Int J Clin Exp Pathol 2019;12(4):1439-1456
www.ijcep.com /ISSN:1936-2625/IJCEP0092210

Original Article

Elevation of miR-191-5p level and its potential signaling pathways in hepatocellular carcinoma: a study validated by microarray and in-house qRT-PCR with 1,291 clinical samples

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Received February 2, 2019; Accepted February 21, 2019; Epub April 1, 2019; Published April 15, 2019

Abstract: Background: The miR-191-5p expression has been reported to increase in hepatocellular carcinoma (HCC), but its clinical value and exact role remain to be further clarified. Thus, a comprehensive analysis was performed in the current study to explore the underlying function of miR-191-5p in HCC. Methods: HCC-related expression data were collected to conduct a thorough analysis to determine the miR-191-5p expression and its clinical significance in HCC, including microarray data from the Gene Expression Omnibus and ArrayExpress database as well as quantitative real-time polymerase chain reaction (qRT-PCR) data of 178 matched clinical samples. The underlying relationship between miR-191-5p and HCC was also explored on the basis of a series of bioinformatics analyses. Results: The overall pooled meta-analysis showed an overexpression of miR-191-5p in the HCC samples (SMD=0.400, 95% CI=0.139-0.663, P=0.003), consistent with the detected result of the clinical HCC samples through the qRT-PCR analysis. Higher miR-191-5p levels were correlated with advanced TNM stages (III and IV), higher pathological grades, and metastasis. Functionally, 64 potential target genes were acquired for further mechanism analysis. Two pathways (p75 neurotrophin receptor and liver kinase B1-mediated signaling pathways), which were likely modulated by miR-191-5p, were regarded to be linked to the deterioration of HCC. Early growth response 1 and UBE2D3 were identified as the most likely targets for miR-191-5p in HCC and were commonly implied in the top enriched pathways and protein-protein network. Conclusions: In summary, miR-191-5p may function as a tumor promoter miRNA of HCC, and the miR-191-5p inhibitor may contribute to the targeted HCC treatment in the future.

Keywords: Hepatocellular carcinoma, MiR-191-5p, signaling pathway, microarray, qRT-PCR

Introduction

Hepatocellular carcinoma (HCC) is one of the top ten cancers around the world, ranking fifth in cancer-related mortality in men, with nearly 21,600 new estimated deaths [1]. HCC is commonly diagnosed in the advanced stages because of its latency, and the currently available therapies for HCC are ineffective, thus leading to unfavorable prognosis [2]. Specifically, less than 20% of HCC patients could survive after five years from the date of diagnosis [3].

MicroRNAs (miRNAs) are endogenous single-stranded, small non-coding RNA molecules containing almost 22 nucleotides, which are implicated in mRNA regulation. Each miRNA may have multiple target genes. Some miRNAs may also regulate the same genes, generally affecting gene expression [4, 5]. MiRNAs act as an essential participant in gene expression, reverse transcription, cell differentiation, proliferation and apoptosis, a series of biological activities associated with immune responses, cardiovascular diseases, and the occurrence and progression of cancers [6-8].

MiR-191-5p (miRBase: MI0000465, miRNA ID: hsa-miR-191-5p, Sequence: caacggaaucccaaaagcagcug) is a miRNA that is abnormally expressed in tissues and cell types from various cancers and is closely related to tumor develop-

ment, cancer diagnosis, and prognosis [9]. Recent studies found that miR-191-5p could be involved in certain biological activities in HCC, including cellular changes, proliferation inhibition, and apoptosis, and it could serve as an oncogene [10]. Moreover, miR-191-5p has been frequently reported to function as an oncogene in cancers such as colon, breast, and gastric [11-13]. Zhang et al. revealed that the suppression of miR-191-5p expression could negatively regulate the proliferation of colorectal cancer cells [11]. Similarly, suppressing the miR-191-5p expression in the gastric cancer cell line HGC-27 reduced cell proliferation and cell cycle progression and then impaired cell migration and invasion [13]. In breast cancer, Nagpal reported that HIF-inducible miR-191-5p could promote the migration of breast cancer by regulating the TGFβ-signaling pathway in a hypoxic microenvironment [12]. Therefore, miR-191-5p has great potential to be a novel diagnostic biomarker and an innovative therapeutic approach for HCC patients. However, current studies are limited by the fact that the specific mechanism between miR-191-5p and HCC has not been clearly clarified. In addition, more evidence remains to be collected for the further application of miR-191-5p in the prediction and early diagnosis of HCC.

Thus, we firstly evaluated the miR-191-5p expression and its potentially clinicopathological and diagnostic significance in HCC by analyzing data from the Gene Expression Omnibus (GEO) and ArrayExpress databases as well as clinical tissue samples. Moreover, we investigated the underlying mechanism of miR-191-5p in HCC through in silico analysis, including potential targets prediction, pathway, gene ontology (GO), and protein-protein interaction (PPI) analyses.

Materials and methods

Data mining of microarray data for the role of miR-191-5p in HCC

We screened and downloaded the miRNA (lastly updated on May 13, 2018) and mRNA (lastly updated on January 7, 2019) microarray profiles of HCC from the GEO and ArrayExpress databases. The keywords used for the search consisted of two parts: (1) hepatocellular OR liver OR hepatic OR HCC and (2) malignan* OR cancer OR tumor OR tumor OR neoplas* OR carcinoma. The microarrays were considered eligible while conforming to the following criteria: (1) the samples were obtained from humans; (2) the profiles should contain two groups, namely, patients diagnosed with HCC as the experimental group and individuals without HCC as the control group; (3) both two groups should include at least three samples; (4) the studies should examine the expression of miR-191-5p or the potential target mRNA in HCC. The profiles were regarded as unqualified if (1) they were not related to human HCC, and (2) the samples were collected from cell lines or other species.

Clinical HCC tissue samples from our institute

In this study, the First Affiliated Hospital of Guangxi Medical University (Nanning, Guangxi, China) provided 89 formalin-fixed, paraffin-embedded (FFPE) HCC tissues and their adjacent non-tumorous liver tissues from 2010 to 2011. The mean age of the included HCC patients was 52 years old. The study design was approved by the Ethical Committee of The First Affiliated Hospital of Guangxi Medical University. Informed consent was collected from all participants. The clinical pathological information of all HCC patients is presented in **Table 1**. The above samples were examined, diagnosed, and confirmed independently by two pathologists (Yu-yan Pang and Gang Chen).

Quantitative real-time polymerase chain reaction (qRT-PCR) to detect miR-191-5p in the HCC clinical sample in house

The miRNeasy Kit (QIAGEN, KJ Venlo, Netherlands) was used to isolate the total RNA, including miRNA from FFPE tissues. A more detailed RNA extraction and normalization was described in our previous reports [14-16]. Previously, RUN6B and let-7a were combined to work as the most reliable endogenous reference for HCC used in in vitro experiments. Moreover, RUN6B and RUN48 were commonly used in the HCC FFPE examination. In the present research, we applied different internal references and included the primers of miR-191, let-7a, RNU48, and RNU6B in the TaqManH MicroRNA Assays (4427975, Applied Biosystems, Life Technologies Grand Island, NY 14072 USA). The TaqMan® MicroRNA Assays provided the primers for miR-191-5p and the controls. The PCR reactions for the miRNAs

Table 1. Correlations between miR-191-5p expression and clinicopathological features in HCC (Mean ± SD)

Parameter	Group	N	miR-191-5p expression	t	P
Tissue	Adjacent liver	89	0.876±0.692	3.441*	0.001
	HCC	89	5.067±8.351		
Age (years)	<50	46	3.173±3.865	2.204	0.032
	≥50	43	7.092±11.045		
Gender	Female	17	3.471±3.714	0.874	0.384
	Male	72	5.443±9.088		
Differentiation	Low	26	2.509±3.379	-	0.022
	Moderate	57	6.336±9.944		
TNM	High	6	4.090±3.077		
	I+II	23	1.807±1.049	-3.721	0.000
	III+IV	66	6.203±9.433		
Metastasis	No	44	2.840±5.675	2.580	0.012
	Yes	45	7.244±9.910		
Cirrhosis	No	46	5.644±9.625	-6.673	0.503
	Yes	43	4.448±6.791		
AFP	Negative	38	7.277±11.824	-2.144	0.038
	Positive	35	2.988±3.359		
Embolus	No	65	4.427±7.443	1.192	0.236
	Yes	24	6.800±10.411		
Capsular	No	44	6.856±10.200	-2.021	0.047
	Yes	45	3.317±5.611		
Nodes	Single	53	3.186±3.269	2.251	0.030
	Multi	36	7.835±12.093		
Size	<5 cm	14	6.901±11.193	-0.843	0.402
	≥5 cm	73	4.8263±7.839		
Survival	Live	49	5.801±7.863	1.440	0.197
	Death	7	15.689±17.927		
Vasoinvasion	No	57	3.674±6.294	1.987	0.054
	Yes	30	7.996±11.009		

SD: standard deviation; *Paired samples t-test was utilized.

were analyzed using PCR7900 (Applied Biosystems) in December 2011. The $2^{-\Delta cq}$ method was applied to examine the miR-191-5p expression of both HCC and its adjacent healthy liver tissues.

Exploration for the prognostic significance of miR-191-5p in HCC based on microarray data

The GEO profiles with the prognostic data of miR-191-5p in HCC were collected from the SurvMicro website [17]. The hazard ratios (HRs) and corresponding confidence intervals (CIs) of each profile were extracted to execute the pooled analysis on Stata 12.0. HCC patients with high miR-191-5p levels were more likely to suffer from poorer outcomes when the pooled HR was greater than 1.

Statistical analysis for the clinical implication of miR-191-5p in HCC

The miR-191-5p expression data and clinical characteristics of HCC patients were retrieved from the qualified included profiles and in-house patient samples. The expression values of miR-191-5p were presented as the mean values and standard deviations (SD), and all statistical calculation was dealt with using SPSS 22.0. Student's paired t-test and independent sample t-test were adopted to calculate the difference of miR-191-5p in the two paired or unpaired groups, respectively. One-way analysis of variance was employed to estimate the values of more than two groups. The correlation analysis was executed by the Spearman's method. The distinguishing potential of miR-191-5p in HCC from non-HCC tissues was evaluated by the receiver operator characteristic (ROC) curve.

To access the underlying value of miR-191-5p in HCC, we utilized Stata 12.0 to merge the standardized mean differences (SMD) and the 95% CIs of all included profiles and institute qRT-PCR data. The pooled results showed marked heterogenicity with a p value of less than 0.05 and I^2 statistics of more than 50%, which pointed to the selection of the random effects model. By contrast, the fixed effects model was employed when the p value was greater than 0.05 and the I^2 was less than 50%. The potential publication bias was estimated by Begg's tests. Sensitivity analysis was adopted to test the effect of each profile on the pooled result. The summary receiver operating characteristic (SROC) curve was drawn using Stata 12.0, and we calculated the concrete value of the area under the curve (AUC) to access the ability of miR-191-5p to distinguish

Table 2. Summary characteristics of the 13 eligible microarray datasets and institute qRT-PCR data

Study ID	First author	Year	Country	Sample type	Cancer N	Cancer M	Cancer SD	Control N	Control M	Control SD	t	p
GSE21362	Sato F	2011	Japan	Tissue	73	3.3716	0.06477	73	3.3199	0.04448	5.279*	0.000
GSE36915	Shih TC	2013	China	Tissue	68	14.2863	0.60812	21	14.1893	0.51409	0.661	0.510
GSE40744	Diaz G	2013	USA	Tissue	26	12.4727	0.18589	50	12.5364	0.20617	-1.32	0.191
GSE41874	Morita K	2013	Japan	Tissue	3	-0.2813	0.15965	3	-0.1226	0.19319	-0.91*	0.459
GSE50013	Shen J	2013	USA	Plasma	20	4.2848	1.9911	20	2.4307	3.05166	2.261*	0.036
GSE54751	Shen J	2014	USA	Tissue	10	-1.9798	0.36558	10	-1.8769	0.73523	-0.558*	0.590
GSE57555	Taguchi Y	2015	Japan	Tissue	5	-0.0429	0.00605	16	-0.0546	0.01826	1.392	0.180
GSE64632	Peng H	2015	USA	Tissue	3	3.3054	0.57741	3	0.6166	0.13044	6.653*	0.022
GSE6857	Budhu A	2008	USA	Tissue	180	11.9961	0.94749	241	11.4932	0.80217	5.747	0.000
GSE74618	Villanueva A	2016	Spain	Tissue	218	9.9458	0.40948	10	9.754	0.10762	4.368	0.000
GSE98269	Xie Z	2017	China	Tissue	3	2.3833	0.01552	3	2.3761	0.02177	0.347*	0.762
E-MTAB-3347	Feng F	2015	China	Tissue	3	2.7215	0.05472	3	2.7648	0.06191,	-6.846*	0.021
E-MTAB-4170	NR	2017	NR	Tissue	24	8.0076	0.78095	24	7.7438	1.2623	1.457*	0.159
In-house qRT-PCR	NR	NR	NR	Tissue	89	5.0665	8.3513	89	0.8735	0.69222	4.765*	0.000

N: Number of samples; M: Mean value of miR-191-5p expression; SD: standard deviation; *Paired samples t-test was utilized.

patients with HCC from individuals without HCC. Furthermore, the pooled specificity and sensitivity, as well as the positive and negative likelihood ratios, were calculated. The level of the two-sided *p* values lower than 0.05 was determined to be statistically significant.

Acquirement of the predicted miR-191-5p targets for and differentially expressed genes in HCC

We applied miRWalk2.0, a platform mainly used for miRNA-targets prediction, to collect the likely targets for miR-191-5p. Twelve databases were involved in the prediction analysis [18]. Only the genes nominated by more than four databases were selected as possible targets for miR-191-5p. The differentially expressed genes (DE-genes) were identified by a GEO microarray (GSE22790), which recorded the values of gene expression in the HCC cell lines after treating with anti-miR-191-5p (the experimental group) and without anti-miR-191-5p (the control group). Then, a part of the DE-genes was recognized as potential miR-191-5p targets when it showed elevated expression levels in this microarray file (|log2 fold changes (FC)|>=1.0, P<0.05).

Signaling pathway analysis for overlapping genes

Finally, the intersection elements of the particularly selected predicted targets of miR-191-5p and the DEGs recognized by GSE22790 were considered the candidate target genes of miR-191-5p. The key genes were adopted to determine the underlying mechanism of miR-191-5p in HCC. Originally, FunRich3.0 was used to perform the GO enrichment and pathway analysis of the target genes [19]. The GO analysis aimed to find the annotations among the biological processes, cellular component, and molecular function in HCC. Pathway enrichment showed the signaling pathways involved in HCC. The detailed files were ultimately downloaded to display the terms involved in the GO and pathway analyses. Moreover, the String database was adopted to construct a visualized and comprehensive PPI network, reflecting the relationship among the total selected genes [20]. The genes commonly involved in the most enriched pathways and the PPI network were considered as miR-191-5p potential targets. As the pooled result of the microarray datasets indicated a high expression trend of miR-191-5p in HCC, its target genes were likely to show down-regulated levels. Thus, we utilized the current microarray data in the GEO and ArrayExpress databases to analyze the expression levels of the candidate targets and the details described above.

Results

Characteristics of the eligible GEO and ArrayExpress profiles

In total, 13 eligible profiles associated with miR-191-5p and HCC, as well as 40 datasets related to mRNA and HCC, were selected for later analysis. The samples used for the experiments of each profile were obtained from the HCC and non-HCC tissues, except one from the plasma specimens (GSE50013). The main characteristics are presented in **Table 2**. The

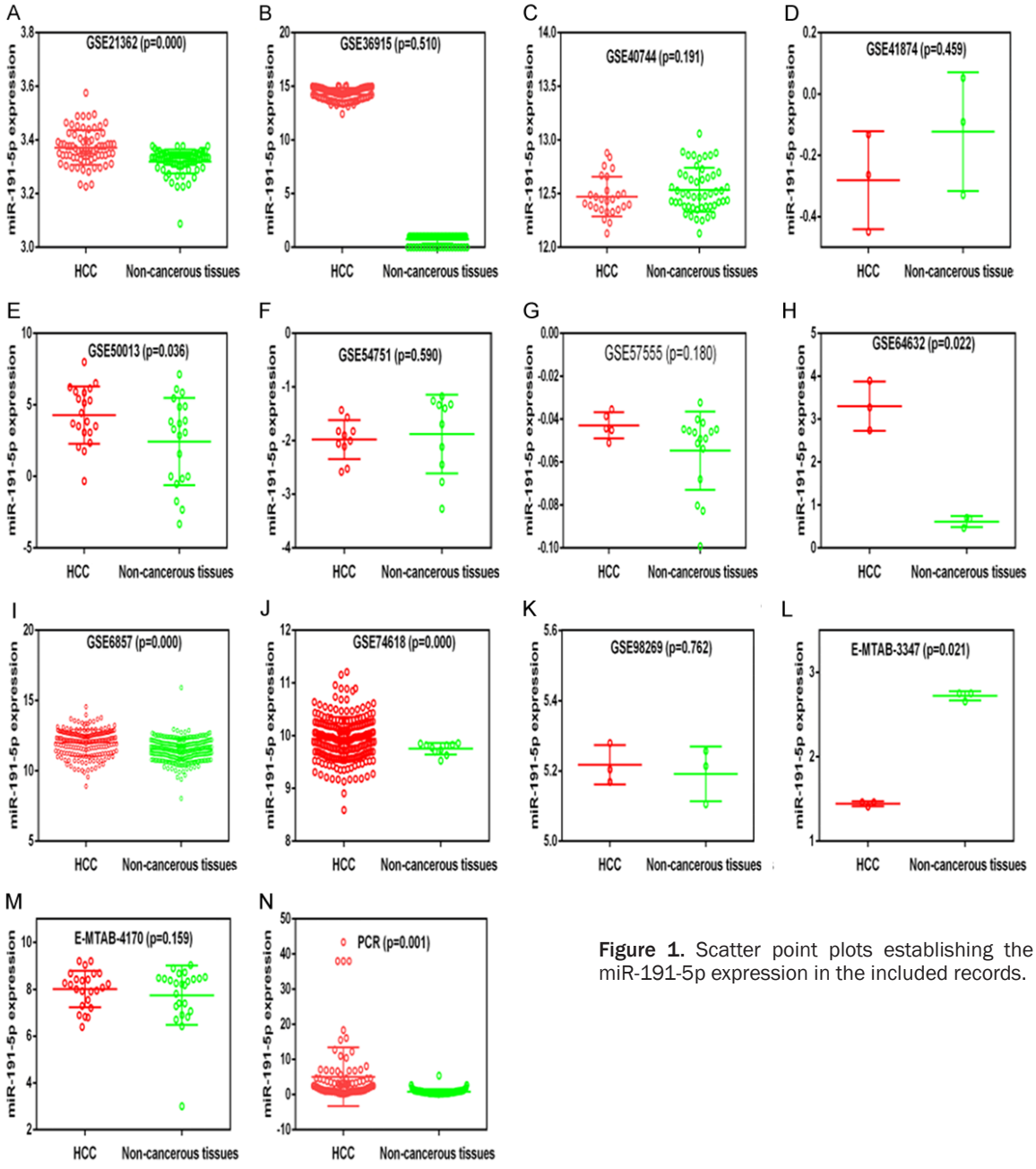


Figure 1. Scatter point plots establishing the miR-191-5p expression in the included records.

scatter point plots of all the qualified profiles are illustrated in **Figure 1**. Nine records exhibited a potential trend of increased miR-191-5p expression in HCC tissues (GSE21362, GSE36915, GSE50013, GSE57555, GSE64632, GSE6857, GSE74618, GSE98269, and E-MTAB-4170). Specifically, the miR-191-5p expression was markedly higher in the HCC tissues than in the control group in five records, namely, GSE21362 (P=0.000), GSE50013 (P=0.036), GSE64632 (P=0.022), GSE6857 (P=0.000), and GSE74618 (P=0.000). By contrast, the miR-191-5p levels were lower in the tissue samples from HCC patients than in the control samples in E-MTAB-3347 (P=0.021).

MiR-191-5p expression in clinical HCC patients

We obtained 178 clinical tissue samples from 89 HCC patients, including cancer and their paired non-cancerous tissues. A significantly higher miR-191-5p expression was identified in the HCC samples (5.067±8.351) than in the

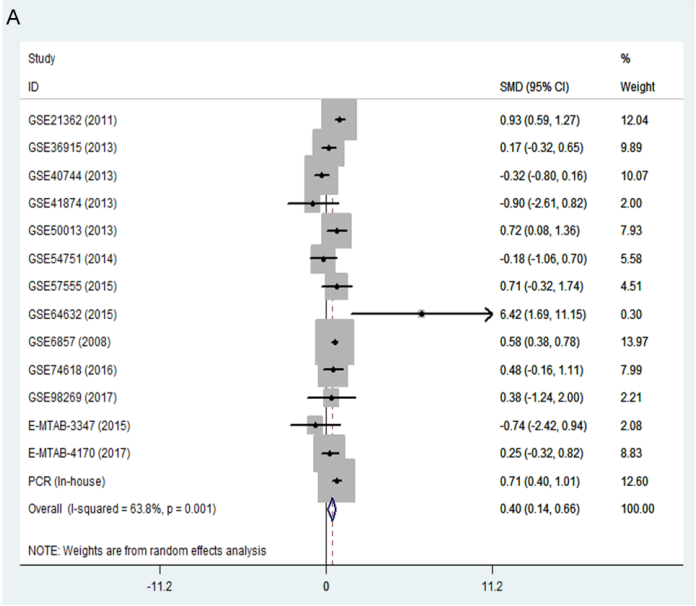
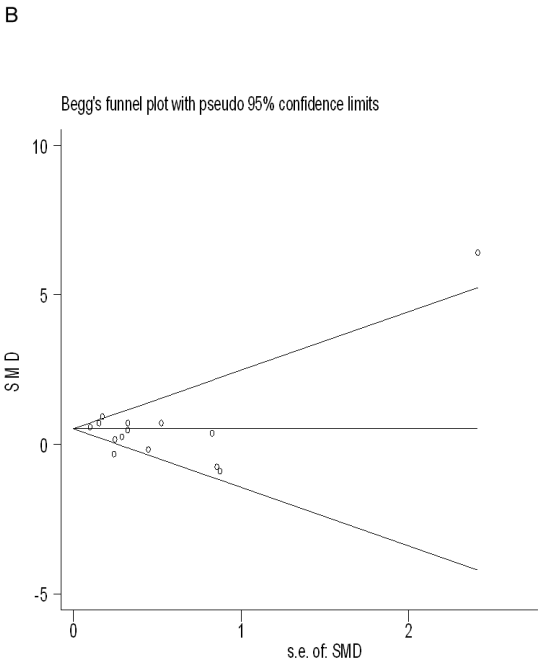
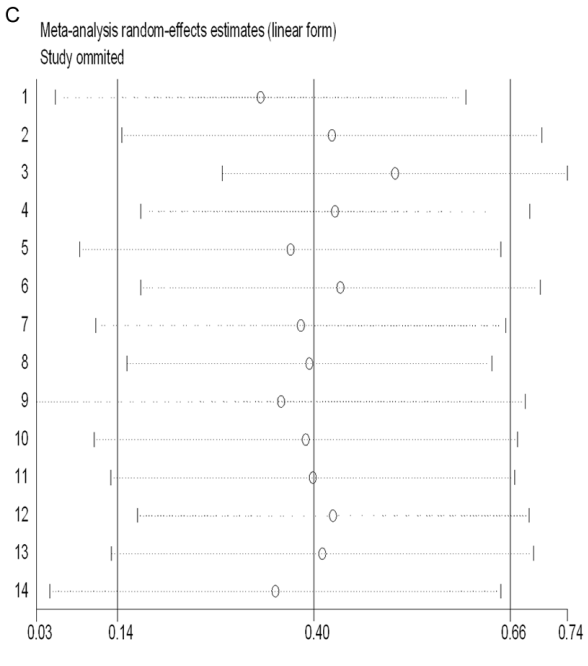


Figure 2. Meta-analysis of the miR-191-5p expression in hepatocellular carcinoma (HCC). A. Forest plot of the 14 included records that detected miR-191-5p in HCC applying a random effects model (I^2=63.80%, P=0.001). The pooled result (SMD=0.400>0, 95% CI: 0.139-0.661) was indicative of the miR-191-5p up-regulation in the HCC samples. B. Begg's funnel plot was used to assess the potential publication bias, and no obvious publication bias was found (P=0.443>0.05). C. Sensitivity analysis was used to evaluate the possible source of heterogenicity.





corresponding non-cancerous hepatic samples (0.876±0.692; P=0.001, **Table 2**; **Figure 1**). The remarkably elevated miR-191-5p expression was detected in the clinically advanced stages (TNM III and IV, 6.203±9.433) rather than in the early stages (TNM I and II, 1.807±1.049, P=0.000). Similarly, the miR-191-5p levels were up-regulated in the HCC tissues with metastasis (7.244±9.91) but not in the non-metastasis tissues (2.84±5.675, P=0.012). A higher miR-191-5p expression was identified in the groups of higher pathological grade (4.090±3.077, P=0.022), multi-nodes (7.835±12.093, P=0.030), non-encapsulated HCC (6.856±10.2, P=0.047), and elderly individuals (7.092±11.045, P=0.032) than in their corresponding control groups. By contrast, with regard to AFP examination, the miR-191-5p levels were down-regulated in the positive AFP groups (2.988±3.359) in comparison with the negative AFP groups (7.277±11.824, P=0.038). However, no obvious correlation was found between the miR-191-5p expression and the rest of the clinical parameters.

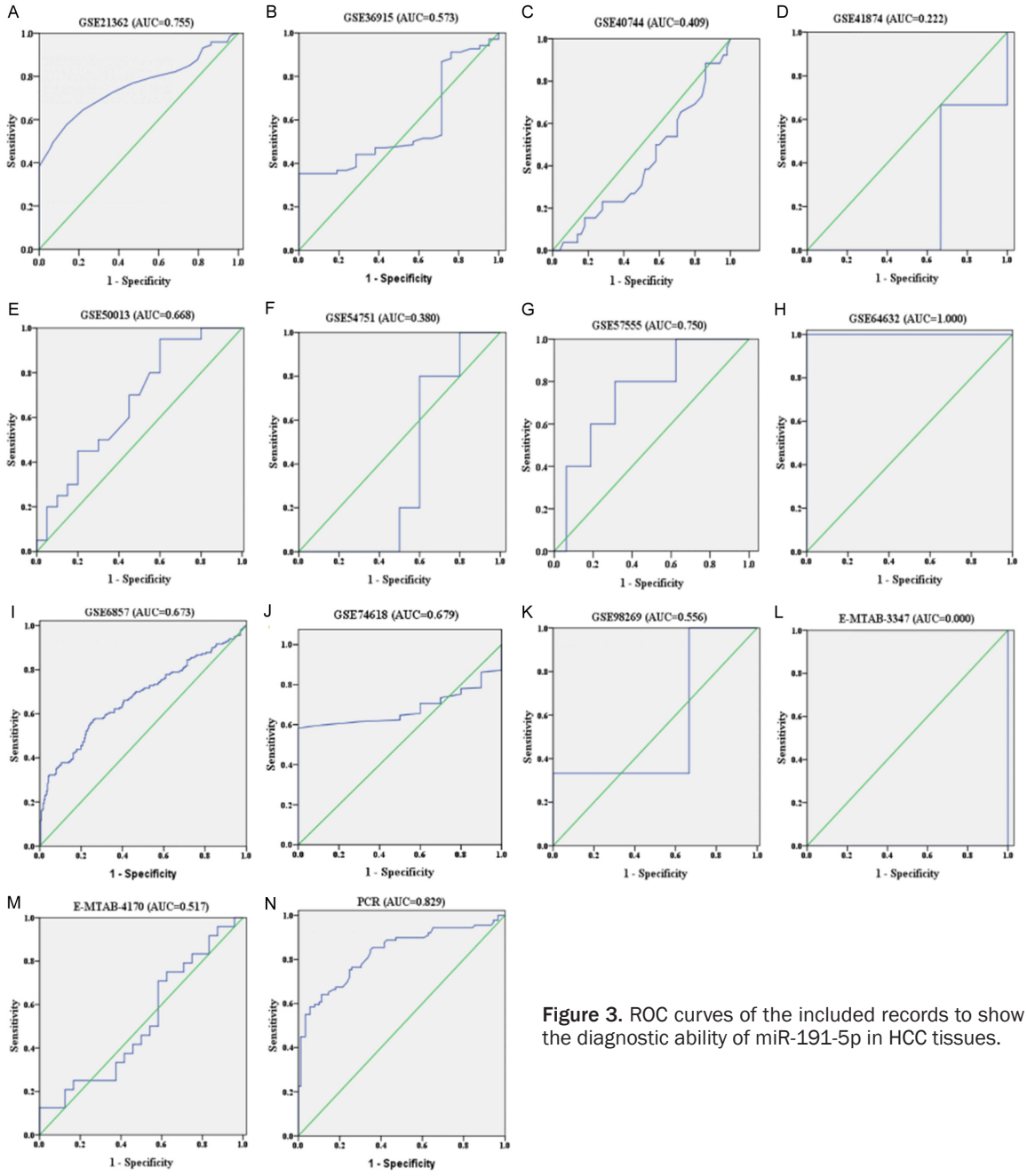


Figure 3. ROC curves of the included records to show the diagnostic ability of miR-191-5p in HCC tissues.

Clinical significance of the miR-191-5p expression in HCC

In total, 1,291 samples were included in a meta-analysis, among which 725 were included in the experimental groups and 566 in the control groups. The pooled results of all included records suggested that the miR-191-5p levels were significantly different among the samples between patients with HCC and the non-cancerous controls (SMD=0.400, 95% CI=0.139-0.663, P=0.003). The random effect model was used because of the obvious heterogeneity (P=0.001, I^2=63.8%), as shown in **Figure 2**. No publication bias was evaluated in this study, as the *p* value of Begg's (P=0.443) test was greater than 0.05 (**Figure 2**). The sensitivity analysis is illustrated in **Figure 2**.

Likely diagnosis ability of miR-191-5p in HCC

The diagnostic value of each eligible record was varied, as identified by the ROC curves (**Figure 3**). The AUC of the plotted sROC curve was

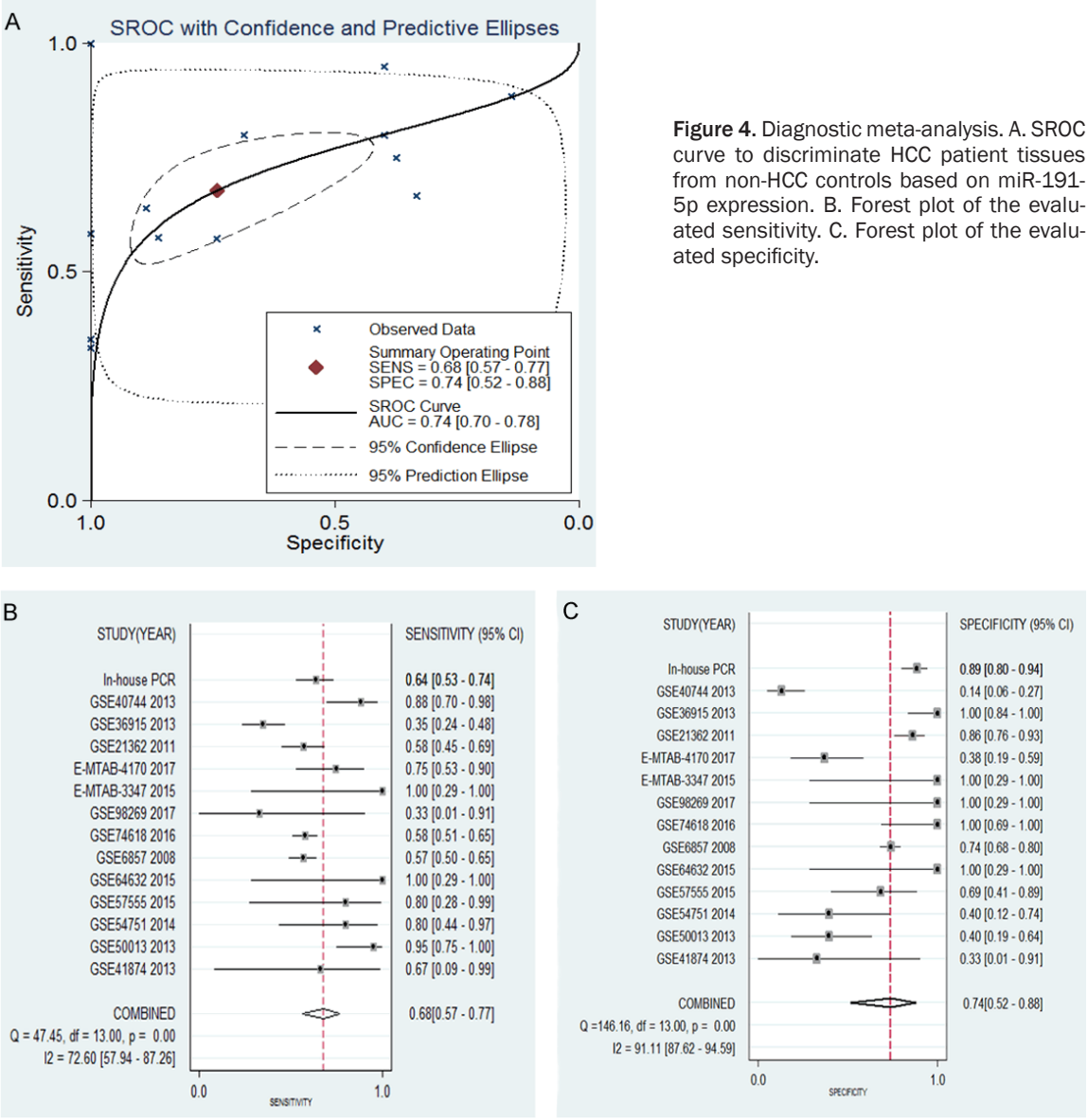


Figure 4. Diagnostic meta-analysis. A. SROC curve to discriminate HCC patient tissues from non-HCC controls based on miR-191-5p expression. B. Forest plot of the evaluated sensitivity. C. Forest plot of the evaluated specificity.

0.740 (95% CI=0.700-0.780), suggesting that miR-191-5p could function as an effective biomarker to diagnose patients with HCC at an earlier stage (**Figure 4**). The merged diagnostic sensitivity (0.678, 95% CI: 0.567-0.772) and specificity (0.741, 95% CI: 0.522-0.882) are shown in **Figure 4**. The pooled results of the diagnostic odds ratio (6.009, 95% CI: 2.975-12.137), positive likelihood ratio (2.614, 95% CI: 1.418-4.819, I^2=78.84%), and negative likelihood ratio (0.435, 95% CI: 0.348-0.544, I^2=42.40%) were also calculated. These results proved the diagnostic ability of miR-191-5p in HCC. The *p* value of the publication bias analysis was 0.539, and thus this study could be considered to have no publication bias.

Prognostic significance of miR-191-5p in HCC

We acquired three GEO profiles, namely, GSE10694 (HR=1.280, 95% CI: 0.880-1.850, P=0.197), GSE31384 (HR=0.694, 95% CI: 0.433-1.11, P=0.131), and GSE6857 (HR=1.204, 95% CI: 0.769-1.887, P=0.413), recording the prognostic data of miR-191-5p for the HCC and non-HCC patients. However, none of the three chips, as well as the pooled result (HR=1.042, 95% CI: 0.721-1.504, P=0.828), had statistical significance. However, there was a tendency in GSE10694, GSE6857, and the pooled result that a higher miR-191-5p level was correlated with poorer outcomes for HCC patients (**Figure 5**).

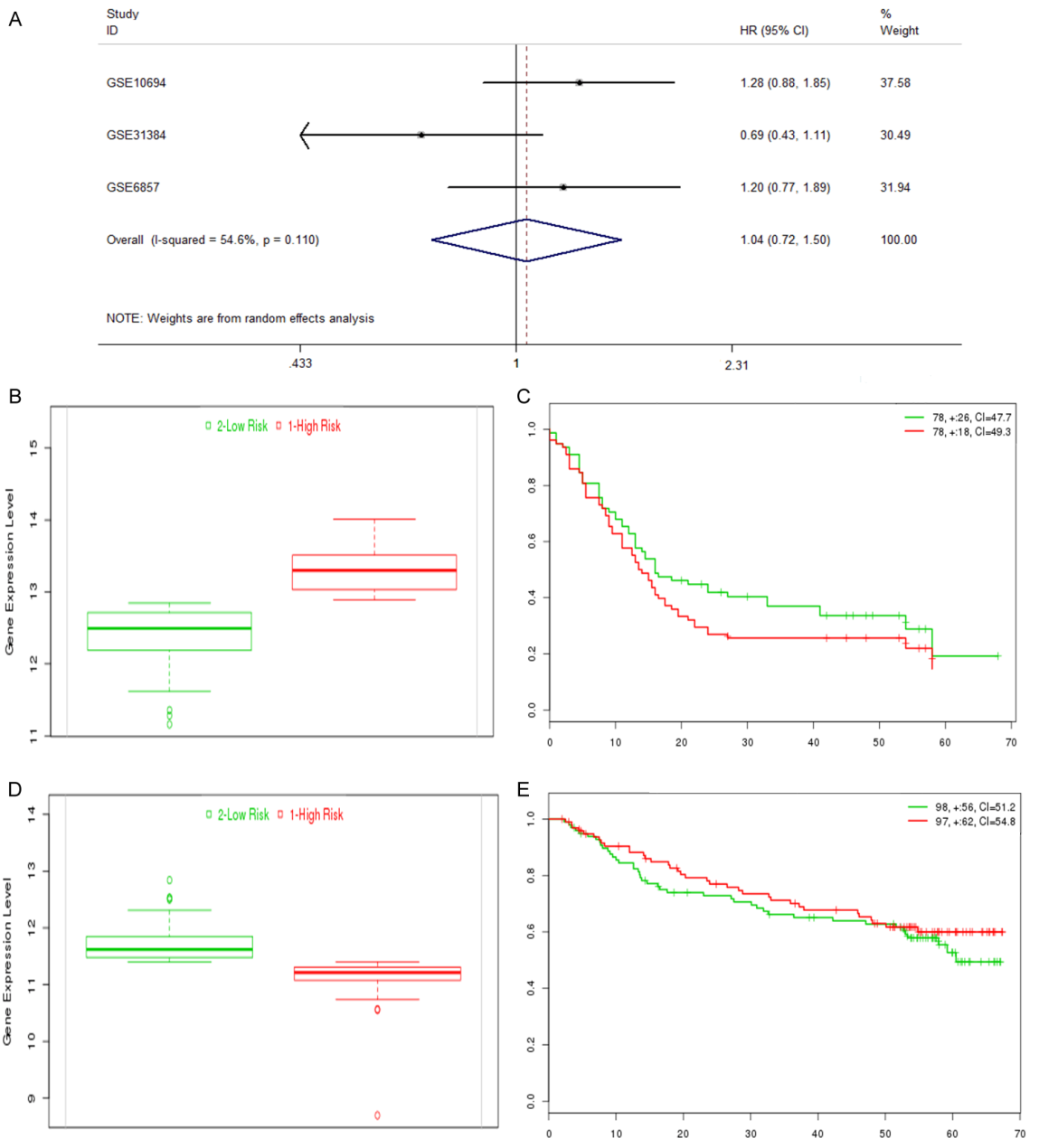


Figure 5. Kaplan-Meier survival curve to show the potential miR-191-5p prognostic significance in HCC. A. Forest plots of the three eligible GEO profiles. B and C. A higher miR-191-5p expression implied likely poorer outcomes in GSE10694 (HR=1.280, 95% CI: 0.880-1.850, P=0.197). D and E. A higher miR-191-5p expression implied likely poorer outcomes in GSE6857 (HR=1.204, 95% CI: 0.769-1.887, P=0.413).

Potential hub genes

In total, 19,034 gene records were predicted by 12 online databases after deleting the repeated genes. To ensure accuracy, the sum of 2,032 genes identified by at least four databases was derived as the most likely target genes. By analyzing the gene expression data of GSE22790, we obtained 491 up-regulated DE-genes to integrate with the selected predicted target genes in HCC. Finally, we collected 64 overlapping genes, which could be considered as the likely miR-191-5p targets in the regulation of HCC. The overlapping outcome is illustrated in a Venn diagram in **Figure 6**.

Underlying mechanism of miR-191-5p in HCC by bioinformatics analysis

The candidate targets were then inputted into FunRich 3.1.3 for the subsequent bioinformatics analysis. The most significant pathways and

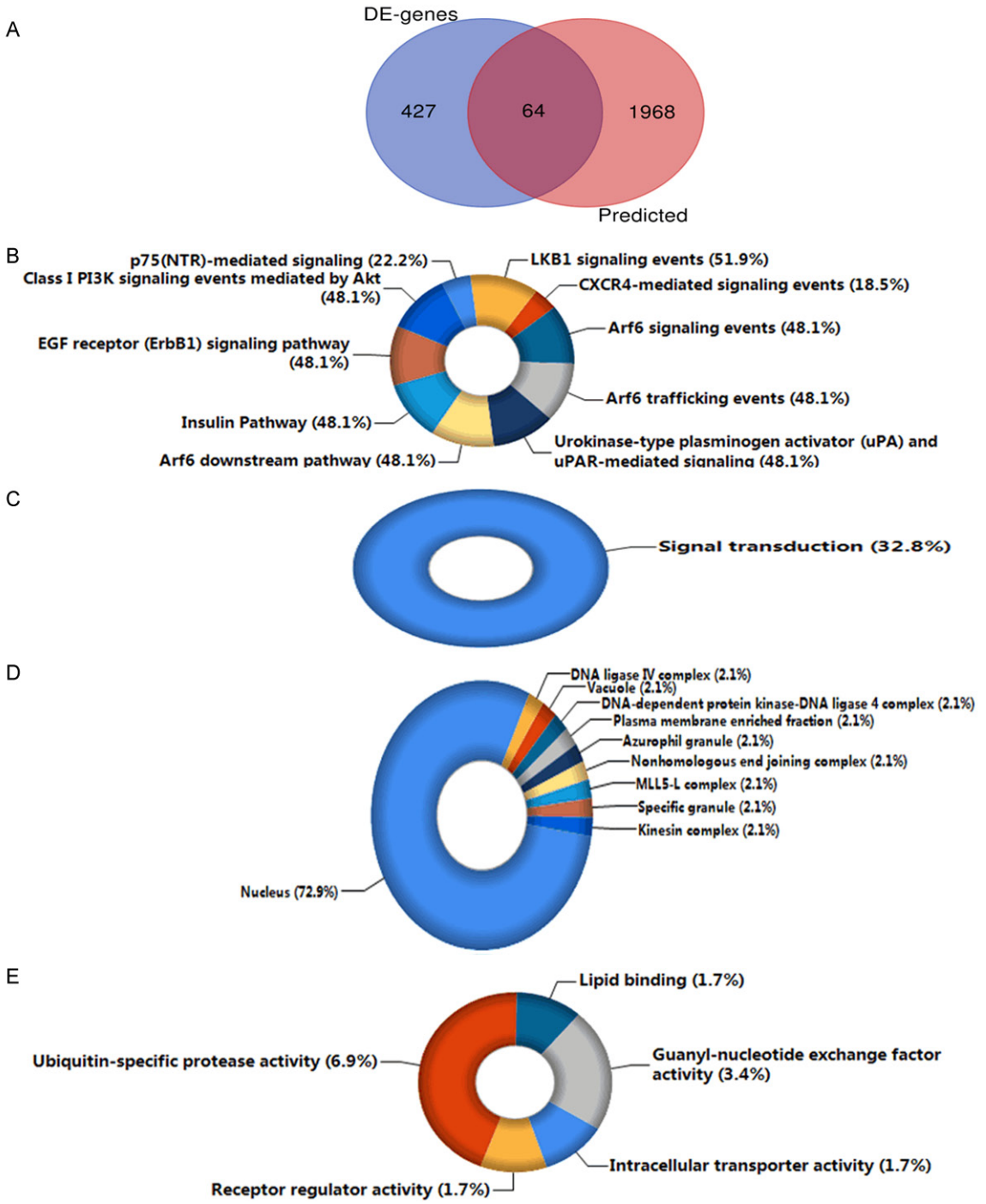


Figure 6. Exploration results for the underlying mechanisms of miR-191-5p in HCC. A. Venn diagram of the crucial genes involved in the bioinformatics analysis. B. The most significant pathways. C. The most significant biological process. D. The most significant cellular component. E. The most significant molecular function.

GO terms ($P<0.05$) are listed in **Table 3**. According to the results, the target genes were highly enriched in several pathways, including p75-neurotrophin receptor (p75-NTR)-mediated signaling, liver kinase B1 (LKB1) signaling events, and CXCR4-mediated signaling events; all were closely related to the growth of HCC cells (**Figure 6**). Additionally, the candidate targets were involved in the biological process of signal transduction and related to activities of certain cellular components, such as the nucleus, DNA ligase IV complex, plasma membrane-

Table 3. The most significantly enriched pathways and gene ontology terms of miR-191-5p

Terms	Count	Percentage (%)	*P*-value
Biological pathway			
p75-NTR-mediated signaling	6	22.222	<0.001
LKB1 signaling events	14	51.852	<0.001
CXCR4-mediated signaling events	5	18.519	0.001
Insulin Pathway	13	48.148	0.001
Internalization of ERBB1	13	48.148	0.001
Urokinase-type plasminogen activator (uPA) and uPAR-mediated signaling	13	48.148	0.001
PDGFR-beta signaling pathway	13	48.148	0.001
EGF receptor (ERBB1) signaling pathway	13	48.148	0.001
Class I PI3K signaling events	13	48.148	0.001
Arf6 signaling events	13	48.148	0.001
Biological process			
Signal transduction	19	32.759	0.034
Cellular component			
Nucleus	35	72.917	<0.001
DNA ligase IV complex	1	2.083	0.007
Plasma membrane enriched fraction	1	2.083	0.013
DNA-dependent protein kinase-DNA ligase 4 complex	1	2.083	0.013
Vacuole	1	2.083	0.013
Non-homologous end joining complex	1	2.083	0.020
Azurophil granule	1	2.083	0.020
Specific granule	1	2.083	0.026
MLL5-L complex	1	2.083	0.026
Kinesin complex	1	2.083	0.029
Molecular function			
Intracellular transporter activity	1	1.724	0.013
Receptor regulator activity	1	1.724	0.019
Ubiquitin-specific protease activity	4	6.897	0.032
Lipid binding	1	1.724	0.035
Guanyl-nucleotide exchange factor activity	2	3.448	0.050

enriched fraction, and DNA-dependent protein kinase-DNA ligase 4 complex (**Figure 6**). Moreover, by targeting the likely targets, miR-191-5p could participate in the regulation of the intracellular transporter activity, receptor regulator activity, ubiquitin-specific protease activity, and other molecular functions (**Figure 6**).

Figure 7 shows the PPI network. The three genes that were greatly involved in the top two enriched pathways and the PPI network could be the vital target genes of miR-191-5p [early growth response 1 (EGR1), NEDD4L, and UBE2D3], as shown in **Figure 7**. According to the pooled result of the profiles and qRT-PCR data, miR-191-5p showed a high expression in HCC. Thus, the genes with decreased expression levels in HCC were recognized as the likely targets of miR-191-5p. Particularly, the expression of EGR1 and UBE2D3 showed a lower level trend in HCC (**Figure 8**). Thus, we hypothesize that EGR1 and UBE2D3 have great potential to be miR-191-5p targets in HCC.

Discussion

In this study, the up-regulation of miR-191-5p was observed in HCC tissues according to the pooled result of our meta-analysis by microarray profiles and qRT-PCR data. The elevated miR-191-5p levels were clearly correlated with the advanced stages, metastasis, and high pathological grade of HCC, suggesting the oncogene role of miR-191-5p. Moreover, miR-191-5p was recognized to be correlated with the modulation of several processes in HCC

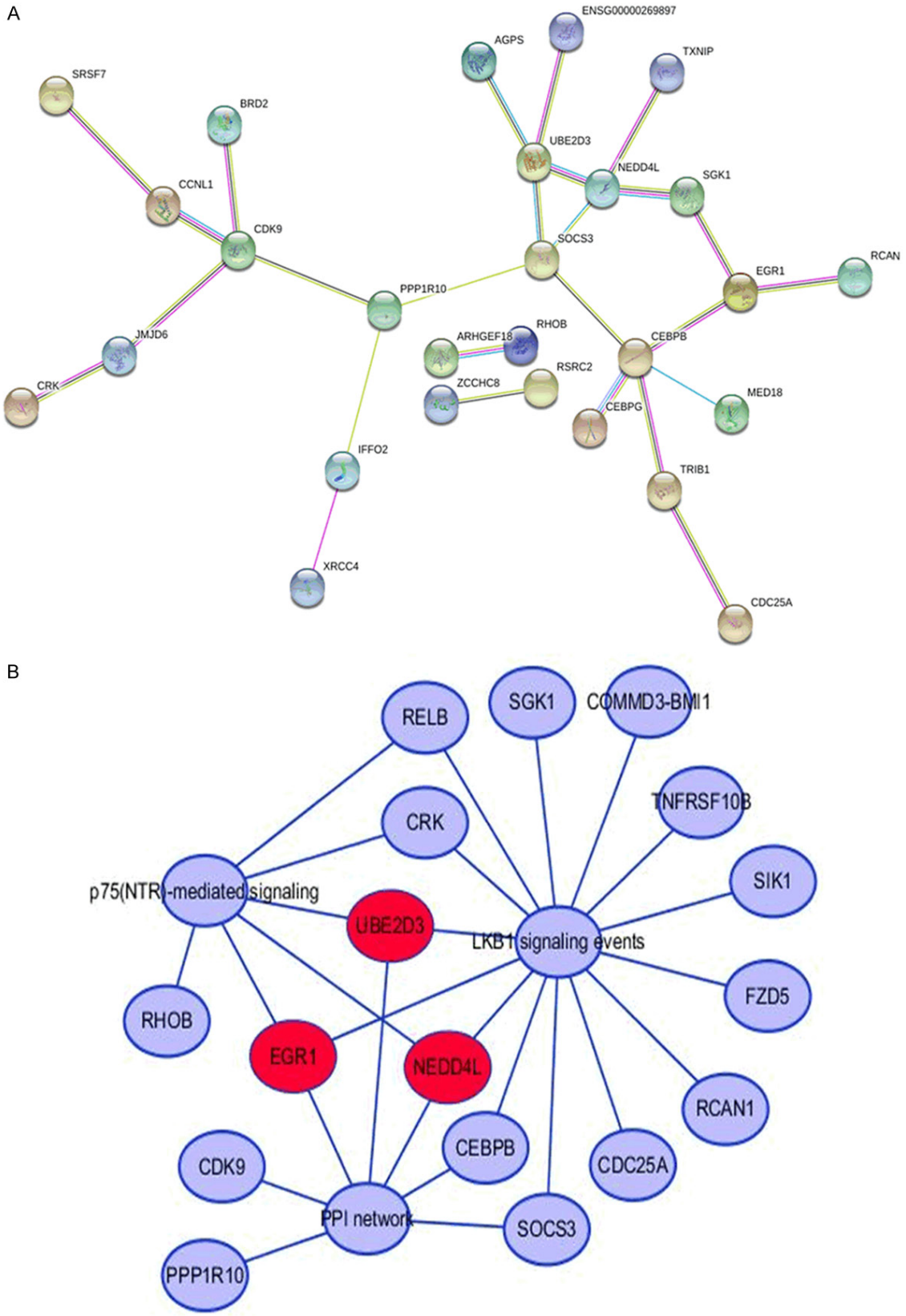


Figure 7. A. Protein-protein interactions of the 64 included genes. B. The likely target genes of miR-191-5p in HCC; we regarded the genes as the miR-191-5p targets that were involved in the top two significant pathways and that frequently interacted with other genes in the PPI network.

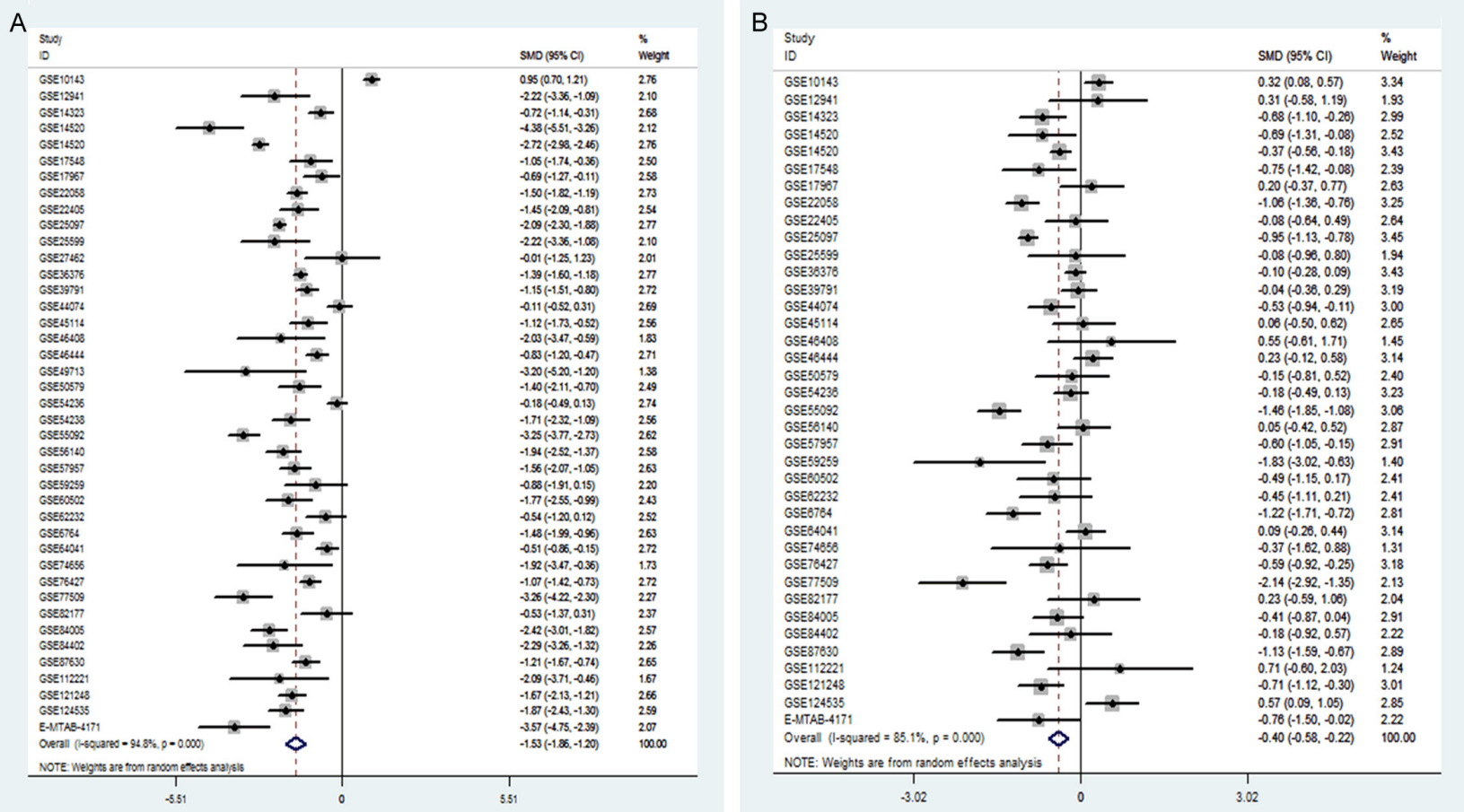


Figure 8. Forest plot of the two target genes. A. UBE2D3 expression was down-regulated in HCC tissues. B. EGR1 expression was decreased in HCC tissues.

cellular metabolism. EGR1 and UBE2D3 were identified as the key genes involved in the modulation network of miR-191-5p in HCC.

Previous studies showed that miR-191-5p was used as an endogenous reference gene based on its suitability for the qRT-PCR analysis of different samples from diverse cancer types, including tissues, serum, or plasma samples, obtained from HCC patients [21, 22]. However, growing evidence reveals that miR-191-5p is differentially expressed in certain cancers. MiR-191-5p has been reported to be significantly elevated in HCC tissues as a carcinogenic factor, thus leading to poor outcomes for patients [23, 24]. He et al. particularly mentioned that the up-regulation of miR-191-5p in HCC patients was potentially correlated with the hypomethylation occurring in the miR-191-5p locus [23]. Similarly, an increased expression of miR-191-5p was previously found in cholangiocarcinoma (CCA). A recent study utilizing qRT-PCR to examine the miRNA levels in CCA confirmed that miR-191-5p was overexpressed and associated with the deterioration of CCA [25]. Another qRT-PCR analysis determined that miR-191-5p was up-regulated in CCA [26]. Consistent with the aforementioned reports, we observed that miR-191-5p showed a high expression in HCC tissues in our analysis of 1,291 clinical samples and that the overexpression of miR-191-5p could lead to an unfavorable outcome for HCC patients.

MiR-191-5p has been applied as a diagnostic biomarker in several cancers. A recent report showed that miR-191-5p was over-expressed in patients diagnosed with pancreatic cancer [27]. Wang et al. found the significant diagnostic capacity of miR-191-5p in osteosarcomas by detecting its expression in the serum of patients [28]. A clinical research also mentioned the early diagnostic value of miR-191-5p in gastric cancer (GC) because it was remarkably up-regulated in the tissues and serum of GC patients in comparison with the normal controls [13]. To date, studies on the diagnostic significance of miR-191-5p for HCC are scarce. Li et al. revealed that miR-191-5p could be used as a specific biomarker combined with other miRNAs, such as miR-221 and miR-let-7a, to diagnose HCC rapidly [29]. Similarly, one of our GEO profiles (GSE50013) showed a higher miR-191-5p level in the HCC plasma samples than in the non-HCC samples. This result implies that miR-191-5p could have a certain diagnostic capacity in HCC through the methods for examining its expression level in the serum or plasma samples of patients and that it is greatly beneficial in the early diagnosis of HCC.

Several studies revealed the underlying mechanism of miR-191-5p in HCC. In 2010, Elyakim et al. initially proposed that the over-expression of miR-191-5p could definitely produce positive effects on HCC growth; by contrast, its suppression induced HCC cell death in an animal experiment, thus implying the potential of miR-191-5p in a new HCC therapy for humans [29]. Afterward, He et al. confirmed the previous results in their report [23]. A new study discovered that an up-regulated miR-191-5p expression was correlated with the induction of epithelial mesenchymal transition, which was possibly related to the tumorigenesis of HCC [30]. Additionally, Huang et al. verified the carcinogenic role of miR-191-5p in HepG2 cells in vitro [31].

In our study, we found that p75-NTR-mediated signaling and LKB1 signaling pathways were greatly enriched through a series of bioinformatics analyses. p75-NTR, a multifunctional receptor that belongs to both the nerve growth factor receptor family and the tumor necrosis factor receptor superfamily, demonstrates several biological activities related to neuronal cells, prominently the meditation of neural cell death [32, 33]. p75-NTR was speculated to function as an inhibitor in the growth and proliferation of tumor cells and as an inducer in cancer cell apoptosis [34]. To date, only He et al. mentioned that a significant decrease in p75-NTR expression was found in clinical liver cancer tissues and that increased p75-NTR levels in vitro could drastically attenuate the growth of HCC cells, mainly by inducing a cell cycle arrest [35]. Thus, they hypothesized that p75-NTR could potentially act as a tumor suppressor in HCC.

LKB1, also called serine/threonine kinase 11, prominently induces the activation of AMP-activated protein kinase after phosphorylation, and it is involved in the suppression of the developmental processes of cancer cells as a tumor inhibitor in many cancers [36-38]. A previous study demonstrated that the LKB1 expression decreased in HCC cells in vitro and that the reduction of LKB1 led to the epithelial-

mesenchymal transition in HCC, suggesting the protective role of LKB1 in liver cancer [39]. Wang et al. established that chemokine ligand 17 is a promoter in HCC progression by blocking the nucleo-cytoplasmic translocation of LKB1 to reinforce the malignant migration and invasion, thus implying the protective role of LKB1 in HCC [40]. Several studies also identified the similar HCC suppressive function of LKB1 [41-43]. Thus, we assumed that miR-191-5p could be a vital participant in promoting the occurrence and development of liver malignant tumors by governing the above two pathways as an oncogene. However, further experiments are required to confirm this speculation.

We observed that EGR1 and UBE2D3 were both involved in the above-described pathways and frequently interacted with other genes in the PPI network. Moreover, the expression levels of the two genes were down-regulated in HCC tissues based on the data mining of the current microarray information, thus implying that miR-191-5p could be implicated in HCC carcinogenesis by targeting the two genes.

Early growth response 1 (EGR1) encodes a nuclear protein that manifests as a vital regulator involved in transcriptional processes, and it activates its target genes to produce proteins associated with differentiation and mitogenesis [44, 45]. Recent studies showed that EGR1 had a suppressive role in certain cancers (e.g., breast cancer and esophageal squamous cell carcinoma) and that miR-191-5p could induce cancer initiation and progression by negatively regulating EGR1 expression [46, 47]. However, research showing the relationship between EGR1 and miR-191-5p in HCC is scarce, and previous studies failed to evaluate the exact function of EGR1 in HCC. Hao et al. found that EGR1 showed a slight or even no expression in HCC cell lines and tissues and that it could be considered a type II tumor inhibitor [48]. They and Wang et al. [49] validated the inhibitory effect of EGR1 in an in vitro experiment. By contrast, the tumorigenic role of EGR1 was also reported [50, 51]. Magee et al. summarized in a review that EGR1 could be an activator or a repressor in liver cancer [52]. We identified a low expression of EGR1 in HCC tissues based on the GEO and ArrayExpress profiles. This low expression could be inversely regulated by miR-191-5p, thus suggesting its potential HCC suppressive role. Therefore, further experiments remain to be conducted.

UBE2D3 encodes the ubiquitin conjugating enzymes E2D3 and is implicated in ubiquitination pathways to modulate a series of cellular activities, including cell growth, apoptosis, DNA damage repair, and carcinogenesis [53, 54]. Accumulating evidence shows that the overexpression of UBE2D3 will decrease cell motility and invasiveness likely by inactivating the human telomerase reverse transcriptase, a risk factor in diverse cancers [55, 56]. Additionally, an elevated UBE2D3 expression was proved to increase the radiosensitivity of breast cancer and esophageal carcinoma cells as a promising molecular target [55, 57, 58]. However, the correlation between UBE2D3 and miR-191-5p in HCC has not been reported yet. Interestingly, we found that miR-191-5p was potentially implied in the regulation of ubiquitin-specific protease activity in our study, thus indicating UBE2D3 to be an inhibitor in HCC through the negative regulation of miR-191-5p. Further studies are required to elucidate our hypothesis.

Conclusion

In sum, our comprehensive analysis of in-house qRT-PCR and microarray data could recognize the over-expressed level of miR-191-5p in HCC. The bioinformatics analysis was suggestive of the oncogenic role of miR-191-5p likely by inversely governing two cancer-related pathways, namely, p75-NTR and LKB1-mediated signaling events, and its underlying target genes, namely, EGR1 and UBE2D3. These findings strengthen our insight into the cancerogenic effects of miR-191-5p on HCC and provide new perspectives for HCC treatment.

Acknowledgements

The authors are thankful for the use of the GEO and ArrayExpress databases and the software employed in the study. The study was supported by the Promoting Project of Basic Capacity for Young and Middle-aged University Teachers in Guangxi (2017KY0111, 2018KY0123), Innovation Project of Guangxi Graduate Education (YCBZ2017045), the National Natural Science Foundation of China (31760319).

Disclosure of conflict of interest

None.

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