Long non-coding RNA HOTTIP promotes hepatocellular carcinoma tumorigenesis and development: A comprehensive investigation based on bioinformatics, qRT-PCR and meta-analysis of 393 cases

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Abstract. HOTTIP functions as an independent biomarker in multiple cancers. However, the role of HOTTIP in hepatocellular carcinoma (HCC) remains unclear. In this study, we sought to investigate the HOTTIP expression in HCC and normal liver. We combined quantitative reverse transcription-polymerase chain reactions (qRT-PCR), Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA), Multi Experiment Matrix (MEM) and Oncomine database to assess the clinical role and the potential molecular mechanism of HOTTIP in HCC. Furthermore, a meta-analysis was performed to evaluate the relationship between HOTTIP and HCC tumorigenesis and development. Additionally, bioinformatics analysis, which contained Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and network analysis, were applied to investigate the underlying functions, pathways and networks of the potential genes. HOTTIP was obviously upregulated in HCC. A statistically significant higher expression of HOTTIP was found in TNM (III +IV), age (≥60), sex (male), race (white) and cirrhosis (no) compared to the control groups ($P<0.05$). Furthermore, the meta-analysis of 393 cases from multiple centers indicated that HOTTIP had high diagnostic value in HCC. Additionally, according to GO and KEGG analyses, we found that the most strongly enriched functional terms were gland development, transcription factor activity and extrinsic to membrane. Also, the HOTTIP co-expressed genes were significantly related to PPAR signaling pathway. We speculate that HOTTIP might play a vital part in HCC via regulating various pathways, especially PPAR signaling pathway. However, the detailed mechanism should be confirmed by functional experiments.

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Abbreviations: HCC, hepatocellular carcinoma; qRT-PCR, quantitative reverse transcription-polymerase chain reactions; GEO, Gene Expression Omnibus; TCGA, The Cancer Genome Atlas; MEM, Multi Experiment Matrix; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; lncRNAs, long non-coding RNAs; ROC, receiver operating characteristic; BP, biological process; CC, cellular component; MF, molecular function; DAVID, Database for Annotation, Visualization and Integrated Discovery; NCBI, National Center of Biotechnology Information; mean ± SD, mean ± standard deviation; SROC, summary receiver operating characteristic

Key words: HOTTIP, hepatocellular carcinoma, qRT-PCR, GO, KEGG, meta-analysis

Introduction

Hepatocellular carcinoma (HCC) is known as the most common liver malignancy worldwide, with extremely high incidence and mortality rate (1-5). In China, HCC frequently occurs owing to chronic infection of hepatitis B virus (HBV) (6-9). Other conditions, such as alcoholic hepatitis, non-alcoholic fatty liver disease, hemochromatosis and diabetes, also contribute to the development of HCC (10-12). Up to now, liver transplantation and tumor resection still have been the most effective treatments for HCC, whereas the high metastasis and postoperative recurrence rates barricade the prognosis of HCC patients, especially HCC patients in advanced stage (13,14). As the current treatment options are limited, it is of great significance to investigate the underlying mechanism of HCC, which might provide novel insights into the diagnosis and treatment of HCC patients.

Long non-coding RNAs (lncRNAs) represent the non-protein coding RNAs with the length from 200 nucleotides to 100 kb (15-17). Recent evidence has clarified that various

lncRNAs could act as key regulators in disease development, epigenetic gene regulation or transcriptional regulation (18-23). Importantly, lncRNAs can regulate the proliferation, invasion, metastasis and apoptosis of malignancies (24-29). In HCC, growing evidence has demonstrated that the differential expression of lncRNAs could affect the development and progression of HCC by regulating the self-renewal ability of liver cancer stem cells and other biological functions (30-34). Moreover, it has been reported that the lncRNA expression might be also associated with chemotherapy resistance of HCC patients (35-37).

lncRNA HOTTIP, also identified as HOXA-AS6, HOXA13-AS1 and NCRNA00213, is located on 7p15.2, with the NCBI Gene ID: 100316868 (38). Moreover, HOTTIP functions as an independent biomarker in multiple cancers, such as breast cancer and esophageal squamous cell carcinoma (39-42). The high HOTTIP expression could inhibit the growth of glioma, increase the chemoresistance of osteosarcoma and promote tumor invasion of gastric cancer (33,43,44). Although it has been shown that HOTTIP was upregulated in HCC compared with normal tissues and could be related to disease progression and predict the outcome of HCC; nonetheless, the detailed functions and mechanism of HOTTIP in HCC remain elusive (45).

In the present study, we investigated the expression of HOTTIP in HCC and normal liver. Furthermore, we combined Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA), Multi Experiment Matrix (MEM) and Oncomine database, quantitative reverse transcription-polymerase chain reactions (qRT-PCR), and meta-analysis to assess the clinical role and the potential molecular mechanism of HOTTIP in HCC. Additionally, bioinformatics analysis, which contain Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and network analysis, were applied to explore the underlying functions, pathways and networks of the potential genes (46-48). A flow chart of this study is shown in Fig. 1.

Materials and methods

Quantitative real-time PCR. A total of 41 HCC cases, between January 2012 and March 2013, were collected from the Department of Pathology, First Affiliated Hospital of the Guangxi Medical University (Nanning, Guangxi, China). The samples were collected randomly from patients undergoing surgical resection without treatment. All methods were performed based on the relevant regulations and guidelines. Also, all experimental protocols have been approved by the Ethical Committee of the First Affiliated Hospital of Guangxi Medical University, and the clinicians and patients have signed the consent forms for the use of their tissues in the study. In this study, the total RNA was extracted via a Takara PrimeScript RT reagent kit based on the manufacturer's instructions. Then, the total RNA was used for cDNA synthesis through the Takara PrimeScript RT reagent kit according to the instructions. Then, qRT-PCR was operated using a LightCycler 480 Real-time PCR system (Roche, Shanghai, China). The specific primers were employed as follows: HOTTIP forward, 5'-CACACTCACATTCGCACACT-3'; reverse, 5'-TCCAGAACTAAGCCAGCCATA-3'. GAPDH (internal control) forward, 5'-AGTGGCAA AGTGGAGATT-3'; reverse, 5'-GTGGAGTCATACTGGAACA-3' (49). Results were normalized to the GAPDH expression and calculated based on the ΔCt method (50,51).

HOTTIP and HCC: a meta-analysis. HCC-related HOTTIP microarray and RNA-seq datasets were downloaded from TCGA, the National Center of Biotechnology Information (NCBI) GEO (http://www.ncbi.nlm.nih.gov/geo/), ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) and Oncomine (https://www.oncomine.org/resource/main.html). In addition, publications associated with the diagnostic value of HOTTIP in HCC were also selected from 12 online databases: PubMed, Web of Science, Science Direct, Google Scholar, Ovid, LILACS, Wiley Online Library, EMBASE, Cochrane Central Register of Controlled Trials, Chong Qing VIP, Chinese CNKI, Wan Fang and China Biology Medicine disc. The retrieval date was up to April 20, 2017 with the following keywords: (HOTTIP or HOXA-AS6 or HOXA13-AS1 or NCRNA00213) and (malignan* OR cancer OR tumor OR tumor OR neoplas* OR carcinoma) and (hepatocellular OR liver OR hepatic OR HCC). The literature retrieval was assessed and cross-checked by two independent investigators (Jia-Cheng Huang and Wen-Ya Pan). Group discussion was carried out if there was disagreement. The number of true-positives (tp), true-negatives (tn), false-positives (fp) and false-negatives (fn) was extracted. When no direct data was found from a study, a basic formula (such as 'sensitivity' = tp/ (tp+fn), 'specificity' = tn/ (tn+fp)) would be used to calculate the incidence.

Validation of the expression of HOTTIP in HCC. TCGA (http://cancergenome.nih.gov/) is a collection of SNP arrays, DNA methylation, miRNA-seq, exome sequencing, RNA-seq, and more (52,53). TCGA can be also applied to investigate the complicated cancer genomics expression and clinical parameters. In this study, RNA-Seq data from individuals with HCC, which were calculated on IlluminaHiSeq RNASeq platform, were achieved from TCGA data portal (https://tcga-data.nci.nih.gov/tcga/), containing 171 HCC tissues and 28 adjacent normal liver tissue samples up to April 1, 2017. The expression data of HOTTIP were displayed as reads per million (RPM) and the HOTTIP expression level was normalized by Deseq package of R language for further analysis. Student's t-test (SPSS Inc., Chicago, IL, USA) was performed for the statistical analysis of the differential expression of HOTTIP between HCC and non-cancerous liver tissues. Also, the relationship between HOTTIP and the clinicopathological parameters in HCC was identified based on the original data from TCGA database. Then, the clinical diagnostic value of HOTTIP was analyzed by a receiver operating characteristic (ROC) curve. Furthermore, the original data in Oncomine database was also applied to verify the HOTTIP expression in HCC (54).

The potential functions and pathways associated with HOTTIP. To further investigate the co-expressed genes associated with HOTTIP, MEM (http://biit.cs.ut.ee/mem/index.cgi), an open-access resource, was utilized to explore the co-expressed genes of HOTTIP based on Affymetrix Gene Chip Human Genome

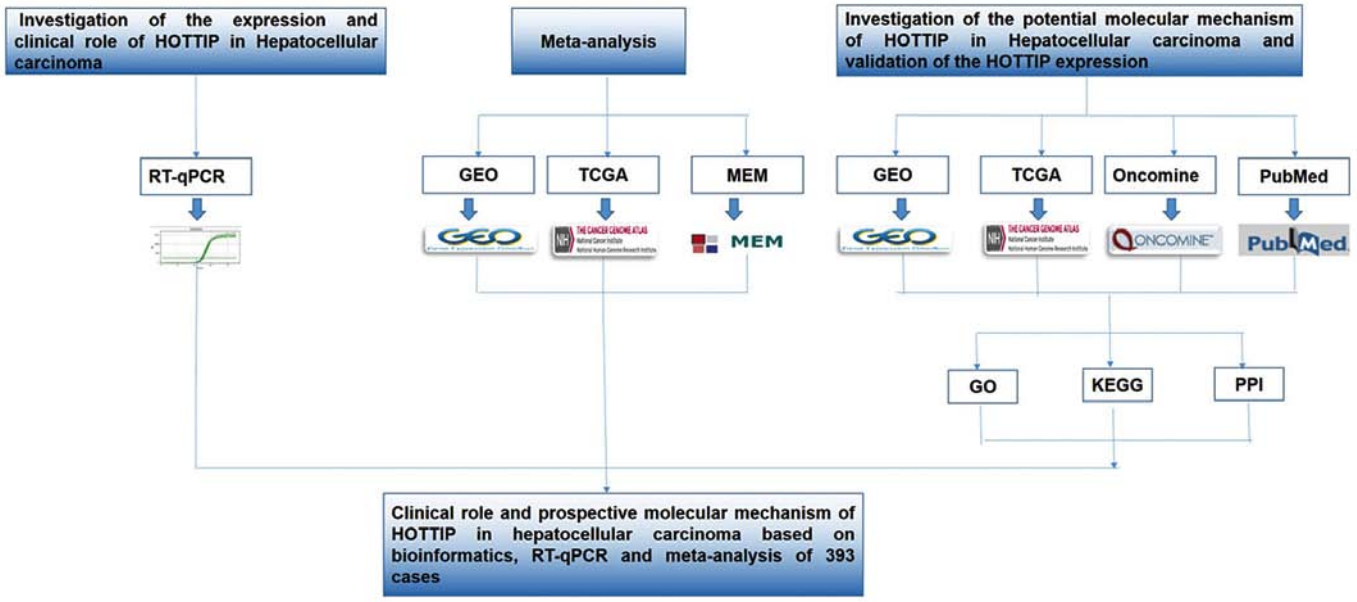

Figure 1. A flow chart of this study.

U133 Plus 2.0 Array platform (55). Differentially expressed genes in TCGA were extracted via R language package DESeq based on the following criteria: adjusted P<0.05 and the absolute log2 fold change >1 (56,57). Also, the genes differentially expressed in GEO (http://www.ncbi.nlm.nih.gov/geo/) database were selected using the GEO2R online tool (58-61). The overlapping genes were identified and compared using Venn diagrams (available online: http://bioinformatics.psb.ugent.be/webtools/Venn/). Then, bioinformatics analyses including GO, KEGG and network analysis were applied to explore the potential functions, pathways and networks of the overlapping genes as described (62,63). In this process, Database for Annotation, Visualization and Integrated Discovery (DAVID: available online: http://david.abcc.ncifcrf.gov/) was applied to perform GO and KEGG analyses. In addition, three independent categories [biological process (BP), cellular component (CC) and molecular function (MF)] were derived from GO analysis. Besides, a functional network was constructed via Cytoscape (version 2.8, http://cytoscape.org).

Construction of protein-protein interaction (PPI) network. The interaction pairs of these co-expressed genes were explored with Search Tool for the Retrieval of Interacting Genes (STRING; version 9.0, http://string-db.org) (64). The STRING database supplies a global perspective for as many organisms as feasible. The known and predicted interactions are integrated and scored. The interaction pairs in PPI network were selected with the combined score >0.4.

Statistical analysis. The high-throughput expression data were log2-transformed. The mean ± standard deviation (mean ± SD) was calculated through SPSS 22.0 (IBM, NY, USA) to estimate the HOTTIP expression level in each dataset. The HOTTIP expression between normal liver tissue and HCC was evaluated by Student's t-test. Student's t-test was also used to evaluate the relationships between HOTTIP expression and the clinicopathological parameters. The comparison between subgroups was performed via one-way analysis of variance (ANOVA). A Mann-Whitney U test or Kruskal-Wallis H test was conducted for non-normally distributed variables. Mining for co-expression genes across hundreds of datasets was carried out via novel rank aggregation and visualization methods. A P-value of <0.05 was identified to be statistically significant (two sides) using SPSS 22.0.

In the diagnostic meta-analysis, all the statistical analysis was performed by STATA 14.0 (STATA Corp., College, TX, USA). Heterogeneity between the included studies was assessed by Cochrane's Q test and I^2 statistic, and I^2 >50% represented significant heterogeneity. Publication bias was evaluated by Deek's funnel plot asymmetry test. A P-value of <0.05 was regarded significant publication bias. To investigate the underlying diagnostic performance of HOTTIP in HCC, we performed summary receiver operating characteristic (SROC) curves and calculated area under the curve (AUC) with 95% CIs and the corresponding sensitivity and specificity by using Meta-DISc software (65). An AUC value of 0.5-0.7 was regarded as low diagnostic capability; an AUC of 0.7-0.9 represented a moderate diagnostic ability; an AUC of >0.9 indicated a high diagnostic accuracy. We also applied STATA 14.0 (STATA Corp.) to conduct continuous variable meta-analysis. Fixed model (Mantel-Haenszel method) was applied at first and random model (Der Simonian and Laird method) was used when there existed significant heterogeneity. Funnel plot was described to test publication bias.

Results

The clinical value of HOTTIP expression in HCC. In the present study, an upregulated trend in HOTTIP level in HCC tissues (4.67-fold) was found when compared to normal liver tissues (P=0.002, Fig. 2A) based on qRT-PCR. We also investigated the relationship between different expression of HOTTIP and clinicopathological parameters. As a result, HOTTIP expression was highly expressed in III +IV and

Table I. Differential expression of HOTTIP of other clinicopathological parameters in HCC tissue based on qRT-PCR.

		HOTTIP expression		
Clinicopathological features	N	Fold change	T	P-value
Tissues				
Normal liver	41	1.00	3.226	0.002
HCC	41	4.67		
Age				
<50	19	3.33	1.233	0.226
≥50	22	6.00		
Sex				
Male	31	5.17	0.775	0.443
Female	10	3.17		
Cirrhosis				
No	27	6.00	-2.212	0.035
Yes	14	2.17		
TNM				
I+ II	25	2.50	-2.255	0.037
III +IV	16	8.17		
Metastasis				
No	38	4.17	-0.754	0.528
Yes	3	10.83		
Tumor diameter (cm)				
<7	11	2.67	-1.126	0.267
≥7	30	5.50		
Vascular invasion				
No	35	5.00	0.772	0.445
Yes	6	2.67		
Complete capsule				
No	16	4.67	0.050	0.961
Yes	25	4.83		
Differentiation				
Low	10	3.17	F=2.194	0.125
Moderate	27	4.67		
High	4	8.33		
AFP				
Negative	22	5.50	-1.409	0.169
Positive	13	2.67		
Embolus				
No	39	4.83	0.791	0.433
Yes	2	0.83		

no cirrhosis groups (P<0.05, Table I and Fig. 2B and C) in HCC, but no statistical significance was found in other clinicopathological parameters. In addition, the diagnostic value of the HOTTIP level in HCC was assessed by a ROC curve and the AUC of HOTTIP was 0.762 (95% CI, 0.660-0.864, P-value of <0.001, Fig. 2D), indicating a moderate diagnostic value of the HOTTIP level in HCC.

HOTTIP and HCC: a meta-analysis. The meta-analysis included 393 cases from multiple centers [two datasets in GEO (GSE58043 and GSE51701), the original data in TCGA, two probe sets (1564069_at and 1564070_s_at) of Wurmbach Liver in Oncomine and one publication (45)]. Thus, the pooled sensitivity and specificity of HOTTIP was 0.88 (0.70-0.96) and 0.59 (0.20-0.89, Fig. 3), respectively. In addition, the

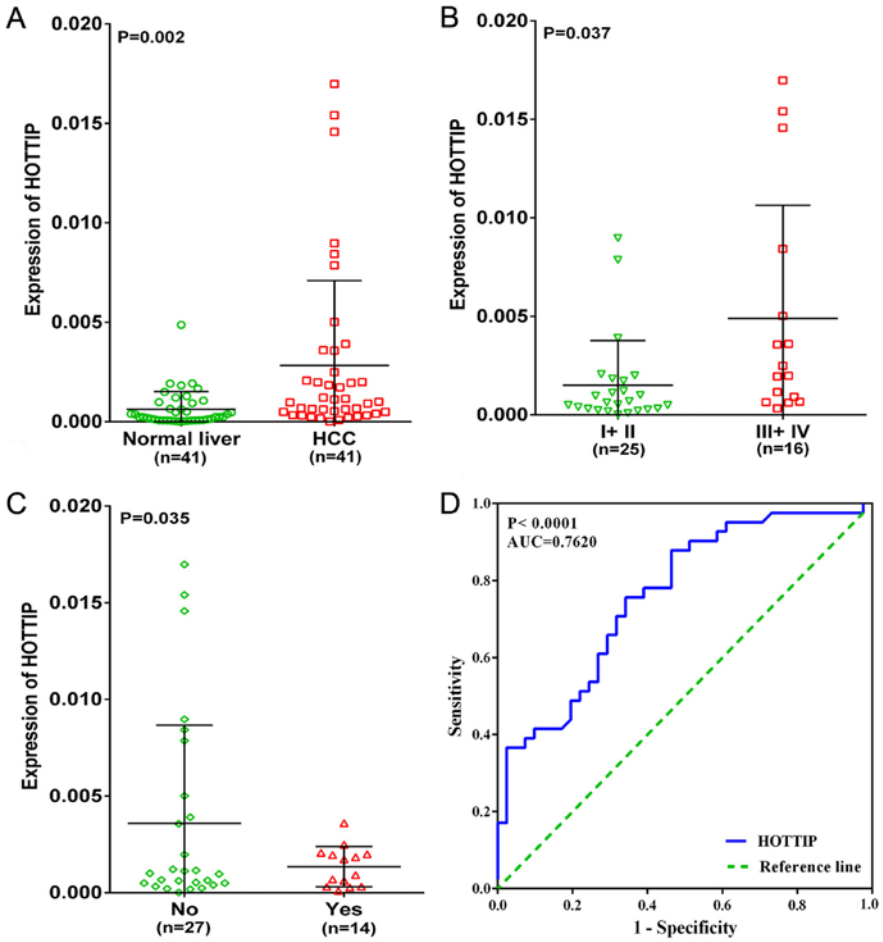

Figure 2. Clinical significance of HOTTIP in HCC based on qRT-PCR. (A) Differential expression of HOTTIP between HCC and non-cancerous liver tissue. (B) Differential expression of HOTTIP in I+ II vs. III +IV. (C) With cirrhosis vs. without cirrhosis. (D) ROC curve of HOTTIP in HCC.

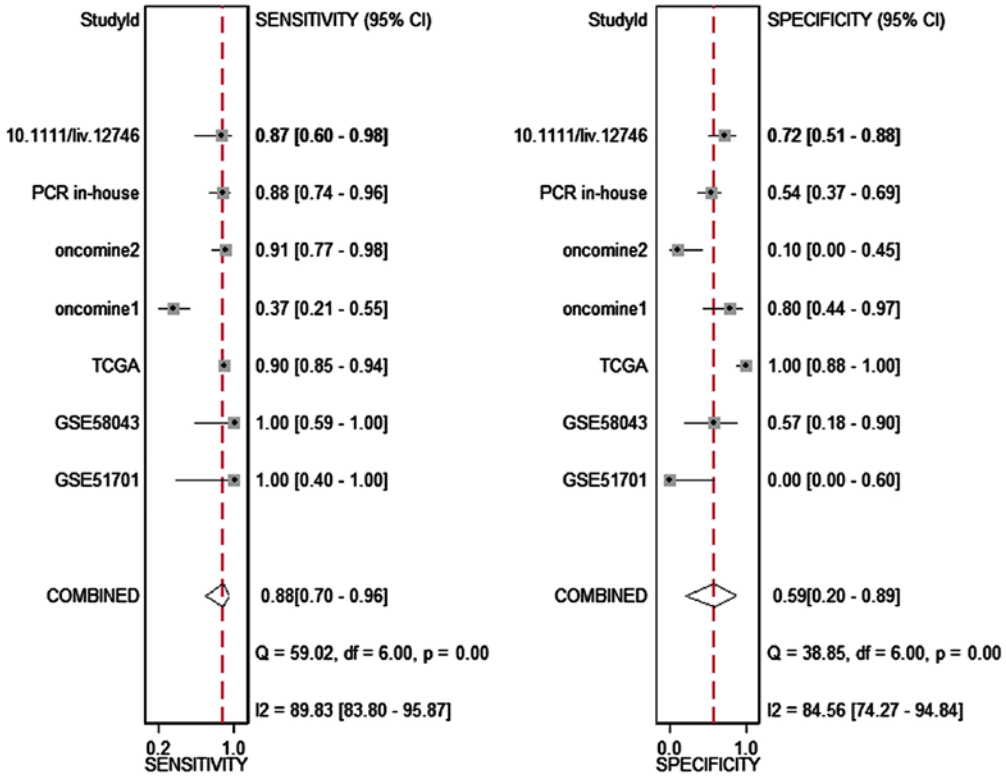

Figure 3. Forest plot showing the pooled sensitivity and specificity of the included studies. The pooled sensitivity and specificity of HOTTIP was 0.88 (0.70-0.96) and 0.59 (0.20-0.89), respectively.

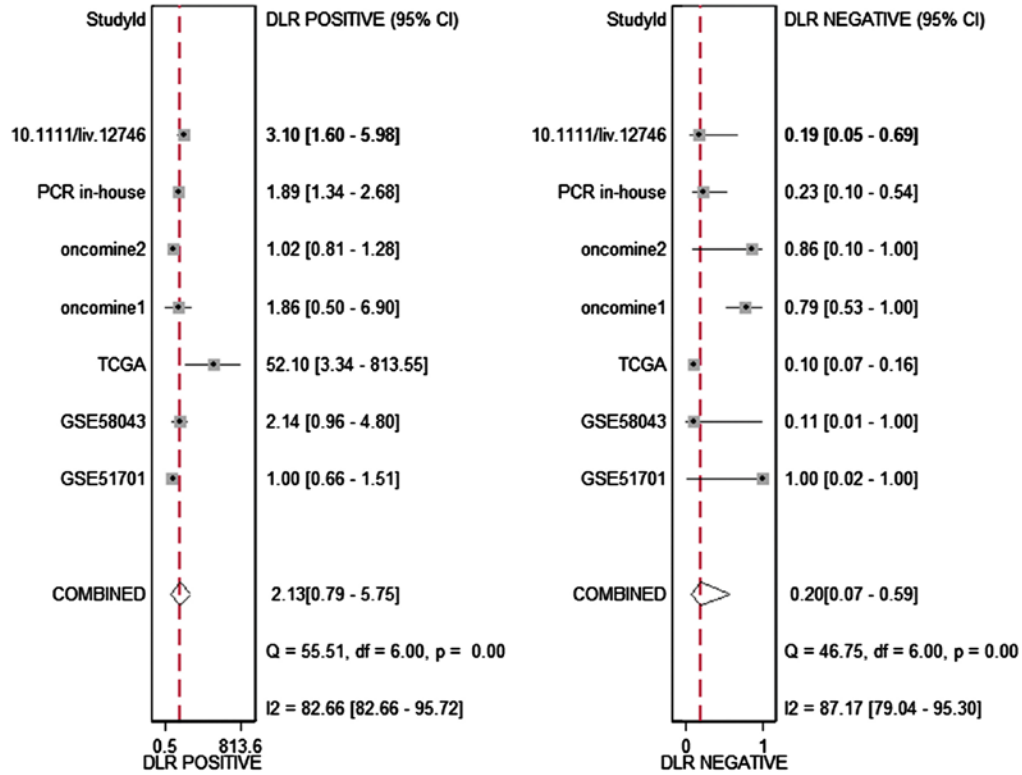

Figure 4. Forest plot showing the pooled positive DLR and negative DLR of the included studies. In addition, the DLR-positive and DLR-negative was 2.13 (0.79-5.75) and 0.20 (0.07-0.59), respectively.

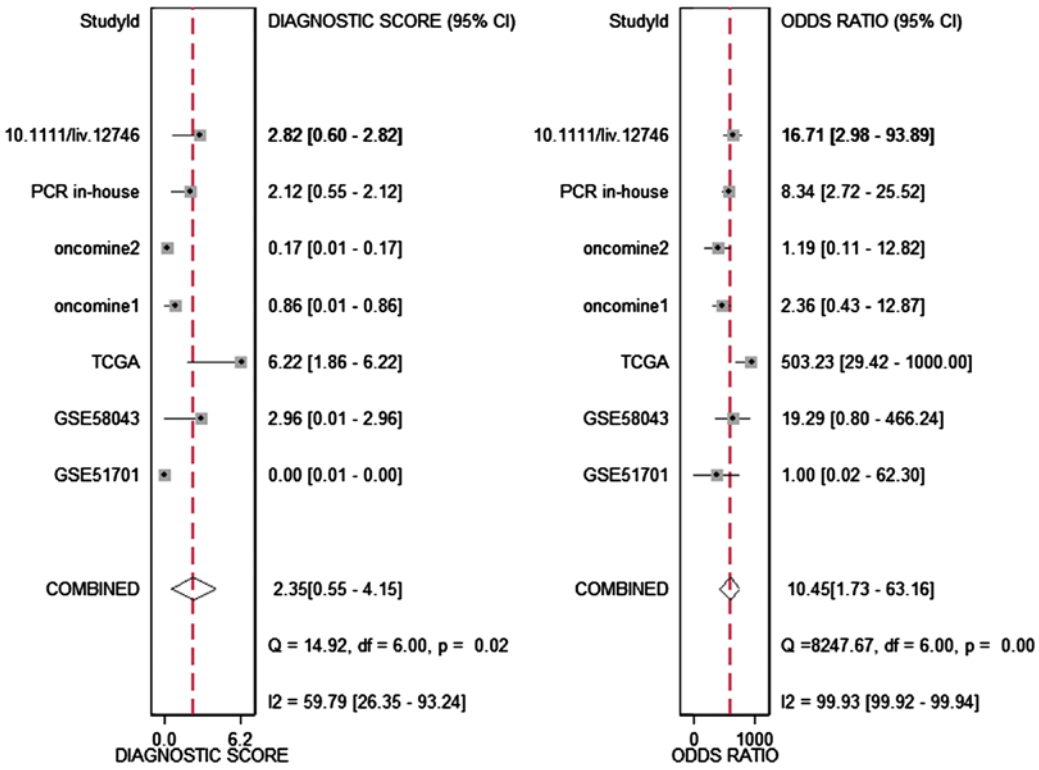

Figure 5. Forest plot showing the pooled diagnostic score and diagnostic odds ratio of the included studies. The diagnostic score and odds ratio was 2.35 (0.55-4.15) and 10.45 (1.73-63.16), respectively.

DLR-positive and DLR-negative were 2.13 (0.79-5.75) and 0.20 (0.07-0.59, Fig. 4), respectively. The diagnostic score and odds ratio were 2.35 (0.55-4.15) and 10.45 (1.73-63.16, Fig. 5), respectively. The AUC of SROC was 0.87 (0.83-0.89, Fig. 6), which indicated a moderate diagnostic value of HOTTIP in HCC. The pre-test probability was 20% when the positive and

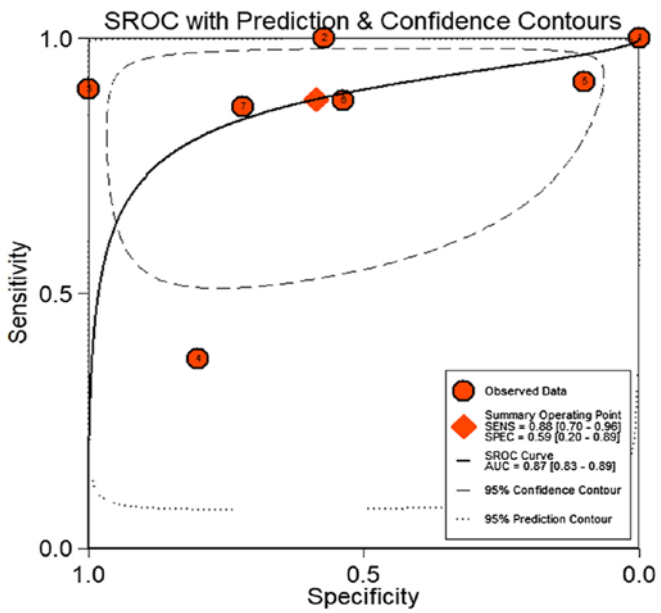

Figure 6. The summary receiver operating characteristic (SROC) curve for assessment of the diagnostic accuracy of HOTTIP for HCC. The AUC of SROC was 0.87 (0.83-0.89), which indicated a moderate diagnostic value of HOTTIP in HCC.

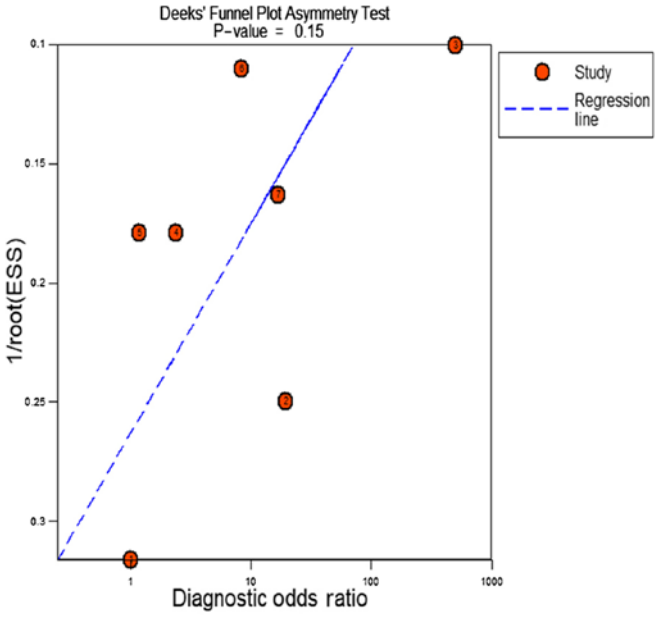

Figure 8. Publication bias of the included studies. 1/root (ESS) meant inverse root of effective sample sizes. Each circle represented an included study.

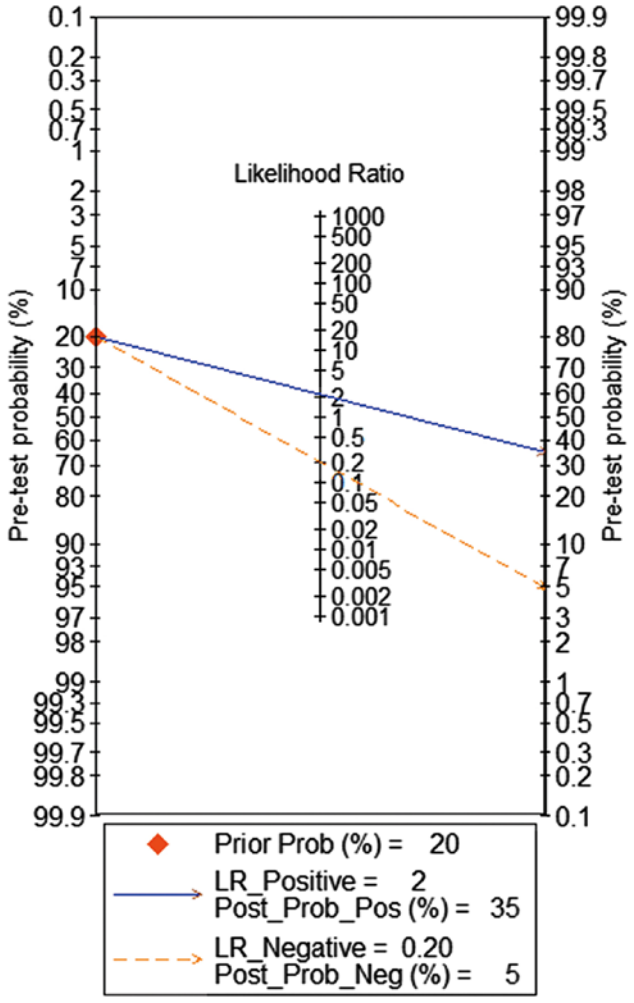

Figure 7. The pre-test and post-test probability of the included studies. The pre-test probability was 20% when the positive and negative pre-test probability was 35 and 5%, respectively.

negative pre-test probability was 35 and 5% (Fig. 7), respectively. As for the publication bias, no significant publication bias was found (P=0.15, Fig. 8).

As for the expression of HOTTIP in HCC compared to non-cancerous group, a fix-effect model was selected to calculate the pooled standard mean deviation (SMD) and 95% CI and the combined SMD reached 0.83 (0.57, 1.09), indicating a statistically significant higher expression of HOTTIP could be found in HCC than in normal control groups (P<0.001, Fig. 9A). In addition, no report bias was found in our study (P>0.05, Fig. 9B). Above all, a flow chart of this meta-analysis is shown in Fig. 10.

Validation of the expression of HOTTIP in HCC. To further explore the differential expression of HOTTIP between HCC and non-cancerous liver tissues, we performed a clinical research based on the original data in TCGA. One HCC cohort, which was comprised of 171 HCC cases and 28 non-cancerous liver cases, was extracted. Increased expression of HOTTIP was observed in HCC tissues (5.001±2.453) compared with the non-cancerous tissues (0.182±0.459, P<0.001, Fig. 11A). With regard to the clinicopathological parameters, we found that HOTTIP expression was highly expressed in age (≥60), sex (male), race (white) compared to that in the control group (P-value of <0.05, Table II and Fig. 11B-D). For the other clinicopathological characteristics, no statistical significance was found based on TCGA database. Moreover, the AUC of HOTTIP reached 0.982 (95% CI, 0.966-0.998, P<0.0001, Fig. 11E), which indicated a high diagnostic value of the HOTTIP level in HCC.

Additionally, two probe sets (1564069_at and 1564070_s_at) of Wurmbach Liver in Oncomine were used to validate the HOTTIP expression. However, an opposite trend was found in the two probe sets (Fig. 12). HOTTIP was downregulated in 1564070_s_at probe set compared to the normal liver, which was inconsistent with the results of qRT-PCR and TCGA.

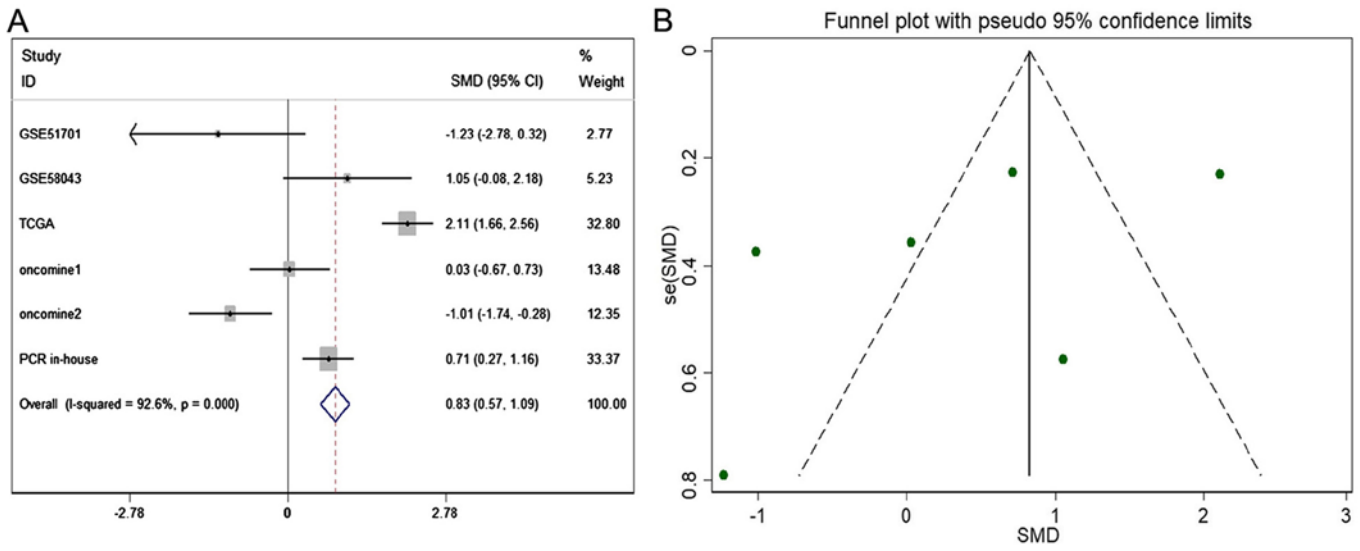

Figure 9. The expression condition of HOTTIP in HCC compared to non-cancerous. (A) Forest plot of datasets evaluating HOTTIP expression between HCC and normal control groups (random-effects model). (B) Funnel plot of datasets and no publication bias was found in our study.

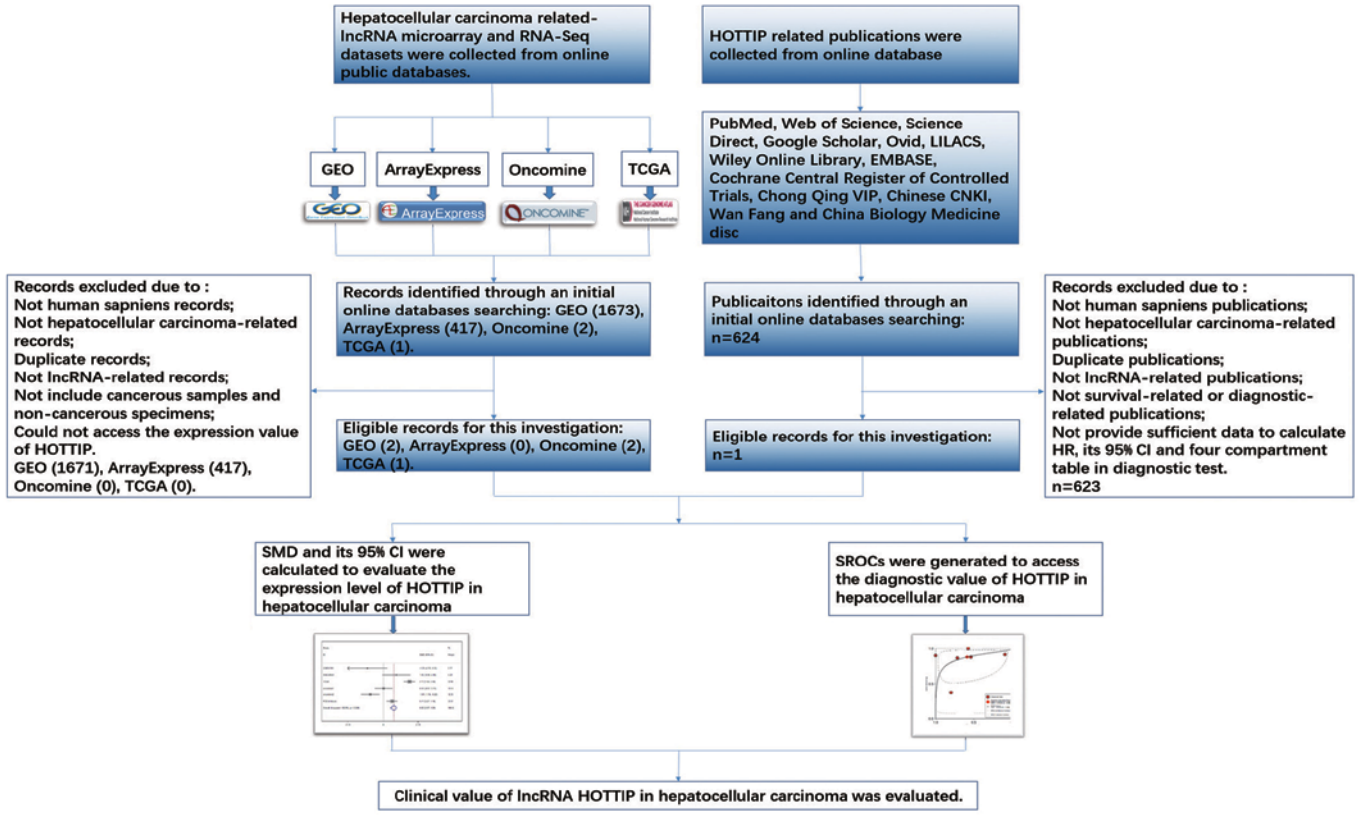

Figure 10. A flow chart of this meta-analysis.

Also, the opposite trend of HOTTIP expression might be a source of heterogeneity in meta-analysis.

The potential pathways associated with HOTTIP. Based on GEO, TCGA and MEM database, 287 overlapped genes were selected (Fig. 13). In addition, GO and pathway analyses were performed using these 287 genes. The most strongly enriched GO terms were identified as follows: embryonic morphogenesis, gland development, transcription factor activity and extrinsic to membrane (Table III). To better affect the functions of these genes, a function network was constructed according to GO analysis (Fig. 14). Besides, the KEGG pathway analysis confirmed that these genes were significantly involved in PPAR signaling pathway, Fc γ R-mediated phagocytosis and endocytosis (Table IV). Altogether, the GO and KEGG pathway analysis revealed that HOTTIP might participate in the biological mechanism of HCC. In addition, a gene network of the 287 genes was

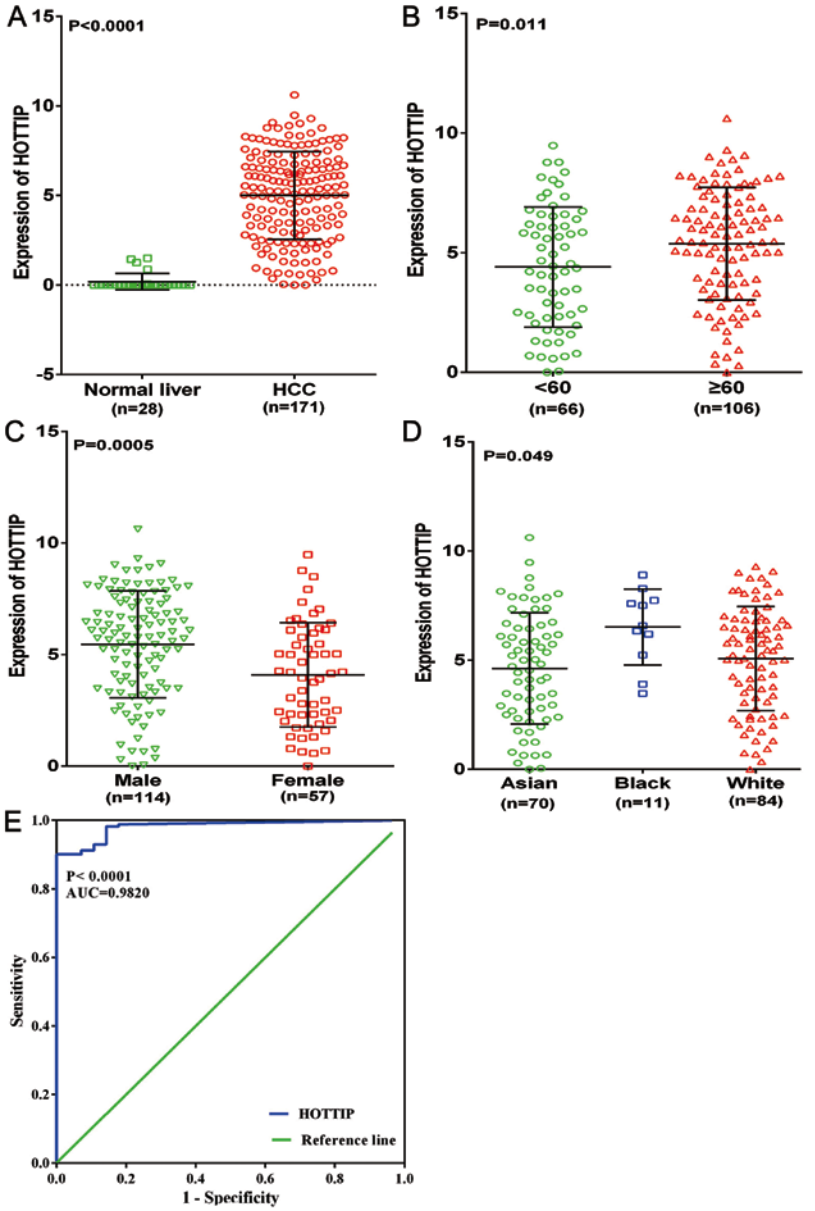

Figure 11. Clinical significance of HOTTIP in HCC based on TCGA database. (A) Differential expression of HOTTIP between HCC and non-cancerous liver tissue. (B) Differential expression of HOTTIP in <60 vs. ≥60. (C) Male vs female. (D) Asian vs. Black vs. White. (E) ROC curve of HOTTIP in HCC.

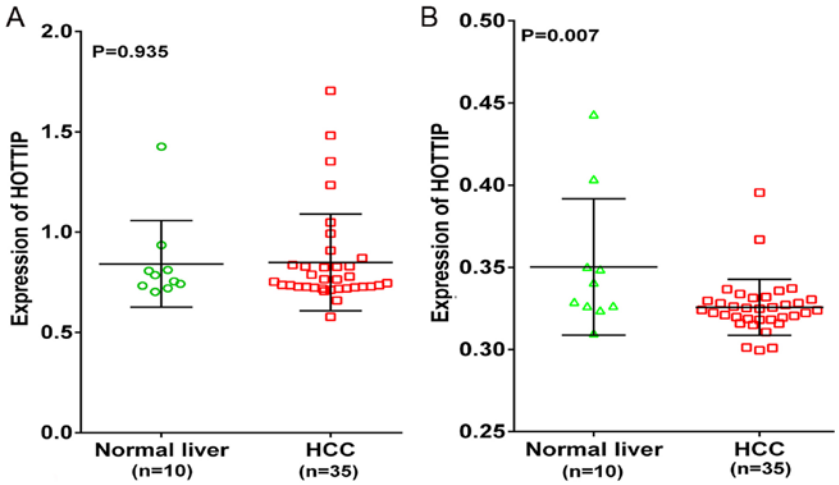

Figure 12. Validation of HOTTIP expression in the cohort of Wurmbach Liver from Oncomine. (A) Normal liver tissues (n=10) and hepatocellular carcinoma tissues (n=35) were included in the cohort of Wurmbach Liver (1564069_at). (B) Normal liver tissues (n=10) and hepatocellular carcinoma tissues (n=35) were included in the cohort of Wurmbach Liver (1564070_s_at).

Table II. Differential expression of HOTTIP of clinicopathological parameters in HCC tissue based on TCGA database.

		HOTTIP expression		
Clinicopathological features	N	Mean ± SD	T	P-value
Tissues				
Normal liver	28	0.182±0.459	8.16	<0.0001
HCC	171	5.001±2.453		
Age				
<60	66	4.405±2.513	-2.563	0.011
≥60	106	5.376±2.349		
Sex				
Male	114	5.457±2.393	3.556	0.0005
Female	57	4.089±2.332		
Race				
White	84	5.077±2.379	F=3.078	0.049
Black	11	6.522±1.740		
Yellow	70	4.622±2.540		
T (tumor)				
T1+ T2	133	5.099±2.372	1.236	0.218
T3+ T4	37	4.539±2.657		
Vascular invasion				
No	95	4.898±2.405	0.521	0.603
Yes	51	5.118±2.509		
Patological grade				
g1+g2	107	5.068±.2.434	0.569	0.570
g3+g4	61	4.847±2.382		
Stage				
I+ II	126	5.065±2.333	1.544	0.125
III +IV	32	4.341±2.520		
Recurrence				
No	151	4.942±2.468	1.334	0.184
Yes	10	6.008±2.029		

Table III. Top 5 enrichment GO terms (BP, CC and MF) of the potential genes of HOTTIP.

GO ID	Term	Ontology	Count	P-value
GO:0048732	Gland development	BP	12	5.32E-06
GO:0007389	Pattern specification process	BP	16	1.08E-05
GO:0003002	Regionalization	BP	13	3.84E-05
GO:0048598	Embryonic morphogenesis	BP	16	5.50E-05
GO:0051216	Cartilage development	BP	8	1.12E-04
GO:0043565	Sequence-specific DNA binding	MF	23	3.61E-04
GO:0003700	Transcription factor activity	MF	31	5.20E-04
GO:0008289	Lipid binding	MF	17	2.81E-03
GO:0004385	Guanylate kinase activity	MF	3	1.31E-02
GO:0042803	Protein homodimerization activity	MF	12	2.08E-02
GO:0019898	Extrinsic to membrane	CC	23	3.54E-06
GO:0045177	Apical part of cell	CC	13	1.32E-05
GO:0016324	Apical plasma membrane	CC	11	2.63E-05
GO:0044459	Plasma membrane part	CC	52	4.28E-04
GO:0005624	Membrane fraction	CC	22	7.97E-03

GO, Gene Ontology. BP, biological process. CC, cellular component. MF, molecular function.

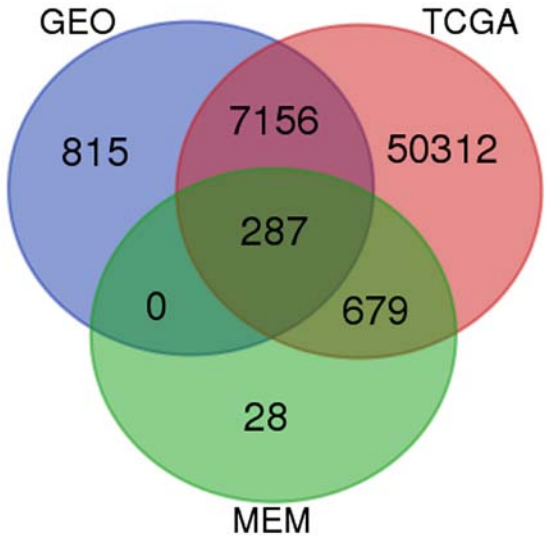

Figure 13. A flow chart to screen the co-expressed genes based on Venn diagrams.

constructed in the present study (Fig. 15), from which we could easily observe relationships between HOTTIP and these potential genes.

The PPI network was constructed through STRING online and a total of 72 PPI pairs with combined score >0.4 were noted (Fig. 16). Also, PPARA had the highest degree (degree 6) according to the PPI network.

In addition, a total of four genes (PPARA, PPARG, FABP2, SCP2) related to PPAR signaling pathway were detected based on KEGG pathway analysis. Moreover, we investigated the expression of these four genes and their correlations with HOTTIP based on the original data in TCGA. We found that both PPARG and FABP2 were highly expressed in HCC compared to normal liver ($P<0.05$, Fig. 17A and B), whereas SCP2 was highly expressed in normal liver (14.99 ± 0.082 vs. 13.74 ± 0.064, $P<0.001$, Fig. 17C). However, only a minor difference was found in PPARA expression between normal liver and HCC (11.88 ± 0.098 vs. 11.73 ± 0.054, $P=0.174$, Fig. 17D). As for the correlation between HOTTIP

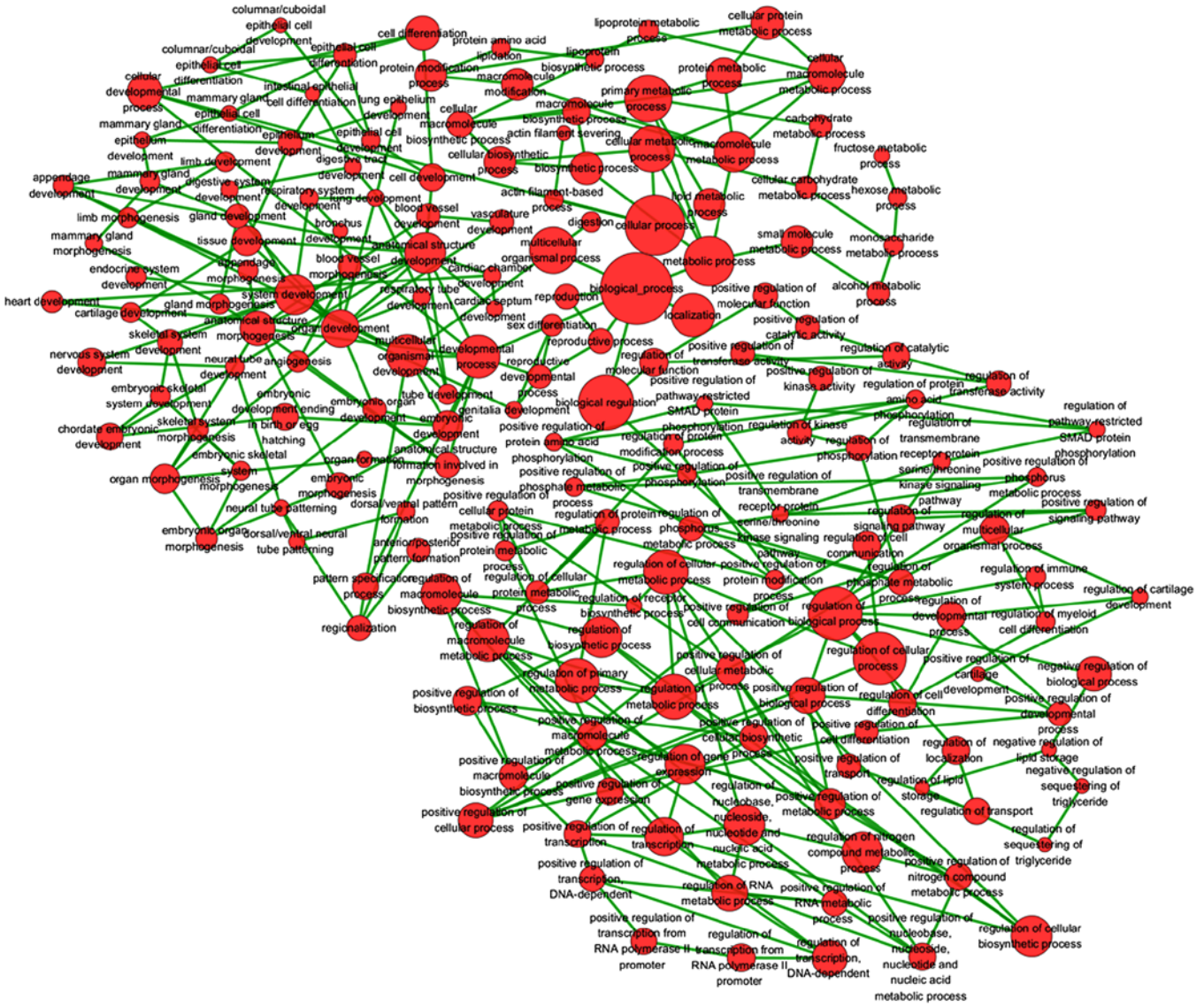

Figure 14. A function network of Gene Ontology (GO) terms for the potential genes of HOTTIP in HCC. Based on GEO, TCGA and MEM database, 287 overlapped genes were selected. In addition, GO analysis was performed using these 287 genes. To further reflect the functions of these genes, a function network was constructed based on GO analysis.

Table IV. KEGG pathway enrichment analysis of the potential genes of HOTTIP.

KEGG ID	KEGG term	Count	P-value	Gene symbol
hsa04666	Fc γ R-mediated phagocytosis	5	5.94E-02	VAV3, SCIN, PIP5K1B, PLA2G4F, SYK
hsa04144	Endocytosis	7	6.52E-02	ACVR1B, EPN3, CHMP4C, ERBB3, RAB4A, PIP5K1B, NEDD4L
hsa03320	PPAR signaling pathway	4	9.13E-02	PPARA, PPARG, FABP2, SCP2

KEGG, Kyoto Encyclopedia of Genes and Genomes.

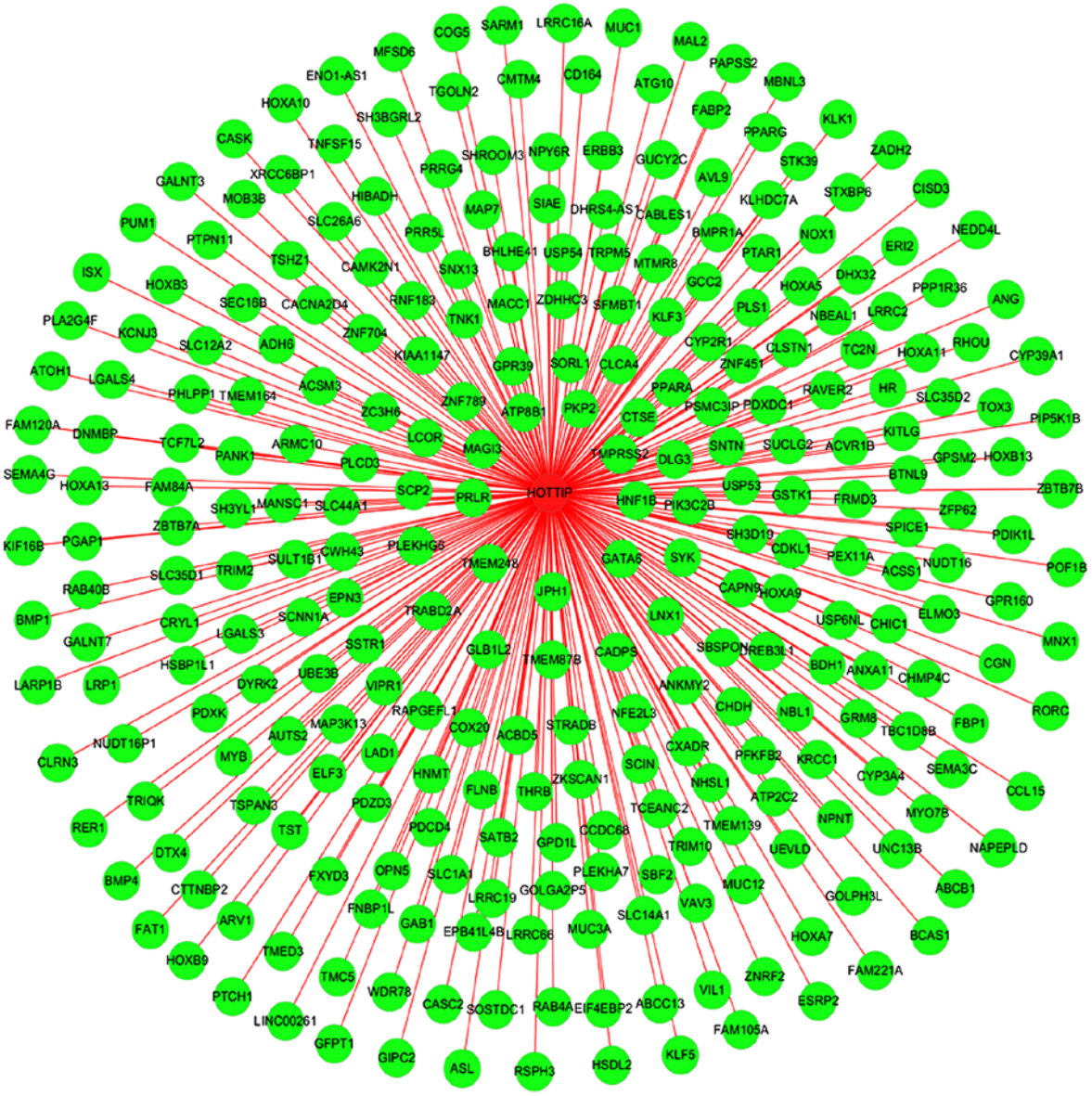

Figure 15. Network analysis between HOTTIP and the potential genes. A gene network of the 287 overlapped genes was constructed. The relationships between HOTTIP and these potential genes were easily observed from this network.

and these genes, we discovered that HOTTIP had a positive correlation with PPARA or SCP2 (P<0.001, Fig. 17E and F), whereas a negative correlation was found between HOTTIP and PPARG and FABP2 (P>0.05, Fig. 17G and H). Based on the aforementioned results, we hypothesized that HOTTIP may influence the SCP2 expression of PPAR signaling

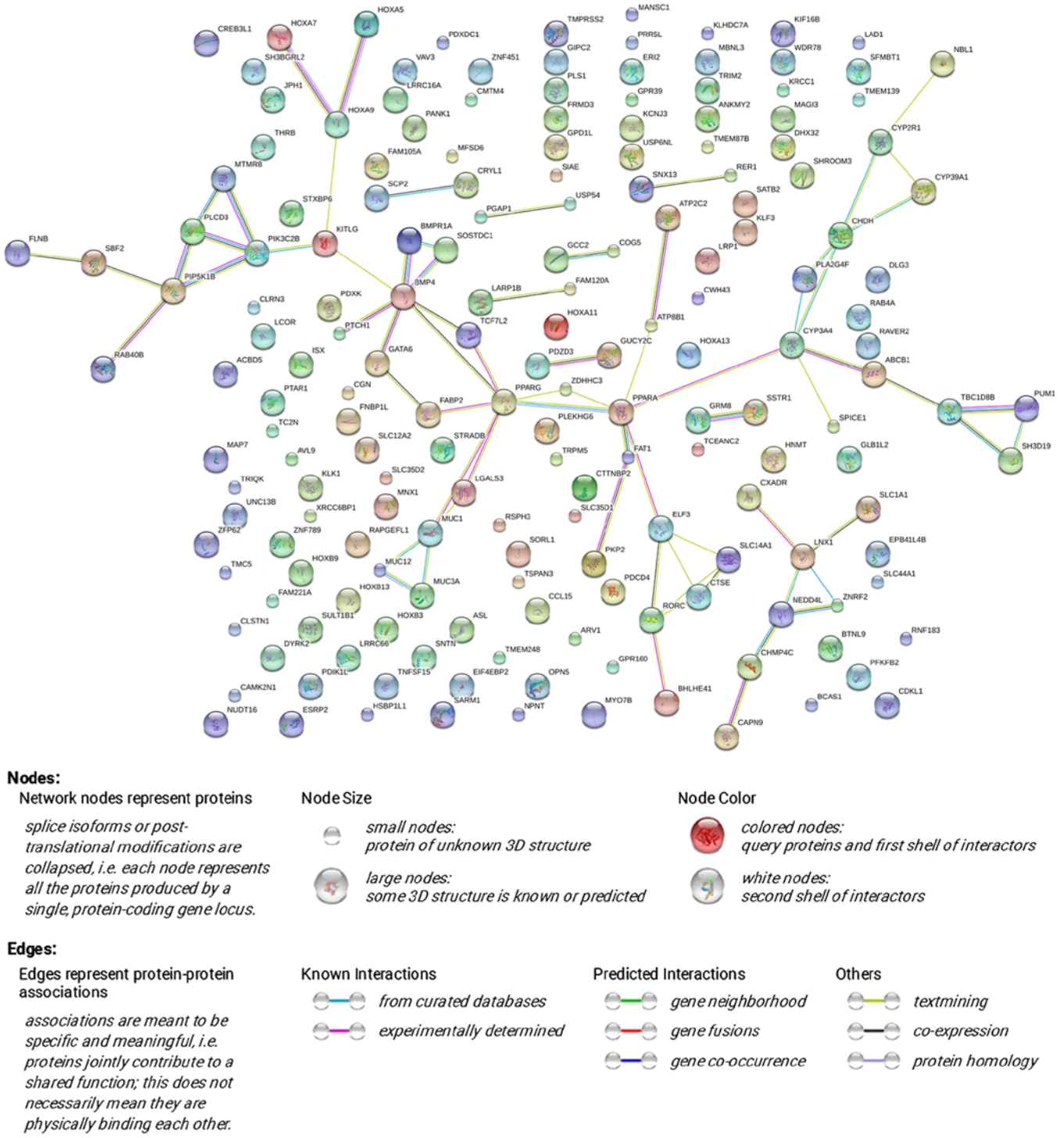

Figure 16. The PPI network of the co-expressed genes. The PPI network was constructed via STRING online and 72 PPI pairs was chosen for further analysis.

pathway to participate in different biological processes of HCC.

Discussion

Up to now, countless studies have confirmed that lncRNAs could participate in various chemical and biological processes, such as chromosome remodeling, transcription, cancer metastasis and posttranscriptional processing (66,67). Many studies have demonstrated that lncRNAs were related to tumorigenesis and development of HCC through various pathways, including regulation of apoptosis, cell cycle and chemotherapy resistance in HCC tissue or cell lines (68-70). lncRNAs have opened an avenue of cancer genomics.

To date, several studies have investigated the effect and potential mechanism of HOTTIP on HCC. Quagliata *et al* (71) found that the HOTTIP expression was associated with HCC progression and HOTTIP could be a predictive biomarker in HCC based on the snap-frozen needle HCC biopsies and their matched non-neoplastic counterparts. They also clarified that HOTTIP could directly control the expression of HOXA locus gene by interacting with the WDR5/MLL complex, but

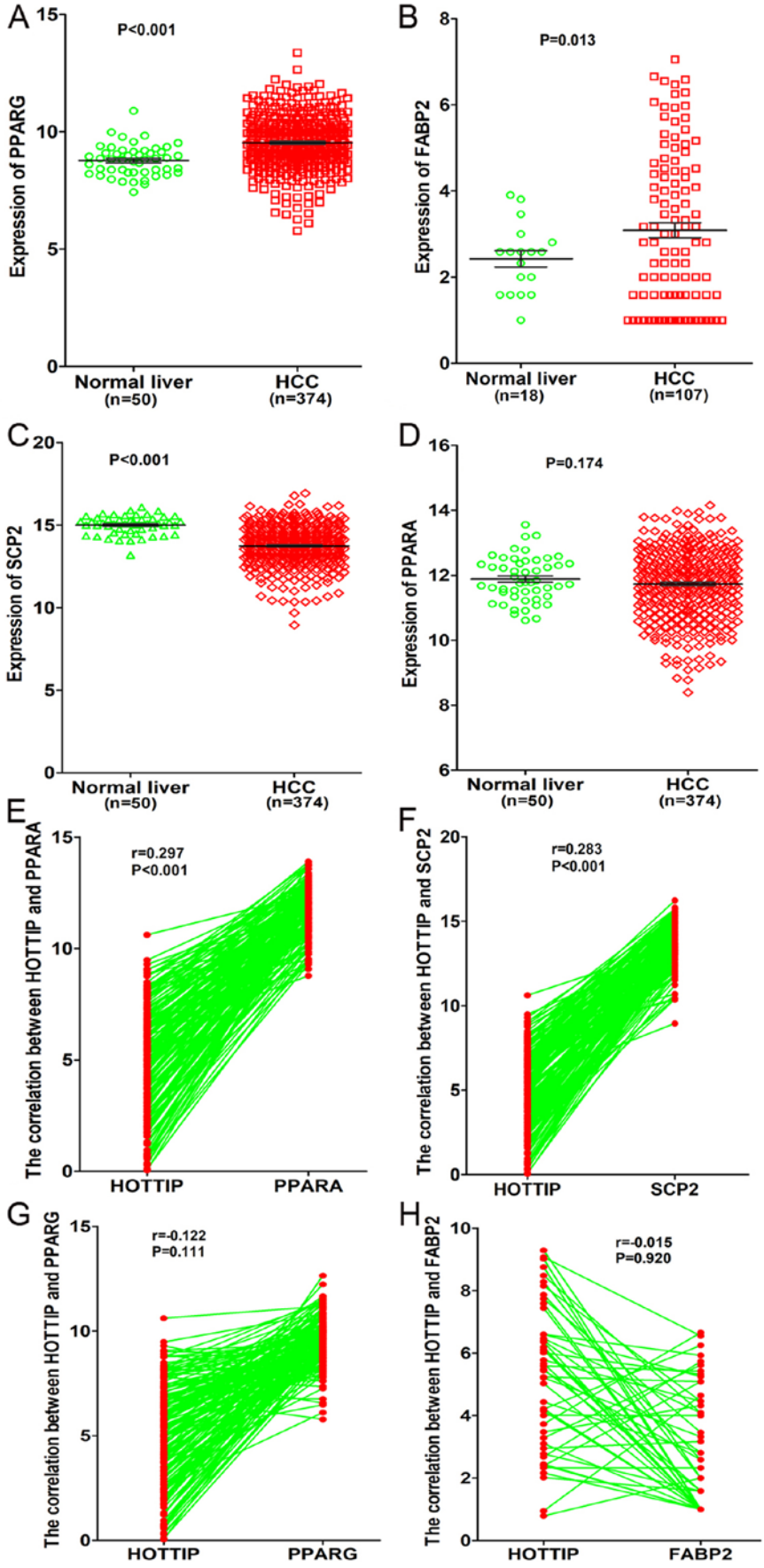

Figure 17. Clinical significance of four related genes (PPARA, PPARG, FABP2, SCP2) in HCC based on TCGA database. (A) Differential expression of PPARG between HCC and non-cancerous liver tissue. (B) Differential expression of FABP2 between HCC and non-cancerous liver tissue. (C) Differential expression of SCP2 between HCC and non-cancerous liver tissue. (D) Differential expression of PPARA between HCC and non-cancerous liver tissue. (E) Positive correlation between HOTTIP and PPARA. (F) Positive correlation between HOTTIP and SCP2. (G) Negative correlation between HOTTIP and PPARG. (H) Negative correlation between HOTTIP and FABP2.

the specific relationship between HOTTIP and HOX genes is still vague. Tsang *et al* (45) found that HOTTIP was an oncogenic lncRNA and highly expressed in HCC tissues based on qRT-PCR, and HOTTIP could contribute to hepatocarcinogenesis via targeting tumor suppressive miR-125b, which was verified by luciferase reporter assay and functional analysis. Also, the migratory ability of HCC cells could be inhibited after silencing HOTTIP expression. Ge *et al* (72), using dual luciferase reporter assays, confirmed that HOTTIP was a significant oncogene in HCC and miR-192/-204-HOTTIP axis was a significant molecular pathway during tumorigenesis of HCC. They also demonstrated the prognostic and potential therapeutic roles of HOTTIP. In comparison, we designed this study using RT-qPCR, meta-analysis and bioinformatics to further investigate the effect of HOTTIP in HCC. Interestingly, we discovered that HOTTIP was a tumorigenic gene and HOTTIP expression was highly expressed in TNM (III +IV), age (≥60), sex (male), race (white) and cirrhosis (no) compared to that in the control groups. In the present study, ROC curve was applied to evaluate the association between HOTTIP expression and the diagnostic value, and the AUC of HOTTIP indicated the potential diagnostic value of HOTTIP level in HCC. Moreover, this is the first meta-analysis to investigate the expression and diagnostic value of HOTTIP in HCC. As a result, the SMD of the meta-analysis validated the higher expression of HOTTIP in HCC. Furthermore, in the diagnostic meta-analysis, 393 cases from GEO, TCGA, Oncomine and publications were included. The meta-analysis was performed to evaluate the validity of HOTTIP for the detection of HCC. The sensitivity of the HOTTIP assay in the included parts ranged from 70 to 96%, and the specificity of HOTTIP range from 20 to 89%. The combined values of sensitivity (0.88) and specificity (0.59) demonstrated the accuracy of HOTTIP for the detection of HCC. Also, our results clarified that the SROC curve was located near the upper left corner. The AUC was 0.87, which indicated a moderate diagnostic accuracy (73). The PLR and NLR were presented to measure the diagnostic accuracy of HOTTIP. Likelihood ratios >10 or <0.1 was identified as high accuracy. A PLR value of 2.13 suggested that patients with HCC had an ~2.13-fold higher chance of being HOTTIP assay-positive. In addition, the NLR (0.20) showed that if the HOTTIP result was negative, the chance that this patient has HCC was ~20%. Hence, the high diagnostic accuracy in meta-analysis confirmed our results. However, there were some limitations in our meta-analysis. The heterogeneity (high I-square values) is unavoidable, partly because of the opposite results in one probe set of Oncomine. Furthermore, blinding in 4 included databases was not certain, which also contributed to the heterogeneity. In addition, only two publications concerned the prognosis of HOTTIP. In this case, the number of studies restricted the prognostic meta-analysis.

As reported, HOX gene encoded-transcription could regulate cell fate and embryonic development (71). According to GO and KEGG analyses, we found that the most strongly enriched functional terms were embryonic morphogenesis, gland development, transcription factor activity and extrinsic to membrane. Also, the HOTTIP co-expressed genes were significantly related to PPAR signaling pathway. Several studies have demonstrated that PPAR signaling pathway played a vital role in HCC, but no studies were found on HOTTIP and PPAR signaling pathway (74,75). In the present study, we hypothesized that HOTTIP could play a significant role in HCC via PPAR signaling pathway, but the specific underlying mechanism of HOTTIP in HCC still needs to be investigated with further research. We also investigated the genes from PPAR signaling pathway and the hub genes from PPI. In addition, we hypothesized that HOTTIP may influence the SCP2 expression of PPAR signaling pathway to participate in different biological processes of HCC. To verify our hypothesis, we intend to perform various experiments, including proliferation, invasion and metastasis assays, chicken embryochorioallantoic membrane and nude mouse models. The clinical significance and the molecular mechanism of HOTTIP on the biological function of HCC will be investigated from the perspectives of molecule, cell, tissue and animal. However, the circulating HOTTIP in HCC patients has higher clinical value in evaluating the real diagnostic potential of HOTTIP than HOTTIP in tissues. We also intend to perform in-depth exploration on the circulating HOTTIP in HCC patients in the future. Focusing on the new insight of HOTTIP, this study aimed to provide a new biomarker or therapeutic target for HCC.

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