Promising significance of the association of miR-204-5p expression with clinicopathological features of hepatocellular carcinoma

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Abstract

Decreased level of miR-204-5p has been documented in various malignancies. However, the expression and clinical significance of miR-204-5p in hepatocellular carcinoma has not been investigated. The aim of this study is to examine the relationship between miR-204-5p expression and clinicopathological features in hepatocellular carcinoma (HCC) as well as to predict the relevant signaling pathways. The miR-204-5p expression level was detected in HCC and in matched paraneoplastic liver from 95 formalin-fixed paraffin-embedded tissues by the real-time reverse transcription polymerized chain reaction (qRT-PCR). The association of miR-204-5p expression with clinicopathological features as well as the prognosis of HCC was examined. Public data portals including the Gene Expression Omnibus and The Cancer Genome Atlas were used to retrieve the HCC-related data in order to perform a comprehensive meta-analysis. Meanwhile, protein–protein interaction (PPI) and enrichment analyses were performed using predicted target genes. The relative expression of miR-204-5p was remarkably reduced in HCC than that in paraneoplastic hepatic tissues. In HCC, the miR-204-5p expression was downregulated in the metastasis, vasoinvasion, and advanced stage (III and IV) subgroups compared with their counterparts. Furthermore, the meta-analysis based on qRT-PCR data demonstrated that miR-204-5p was markedly downregulated in HCC with a standardized mean difference of −5.19 ($P < .001$). However, no significant association was observed between miR-204-5p and survival outcomes. The potential target genes of miR-204-5p were significantly enriched in several pathways which might be associated with HCC, such as "cell proliferation" from GO terms and "pathways in cancer" from the KEGG analysis. A PPI network of miR-204-5p potential target genes identified prospective core genes potentially involved in the regulation of HCC oncogenesis and progression. Our findings suggested that miR-204-5p might act as a tumor-suppressive gene in the tumorigenesis and progression of HCC via vital signaling pathways and that miR-204-5p could be regarded as a protective factor in HCC.

Abbreviations: AUC = area under the curve, CI = confidence interval, DEGs = differential expression genes, FFPE = formalin-fixed paraffin-embedded, GEO = Gene Expression Omnibus, GO = Gene Ontology, HBV = hepatitis B virus, HCC = hepatocellular carcinoma, KEGG = Kyoto Encyclopedia of Genes and Genomes, NLP = natural language processing, OS = overall survival, PPI = protein–protein interaction, RFS = relapse-free survival, ROC = receiver operating characteristic, SMD = standardized mean difference, TCGA = The Cancer Genome Atlas.

Keywords: GEO, hepatocellular carcinoma, miR-204-5p, pathway analysis, qRT-PCR, TCGA

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1. Introduction

Hepatocellular carcinoma (HCC) ranks the sixth as the most frequent cancers and is the third most common cause of cancer-related mortalities in the world.[1] The most common risk factor for HCC is chronic hepatitis B virus (HBV) infection, which causes over 50% of all cases. The relative risk of tumor development is higher among HBV-carriers than noncarriers, and HBV-carriers with cirrhosis share an even higher risk.[2] Hence, it is urgently demanded to seek effective biomarkers which are significantly associated with the pathological characteristics and patients' survival outcomes. However, the potential tissue-based biomarkers for the diagnosis and prognosis remain obscure and need further characterization.[3]

Recent advances in genomics, proteomics, and metabolomics technologies have led to the discovery of novel biomarkers in HCC. MicroRNAs are considered to be ideal biomarkers because they are easy to detect, stable, and are strongly related to clinical outcomes compared with other biomarkers such as genetic and epigenetic alterations, posttranslational protein modifications, and metabolites.[4] microRNAs are a variety of small noncoding RNAs that are involved in multiple oncological processes. miR-204-5p is a type of microRNA, and recent studies have reported that miR-204-5p was decreased in tumors and may serve as a prospective tumor suppressor in several types of malignancies, such as head and neck squamous cell carcinoma,[5] colorectal cancer,[6,7] and acute myeloid leukemia,[8] among others. In regards to the correlation between miR-204-5p and the occurrence and progression of HCC, only few papers have been published on this topic.[9–13] The sample sizes of single studies were small, and thus, the clinical value of miR-204-5p has not yet been confirmed in HCC. Moreover, the potential molecular implication and mechanism of miR-204-5p in HCC remain largely unclear.

Thus, in order to comprehensively explore the significance of miR-204-5p in HCC, the expression pattern of miR-204-5p was detected using real-time reverse transcription polymerized chain reaction (qRT-PCR), which was followed by a meta-analysis featuring the combination of the literature and Gene Expression Omnibus (GEO) microarray data. Prediction of potential miR-204-5p target genes was made using 9 online solutions, and they were filtered using natural language processing (NLP) and differential expression genes (DEGs) in HCC based on The Cancer Genome Atlas (TCGA) data. Lastly, a protein–protein interaction (PPI) network was generated, and terms of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichments were evaluated to understand the potential signaling pathways mediated by miR-204-5p in HCC.

2. Materials and methods

2.1. Tissue samples

The samples used in this study contained a total of 95 tissues collected at the First Affiliated Hospital of Guangxi Medical University from March 2010 to December 2011; these samples comprised HCC tissues and matched formalin-fixed paraffin-embedded (FFPE) paraneoplastic hepatic tissues. All the patients underwent primary hepatectomies with no prior treatment and were selected on a random basis. Tumor sizes varied between 1 and 11 cm with the mean size of 6.4 cm. The mean ages of the HCC patients ranged from 29 to 82 years (the mean age: 52 years). Written informed consents were acquired from all the participants for the scientific use of biological material. Ethical

approval was obtained for the retrospective investigation from the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University.

2.2. qRT-PCR for the determination of miR-204-5p expression

Total RNAs, with microRNA (miRNAs) included, were extracted from samples using the miRNeasy FFPE Kit (QIAGEN, the Netherlands) in accordance with the methods previously published.[14,15] We determined the RNA concentrations by NanoDrop 2000 spectrophotometer (Thermo Scientific, DE). Both RUN6B and RUN48 were used as housekeeping genes for the miR-204-5p detection. The primers for miR-204-5p, RNU6B, and RNU48 were added in the TaqMan MicroRNA assays. The miRNA sequences and references used are listed below: miR-204-5p: UUCCCUUU-GUCAUCCUAUGCCUG; RNU6B: CGCAAGGATGACACG-CAAATTCGTGAAGCGTTCCATATTTTT; RNU48: GATGA CCCCAGGTAACTCTGAGTGTGTCGCTGATGCCATCACC GCAGCGCTCTGACC. Reverse transcription included the use of reverse primers with a TaqMan MicroRNA Reverse Transcription Kit in the entire volume of 10 μL. qRT-PCR for miRNAs was conducted in 7900HT Fast Real-Time PCR System. The abundance of miR-204-5p in each sample was standardized to the expression of the reference genes. The formula $2^{-\Delta Cq}$ was employed for the calculation of miR-204-5p level in FFPE samples.

2.3. Analysis of GEO datasets and human TCGA data

We downloaded the original miRNA expression data from GEO (http://www.ncbi.nlm.nih.gov/geo/) on September 30, 2016. Data related to the following search keywords were obtained:[16] HCC, liver, hepatocellular, hepatic; malignan*, cancer, tumor, tumour, neoplas*, carcinoma; miRNAs and noncoding RNAs. Datasets were included if the following requirements were fulfilled: the samples in the test group and the control group were human HCC tissues and noncancerous hepatic tissues, respectively; both the test group and the control group contained more than 3 samples; and the expression profiling data of miRNAs were available or calculable. In the present study, noncancerous liver tissues included liver tissues adjacent to HCC and liver tissues from healthy donors. The following data were extracted by 3 authors independently (Y-HL, Y-WD, and M-HR): the expression values of miR-204-5p; the sample sizes of both the control group and the test group; the country and publication year of the microarray datasets; and the platform of the microarray datasets. To further explore the potential involvement of miR-204-5p, additional analyses were conducted with regard to the expression of miR-204-5p in accordance with the TCGA (http://cancergenome.nih.gov) RNAseq profiles.

2.4. Meta-analysis of the literatures and GEO microarrays

A comprehensive search was performed within the PubMed, ISI Web of Science, EMBASE, WanFang, and China National Knowledge Infrastructure databases according to the PRISMA guideline.[17] The search strategy and screening processes were consistent with the screening of the datasets. The normalized miRNAs expression data matrixes were obtained from GEO database. Subsequently, a second median-normalization was performed for miR-204-5p expression data to eliminate the heterogeneity across GEO datasets. The standardized mean difference (SMD) with its 95% confidence interval (CI) was

evaluated from all the data in the literature, the GEO microarray expression data and TCGA miRNA-seq data. For studies that detected the miR-204-5p level in HCC and in noncancerous tissues, but failed to estimate the SMD values and their 95% confidence intervals (CIs), we made our best effort to contact the authors in order to obtain the raw data. Finally, a microarray data-based meta-analysis was conducted and a random effect model was used in order to account for interstudy heterogeneity. A sensitivity analysis was performed to detect the stability of the included studies, and Begg test was conducted to examine potential publication bias.[18] For the meta-analysis, Stata Statistical software version 13.0 (StataCorp, College Station, TX) was adopted for the evaluation.

2.5. Prediction of target genes and visualization of the PPI network

Prediction of potential miR-204-5p target genes was performed using the following software solutions: TargetScan, miRDB, DIANA-mT, RNAhybrid, miRanda, PITA, RNA22, PICTAR5, and miRWalk.[19] The genes that were retrieved by 6 or more online software programs were considered potential target genes of miR-204-5p. Moreover, the gene expression data on HCC from TCGA was downloaded, and significant DEGs were further screened. An NLP analysis of liver cancer was performed according to the method used in our previous reports.[20] Finally, the intersection of the candidate genes mentioned above was further analyzed. To further explore the interactions among these genes, a PPI network was constructed within the STRING (http://string-db.org/) Interacting Genes/Proteins Database.[21]

2.6. Functional and pathway enrichment analysis

To understand the underlying functions of miR-204-5p in the tumorigenesis of HCC, GO enrichment, and KEGG pathway analyses were conducted using the KOBAS 3.0 (http://kobas.cbi.pku.edu.cn/) amongst selected genes.[22] For the GO analysis, the most significant 20 functions were selected. For the KEGG pathway analysis, the pathways with a corrected P-value $<.001$ were considered statistically significant.

2.7. Statistical analysis

All the statistical analyses of this study were carried out with SPSS 22.0. Paired and unpaired Student's t tests were applied to assess the significance between paired and unpaired groups, respectively. A 1-way analysis of variance test was taken to evaluate the significance among groups of different variations. We presented the values in form of the mean $\pm$ the standard deviation (SD). Survival curve and log-rank test were performed to investigate the prognostic power of miR-204-5p in HCC. We drew the receiver operating characteristic (ROC) curve to reveal the diagnostic ability of miR-204-5p in HCC. It was regarded to be statistically significant when a 2-tailed P-value was $<.05$.

3. Results

3.1. Decreased miR-204-5p expression in HCC tissues and its correlation with clinicopathological features

As shown in Table 1, the miR-204-5p expression in HCC tissues was pronouncedly downregulated as compared to that in

Table 1

Relationship between miR-204-5p expression and clinicopathological features of HCC.

Clinicopathological features		N (patients)	miRNA-204 relevant expression ($2^{-\Delta Cq}$)		
			Mean $\pm$ SD	t	P
Tissue	Adjacent noncancerous liver	95	5.5429 $\pm$ 2.4099	−4.242	<.001
	HCC	95	4.1853 $\pm$ 1.9806		
Age, years	<50	49	4.2367 $\pm$ 2.0207	−0.260	.795
	≥50	46	4.1304 $\pm$ 1.9578		
Gender	Male	75	4.1760 $\pm$ 1.9792	−0.088	.930
	Female	20	4.2200 $\pm$ 2.0369		
Differentiation	Well	6	3.2167 $\pm$ 0.4355	F=0.780	.461*
	Moderate	60	4.2783 $\pm$ 2.1128		
	Poor	29	4.1931 $\pm$ 1.8735		
Size, cm	<5	77	4.0818 $\pm$ 1.9389	−1.054	.295
	≥5	18	4.6278 $\pm$ 2.1513		
Tumor nodes	Single	52	4.1846 $\pm$ 2.0162	−0.003	.997
	Multiple	43	4.1860 $\pm$ 1.9605		
Metastasis	−	49	4.7500 $\pm$ 2.0280	2.788	.006
	+	46	3.6551 $\pm$ 1.7983		
Clinical TNM stage	I–II	22	5.4227 $\pm$ 2.4180	2.920	.007
	III–IV	73	3.8123 $\pm$ 1.6755		
Portal vein tumor embolus	−	63	4.3984 $\pm$ 2.0696	1.481	.142
	+	32	3.7656 $\pm$ 1.7477		
Vasoinvasion	−	59	4.5186 $\pm$ 2.0659	2.140	.035
	+	36	3.6389 $\pm$ 1.7228		
Tumor capsular infiltration	With complete capsule	45	4.2733 $\pm$ 1.9767	0.409	.683
	Infiltration or not capsule	50	4.1060 $\pm$ 2.0008		
AFP	−	41	4.3146 $\pm$ 2.0957	0.008	.994
	+	38	4.3184 $\pm$ 2.0783		
Cirrhosis	−	45	4.2644 $\pm$ 1.9722	0.368	.714
	+	50	4.1140 $\pm$ 2.0054		

AFP = alpha fetal protein, HCC = hepatocellular carcinoma, miRNA = microRNA, SD = standard deviation.

* A 1-way analysis of variance test.
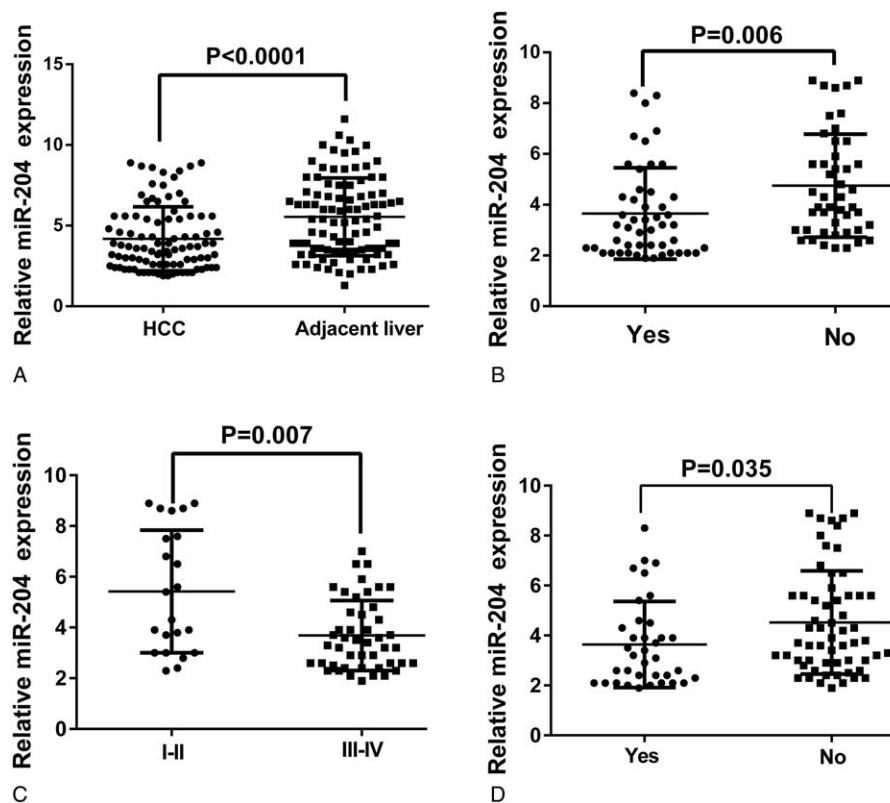
Figure 1. The association of miR-204-5p expression with clinicopathological features by qRT-PCR. (A) Tissue; (B) metastasis; (C) clinical TNM stage; (D) vasoinvasion. qRT-PCR = real-time reverse transcription polymerized chain reaction.

paraneoplastic hepatic tissues (P<.001) (Fig. 1A). And the miR-204-5p expression in individual HCC tissues and controls by qRT-PCR is shown in Table S1, http://links.lww.com/MD/B813. For the clinical TNM stage, miR-204-5p was clearly expressed at a lower level in advanced stages (III and VI) compared with the early stages (I and II) (P=.007) (Fig. 1C). In addition, we demonstrated that the expression of miR-204-5p was predominantly reduced in HCC with metastasis (P=.006) (Fig. 1B) and vasoinvasion (P=.035) (Fig. 1D). Moreover, our results showed that miR-204-5p was not associated with age, gender, differentiation, size, portal vein tumor embolus, tumor nodes, tumor capsular infiltration, alpha fetal protein (AFP) level, or cirrhosis (Table 1). To explore the diagnostic value of miR-204-5p, ROC analysis was conducted and the area under the curve (AUC) of ROC curve was calculated. The AUC of miR-204-5p expression in the diagnosis of HCC was 0.671 (95% CI: 0.595–0.747, P<.001) (Fig. 2A). The cut-off value for miRNA-204-5p was 5.75. In relation to the clinical TNM stage of HCC, the AUC of the downregulated expression of miR-204-5p was 0.705 (95% CI: 0.579–0.831, P=.004) (Fig. 2B). The cut-off value for miRNA-204-5p was 3.65. For metastasis and vasoinvasion, the AUC values of miR-204-5p in HCC were 0.689 (95% CI: 0.583–0.794, P=.002) (Fig. 2C) and 0.639 (95% CI: 0.523–0.755, P=.023) (Fig. 2D). The cut-off values were 2.45 and 2.70, respectively.

3.2. Survival analysis of miR-204-5p in HCC

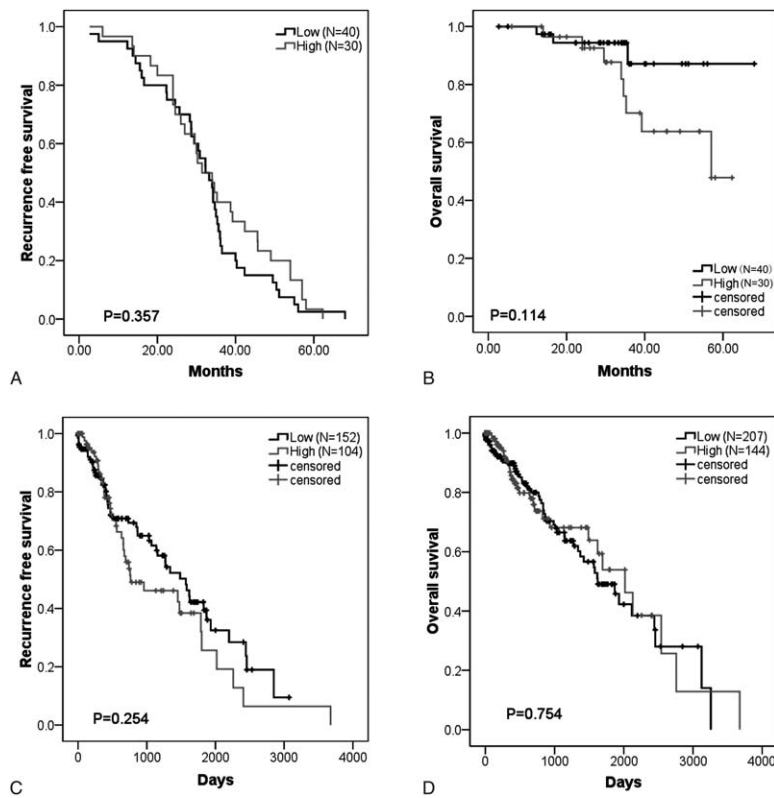To study the correlation of miR-204-5p expression with survival outcomes, patients were divided into either low-expression or high-expression group in accordance with the mean value of miR-204-5p expression. The survival analysis based on qRT-PCR data suggested no statistically significant difference between miR-204-5p expression and relapse-free survival (RFS) (P=.357, Fig. 3A) as well as overall survival (OS) (P=.114, Fig. 3B). Similarly, the results were also not statistically significance when we analyzed the association of miR-204-5p expression with RFS (P=.254, Fig. 3C) and OS (P=.754, Fig. 3D), which was based on TCGA data.

### 3.3. Validation of miR-204-5p expression based on TCGA data and meta-analysis

With respect to the TCGA data, we included a total of 353 HCC patients in the study. The expression of miR-204-5p in HCC tissues was decreased as compared to that in normal hepatic tissues (P<.001) (Fig. 4A). The AUC of the downregulated miR-204-5p expression in the diagnosis of HCC was 0.701 (Fig. 4B). The cut-off value for miRNA-204-5p was 6.31. For the literature and GEO datasets search, a total of 2 studies and 9 GEO datasets were included in our meta-analysis (Table 2). Considering the heterogeneity among studies, a random effect model was used for meta-analysis. The result of the meta-analysis based on literature with qRT-PCR data suggested that the relative expression of miR-204-5p was significantly lower in HCC tissues as compared to that in noncancerous liver tissues (SMD=−5.19, 95%CI: −6.69 to 3.69, P<.001) (Fig. 5B) with significant heterogeneity (I^2=85.8%, P=.008) observed. However, no significant difference was observed in the meta-analysis of microarrays (SMD= 0.096, 95%CI: −0.366 to 0.557, P=.685) (Fig. 5A). We did not test publication bias due to the scarce number of datasets (<10).
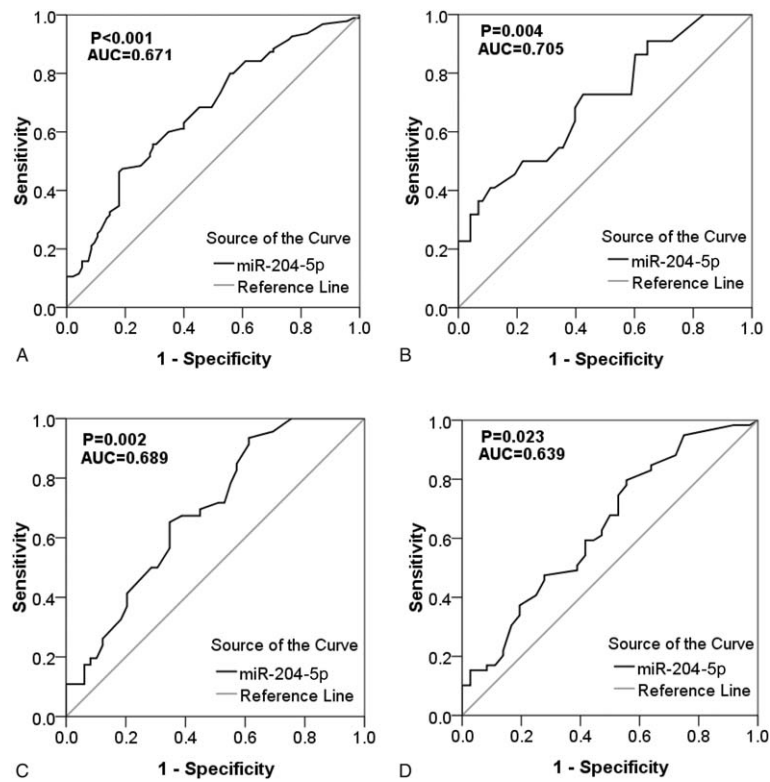
Figure 2. Diagnostic significance of relative miR-204-5p expression for the distinction of clinicopathological features in HCC by qRT-PCR. (A) Tissue; (B) clinical TNM stage; (C) metastasis; (D) vasoinvasion. qRT-PCR = real-time reverse transcription polymerized chain reaction.

Figure 3. The association of miR-204-5p expression with RFS and OS of HCC patients. (A) RFS of HCC patients according to TCGA data; (B) OS of HCC patients according to TCGA data; (C) RFS of 70 HCC patients in our study; (D) OS of 70 HCC patients in our study. HCC = hepatocellular carcinoma, OS = overall survival, RFS = relapse-free survival, ROC = receiver operating characteristic, TCGA = The Cancer Genome Atlas.
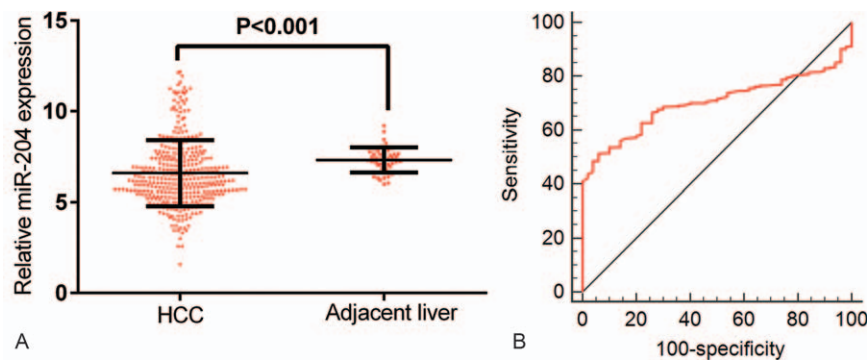
Figure 4. Diagnostic significance of relative miR-204-5p expression for the distinction of HCC and noncancerous liver tissue according to TCGA data. (A) Scatter diagram; (B) ROC curve. HCC = hepatocellular carcinoma, ROC = receiver operating characteristic, TCGA = The Cancer Genome Atlas.

3.4. Target genes of miR-204-5p and construction of the PPI network

The target genes were predicted using the miRWalk website online tool (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/), which summarized the results of multiple prediction software.[19] A total of 9987 genes were predicted via computational algorithms, of which 73 genes were predicted in 6 or more online platforms. With regard to the gene expression data from TCGA, 13,934 DEGs were screened. In addition, 1800 HCC-related genes were identified via NLP analysis, as mentioned in our previous study.[20,23] After examining the intersection of genes, 73 genes were finally selected for subsequent analysis. Subsequently, the selected 73 genes were imported into the STRING database and a graph of the intersections within the PPI network was constructed online to

determine the associations among the genes. The isolated nodes that were not in the network were removed for the lack of biological significance. As a result, 58 nodes and 126 edges participated in the PPI network (Fig. 6A) of the selected genes. Out of the genes with the highest degree of intersections in the PPI networks discussed above, the top 10 genes were identified as core genes (Fig. 6B).

### 3.5. Enrichment analyses of GO term annotations and KEGG pathways

Annotations of GO terms and KEGG pathways of the potential target genes of miR-204-5p in HCC were obtained by gene set enrichment analysis in KOBAS 3.0 (http://kobas.cbi.pku.edu.cn/).[22,24] Modified Fisher's exact test was employed to determine the P value. In relation to GO terms, the most significant 20 terms were chosen. The

Table 2

The diagnostic role of miR-204-5p expression in HCC.

Datasets	Year	Country	Platform/method	Tissue	N	Mean±SD
GSE57555	2014	Japan	GPL18044	HCC	5	2.2674±0.0370
				Noncancerous	16	2.2089±0.0890
GSE69580	2015	Taiwan	GPL10850	HCC	5	2.5530±1.2475
				Noncancerous	5	2.3554±0.8691
GSE54751	2014	USA	GPL18262	HCC	13	2.2496±0.0171
				Noncancerous	7	2.2618±0.0373
GSE41874	2012	Japan	GPL7722	HCC	6	2.3008±0.6608
				Noncancerous	4	2.4893±0.7941
GSE40744	2012	USA	GPL14613	HCC	39	2.2175±0.3031
				Noncancerous	18	2.2524±0.1818
GSE21362	2010	Japan	GPL10312	HCC	73	2.2832±0.7596
				Noncancerous	73	2.2026±0.3493
GSE22058	2010	USA	GPL10457	HCC	96	2.0278±1.8543
				Noncancerous	96	2.6057±0.7544
GSE12717	2008	USA	GPL7274	HCC	10	2.3154±0.5143
				Noncancerous	6	2.3166±0.1769
GSE6857	2007	USA	GPL4700	HCC	82	2.4239±0.2139
				Noncancerous	421	2.2472±0.1815
Ge et al[9]	2015	China	RT-PCR	HCC	48	6.6667±1.2821
				Noncancerous	48	13.5897±1.7948
Li et al[12]	2015	China	RT-PCR	HCC	60	2.6130±0.6790
				Noncancerous	60	8.9310±1.3350
Our data	–	China	RT-PCR	HCC	95	4.1853±1.9806
				Noncancerous	95	5.5429±2.4099
TCGA data	–	–	–	HCC	353	8.9535±0.6586
				Noncancerous	50	8.6283±0.6762

HCC = hepatocellular carcinoma, RT-PCR = real-time polymerized chain reaction, SD = standard deviation, TCGA = The Cancer Genome Atlas.
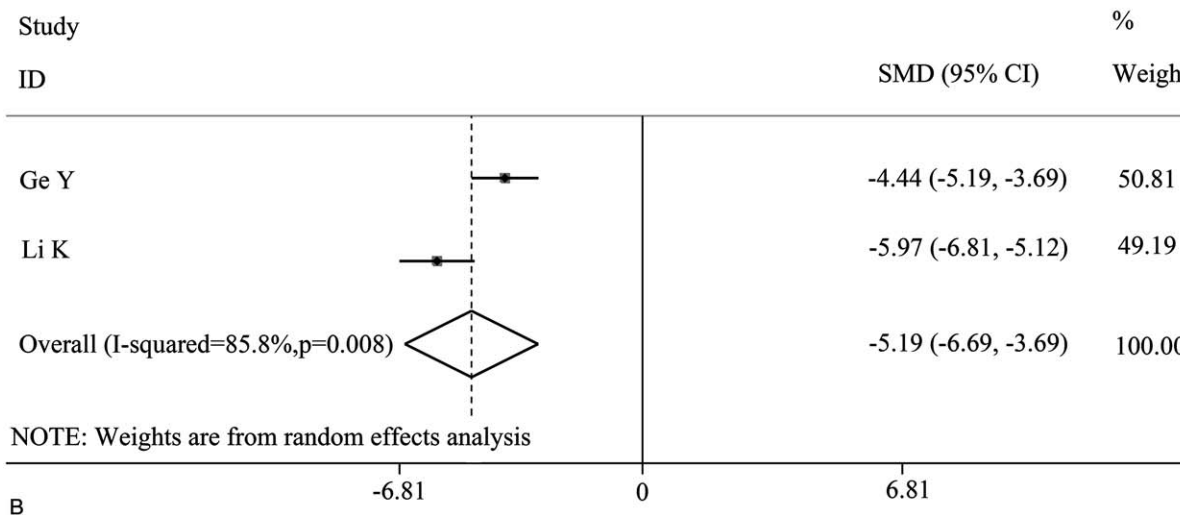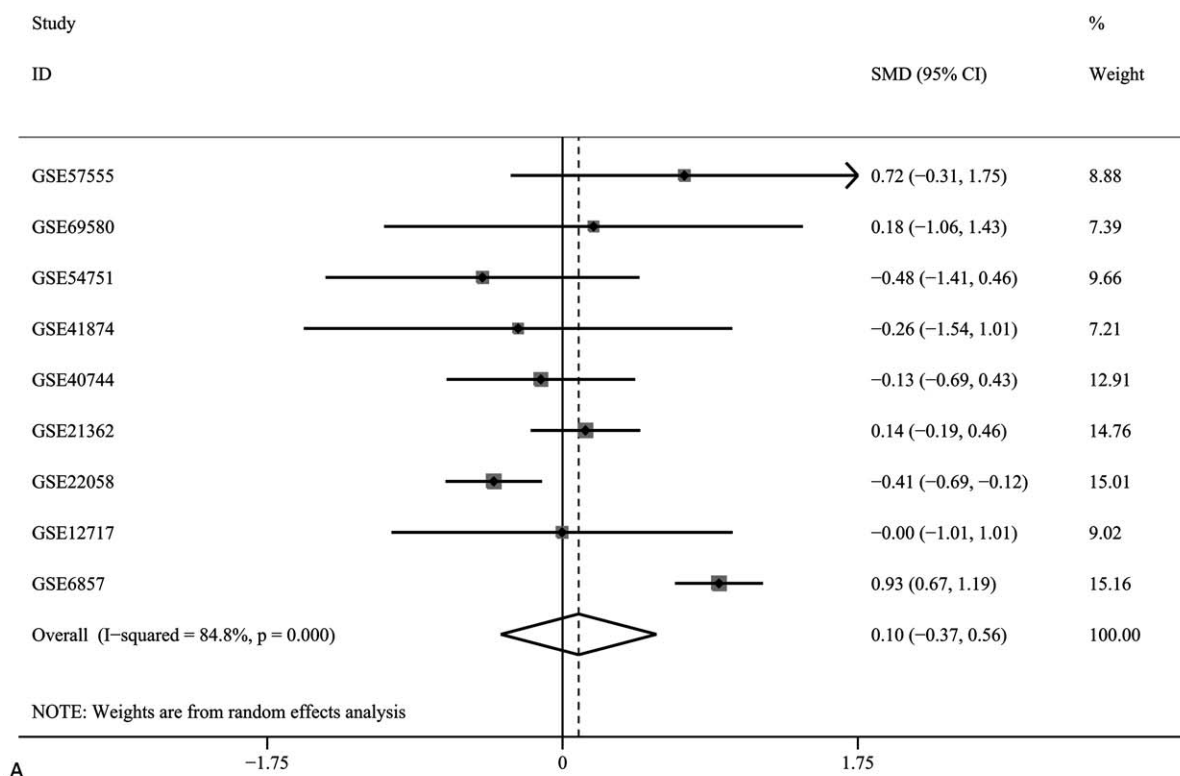
Figure 5. Forest plot for the diagnostic role of miR-204-5p expression in the distinction of HCC from noncancerous liver tissue. (A) Microarray; (B) qRT-PCR. HCC = hepatocellular carcinoma, qRT-PCR = real-time reverse transcription polymerized chain reaction.

potential targets of miR-204-5p showed significant involvement in cell proliferation, cell surface receptor signaling pathway, apoptotic process, and so on (Table 3). With respect to the KEGG pathway analysis, the potential targets of miR-204-5p were notably related to microRNAs in cancer, pathways in cancer, cell cycle, and so on (Table 4).

4. Discussion

Recently, several published studies[9,10,12,13] have shown that miR-204-5p expression was at lower levels in HCC compared with that in adjacent liver tissue and that it served as a contributor

to HCC metastasis; however, the sample size of a single study was limited. In our study, the miR-204-5p expression was detected by qRT-PCR in 95 HCC tissues and in corresponding normal liver tissues. In addition, further analysis based on TCGA data and a comprehensive meta-analysis was conducted to explore the diagnostic significance of miR-204-5p in HCC. The results of qRT-PCR and TCGA data indicated that the level of miR-204-5p expression was evidently lower in HCC tissue than that in adjacent hepatic tissue, which is consistent with results of previous studies. In terms of the meta-analysis, the pooled SMD of qRT-PCR demonstrated that the miR-204-5p expression was downregulated in HCC tissue as compared to noncancerous liver
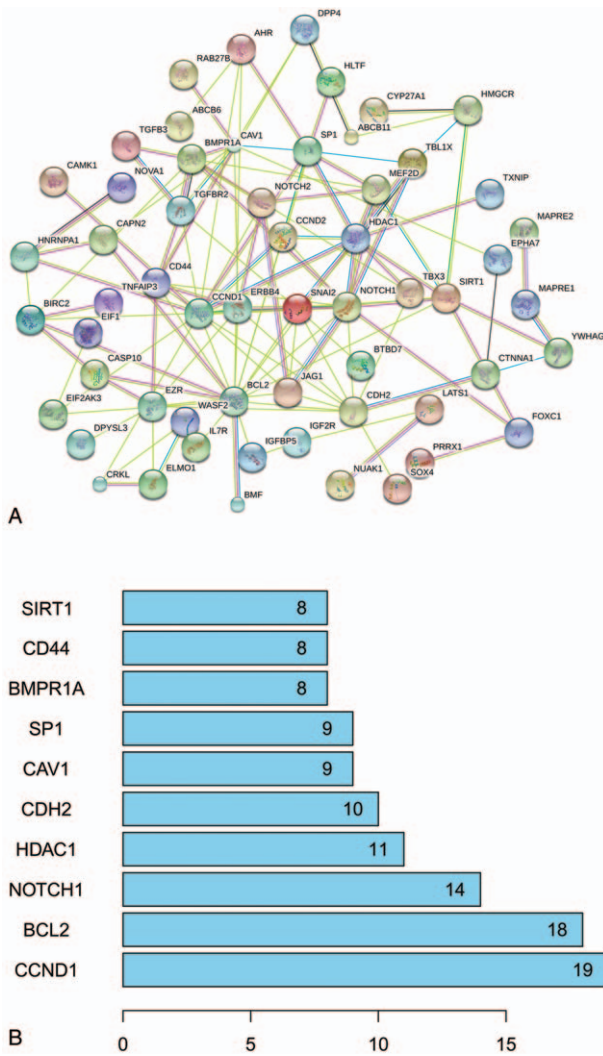
Figure 6. PPI network of genes. (A) Genes interaction; (B) the degree of core genes. PPI = protein–protein interaction.

KEGG analysis for the potential target genes of miR-204-5p in HCC (corrected $P < .001$).

ID	KEGG pathways	No. of targets	Corrected P
hsa05206	MicroRNAs in cancer	12	1.14E–09
hsa04390	Hippo signaling pathway	10	1.46E–09
hsa01522	Endocrine resistance	7	3.93E–07
hsa04510	Focal adhesion	7	2.63E–05
hsa04068	FoxO signaling pathway	6	3.20E–05
hsa05220	Chronic myeloid leukemia	5	3.37E–05
hsa05100	Bacterial invasion of epithelial cells	5	4.53E–05
hsa04330	Notch signaling pathway	4	1.17E–04
hsa05200	Pathways in cancer	8	1.52E–04
hsa05169	Epstein–Barr virus infection	6	2.12E–04
hsa04110	Cell cycle	5	2.36E–04
hsa05210	Colorectal cancer	4	2.94E–04
hsa05131	Shigellosis	4	3.24E–04
hsa05162	Measles	5	3.64E–04
hsa04210	Apoptosis	5	4.10E–04
hsa04520	Adherens junction	4	4.70E–04
hsa04630	JAK-STAT signaling pathway	5	6.47E–04
hsa04350	TGF-β signaling pathway	4	6.94E–04
hsa04144	Endocytosis	6	7.02E–04

HCC = hepatocellular carcinoma, KEGG = Kyoto Encyclopedia of Genes and Genomes, TGF-β = transforming growth factor-beta.

tissue, and significant heterogeneity was observed. Differences in the detection methods and reagents might be the main source of heterogeneity. However, a negative result was observed when the miR-204-5p expression data of microarrays were combined. As generally believed, the results of qRT-PCR are considered more credible and accurate as compared to microarrays. Specific signatures of miRNA expression in different types of cancer have been reported.[25] MiR-204-5p was found to be downregulated not only in HCC but also in colorectal cancer,[7] nasopharyngeal carcinoma,[26] non-small-cell lung carcinoma,[27] gastric tumors,[28] and breast cancer[29] among others. In light of the especial expression signature of miR-204-5p, its diagnostic value was further investigated. In our study, the AUC of miR-204-5p in the diagnosis of HCC was 0.671 and 0.701 according to our

GO enrichment analysis for the potential target genes of miR-204-5p in HCC (top 20 terms).

ID	GO terms	No. of targets	Corrected P
GO:0048522	Positive regulation of cellular process	52	3.88E–20
GO:0007275	Multicellular organism development	52	1.52E–19
GO:0048518	Positive regulation of biological process	53	1.75E–19
GO:0048731	System development	49	3.19E–19
GO:0048513	Animal organ development	43	1.06E–18
GO:0048856	Anatomical structure development	52	7.86E–18
GO:0044767	Single-organism developmental process	53	8.04E–18
GO:0032502	Developmental process	53	1.48E–17
GO:0044707	Single–multicellular organism process	53	4.20E–17
GO:0050794	Regulation of cellular process	65	5.10E–17
GO:0008283	Cell proliferation	34	6.99E–17
GO:0007166	Cell surface receptor signaling pathway	38	8.05E–17
GO:0050896	Response to stimulus	59	5.63E–16
GO:0050789	Regulation of biological process	65	7.17E–16
GO:0006915	Apoptotic process	32	7.17E–16
GO:0012501	Programmed cell death	32	9.55E–16
GO:0065007	Biological regulation	66	1.27E–15
GO:0051716	Cellular response to stimulus	54	3.08E–15
GO:0032501	Multicellular organism process	54	3.58E–15
GO:0008219	Cell death	32	3.89E–15

GO = Gene Ontology, HCC = hepatocellular carcinoma.

qRT-PCR data and TCGA data, respectively. Collectively, more experiments with larger patient cohorts are required to illuminate the diagnostic role of miR-204-5p in HCC.

Our study verified that the downregulation of miR-204-5p in HCC was related to metastasis, which corresponded to the report by Zeng and others.[13] Additionally, the decreased expression of miR-204-5p was related to advanced TNM stage and vaso-invasion, which showed the significant correlation of miR-204-5p with a more aggressive tumor phenotype. This indicated the promising value of miR-204-5p as a biomarker for recognizing tumor progression. Previous studies have reported that miR-204-5p was an independent biomarker in gastric tumors,[28] colorectal cancer,[7] and non-small-cell lung carcinoma.[27] Based on our qRT-PCR data and TCGA data, however, no significant association between miR-204-5p and RFS or OS was observed in the study. The result conflicts with that of the previous study published by Ge and others,[9] which concluded that patients with HCC and a low miR-204-5p level exhibited a worse prognosis according to a multivariate analysis. Differences in samples size and clinicopathological features of the included patients might be possible reasons for these inconsistent results. Therefore, until now, we could not establish a firm conclusion as to the association between miR-204-5p and survival in HCC. Prospective studies with a large cohort of patients and quantitative meta-analyses are needed to draw a credible conclusion.

The underlying molecular mechanism of HCC development is currently unclear and urgently requires clarification. miRNAs can exert ample effects via the repression of gene expression through interactions with their target messenger RNAs.[30] Previously, some studies have concluded that miR-204-5p may act as an anti-oncogene through the regulation of target genes in various cancers. In HCC, Zeng and others[13] predicted that miR-204-5p would be one of the crucial regulatory modules in tumor metastasis; this miRNA is involved with 10 target genes, including SRXN1, TOMM70A, CHD5, ATF2, FAM168B, POU2F2, WDR26, WASF2, SPOP, and PLAA. The study published by Ge and others[9] demonstrated that miR-204-5p and miR-192 could remarkably suppress HOTTIP and interrupt glutaminolysis mediated by GLS1; they also found that the miR-204-5p/192-HOTTIP axis may be a vital pathway in liver tumorigenesis. Chronic HBV infection, which is common in developing countries, can initiate and accelerate the processes that are involved in the progression of liver cirrhosis to liver cancer.[31] Interestingly, a feed-forward loop among miR-204-5p, HBV, and STAT3 was identified by Huang and others.[10] They observed that the expression of miR-204-5p suppressed encapsidation as well as the assembly of the capsid of HBV and pregenomic RNA. Conversely, HBV can inhibit the miR-204-5p level via the activation of the transcription factor STAT3. The feed-forward loop might be a potential mechanism that plays a role in HCC incidence and development. In HCC cell lines, Jiang and others[11] found that miR-204-5p significantly inhibited cell proliferation and thus invasion via targeting SIRT1. Li and others[12] also obtained a similar result in that the overexpression of miR-204-5p inhibited cell growth and promoted apoptosis by targeting BCL2 and SIRT1. Collectively, published studies have provided evidence that miR-204-5p functions as a tumor-suppressive gene that is involved in tumor progression through the regulation of target genes or by affecting HBV replication.

In our study, we conducted a comprehensive prediction of target genes of miR-204-5p using in silico methods; as a result, a total of 73 potential genes that were selected after the intersections were considered. The GO enrichment analysis demonstrated that these genes were the most enriched in the processes of cell proliferation, apoptosis, and intercellular signal transduction, which implied that miR-204-5p might be a regulator of abnormal proliferation, anti-apoptosis, and the keen aggressiveness of tumor cells, and membrane-associated communication might be regulated by these genes as well. Interestingly, in the KEGG pathway analysis, we discovered that the potentially targeted genes were most correlated with several cancer-related pathways, which suggested that miR-204-5p might participate in key cancer-related pathways by targeting these genes. With respect to the PPI network, 10 core genes were identified out of the selected genes, and these genes were found to participate in multiple cancer-associated pathways. These core genes were believed to be of great importance in the network regulated by miR-204-5p in HCC. Moreover, the core genes BCL2 and the SIRT1 were validated in previous studies,[11,12] which indicated that prediction using in silico methods, to some extent, is credible and valuable. Therefore, we provided the most probable target genes for forthcoming researchers who intend to investigate and validate the target of miR-204-5p. On the flip side, we cannot exclude the possibility that the progression of HCC might affect miR-204-5p expression and its regulatory network. Hence, further experiments need to be performed to better understand the molecular involvement and mechanism of this miRNA in HCC.

It should not be neglected that this study holds several limitations. Firstly, our study only investigated the aberrant expression of miR-204-5p in HCC tissues, and no in vitro experiments with cell lines of HCC were performed. It would be more credible if the research findings were established in both in vitro and in vivo experiments. Second, the samples used in the present study consisted of tissues that were collected from HCC patients. It would be more attractive to investigate whether miR-204-5p can be used to screen patients in early or premorbid stages of the disease. Third, the included datasets from the GEO were obtained by different platforms; this may have caused methodological heterogeneity when we pooled the results. Finally, the prediction of target genes based only on computer arithmetic might produce false-positive or false-negative results. Our research group will conduct further experiments to validate the targeted regulation of these genes by miR-204-5p.

In summary, the study demonstrated that the downregulation of miRNA-204-5p might participate in HCC progression and that miR-204-5p might act as a tumor suppressor by targeting prospective hub genes in cancer-related pathways. To strengthen the findings of the present study, multicenter studies with large sample sizes are needed. Moreover, future validations are required to elucidate the molecular mechanism of miR-204-5p in HCC.

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