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Original Article

Down-regulation of miR-133a-3p in hepatocellular carcinoma tissues and its potential regulatory molecular mechanism: a study of qRT-PCR and bioinformatics analysis

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Abstract: MicroRNAs have recently been discovered to be of great importance in regulating biological processes including proliferation differentiation, apoptosis necrosis and invasion. Here, we aimed to verify the clinical implication of miR-133a-3p in hepatocellular carcinoma (HCC) and to explore the underlying regulatory molecular mechanism. Clinical HCC samples (n=95) were enrolled in the present study. The expression of miR-133a-3p was detected by RT-qPCR and its associations with clinicopathological parameters were further analyzed. We next utilized 14 online prediction databases to seek the potential target genes of miR-133a-3p in HCC. The overlapping genes were further evaluated through bioinformatics methods including Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), PANTHER and protein-protein interaction network (PPI). We discovered that the expression of miR-133a-3p in HCC was obviously lower than that in non-HCC liver tissues (P<0.001). Besides, the Receiver Operating Characteristic (ROC) curve analysis exhibited the Area Under Curve (AUC) was 0.683. Additionally, the expression of miR-133a-3p also showed statistical correlation with tumor nodes, metastasis, portal vein tumor embolus and vascular invasion (all P<0.05). GO analysis displayed three items, including regulation of cell proliferation, plasma membrane part, protein kinase activity, which were predominant respectively in biological process (BP), cellular component (CC), molecular function (MF). Through PPI, we also achieved 30 hub genes which were the key target genes of miR-133a-3p in HCC. The information we obtained here might offer new perspectives in clinical diagnosis and elucidate part of regulatory molecular mechanism of miR-133-3p in HCC.

Keywords: MiR-133a-3p, hepatocellular carcinoma, RT-qPCR, bioinformatics, target gene

Introduction

Hepatocellular carcinoma (HCC) accounts for approximately 90% of all patients of primary liver cancer and is the second leading cause of death from malignancies globally [1]. It has been reported that, annually, approximately 65,000 cases of HCC are confirmed and about 60240, patients succumb to the disease in Europe [2]. Well known hazard issues of HCC include viral hepatitis, alcoholism and non-alcoholic fatty liver disease [3-7]. Surgical resection and liver transplantation are the only effective treatments for most patients with HCC. However, only 10 to 20% of the patients are suitable for surgical techniques, due to the difficulty of effectively diagnosing HCC in its early periods [8-12]. Patients with HCC have poor prognosis with a five-year survival ratio of approximately 5% after surgical intervention due to frequent tumor metastasis and recurrence of the disease [13-15]. Thus, it is imperative to discover reliable biomarkers and to identify the key players that suppress these processes, for the purpose of the promotion of groundbreaking therapeutic molecular targets for accelerating the prognosis of HCC patients.

MicroRNAs (miRNAs), which are small, single-stranded non-coding RNAs of 20-25 nucleotides, play crucial parts in many biological processes comprising cell apoptosis, proliferation, development and differentiation. Studies have shown that aberrant miRNAs have oncogenic or

Table 1. Relationship between the expression of miR-133a-3p and clinicopathological features in HCC

Clinicopathological Feature		n	miR-133a-3p expression ($2^{-\Delta Cq}$)		
			Mean ± SD	t	P
Tissue	Non-HCC	95	4.4971 ± 2.19456	3.649[A]	<0.001
	HCC	95	3.2852 ± 2.37943		
Age	<50	49	3.0412 ± 2.1413	-1.032	0.305
	≥50	46	3.5450 ± 2.6080		
Gender	Male	75	3.3707 ± 2.3952	0.676	0.501
	Female	20	2.9645 ± 2.3513		
Differentiation	Well	6	5.1167 ± 2.5841	F=2.052[B]	0.134
	Moderate	60	3.2440 ± 2.5733		
	Poor	29	2.9914 ± 1.7464		
Size	<5 cm	18	3.0939 ± 1.8463	-0.377	0.707
	≥5 cm	77	3.3299 ± 2.4959		
Tumor nodes	Single	52	3.9637 ± 2.6226	3.323	0.001
	Multiple	43	2.4647 ± 1.7494		
Metastasis	No	46	4.1115 ± 2.7144	3.419	0.001
	Yes	49	2.5094 ± 1.7062		
Clinical TNM stage	I~II	22	4.0214 ± 2.9106	1.671	0.098
	III~IV	73	3.0633 ± 2.1691		
Portal vein tumor embolus	No	63	3.7349 ± 2.5418	3.008	0.003
	Yes	32	2.3997 ± 1.7393		
Vascular invasion	No	59	3.8229 ± 2.3915	2.932	0.004
	Yes	36	2.4039 ± 2.1076		
Tumor capsular infiltration	With complete capsule	45	3.7069 ± 2.7309	1.654	0.102
	Infiltration or No capsule	50	2.9056 ± 1.9632		
AFP[C]	Negative	41	3.7541 ± 2.5620	1.796	0.076
	Positive	38	2.7950 ± 2.1483		
Cirrhosis	No	50	3.3396 ± 2.3900	0.234	0.816
	Yes	45	3.2247 ± 2.3931		

A: Student's paired t test. B: One-way analysis of variance (ANOVA) test. C: 16 cases without AFP data.

tumor suppressor abilities while also being correlated with the growth and progression of cancer, depending on the target mRNAs that they regulate [16-19]. Therefore, it is critical to identify their target mRNAs in order to elucidate the role of miRNA in tumorigenesis. Furthermore, miRNAs can act as biomarkers for the diagnosis and prognosis of cancer while also being targets for molecular therapy of cancer [20-25]. MiR-133a-3p is publicized as one of the most commonly downregulated miRNAs in human malignancies such as gastric cancer [26], non-small cell lung cancer [27] esophageal squamous cell carcinoma [28], colorectal cancer [29], cervical cancer [30], ovarian cancer [31] and pancreatic cancer [32] et al. Furthermore, a most recent meta-analysis performed by Xiao et al revealed that decreased miR-133a-3p level was related to poor overall survival with some solid cancers, including osteosarcoma, lung cancer, esophageal cancer, colorectal cancer and pancreatic cancer [33].

Thus far, only two clinical studies explored the role of miR-133a-3p with respect to HCC [34, 35], which showed that miR-133a-3p was down-regulated in HCC tissues and the expression of miR-133a-3p could discriminate between patients with and without HCC. Nevertheless, the sample size was quite small and the clinical role of miR-133a-3p needs additional confirmation. In order to further elucidate the importance of miR-133a-3p in HCC, the purpose of the current study was to explore the clinical implication of miR-133a-3p in HCC with RT-qPCR, also to predict its possible

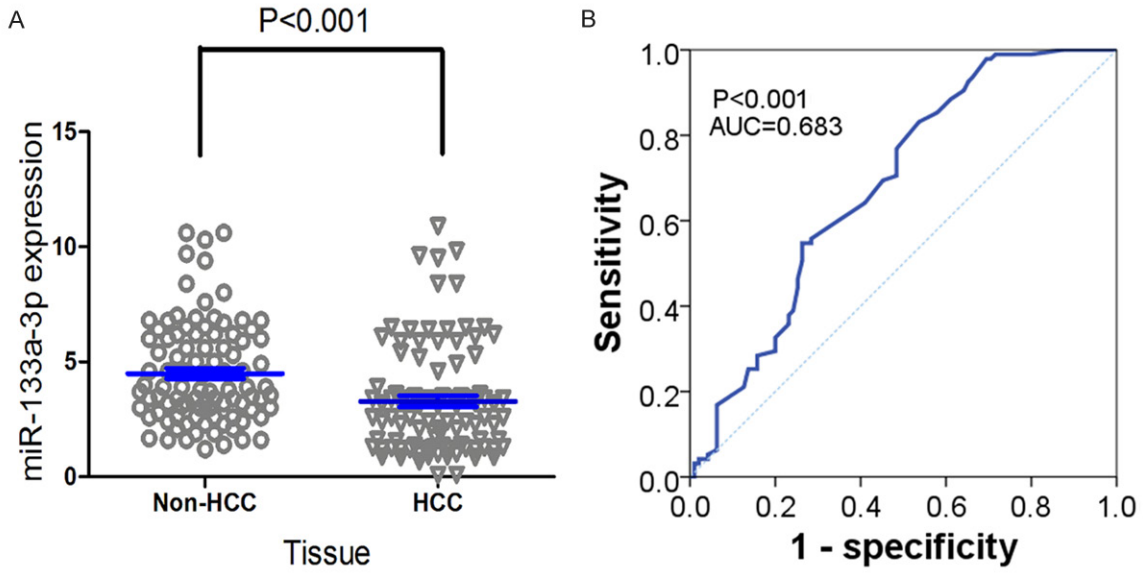

Figure 1. Diagnostic value of miR-133a-3p expression in hepatocellular carcinoma (HCC) tissue. A: Plot diagram of miR-133a-3p expression in non-HCC and HCC tissues. B: Receiver Operating Characteristic (ROC) curve of miR-133a-3p for diagnosis of HCC.

molecular mechanisms with bioinformatics signaling pathway analyses. This is especially vital in the context of biomarker selection for diagnosis and progression prediction of HCC in the future.

Methods and materials

Tissue samples

A total of 95 HCC patients (75 males and 20 females; mean age: 52 years old; range: 29 to 82 years) who underwent hepatectomy in the First Affiliated Hospital of Guangxi Medical University were admitted in our study from March 2010 to December 2011. All formalin-fixed, paraffin-embedded (FFPE) clinical samples specimens were histopathologically confirmed by two pathologists and the homologous non-cancerous liver tissue samples were collected from >5 cm away from the tumors. All the clinicopathological features were included in **Table 1**. This study was approved by the ethics committee of the First Affiliated Hospital of Guangxi Medical University and written informed consent was given by all patients or legal representative.

RT-qPCR

Total RNA, miRNAs included, were obtained from the sections from the clinical samples specimens of 95 HCC patients and the miRNeasy FFPE kit (QIAGEN, KJ Venlo, the Netherlands) was used to conduct RNA isolation as described previously [15, 36-39]. NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA) was used to measure RNA concentration with the OD260/OD280 and OD260/OD230 ratio of total (miRNAs included) isolated from the FFPE tissues.

The combination of RUN6B and RUN48 were considered as the most stable house-keeping reference. The primers of miR-133a-3p, RNU6B as well as RNU48 were performed by TaqMan® MicroRNA Assays. Sequence of miR-133a-3p was as follows: UUGGUCCCCUUCAACCAGCUGU (Applied Biosystems Cat. No. 4427975-000458). The reverse primers were also given by the reverse transcription with TaqMan® MicroRNA Reverse Transcription Kit in a volume of 10 μL. Furthermore, we implemented our real-time qPCR for miRNA trial with Applied Biosystems PCR7900. The expression level of miR-133a-3p was reckoned with the formula $2^{-\Delta cq}$.

Collection of potential target genes of miR-133a-3p

The target mRNAs of miR-133a-3p were predicted based on 14 online prediction databases such as miRWalk; Microt4; miRanda; mir-

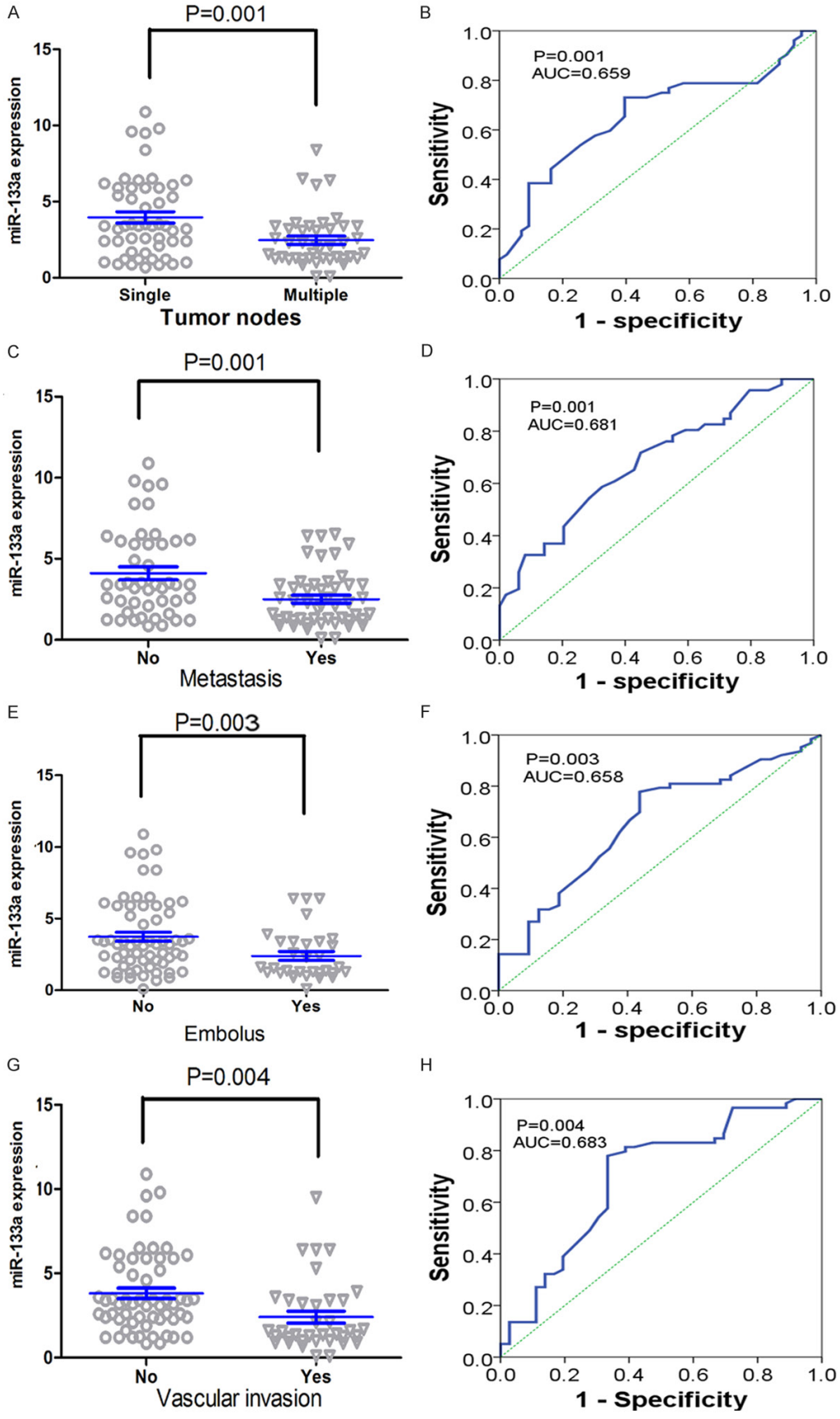
A
P=0.001
miR-133a expression
Single
Multiple
Tumor nodes
B
P=0.001
AUC=0.659
Sensitivity
1 - specificity
C
P=0.001
miR-133a expression
No
Yes
Metastasis
D
P=0.001
AUC=0.681
Sensitivity
1 - specificity
E
P=0.003
miR-133a expression
No
Yes
Embolus
F
P=0.003
AUC=0.658
Sensitivity
1 - specificity
G
P=0.004
miR-133a expression
No
Yes
Vascular invasion
H
P=0.004
AUC=0.683
Sensitivity
1 - Specificity

Figure 2. The relationship between miR-133a-3p and progression of hepatocellular carcinoma (HCC). Scatter plots (A: Tumor nodes, C: Metastasis, E: Portal vein tumor embolus, G: Vascular invasion). Receiver Operating Characteristic (ROC) curves to speculate the status of progression (B: Tumor nodes, D: Metastasis, F: Portal vein tumor embolus, H: Vascular invasion).

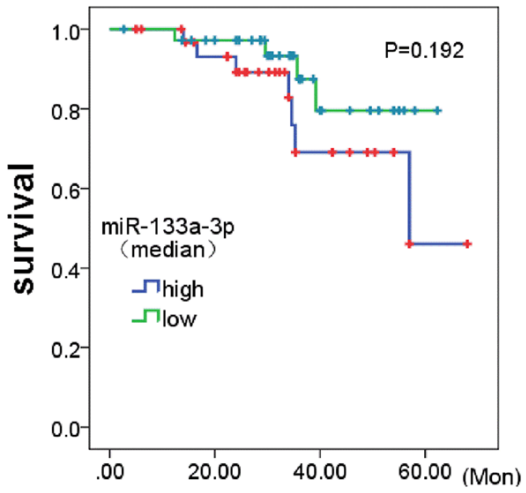

Figure 3. The prognostic implication of miR-133a-3p in hepatocellular carcinoma (HCC) patients. Kaplan-Meier (K-M) curve was drawn to assess the relationship between miR-133a-3p expression and recurrent-free survival.

bridge; miRDB; miRMap; miRNAMap; PicTar; PITA; RNA22; RNAhybrid; Targetscan; mirTarbase and PolymiRTS. We also accepted other validated genes targeted by miR-133a-3p throughout literature review. The selected predicted target genes were further intersected with the key genes assessed by natural language processing (NLP) analysis as reported previously [40, 41].

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses

The potential target genes were next sent to GO analysis, which is based on functional annotation summaries provided by DAVID (Database for Annotation, Visualization, and Integrated Discovery, http://david.abcc.ncifcrf.gov/), and demonstrates the predominant functions of the differentially expressed genes from three aspects: biological process, molecular function as well as cellular component. KEGG pathway analysis (http://www.genome.jp/kegg/) was used to excavate remarkable pathways associated with those target genes. Fisher's exact test and χ^2 test both <0.01 was supposed to be significant.

PPI network construction

The PPI network, which was framed based on the STRING database analysis, was also applied to indicate the association among the potential target genes. The number of nodes and edges was of remarkable significance to show the most important target genes for miR-133a-3p in HCC. The *P* value <0.05 was considered gaining statistical significance.

Statistical analysis

All statistical analyses were adopted using the SPSS 22.0 software package (SPSS, Chicago, IL, USA). The clinical role of miR-133a-3p was evaluated by using student's paired or unpaired t-test. Quantitative values are presented as mean ± SD (range); the spearman correlation was used to examine possible correlations between miR-133a-3p expression and clinical features. Survival curves were plotted by the Kaplan-Meier method and compared using the log-rank test. All tests were two-tailed. Differences were considered to be statistically significant when P was less than 0.05.

Results

Clinical value of miR-133a-3p expression in HCC

It was indicated that the amplification efficiency of all the quantitative real-time PCR (RT-qPCR) reactions, which was supposed to exam the expression of miR-133a-3p, could account from 91.0% to 95.2%.

In the studied population of 95 cases of HCC patients, the expression of miR-133a-3p was prominently downregulated in HCC tissues (3.2852 ± 2.3794) compared with that in noncancer hepatic tissues (4.4971 ± 2.1946, P<0.001, **Table 1**; **Figure 1A**). The area under the ROC curve of low miR-133a-3p was 0.683 (95% CI: 0.611-0.748; P<0.001), with sensitivity of 46.32% and specificity of 83.16% in distinguishing the HCC from noncancerous liver tissues (**Figure 1B**).

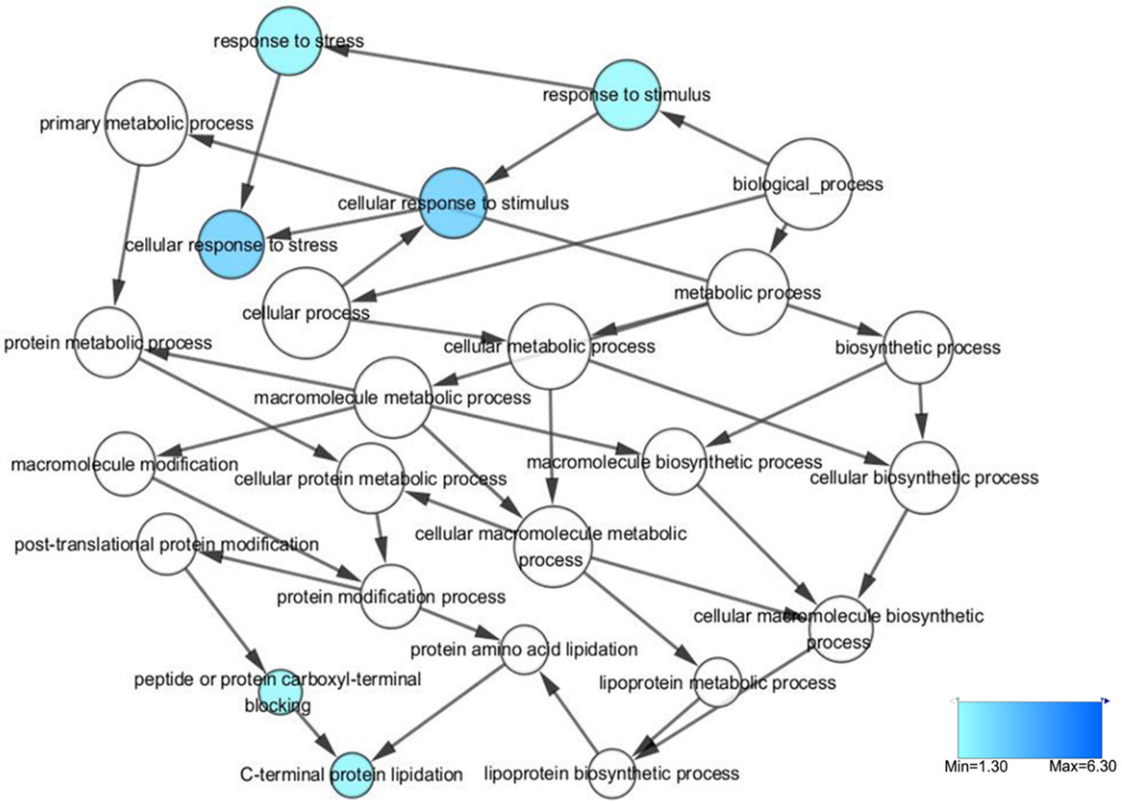

Figure 4. Related signaling pathways of prospective target genes from term of biological process (BP). Gene network analysis with the prospective target genes of miR-133a-3p of BP was drawn by Cytoscape. The circles represented different terms of BP. The relationships among terms were represented by arrows. The significance level of 0.05 was selected for the current Direct Acyclic Graph (DAG) with 25 nodes and 38 edges included. The darker the color appeared the greater significance the term demonstrated.

The level of miR-133a-3p was also pronouncedly reduced in patients with multiple tumor nodes, metastasis, portal vein tumor embolus and vascular invasion, when compared with each counterpart (**Table 1**; **Figure 2**). The association of miR-133a-3p with all clinicopathological parameters was further supported by ROC curves (**Figure 2**) and Spearman analyses. From the correlation test, miR-133a-3p was shown to be negatively associated with tumor nodes (t=3.323; P=0.001), metastasis (t=3.419; P=0.001), portal vein tumor embolus (t=3.008; P=0.003), and vascular invasion (t=2.932; P=0.004), which indicated that low expression of miR-133a-3p could contribute to the progression of HCC. We also inquired into the association between miR-133a-3p and recurrent-free survival of HCC in 70 patients. The follow-up time of HCC patients range from 2.68 to 68 months. The mean recurrent-free survival time was 33.13 ± 14.28 months for all 70 patients. We divided these 70 patients with follow-up information into two groups based on the median level of miR-133a-3p expression which was 2.6. However, there was no remarkable difference of the survival time between patients with high (n=33, 31.7855 ± 15.2347) and low miR-133a-3p level (n=37, 34.3322 ± 13.4732, P=0.192, **Figure 3**).

Bioinformatics analysis of the potential target genes of miR-133a-3p

We continued to explore the potential role and target genes of miR-133a-3p in HCC. The NLP gained a total of 1,800 HCC-related genes, as we previously reported [40, 41]. Meanwhile, 14 online software predicted 18103 target genes and 3995 genes showing up for more than five times were regarded as potential target genes of miR-133a-3p. After 1,800 HCC-related genes from NLP and 3995 potential target genes were overlapped, we eventually achieved 425 genes for the next signaling pathways analyses. For the GO pathway analysis, the potential targets of miR-133a-3p were notably asso-

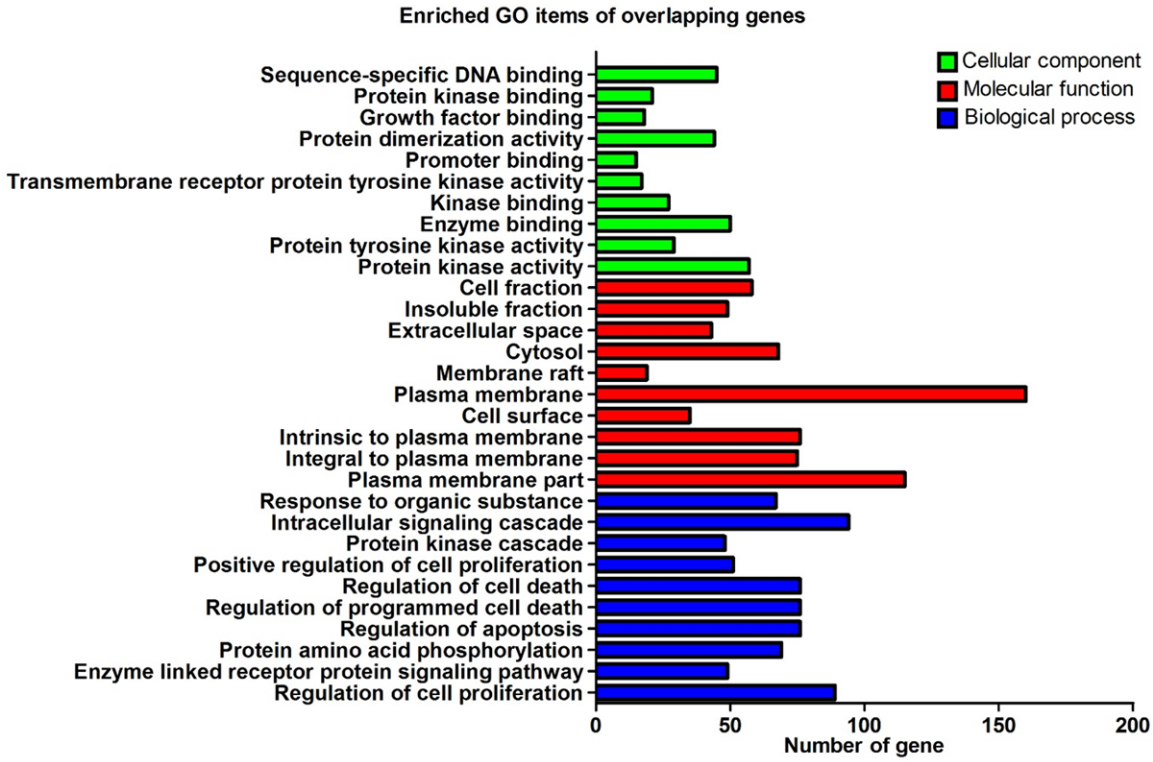

Figure 5. Enriched Gene Ontology (GO) items of overlapping target genes of miR-133a-3p. The significantly enriched annotation of GO categories of the potential mRNAs targeted by miR-133a-3p in HCC. The length of the line delegated the number of overlapping genes in GO.

ciated with regulation of cell proliferation for biological process (P=3.41E-28, **Figures 4**, **5**; **Table 2**), plasma membrane part for cellular component (P=2.04E-12, **Figures 5**, **6**; **Table 2**) and protein kinase activity for molecular factors (P=2.66E-14, **Figures 5**, **7**; **Table 2**) in the GO analyses. And for the KEGG pathway analysis, the potential targets of miR-133a-3p were particularly related to Pathways in cancer, Pancreatic cancer, Neurotrophin signaling pathway, Adherens junction, and Prostate cancer (**Table 3**; **Figure 8**). Furthermore, the top three pathways revealed by PANTHER analysis were kinase/MAP kinase cascade, Ras Pathway and Angiogenesis (**Table 3**; **Figure 8**). To know the core genes among the 425 target genes, PPI network was performed by STRING 10.0 and TP53, SRC and STAT3 with 170, 136 and 97 interaction lines, respectively, ranked as the top three (**Figure 9**).

Discussion

In our study, we observed obvious lower expression of miR-133a-3p in HCC tissues than that in non-cancerous tissues according to data from RT-qPCR. Meanwhile, statistical correlation was also indicated between down-regulation of miR-133a-3p and several clinicopathological parameters such as tumor nodes, metastasis, vascular invasion and tumor embolus, which suggested poor prognosis. To further investigate the function of miR-133a-3p, we predicted its targets via target-predicting database as well as NLP analysis, and then we carried out GO, KEGG, PANTHER, and PPI analysis.

The regulatory function of miR-133a-3p has been highlighted by researchers during the past few years both in malignancies and other diseases. With regards to tumor, Xiao et al [33] conducted a meta-analysis and further demonstrated that down-regulation of miR-133a-3p appeared in osteosarcoma, non-small cell lung cancer, esophageal cancer, colorectal cancer as well as pancreatic cancer, thus extending the decreased expression to all solid cancer. Reports about decreased miR-133a-3p were also seen in cervical cancer [30], bladder cancer [42], ovarian cancer [31], gastric cancer

Table 2. Gene Ontology (GO) functional annotation for the potential targets of miR-133a-3p

Category	Term	Count	*P* Value
GOTERM_BP_FAT	GO:0042127~regulation of cell proliferation	89	3.41E-28
GOTERM_BP_FAT	GO:0007167~enzyme linked receptor protein signaling pathway	49	1.60E-19
GOTERM_BP_FAT	GO:0006468~protein amino acid phosphorylation	69	1.68E-19
GOTERM_BP_FAT	GO:0042981~regulation of apoptosis	76	3.12E-19
GOTERM_BP_FAT	GO:0043067~regulation of programmed cell death	76	5.51E-19
GOTERM_BP_FAT	GO:0010941~regulation of cell death	76	6.80E-19
GOTERM_BP_FAT	GO:0008284~positive regulation of cell proliferation	51	1.69E-17
GOTERM_BP_FAT	GO:0007243~protein kinase cascade	48	2.27E-17
GOTERM_BP_FAT	GO:0007242~intracellular signaling cascade	94	4.46E-17
GOTERM_BP_FAT	GO:0010033~response to organic substance	67	1.51E-16
GOTERM_CC_FAT	GO:0044459~plasma membrane part	115	2.04E-12
GOTERM_CC_FAT	GO:0005887~integral to plasma membrane	75	1.31E-11
GOTERM_CC_FAT	GO:0031226~intrinsic to plasma membrane	76	1.43E-11
GOTERM_CC_FAT	GO:0009986~cell surface	35	1.39E-10
GOTERM_CC_FAT	GO:0005886~plasma membrane	160	3.13E-10
GOTERM_CC_FAT	GO:0045121~membrane raft	19	7.82E-08
GOTERM_CC_FAT	GO:0005829~cytosol	68	7.17E-07
GOTERM_CC_FAT	GO:0005615~extracellular space	43	9.58E-07
GOTERM_CC_FAT	GO:0005626~insoluble fraction	49	1.12E-06
GOTERM_CC_FAT	GO:0000267~cell fraction	58	1.43E-06
GOTERM_MF_FAT	GO:0004672~protein kinase activity	57	2.66E-14
GOTERM_MF_FAT	GO:0004713~protein tyrosine kinase activity	29	7.25E-14
GOTERM_MF_FAT	GO:0019899~enzyme binding	50	7.85E-13
GOTERM_MF_FAT	GO:0019900~kinase binding	27	1.99E-11
GOTERM_MF_FAT	GO:0004714~transmembrane receptor protein tyrosine kinase activity	17	8.01E-11
GOTERM_MF_FAT	GO:0010843~promoter binding	15	8.30E-10
GOTERM_MF_FAT	GO:0046983~protein dimerization activity	44	4.18E-09
GOTERM_MF_FAT	GO:0019838~growth factor binding	18	1.22E-08
GOTERM_MF_FAT	GO:0019901~protein kinase binding	21	1.38E-08
GOTERM_MF_FAT	GO:0043565~sequence-specific DNA binding	45	4.13E-08

Only the top ten pathways were listed for each item of GO.

[26], renal carcinoma [17], breast cancer [43] as well as head and neck squamous cell carcinoma [44]. However, in vulvar carcinoma [45], up-regulated miR-133a-3p was reported to have a correlation with advanced the International Federation of Gynecology and Obstetrics (FIGO) stage, which may suggest that miR-133a-3p plays distinct roles in different cancers.

Up until now, there are only two studies concentrating on the clinical value of miR-133a-3p in HCC. Wang et al [34] discovered that miR-133a-3p was down-regulated in only 10 cases of HCC. And Zhang et al [35] also identified that miR-133a-3p was under-expressed in HCC tissues (n=40) and cell lines (n=4, SMMC-7721, Hep3B, HepG2, and Huh-7). Consistent with the previous reports [34, 35], pronouncedly lower expression level of miR-133a-3p was verified with a larger cohort (n=95) by RT-qPCR in the current study. All these three studies revealed that miR-133a-3p could be closely related to carcinogenesis of HCC. Back to the clinical significance, the AUC of ROC reached 0.683 for the under-expression of miR-133a-3p, which indicated that miR-133a-3p could function as a diagnostic marker with a moderate value for the screening of HCC.

When concerning the role of miR-133a-3p in the development of HCC, only Zhang et al [12] reported that miR-133a-3p was negatively related to the histology differentiation, clinical

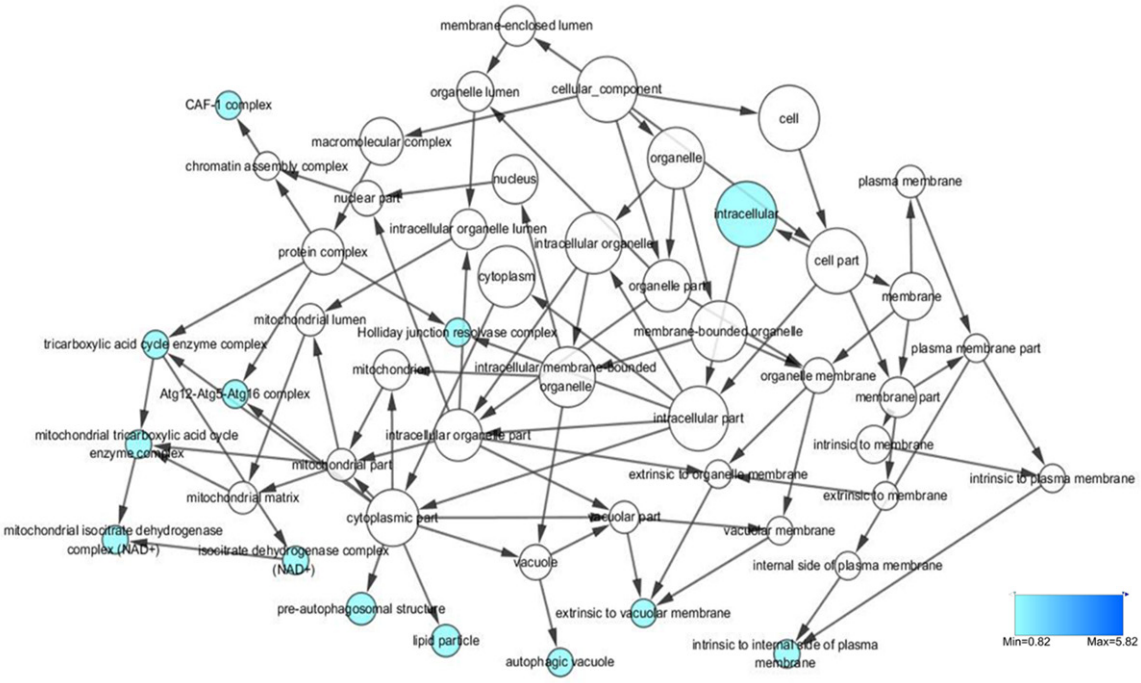

Figure 6. Gene network analysis with the prospective target genes of miR-133a-3p from cellular component (CC). The network was drawn by Cytoscape. The circles were on behalf of different terms of CC. The relationships among terms were represented by arrows. The significance level of 0.15 was selected for the current Direct Acyclic Graph (DAG) which harbored 50 nodes and 86 edges. The color depth suggested that the significance of the corresponding term.

TNM stage, as well as the status of lymph node metastasis of HCC. Accordantly, we also found that the miR-133a-3p was inversely correlated with metastasis. Furthermore, there was also a remarkable relationship between miR-133a-3p and tumor embolus and vascular invasion, which could reflect the status of metastasis and disease progression in the present study. Interestingly, we also noted that in the HCC patients with multiple tumor nodes, the miR-133a-3p was notably lower than that with single tumor node, which indicated a probable correlation of this miRNA with the number of tumor nodes of HCC. We are the first to investigate the prognostic role of miR-133a-3p in HCC. However, no significant result was achieved. The effect of miR-133a-3p on the survival estimate for HCC remains to be explored with larger cohorts. Even though, our current finding, together with Zhang et al [12], suggests that miR-133a-3p might play a crucial part in the development of HCC.

To preliminarily explain the clinical role of miR-133a-3p, several biological functional experiments have been employed. Zhang et al [35] found that under-expressed miR-133a-3p was able to promote proliferation, colony formation, migration, invasion and decrease cell cycle arrest at G0/G1 stage and cell apoptosis. *In Vivo* experiments further revealed that low expression of miR-133a-3p could reduce tumor size and weight in HepG2 nude mouse xenograft model. Wang et al [34] also found that inhibited miR-133a-3p could promote proliferation, migration, invasion and inhibit apoptosis. Likewise, Chen et al [46] stated the similar findings that decreased miR-133a-3p suppressed proliferation, colony formation, migration and invasion in HepG2 and SMMC-7721 cells. All these findings could assist to interpret the mechanism of miR-133a-3p in the progression of HCC, which is closely related to the inhibition of cell growth, migration, invasion and metastasis.

Since a miRNA needs to bind their specific target mRNAs to exert their biological function, including cell growth, migration, invasion and metastasis. We are also wondering what genes miR-133a-3p could target. Only several target genes of miR-133a-3p in HCC have been reported, such as IGF-1R, FSCN1, Versican and MMP-9 [34, 35, 46, 47]. It still remains large

Figure 7. Gene network analysis with the prospective target genes of miR-133a-3p of molecular functions (MF). The network was drawn by Cytoscape and the circles represented different terms of MF. The relationships among terms were represented by arrows. The significance level of 0.06 was selected for the current Direct Acyclic Graph (DAG), which possessed 70 nodes and 77 edges. The color depth demonstrated the significance of the corresponding term.

unknown about the exact target genes of miR-133a-3p in HCC. Therefore, we used online prediction databases to predict the potential target genes of miR-133a-3p in HCC. A total of 425 overlapping target genes were screened out. Further PPI network analysis helped identify 30 hub genes which were the key target genes of miR-133a-3p in HCC. It is worthy of noticing that the verified target gene MMP-9 also exists in the 30 hub genes. This proves the accuracy of our target gene prediction to some extent. Among the 30 hub genes, TP53, CREB1 and STAT3 were the most significant three genes. We further focus on the roles and functions of TP53, CREB1 and STAT3 in HCC for detailed discussion.

Tumor protein 53 (TP53) encodes a tumor suppressor protein consisting of transcriptional activation, DNA binding, and oligomerization domains. Mutated TP53 is related to various cancers. Take HCC as an example, TP53 has recently been reported to be involved in HCC [48-50]. Bae et al found that mutated TP53 interacted with PIN1 was able to promote HCC cells proliferation, migration and invasion [51]. Besides, TP53 mutation indicated poor survival for patients with HCC [52-55]. However, the relationship of TP53 and miR-133a-3p has not been reported. Since TP53 is the most significant core gene of miR-133a-3p in HCC as predicted, further studies are needed to verify this association between TP53 and miR-133a-3p in HCC.

Sarcoma (SRC), e.g., SRC proto-oncogene, non-receptor tyrosine kinase, encodes a tyrosine-protein kinase which can be suppressed by phosphorylation. Recently studies have reported that SRC was involved in HCC through various signal pathways [56-58]. Ito Y et al discovered activated SRC played a role in early stages of HCC [59]. Moreover, Ran et al and Lau et al documented that SRC expression was activated and elevated in HCC [60, 61]. And activated SRC may promote HCC progression such as metastasis [60, 61]. Another study found that activated SRC might serve as an independent prognostic marker in HCC [62]. In sum, SRC is of great importance in HCC. However, the rela-

Table 3. KEGG and PANTHER Pathway of overlapping target genes of miR-133a-3p

Category	Term	Count	*P* Value
KEGG_PATHWAY	hsa05200:Pathways in cancer	49	1.74E-13
KEGG_PATHWAY	hsa05212:Pancreatic cancer	21	3.24E-11
KEGG_PATHWAY	hsa04722:Neurotrophin signaling pathway	24	6.5E-09
KEGG_PATHWAY	hsa04520:Adherens junction	18	4.61E-08
KEGG_PATHWAY	hsa05215:Prostate cancer	19	7.78E-08
KEGG_PATHWAY	hsa04010:MAPK signaling pathway	32	1.29E-06
KEGG_PATHWAY	hsa05219:Bladder cancer	12	1.95E-06
KEGG_PATHWAY	hsa05220:Chronic myeloid leukemia	15	6.2E-06
KEGG_PATHWAY	hsa04062:Chemokine signaling pathway	24	1.3E-05
KEGG_PATHWAY	hsa05210:Colorectal cancer	15	2.42E-05
PANTHER_PATHWAY	P00032:Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade	12	7.51E-05
PANTHER_PATHWAY	P04393:Ras Pathway	16	0.000245
PANTHER_PATHWAY	P00005:Angiogenesis	28	0.000256
PANTHER_PATHWAY	P00048:PI3 kinase pathway	17	0.001231
PANTHER_PATHWAY	P00006:Apoptosis signaling pathway	18	0.001608
PANTHER_PATHWAY	P00018:EGF receptor signaling pathway	19	0.001699
PANTHER_PATHWAY	P00010:B cell activation	13	0.00446
PANTHER_PATHWAY	P05918:p38 MAPK pathway	10	0.005022
PANTHER_PATHWAY	P00033:Insulin/IGF pathway-protein kinase B signaling cascade	13	0.005936
PANTHER_PATHWAY	P00034:Integrin signaling pathway	23	0.011014

Only the top ten pathways were listed for each category.

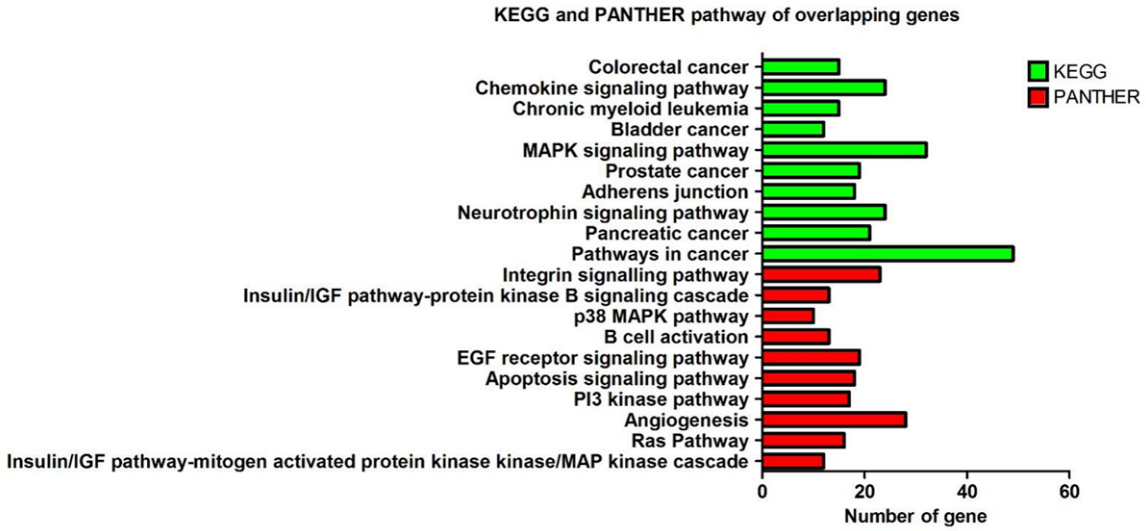

Figure 8. Kyoto Encyclopedia of Genes and Genomes (KEGG) and Protein Analysis Through Evolutionary Relationships (PANTHER) pathway enrichment analysis of miR-133a-3p target genes. The length of the line represented the number of overlapping genes in KEGG and PANTHER pathways.

tionship between SRC and miR-133a-3p has not been reported, which requests further studies.

Signal transducer and activator of transcription 3 (STAT3) encodes the protein which is a member of STAT protein family. STAT3 plays a pivotal role in various cellular processes including cell growth and apoptosis. Different STAT3 signal pathways have been widely reported to play a crucial role in HCC [63-66]. STAT3 has also been reported to exert regulatory function in the expression of miRNAs such as miR-146a and miR-21 in HCC [67, 68]. Furthermore, Lu et al found that STAT3 was targeted by miR-124 which acted as a tumor suppressor in HCC [69].

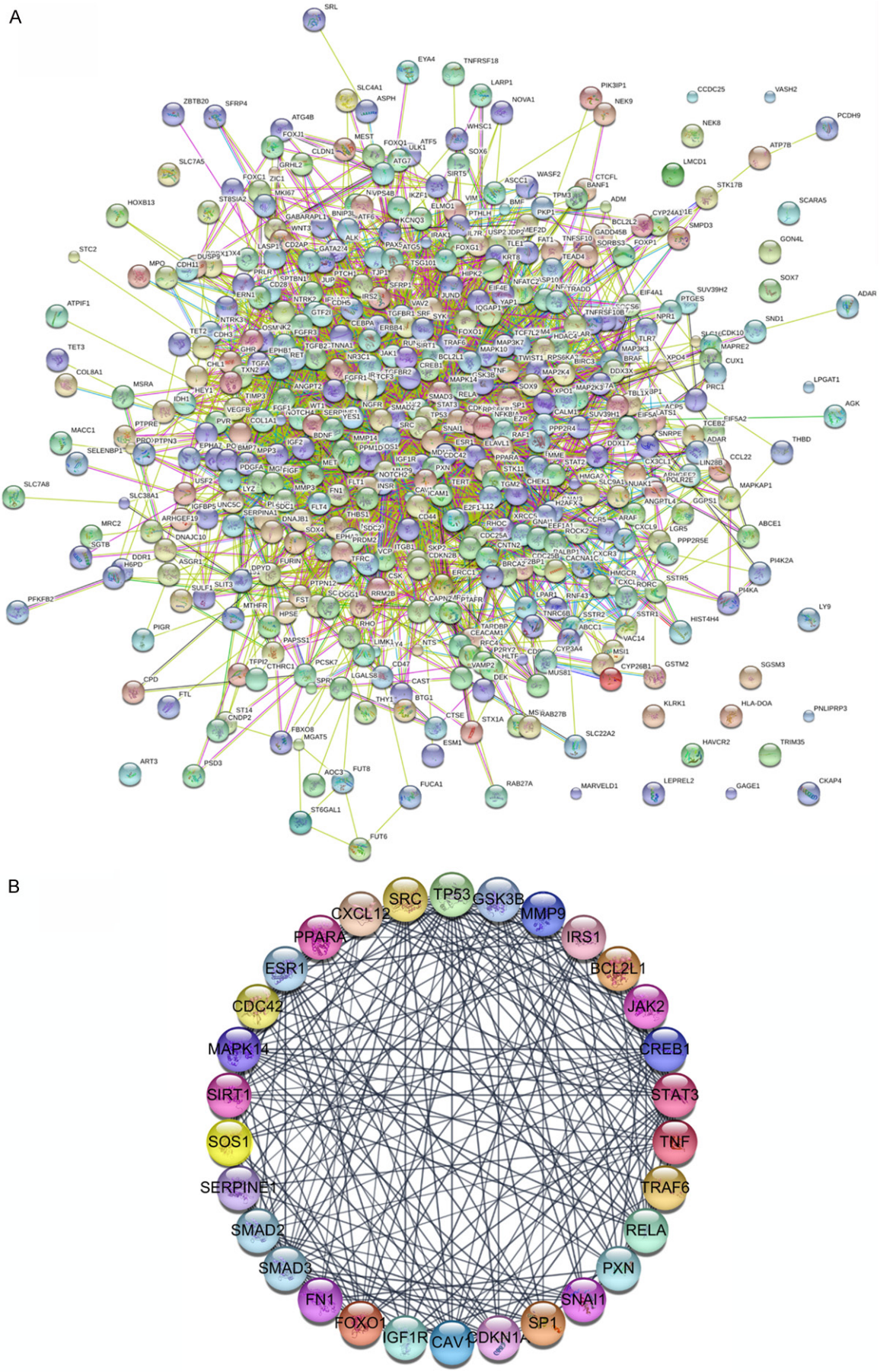
A
B
SRC
TP53
GSK3B
MMP9
IRS1
BCL2L1
JAK2
CREB1
STAT3
TNF
TRAF6
RELA
PXN
SNAI1
SP1
CDKN1A
CAV1
IGF1R
FOXO1
FN1
SMAD3
SMAD2
SERPINE1
SOS1
SIRT1
MAPK14
CDC42
ESR1
PPARA
CXCL12

Figure 9. Protein-protein interaction (PPI) network of the potential mRNAs targeted by miR-133a-3p in HCC. A. Target gene network of PPI consisting of all 425 potential target genes. B. The top 30 hub genes with the largest number of connections in the PPI target gene network.

However, no report about the correlation of STAT3 and miR-133a-3p was available. Since STAT3 has been widely confirmed to play vital parts in HCC, and miR-133a-3p may also contribute to the hepatocarcinogenesis, we could speculate that miR-133a-3p is possible to function via target STAT3 in HCC. Nevertheless, this hypothesis needs further exploration.

Based on the above findings, it was promising to discern the clinical role and potential molecular mechanisms of miR-133a-3p in HCC. Due to the notable down-regulation in HCC tissues and the close relationship with tumor progression, miR-133a-3p could be assessed as a probable candidate for molecular biomarker for HCC in clinic; even this finding needs to be validated in larger prospective studies in the future. The signaling pathways analyses also revealed that miR-133a-3p could target some key pathways in HCC, including the most pivotal hub genes like TP53, SRC and STAT3. Since these potential targets genes have never been confirmed in HCC, their relevant underlying mechanisms need further validation with *In Vitro* and *In Vivo* investigation.

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Disclosure of conflict of interest

None.

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