

Original Article

Down-regulation of MiR-365 as a novel indicator to assess the progression and metastasis of hepatocellular carcinoma

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Abstract: MicroRNAs (miRNAs) are involved in the pathogenesis of diverse types of malignancies, including hepatocellular carcinoma (HCC). However, miR-365 has rarely been reported in HCC. The purpose of the current study was to identify the clinical relevance of miR-365 in HCC and examine the potential downstream signaling effectors. Using real-time RT-qPCR, we confirmed that miR-365 expression was markedly decreased in HCC tissues (3.5138 ± 2.2527) compared to that in paraneoplastic liver tissues (6.5950 ± 4.1230 , $P < 0.001$). Receiver operating characteristic curves to assess the diagnostic value of miR-365 in HCC demonstrated that the area under the curve was 0.757. Furthermore, down-regulation of miR-365 was remarkably correlated to the number of tumor nodes, status of metastasis, clinical TNM stage, portal vein tumor embolus and vaso-invasion. In addition to the clinical value of miR-365, a total of 238 downstream direct targets selected by online predictive algorithms and key genes generated from natural language processing and the Cancer Genome Atlas (TCGA) were pooled for bioinformatics analysis. These potential targets were mainly enriched in the Ras Pathway using PANTHER analysis and the 'Pathways in Cancer' using Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. In conclusion, down-regulated miR-365 may contribute to the progression and metastasis of HCC via targeting multiple signaling pathways, and miR-365 may act as a novel biomarker for HCC.

Keywords: Hepatocellular carcinoma, miR-365, progression, metastasis, target genes, bioinformatics enrichment analysis

Introduction

Hepatocellular carcinoma (HCC) remains the leading histologic subtype of primary liver cancer (PLC) and the second most frequent cause of cancer-related mortality, causing nearly one million deaths each year [1-4]. In 2015, liver cancer was still one of the top five leading causes of cancer deaths in both Chinese men and women. A total of 466,100 new liver cancer cases and 422,100 deaths caused by liver cancer were estimated [5]. In 2016, in the United States, 39,230 new cases of liver cancer were predicted, and the number of deaths due to liver cancer reached 27,170 [6]. On account of the high incidence and death rates, diagnosis in the early stage and effective treatments for HCC are essential.

MiRNAs belong to the big family of non-coding small RNAs and consist of approximately 22

nucleotides. In addition, on the basis of base-pairing with the 3'-untranslated region (3'-UTR), miRNAs are capable of adjusting the translation progression of target mRNAs [7-9]. Meanwhile, biological functions, including cell proliferation, apoptosis and differentiation, are widely regulated by miRNAs [10, 11].

As a member of miRNAs, growing evidence has proven that miR-365 plays crucial roles in various types of cancers via targeting different genes. To date, however, only one available study has explored the correlation between miR-365 and the progression of HCC. Chen Z et al. found that miR-365 was expressed at lower levels in HCC tissues than in noncancerous tissues based on a small sample size of patients (n=15) [8]. Furthermore, no studies focusing on the potential target genes of miR-365 in HCC have been published. For this reason, the

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Table 1. Correlation between clinicopathological features and expression of miR-365 in HCC

Clinicopathological feature		n	miR-365 expression condition (2 ^{-ΔCq})		
			($\bar{x} \pm SD$)	t	P-value
Tissue	HCC	101	3.5138 ± 2.2527	-6.591	<0.001
	Adjacent noncancerous liver	101	6.5950 ± 4.1230		
Age, years	<50	51	3.6463 ± 2.5742	0.595	0.553
	≥50	50	3.3786 ± 1.8861		
Gender	Male	80	3.3773 ± 2.1118	-1.028	0.313
	Female	21	4.0338 ± 2.7198		
Differentiation	High	7	3.2418 ± 1.3391	-0.177	0.860
	Moderate	64	3.5061 ± 2.2913		
Size (cm)	<5 cm	21	3.7295 ± 2.6558	0.491	0.624
	≥5 cm	80	3.4571 ± 2.1499		
Tumor nodes	Single	57	4.0514 ± 2.6486	3.051	0.003
	Multi	44	2.8173 ± 1.3363		
Metastasis	-	49	4.2535 ± 2.8033	3.297	0.001
	+	52	2.8167 ± 1.2379		
Clinical TNM stage	I-II	25	5.3872 ± 3.1153	3.861	0.001
	III-IV	76	2.8975 ± 1.4510		
Portal vein tumor embolus	-	69	3.8938 ± 2.4727	3.104	0.003
	+	32	2.6944 ± 1.3937		
Vaso-invasion	-	63	3.9581 ± 2.5914	3.071	0.003
	+	38	2.7771 ± 1.2523		
Tumor capsular infiltration	+	49	3.8849 ± 2.4052	1.620	0.108
	-	52	3.1640 ± 2.0611		
AFP	Positive	39	3.8567 ± 2.4387	1.457	0.149
	Negative	46	3.1578 ± 1.9833		
Cirrhosis	+	47	3.4700 ± 2.0554	-0.181	0.857
	-	54	3.5519 ± 2.4299		

Note: t, Student's t-test. Abbreviations: SD, standard deviation; TNM, tumor-node-metastasis; AFP, alpha-fetoprotein.

clinical value of miR-365 as well as its potential molecular mechanisms remains largely unknown.

To identify a more comprehensive association between miR-365 expression and the development of HCC, we investigated the clinical role of miR-365 expression by real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR). We further performed target prediction and bioinformatical enrichment annotations to reveal the prospective signaling pathways of miR-365 in HCC.

Materials and methods

Clinical tissue samples

A total of 101 HCC cases and the clinicopathological data of consecutive patients were col-

lected prospectively from January 2012 to February 2014 in the First Affiliated Hospital of Guangxi Medical University, P. R. China. Cancerous and paraneoplastic liver tissue from 80 male and 21 female HCC patients were resected for formalin-fixed paraffin-embedded (FFPE) blocks. The clinical parameters of the selected patients were sorted and are displayed in **Table 1**. The local ethics committee approved the research protocol. Written informed consents were signed by all of the patients involved for the agreement for the use of the samples for research.

Isolation of total RNA and RT-qPCR

As in our preceding reports, the miRNeasy FFPE Kit (QIAGEN, the Netherlands) was adopted to extract total RNA from both the HCC and paraneoplastic liver tissue samples [12-23].

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The reverse transcription step was performed with a TaqMan MicroRNA Reverse Transcription Kit (4366596, Applied Biosystems). Then, the expression of miR-365 was detected using RT-qPCR. In each sample, the normalization of miR-365 quantity was performed by comparison with its inner reference, which was the combination of RNU6B (Applied Biosystems, cat no 4427975-001093) and RNU48 (Applied Biosystems, cat no 442975-001006) as confirmed previously [15-23]. The primers of miR-365, RNU6B and RNU48 were obtained from Applied Biosystems. RT-qPCR for miRNA was carried out on Applied Biosystems PCR7900. Ultimately, the expression of miR-365 was calculated by the formula of $2^{-\Delta\text{Ct}}$ [12, 15-23].

Statistical analysis

Statistical analysis was performed by SPSS 22.0. Values were all shown as the mean \pm standard deviation (SD), including data from RT-qPCR. The difference of miR-365 among three or more groups was analyzed by a one-way analysis of variance (ANOVA) test. In addition, a student's *t*-test was employed to analyze the difference between two paired or unpaired values. Differences were considered statistically significant when the *P* value was under 0.05.

Key HCC genes identified by natural language processing analysis

As we previously reported, natural language processing (NLP) analysis was performed for HCC to identify all specific genes that have been validated from published studies from January 1980 to May 2015 [16, 24].

Identification of differentially expressed genes from the TCGA database

The Cancer Genome Atlas (TCGA) data portal (<https://tcga-data.nci.nih.gov/tcga/tcgaHome-2.jsp>) was used to download RNA sequence data of liver hepatocellular carcinoma (LIHC) directly based on the mRNA level in April 2016. The data sequences were obtained using the Illumina Genome Analyzer Sequencing platform. A total 369 HCC and 50 normal liver tissue samples were included in the downloaded data. In the current study, the differentially expressed genes (DEGs) with a *P*-value <0.05 and a fold change (FC) >2 or <0.5 were selected.

Prediction of potential target genes of miR-365 in HCC

Target genes of miR-365 were predicted via the miRWalk online database (<http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/index.html>), which contains nine predictive programs, including DIANA-mT, miRanda, miRDB, miR-Walk, RNAhybrid, PICTAR5, PITA, RNA22 and TargetScan.

Bioinformatics analysis of potential target genes

To study the potential function and molecular mechanisms of the target genes of miR-365, Gene Ontology (GO) terms, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and PANTHER pathway annotations were employed using DAVID resources (<http://david.abcc.ncifcrf.gov/>) [25]. The pathways of the enrichment annotation analyses were mapped by software Cytoscape 3.4.0. Furthermore, protein-protein interaction (PPI) networks of the genes were produced via the online software STRING (<http://www.string-db.org/>) to demonstrate the correlations among these genes.

Results

Down-regulation of miR-365 detected by RT-qPCR

MiR-365 expression was significantly down-regulated in HCC tissues (3.5138 ± 2.2527), compared with that in paraneoplastic liver tissues (6.5950 ± 4.1230 , $P < 0.001$). Moreover, miR-365 also showed a significantly lower expression pattern in tissue in the III-IV stage (2.8975 ± 1.4510) than that in the I-II stage (5.3872 ± 3.1153 , $P = 0.001$). Compared with the expression level in patients without metastasis (4.2535 ± 2.8033), miR-365 expression was markedly lower in metastatic patients (2.8167 ± 1.2379 , $P = 0.001$). The miR-365 expression level in cases with portal vein tumor embolus (2.6944 ± 1.3937) was largely down-regulated compared to that in cases with no portal vein tumor embolus (3.8938 ± 2.4727 , $P = 0.003$). MiR-365 expression was significantly lower in patients with vascular invasion (2.7771 ± 1.2523) than patients free from vascular invasion (3.9581 ± 2.5914 , $P = 0.003$). The level of miR-365 was apparently reduced in patients with multiple tumor nodes (2.8173 ± 1.3363 , $P = 0.003$) compared with that in patients with a

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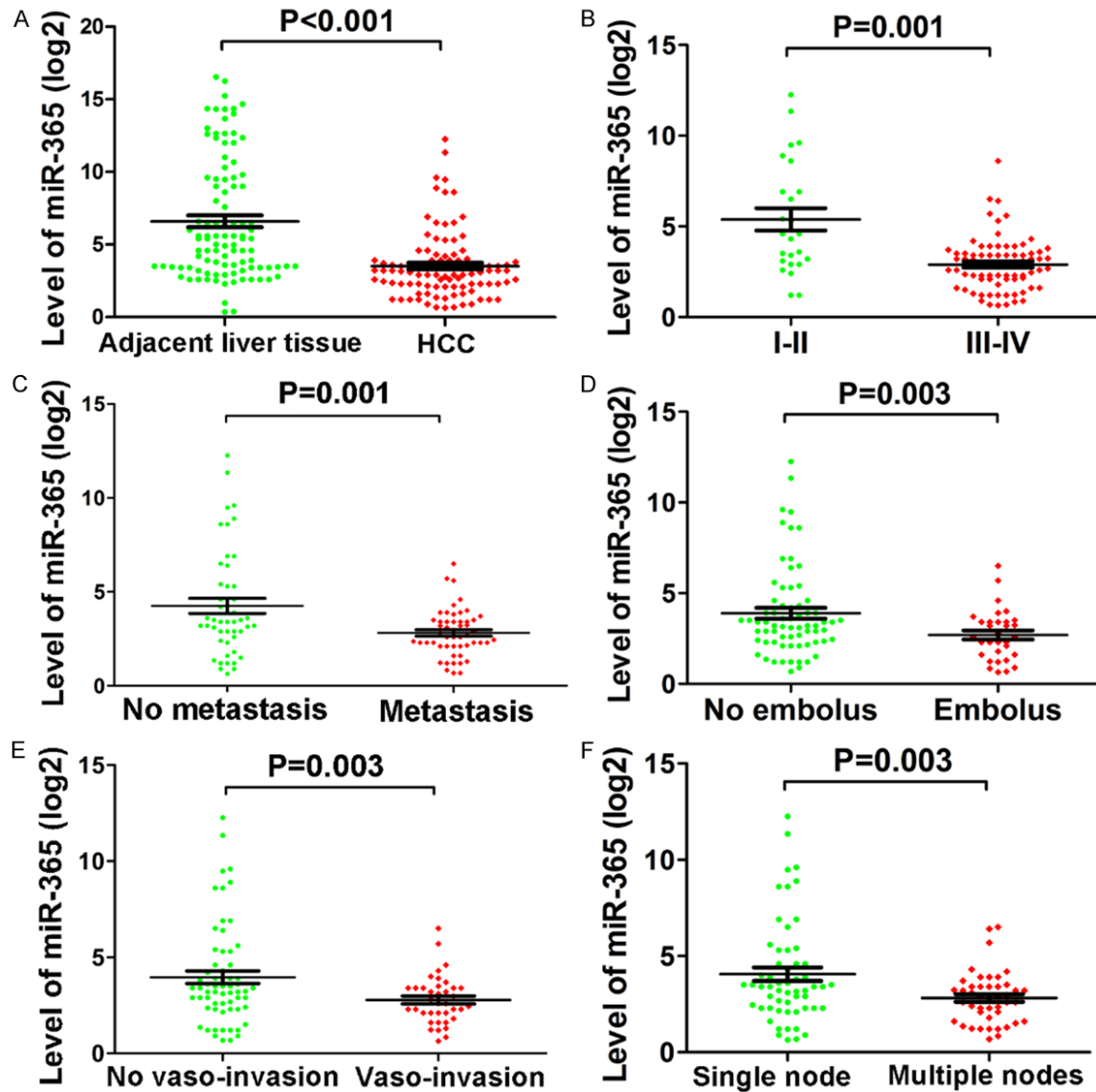


Figure 1. MiR-365 expression in HCC detected by RT-qPCR. Note: A: Tissue, B: Clinical TNM stage, C: Metastasis, D: Portal vein tumor embolus, E: Vaso-invasion, F: Tumor node.

single tumor node (4.0514 ± 2.6486 , $P=0.003$, **Figure 1**). ROC curves were conducted to assess the diagnostic and predictive value of miR-365 for the progression of HCC (**Figure 2**; **Table 2**).

Screening of target genes of miR-365

A flow chart of the process for target prediction is shown in **Figure 3**. Nine online predicting programs from the miRWalk database resulted in the identification of a total of 11,525 target genes of miR-365. Among these predicted genes, 4,762 genes that occurred in no less than three different programs were selected for

further analysis. Simultaneously, 1,800 genes identified from NLP and 4159 DEGs identified from the TCGA database were merged to obtain the potential target genes of miR-365. Ultimately, 238 overlapped genes were identified for further bioinformatics analysis (**Figure 3**).

Enrichment analysis and pathway annotations

The top five significant results of the enrichment analysis and pathway annotations obtained from DAVID and BINGO in the GO analysis are shown in **Table 3**. The most concentrated biological processes (BP) were response to

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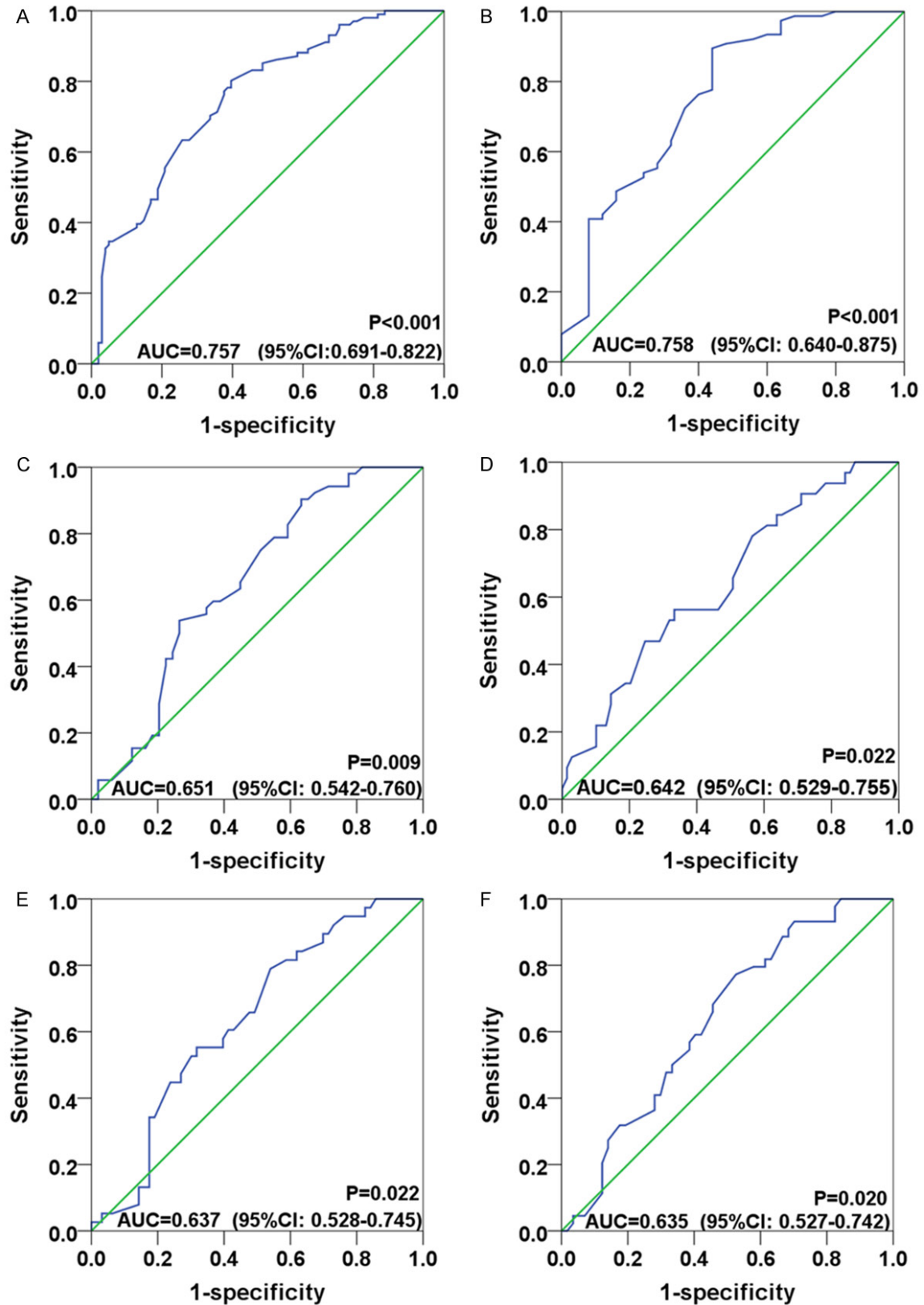


Figure 2. ROC curves of miR-365 expression for different clinicopathological features of HCC. Note: A: HCC, B: Clinical TNM stage, C: Metastasis, D: Portal vein tumor embolus, E: Vaso-invasion, F: Tumor nodes.

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Table 2. ROC analyses of miR-365 expression in HCC

	AUC	P-value	95% CI (LL)	95% CI (UL)	Sensitivity	Specificity	Cut-off value
HCC	0.757	P<0.001	0.691	0.822	80.2%	60.4%	0.406
TNM stage	0.758	P<0.001	0.640	0.875	89.5%	56%	0.455
Metastasis	0.651	P=0.009	0.542	0.760	53.8%	73.5%	0.538
Portal vein tumor embolus	0.642	P=0.022	0.529	0.755	53.6%	43.8%	0.229
Vaso-invasion	0.637	P=0.022	0.528	0.745	78.9%	25%	0.250
Tumor nodes	0.635	P=0.020	0.527	0.742	77.3%	47.4%	0.246

Note: LL, lowerlevel; UL, upperlevel; CI, confidence interval. Abbreviations: AUC, area under curve; HCC, hepatocellular carcinoma; TNM, tumor-node-metastasis.

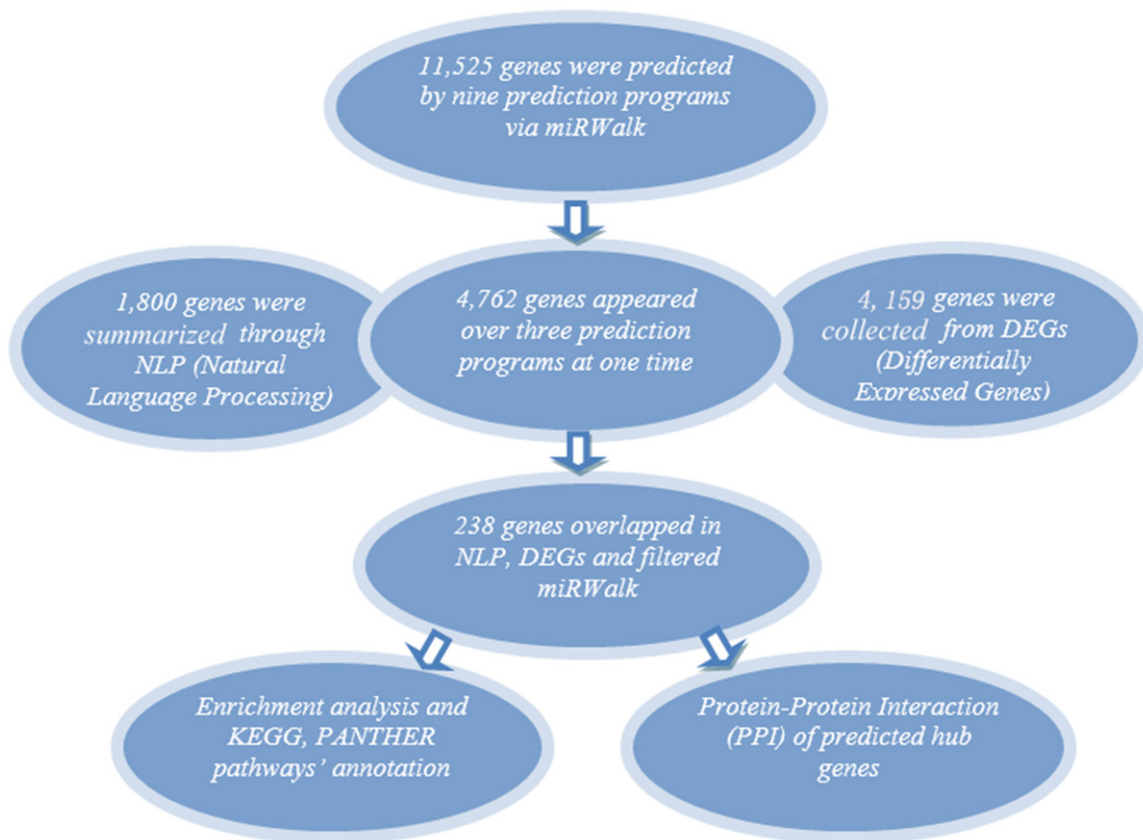


Figure 3. Flow chart of database screening and selection.

organic substance, regulation of cell proliferation, and regulation of programmed cell death ($P<0.001$, **Figure 4**). For cellular components (CCs) from the GO analysis, the genes were most enriched in the CCs of cell surface, cell fraction, and organelle lumen ($P<0.001$, **Figure 5**). For molecular functions (MFs), the genes were prominently involved in enzyme binding, protein heterodimerization activity, protein kinase activity, etc. ($P<0.001$, **Figure 6**). Moreover, potential targets were mainly enriched in the Ras Pathway ($P=2.09E-05$) using PANTHER

analysis, and the 'Pathways in Cancer' ($P=1.45E-16$) was the most remarkable pathway identified in the KEGG analysis. In addition, 27 genes that interacted with two or more other genes were regarded as hub genes, and the protein-protein interaction (PPI) network was mapped with these hub genes (**Figure 7A**). Since the 'Pathways in Cancer' is the most essential pathway for malignancies, we also created the PPI network to identify the hub genes within the 41 genes in this pathway (**Figure 7B**).

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Table 3. Bioinformatical analysis of the potential targets of miR-365

Category	Term	Count	Genes	P-Value	FDR
GOTERM_BP_FAT	GO:0010033~response to organic substance	53	TGFB3, FOXO1, DEK, TLR4, RPS6KB1, MMP3, PTEN, GSTM3, MYD88, CD44, etc.	4.02E-20	7.02E-17
GOTERM_BP_FAT	GO:0042127~regulation of cell proliferation	51	TGFB3, FOXO1, RPS6KB1, TNFSF12, PTEN, PGR, CD47, BDNF, MYD88, CDKN2A, etc.	4.79E-17	8.37E-14
GOTERM_BP_FAT	GO:0043067~regulation of programmed cell death	50	PDIA3, TGFB3, FOXO1, TLR4, TNFSF12, FOXO3, PTEN, BDNF, CDKN2A, MYD88, etc.	1.03E-15	1.74E-12
GOTERM_BP_FAT	GO:0010941~regulation of cell death	50	PDIA3, TGFB3, FOXO1, TLR4, TNFSF12, FOXO3, PTEN, BDNF, CDKN2A, MYD88, etc.	1.15E-15	1.94E-12
GOTERM_BP_FAT	GO:0009725~response to hormone stimulus	34	ERBB4, GRB2, TGFB3, FOXO1, RPS6KB1, SRF, PTEN, IGF1R, GSTM3, KRAS, etc	1.39E-15	2.33E-12
GOTERM_CC_FAT	GO:0009986~cell surface	20	ICAM1, KLRK1, TGFB3, TLR4, RPS6KB1, KIT, CX3CL1, PROM1, NOTCH2, CD44, etc.	9.64E-07	0.001275
GOTERM_CC_FAT	GO:0000267~cell fraction	32	HMGCR, RPS6KB1, FOXO3, IGF1R, GSTM3, KRAS, BCL2, CYP26B1, RAC1, FUT4, etc.	2.66E-04	0.350684
GOTERM_CC_FAT	GO:0043233~organelle lumen	46	ING3, PDIA3, LIN9, SRL, DNAJC10, TGFB3, FOXO1, TBP, NR3C1, KIT, etc.	2.79E-04	0.367937
GOTERM_CC_FAT	GO:0031974~membrane-enclosed lumen	46	ING3, PDIA3, LIN9, SRL, DNAJC10, TGFB3, FOXO1, TBP, NR3C1, KIT, etc.	4.32E-04	0.569703
GOTERM_CC_FAT	GO:0044459~plasma membrane part	52	MICB, MPZL1, TGFB3, RPS6KB1, TLR4, TNFSF12, CD47, CXCR5, CD44, ROBO1, etc.	4.89E-04	0.645476
GOTERM_MF_FAT	GO:0019899~enzyme binding	32	PFKFB2, TLR4, FOXO3, IGF1R, CDKN2A, SERPINA5, BCL2, RAC1, MAP2K7, TOP2A, etc.	2.59E-10	3.75E-07
GOTERM_MF_FAT	GO:0046982~protein heterodimerization activity	19	EGFR, IRAK1, EPAS1, TGFB3, ADIPOR2, TGFB3, ADIPOR1, HGF, SOX6, FOXP1, etc.	6.22E-09	9.00E-06
GOTERM_MF_FAT	GO:0004672~protein kinase activity	30	STK33, ERBB4, NUAQ1, RPS6KB1, KIT, IGF1R, TGFA, CSK, MAP2K7, AKT3, etc.	1.21E-07	1.76E-04
GOTERM_MF_FAT	GO:0004714~transmembrane receptor protein tyrosine kinase activity	10	EGFR, NTRK3, IGF1R, RET, ERBB4, NTRK2, PDGFRA, PDGFRB, KIT, IRS1	1.12E-06	0.00162
GOTERM_MF_FAT	GO:0046983~protein dimerization activity	26	EGFR, IRAK1, CARD9, EPAS1, HMGCR, TGFB3, ADIPOR2, ADIPOR1, SOX6, etc.	1.91E-06	0.002761
PANTHER_PATHWAY	P04393:Ras Pathway	13	GRB2, SRF, STAT3, MAPK1, RPS6KA3, KRAS, CDKN2A, ETS1, SOS1, RAC1, MAP2K7, AKT3, AKT2	2.09E-05	0.021255
PANTHER_PATHWAY	P00048:PI3 kinase pathway	14	IRS2, GRB2, FOXO1, RPS6KB1, FOXO3, IRS1, PTEN, FOXP1, CCND1, KRAS, SOS1, AKT3, AKT2, PIK3R2	5.61E-05	0.057011
PANTHER_PATHWAY	P00032:Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade	9	IGF1R, MAPK1, RPS6KA3, IRS2, GRB2, SOS1, RPS6KB1, MAP2K7, IRS1	9.54E-05	0.096863
PANTHER_PATHWAY	P00018:EGF receptor signaling pathway	15	EGFR, MAPK1, DAB2IP, KRAS, ERBB4, GRB2, SOS1, RAC1, CBL, TGFA, etc.	1.21E-04	0.122745
PANTHER_PATHWAY	P04398:p53 pathway feedback loops 2	10	CDKN1A, PPM1D, CDKN2A, KRAS, RB1, CCNG1, PTEN, AKT3, PIK3R2, AKT2	1.25E-04	0.126658
KEGG_PATHWAY	hsa05200:Pathways in cancer	41	GRB2, TGFB3, KITLG, FOXO1, KIT, PTEN, TPM3, IGF1R, KRAS, CDKN2A, etc.	1.45E-16	1.33E-13
KEGG_PATHWAY	hsa04722:Neurotrophin signaling pathway	24	IRAK1, IRS2, GRB2, FOXO3, IRS1, PTPN11, NTRK3, MAPK1, RPS6KA3, BDNF, etc.	1.50E-13	1.74E-10
KEGG_PATHWAY	hsa05214:Glioma	18	EGFR, GRB2, CDK6, RB1, PTEN, MAPK1, IGF1R, CCND1, CDKN1A, CDKN2A, etc.	4.39E-13	5.09E-10
KEGG_PATHWAY	hsa05218:Melanoma	18	EGFR, CDK6, RB1, HGF, PTEN, IGF1R, MAPK1, CCND1, CDKN1A, CDKN2A, etc.	3.72E-12	4.31E-09
KEGG_PATHWAY	hsa05220:Chronic myeloid leukemia	18	GRB2, TGFB3, CBL, TGFB3, CDK6, RB1, PTPN11, MAPK1, CCND1, CDKN1A, etc.	9.70E-12	1.12E-08

Discussion

HCC represents the fifth most frequent malignant tumor and the third most frequent cancer-related cause of mortality in the world. In this

study, decreased expression of miR-365 in HCC tissues was detected by RT-qPCR. In addition, ROC curves indicated a diagnostic value of the lower miR-365 level in HCC. Furthermore, down-regulation of miR-365 was closely corre-

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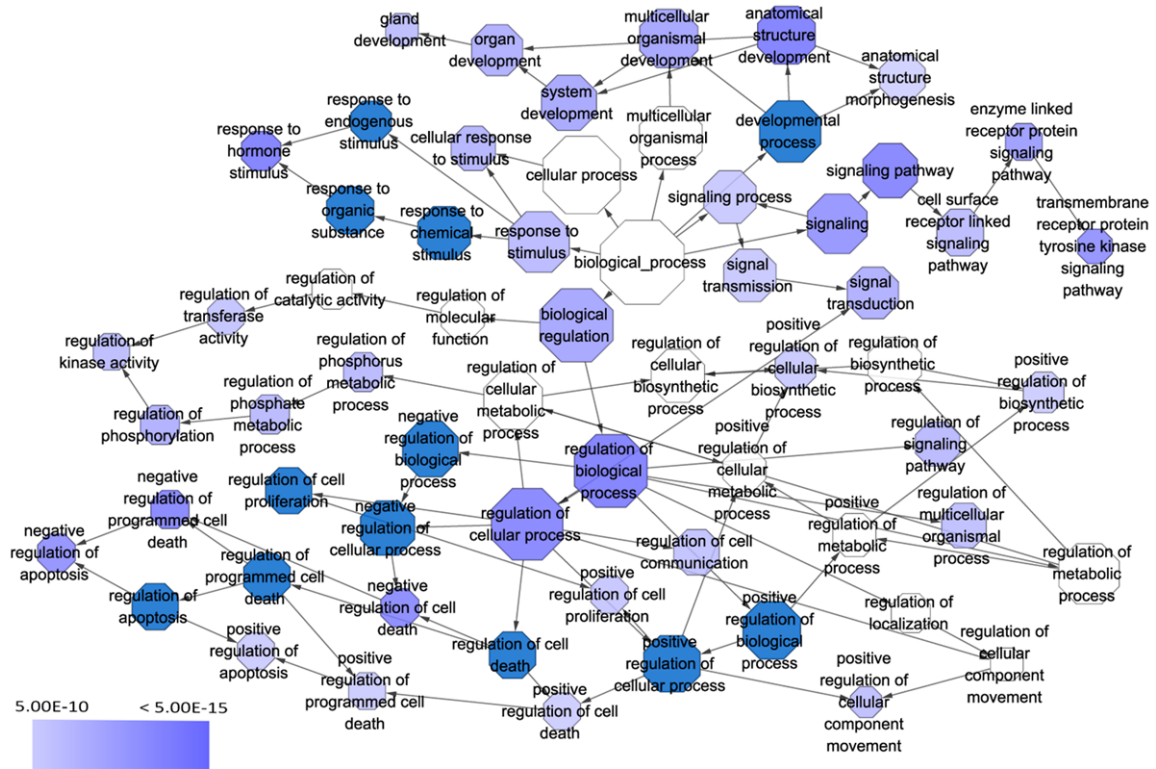


Figure 4. Network of biological processes (BPs) of predicted targets of miR-365. Note: Each octagon represents a biological process in which potential target genes of miR-365 participated. The deeper color of the octagons indicates a higher significance in BPs.

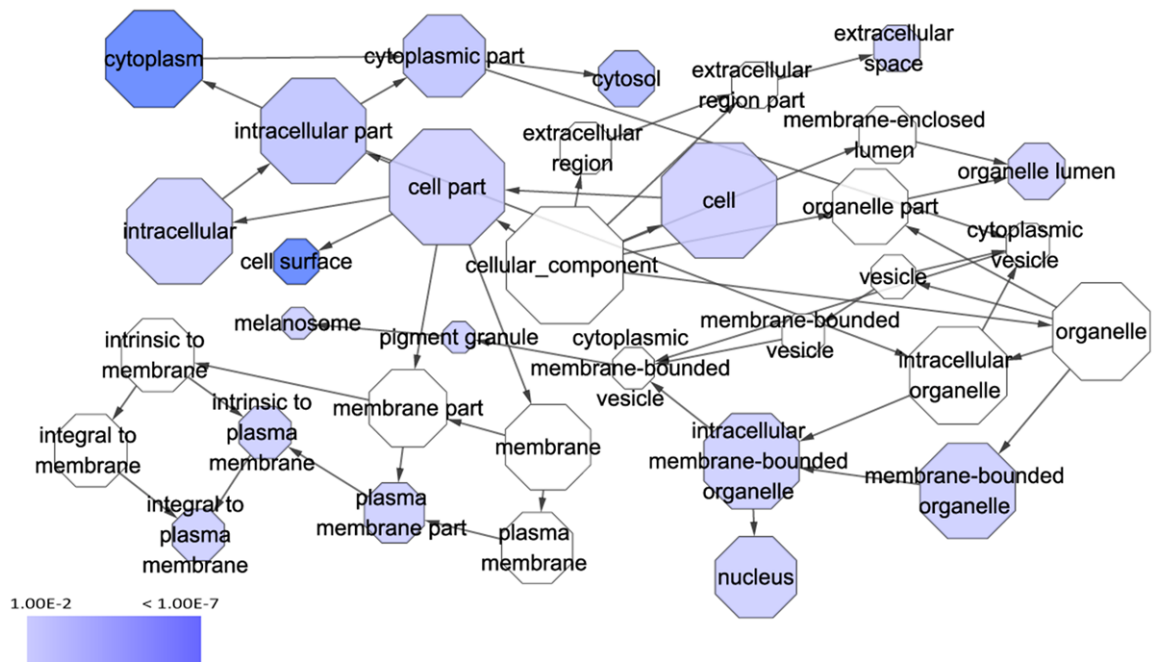


Figure 5. Cellular component (CC) network of predicted targets of miR-365. Note: Each octagon represents the cellular components in which potential target genes of miR-365 participated. The deeper color of the octagons indicates a higher significance in CCs.

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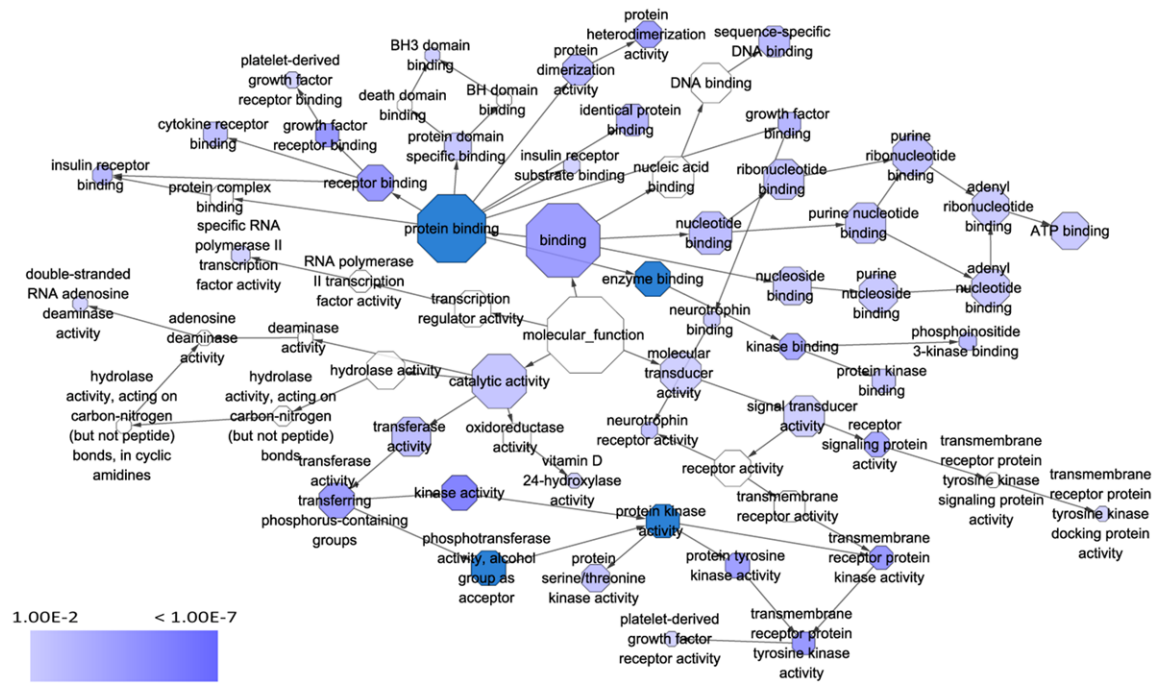


Figure 6. Molecular function (MF) network of predicted targets of miR-365. Note: Each octagon represents the molecular functions in which potential target genes of miR-365 participated. The deeper color of the octagons indicates a higher significance in MFs.

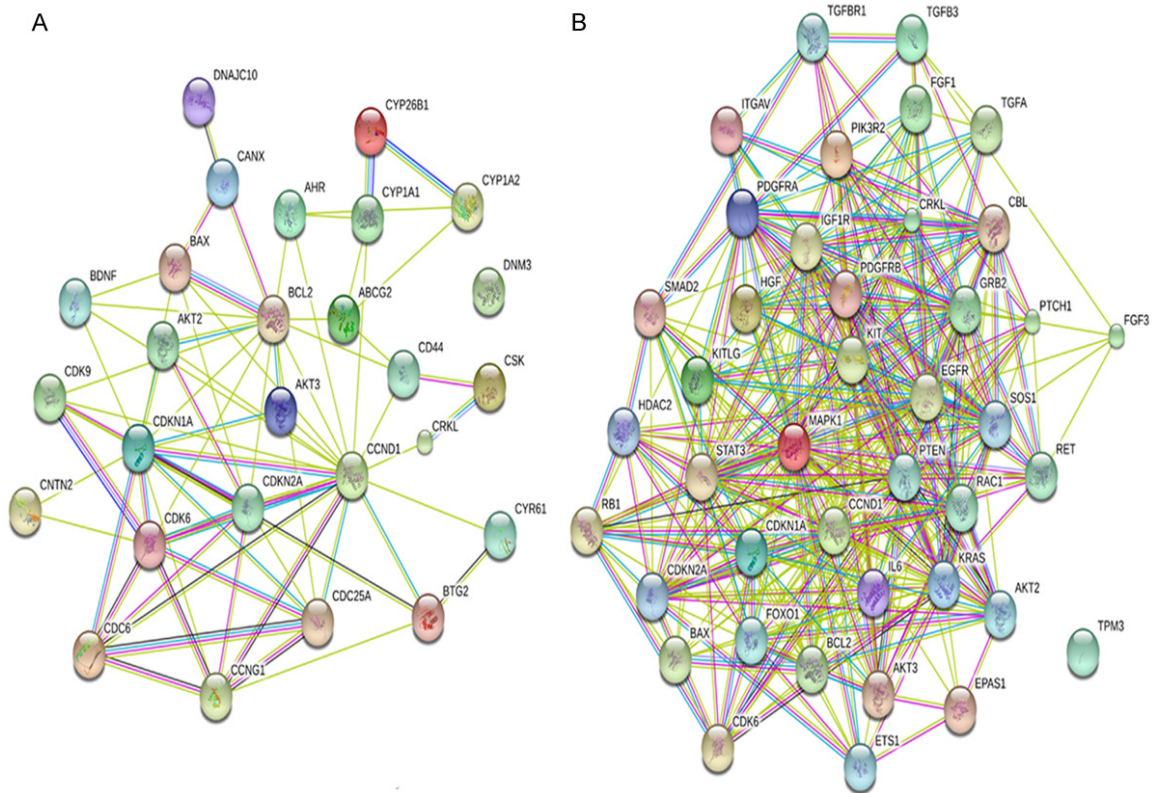


Figure 7. The protein-protein interaction (PPI) networks of overlapped predicted target genes of miR-365. Each node stands for various genes. A line is representative of a connection between two different genes. Note: A. Network of 27 hub genes of miR-365 in HCC. B. Map of 41 predicted target genes from the 'Pathways in Cancer' from the KEGG analysis.

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lated to the progression of HCC in the clinic, and this link could be due to various prospective pathways and target genes, which require further validation in the future.

MiR-365 has been identified as a multifunctional non-coding small RNA involved in different diseases. MiR-365 might play an essential role in both the development of human placenta and semiallogenic embryo immunoprotection [26]. As a potential potent therapeutic target of osteoarthritis, mechanical stress and pro-inflammatory responses are mediated by miR-365 [27]. Furthermore, ectopic expression of miR-365 might also be a novel diagnostic or prognostic biomarker in different types of cancers. For instance, in pancreatic invasive ductal adenocarcinoma, the miR-365 level was reported to be up-regulated. MiR-365 could enhance gemcitabine resistance in pancreatic cancer cells [28]. However, in NSCLC patients, down-regulated miR-365 was closely associated with poor differentiation, advanced TNM stage and lymph node metastasis [29]. Low expression of miR-365 in malignant melanoma was strongly correlated to lymph node metastasis status and clinical stage. MiR-365 could inhibit growth, invasion and metastasis of malignant melanoma by targeting neuropilin 1 (NRP1) [30]. MiR-365 was also down-regulated in other cancers, such as cutaneous squamous cell carcinoma [31] and colorectal cancer [32]. All of these growing pieces of evidence show an important but varied role of miR-365 in different tumors.

As for the clinical role of miR-365 in HCC, only one study has been published. Chen et al. [8] reported that the miR-365 level was remarkably lower in HCC tissues than in non-cancerous liver tissues; however, the finding was based on the analysis of an extremely small sample size (n=15). Additionally, an ROC analysis was not conducted to assess the diagnostic value of miR-365 in HCC by Chen et al. [8]. We had attempted to analyze high-throughput data from miRNA-seq based on a TCGA dataset. However, no significant difference between the miR-365 level in HCC and non-HCC tissues was detected (data not shown). However, in this study, down-regulation of miR-365, in accordance with that reported by Chen et al. [8], was confirmed by RT-qPCR with a larger sample size of 101 cases. The data showed that the

level of miR-365 in HCC tissues was 53.28% (3.5138/6.5950) of that in non-cancerous liver tissues. Furthermore, ROC analysis demonstrated a fair accuracy of HCC diagnosis based on the miR-365 level with an AUC of 0.757. Therefore, together with the previous publication [8], we verified the reduction in the miR-365 level in HCC, which suggests that miR-365 could play an essential part in hepatocellular carcinogenesis and gain the potential to become a diagnostic biomarker for HCC.

Since miRNAs are greatly stable in the circulation, this signature might be used for the early diagnosis of human cancers. The circulating level of miR-365 has been reported in several cancers, including pancreatic cancer [33], breast cancer [34, 35], esophageal squamous cell carcinoma [36] and non-small cell lung cancer [29]. However, no study on the circulating level of miR-365 in HCC has been performed. The diagnostic role of miR-365 based on non-invasive approaches is urgently required in the future.

In addition to the pivotal role of miR-365 in the tumorigenesis and clinical diagnosis of HCC, the reduction in the miR-365 level was also related to the exacerbation of HCC as found previously [8] and confirmed by our current study. According to the report by Chen et al., patients with a low level of miR-365 suffered a poorer survival than those with a high level of miR-365. Additionally, a low level of miR-365 was noted to markedly correlate to tumor size, clinical stage and tumor differentiation [8]. In the current study, down-regulation of miR-365 was also verified to be significantly related to the exacerbation of HCC, as shown by several clinicopathological features, including the number of tumor nodes, the status of metastasis, clinical TNM stage, and the status of portal vein tumor embolus and vaso-invasion. Hence, the level of miR-365 in HCC could predict the progression, especially the status of metastasis, of the disease. Unfortunately, we failed to assess the prognostic role of miR-365 in the current cohort due to the short follow-up time for all patients. Another independent cohort with a longer follow-up time is required in the future.

The *in vitro* investigation of Chen et al. [8] could partly explain the clinical role of miR-365 in

HCC. Overexpression of miR-365 in the HCC cell line HepG2 could inhibit cell proliferation and cell migration, which mimics the clinical phenomenon in which patients with higher miR-365 expression tend to be in the early stage of the disease or are unlikely to progress, and vice versa. Importantly, in the clinic, HCC cases with a higher level of miR-365 were not likely to suffer metastasis, most likely due to the suppressive effect of miR-365 on cell migration, as witnessed by the *in vitro* tests [8]. Nevertheless, to date, the in-depth molecular mechanism of miR-365 in HCC remains largely undetermined. No study has unveiled the target genes of miR-365 in HCC. However, the targets of miR-365 have been reported in several cancers. For example, miR-365 has been shown to target adaptor protein Src homology 2 domain-containing 1 (SHC1) and apoptosis-promoting protein BAX in pancreatic cancer cells [28], neuropilin 1 (NRP1) in malignant melanoma [37], thyroid transcription factor 1 (TTF-1) in non-small cell lung cancer (NSCLC) [29], ADAMTS-1 in breast cancer [38], nuclear factor I/B (NFIB) in cutaneous squamous cell carcinoma [39], PAX6 in retinoblastoma [40], and NKX2-1 in lung cancer [41]. To gain a deeper insight into the prospective target genes of miR-365 in HCC, we combined the genes predicted by online software and those key genes closely related to HCC from the NLP and TCGA analysis with *in silico* methods. Eventually, 238 overlapping genes, which were the most significant target genes of miR-365 in HCC, were identified to be enriched in several classical signaling pathways related to the progression and metastasis of HCC, especially in the regulation of cell proliferation and regulation of programmed cell death from the GO analysis, the Ras Pathway from the PANTHER analysis, and the 'Pathways in Cancer' from the KEGG analysis [42-47]. However, dual-luciferase reporter assays are still needed to ascertain the relationship between miR-365 and these prospective target genes in the future.

Conclusion

A reduction or absence of miR-365 was remarkably associated with the occurrence, progression and metastasis of HCC, probably via targeting various genes and pathways. Therefore, we show that the miR-365 level deserves attention as a potential biomarker in the diagnostic and prognostic setting of HCC. More experi-

ments are desired to inspect the molecular mechanism of miR-365 in HCC.

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Disclosure of conflict of interest

None.

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