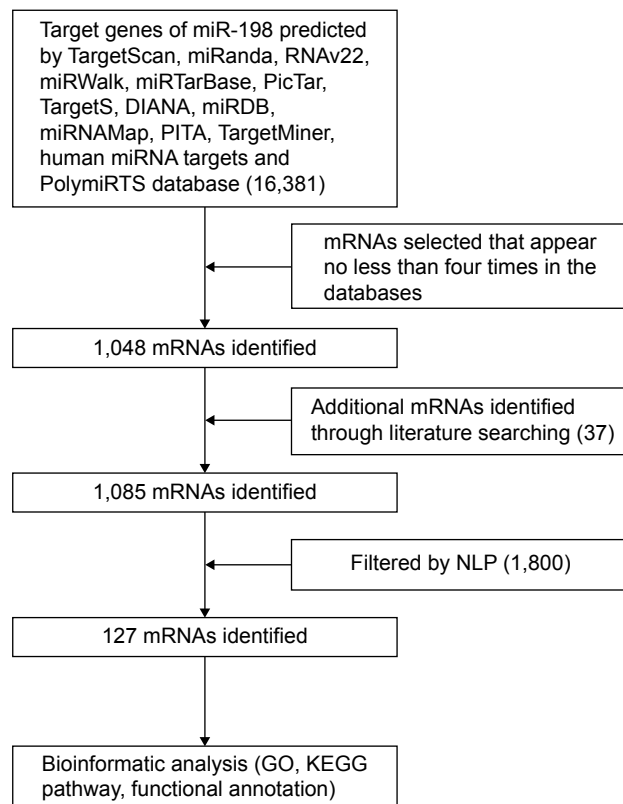


Figure 5 The flow diagram of the bioinformatics study for miR-198 in HCC.

Abbreviations: GO, Gene Ontology; HCC, hepatocellular carcinoma; KEGG, Kyoto Encyclopedia of Genes and Genomes; mRNA, messenger RNA; miR-198, microRNA-198; NLP, natural language processing.

127 mRNAs were selected for gene-annotation enrichment analysis and KEGG pathway annotation, which could be largely representative of the molecular mechanism of miR-198 in HCC. The flow diagram is shown in Figure 5.

Gene-annotation enrichment analysis and KEGG pathway annotation

According to the functional annotation summaries with the background of Homo sapiens listed by DAVID, we gained GO term annotations and KEGG pathways of all the selected target genes of miR-198 in HCC. A modified Fisher's exact test or the EASE score was used to calculate the P -value. For biological process in GO results, the potential targets of miR-198 were significantly involved in the regulation of cell motion, cell migration, cell motility, regulation of cell proliferation, enzyme-linked receptor protein signaling pathway, and transmembrane receptor protein tyrosine kinase signaling pathway, and so on ($P < 0.001$, Table 3, Table S1); for cellular component, these genes were most enriched in organelle lumen ($n=34$), membrane-enclosed lumen ($n=34$), nuclear lumen ($n=29$), plasma membrane ($n=49$), nucleoplasm ($n=18$), cytosol ($n=26$), ruffle ($n=6$), and caveola ($n=5$)

($P < 0.001$, Table 3, Table S2); as for molecular function, the selected mRNAs were markedly involved in enzyme binding, protein domain-specific binding, and protein kinase activity, and so forth ($P < 0.001$, Table 3, Table S3); and for the KEGG pathway analysis, the potential targets of miR-198 were notably associated with focal adhesion, pathways in cancer, p53 signaling pathway, and adheres junction ($P < 0.005$, Table 4). The overview schematic of the analysis is shown in Table 2, Figures 6 and 7.

Validation of some potential target gene expression based on TCGA dataset

Among all the significant pathways in the current study, the organelle lumen (GO: 0043233) was randomly selected. There were 34 genes involved in this pathway. We then analyzed the relative expression (on a log 2 scale) of these 34 predicted genes of miR-198 by using TCGA LIHC dataset. Eleven genes were downregulated, and they could not be real targets of miR-198 due to same trend of the expression in liver tumor (Table 5). And 22 among the rest 23 genes yielded significant overexpression in liver cancers as compared to that in the adjacent liver tissues ($P < 0.05$, Table 5). More interesting, ten among these 22 genes were extremely overexpressed in liver tumor tissues as their FC value was >1 ($P < 0.001$, Table 5, Figure 8A–J), such as *BIRC5*, *FANCD2*, *EGF*, *HMGAI*, *GPSM2*, *H2AFX*, *POLD1*, *HOXB13*, *HDAC4*, *SPDYA*.

Discussion

In the present study, we found relevant lower expression of miR-198 in HCC, as compared to that in their para-noncancerous liver tissues. Downregulated miR-198 level was closely related to the disease progression of HCC. Our experiment showed that miR-198 had a close relationship with differentiation, tumor nodes, metastasis, clinical TNM stage, vaso-invasion, tumor capsular infiltration, and HCV infection. And these clinicopathological features could reflect a trend of poor prognosis. We found that the expression of miR-198 was not obviously changed in precancerous lesions of HCC, once liver tissue turned into cancerous tissue, the expression level of miR-198 was downregulated remarkably. Thus we deduce that miR-198 could have a key role in the late stage of tumorigenesis of HCC, not the period of development from normal liver to cirrhosis. Furthermore, we also performed in silico prediction for the prospective targets of miR-198 in HCC and found that miR-198 was involved in a few key pathways related to the tumorigenesis and progression of HCC. As for tissue origin, it could have been better

Table 3 GO functional annotation for most significantly related targets of miR-198

GO ID	GO term	Count (%)	P-value	Gene symbol
Biological process				
GO:0007167	Enzyme-linked receptor protein signaling pathway	20 (1.3)	1.82E-10	MTSSI, MPZLI, FUT8, NLK, CBL, MET, SMAD2, PTEN, SRC, etc
GO:0007169	Transmembrane receptor protein tyrosine kinase signaling pathway	15 (1.0)	1.23E-08	MTSSI, MPZLI, CBL, MET, PTEN, SRC, NTRK3, DDR1, CTGF, etc
GO:0006928	Cell motion	20 (1.3)	4.06E-08	PVR, MTSSI, TLN1, FUT8, FOXJ1, MET, ZEB2, PTEN, SRC, etc
GO:0016477	Cell migration	14 (0.9)	1.10E-06	PVR, FUT8, FOXJ1, MET, ZEB2, PTEN, SRC, ULK1, ITGA5, etc
GO:0042127	Regulation of cell proliferation	23 (1.5)	1.59E-06	CTBP2, FOXJ1, CSF1, TNFRSF8, CDK6, SMAD2, BCL2L1, CDK4, etc
GO:0051674	Localization of cell	14 (0.9)	3.59E-06	PVR, FUT8, FOXJ1, MET, ZEB2, PTEN, SRC, ULK1, ITGA5, etc
GO:0048870	Cell motility	14 (0.9)	3.59E-06	PVR, FUT8, FOXJ1, MET, ZEB2, PTEN, SRC, ULK1, ITGA5, etc
GO:0042325	Regulation of phosphorylation	17 (1.1)	3.90E-06	CSF1, MET, ZEB2, PTEN, SPDYA, PPP1R9B, SPRY2, YWHAG, etc
GO:0051174	Regulation of phosphorus metabolic process	17 (1.1)	6.48E-06	CSF1, MET, ZEB2, PTEN, SPDYA, PPP1R9B, SPRY2, YWHAG, etc
GO:0019220	Regulation of phosphate metabolic process	17 (1.1)	6.48E-06	CSF1, MET, ZEB2, PTEN, SPDYA, PPP1R9B, SPRY2, YWHAG, etc
Cellular component				
GO:0043233	Organelle lumen	34 (2.2)	5.11E-06	POLR2E, HOXB13, DGCR8, GPM2, CD4, H2AFX, OGT, THBS1, etc
GO:0031974	Membrane-enclosed lumen	34 (2.2)	7.82E-06	POLR2E, HOXB13, DGCR8, GPM2, CD4, H2AFX, OGT, THBS1, etc
GO:0031981	Nuclear lumen	29 (1.9)	1.07E-05	POLR2E, HOXB13, DGCR8, GPM2, H2AFX, OGT, MYB, NOVA1, etc
GO:0005829	Cytosol	26 (1.7)	5.67E-05	TLN1, INPPL1, CBL, CYCS, BIRC5, SMAD2, BCL2L1, ARHGEF9, etc
GO:0070013	Intracellular organelle lumen	31 (2.0)	6.56E-05	POLR2E, HOXB13, DGCR8, GPM2, CD4, H2AFX, OGT, MYB, etc
GO:0001726	Ruffle	6 (0.4)	2.19E-04	MTSSI, TLN1, TLN2, ITGA5, ARHGAP1, CDK6
GO:0005886	Plasma membrane	49 (3.1)	3.18E-04	PVR, HDLBP, TLN1, MPZLI, MSRI, TLN2, CSF1, HFE, TNFRSF8, etc
GO:0005654	Nucleoplasm	18 (1.2)	8.06E-04	CTBP2, ADARBI, POLR2E, HOXB13, SMAD2, DACH1, RAD52, CDK4, etc
GO:0005901	Caveola	5 (0.3)	8.60E-04	CBL, SLC2A1, CACNA1C, SRC, BMPRI A
GO:0031252	Cell leading edge	7 (0.4)	9.53E-04	MTSSI, TLN1, TLN2, ITGA5, ARHGAP1, CDK6, PAFAH1B1
Molecular function				
GO:0019899	Enzyme binding	21 (1.3)	1.55E-08	NLK, BIRC5, SMAD2, HMGAI, SPDYA, HDAC4, PPP1R9B, etc
GO:0019904	Protein domain-specific binding	13 (0.8)	2.54E-05	PPARA, MTSSI, TLN1, YWHAG, FUT8, VCP, INPPL1, NLK, etc
GO:0004672	Protein kinase activity	16 (1.0)	1.94E-04	PAK6, NTRK3, DDR1, POLR2E, ULK1, NLK, MET, NEK8, etc
GO:0017124	SH3 domain binding	6 (0.4)	1.38E-03	MTSSI, FUT8, INPPL1, CBL, ARHGAP1, ADAM12
GO:0004713	Protein tyrosine kinase activity	7 (0.4)	2.99E-03	NTRK3, DDR1, MET, TNK2, MST1R, MAP2K7, SRC
GO:0019900	Kinase binding	7 (0.4)	4.33E-03	SPDYA, YWHAG, CDKN2B, CCND2, SLC2A1, CD4, MAP2K7
GO:0032403	Protein complex binding	7 (0.4)	6.70E-03	ULK1, ITGA5, CTGF, SPTBN1, PTPN1, THBS1, FRS2
GO:0004857	Enzyme inhibitor activity	8 (0.5)	8.43E-03	PPP1R9B, YWHAG, CDKN2B, SERPINF2, SERPINA5, BIRC5, BCL2L1, TNK2
GO:0019901	Protein kinase binding	6 (0.4)	8.53E-03	SPDYA, YWHAG, CDKN2B, CCND2, CD4, MAP2K7

Abbreviations: GO, Gene Ontology; miR-198, microRNA-198.

Table 4 KEGG pathway enrichment analysis of miR-198 target genes

KEGG ID	KEGG term	Count (%)	P-value	Gene symbol
hsa04510	Focal adhesion	12 (0.8)	5.93E-05	SPDYA, PAK6, TLN1, CCND2, TLN2, ITGA5, MET, EGF, CAPN2, etc
hsa05200	Pathways in cancer	14 (0.9)	3.25E-04	CTBP2, CBL, MET, CYCS, CDK6, BIRC5, SMAD2, BCL2L1, CDK4, etc
hsa04115	p53 signaling pathway	6 (0.4)	2.00E-03	CCND2, CYCS, CDK6, CDK4, THBS1, PTEN
hsa04520	Adherens junction	6 (0.4)	3.46E-03	NLK, MET, PVRL2, SMAD2, PTPN1, SRC
hsa05222	Small cell lung cancer	6 (0.4)	5.03E-03	CDKN2B, CYCS, CDK6, BCL2L1, CDK4, PTEN
hsa05218	Melanoma	5 (0.3)	1.45E-02	MET, CDK6, EGF, CDK4, PTEN
hsa05212	Pancreatic cancer	5 (0.3)	1.52E-02	CDK6, SMAD2, BCL2L1, EGF, CDK4
hsa05220	Chronic myeloid leukemia	5 (0.3)	1.75E-02	CTBP2, CBL, CDK6, BCL2L1, CDK4
hsa04110	Cell cycle	6 (0.4)	2.52E-02	YWHAG, CDKN2B, CCND2, CDK6, SMAD2, CDK4
hsa04350	TGF-beta signaling pathway	5 (0.3)	2.84E-02	CDKN2B, SPI, SMAD2, THBS1, BMPRI A
hsa04012	ErbB signaling pathway	5 (0.3)	2.84E-02	PAK6, CBL, EGF, MAP2K7, SRC
hsa04514	CAMs	6 (0.4)	3.09E-02	PVR, MPZLI, PVRL2, CNTN2, CD4, PDCD1LG2

Abbreviations: CAMs, cell adhesion molecules; KEGG, Kyoto Encyclopedia of Genes and Genomes; miR-198, microRNA-198.

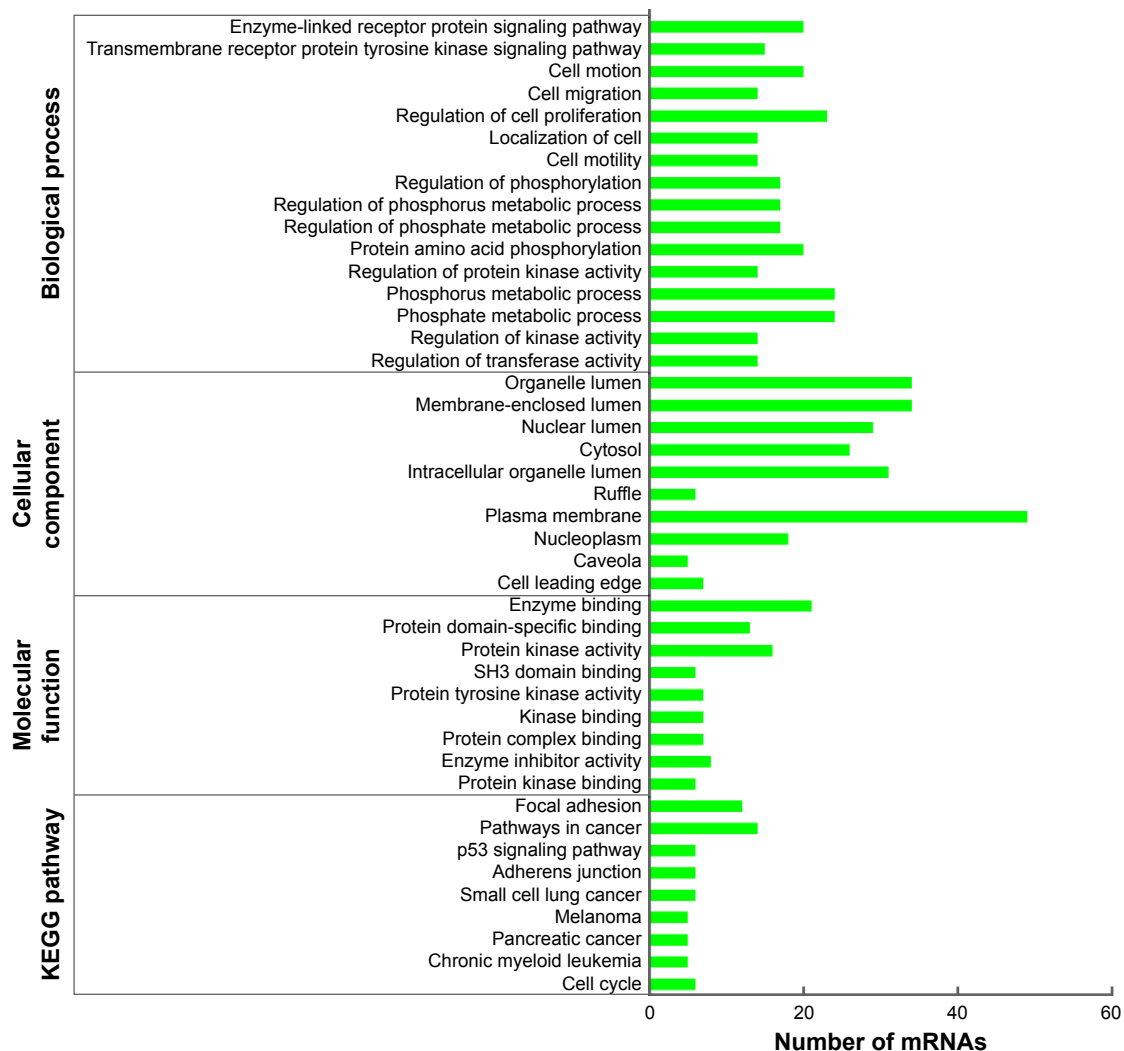


Figure 6 The significantly enriched annotation of GO categories and KEGG pathway of the potential mRNAs targeted by miR-198 in HCC.

Abbreviations: GO, Gene Ontology; HCC, hepatocellular carcinoma; KEGG, Kyoto Encyclopedia of Genes and Genomes; mRNA, messenger RNA; miR-198, microRNA-198.

if both normal liver tissues and adjacent noncancerous tissues had been collected in this study. Despite the adjacent noncancerous tissue from the same HCC patient, the absence of normal liver tissues is a limitation in our study.

Besides HCC, recent studies have focused on the expression of miR-198 and its regulatory roles in other diseases. Up to now, lower expression of miR-198 has been reported in lung adenocarcinoma,⁴ pancreatic cancer,²⁰ and colorectal cancer.¹⁰ However, higher expressed miR-198 has been confirmed in other malignancies such as retinoblastoma,⁷ squamous cell carcinoma of tongue,⁶ esophageal cancer,⁵ pancreatic adenocarcinoma or ampullary adenocarcinoma,⁸ prostate cancer,¹¹ and multiple myeloma.⁹ Overexpression of miR-198 has also been found in noncancerous diseases such as severe preeclampsia,²¹ lupus nephritis,²² and human anencephaly.²³ In lung cancer, Yang et al reported that miR-198 could suppress proliferation and induce apoptosis by

targeting fibroblast growth factor receptor 1 in vitro,²⁴ while Wu et al validated that serine hydroxymethyltransferase 1, another target of miR-198, could exert similar functions in lung adenocarcinoma.²⁵ In colorectal cancer, Wang et al demonstrated that miR-198 targeted fucosyltransferase 8 to inhibit tumor growth and metastasis.¹⁰ And in pancreatic cancer, Marin-Muller et al identified miR-198 as a crucial tumor suppressor which linked a tumor network signature including mesothelin, OCT-2, pre-B-cell leukemia homeobox 1, and valosin containing protein and prognosticates poor patient outcome.²⁰ miR-198 could play versatile roles in different diseases via influencing various biological processes.

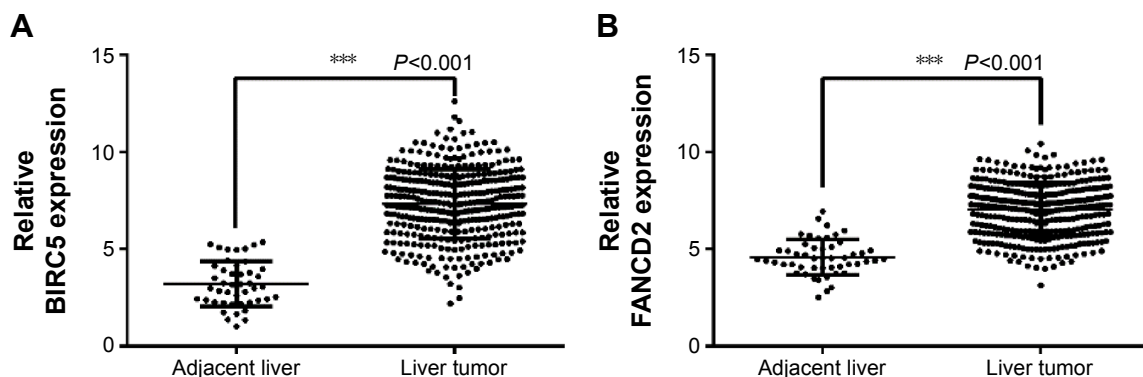
So far, several studies have been conducted on the roles of miR-198 in HCC. Varnholt et al first reported that miR-198 was downregulated in HCV-associated HCC compared to normal hepatic parenchyma in Caucasian patients,¹² but the study was confined to the limitation of racial difference.

Table 5 Expressions of 34 genes based on TCGA dataset (369 liver tumor tissues and 50 adjacent nontumor tissues)

Gene name	Liver tumor (Mean \pm SD)	Adjacent liver (Mean \pm SD)	t	P-value	FC
BIRC5	7.34 \pm 1.78	2.89 \pm 1.47	16.87	4.77E-49	4.45
FANCD2	7.05 \pm 1.38	4.59 \pm 0.91	16.67	3.16E-28	2.46
EGF	1.91 \pm 2.84	0.28 \pm 0.65	9.34	1.45E-18	1.63
HMGA1	10.53 \pm 1.20	8.98 \pm 0.71	13.15	6.28E-23	1.55
GPSM2	7.06 \pm 1.09	5.58 \pm 0.63	14.08	5.19E-25	1.48
H2AFX	9.34 \pm 1.00	8.06 \pm 0.49	14.84	2.08E-28	1.29
POLD1	9.19 \pm 0.79	7.92 \pm 0.43	17.33	4.57E-32	1.27
HOXB13	1.31 \pm 2.46	0.05 \pm 0.51	8.61	2.30E-16	1.27
HDAC4	7.85 \pm 0.78	6.81 \pm 0.56	11.60	1.39E-18	1.04
SPDYA	3.09 \pm 1.03	2.06 \pm 0.90	6.73	5.75E-11	1.03
SETDB1	9.90 \pm 0.59	8.93 \pm 0.30	18.55	1.12E-35	0.97
MYB	2.97 \pm 1.81	2.04 \pm 1.54	3.91	2.17E-04	0.93
CDK4	10.96 \pm 0.69	10.27 \pm 0.25	13.79	5.06E-30	0.69
OGT	10.80 \pm 0.63	10.13 \pm 0.43	9.63	4.99E-15	0.67
NCOR2	11.01 \pm 0.61	10.45 \pm 0.37	9.28	8.65E-15	0.57
MLL2	10.06 \pm 0.74	9.53 \pm 0.57	5.92	9.52E-08	0.53
RAD52	6.46 \pm 0.71	6.00 \pm 0.59	4.37	1.58E-05	0.46
SMAD2	10.09 \pm 0.47	9.74 \pm 0.22	8.63	3.09E-14	0.35
CYCS	11.63 \pm 0.73	11.33 \pm 0.37	4.52	1.61E-05	0.29
NLK	8.92 \pm 0.46	8.67 \pm 0.29	5.26	1.08E-06	0.25
VCP	12.96 \pm 0.40	12.76 \pm 0.23	5.16	1.44E-06	0.20
DGCR8	9.23 \pm 0.44	9.04 \pm 0.32	3.65	4.70E-04	0.18
SPI	10.63 \pm 0.44	10.57 \pm 0.27	1.36	1.78E-01	0.06
POLR2E	12.08 \pm 0.54	12.16 \pm 0.39	-1.28	2.05E-01	-0.08
PPPIR9B	9.60 \pm 0.86	9.70 \pm 0.48	-1.24	2.18E-01	-0.10
ADARBI	7.93 \pm 0.79	8.04 \pm 0.35	-1.56	1.20E-01	-0.10
NOVA1	3.59 \pm 2.45	3.77 \pm 0.79	-1.08	2.82E-01	-0.18
SPTBN1	13.44 \pm 0.66	13.66 \pm 0.46	-2.97	3.97E-03	-0.22
CTBP2	7.45 \pm 1.27	7.82 \pm 0.47	-3.88	1.50E-04	-0.36
CBS	11.90 \pm 1.64	13.49 \pm 0.42	-15.18	9.33E-39	-1.58
SERPINF2	14.40 \pm 1.82	16.14 \pm 0.35	-16.27	1.39E-45	-1.74
THBS1	10.55 \pm 1.75	12.68 \pm 1.11	-11.75	1.33E-19	-2.13
CD4	9.89 \pm 1.23	12.06 \pm 0.70	-18.55	1.13E-33	-2.18
DACH1	2.64 \pm 1.61	6.01 \pm 0.65	-27.11	9.98E-60	-3.37

Abbreviations: FC, fold change; SD, standard deviation; TCGA, The Cancer Genome Atlas.

on the function based on in silico methods. Although some studies have reported the method of miRNA targets prediction through two or three public databases online; however, from the perspective of artificial prediction, there still can be some inevitable insufficiencies in the single computational algorithm. Therefore, in this study, we used 14 computational algorithms online to predict the putative targets of miR-198, and screened the possible targets in a high probability. Moreover, to achieve potential target mRNAs especially expressed in HCC, we further narrowed down the list by performing NLP analysis in HCC. Having identified 127 mRNAs as potential targets for miR-198, we performed GO enrichment analysis and KEGG pathway annotation to further explore the regulatory function of these mRNAs in HCC. Significant results were adopted according to the *P*-value provided by DAVID.²⁰ We observed that these mRNAs were most enriched in organelle lumen, membrane-enclosed lumen, nuclear lumen, and plasma membrane, which indicated a potential membrane-associated metabolism regulated by these target genes. Besides, for biological process, we found these targets were significantly involved in regulation of cell proliferation, cell migration, cell motion, cell motility, and localization of cell, which may contribute to the clinicopathological manifestation of multiple tumor nodes, vaso-invasion, metastasis, and capsular formation, respectively. As for molecular function, the selected mRNAs were significantly involved in enzyme binding, protein domain-specific binding, and protein kinase activity, which showed high tendency of cellular invasion. Furthermore, in KEGG pathway analysis, we found that the targeted mRNAs were most associated with focal adhesion, pathways in cancer, p53 signaling pathway, and adheres junction, which indicated that the potential miR-198 targets may participate in tumor-related intercellular signaling molecules exchange

**Figure 8** (Continued)

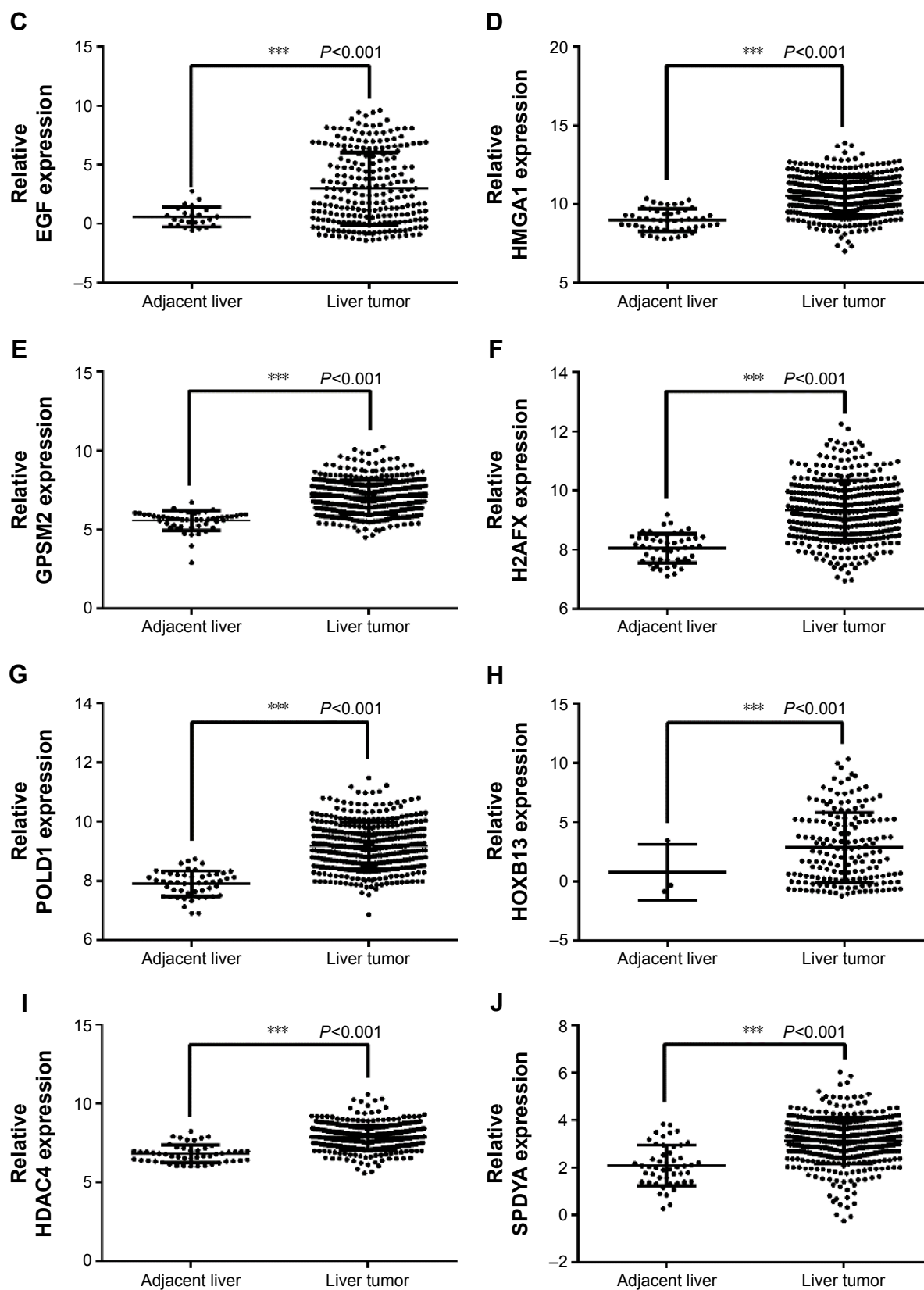


Figure 8 Expressions of the top ten genes with most significant fold changes from organelle lumen pathway in liver tumor based on TCGA dataset.

Notes: The TCGA RNAseq profiles of LIHC were extracted to explore the relative expressions of ten selected genes from organelle lumen pathway in liver tumor tissue and adjacent liver tissue (on a log₂ scale). *** $P < 0.001$. (A) BIRC5, (B) FANCD2, (C) EGF, (D) HMGA1, (E) GPM2, (F) H2AFX, (G) POLD1, (H) HOXB13, (I) HDAC4, (J) SPDYA.

Abbreviations: RNAseq, RNA sequencing; TCGA, The Cancer Genome Atlas; LIHC, Liver Hepatocellular Carcinoma.

and signal transduction, thus contributing to the stepwise development of tumorigenesis in HCC.

To better explore the possibility of the target candidates from simple prediction, we further inspected liver data on TCGA dataset and confirmed the overexpression of 23 of 34 genes described in the organelle lumen (GO: 0043233), which was the most significant GO term for cellular component. Especially, ten genes were dramatically upregulated in liver tumor tissues, giving a FC of >1. These genes may have more possibility to be the real targets of miR-198 in HCC due to their noteworthy overexpression in cancer tissues. Of course, the results need to be further confirmed with other experiments.

Conclusion

In this study, we confirmed the lower expression of miR-198 in HCC by using RT-qPCR on paraffin-embedded tissues; potential targets and pathways of miR-198 were achieved through bioinformatics methods, which further clarified the potential role of miR-198 in the processes of hepatocellular carcinogenesis. Additionally, we provided guidelines for clinical reference and scientific research in the future. In conclusion, miR-198 might play essential regulatory roles via different target genes and relevant pathways in the development of HCC. Further investigation of the molecular mechanisms of miR-198 is needed to be explored in malignant phenotype of HCC.

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Disclosure

The author reports no conflicts of interest in this work.

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