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

Yi-Huan Luo, Wei Tang, MM, Xin Zhang, Zhong Tan, Wen-Liang Guo, Na Zhao, Si-Min Pang, Yi-Wu Dang, MM, Min-Hua Rong, MD, Ji Cao, MM, Ji Cao, MMed, Guangxi Medical University, Key Laboratory for High-Incidence Tumor Prevention and Treatment, Ministry of Education, Guangxi Medical University, Nanning, Guangxi

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, vaso-invasion, 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 in 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

Editor: Huitao Fan.
Y-HL and WT contributed equally.

Funding: The study was funded by the Ministry of Education (GKE2015-ZZ04), the National Natural Science Foundation of China (NSFC 81260222), the Key Laboratory for High-Incidence Tumor Prevention and Treatment Foundation, the Scientific Research Project of the Department of Education in Guangxi Zhuang Autonomous Region (No. LX2014064 and No.201204LX044), Sponsoring Projects of Scientific Research for Universities in Guangxi (201204LX044), and Future Academic Stars of Guangxi Medical University (WLXSZX16001). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

The authors have no conflicts of interest to disclose.

Supplemental Digital Content is available for this article.

* Correspondence: Min-Hua Rong, Research Department, Affiliated Cancer Hospital, Guangxi Medical University, Nanning, Guangxi Zhuang Autonomous Region, People’s Republic of China (e-mail: tourtair@163.com); Ji Cao, Key Laboratory for High-Incidence Tumor Prevention and Treatment, Ministry of Education, Guangxi Medical University, Nanning, Guangxi Zhuang Autonomous Region, People’s Republic of China (e-mail: caojicn@163.com).
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. 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. 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.

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. MicroRNAs are a variety of small noncoding RNAs that are involved in multiple ontological 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, colorectal cancer, and acute myeloid leukemia, 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. 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. 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, RUN6B, and RUN48 were added in the TaqMan MicroRNA assays. The mRNA sequences and references used are listed below: miR-204-5p: UUCCUUUGUCUAUCUAUGCCUG; RUN6B: CGCAAGGATGACACGCACAATTTCCGTGAAGCCTCATATTCC; RUN48: GATGACCCAGGGTAACTCTGAGTGTGTCGCTGATGCCATCACC GCAGGCGCTCTGACC. 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−ACt 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: HCC, liver, hepatocellular, hepatic, malignant*, 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. We searched strategy and screening processes were consistent with the screening of the datasets. The normalized miRNAs expression data matrices 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-miT, 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± 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

| Clinicopathological features | N (patients) | miRNA-204 relevant expression (2\(^{-\Delta\Delta Cq}\)) | Mean± SD | t | P |
|-----------------------------|--------------|--------------------------------------------------|----------|---|---|
| Tissue                      |              |                                                  | 5.5429±2.4099 | -4.242 | <.001 |
| Adjacent noncancerous liver | 95           |                                                  | 4.1833±1.9806 | \_     | \_   |
| HCC                        | 95           |                                                  | 4.2367±2.0207 | -0.260 | .795  |
| Age, years                  |              |                                                  | 4.1304±1.9578 | \_     | \_   |
| <50                         | 49           |                                                  | 4.1760±1.9792 | -0.088 | .930  |
| ≥50                         | 46           |                                                  | 4.2200±2.0369 |      |      |
| Gender                      |              |                                                  | 3.2167±0.4355 | F=0.780 | .461  |
| Male                        | 75           |                                                  | 4.2783±2.1128 | \_     | \_   |
| Female                      | 20           |                                                  | 4.1931±1.8735 |      |      |
| Differentiation             |              |                                                  | 4.0819±1.9389 | -1.054 | .295  |
| Well                        | 6            |                                                  | 4.6279±2.1513 | \_     | \_   |
| Moderate                    | 60           |                                                  | 4.1846±2.0162 | -0.003 | .997  |
| Poor                        | 29           |                                                  | 4.180±1.9605 |       |      |
| Size, cm                    |              |                                                  | 4.7500±2.0280 | 2.788  | .006  |
| <5                          | 77           |                                                  | 3.6551±1.7983 |       |      |
| ≥5                          | 53           |                                                  | 4.5227±2.4180 | 2.920  | .007  |
| Tumor nodes                 |              |                                                  | 3.8123±1.6755 |       |      |
| Single                      | 52           |                                                  | 4.3994±2.0696 | 1.481  | .142  |
| Multiple                    | 43           |                                                  | 3.7656±1.7477 |       |      |
| Metastasis                  |              |                                                  | 4.518±2.0659 | 2.140  | .035  |
| –                           | 49           |                                                  | 3.6389±1.7228 |       |      |
| +                           | 46           |                                                  | 4.7500±2.0280 | 2.788  | .006  |
| Clinical TNM stage           |              |                                                  | 3.8123±1.6755 |       |      |
| I–II                        | 22           |                                                  | 3.6551±1.7983 |       |      |
| III–IV                      | 73           |                                                  | 4.5227±2.4180 | 2.920  | .007  |
| Portal vein tumor embolus   |              |                                                  | 3.8123±1.6755 |       |      |
| –                           | 63           |                                                  | 4.3994±2.0696 | 1.481  | .142  |
| +                           | 32           |                                                  | 3.7656±1.7477 |       |      |
| Vaso invasion               |              |                                                  | 4.518±2.0659 | 2.140  | .035  |
| –                           | 59           |                                                  | 3.6389±1.7228 |       |      |
| +                           | 36           |                                                  | 4.7500±2.0280 | 2.788  | .006  |
| Tumor capsular infiltration |              |                                                  | 3.8123±1.6755 |       |      |
| With complete capsule       | 45           |                                                  | 4.2733±1.9767 | 0.409  | .683  |
| Infiltration or not capsule | 50           |                                                  | 4.106±2.0008 |       |      |
| AFP                         |              |                                                  | 4.3146±2.0587 | 0.008  | .994  |
| –                           | 41           |                                                  | 4.3146±2.0587 |       |      |
| +                           | 38           |                                                  | 4.2644±1.9722 | 0.368  | .714  |
| Cirrhosis                   |              |                                                  | 4.1140±2.0054 |       |      |

\(\alpha\text{-}\text{fetal protein, HCC=hepatocellular carcinoma, miRNA = microRNA, SD=standard deviation.}\)

\(^{*}\) A 1-way analysis of variance test.
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) \((P = .357, \text{Fig. 3A})\) as well as overall survival (OS) \((P = .114, \text{Fig. 3B})\). Similarly, the results were also not statistically significance when we analyzed the association of miR-204-5p expression with RFS \((P = .254, \text{Fig. 3C})\) and OS \((P = .754, \text{Fig. 3D})\), 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 \((\text{SMD} = -5.19, 95\% \text{CI}: -6.69 \text{ to } 3.69, \ P < .001)\) (Fig. 5B) with significant heterogeneity \((I^2 = 85.8\%, \ P = .008)\) observed. However, no significant difference was observed in the meta-analysis of microarrays \((\text{SMD} = 0.096, 95\% \text{CI}: -0.366 \text{ to } 0.557, \ P = .685)\) (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) vaso-invasion. 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.
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   |
| GSE69580      | 2015 | Taiwan  | GPL10850        | Noncancerous | 16 | 2.2089 ± 0.0890   |
| GSE54751      | 2014 | USA     | GPL18262        | HCC     | 5  | 2.5530 ± 1.2475   |
| GSE41874      | 2012 | Japan   | GPL7722         | Noncancerous | 5  | 2.3554 ± 0.8691   |
| GSE40744      | 2012 | USA     | GPL14613        | HCC     | 13 | 2.2494 ± 0.0171   |
| GSE21362      | 2010 | Japan   | GPL10312        | Noncancerous | 18 | 2.2175 ± 0.3031   |
| GSE22058      | 2010 | USA     | GPL10457        | HCC     | 73 | 2.2832 ± 0.7596   |
| GSE12717      | 2008 | USA     | GPL7274         | Noncancerous | 96 | 2.0279 ± 0.8543   |
| GSE6857       | 2007 | USA     | GPL4700         | HCC     | 10 | 2.3154 ± 0.5143   |
| Ge et al[9]   | 2015 | China   | RT-PCR          | Noncancerous | 421 | 2.2472 ± 0.1815   |
| Li et al[22]  | 2015 | China   | RT-PCR          | HCC     | 48 | 6.6667 ± 1.2821   |
| Our data      | –    | China   | RT-PCR          | Noncancerous | 95 | 4.1853 ± 1.9806   |
| TCGA data     | –    | –       | –               | HCC     | 353 | 8.9535 ± 0.6586   |

HCC = hepatocellular carcinoma, RT-PCR = real-time polymerized chain reaction, SD = standard deviation, 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.
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

| Study ID | SMD (95% CI) | Weight |
|----------|--------------|--------|
| GSE7555  | 0.72 (-0.31, 1.75) | 8.88   |
| GSE69350 | 0.18 (-1.06, 1.43)  | 7.39   |
| GSE54715 | -0.48 (-1.41, 0.46) | 9.66   |
| GSE41874 | -0.26 (-1.54, 1.01) | 7.21   |
| GSE40744 | -0.13 (-0.69, 0.43) | 12.91  |
| GSE21356 | 0.14 (-0.19, 0.46)  | 14.76  |
| GSE20258 | -0.41 (-0.69, -0.12) | 15.01  |
| GSE12717 | -0.09 (-1.01, 1.01) | 9.02   |
| GSE65857 | 0.93 (0.67, 1.19)   | 15.16  |
| Overall (I-squared = 84.8%, p = 0.000) | 0.10 (-0.37, 0.56) | 100.00 |

NOTE: Weights are from random effects analysis.

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.
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 Figure 6.

### Table 3

| 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:0048701 | System development                     | 49             | 3.15E–19     |
| GO:0048513 | Animal organ development               | 43             | 1.08E–18     |
| GO:0048856 | Anatomical structure development       | 52             | 7.86E–18     |
| GO:004767  | Single-organism developmental process  | 53             | 8.04E–18     |
| GO:0032502 | Developmental process                  | 53             | 1.48E–17     |
| GO:004707  | 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:007166  | Cell surface receptor signaling pathway| 38             | 8.05E–17     |
| GO:000896  | Response to stimulus                   | 59             | 5.63E–16     |
| GO:000789  | 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:001716  | 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.

### Table 4

| 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 | Fox0 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              | 4.76E–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.
on our 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 vasculo-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.\[10\] 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 premorbidity 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 prosurvival 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.

References

[1] Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin 2012;62:10–29.
[2] Llovet JM, Zucman-Rossi J, Pikarsky E, et al. Hepatocellular carcinoma. Nat Rev Dis Primers 2016;2:16018.
[3] Chauhan R, Lahiri N. Tissue- and serum-associated biomarkers of hepatic cellular carcinoma. Nat Rev Dis Primers 2016;2:16018.
[4] Fiorino S, Bacchi-Reggiani ML, Visani M, et al. MicroRNAs as possible biomarkers for diagnosis and prognosis of hepatitis B- and C-related-hepatocellular carcinoma. World J Gastroenterol 2016;22:3907–36.
[5] Lee Y, Yang X, Huang Y, et al. Network modeling identifies molecular functions targeted by miR-204 to suppress head and neck tumor metastasis. PLoS Comput Biol 2010;6:e1000730.
[6] Sumbul AT, Gogebakan B, Ergun S, et al. miR-204-5p expression in colorectal cancer: an autophagy-associated gene. Tumour Biol 2014;35:12713–9.
Yin Y, Zhang B, Wang W, et al. miR-204-5p inhibits proliferation and invasion and enhances chemotherapeutic sensitivity of colorectal cancer cells by downregulating RAB22A. Clin Cancer Res 2014;20:6187–99.

Butrym A, Rybka J, Baczynska D, et al. Low expression of microRNA-204 (miR-204) is associated with poor clinical outcome of acute myeloid leukemia (AML) patients. J Exp Clin Cancer Res 2015;34:68.

Ge Y, Yan X, Jin Y, et al. MiRNA-192 [corrected] and miRNA-204 directly suppress IncRNA HOXT13 and disrupt GLI1-mediated glutaminolysis in hepatocellular carcinoma. PLoS Genet 2013;11:e1005726.

Huang JT, Chen HL, Shih C. MicroRNA miR-204 and miR-1236 inhibit hepatitis B virus replication via two different mechanisms. Sci Rep 2016;6:34740.

Jiang G, Wen L, Zheng H, et al. miR-204-5p targeting SIRT1 regulates hepatocellular carcinoma progression. Cell Biochem Funct 2016;34:505–10.

Li K, Xyu Q, Liu X, et al. Growth inhibition of human hepatocellular carcinoma by miRNA-204 via down-regulation of Bcl-2 and Sirt1 expression. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 2015;31:168–72.

Zeng L, Yu J, Huang T, et al. Differential combinatorial regulatory network analysis related to venous metastasis of hepatocellular carcinoma. BMC Genomics 2012;13(suppl 8):S14.

Liu Y, Ren F, Rong M, et al. Association between underexpression of microrna-203 and clinicopathological significance in hepatocellular carcinoma tissues. Cancer Cell Int 2015;15:62.

Rong M, He R, Dang Y, et al. Expression and clinicopathological significance of miR-146a in hepatocellular carcinoma tissues. Ups J Med Sci 2014;119:19–24.

Zhang X, Ye ZH, Liang HW, et al. Down-regulation of miR-146a-5p and its potential targets in hepatocellular carcinoma validated by a TCGA- and GEO-based study. FEBS Open Biol 2017;7:504–21.

Liberati A, Altman DG, Tetzlaff J, et al. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate healthcare interventions: explanation and elaboration. BMJ 2009;339:b2700.

Begg CB, Mazumdar M. Operating characteristics of a rank correlation test for publication bias. Biometrics 1994;50:1088–101.