Clinical significance of microRNA-449a in hepatocellular carcinoma with microarray data mining together with initial bioinformatics analysis

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Abstract. Increasing evidence has demonstrated that microRNA (miR)-449a expression is reduced in various types of tumors and that it serves as a tumor suppressor. However, the molecular mechanism of miR-449a in hepatocellular carcinoma (HCC) has not been thoroughly elucidated and is disputed. Therefore, the aim of the present work was to systematically review the current literature and to utilize the public Gene Expression Omnibus database to determine the role of miR-449a and its significance in HCC. A total of eight original papers and seven microarrays were included in the present study. Based on the evidence, miR-449a was reduced in HCC. miR-449a is likely involved in various signaling pathways and is targeted to multiple mRNA as part of its function in HCC. In addition, a preliminary bioinformatic analysis was conducted for miR-449a to investigate the novel potential pathways that miR-449a may participate in regarding HCC.

Introduction

The development of high-throughput RNA sequencing technology has led to the discovery of thousands of non-coding RNA (ncRNA) genes (1,2). Until now, the number of newly identified ncRNA genes has been increasing and outnumbers the number of coding transcripts (3,4). Additionally, ncRNAs have been demonstrated to participate in a plethora of physiological and pathological processes (5-7). Previously, ncRNAs were believed to be the product of random transcription without any intrinsic function (8,9). With the development of ncRNA research, accumulating evidence has unveiled that ncRNAs are functionally important molecules in the cell (5). In general, ncRNAs are a heterogeneous group of molecules and may be categorized into microRNA (miR), long ncRNA (lncRNA), circular RNA (circRNA), endogenous small interfering RNA, Piwi-associated small RNA, small nucleolar RNA (snoRNA), sno-derived RNA, transcription initiation RNA and miR-offset RNA (10). Among them, the function of miR, lncRNA and circRNA have attracted attention from academia. To date, miRs are the most studied ncRNAs, with hundreds of potential target genes, most of which have been bioinformatically predicted (11-13).

miR-449a, located in the first intron of cell division cycle (CDC)20B (chromosome 5q11), belongs to the miR-34/miR-449 families and has been reported to be downregulated in tumors (14). Previous studies also revealed that miR-449a functions as a tumor suppressor by regulating cell proliferation, cycle procession, apoptosis, migration and invasion in multiple human malignances (15-17). As studies focusing on miR-449a and hepatocellular carcinoma (HCC) are currently rather scarce, the role of miR-449a in HCC tumorigenesis remains unclarified.

The incidence of liver cancer is high throughout the world, particularly in East and South-East Asia and Northern and Western Africa (18). Every year, cases in China alone account for ~50% of the total number of cases and mortalities (19). It has been reported that HCC is the most common type (~80%) of primary liver cancer in the world (18). Currently, with the improvement of hygiene and sanitation, hepatitis B virus (HBV) and hepatitis C virus (HCV) infection are decreasing (20). The understanding of the molecular pathogenesis of HCC has also significantly improved. The incidence of liver cancer is decreasing in some high-risk areas, including China and Japan (18,20). However, HCC remains to have a high incidence rate; therefore, it is urgent that more effective

diagnostic techniques or replacement therapies are identified for patients with HCC. Collective data from high-throughput analyses of a large number of samples have offered a precise landscape of HCC genetic alterations (21). Indeed, it has enabled the delineation of some key events that may dominate tumor development and progression. It is hoped that translation of this knowledge into targets and biomarkers may impact HCC decision-making and ultimately improve patient outcomes.

The aim of the present work was to systematically review the current literature and to utilize the public Gene Expression Omnibus (GEO) databases to determine the role of miR-449a and its significance in HCC, thereby helping to understand and explore the pathogenesis of miR-449a in HCC. In addition, a preliminary bioinformatics analysis was conducted for miR-449a to investigate the novel potential pathways that miR-449a may participate in.

Materials and methods

Literature retrieval. To identify the role of miR-449a in HCC, a systematic literature search was conducted in the electronic databases PubMed (https://www.ncbi.nlm.nih.gov/pubmed), Embase (https://www.embase.com/), Web of Science (https://apps.webofknowledge.com/), China Biology Medicine disc (CBMdisc) (http://www.sinomed.ac.cn/zh/), China National Knowledge Infrastructure (CNKI) (http://www.cnki.net/), WANFANG DATA (http://g.wanfangdata.com.cn/), Chongqing VIP (http://qikan.cqvip.com/) and Google Scholar (https://scholar.google.com) until October 2017. The key words were as follows: 'Malignan* OR cancer OR tumor OR tumor OR neoplas* OR carcinoma,' 'hepatocellular OR liver OR hepatic OR HCC' and 'miR-449a OR miRNA-449a OR microRNA-449a OR miR 449a OR miRNA 449a OR microRNA 449a.' The articles were selected for the study if they met the following criteria: i) Studies published in Chinese or English; ii) studies assessing the dysregulation of miR-449a in HCC; iii) the expression profiles of miR-449a in patients with HCC and healthy controls were available; and iv) studies conducted on human tissues or body fluids, such as serum and urine. A total of 9 papers from PubMed, 5 from Google Scholar and 19 from the Web of Science were identified in the initial search, while only one study was found in the four Chinese databases (CBMdisc, CNKI, WANFANG DATA and Chongqing VIP). Following the elimination of duplicate studies, eight studies met the inclusion criteria. Review articles were not included in the present study. In the second step, the references and related citations of the eight articles were checked for additional information.

GEO microarray chip screening. To guarantee more comprehensive data collection, the GEO database of the National Center for Biotechnology Information of the National Institute of Health of USA (http://ncbi.nlm.nih.gov/geo/) was searched, which is a public functional genomics data repository. The following terms were used: 'Malignan* OR cancer OR tumor OR tumor OR neoplas* OR carcinoma' AND 'hepatocellular OR liver OR hepatic OR HCC.' Eligible microarrays were downloaded to further extract the miR-449a expression values, which contained two sides of the miR-449a expression level

(HCC and normal control). In each dataset, the mean value and standard deviation were calculated. Following this, all datasets were gathered for the assessment of miR-449a expression in HCC with STATA 12.0 (StataCorp LLC, College Station, TX, USA). The standardized mean difference (SMD) and 95% confidence interval (CI) were determined for the pooled values. As the SMD is the mean difference (MD) divided by the standard deviation (SD) and MD is the mean difference of the tumor group and the control group (22), SMD=0 meant that there was no significant difference of miR-449a expression in HCC tissues and normal control, SMD<0 means that miR-449a was downregulated in HCC and SMD>0 means that miR-449a was upregulated in HCC. A sensitivity analysis was also conducted with STATA 12.0 'metainf' command line (23).

Screening differentially expressed genes and predicting target genes. GEO Profiles (https://www.ncbi.nlm.nih.gov/geoprofiles/) was searched to explore the differentially expressed genes associated with miR-449a in HCC using the terms 'HCC AND miR-449a.' Finally, one dataset (GSE74710) (24) demonstrated that the transient transfection of miR-449a mimics into an HCC cell line, HLE, identified putative target genes of miR-449a. The dataset was downloaded to calculate the fold change (FC) between the group transfected with the miR-449a mimics and the negative control by R/Bioconductor (version 3.4.2, https://www.R-project.org/) FC<0.5 and P<0.05 were selected.

On the other hand, target genes of miR-449a were predicted using miRWalk 2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) (25), which combined 12 existing miR-target prediction programs (miRWalk, Microt4, miRanda, mirbridge, miRDB, miRMap, miRNAMap, Pictar2, PITA, RNA22, RNAhybrid and TargetScan) to provide comprehensive potential targets for miR-449a. The genes predicted in more than five prediction software programs were regarded as reliable targets of miR-449a.

In addition, a novel computerized approach, Natural Language Processing (NLP), was performed to obtain all the genes related to HCC (details have been described previously) (26). Finally, three components that were overlapped were identified for further bioinformatics analysis.

Functional and signaling pathway analysis. The functional and signaling pathway analyses of the selected genes were performed on a public database platform, the Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.ncifcrf.gov/). The analyses included Gene Ontology (GO) function analysis (http://geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/) analysis. The GO function analysis included three categories, namely, the biological processes (BP), cellular components (CC) and molecular functions (MF). The results of the GO analysis were visualized as co-expression networks via Cytoscape v3.4.0 (http://cytoscape.org/) with the BiNGO plug-in, and the result of the KEGG analysis was visualized as a bar chart.

Protein-protein interaction (PPI) networks analysis. The potential targets were also input into the STRING database (http://string-db.org/) to explore the hub genes involved in

Table I. Characteristics of the eight included studies.

Author, year	Country	Tissue	Sample, n (HCC/control)	miR	Targets	(Refs.)
Wang *et al*, 2017	China	Liver cancer	18/18	miR-449a	CXCL5	(29)
Liu *et al*, 2016	China	HCC	40/40	miR-449a	ADAM10	(30)
Chen *et al*, 2015	China	HCC	66/18	miR-449a	FOS, MET	(31)
Liu *et al*, 2016	China	Liver cancer	48/48	miR-449a	POU2F1, CAPN6	(32)
Zhang *et al*, 2016	China	HCC cell lines	-	miR-449a	CREB5	(33)
Buurman *et al*, 2012	Germany	HCC	23/0	miR-449 family	C-MET, SOX4	(36)
Sandbothe *et al*, 2017	Germany	Liver cancer	61/4	miR-449 family	SOX4	(24)
Sarma *et al*, 2012	America	HCV, AH, NASH	10 HCV, 10 AH 10 NASH, 10 control	miR-449a	NOTCH1	(34)

HCC, hepatocellular carcinoma; miR, microRNA; HCV, hepatitis C virus; AH, alcoholic hepatitis; NASH, non-alcoholic steatohepatitis.

HCC. Network nodes represented the proteins, and edges represented the protein-protein associations. All the parameter settings were selected as defaults.

Statistical analysis. The scatterplots that exhibited miR-449a expression from all the GEO datasets were generated using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). The significance of the difference between the HCC and non-cancerous liver tissues was analyzed using Student's t-tests. $P<0.05$ was considered to indicate a statistically significant difference. For meta-analysis, fixed-effects models or random-effects models were used for pooling SMD depending on inconsistency statistics (I^2). When $I^2 >50\%$, a random-effects model was selected; when $I^2 <50\%$, a fixed-effects model was preferentially used (27). Furthermore, Begg's funnel plot was applied for calculating publication bias (28).

Results

Systematic review of original literature. With the search and screen criteria, eight studies exploring the role of miR-449a in HCC were obtained. The data from the selected studies included HCC cell lines, animal models and human biopsy specimens. However, the studies on miR-449a are currently limited and effective data could not be extracted from the articles. Thus, it was not possible to perform a meta-analysis. The characteristics of the eight included studies are presented in Table I.

The first study conducted by Wang *et al* (29) collected 18 liver cancer tissues along with their adjacent normal controls to explore the effect of miR-449a on liver cancer migration and invasion. This study provided preliminary evidence that miR-449a could serve as a tumor suppressor to influence the migration and invasion of liver cancer through targeting C-X-C motif chemokine 5 (CXCL5). Consistent results were also observed in four HCC cell lines.

Another study conducted by Liu *et al* (30) measured miR-449a expression in 40 pairs of HCC tissues and adjacent normal tissues, as well as four HCC cell lines, and demonstrated that miR-449a expression was decreased in the

HCC tissues and four cell lines. They further explored the mechanism underlying the inhibitory effects of miR-449a on the growth and metastasis of HCC cells and revealed that miR-449a functioned as a tumor suppressor miR by inhibiting the cell proliferation, colony formation, migration and invasion of HCC by partially repressing a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) expression.

Chen *et al* (31) investigated the miR-449a expression level in 77 HCCs and 18 normal controls. They found that the level of miR-449a in HCC was notably lower when compared to that in the normal controls. Furthermore, the portal vein tumor thrombus tissues displayed a more significant reduction of miR-449a expression, which indicated that the reduction of miR-449a in HCCs was notably associated with a more aggressive tumor phenotype. They also conducted a log-rank test, which revealed that the reduction of miR-449a was associated with short disease-free survival in patients with HCC. The study revealed that miR-449a may suppress the epithelial-mesenchymal transition (EMT) and the metastasis of HCC by inhibiting FOS and Met expression and subsequently suppressing the downstream signaling.

Another study by Liu *et al* (32) explored the cellular function of miR-449a in 48 cases of liver cancer. By comparison with adjacent tissues, it was revealed that miR-449a was decreased in the liver cancer tissues, and the loss of miR-449a in the liver cancer tissues was associated with tumor progression, which suggested that miR-449a may be a suppressor of cancer metastasis. The results obtained from four human liver cancer cell lines (HepG2, 7404, 7721 and 7405) were consistent with the liver tissues. Additionally, they performed a series of bioinformatics analyses and validated experiments, which revealed that miR-449a could induce G_1 arrest of the liver cancer cells, suppress cell proliferation and promote cell apoptosis. It was also identified that miR-449a affected the biological behavior of liver cancer cells by the downregulation of calpain 6 (CAPN6) and POU class 2 homeobox 1 (POU2F1).

The study conducted by Zhang *et al* (33) explored the molecular mechanisms of the interactions between miR-449a and HBV infection and identified that miR-449a expression was downregulated in the HCC cells. Ectopic expression of miR-449a
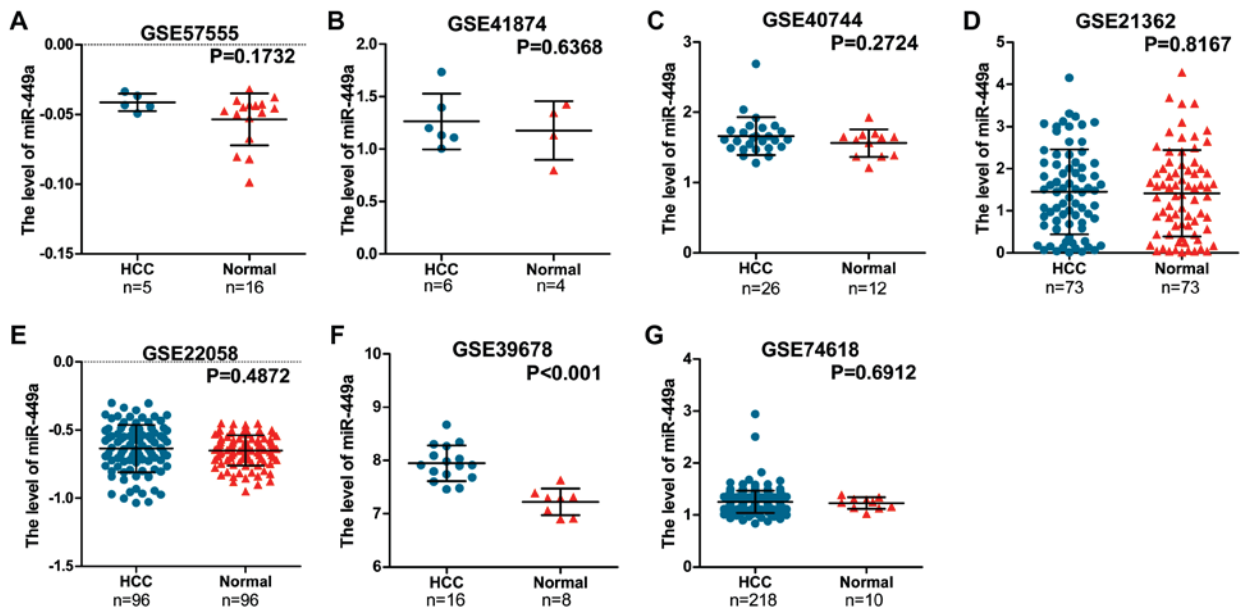
Figure 1. Scatter diagrams of miR-449a expression in seven evaluable microarray chips. (A) miR-449a expression in GSE57555, P=0.1732; (B) miR-124-3p expression in GSE41874, P=0.6368; (C) miR-124-3p expression in GSE40744, P=0.2724; (D) miR-124-3p expression in GSE21362, P=0.8167; (E) miR-124-3p expression in GSE22058, P=0.4872; (F) miR-124-3p expression in GSE39678, P<0.001; and (G) miR-124-3p expression in GSE74618, P=0.6912. miRNA, microRNA.

in HCC cells, to a large extent, boosted HBV replication, transcription, progeny virion secretion and antigen expression in a dose-dependent manner. They reported that miR-449a directly targeted the cyclic adenosine monophosphate (cAMP)-responsive factor and bound to cAMP responsive element binding protein 5 (CREB5), which influenced Farnesol-X-Receptor (FXR)α expression and facilitated HBV replication. Similarly, Sarma *et al* (34) analyzed the genome-wide miR in liver biopsies obtained from patients with chronic HCV infection and observed a downregulation of miR-449a compared with the level in the normal liver. It is well known that patients with HCV infection have a high risk of developing HCC (35).

Buurman *et al* (36) investigated 23 primary HCCs of various Gleason grades, as well as HCC cell lines and tumor xenografts, all of which demonstrated a reduced expression of miR-449a and an increased expression of c-MET in the samples. This indicated that miR-449a may function through targeting c-MET in hepatocarcinogenesis. Furthermore, analysis of the tissue samples revealed that the lowest concentrations of miR-449 were associated with high levels of c-MET, which appeared in Gleason grade 1, suggesting that the deregulation of miR-449a was mainly effective in the early stages of HCC tumorigenesis.

Recently, Sandbothe *et al* (24) further investigated the function of the miR-449 family using microarray analysis and public databases to identify their binding specificities, putative target genes and regulated pathways. They demonstrated that miR-449 family members significantly regulated cell cycle control, transforming growth factor (TGF)-β signaling, hepatocyte growth factor signaling and the Wnt/β-catenin signaling pathways that were frequently altered in HCC. Following comprehensive analysis, they focused their study on the signaling of TGF-β by targeting SOX4, which served a dual role in HCC, acting as a tumor suppressor during the early stages of liver damage, but promoting tumor progression and metastasis in advanced HCC.

Determining the expression of miR-449a in HCC by gathering seven GEO datasets. GEO datasets were searched to assess the expression of miR-449a between HCC and non-cancer tissues. miR-449a levels in all seven microarray chips [GSE57555 (37), GSE41874 (38), GSE40744 (39), GSE21362 (40), GSE22058 (41), GSE39678 (42) and GSE74618 (43)] were presented in Fig. 1. Meta-analysis was conducted with seven datasets to assess the miR-449a expression level in patients with HCC in another manner, on account of the fact that the GEO microarrays could not present the role of miR-449a in HCC directly. In total, 440 patients with HCC and 219 normal controls were selected for the meta-analysis. A random-effects model was selected to calculate the pooled SMD and 95% CI, and significant heterogeneity was identified among individual datasets (I^2=65.8%; P=0.007). The results demonstrated that a significant difference was observed between the HCC groups and the normal controls (SMD=0.40; 95% CI, 0.01-0.79; P=0.046), suggesting that the miR-449a expression levels were increased in patients with HCC rather than in the normal controls (Fig. 2).

The results of the sensitivity analysis revealed that heterogeneity may be confounded by the GSE39678 dataset (Fig. 3). The heterogeneity disappeared when the GSE39678 dataset was removed (I^2=0.0%; P=0.813). Hence, the SMD and 95% CI were pooled again as a fixed-effects model. However, no significant difference was observed in the miR-449a expression level between the patients with HCC and healthy controls (SMD=0.13; 95% CI, -0.06-0.32; P=0.179) (Fig. 4). The results suggested that the conclusion for the present meta-analysis was unreliable according to the inconsistent values when pooled by the separate random- and fixed-effects models.

Publication bias analysis revealed that the Begg's funnel plots (Fig. 5) were almost symmetrical, with the obtained Pr>|z|=0.072>0.05 for miR-449a (where Pr represented the Begg's P-value and z represented the standard normal variate for the confidence level specified by option level), suggesting
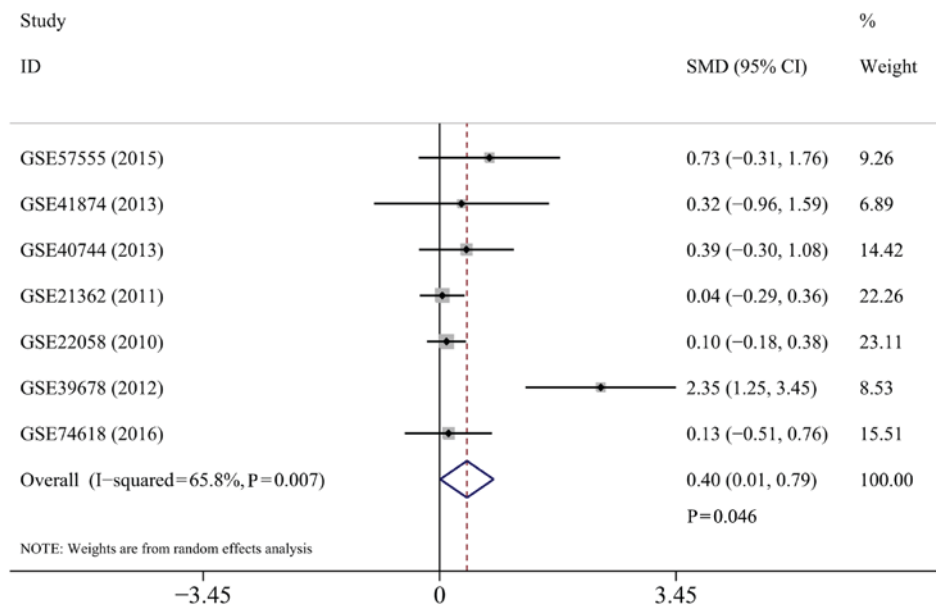
Figure 2. Forest plot of the seven evaluated datasets assessing microRNA-449a expression in HCC. A random-effects model was selected to evaluate the pooled SMD with a 95% CI. The combined SMD suggested a marked difference between the HCC group and the normal control group (SMD=0.40; 95% CI, 0.01-0.79; P=0.046). SMD, standardized mean difference; CI, confidence intervals; HCC, hepatocellular carcinoma.
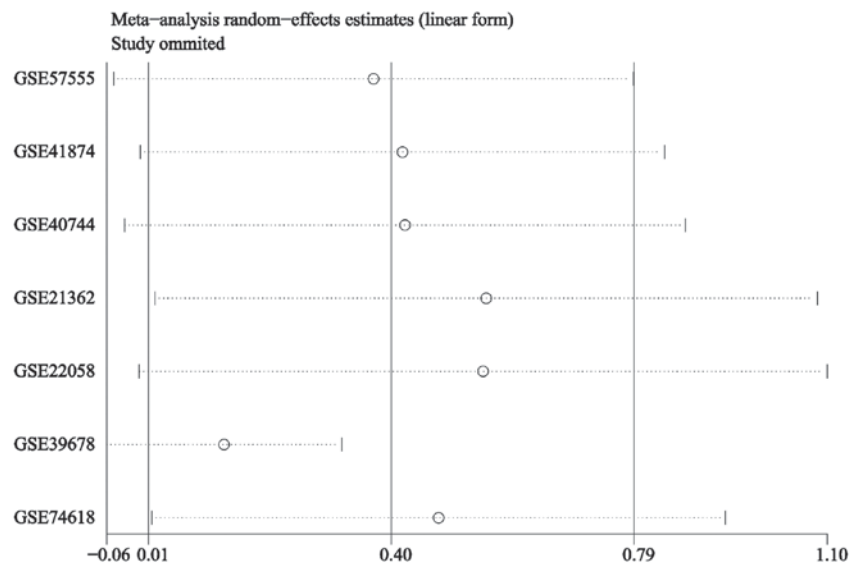

Figure 3. Sensitivity analysis of the seven evaluated datasets assessing the expression of microRNA-449a in hepatocellular carcinoma. Significant heterogeneity existed among the individual datasets (I^2=65.8%; P=0.007), and the datasets from GSE39678 presented an obvious deviation from the estimate.

that publication bias from the included studies was absent in the present study.

Potential target genes of miR-449a in HCC. In the GSE74710 dataset, since the HCC cell line, HLE, was transfected with miR-449a mimics, the downregulated genes (FC<0.5 and P<0.05) were screened as potential miR-449a target genes, and 617 eligible genes were ultimately extracted. Additionally, potential targets of miR-449a *in silico* were also predicted. A total of 3,662 genes that appeared in more than five databases were selected. Furthermore, a total of 1,800 HCC-related genes were recognized from NLP.

To narrow down the area and obtain more reliable miR-449a targets, three kinds of genes were overlapped. Finally, 23 genes

were left for further GO and KEGG pathway analysis and PPI network analysis, which were largely representative of the prospective molecular mechanism of miR-449a in HCC (data not shown).

Potential pathways of miR-449a in HCC. The GO and KEGG enrichment analyses were performed on the potential target genes of miR-449a that were associated with HCC by the functional annotation tool of the DAVID database. The most enriched GO and KEGG terms were obtained. It was revealed that in the GO_BP category, these genes were predominantly involved in 'positive regulation of transcription from RNA polymerase II promoter' (GO: 0045944; P=1.778×10^{-4}), 'positive regulation of apoptotic process' (GO: 0043065;
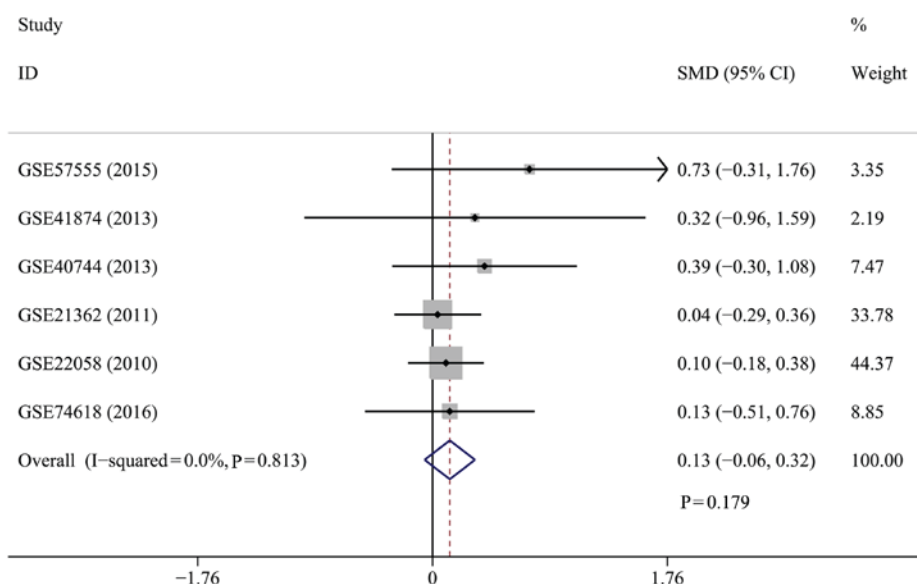
Figure 4. Forest plot of the six evaluated datasets assessing expression of microRNA-449a in hepatocellular carcinoma. A fixed-effects model was selected to evaluate the pooled SMD with a 95% CI, and the combined SMD with a 95% CI, following the removal of the data from GSE39678 (SMD=0.13; 95% CI, -0.06-0.32; P=0.179; I^2=0.0%; P=0.813). No significant difference between the tumor and normal groups was identified. SMD, standardized mean difference; CI, confidence interval.
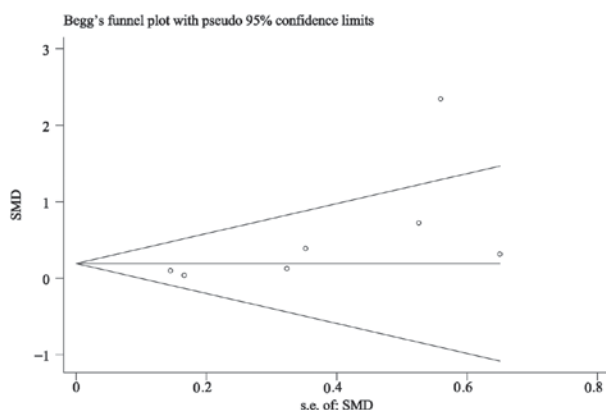

Figure 5. Begg's plot of the publication bias test of the seven studies. SMD, standardized mean difference; s.e., standard error.
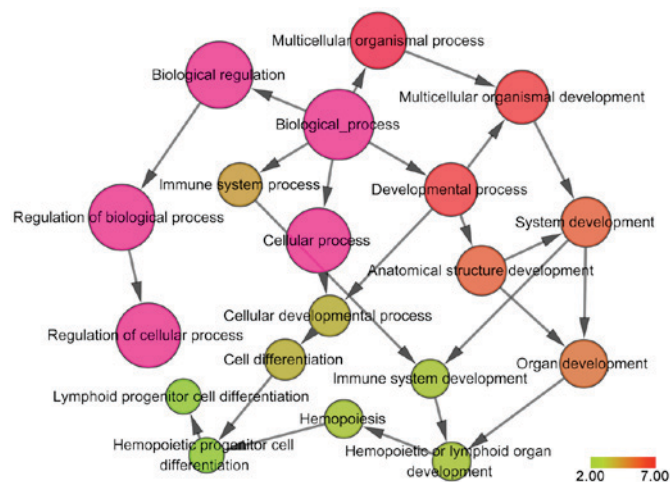

Figure 6. Gene Ontology pathway interaction maps were drawn using the Cytoscape v3.4.0 BiNGO plug-in. Color and size intensity denote the significance of the functional pathway and the enrichment of the gene. The green color represents the most significant pathway terms, and the larger size represents the more enriched genes.

P=5.67x10^{-4}) and 'negative regulation of cell proliferation' (GO: 0008285; P=0.0016). In the GO_CC category, the genes tended to affect the 'cell surface' (GO: 0009986; P=0.0265), 'cytoplasm' (GO: 0005737; P=0.0279) and 'nucleus' (GO: 0005634; P=0.0361), while in the GO_MF category, they mainly participated in 'transmembrane receptor protein tyrosine kinase activity' (GO: 0004714; P=0.0011), 'protein binding' (GO:0005515; P=0.0038) and 'transcription factor activity, and sequence-specific DNA binding' (GO: 0003700; P=0.0069). The results of the GO analysis were presented in Table II and visualized as co-expression networks using Cytoscape v3.4.0 with the BiNGO plug-in, as demonstrated in Fig. 6.

KEGG pathway investigation indicated that these target genes participated in the following categories: 'MicroRNAs in cancer' (hsa05206; P=0.0146), 'Cell cycle' (hsa04110; P=0.0219), 'PI3K-Akt signaling pathway' (hsa04151; P=0.0243) and 'N-Glycan biosynthesis' (hsa00510; P=0.0884) (Table III and Fig. 7).

In the PPI network analysis, 23 genes were input to construct an interaction network, and the PPI enrichment P-value was 0.0096, with 23 interaction edges (Fig. 8). Meanwhile, the local clustering coefficient was 0.23. With comprehensive analysis of the network, the hub nodes, MYB, E2F5, CDK6 and CDC25A, were identified as the most essential genes in HCC, which may also function as novel targets for medical treatment.

Discussion

Accumulating evidence (44-47) has revealed that the altered expression of miR contribute to the initiation and progression of various diseases, including cancer. miR are post-transcriptional

Table II. Top ten GO functional annotation for most significantly related targets of microRNA-449a.

GO ID	GO term	Count (%)	P-value	Gene symbol
Biological process				
GO:0009615	Response to virus	6 (13.3)	9.75×10^{-6}	DUOX2, CDK6, HMGA2, FOXP3, CXCL12, FOSL1
GO:0043065	Positive regulation of apoptotic process	8 (17.8)	1.18×10^{-5}	TXNIP, DAB2IP, NOTCH1, DIABLO, SOX4, ARHGEF9, HMGA2, FOSL1
GO:0045944	Positive regulation of transcription from RNA polymerase II promoter	11 (24.4)	1.78×10^{-4}	DAB2IP, NOTCH1, E2F5, MET, SOX4, CTCFL, HMGA2, MYB, FOXP3, FOSL1, FOXP1
GO:0051591	Response to cAMP	4 (8.9)	2.36×10^{-4}	DUOX2, COL1A1, AREG, FOSL1
GO:0042542	Response to hydrogen peroxide	4 (8.9)	3.20×10^{-4}	TXNIP, COL1A1, AREG, FOSL1
GO:0008285	Negative regulation of cell proliferation	7 (15.6)	5.48×10^{-4}	SPRY1, DAB2IP, NOTCH1, SOX4, CDK6, FOXP3, FOSL1
GO:0035019	Somatic stem cell population maintenance	4 (8.9)	6.55×10^{-4}	ZHX2, SOX4, KIT, HMGA2
GO:0045892	Negative regulation of transcription, DNA-templated	7 (15.6)	1.81×10^{-3}	DAB2IP, NOTCH1, ZHX2, HMGA2, MYB, FOXP3, FOXP1
GO:0045893	Positive regulation of transcription, DNA-templated DNA	7 (15.6)	2.12×10^{-3}	NOTCH1, SOX4, CTCFL, COL1A1, HMGA2, MYB, FOXP3
GO:0000122	Negative regulation of transcription from RNA polymerase II promoter	8 (17.8)	2.48×10^{-3}	TXNIP, DAB2IP, NOTCH1, ZHX2, HMGA2, MYB, FOXP3, FOXP1
Cellular component				
GO:0009986	Cell surface	7 (15.6)	1.82×10^{-3}	CLCN3, NOTCH1, MET, AXL, TGFA, CFTR, AREG
GO:0000139	Golgi membrane	6 (13.3)	1.35×10^{-2}	CLCN3, NOTCH1, ST6GAL1, FUT8, TGFA, AREG
GO:0030141	Secretory granule	3 (6.7)	1.38×10^{-2}	CLCN3, COL1A1, RAB27B
GO:0005615	Extracellular space	9 (20.0)	1.42×10^{-2}	LPO, AXL, TGFA, IGFBP1, COL1A1, KIT, AREG, CXCL12, TIMP3
GO:0016324	Apical plasma membrane	4 (8.9)	3.30×10^{-2}	NOTCH1, DUOX2, CFTR, RAB27B
GO:0005634	Nucleus	20 (44.4)	3.97×10^{-2}	TXNIP, GLRX5, E2F5, ZHX2, SOX4, CTCFL, CDK6, HMGA2, FOXP3, TIMP3, CDC25A, FOXP1, MBP, NOTCH1, HPSE, MSI1, TGFA, AREG, MYB, FOSL1
GO:0070062	Extracellular exosome	12 (26.7)	6.662×10^{-2}	GSTM2, LPO, DAB2IP, ST6GAL1, FUT8, DUOX2, ARHGAP1, AXL, CFTR, RAB27B, CXCL12, TIMP3
GO:0016323	Basolateral plasma membrane	3 (6.7)	7.01×10^{-2}	LPO, TGFA, CFTR
GO:0005829	Cytosol	13 (28.9)	9.0×10^{-2}	TXNIP, DAB2IP, CDK6, CFTR, ARHGEF9, CDC25A, GSTM2, SPRY1, NOTCH1, ATG4B, ARHGAP1, DIABLO, FOSL1
GO:0009897	External side of plasma membrane	3 (6.7)	9.35×10^{-2}	CLCN3, KIT, CXCL12

Table II. Continued.

GO ID	GO term	Count (%)	P-value	Gene symbol
Molecular function				
GO:0005515	Protein binding	35 (77.8)	5.07×10^{-4}	CLCN3, E2F5, SOX4, CTCFL, KIT, TIMP3, MBP, GSTM2, SPRY1, HPSE, ARHGAP1, TGFA, DIABLO, RAB27B, MYB, etc.
GO:0001077	Transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding	5 (11.1)	3.26×10^{-3}	SOX4, CTCFL, HMGA2, MYB, FOSL1
GO:0004714	Transmembrane receptor protein tyrosine kinase activity	3 (6.7)	4.40×10^{-3}	MET, AXL, KIT
GO:0001047	Core promoter binding	3 (6.7)	1.21×10^{-2}	NOTCH1, HMGA2, FOXP3
GO:0002020	Protease binding	3 (6.7)	2.85×10^{-2}	KIT, TIMP3, MBP
GO:0046982	Protein heterodimerization activity	5 (11.1)	3.24×10^{-2}	CLCN3, NOTCH1, AXL, ZHX2, SOX4
GO:0003700	Transcription factor activity, sequence-specific DNA binding	7 (15.6)	3.73×10^{-2}	NOTCH1, E2F5, ZHX2, SOX4, FOXP3, FOSL1, FOXP1
GO:0017124	SH3 domain binding	3 (6.7)	3.85×10^{-2}	DAB2IP, FUT8, ARHGAP1
GO:0042803	Protein homodimerization activity	6 (13.3)	4.03×10^{-2}	GSTM2, CLCN3, DAB2IP, ZHX2, KIT, FOXP3
GO:0008191	Metalloendopeptidase inhibitor activity	2 (4.4)	4.09×10^{-2}	RECK, TIMP3

GO, Gene ontology.

Table III. KEGG functional annotation for most significantly related targets of microRNA-449a.

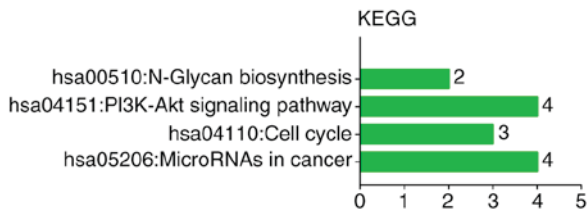| KEGG ID	KEGG term	Count (%)	P-value	Gene symbol
hsa05206	MicroRNAs in cancer	7 (15.6)	5.35×10^{-4}	RECK, NOTCH1, MET, CDK6, HMGA2, TIMP3, CDC25A
hsa02151	PI3K-Akt signaling pathway	5 (11.1)	3.83×10^{-2}	MET, CDK6, COL1A1, KIT, MYB
hsa05200	Pathways in cancer	5 (11.1)	5.71×10^{-2}	MET, TGFA, CDK6, KIT, CXCL12
hsa04110	Cell cycle	3 (6.7)	7.85×10^{-2}	E2F5, CDK6, CDC25A

KEGG, Kyoto Encyclopedia of Genes and Genomes.

Figure 7. KEGG pathway analysis of the potential target genes of microRNA-449a in hepatocellular carcinoma. The results of the KEGG analysis were visualized as a bar chart. KEGG, Kyoto Encyclopedia of Genes and Genomes; PI3K, phosphoinositide 3 kinase.
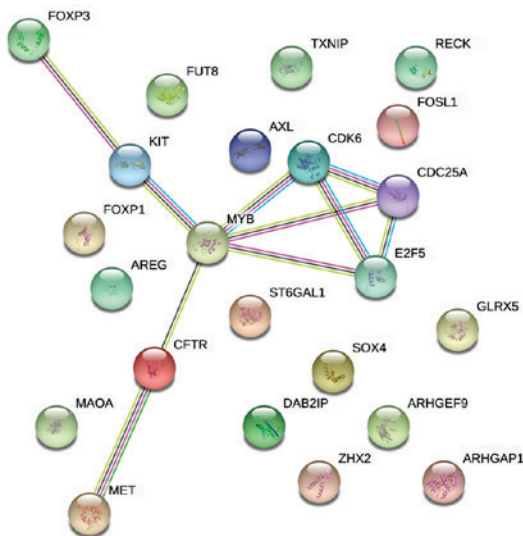

Figure 8. Protein-protein interaction networks of the potential target genes of microRNA-449a in hepatocellular carcinoma. Interactions among the 23 genes were illustrated using the STRING online database. The network nodes represent proteins and the edges represent the protein-protein associations.

regulators of gene expression and are involved in the silencing of mRNA translation (48). Investigating the altered expression of miR in malignancies may provide a novel and effective therapeutic target for HCC in the future.

The present systematic review and meta-analysis summarized eight original studies and seven microarray chips to investigate the function of miR-449a in HCC. In the original studies, all the researchers held the same viewpoint, that the miR-449a expression level was reduced in HCC. However, there were still some differences in their research. Wang *et al* (29) explored the effect of miR-449a on liver cancer and found that

miR-449a predominantly regulated CXCL5 expression, which encodes a chemokine that was suggested to bind the G-protein coupled receptor chemokine (C-X-C motif) receptor 2 to recruit neutrophils, promote angiogenesis and remodel connective tissues (49). The increased expression of this gene would promote cancer cell proliferation, migration and invasion. Liu *et al* (30) also found that miR-449a functioned as a tumor suppressor by obstructing cell proliferation, colony formation, migration and invasion of HCC by partially repressing ADAM10 expression. However, different from these two findings, Chen *et al* (31) documented the involvement of EMT in HCC progression. They revealed that miR-449a likely suppressed EMT and metastasis of HCC by inhibiting FOS and Met expression and suppressing the downstream signaling. The mechanism of EMT in HCC metastasis has been presented by Yang *et al* (50). In addition, Liu *et al* (32) reported that miR-449a may function by down-regulating CAPN6, which is a potential oncogene in tumors and may serve a role in tumor formation by inhibiting apoptosis and promoting angiogenesis.

Another notable discovery was documented by Zhang *et al* (33) and Sarma *et al* (34). Zhang *et al* dissected the relationship between miR-449a and HBV infection and revealed that miR-449a acted as a linker between histone deacetylation and HBV replication, directly targeting CREB5, and CREB5 knockdown induced FXRα expression. FXRα also referred to as nuclear receptor subfamily 1 group H member 4, which is a transcription factor that binds two motifs within the HBV enhancer II and the core promoter regions to promote HBV transcription and replication (51). Sarma *et al* (34) demonstrated that HCV downregulated miR-449a expression in human livers, which could elevate the levels of transcription factors, leading to an increase in the inflammatory response and promoting cell proliferation, which resulted in HCC.

Buurman *et al* (36) investigated the miR-449 family for several years, and identified at least one member (miR-449a, miR-449b or miR-449c) of the miR-449 family-targeted pathways, including Wnt/β-catenin, p53/cell cycle control, mitogen-activated protein kinase and TGF-β. These are promising targets for HCC therapy (52). Buurman *et al* (36) focused on the signaling of TGF-β and discovered that this pathway served a dual role in HCC, acting as a tumor suppressor during early stages of liver damage, but promoting tumor progression and metastasis in advanced HCC. Furthermore, their first clinical trial for miR replacement therapy was initiated in 2013 to evaluate the safety of miR-449a in patients with unresectable primary liver cancer or other selected solid or

hematologic malignancies (53). miR replacement therapy may provide hope for patients with liver cancer in the future.

In summary, previous original studies have shared the same views, that miR-449a is downregulated in HCC tissues and involved in various pathways by targeting multiple mRNA. However, to our surprise, the pooled results of seven microarrays revealed that miR-449a was overexpressed in HCC, which was completely contrary to the original studies. The causes of this discrepancy were analyzed, and it was speculated that microarray analysis was not sensitive enough to detect the RNA expression level compared to the method of reverse transcription-quantitative polymerase chain reaction. The majority of the microarrays gathered in the present study demonstrated no significant difference in the miR-449a expression between HCC and normal controls.

Since the original studies indicated that miR-449a may be involved in multiple signaling pathways and target multiple mRNAs, the potential targets of miR-449a in HCC were over-lapped to explore whether miR-449a may exert its function in other pathways. The potential targets, including GEO downreg-ulated genes, *in silico* predicted genes, as well as HCC-related genes recognized from NLP, were merged with three different resources to obtain more reliable targets. However, limitations remained in the present study. The GEO downregulated genes were required to have an FC value of <0.5 and a P-value of <0.05 for inclusion, and the adjusted P-value was also taken into consideration, which provides a more accurate indication of the level of false positives for a given cut-off value. However, the adjusted P-values of all the eligible genes were distributed from 0.1-0.3. The results revealed that more false positives may exist in these included genes. Therefore, the miRWalk database, which contains 12 prediction algorithms, and NLP were used to identify more reliable targets. The present results revealed that, in addition to 'cell cycle pathway' (hsa04110), miR-449a may participate in 'MicroRNAs in cancer' (hsa05206), 'PI3K-Akt signaling pathway' (hsa04151) and 'N-Glycan biosynthesis pathways' (hsa00510) to regulate HCC tumorigenesis.

In addition, PPI network analysis was conducted, and hub nodes, including MYB, E2F5, CDK6 and CDC25A, were identi-fied as the most essential genes in HCC. Among them, MYB encoded a protein with three helix-turn-helix DNA-binding domains, which functioned as transcription regulators (54). It was reported that MYB expression was increased at the mRNA level in HCC (55). E2F5 is a member of the E2F transcrip-tion factor family and binds to the promoters of target genes involved in cell cycle control, and consequently modulates the expression of these targets (56). The members of the E2F family have been divided into the activator (E2F1-E2F3) and repressor (E2F4-E2F8) subclasses (57). The overexpression or amplification of the E2F5 gene has been reported in various solid tumors, such as HCC. For example, Jiang *et al* (58) provided evidence that E2F5 was commonly upregulated in HCC and E2F5 knockdown notably inhibited the growth of HCC cells. Whether MYB or E2F5 deregulation in HCC is targeted by miR-449a requires further investigation. The phosphorylation of CDKs is required for timely progression through the cell division cycle (59). CDK6 has been demonstrated to phosphory-late and regulate the activity of the tumor suppressor protein, retinoblastoma (60,61). Altered expression of this gene has been observed in multiple human cancer types (62-64). Likewise,

the dysregulation of CDK6 in HCC has also been previously reported (65,66). CDC25A, a member of the CDC25 family of phosphatases, is a cell cycle and apoptotic regulator (67). Previous research has reported that dephosphorylation of CDC25 is the rate-limiting step for CDK activation. Their phos-phorylation removes the inhibitory phosphorylation on the CDK and regulates the G_1-S phase progression in the cell cycle (68).

In conclusion, as the number of studies in the present systematic review was small and these studies provided limited data to conduct a meta-analysis, it is difficult to deduce any definite conclusion. Based on the evidence presented here, miR-449a was revealed to be reduced in HCC and is likely to be involved in various kinds of signaling pathways that target multiple mRNAs to exert its function in HCC. Larger samples and further investigation are required to validate this conclusion.

Competing interests

The authors declare that they have no competing interests.

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