A miR-9-5p/FOXO1/CPEB3 feed-forward loop drives the progression of hepatocellular carcinoma

Hui Hu ('huhui@hust.edu.cn')
Huazhong University of Science and Technology - Main Campus: Huazhong University of Science and Technology

Wei Huang
Huazhong University of Science and Technology - Main Campus: Huazhong University of Science and Technology

Jianye Li
Huazhong University of Science and Technology

Qiong Zhang
Huazhong University of Science and Technology

Ya-Ru Miao
Huazhong University of Science and Technology

Fei-Fei Hu
Huazhong University of Science and Technology

Lu Gan
Huazhong University of Science and Technology

Xiangliang Yang
Huazhong University of Science and Technology

An-Yuan Guo
Huazhong University of Science and Technology

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Abstract

Background

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide, but its regulatory mechanism remains unclear. Although many TFs and miRNAs are reported to be important in HCC, their co-regulation and FFL modules in HCC development are needed to be investigated.

Methods

The feed-forward loop (FFL) regulatory module was identified by analyzing the miRNA and transcription factor co-regulatory network for differentially expressed genes between tumors and matched adjacent tissue samples. Gene expression and regulatory role of HCC development by key FFL in vitro and in vivo were validated by qPCR, Western blotting, cell proliferation assay, migration and invasion assay and experiments in nude mice with hepatoma xenografts.

Results

Here, by bioinformatics analysis, we identified FFL regulatory module miR-9-5p/FOXO1/CPEB3 may play critical roles in HCC progression. Gain- and loss-of-function studies demonstrated that miR-9-5p promote hepatocarcinogenesis, while FOXO1 and CPEB3 inhibit hepatocarcinoma growth. Furthermore, CPEB3 was firstly identified as a direct downstream target of miR-9-5p and FOXO1 by luciferase reporter assay and ChIP-Seq data, which was negatively regulated by miR-9-5p and activated by FOXO1. Following, the miR-9-5p/FOXO1/CPEB3 FFL was associated with poor prognosis and promoted cell growth and tumorigenesis of HCC in both in vitro and in vivo experiments.

Conclusion

Our study newly identified the existence of miR-9-5p/FOXO1/CPEB3 FFL and revealed its regulatory role in HCC progression, which may represent a new potential therapeutic target for cancer treatment.

Background

Hepatocellular carcinoma (HCC) is the sixth most common and the third most lethal cancer in the world [1], which poses a serious threat to human health. The major risk factors of HCC include HBV, HCV, diabetes, obesity, alcoholism, and metabolic diseases. Although many patients have been diagnosed and treated in early stage, HCC is characterized by a high recurrence rate and poor prognosis [2, 3]. In addition, the high prevalence and mortality rate of HCC make it the main healthy burden at present [4]. Thus, these challenges give rise to the urgent need to systematically investigate the pathogenesis and to identify potential biomarkers for HCC.
MicroRNAs (miRNAs) and transcription factors (TFs) play crucial roles in multiple biological processes and participate in the development of human diseases including cancers [5, 6]. The interaction between miRNAs and TFs has been proved to serve as a feed-forward loop (FFL) mode that involve in biological processes and diseases [7]. Previous studies have revealed the role of miR-19/CYLD/NFKB and miR-429/MYCN/MFHAS1 FFLs in the development or relapse of T-lineage acute lymphoblastic leukemia (T-ALL) [8, 9]. FOXP3-miR-7/miR-155-SATB1 FFL is reported to prevent the transformation of healthy mammary epithelium into a cancerous phenotype [10]. Src/Sox2/miR-302b FFL increased metastatic progression of breast cancer [11]. In addition, our previous projects revealed key factors or FFL module involved in promoting cancer cell stemness [12] and CD19-CAR-T immunotherapy [13]. To detect key factors or FFL modules in biological processes and diseases, we have developed an online analysis server FFLtool [14].

In the last decade, various studies have shown that the abnormal expression of miRNAs or TFs is broadly associated with the pathogenic mechanism of HCC. For example, miR-206 is a powerful tumor suppressor that regulated cell-cycle progression of HCC [15]. The miR-449 family inhibits cell migration by targeting SOX4 [16]. In addition, circular RNA circMT01 suppresses HCC progression by down-regulated the target of oncogenic miR-9 [17]. FOX01 is a TF that acts as a tumor suppressor in regulating cell cycle, progression, differentiation, metabolism and survival [18, 19]. Recent evidence suggested that miR-96 promotes cell growth and migration by inhibiting FOX01 in HCC [20]. Up-regulated SOX12 promotes HCC invasion and metastasis [21]. Besides, HNF4A, TP53, STATs and NFKB were also reported as key TFs related to the development of HCC [22]. Although many TFs and miRNAs were reported to be important in HCC, their co-regulation and FFL modules in HCC has not been studied, which may identify novel driving modules in HCC development.

In this study, we analyzed the expression of gene and miRNA in HCC and performed miRNA-TF-gene network analysis. The results firstly identified CPEB3 as a target gene of miR-9-5p and FOX01, and further revealed a novel miR-9-5p/FOX01/CPEB3 FFL may involve in the progression of HCC, which promoted proliferation of HCC cell lines and enhanced tumor burden by in vivo tumorigenesis experiments. Altogether these results highlight the important role of this FFL in HCC progression.

Methods

Data source

RNA-seq (V2) and miRNA-seq data (level3) of 374 HCC cases were downloaded from The Cancer Genome Atlas (TCGA) data portal (https://portal.gdc.cancer.gov/). Expression data (RSEM normalized) of paired cancer and adjacent tissue samples (50 paired for RNA-Seq, 49 paired for miRNA-Seq) were used to analyze differentially expressed genes (DEGs) and differentially expressed miRNAs (DEM).
The DEGs in our study were carried out by Bioconductor-package NOISeq [23]. Genes expression higher than 20 normalized RSEM count, and miRNAs above 10 normalized RSEM count in any sample were remained for differential analysis. The cutoffs for DEGs were \( \text{prob} > 0.99 \) and \( |\text{Fold Change}| \geq 2 \). While TFs and miRNAs are regulatory factors with amplificatory effects for their functions, we set \( \text{prob} > 0.99 \) and \( |\text{Fold Change}| \geq 1.6 \) as the cutoffs of differentially expressed TFs or miRNA (DEMs) (Table S2). All heatmaps were normalized by genes in a row and hierarchically clustered through Euclidean distance by R pheatmap package.

**Enrichment analysis**

Gene ontology (GO) and KEGG pathway enrichment analyses of all DEGs were conducted by tool Metascape (http://metascape.org/gp/index.html) \((P \leq 0.05)\) [24]. Functional enrichment analysis of DEMs were performed by the DIANA-miRPath version (v.)3.0 [25]. All results were plotted by R package ggplot2 (https://ggplot2-book.org/).

**Survival Analysis**

Kaplan-Meier survival analysis was performed based on all tumor samples of TCGA HCC data. For each gene, low- and high-expression samples were defined by expression levels of the given gene lower than 25th percentile and higher than 75th percentile, respectively. For multi-gene survival analysis, samples were divided into low- and high-expression groups by median gene expression levels. We compared the best prognostic subgroups with the worst prognostic subgroups. Statistical significance \((P\text{ value})\) was calculated by log-rank (Mantel-Cox) test. Data was removed when the patients were lost to follow-up.

**Regulatory networks and hub analysis**

We constructed miRNA-TF co-regulatory networks for all DEGs and DEMs based on interaction data collected in previous work [7, 26], including experimental and predicted TF-target/miRNA and miRNA-target/TF interaction pairs. All networks were visualized by Cytoscape (version 3.4.0). To determine hub nodes (TFs, miRNAs and genes) in networks, we calculated eight centrality feature values of network nodes based on CytoNCA [27]. A higher value meant the node was more important in the network. We retained the top 50% of genes for each centrality feature value, and then obtained 30 hub nodes (including 10 miRNAs, 7 TFs and 13 genes) in the network by the intersection of eight features.

**Cell lines and cell culture**

Human hepatocellular carcinoma cell line HepG2 was purchased from Cell Bank, Chinese Academy of Sciences. Human hepatocarcinoma cell line Bel7402 was purchased from China Center for Type Culture Collection (CCTCC, Wuhan, China). Human liver cell line HL7702, human hepatocarcinoma cell lines Huh7 and LM3 were kindly provided by Prof. Bixiang Zhang [28]. Cells were cultured on the plastic plate in DMEM (high glucose Dulbecco’s modified Eagle’s medium) with 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA), and 100 mg/ml penicillin/streptomycin (Gibco) at 37°C with 5% CO₂. The cells were randomly assigned to each experimental group and the potential presence of mycoplasma was monitored via continuous microscopic imaging.
Transfection

Cells were transfected with small interfering RNA (siRNA) using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) as manufacturer's instructions. The negative control siRNA, siRNA duplexes and shRNA were synthesized from Genephama Biotech (Suzhou, China). All the siRNA and shRNA sequences were listed in Table S3. miR-9-5p mimic, specific inhibitor molecules and appropriate negative control molecules were purchased from Genephama Biotech (Suzhou, China). All the mimic, inhibitor or negative control molecules were transfected using X-treme GENE siRNA Transfection Reagent (Roche, Germany), based on the manufacturer's protocol. The FOXO1 overexpressed plasmids were supplied by Prof. Lu Gan. The CPEB3 expression construct was generated by subcloning PCR-amplified full-length human CPEB3 cDNA into the pBABE (Plasmid #21836, addgene).

Migration and Invasion assay

For the migration assay, $4 \times 10^4$ Bel7402 cells in serum-free medium were seed in the upper compartment of an 8 µm transwell chamber (Corning, NY, USA). For the invasion assay, the upper chambers were previously coated with 50 µg extracellular matrix gel (Corning, NY, USA). After incubation for 24–48 hours, the migrated or invaded cells on the lower membrane were stained with 4% paraformaldehyde, then stained with 0.1% crystal violet. The upper cells were moved gently by the soft medical cotton ball. Each group random took 10 pictures under a microscope with 400× magnification (Olympus, Japan). Cell numbers per field were calculated by ImageJ.

Cell proliferation assay

Cell proliferation was measured with the Cell Counting Kit-8 (CCK-8) assay kit (Dojindo Corp., Japan). HepG2 cells or Bel7402 cells were seeded in 96-well plates ($8 \times 10^3$ cells/well). Vectors or RNAi were transfected for 24h (HepG2) and 48h (Bel7402). 10 µL CCK-8 reagent was added to each well. After incubating for 4 hours, the cell density was measured indirectly through quantification of the solubilized formazan product at 450 nm with Multiskan GO (ThermoFisher, US). Three independent experiments were performed.

Animals

Four-week old BALB/c Nude male mice were obtained from Beijing HFK (China). The mice were randomly assigned to control or treated group, each group had eight mice. The experimentalists were blinded from the expected outcome of the treatment. All animals received humane care in compliance with the Principles of Laboratory Animal Care Formulated by the National Society of Medical Research and the guide for the US National Institutes of Health. The protocol was approved by the Animal Care and Use Committee of Huazhong University of Science and Technology.

Statistical analysis

Data was described as the mean ± s.e.m. from at least three independent experiments. Association among FOXO1, CPEB3 and miR-9-5p were assessed using Pearson's correlation test. Comparisons
between different groups in expression and tumor weight were performed using Student's two-sided t-test. One-way ANOVA test was used for intergroup comparison for tumor volume. A two-way analysis of variance (ANOVA) was used to conduct multiple group variables with similar variance. Single, double and triple asterisks indicating statistical significance: * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \); while ns indicates a non-significant result. Statistical analyses were done by R package (https://www.r-project.org/). The detailed procedures of analyses, including high-throughput sequencing data, luciferase reporter gene assay and chromatin immunoprecipitation, real-time qPCR analysis and western blotting assays were presented in Supplementary Material.

**Results**

**The gene expression and regulatory network analyses revealed the miR-9-5p/FOXO1/CPEB3 FFL in HCC**

To study the gene expression and regulation in HCC, RNA-Seq and miRNA-Seq data (level 3) of 50 paired HCC tumor and adjacent tissue samples from TCGA were analyzed. As a result, 387 significantly DEGs and 17 DEMs were detected in the comparison of tumor vs. adjacent sample (Figure S1A-B). About 8% (32) of DEGs were TFs, as shown in the Fig. 1A. Functional enrichment analysis revealed that the largest amount of up-regulated DEGs were involved in cell cycle, oocyte meiosis and pathways in cancer, while most of down-regulated DEGs were associated with cytokine-cytokine receptor, drug metabolism, and HTLV-I infection pathways, which may suggest their pilot roles in the development of HCC (Fig. 1B). For example, LYVE1, a down-regulated gene in HCC, may constitute as an early biomarker of postoperative survival in HCC patients [29]. And VCAN, an up-regulated gene in HCC, could serve as a potential biomarker for early-HCC diagnosis [30]. Besides, target genes of DEMs were mainly enriched in the processes of cancer, such as glioma, hippo signaling pathway, and signaling pathways regulating pluripotency of stem cells (Figure S1C).

Next, to explore the mechanism of gene expression regulation in HCC, we built miRNA-TF-gene regulatory networks based on the DEGs and DEMs mentioned above. There were 118 nodes (17 TFs, 13 miRNAs, and 88 genes) with 481 edges in the network (Fig. 1C). Based on 30 hub nodes (7 TFs, 10 miRNAs, and 13 genes) detected by CytoNCA [27] in the network, a sub regulatory network (30 hub nodes with 138 edges) were constructed (Figure S1D). Hub node, which regulates lots of genes or is regulated by numerous factors, may play important roles in the development of liver cancer. For example, hub node gene CPEB3 has been described as a newly discovered tumor suppressor in HCC [31], and hub TF FOXO1 was responsible for blockage of HCC proliferation [32]. In our results, the hub regulator miR-9-5p is the most significantly DEM (log\(_2\)FoldChange = 4.012) between tumor and adjacent samples among all DEMs (Table S2), which was reported to promote cell proliferation of HCC [33]. The sub-network of miR-9-5p consisted of 16 genes and 14 TFs (Fig. 1D). Interestingly, as target genes of miR-9-5p in the network, both FOXO1 and CPEB3 were reported to be associated with hepatocellular carcinoma and down-regulated in HCC (Fig. 1E), and their expression levels were not affected by DNA methylation or copy number variation.
(Figure S1E-F). In addition, CPEB3 was regulated by FOXO1 in our regulatory network, thus forming a feed-forward loop (FFL) with FOXO1 and miR-9-5p (Fig. 1E). Kaplan–Meier analyses indicated that the downregulations of FOXO1 and CPEB3 were significantly correlated with better prognosis, while high expression of miR-9-5p was associated with poor prognosis (Fig. 1F and Figure S2A). Although miR-9-5p, CPEB3, and FOXO1 have been studied alone in HCC, the FFL consisting of these three elements has not been studied and it may be more powerful in explaining HCC disease progression. Thus, we focused on the role of the FFL formed by FOXO1, miR-9-5p and CPEB3 in HCC progression.

miR-9-5p promotes tumor cells proliferation, while FOXO1 and CPEB3 play tumor-suppressive roles in HCC

As we shown on Fig. 1F, miR-9-5p had higher expression in tumor tissues compared to adjacent tissues, while FOXO1 and CPEB3 were in higher expression in adjacent tissues. To further investigate roles of miR-9-5p, FOXO1 and CPEB3 in HCC, we detected their expression in several HCC cell lines and liver cell line HL7702 (Fig. 2A). miR-9-5p expression was elevated in HepG2, Bel7402 and Huh7, compared to liver cell line HL7702. The expression levels of FOXO1 and CPEB3 were significantly decreased in HepG2, Huh7, Bel7402, and LM-3 cells. To further explore their biological roles in HCC, we performed gain- and loss-of-function experiments in Bel7402 and HepG2 cell lines, respectively (Fig. 2B-C and Figure S2). The inhibition of miR-9-5p or overexpression of FOXO1 and CPEB3 significantly suppressed cell growth (Fig. 2B), decreased cell invasion ability in vitro by transwell assay (Fig. 2C) and enhanced cell apoptosis of Bel7402 and HepG2 cells (Fig. 2D and Figure S2C). Conversely, overexpressed miR-9-5p or inhibition of FOXO1 and CPEB3 markedly promoted cell proliferation (Fig. 2B and Figure S2B), which was also confirmed by the in vitro transwell assay results (Fig. 2C). Furthermore, co-transfection FOXO1 or CPEB3 vector and siRNA significantly inhibited their inhibitory effects on cell proliferation in HCC (Fig. 2B and Figure S2B).

In a nutshell, miR-9-5p, FOXO1 and CPEB3 may affect progression of HCC through impact cell proliferation and invasion. Past efforts have demonstrated that miR-9-5p and FOXO1 were involved in the progression of HCC in vivo [17, 18, 33] and CPEB3-silencing increased tumor size and weight in HCC [31]. In this work, we observed that the tumor formation ability of the CPEB3 overexpressing cells was reduced in nude mice when compared with control (Fig. 2E). These results suggested that miR-9-5p promotes HCC cells proliferation and invasion in vitro, whereas FOXO1 and CPEB3 had opposite effects.

The existence of FFL among miR-9-5p, FOXO1 and CPEB3

Further, we aim to study the relationship among miR-9-5p, FOXO1 and CPEB3, and prove the existence of the FFL formed by them. Firstly, we performed gene expression correlation analysis based on the mRNA expression in TCGA. The results showed that FOXO1 expression was significantly positively associated with CPEB3 (Figure S2D; Pearson correlation coefficient = 0.34, \(P = 7.02\times10^{-11}\)), and these two genes were slightly negatively correlated with miR-9-5p expression. Next, qRT-PCR results in Bel7402 cells indicated FOXO1 act as a negative regulator of miR-9-5p and possibly a positive modulator of CPEB3 (Fig. 3A). Besides, CPEB3 expression was significantly suppressed with miR-9-5p upregulation in Bel7402 cells,
whereas CPEB3 expression in miR-9-5p inhibitor group showed the contrary result (Fig. 3A). As FOXO1 was reported to be a target gene of miR-9-5p [34], we next verified whether CPEB3 is a direct target of miR-9-5p. We found that CPEB3 was predicted as a target of miR-9-5p through TargetScan algorithm (Fig. 3B). Two loci on the 3’ UTR of CPEB3 were predicted as potential binding sites, named as CPEB3-1 and CPEB3-2, respectively. The luciferase activities of wild-type CPEB3-1 (CPEB3-1 WT), wild-type CPEB3-2 (CPEB3-2 WT) and mutant-type CPEB3-2 (CPEB3-2 Mut) were significantly inhibited ($P < 0.05$) by transfecting miR-9-5p, while that of mutant-type CPEB3-1 (CPEB3-1 Mut) was rarely changed (Fig. 3C). These may indicate that miR-9-5p downregulated CPEB3 expression by directly targeting the locus 1 (position 437–443) of 3’ UTR of CPEB3.

Next, ChIP-Seq analysis was employed to evaluate whether FOXO1 directly regulates CPEB3 transcription. ChIP-Seq with FOXO1 antibodies showed a strong peak of FOXO1 binding associated with the two CPEB3 promoters in HepG2 cell (Fig. 4A). Besides, the result by ChIP-qPCR demonstrated that FOXO1 binding was significantly enhanced after immunoprecipitation at CPEB3 transcript isoform 2 (NCBI: NM_001178137.1) but not the CPEB3 transcript isoform 1 (NM_014912.5) (Fig. 4B). In addition, CPEB3 expression was remarkably up-regulated after FOXO1 overexpression both in gene level (Fig. 4C) and protein expression (Fig. 4D). These indicated FOXO1 was a positive modulator of CPEB3. Furthermore, FOXO1 overexpression affected not only CPEB3 expression but also a huge number of pathways. Functional enrichment analysis demonstrated that DEGs of FOXO1 overexpression compared with control (Table S1) were associated with Non-alcoholic fatty liver disease, FoxO signaling pathway, cell cycle and autophagy pathways, which were closely related to the occurrence and development of HCC (Fig. 4E). The above results suggest regulatory relationships between miR-9-5p, FOXO1 and CPEB3 and further reveal the existence of miR-9-5p/FOXO1/CPEB3 FFL (Fig. 1E).

The function of miR-9-5p/FOXO1/CPEB3 FFL in vivo

How does the miR-9-5p/FOXO1/CPEB3 FFL affect HCC patients? We performed Kaplan-Meier survival analysis for two or three genes combination in miR-9-5p/FOXO1/CPEB3 FFL. Patients with the combination of low miR-9-5p and high FOXO1 had a significantly longer overall survival at 60 months ($P = 0.0092$, Fig. 5A) and 100 months ($P = 0.0152$, Figure S3B) versus the opposite group. Same as the combinations of low miR-9-5p and high CPEB3 group or high FOXO1 and high CPEB3 group, the survival was longer than their respective corresponding opposite groups (60 months: $P = 0.0002$; $P = 0.0153$, Fig. 5A, 100 months: $P = 0.0024$; $P = 0.0392$, Figure S3B). Likewise, patients with low miR-9-5p and high FOXO1 and high CPEB3 had significantly longer overall survival than the opposite expression group ($P = 0.009$ for 60 months, Fig. 5A, $P = 0.018$ for 100 months, Figure S3B).

To further explore the function of miR-9-5p/FOXO1/CPEB3 FFL in vivo, we build the hepatoma xenografts in nude mice. Bel7402 cells transfected or co-transfected with NC, miR-9-5p inhibitor, FOXO1, and CPEB3 shRNA were injected subcutaneously into the flank of each nude mouse (Figure S3A). As expected, tumor volumes (since day 17) and weights were significantly reduced with the miR-9-5p silencing or FOXO1 overexpression, while the tumor suppression was sustained after miR-9-5p-silencing along with FOXO1-
overexpression (Fig. 5B-C, E). Tumor grown faster when silenced CPEB3 (Fig. 5D) and the tumor-promoting action was not suppressed by miR-9-5p-silencing and FOXO1-overexpression (Fig. 5F). This may be due to the fact that alterations in downstream effectors have a greater impact on tumor progression compared to upstream regulators. These all indicated the miR-9-5p/FOXO1/CPEB3 FFL may promote progression of HCC in vivo through downregulation of effector gene CPEB3 (Fig. 5).

Discussion

HCC is an important worldwide cause of cancer related mortality [35]. The poor prognosis of liver cancer and the high recurrence ratio [3] make it imperative to investigate the mechanisms of HCC. As the fact that many TFs and miRNAs play important roles in liver cancer and the successful application of FFL analysis in disease mechanisms, a novel avenue has been opened to investigate the molecular mechanisms of HCC pathogenesis. The TF-miRNA co-regulation network constructed base on DEGs and DEMs gives us a clue on identifying potential functional regulators in HCC. In this research, we detected key FFL module through bioinformatics analyses, the miR-9-5p/FOXO1/CPEB3 FFL was further verified contributing to the progression of HCC in vivo and in vitro.

Our study firstly revealed the existence of miR-9-5p/FOXO1/CPEB3 FFL in HCC (Fig. 1E). We demonstrated that miR-9-5p was highly expressed in HCC, whereas FOXO1 and CPEB3 were significantly down-regulated (Fig. 1F, 2A), and that the three could form FFL by network analysis (Fig. 1E). Further, given the expression of FOXO1 or CPEB3 was not affected by DNA methylation as well as mutations in TCGA HCC (Figure. S1E and S1F), we speculated that expression changes of the two genes were mainly due to the regulation. More importantly, we firstly demonstrated that CPEB3 is a functional target gene of miR-9-5p and FOXO1 by luciferase reporter (Fig. 3C) and ChIP-Seq assays (Fig. 4A). In addition, FOXO1 was reported to be a target gene of miR-9-5p [34]. Interestingly, experimental studies showed that FOXO1 may in return downregulated miR-9-5p (Fig. 3A). In line with that 3’ UTR of FOXO1 may function as a miR-9-inhibitor to regulate miR-9-5p activity [36]. Therefore, we confirmed the existence of miR-9-5p/FOXO1/CPEB3 FFL.

Results showed that low expression of FOXO1 and CPEB3 or high expression of miR-9-5p was correlated with poor prognosis in HCC (Fig. 1F and Figure S2A), which was same as two- or three-factors combinations groups among low FOXO1, low CPEB3 and high miR-9-5p (Fig. 5A and Figure S3B). This implied the miR-9-5p/FOXO1/CPEB3 FFL could be a bad prognostic biomarker for HCC. Gain- and loss-of-function analyses indicated that high expression of miR-9-5p was associated with aggressive tumor phenotypes and poor clinical diagnosis (Fig. 1F, 2B-D), while FOXO1 and CPEB3 were act as tumor suppressers (Fig. 2B-D). Similar to in vitro results, animal experiments indicated that miR-9-5p silencing and FOXO1 overexpression could suppress the growth of hepatoma xenografts (Fig. 5B-C, E), while silenced CPEB3 promoted tumor growth in vivo (Fig. 5B, D), which was consistent with the previous reports in HCC [18, 31, 33]. Furthermore, co-transfection of miR-9-5p inhibitors, FOXO1, and CPEB3 shRNA significantly increased the growth of hepatoma xenografts, which was similar to the effect of transfection with CPEB3 shRNA alone (Fig. 5B). This implied that expression change of downstream
effector gene of the FFL maybe has a greater effect on cancer than the upstream regulators. With CPEB3 silencing, the effects of promoting CPEB3 expression by miR-9-5p silencing and FOXO1 overexpression were masked. In conclusion, we speculated that miR-9-5p/FOXO1/CPEB3 FFL may promote the progression of HCC due to the downregulated of the effector gene CPEB3.

Given the importance of miR-9-5p/FOXO1/CPEB3 FFL in HCC, we tried to explore the downstream pathways of this regulatory loop. CPEB3, cytoplasmic polyadenylation element binding protein 3, that act as the downstream effector gene of the FFL involved in downstream pathways. CPEB3 promoted human HCC cell proliferation and metastasis [31] and was downregulated in HCC [37], which were also verified by our work. Through Western blot experiments, we demonstrated that CPEB3 induced upregulation of NCK2 and LIMS1, suggesting that these two genes may be downstream genes of CPEB3 (Fig. 6A). NCK2 is an Nck-related adaptor protein involved in growth factor receptor kinase signaling pathways, which was approved to interact with LIMS1 to participate in ILK signaling and downregulate EGFR protein [38, 39]. Besides, previous outlined that FOXO1 activation suppressed the ILK pathway [40], and the carcinogenic ability of ILK in vivo in HCC cells has been confirmed [41]. While EGFR is correlated with poor prognosis, drug resistance, and cancer metastasis in many different cancers including HCC [31, 42]. In addition, the EGFR signaling pathway was negatively regulated by CPEB3 in HCC [31]. Furthermore, RSU1, a suppressor of Ras-dependent oncogenic transformation, was reported to interact with LIMS1, and was attributed to inhibit cell proliferation and invasion in hepatocytes and tumor cells [38, 43]. These results indicated that miR-9-5p/FOXO1/CPEB3 FFL may facilitate HCC pathogenesis through the EGFR, and ILK signaling pathway (Fig. 6B).

**Conclusion**

In summary, we performed miRNA-TF-gene regulatory network analysis to investigate the possible mechanisms for HCC. Three hub nodes including miR-9-5p, FOXO1 and CPEB3 were identified, and their function in carcinogenesis were investigated. CPEB3 was firstly identified as a direct target of miR-9-5p and FOXO1. Most importantly, we firstly demonstrated the existence of miR-9-5p/FOXO1/CPEB3 FFL and validated the regulation role of it in hepatocarcinogenesis in vivo and in vitro. This study provides us with a deeper understanding of the transcriptional and post-transcriptional regulatory mechanisms underlying HCC progression.

**Abbreviations**

HCC
hepatocellular carcinoma
DEG
differentially expressed gene
DEM
differentially expressed miRNA
miRNA
Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Sequencing data of RNA-Seq and ChIP-Seq of FOXO1 overexpressed in HepG2 Cells during the current study are available in the Genome Sequence Archive at the BIG Data Center, Beijing Institute of Genomics (BIG), Chinese Academy of Sciences, under the accession number CRA003225, which is publicly accessible at http://bigd.big.ac.cn/gsa.

Competing interests

The authors declare that they have no competing interests.
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Author information

Hui Hu and Wei Huang contributed equally to this work.

Affiliations

Center for Artificial Intelligence Biology, Hubei Bioinformatics & Molecular Imaging Key Laboratory, Key Laboratory of Molecular Biophysics of the Ministry of Education, College of Life Science and Technology, Huazhong University of Science and Technology; Wuhan, 430074, China

Hui Hu, Qiong Zhang, Ya-Ru Miao, Fei-Fei Hu, An-Yuan Guo

National Engineering Research Center for Nanomedicine, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, China

Wei Huang, Jianye Li, Lu Gan, Xiangliang Yang

School of Chemistry, Chemical Engineering and Life Sciences, Wuhan University of Technology, Wuhan, Hubei, 430070, China

Wei Huang

Research Center of Clinical Medicine, Affiliated Hospital of Nantong University, Nantong, 226001, China

Qiong Zhang, An-Yuan Guo

Brain Science and Advanced Technology Institute, School of Medicine, Wuhan University of Science and Technology, Wuhan, Hubei 430081, China

Fei-Fei Hu

Authors' contributions
AY.G. and X.Y. conceived the project. AY.G., X.Y., and L.G. supervised the study. W.H. and J.L. performed the clinical trial and biochemical analysis. H.H. performed the bioinformatics analysis with the help of Q.Z., YR.M., and FF.H. H.H. and W.H. drafted the manuscript with the help of AY.G. and X.Y. H.H. and W.H. revised the manuscript. All authors read and approved the final manuscript.

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Figures
Figure 1

The miRNA–TF–gene regulatory network and hub nodes in HCC. (A) Heatmap for differentially expressed TFs in HCC tumor vs. adjacent tissues. The color gradient from blue to red indicates the scaled expression (Z score) from low to high. (B) Result of KEGG pathway enrichment analysis for DEGs. (C) miRNA–TF–gene FFL regulatory network for all DEGs and DEMs. TFs, miRNAs and their target gene(s) are indicated in triangles, rounded rectangle and circles, respectively. Genes up-regulated and down-
regulated in all patients are indicated in red and blue, respectively. Purple edges indicated TF–target regulation, while red edges indicated miRNA–target regulation. (D) The miR-9-5p–TF–gene regulatory network. (E) The miR-9-5p–FOXO1–CPEB3 FFL. (F) Boxplots indicate the expression of miR-9-5p, FOXO1 and CPEB3 in TCGA paired HCC and adjacent tissue samples (paired t test). Survival curves are results of Kaplan-Meier survival analyses of miR-9-5p, FOXO1 and CPEB3 at 60 months (log-rank test). L: Low-expression samples, H: High-expression samples. * P < 0.05, ** P < 0.01, *** P < 0.001, ns: non-significant result.
Figure 2

miR-9-5p promotes the growth of HCC cells in vitro and FOXO1, CPEB3 inhibit cell growth. (A) qRT-PCR expression analysis of miR-9-5p, FOXO1 and CPEB3 expression in liver (HL7702) and HCC cell lines. Significant test was performed by Student's t-test. Mean ± s.e.m., n = 3/group. If not otherwise stated, the subsequent tests are carried out in the same way. (B) Cell viability of Bel7402 cells after transfection with Vector, NC, miR-9-5p mimics, inhibitor as indicated (top); Transfected with Vector, NC, FOXO1 siRNA or FOXO1 vector with or without siRNA (middle) and transfected with Vector, NC, CPEB3 siRNA or CPEB3 vector with or without siRNA (bottom). n = 4/group in cell viability analyses, while n=9/group in cell number analyses. (C) Transwell assays of cellular invasion and migration after transfection with miR-9-5p mimic or its inhibitor, FOXO1 or siFOXO1, and CPEB3 or siCPEB3 in Bel7402 cells. Representative images (right) and quantification of 10 randomly selected fields (left) are shown. (D) Apoptosis induced by miR-9-5p inhibitor, FOXO1, and CPEB3 is detected using flow cytometry in Bel7402 cells. n = 3/group. (E) The in vivo effect of CPEB3 was evaluated in xenograft mouse models bearing tumors originating from Bel7402 cells, tumor volume and tumor weight (TW) was periodically measured for each mouse and tumor growth curves or box plot was plotted. One-way ANOVA was used for significant test of tumor volume and Student's t-test for TW. Mean ± s.e.m. (standard error of mean), n=5/group. ∗ P < 0.05, ∗∗ P < 0.01, ∗∗∗ P < 0.001, ns: non-significant result.
miR-9-5p is a negative regulator of CPEB3. (A) Levels of miR-9-5p, CPEB3 were detected by qPCR in miR-9-5p mimic, miR-9-5p inhibitor or FOXO1, siFOXO1 treated Bel7402 cells. One-way ANOVA and Dunnett's multiple comparison tests were used for one-to-one and one-to-many tests, respectively. Mean ± s.d., n = 3/group. (B) Schematic representation of the 3' UTR regions of CPEB3 (CPEB3 transcript variant 2, NCBI: NM_001178137.1) with the putative miR-9-5p binding sites (CPEB3-1, position 437-443 of CPEB3 3' UTR; CPEB3-2, position 1367-1373 of CPEB3 3' UTR), including wild-type (CPEB3-1 WT, CPEB3-2 WT) and mutant (CPEB3-1 Mut-1, CPEB3-2 Mut-2). (C) Relative luciferase activity of the indicated CPEB3 3' UTR vectors in 293T cells transfected with miR-9-5p plasmids. Renilla luciferase activity was normalized to firefly activity and is presented as relative luciferase activity. Student's t-test. Mean ± s.d., n = 3/group. * P < 0.05, ** P < 0.01, *** P < 0.001, ns: non-significant result.
Figure 4

FOXO1 is a positive regulator of CPEB3. (A) ChIP-seq tag profiles for FOXO1 normalized read count at the CPEB3 promoters of two isoforms (NCBI: NM_014912.5 for isoform 1, NM_001178137.1 for isoform 2) in HepG2 cells under no-immunoprecipitation (IN) or immunoprecipitation (IP) conditions. Locations of FOXO1-binding sites (magenta color) are indicated. P value of each binding site was calculated by MACS2. Light yellow indicated the promoter region of CPEB3. (B) Estimation of ChIP-seq via quantitative
ChIP-PCR in HepG2 cells normalized by input control, IgG serves as a negative control. CPEB3-1 and CPEB-2 indicate CPEB3 transcript isoform 1 and CPEB3 transcript isoform 2 as shown in figure A; n = 3. Error bars indicate s.e.m. (C) Levels of FOXO1 and CPEB3 were detected by qPCR in FOXO1 treated HepG2 and Bel7402 cells. One-way ANOVA and Dunnett’s multiple comparison tests were used for one-to-one and one-to-many tests, respectively. Mean ± s.d., n = 3/group. (D) Protein expression of FOXO1 and CPEB3 assessed by Western blot assays in HepG2 cells. (E) Significantly enriched terms (P< 0.01) in Gene Ontology and KEGG pathway for DEGs of FOXO1 overexpression compared with control. CC: cellular component. BP: biological process. MF: molecular function. * P < 0.05, ** P < 0.01, *** P < 0.001, ns: non-significant result.

Figure 5
Effects of miR-9-5p/FOXO1/CPEB3 FFL on survival and tumor growth in vivo. (A) Kaplan-Meier survival analysis of combinations of two or three genes in miR-9-5p/FOXO1/CPEB3 FFL at 60 months (log-rank test). (B) Tumor formation experiment with different treated Bel7402 cells. The vectors were different in miR-9-5p inhibitor, FOXO1 and CPEB3-shRNA, we used tumor weight to evaluate the various combinations of miR-9-5p inhibitor/FOXO1/CPEB3-shRNA in xenograft mouse models for each mouse. Tumor volume of miR-9-5p inhibitor (C), CPEB3 shRNA (D), FOXO1 or FOXO1 and miR-9-5p inhibitor combination (E) and three genes miR-9-5p inhibitor, FOXO1 and CPEB3 shRNA combination (F) were periodically measured for each mouse and growth curves were plotted. Scale bar = 1 cm. Significant test in B was performed with Student’s t-test. Others were One-way ANOVA. Mean ± s.e.m., n=6/group. * P < 0.05, ** P < 0.01, *** P < 0.001, ns: non-significant result.
Figure 6

A potential pathway mediated by miR-9-5p/FOXO1/CPEB3 FFL. (A) Western blot analysis of CPEB3, LIMS1, NCK2 and GAPDH protein expression (left) and quantification (right) in HepG2 cells. (B) Schematic illustration of miR-9-5p/FOXO1/CPEB3 FFL and its possible downstream pathways in hepatocellular carcinoma. Significant test was performed by Student’s t-test. Mean ± s.e.m., n = 3/group. ** P < 0.01.
Supplementary Files

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- HCCFFLSupplementaryMaterialJEXPCLINCANCRES.doc
- TableS2.DifferentiallyexpressedgenesandmiRNAs.xlsx