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ORIGINAL RESEARCH

Upregulation of long non-coding RNA FOXP4-AS1 and its regulatory network in hepatocellular carcinoma

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Objective: FOXP4-AS1 (FOXP4 antisense RNA 1) is putatively a functional oncogene in colorectal cancer. This study constructed a regulatory network involving FOXP4-AS1 for better understanding of its function in hepatocellular carcinoma (HCC).

Methods: FOXP4-AS1 was assessed in HCC and adjacent normal (control) liver samples via quantitative real-time PCR. Differentially expressed micro RNAs (DEmiRNAs) were predicted. Their target genes were verified via the gene expression profiling interaction analysis (GEPIA) database, and subjected to gene ontology (GO) annotation and KEGG (Kyoto Encyclopedia of Genes and Genome) pathway enrichment analysis. Protein-protein interaction (PPI) networks were established and hub genes identified with Cytoscape software. The GEPIA database was used to assess the prognostic roles of 20 hub genes in liver cancer. The cBioPortal database was used to exhibit alterations of the genes.

Results: The HCC samples had significantly higher levels of FOXP4-AS1 compared with the control (P=0.001). Six upregulated and 4 downregulated DEmiRNAs were identified. Over- and under-expressed predicted target genes (183 and 147, respectively) were selected for GO annotation and KEGG pathway enrichment analysis. The downregulated genes were significantly prominent in the PI3K-Akt signaling pathway; the upregulated genes in the cell cycle. The PPI networks indicated IGFBP3 and PRC1 as hub genes with the highest node degrees. Higher expressions of 9 (6) genes were associated with worse (better) prognosis in HCC.

Conclusion: An HCC-associated FOXP4-AS1-miRNA-mRNA regulatory network was constructed, and molecular mechanisms involved in HCC development were elucidated. This work provides direction for finding new HCC therapeutic targets.

Keywords: quantitative real-time PCR, hepatocellular carcinoma, long non-coding RNA, FOXP4-AS1

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Introduction

Hepatocellular carcinoma (HCC) globally accounts for more than 90% of primary liver cancer cases, and HCC is one of the most common malignancies.[1] Despite significant advances in therapy, the 5-year survival rate for patients with HCC remains low, mainly due to high recurrence and metastasis rates.[2] Further understanding of the pathogenesis of HCC and new prevention and treatment strategies are urgently needed.

Long non-coding RNAs (lncRNAs) are a class of transcripts longer than 200 nucleotides. Thousands of lncRNAs have been identified, and some have key roles

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in various cellular processes.[3,4] LncRNAs associated with HCC are important for the regulation of tumorigenesis and progression, and have become markers of HCC that can be used for early diagnosis and evaluation of prognosis and therapy.[5,6] A significant association between the lncRNA-miRNA-mRNA regulatory network and the liver is believed to exist; deregulation of the lncRNA-miRNA-mRNA regulatory network in the liver leads to a variety of human diseases, such as cirrhosis and liver cancer.[3,5] A comprehensive lncRNA-miRNA-mRNA regulatory network in liver cancer has not been established.

The lncRNA FOXP4-AS1 (forkhead box protein P4 antisense RNA 1) promotes the progression of various cancers, including osteosarcoma and colon cancer.[7,8] However, associations between FOXP4-AS1 and either the progression/metastasis of liver cancer or clinical features of patients have rarely been reported.

The present study evaluated the expression of FOXP4-AS1 in HCC via quantitative real-time PCR (qRT-PCR), and investigated an association between FOXP4-AS1 and clinical features. The potential mechanism of FOXP4-AS1 in HCC was explored by predicting the genes targeted by FOXP4-AS1 and performing an enrichment analysis. In addition, we screened for hub genes and validated their expressions and prognostic roles using the gene expression profiling interaction analysis (GEPIA) database. Finally, an HCC-associated FOXP4-AS1-miRNA-mRNA regulatory network was constructed that explores the molecular mechanisms involved in HCC development.

Methods

The hospital ethics committee approved this study, and all patients signed the relevant informed consent forms.

Data collection and quantitative real-time PCR

Affiliated Tumor Hospital of Guangxi Medical University provided 213 pairs of HCC and matched normal liver tissues from 213 respective patients. These were preserved by formalin fixation and paraffin embedding. The FOXP4-AS1 gene expressions in the 213 sample pairs were detected by qRT-PCR using Applied Biosystems 7900HT Fast Real-Time PCR System software. For analysis, the gene expression in the HCC tissue of each patient was compared relative to that of the average of the adjacent normal tissues, and the patients were accordingly apportioned to high- and low-expression groups.

Patients were followed-up in the short term (within one year) once every three months mainly by outpatient reexamination, and in the long term (after one year) by telephone. The follow-up deadline was May 2018. The median follow-up was 31 (2 to 53) months. The characteristics of the 213 patients were recorded (Table S1). Cut-off values of clinical indicators were determined based on previous studies and Chinese guidelines for HCC.[9,10]

Prediction of potential miRNA

DIANA tools (http://carolina.imis.athena-innovation.gr/diana_tools/) and LncRNASNP2 (http://bioinfo.life.hust.edu.cn/lncRNASNP/) were used to predict target miRNAs for lncRNA FOXP4-AS1. Differentially expressed miRNAs (DEmiRNAs) in HCC, relative to normal samples, were identified with OncomiR (http://www.oncomir.org/). The intersection of the predicted miRNA in LncRNASNP2 and DIANA tools, with the miRNA in OncomiR, was then considered a target DEmiRNA of FOXP4-AS1 in HCC.

Prediction of target genes of demirnas

The target gene was predicted by miRWalk 3.0. To improve the reliability of the subsequent analysis of the screened DEmiRNA target genes, the expression of these target genes was confirmed using the GEPIA database. The intersection of the upregulated and downregulated genes with the predicted target genes was considered a target gene for FOXP4-AS1 in HCC.

Gene ontology (GO) annotation and KEGG pathway enrichment analysis

The Enrichr database (http://amp.pharm.mssm.edu/Enrichr/) was used to perform GO functional annotation and KEGG pathway enrichment analysis, for the target genes and the most frequently altered neighboring genes.

Establishment and analysis of protein-protein interaction (PPI) network

The PPI networks were established using the STRING database (https://string-db.org/). The hub genes were identified according to nodal degree (number of genes correlated with the target gene), using Cytoscape software (version 3.6.1).

Validation of hub gene

The expression levels of the top 20 hub genes were further validated using the GEPIA database. Hub genes with $|\log_2$ (fold change)| >1 and $P<0.05$ were considered statistically significant.

GEPIA database analysis

The prognostic roles of the screened 20 hub genes in HCC were analyzed using the GEPIA database. Cox $P<0.05$ was considered significant.

Analysis via cbioportal

The alterations of hub genes and neighboring genes of hub genes in the HCC sample were analyzed using the cBioPortal database. GO and KEGG pathway enrichment analyses were conducted of the most frequently altered neighbor genes using the Enrichr database.

Statistical analysis

Most of the statistical analyses were conducted using the bioinformatic tools mentioned above. Genes or miRNAs with $|\log_2$(fold change)| >1 and $P<0.05$ were considered as statistically significant. Cox $P<0.05$ was considered as statistically significant for survival analysis.

Results

FOXP4-AS1 in HCC samples

Using RT-qPCR, the clinical expression of FOXP4-AS1 in 213 HCC tissues was evaluated relative to adjacent matched normal tissues (Figure 1). The levels of FOXP4-AS1 were significantly higher in the HCC samples compared with the normal liver samples (P=0.001).

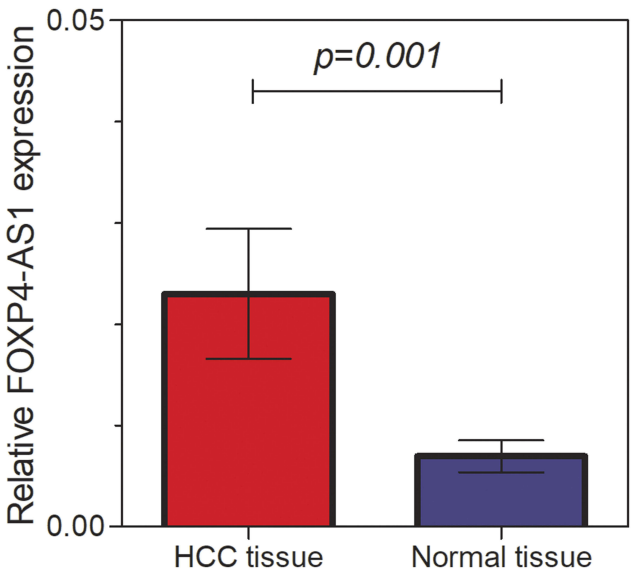

Figure 1 Levels of FOXP4-AS1 in HCC and control groups, based on quantitative real-time PCR data.

Associations between FOXP4-AS1 level in HCC and patients' clinical characteristics

The chi-squared analysis of the qRT-PCT data showed that the levels of FOXP4-AS1 in HCC samples were associated with the following clinical characteristics (Table S1): serum alpha-fetoprotein (AFP), serum aspartate aminotransferase (AST), and size of tumor (P=0.001, each); and liver cirrhosis, BCLC stage, and patient age (P=0.043, 0.024, and 0.003, respectively).

All indexes were included in the multivariate COX regression analysis. Multivariate analyses showed that FOXP4-AS1 was an independent risk factor for

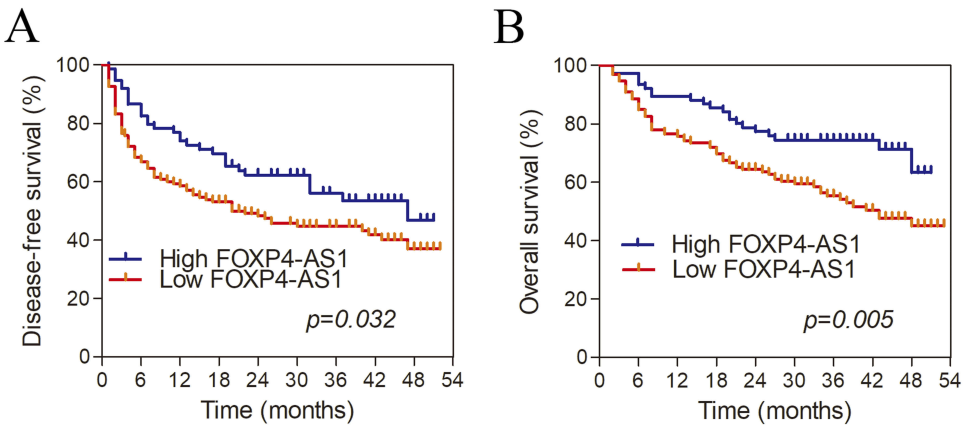

Figure 2 Kaplan-Meier curves for FOXP4-AS1 in HCC based on qRT-PCR. (**A**) Disease-free survivals of groups with low and high levels of FOXP4-AS1. (**B**) Overall survival of groups with low and high levels of FOXP4-AS1.

disease-free and overall survival times of patients after hepatectomy (P=0.037 and 0.007, respectively; Table S2).

A Kaplan-Meier curve was used to identify the effects of FOXP4-AS1 level on survival time and disease-free survival (Figure 2). The P-values of the Kaplan-Meier curves were less than 0.05 in HCC. This indicated that patients with higher-than-normal levels of FOXP4-AS1 had significantly shorter overall survival time (P=0.005) and disease-free survival (P=0.032) compared with the patients in the group with lower levels.

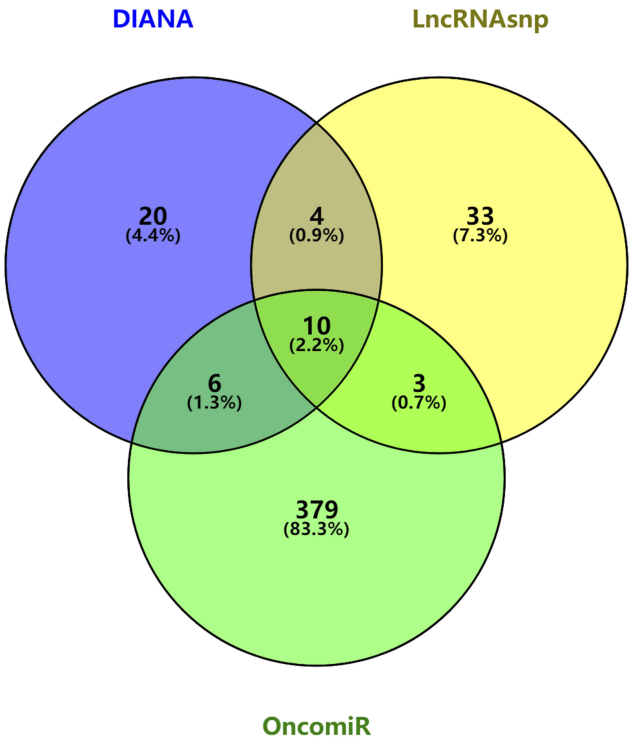

Figure 3 Venn diagram of DEmiRNAs of FOXP4-AS1.

Identification of candidate DEmiRNAs

From DIANA tools and LncRNASNP2, 14 target miRNAs were predicted for FOXP4-AS1. Based on the OncomiR database, 398 DEmiRNAs in HCC were respectively selected. After intersection, 6 upregulated and 4 downregulated DEmiRNAs were selected (Figure 3). Specifically, the 6 upregulated DEmiRNAs were: miR-10b-3p, miR-652-3p, miR-500a-3p, miR-526b-5p, miR-944, and miR-491-3p. The 4 downregulated DEmiRNAs were: miR-542-3p, miR-3199, miR-655-3p, and miR-136-5p.

Prediction of target genes of DEmiRNAs

From miRWalk 3.0, 3437 and 6026 target genes were predicted for the upregulated and downregulated DEmiRNAs, respectively. Based on the GEPIA database, 1484 and 732 over- and under-expressed genes in HCC were selected. After intersection, 183 over-expressed and 147 under-expressed predicted target genes were selected (Figure 4). The miRNA-mRNA regulatory network involved with development of HCC was established according to the predicted miRNA-mRNA pairs (Figure 5, 6).

Functional annotation and pathway enrichment analysis

The top 10 enriched GO items are listed in Figure 7. GO biological process (BP) analysis revealed that target genes of upregulated DEmiRNAs were prominent in mitotic cytokinesis, mitotic spindle elongation, and cytoskeleton-dependent cytokinesis (Figure 7A). For cellular component (CC) analysis, these genes were significantly enriched in the spliceosomal tri-small nuclear RNA (snRNP) complex, spindle, and microtubule (Figure 7B). The molecular function (MF) analysis for

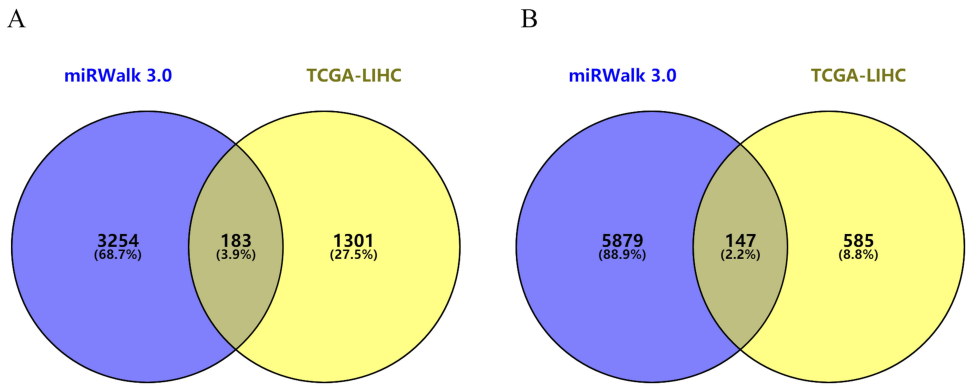

Figure 4 Venn diagram of target genes of FOXP4-AS1. (**A**) Upregulated target genes; (**B**) downregulated target genes.

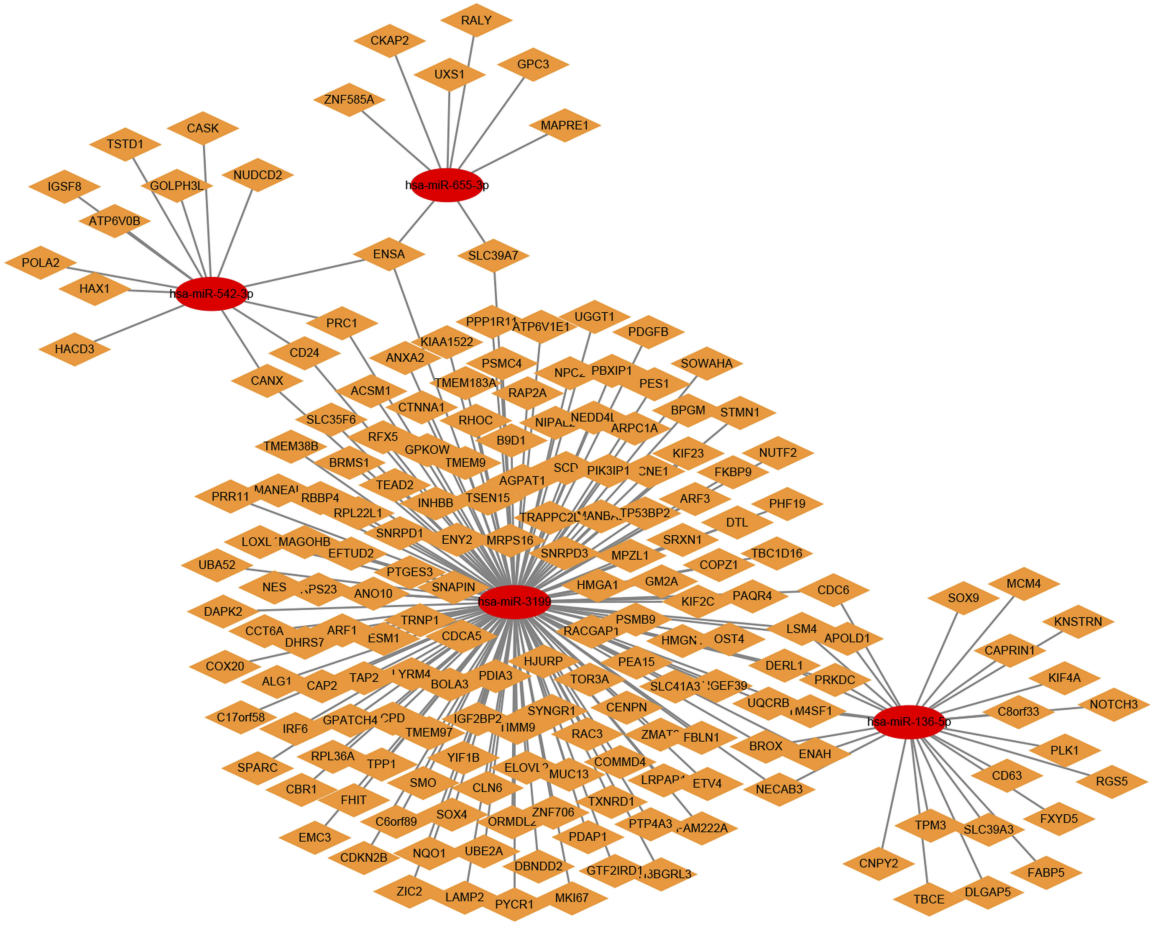

Figure 5 MiRNA-mRNA regulatory network of 6 upregulated miRNAs associated with FOXP4-AS1.

these genes included RNA binding, microtubule plus-end binding, and oxidoreductase activity, and acting on NAD (P)H, quinone, or a similar compound as acceptor (Figure 7C).

GO BP analysis showed that candidate target genes of downregulated DEmiRNAs were significantly prominent in the monocarboxylic acid metabolic process, positive regulation of peptidyl-tyrosine phosphorylation, and regulation of peptidyl-tyrosine phosphorylation (Figure 7E). For CC analysis, these genes were significantly concentrated in the integral component of the plasma membrane, platelet alpha granule lumen, and platelet alpha granule (Figure 7F). The MF analysis for these genes revealed that they were significantly involved in growth factor activity, alpha-adrenergic receptor activity, and metal ion binding (Figure 7G).

KEGG pathway enrichment analysis was conducted for target genes of the upregulated and downregulated DEmiRNAs. Candidate target genes of the upregulated DEmiRNAs were prominent in the lysosome, cell cycle, spliceosome, biosynthesis of unsaturated fatty acids, antigen processing and presentation, pathways in cancer, *Vibrio cholerae* infection, and phagosomes, ribosomes, and miRNAs in cancer (Figure 7D). The enriched pathways for candidate target genes of downregulated DEmiRNAs were the following: cytokine-cytokine receptor interaction; inflammatory mediator regulation of TRP (transient receptor potential) channels; cholinergic synapse; cancer; fatty acid degradation; and MAPK, PI3K-Akt, rap1, JAK-STAT, and Ras signaling (Figure 7H).

Screen of hub genes

We constructed a PPI network of these genes (Figures 8 and 9). To obtain the hub genes in the PPI network, these node pairs were entered into the Cytoscape software. The top 10 hub genes were listed in Table S3. For the target genes of upregulated DEmiRNAs, the hub genes were:

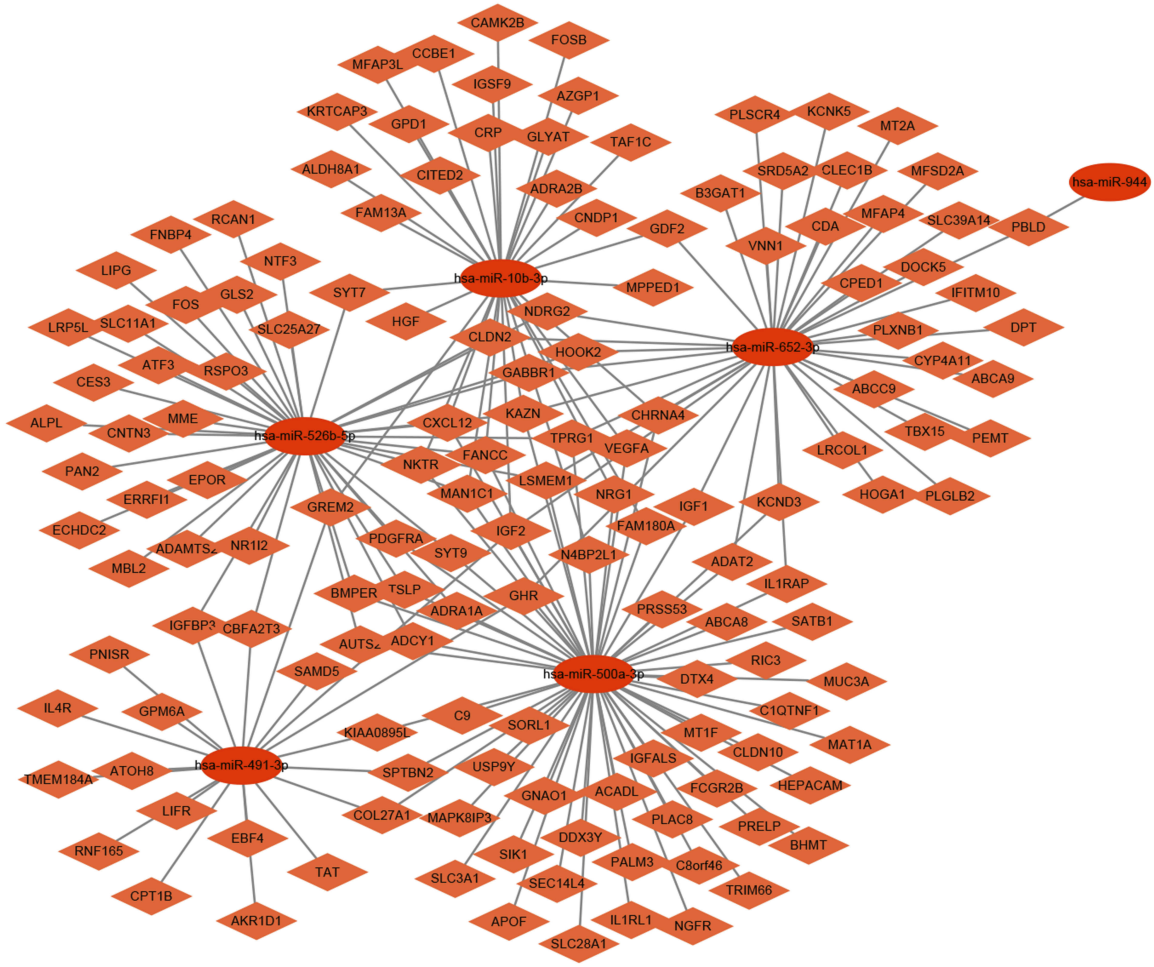

Figure 6 MiRNA-mRNA regulatory network of 4 downregulated miRNAs associated with FOXP4-AS1.

IGFBP3, NTF3, GABBR1, GHR, NGFR, ADRA2B, GDF2, ADRA1A, SLC11A1, and FOSB. For the target genes of downregulated DEmiRNAs, the hub genes were PRC1, MCM4, CDCA5, HJURP, CENPN, DTL, CKAP2, RPL36A, SNRPD1, and KNSTRN.

Confirmation of potential hub genes

The GEPIA database was used to detect the expressions in HCC of the 20 hub genes. The levels of all the 10 hub genes of downregulated DEmiRNAs were significantly higher in HCC tissues than in normal tissues (Figure 10). The levels of all the 10 hub genes of upregulated DEmiRNAs genes were significantly lower in the HCC tissues than in the normal tissues (Figure 11).

For further identifying potential hub genes, the prognostic roles of these 10 hub genes in HCC were assessed using the GEPIA database. Elevated expressions of PRC1, MCM4, CDCA5, HJURP, CENPN, DTL, CKAP2, SNRPD1, and KNSTRN indicated a worse prognosis (Figure 12), whereas higher expressions of IGFBP3, NTF3, GHR, ADRA2B, ADRA1A, and SLC11A1 were associated with a better prognosis (Figure 13).

Biological interaction network of hub gene alterations in HCC

A biological interaction network of the 20 hub genes in HCC was investigated. The tab Network in cBioPortal was used to show neighboring genes that were altered at frequencies >20% (Figure 14A and B). The 10 overexpressed hub genes were altered in HCC, and DTL, MCM4, and PRC1 were altered most often (19%, 14%, and 9%, respectively). The 10 underexpressed hub genes were altered in HCC, and NGFR and GABBR1 were altered most often (both 9%; Figure 14C and D).

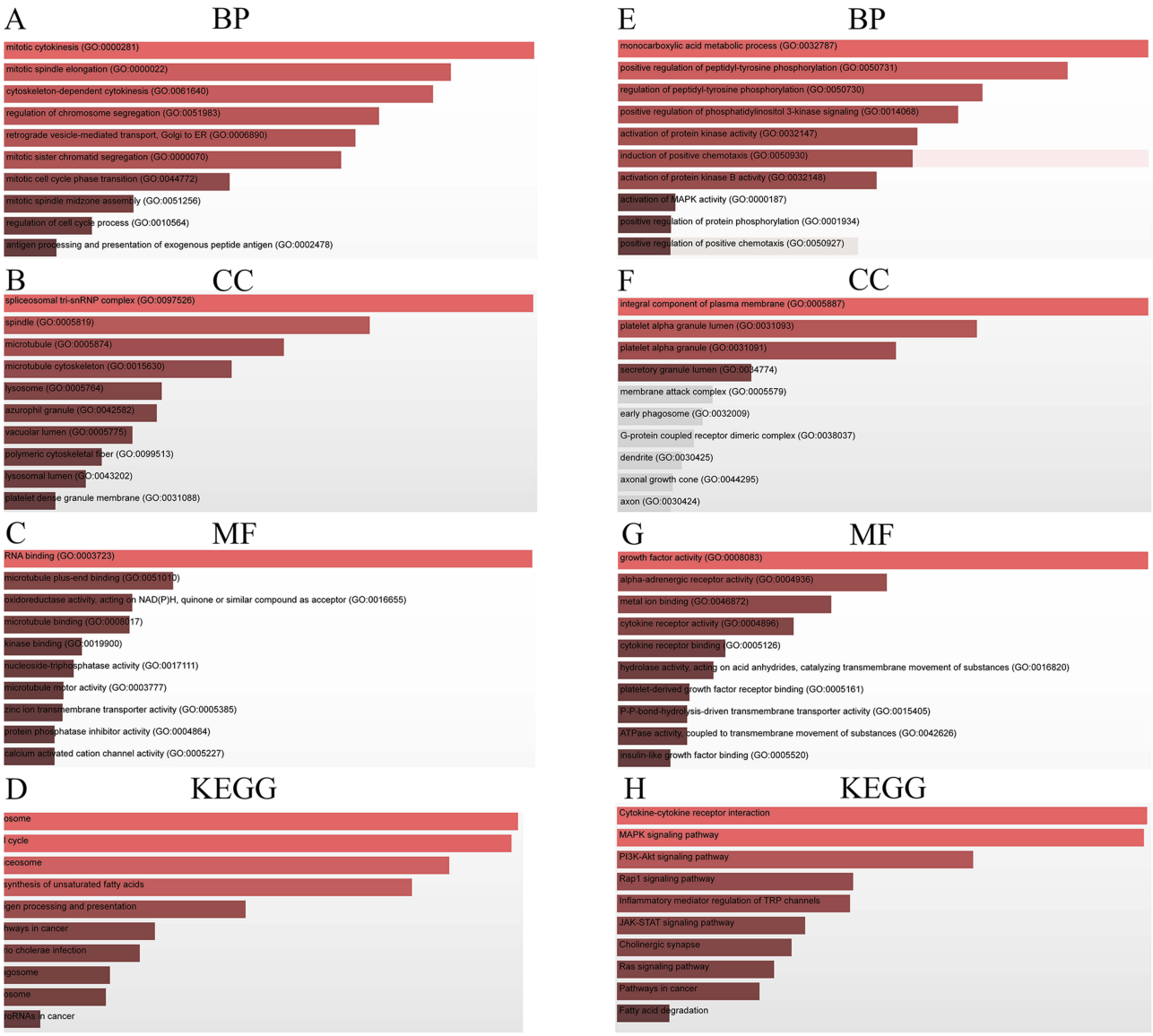

Figure 7 GO functional annotation and KEGG pathway analysis. Most prominent BP, CC, MF, and KEGG pathways associated with (**A**–**D**) upregulated and (**E**–**H**) downregulated candidate genes of FOXP4-AS1. (**A, E**) BP; (**B, F**) CC; (**C, G**) MF; and (**D, H**) KEGG pathways.

GO and KEGG pathway enrichment analyses were conducted of the most frequently altered neighboring genes. The GO terms indicated that the overexpressed 10 hub genes encode proteins that are localized mainly in the condensed chromosome kinetochore, condensed chromosome centromere region, and chromosome centromere region. These proteins are primarily involved in RNA binding, signal recognition particle binding, and mRNA binding (Figure 15A–C). Similarly, the KEGG pathway analysis indicated enrichment in the mRNA surveillance pathway, RNA transport, and protein export (Figure 15D).

The GO terms indicated that the underexpressed 10 hub genes encode proteins localized mainly to the gamma-secretase complex, integral component of the plasma membrane, and axon. These proteins are primarily involved in peptidase activity, acting on L-amino acid peptides, receptor tyrosine kinase binding, and protein tyrosine kinase binding (Figure 15E–G). Similarly, the KEGG pathway analysis showed enrichment in the neurotrophin, notch, and estrogen signaling pathways (Figure 15H). The tab OncoPrint displays an overview of genetic alterations per sample in hub genes.

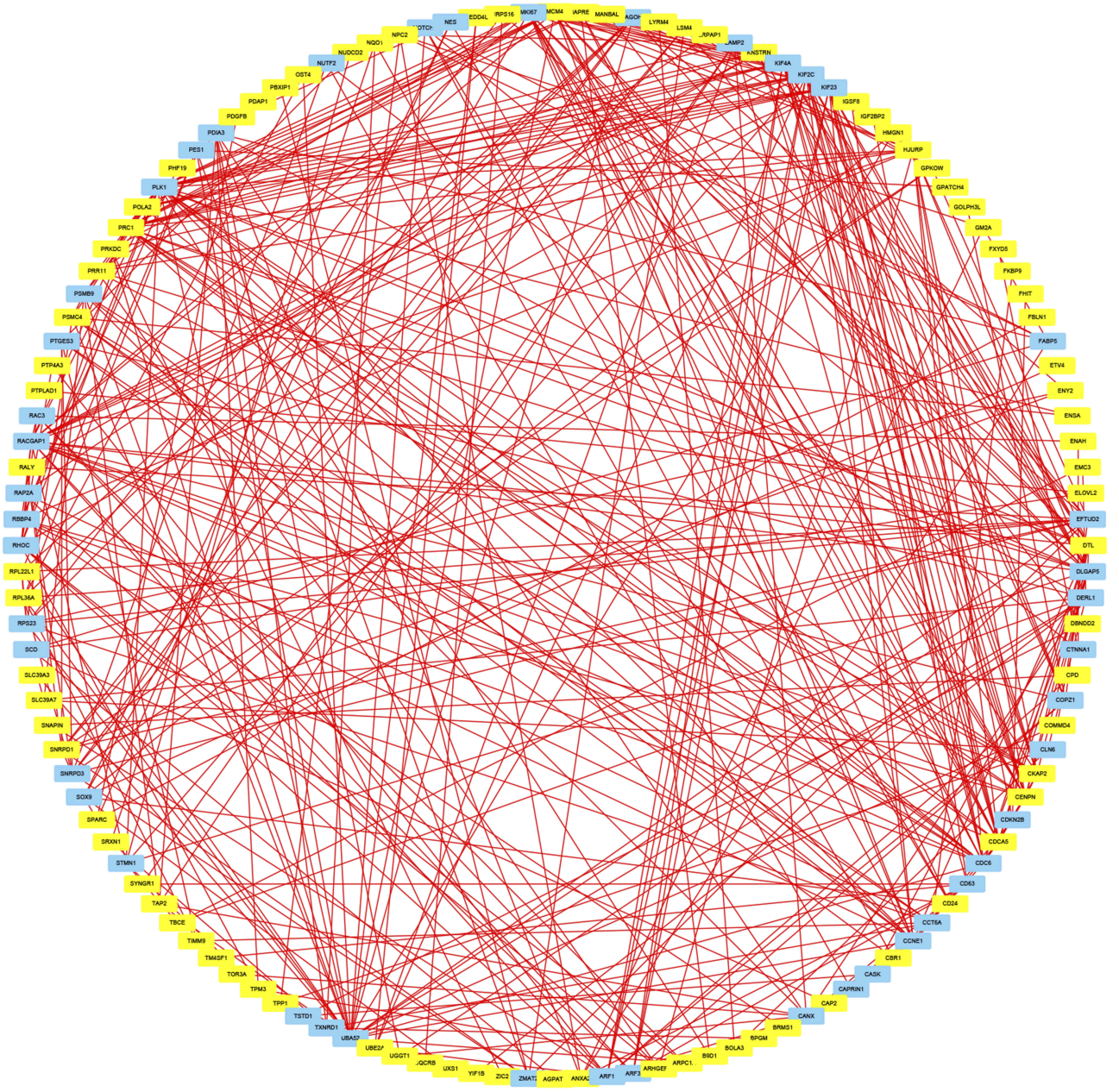

Figure 8 PPI network of upregulated target genes of FOXP4-AS1 in HCC.

Identification of potential miRNA-mRNA regulatory pathways

Analysis of the levels and prognostic roles of hub genes indicated that the following were oncogenic in HCC: PRC1, MCM4, CDCA5, HJURP, CENPN, DTL, CKAP2, SNRPD1, and KNSTRN. The following were tumor suppressive in HCC: IGFBP3, NTF3, GHR, ADRA2B, ADRA1A, and SLC11A1. This is consistent with the previous prediction that these genes were potential targets of tumor suppressive and oncogenic miRNAs. Based on these findings, an miRNA-mRNA regulatory network that may contribute to HCC onset and progression was established that included the following regulatory pathways (Figure 16): miR-652-3p/miR-491-3p/miR-500a-3p-GHR; miR-491-3p/miR-526-5p-IGFBP3; miR-10b-3p-ADRA2B; miR-526b-5p/miR-500a-3p-ADRA1A; miR-526-5p-NTF3/SLC11A1; miR-136-5p-MCM4/KNSTRN; miR-655-3p-CKAP2; miR-3199-DTL/CDCA5/HJURP/SNRPD1/CENPN/PRC1; and miR-3199/miR-542-3p-PRC1.

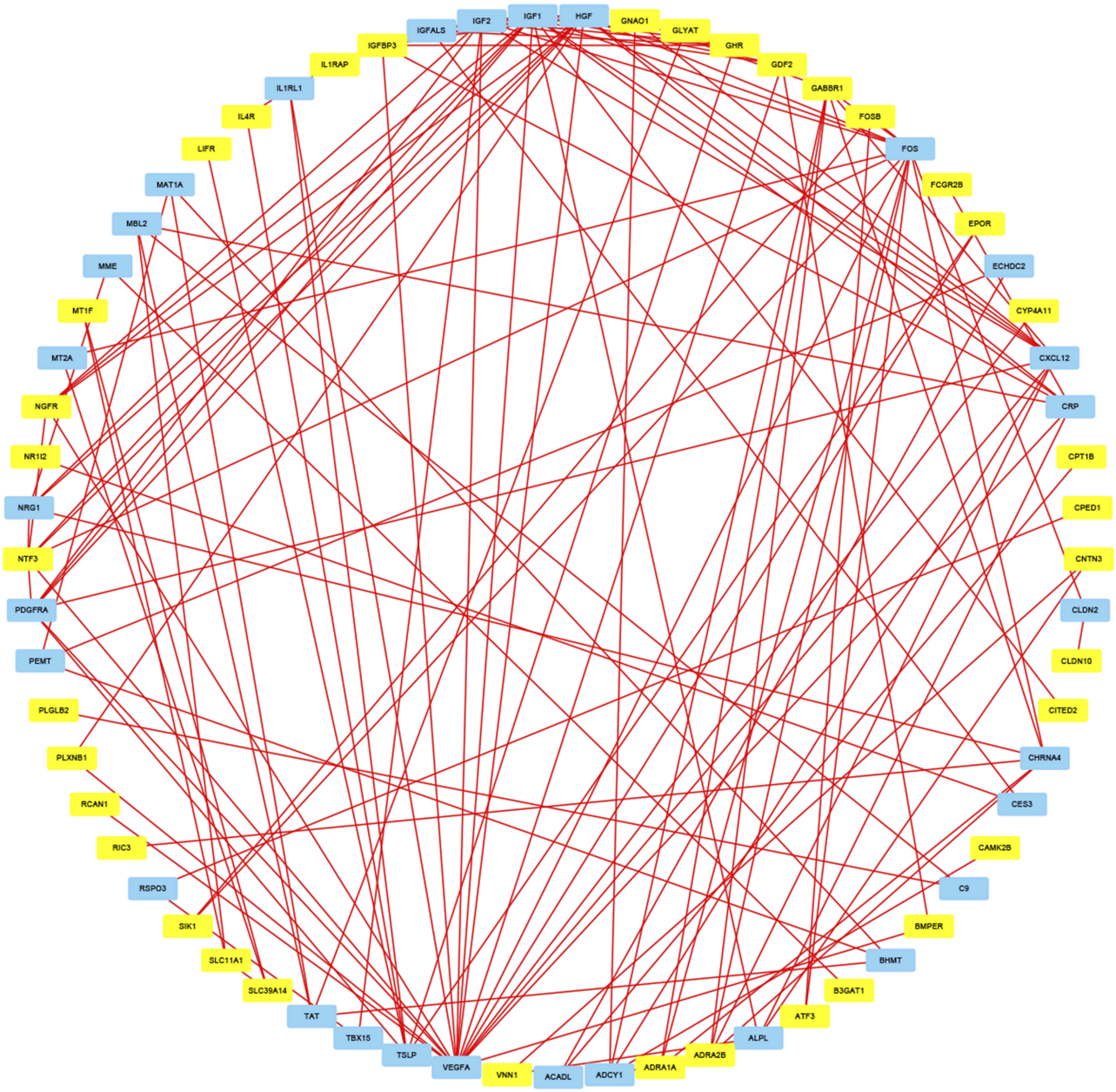

Figure 9 PPI network of downregulated target genes of FOXP4-AS1 in HCC.

Discussion

In the present study, FOXP4-AS1 data from qRT-PCR was used to determine the expression of FOXP4-AS1 in HCC. Six upregulated and four downregulated DEmiRNAs were identified by predicting miRNAs that bind to a lncRNA.

Previous studies are in accord with most of the DEmiRNAs in this analysis. For example, miR-10-3p is overexpressed in HCC tumor tissues,[11] miR-500a-3p is significantly elevated in HCC tissues and cells, and high expression of miR-500a-3p is associated with overall and recurrence-free survival in patients with HCC.[12] In addition, miR-944 directly targets IGF-1R by downregulating PI3K/Akt signaling and inhibits HCC invasiveness in vitro and in vivo.[13] MiR-542-3p inhibits HCC metastasis and epithelial-mesenchymal transition by targeting UBE3C.[14] MiR-655-3p and miR-136-5p are significantly downregulated in HCC tissues compared with normal adjacent liver tissue, and are associated with worse prognosis.[15,16]

For the DEmiRNAs identified in the present study, 183 and 147 upregulated and downregulated target

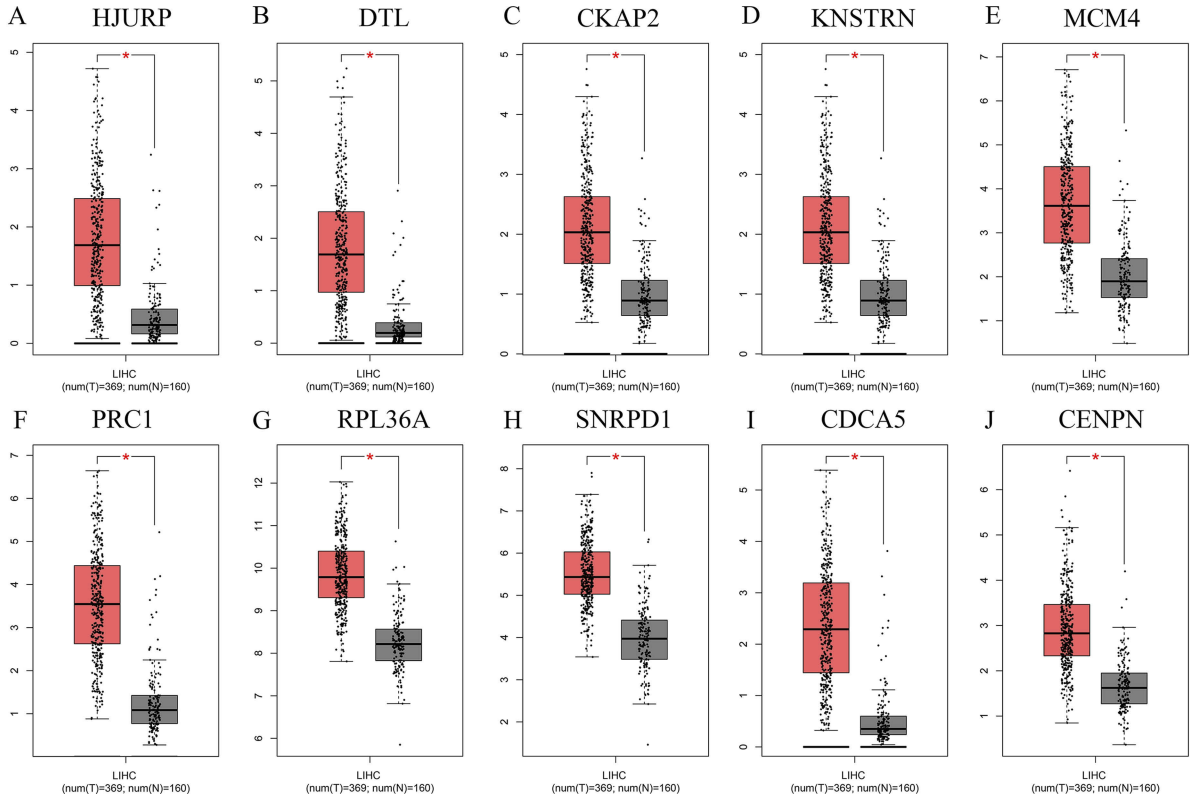

Figure 10 Expressions of 10 upregulated hub genes in HCC and normal tissues, based on GEPIA. (**A**) HJURP. (**B**) DTL. (**C**) CKAP2. (**D**) KNSTRN. (**E**) MCM4. (**F**) PRC1. (**G**) RPL36A. (**H**) SNRPD1. (**I**) CDCA5. (**J**) CENPN.

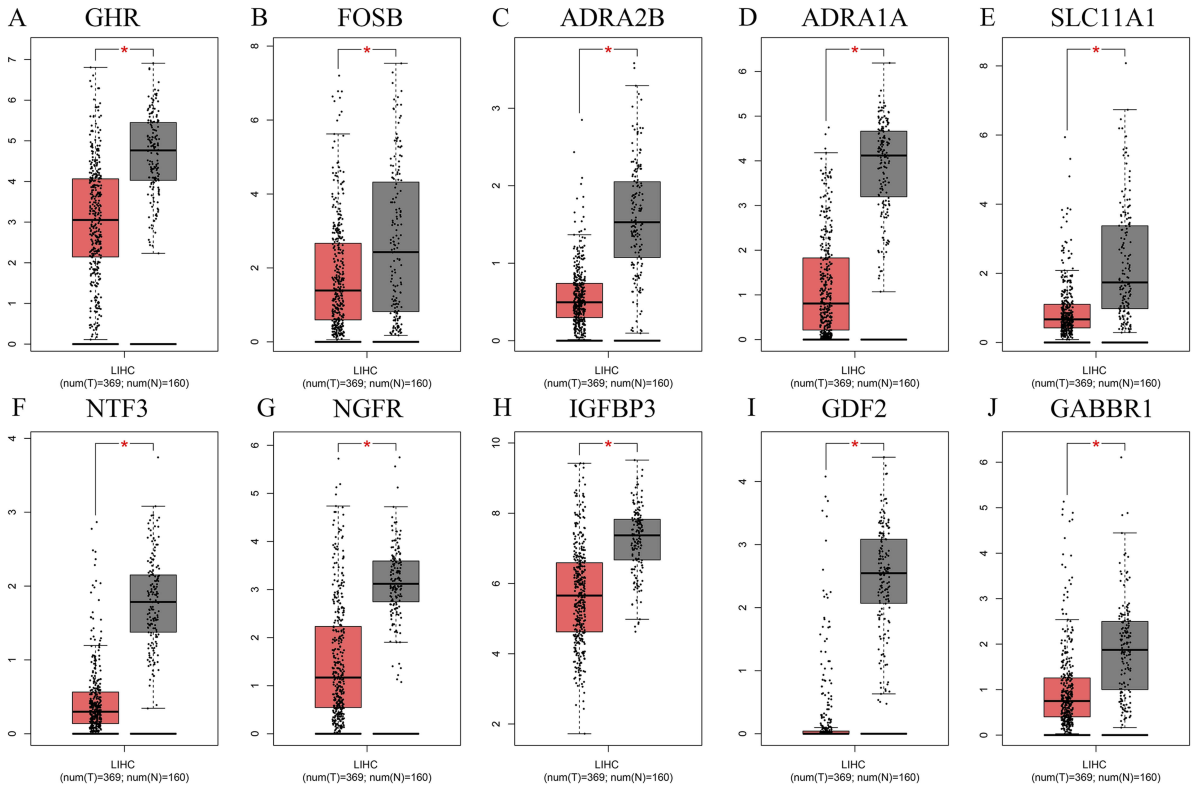

Figure 11 Expressions of 10 downregulated hub genes in HCC and normal tissues, based on GEPIA. (**A**) GHR. (**B**) FOSB. (**C**) ADRA2B. (**D**) ADRA1A. (**E**) SLC11A1. (**F**) NTF3. (**G**) NGFR. (**H**) IGFBP3. (**I**) CDF2. (**J**) GABBR1.

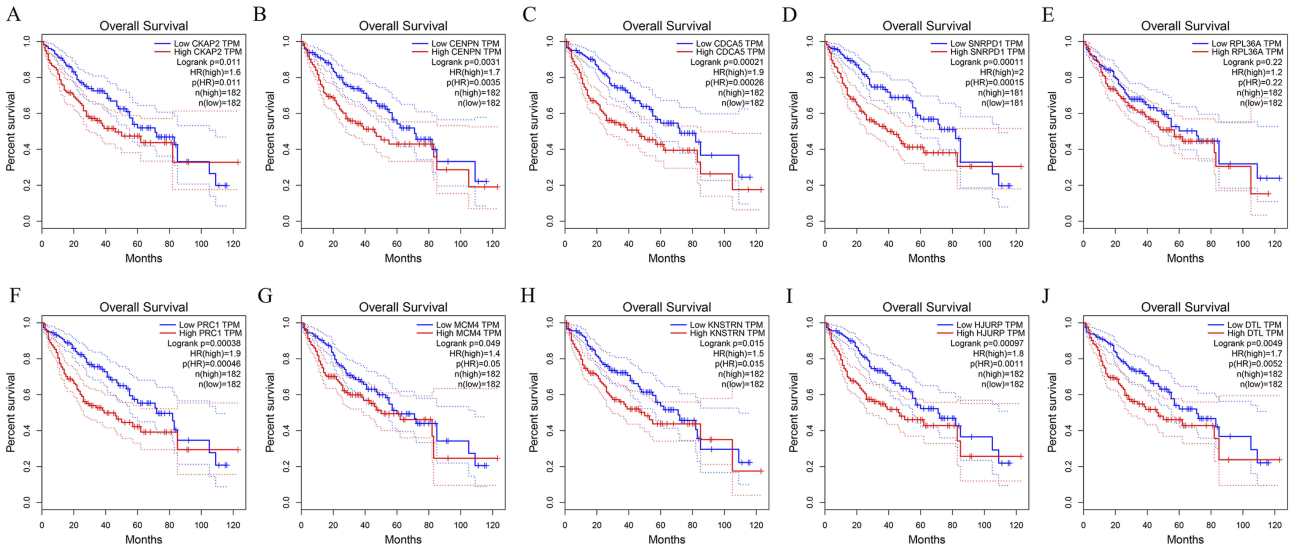

Figure 12 Association of expressions of 10 upregulated hub genes and the survival of patients with HCC. (**A**) CKAP2. (**B**) CENPN. (**C**) CDCA5. (D) SNRPD1. (**E**) RPL36A. (**F**) PRC1. (**G**) MCM4. (**H**) KNSTRN. (**I**) HJURP. (**J**) DTL.

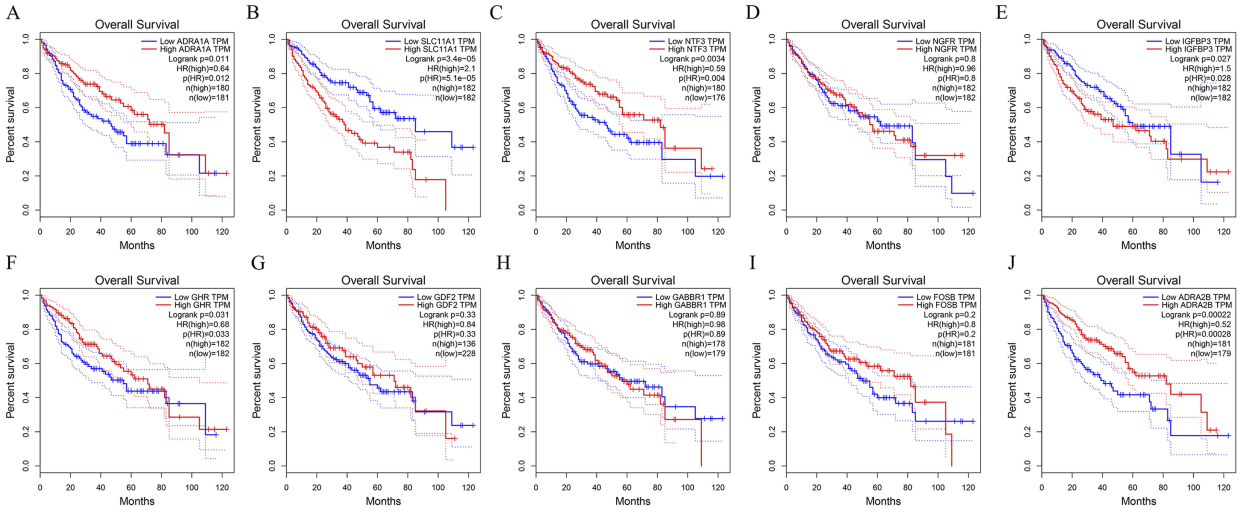

Figure 13 Association of expressions of 10 downregulated hub genes and survival of patients with HCC. (**A**) ADRA1A. (**B**) SLC11A1. (**C**) NTF3. (**D**) NGFR. (**E**) IGFBP3. (**F**) GHR. (**G**) GDF2. (**H**) GABBR1. (**I**) FOSB. (**J**) ADRA2B.

genes, respectively, were predicted. The KEGG pathway enrichment analysis revealed that the downregulated genes are involved mainly in lysosome, cell cycle, and spliceosome pathways; the upregulated genes in inflammatory mediator regulation of TRP channels and the following signaling pathways: PI3K-Akt, rap1, and JAK-STAT. Numerous studies have also shown that the cell cycle pathway is associated with human cancer, including HCC.[17] Activation of the PI3K-Akt signaling pathway has been implicated in HCC progression and poor prognosis.[18] These reports further support our previous analysis.

After determining target genes of the DEmiRNAs, a PPI network was constructed and the top 20 central genes, comprising 10 upregulated and 10 downregulated genes, were identified. The GEPIA database was employed to assist the analysis of these central genes in HCC. These 20 genes determined by our analysis were, in the main, consistent with TCGA (Cancer Genome Atlas) mRNA data, and most have been

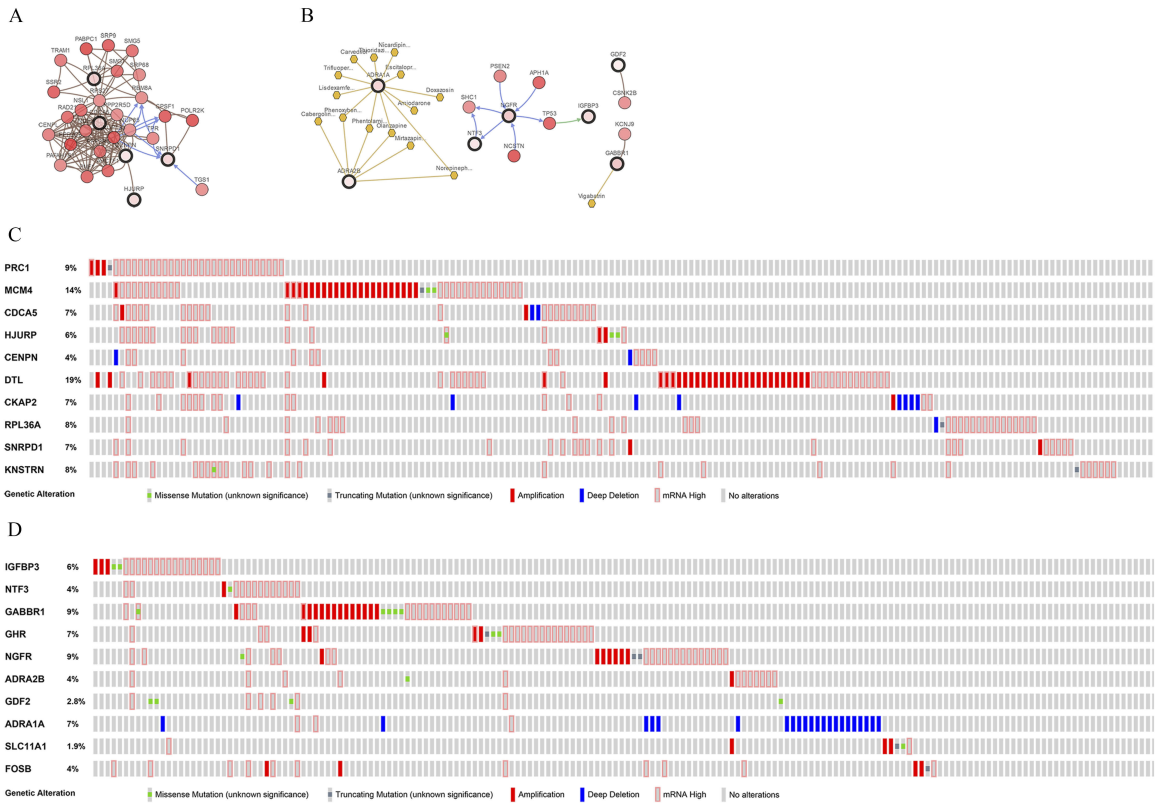

Figure 14 Network review of genes neighboring hub genes and genetic alteration of hub genes in HCC. (**A**) Ten upregulated hub genes. (**B**) Ten downregulated hub gene. (**C**) Genetic alteration of 10 upregulated hub genes. (**D**) Genetic alteration of 10 downregulated hub genes. Association between hub genes and drugs.

identified as key regulators in HCC. For example, the microtubule-associated protein PRC1 promotes early recurrence of HCC associated with the Wnt/β-catenin signaling pathway;[19] and CDCA5 overexpression is an indicator of poor prognosis in patients with HCC.[20] In addition, HJURP via the MAPK/ERK1/2 and AKT/GSK3β signaling pathway to stabilize p21 promotes HCC proliferation;[21] CKAP2 is a potential predictor of early and extensive recurrence of HCC after surgical resection;[22] and IGFBP3 impedes invasive growth of pediatric liver cancer and is epigenetically silenced in vascular invasive and metastatic tumors.[23]

Analysis of the prognostic effects of the central genes revealed that the following significantly promote HCC: PRC1, MCM4, CDCA5, HJURP, CENPN, DTL, CKAP2, SNRPD1 and KNSTRN,and the following significantly inhibit HCC:IGFBP3, NTF3, GHR, ADRA2B, ADRA1A, and SLC11A1. Based on all of the above findings, a potential miRNA-mRNA regulatory network was envisioned. While the expressions and roles of these miRNAs and mRNAs in cancer have been demonstrated, little is known of their role specifically in HCC. Study of the miRNA-mRNA pairs that potentially contribute to the pathogenesis of HCC may lead to new therapeutic goals.

In this study, a potential lncRNA-miRNA-mRNA regulatory network in HCC was constructed. In vitro and in vivo functional experiments of the regulatory pathways are planned for our future work.

Conclusion

We established a potential HCC-associated FOXP4-AS1-miRNA-mRNA regulatory network that explores the molecular mechanisms involved in HCC development, and provides direction for finding new HCC therapeutic targets. Experiments are needed to validate these results.

Abbreviations

BP, Biological process; CC, Cellular component; CI, Confidential interval; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; MF, Molecular function; HCC, Hepatocellular carcinoma; PPI, Protein–protein interaction network; qRT-PCR, Quantitative real-time PCR.

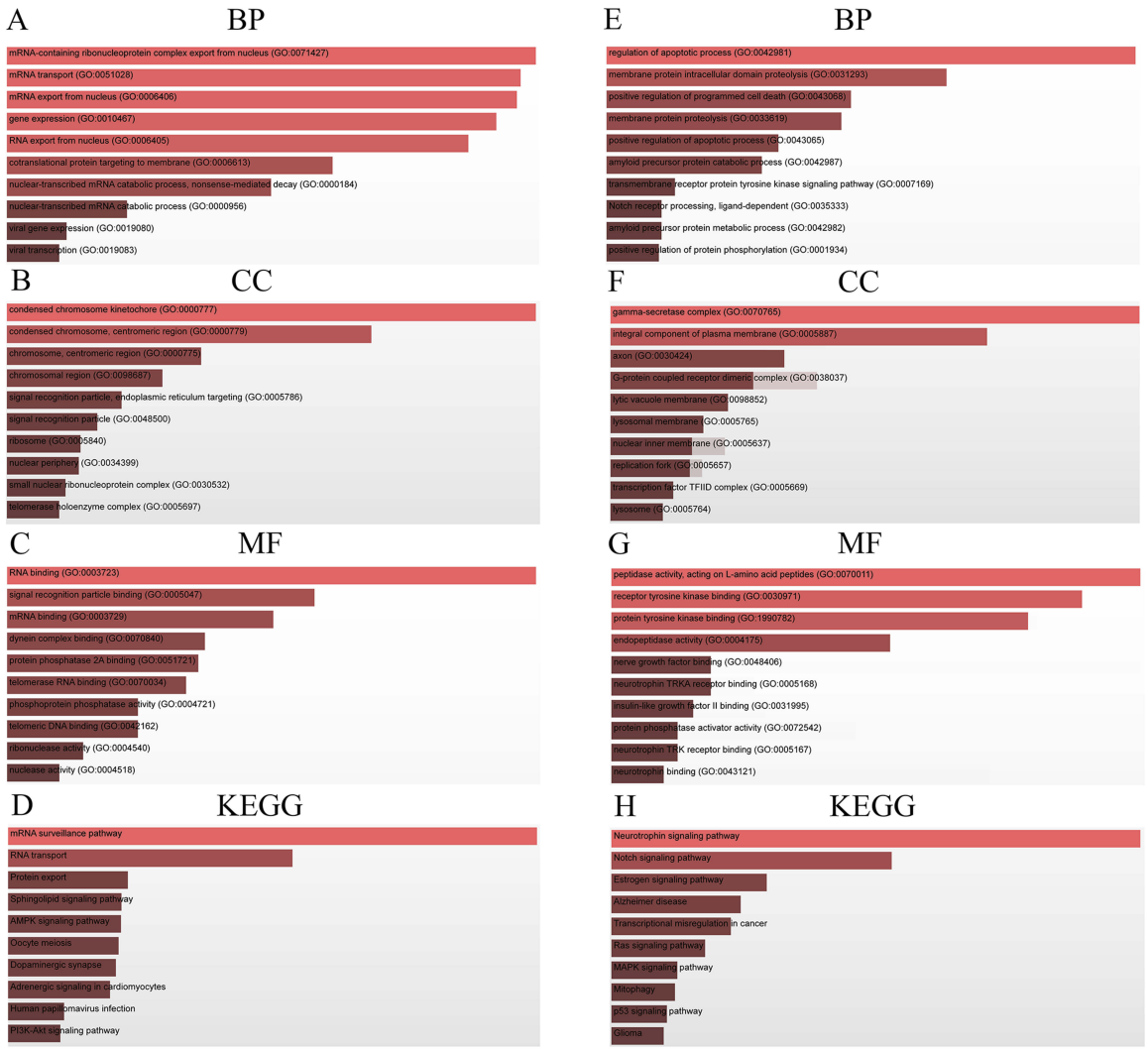

Figure 15 Enrichment analysis of the genes altered in the 20 hub neighboring genes in HCC. Most prominent BP, CC, MF, and KEGG pathways associated with (**A**–**D**) upregulated and (**E**–**H**) downregulated hub neighboring genes of FOXP4-AS1. (**A, E**) BP; (**B, F**) CC; (**C, G**) MF; and (**D, H**) KEGG pathways.

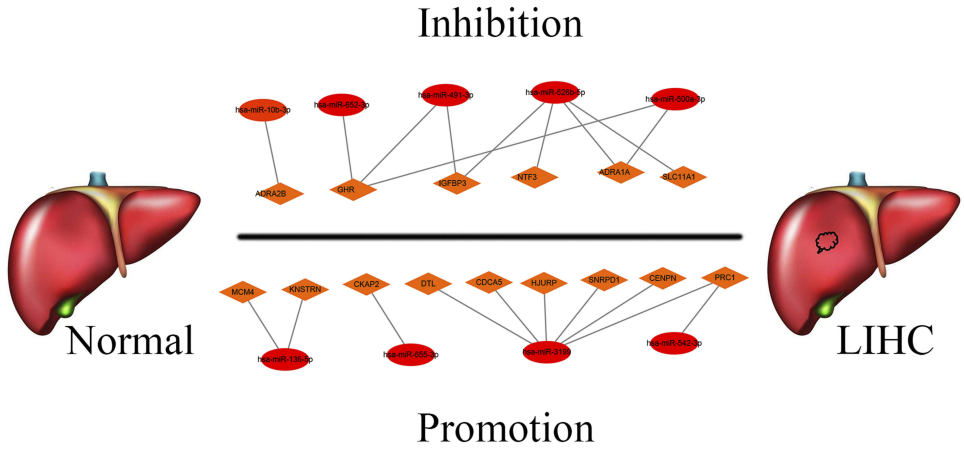

Figure 16 The candidate miRNA-hub gene regulatory network in HCC.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Affiliated Tumor Hospital of Guangxi Medical University.

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Disclosure

The authors declare that they have no conflicts of interest in this work.

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