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# miRNA-mRNA network contributes to HBV-related hepatocellular carcinoma via immune infiltration induced by GRB2

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**Abstract.** Chronic hepatitis B virus (HBV) infection is a critical causative factor in the tumorigenesis and progression of hepatocellular carcinoma (HCC). MicroRNAs (miRNAs) serve a critical role in the process of viral infection. However, there has been insufficient evaluation of HBV-associated miRNA-mRNA regulatory networks in HCC. The differential expression levels of miRNAs were compared in HBV-associated HCC tumor and normal tissues using the Gene Expression Omnibus database. The present study evaluated potential target genes of differentially expressed miRNAs using protein-protein interaction network, hub gene, Gene Ontology, Kyoto Encyclopedia of Genes and Genomes, gene set enrichment and immune infiltration analysis. A total of five miRNAs and seven target genes were identified in the HBV-associated miRNA-mRNA network. miRNA-93 could positively regulate the growth factor receptor bound protein 2 (GRB2) gene, while there was a positive correlation between GRB2 and cancer immune infiltrate function in Tumor Immune Estimation Resource. Collectively, the present study investigated the miRNA-mRNA regulatory network in HCC with HBV infection and showed that miRNA-93 positively regulated immune infiltration-related GRB2. Restoring GRB2 may be a candidate strategy for the treatment of HBV-related HCC.

## Introduction

Hepatocellular carcinoma (HCC) is the sixth most common type of malignant tumor and the fourth leading cause of cancer death worldwide. In 2018, global incidence of liver cancer was 4.7% and the mortality rate was 8.2% (1). Incidence of HCC has been increasing and the mortality rate has been growing faster than that of any other malignant tumor (2). Especially in developing countries, the prevalence of hepatitis B virus (HBV)-associated HCC remains high and is associated with local HBV epidemics (3).

HBV is a hepatotropic DNA virus infecting ~240 million people worldwide (4). HBV DNA is detected in serum and tissue samples from patients with HBV-related HCC (5). The mechanism by which HBV promotes HCC is unclear, but it is hypothesized that the pathogenesis of HCC is caused by chronic HB triggering cirrhosis (6). The mortality related to HBV-associated diseases, which account for ~600,000 deaths each year, is mainly caused by decompensated cirrhosis and HCC (7). HBV mediates cell proliferation and DNA damage by increasing oxidative stress and inflammation, which are key links in the pathological changes of HCC (8). A study also revealed that the high expression of the HBV X gene leads to HCC in transgenic mice (9). Therefore, it is necessary to study the uncovered mechanism of HBV in pathological changes in the liver.

MicroRNAs (miRNAs or miRs) have begun to receive widespread attention in the field of virology (10-12). miRNAs are non-coding RNAs with a length of ~22 nt that primarily control gene expression via post-transcriptional regulation patterns (13). Abnormally expressed miRNAs are often detected in patients with HCC and HBV infection and may be considered biomarkers for diagnosis and prognosis assessment, such as miR-375, miR-92a, miR-25 and let-7f (14,15). miRNAs serve a number of functions *in vivo* to regulate cell invasion, migration, proliferation, apoptosis and metabolism (16-19). Additionally, miRNAs serve a critical role in the process of oxidative stress, inflammation and autophagy (20,21). HBV can influence the expression of miRNAs at multiple stages following infection (22). However, expression patterns of numerous miRNAs, such as miR-125b and miR-210, remain controversial in patients with HBV-associated HCC: Some researchers believe that miR-125b and miR-210 are highly expressed in HCC, while others believe that the expression of the two miRNAs is low in HCC (23-27).

Researchers have reported that miR-125b and miR-210 can be up- or downregulated in patients with HBV-associated HCC (23-25). Certain miRNAs have only been evaluated

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in a few studies of HBV-related HCC, and their biological changes and mechanisms need to be further elucidated, such as miR-374b (28,29). Therefore, the regulatory mechanism of miRNAs in HCC needs to be further studied.

Proteins serve a vital role in the occurrence and development of tumors (30). Previous studies have demonstrated that miRNAs in the cytoplasm inhibit gene expression by binding to the 3' untranslated region (UTR) of the gene to inhibit translation or direct degradation of mRNAs (31,32). However, unlike the role of cytoplasmic miRNA, miRNAs in the nucleus are primarily involved in regulating transcription (33). miRNAs, such as miR-373, miR-744 and miR-1186, are located in the nucleus and can combine with promoter or enhancer regions to activate gene expression (33). The present study aimed to use bioinformatics methods using the Gene Expression Omnibus (GEO) database and R programming language to identify the HBV-associated miRNA-mRNA regulatory network.

## Materials and methods

*Analysis with R programming language*. The 'limma' package was obtained from Bioconductor (bioconductor.org/biocLite) and was employed to analyze the data via R software (version 4.0.0). RNA sequencing (-seq) data was imported into the R environment and converted into a data structure supported by 'DESeq2' package (bioconductor.org/biocLite). Original data was preprocessed by standardizing the data, removing low-expression genes and batch effects. R language 'limma', 'dplyr' and 'tidyr' packages (bioconductor.org/biocLite) were used for differential expression gene analysis. R package 'ggplot2' was applied to visualize the results. Heatmap results of the miRNAs and differential gene expression were produced with TBtools (version 1.09876) (34).

*Non-coding RNA profiling by array*. The miRNA expression database was established by searching GEO Data Sets (ncbi.nlm.nih.gov). The inclusion criteria were samples must be pathologically confirmed as HCC patients and miRNA expression profiles are detected in tumor and adjacent normal tissues. Through screening, two datasets containing samples from tissues of patients with HCC were included: GSE67882 and GSE69580. A total of 13 HCC and nine non-tumor tissues were included in the present study. These patients with HCC had diagnosis confirmed through pathological examination. The 'limma' R package was applied to compare expression levels of miRNAs between HBV-related HCC and the corresponding non-tumor tissues from the two series of matrix files. After data normalization, expression levels of the miRNAs in the two datasets were analyzed.

*Analysis of prognostic value of differentially expressed miRNAs (DE-miRNAs)*. The prognostic values of DE-miRNAs in HCC were analyzed using the Kaplan-Meier Plotter database (kmplot.com/analysis/index.php?p=service&cancer=liver_mirna). Through comparing the relationship between miRNA expression levels and the survival rate of HCC patients, the DE-miRNAs with an impact on the prognosis of patients with HBV-related HCC were selected for further analysis.

*DE-miRNA target gene prediction*. miRNet (mirnet.ca/miRNet/home.xhtml), an easy-to-use analysis tool based on an online network, was used to predict the miRNA target genes (35). miRNet integrates data from 11 high-quality miRNA-target interaction databases and displays the results through a network visualization system. Gephi software (version 0.9.2, gephi.org/users/), a network visualization software based on the JAVA working environment, displayed the regulatory association between miRNAs and their target genes.

*miRNA-hub gene prediction*. Cytoscape software (version 3.7.1, https://github.com/cytoscape/cytoscape) was used to evaluate the miRNA hub genes. For all DE-miRNA target genes, Molecular Complex Detection plug-in (version 3.3.10, http://apps.cytoscape.org/) was used for scoring according to the interaction between the genes. The higher the score, the more key the role the hub genes play in the entire gene network.

*Functional annotation and signal pathway enrichment analysis*. Database for Annotation, Visualization and Integrated Discovery (DAVID; david.ncifcrf.gov/home.jsp) was applied for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of DE-miRNA target genes. For GSE101728 data, containing the expression profiling of lncRNA and mRNA in HCC (36), the enrichment of KEGG pathway according to the top 25% of the highest and lowest GRB2 expression patients' genes expression profiling was assessed.

*HBV-associated HCC target gene prediction*. HBV-associated experimental data were screened through the GEO database. In the GSE100400 dataset, the researchers combined circularized chromosome conformation capture (4C) with RNA-seq and chromatin immunoprecipitation (ChIP)-seq to illuminate the nuclear landscape associated with HBV episomes and HBx (37). To the best of our knowledge, the aforementioned study is the first to use 4C to identify regional virus-host genome interactions. By using R programming language tool, expression matrix of RNA-seq data in GSE100400 was obtained and the difference in gene expression was calculated.

*The Cancer Genome Atlas (TCGA) database analysis*. UALCAN database (ualcan.path.uab.edu/) is an easy-to-use interactive web portal that for in-depth analysis of TCGA gene expression data. UALCAN includes TCGA RNA-seq and clinical data from 31 types of cancer (38). This was used to select genes with significant differences in expression and prognosis between tumors and corresponding adjacent tissues.

*Immune infiltrate analysis*. The Tumor Immune Estimation Resource (TIMER) database (cistrome.shinyapps.io/timer/) is designed to investigate the molecular characteristics of tumor-immune interactions and analyze the correlation between gene expression and the abundance of immune infiltrates (39). This was used to screen genes related to immune infiltration.

*Cell culture and transfection*. Human hepatoblastoma cell lines (HepG2 and HepG2.2.15) were purchased from Guangzhou Cellcook Biotech Co., Ltd. and were authenticated

via STR profiling. The hepatoblastoma cells were grown in Minimal Essential Medium supplemented with non-essential amino acids and 10% fetal bovine serum (all WISENT, Inc.). Cells were placed in a cell incubator (Thermo Fisher Scientific, Inc.) at 37˚C with 5% carbon dioxide. miRNA-93 mimics and inhibitors and negative controls (Guangzhou RiboBio Co., Ltd.) were prepared for cell transfection. The confluence of the cells was maintained at ~60% before transfection. Cells were starved in serum-free Minimal Essential Medium (WISENT, Inc.) at 37˚C with 5% carbon dioxide for 2 h and transfected with 1 $\mu$g miRNA-93 mimics and inhibitors and their respective blank vectors using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for 36 h at 37˚C. Subsequent experiment was conducted within 72 h. The sequences of the miRNA-93 mimics/inhibitors are displayed in Table SI. The sequence of mature miRNA-93 was 5'-CAAAGUGCUGUUCGUGCAGGUAG-3'. The sequence of mimics negative control was 5'-UUGUACUACACAAAA GUACUG-3' and inhibitor negative control was 5'-CAGUAC UUUUGUGUAGUACAA-3'.

*RNA extraction and reverse transcription-quantitative PCR (RT-qPCR).* Total RNA of the cells was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). RT was performed using a PrimeScript™ RT reagent Kit (Takara Bio, Inc., No RR047A) according to the manufacturer's instructions. A SYBR Premix Ex Taq II kit (Takara Bio, Inc.) was used for RT-qPCR with thermocycling conditions as follows: initial denaturation 95˚C, 30 sec; 40 cycles of denaturation 95˚C, 30 sec, annealing at 60˚C 30 sec and extension 72˚C, 30 sec. StepOne Plus system (Applied Biosystems; Thermo Fisher Scientific, Inc.) was employed. Target gene expression was normalized using the $2^{-\Delta\Delta Cq}$ method (40). The sequences of mature miRNAs are presented in Table SII; RT-qPCR primers (Shanghai GenePharma Co., Ltd.) are displayed in Table SIII.

*Statistical analysis.* All experimental data with at least 3 times are presented as the mean ± standard deviation using GraphPad Prism (version 5.0; Dotmatics) and the statistical analysis was performed using SPSS 20.0 (IBM Corp.). Differences between the two groups was analyzed using paired t tests. For >2 groups in TCGA database, One-way ANOVA was used followed by Fisher's least significant difference post hoc test. The correlation analysis between two genes was evaluated using the Spearman rank correlation coefficient. All plots were drawn with GraphPad Prism software. For the results of miRNA microarray, $P<0.05$ and $|\log_2$fold-change (FC)$|>1$ were considered to define the DE-miRNAs. The association between expression of DE-miRNAs and the prognostic value was analyzed using log-rank test. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Screening of potential DE-miRNAs in HBV-associated HCC.* The original data of miRNA arrays GSE67882 and GSE69580 were obtained and normalized (Fig. S1). A total of 13 HCC and nine non-tumor tissues were included for further analysis. The 'limma' R package was used to generate volcano plots of miRNA expression (Fig. S2). The top 10 up- and downregulated genes are displayed as heatmaps (Fig. 1A and B). Intersection analysis showed that there were 63 up- and 22 downregulated genes (Fig. 1C and D). The candidate DE-miRNAs were defined according to two indicators $|\log_2 FC|>1$ and $P<0.05$. Following screening, nine up- and one downregulated DE-miRNA were obtained (Table I).

*Prognostic value of DE-miRNAs.* To evaluate candidate DE-miRNAs, their prognostic value was analyzed. Kaplan-Meier Plotter database showed that among nine upregulated miRNAs, only four miRNAs, namely miRNA-93, miRNA-106b, miRNA-210 and miRNA-374b, had a significant effect on prognosis of patients with HCC (Fig. 2A-D). The prognostic analysis of miRNA-125b showed that the lower the expression of miRNA-125b, the worse the prognosis (Fig. 2E). Finally, five miRNAs whose expression levels and prognosis were significantly different between HCC and normal tissue were identified as candidate DE-miRNAs for further study.

*Prediction of DE-miRNAs target and hub genes.* The target genes of DE-miRNAs were predicted in miRNet database. miRNA-93 had 1,220, miRNA-106b had 1,091, miRNA-210 had 124, miRNA-374b had 234 and miRNA-125b had 432 potential target genes (Fig. S3A and B; Table II). Protein-protein interaction network analysis was performed on predicted target genes of DE-miRNA using the STRING database and Cytoscape software (version 3.7.1; Fig. 3A and B). The top 20 resulting hub genes are displayed, as well as their scores (Table SIV).

*Functional annotation and pathway enrichment analysis.* The present study evaluated the terms involved in these target genes through GO analysis. DAVID (version 6.8) database was used to perform GO enrichment analysis of 2,669 predicted target genes of up- and 432 predicted target genes of downregulated DE-miRNAs. The results were divided into three categories: Biological process, cellular component and molecular function. The top ten terms are listed according to P-value (Fig. 4A and B). KEGG pathway enrichment analysis of target genes was performed using the DAVID database to evaluate the signaling pathways. The top 20 terms are displayed according to P-value (Fig. 4C and D). Notably, three signal pathways ('pathway in cancer', 'hepatitis B' and 'microRNAs in cancer') were co-enriched, which were associated with HBV-related HCC. To identify potential target genes regulated by HBV, these signaling pathways were selected for intersection analysis, resulting in 11 and 8 sets of intersecting genes for up- and downregulated genes, respectively (Fig. 4E and F). For these predicted target genes, experimental data from GEO database were further investigated. In GSE100400 dataset, RNA-seq, ChIP-seq and 4C methods were combined to identify target genes regulated by HBV (37). Since this dataset provided a reliable and well-analyzed differential gene expression profile, it was cross-analyzed with the present findings to predict the potential miRNA-mRNA regulatory relationship network in HBV-related HCC. An intersection analysis of 11 up- and 8 downregulated target genes with the results of GSE100400 was conducted. A total of seven HBV-associated intersecting genes was obtained: CCND1, MYC, E2F1, E2F2, E2F3,

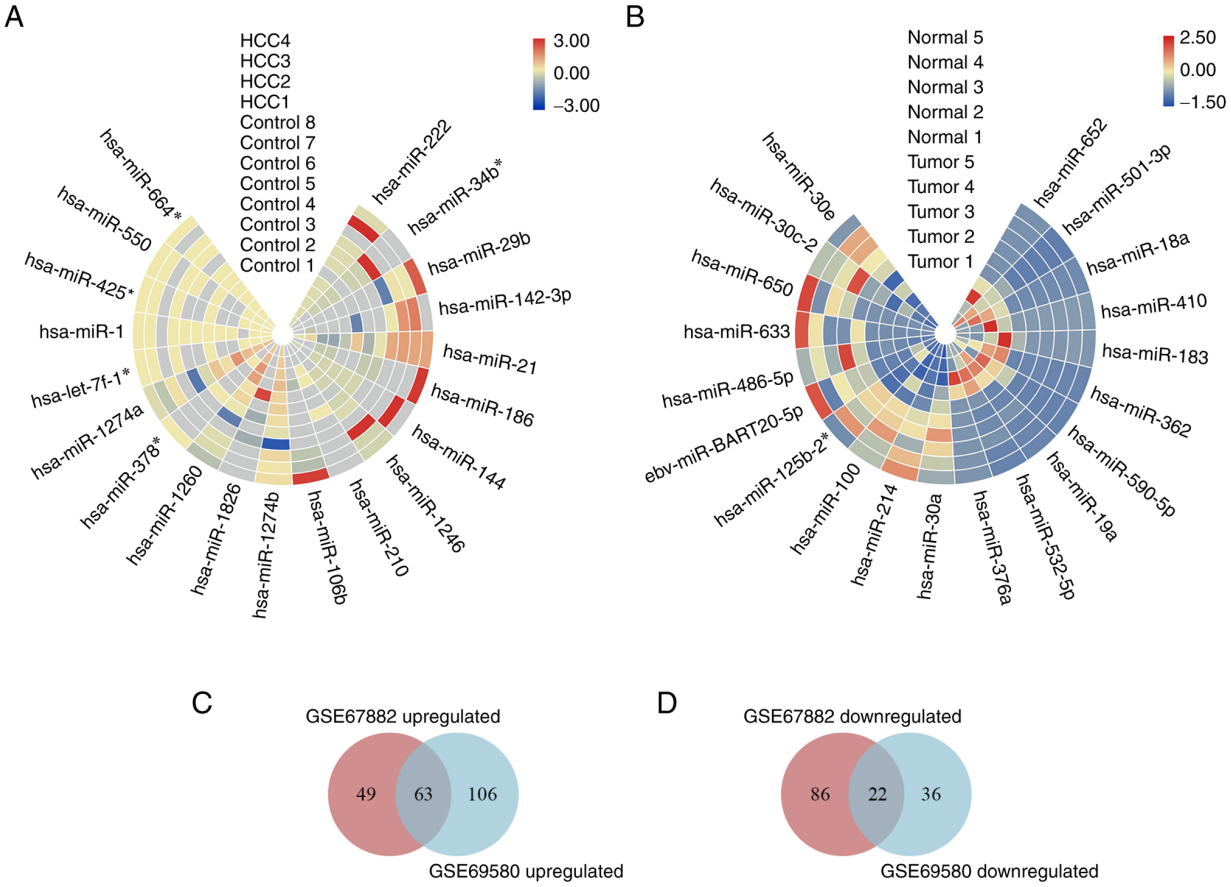


Figure 1. Candidate DE-miRNAs identified by GSE67882 in hepatitis B virus-related HCC. Top 20 up- and downregulated miRNAs in (A) GSE67882 and (B) GSE69580 data. (C) Intersection analysis of miRNAs (C) up and (D) downregulated in both datasets. DE-miRNA, differentially expressed microRNA; HCC, hepatocellular carcinoma.

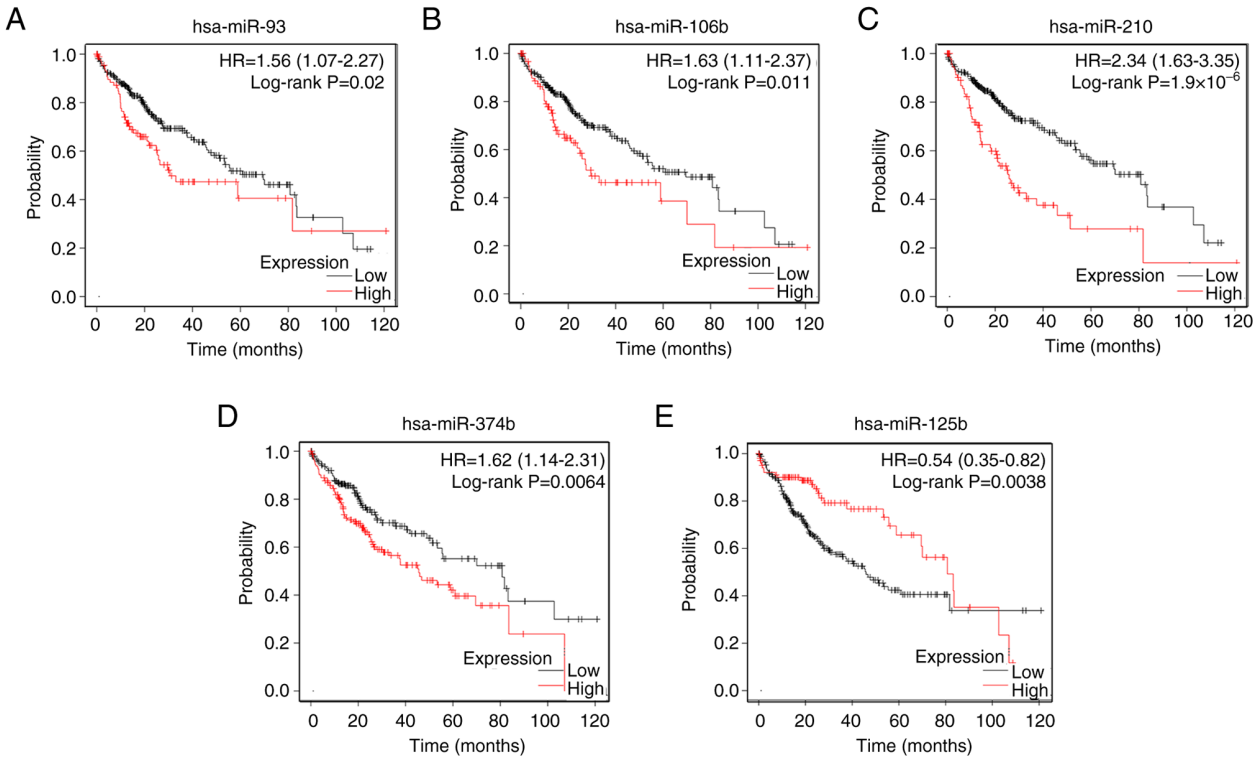


Figure 2. Association between expression of candidate DE-miRNAs and prognostic value in HCC. (A) Expression levels of miRNA-93, (B) miRNA-106, (C) miRNA-210, (D) miRNA-374 and (E) miRNA-125 and prognosis of patients with HCC. DE-miRNA, differentially expressed microRNA; HCC, hepatocellular carcinoma.

Table I. DE-miRNAs in hepatitis B virus-associated hepatocellular carcinoma.

A, Upregulated

| | GSE67882 | | GSE69580 | |
|---|---|---|---|---|
| DE-miRNA | P-value | $log_2FC$ | P-value | $log_2FC$ |
| hsa-miR-15a | 0.007808345 | 1.611184956 | 0.019320684 | 1.29726588 |
| hsa-miR-29b | $1.82x10^{-6}$ | 3.659264924 | 0.027230532 | 2.09606417 |
| hsa-miR-93 | 0.030902439 | 1.22769598 | 0.005843196 | 1.842708148 |
| hsa-miR-106b | 0.002537174 | 2.156166989 | 0.000566846 | 2.676683779 |
| hsa-miR-146b | 0.003197486 | 2.058917809 | 0.004808375 | 2.831128268 |
| hsa-miR-210 | 0.000261703 | 2.486264664 | 0.003623182 | 3.288727003 |
| hsa-miR-374b | 0.021895978 | 1.863305503 | 0.045327223 | 3.113135194 |
| hsa-miR-660 | 0.017308666 | 1.21052014 | 0.029714338 | 5.260854 |
| hsa-miR-886 | 0.032721809 | 1.547302991 | 0.013391202 | 1.871457118 |

B, Downregulated

| | GSE67882 | | GSE69580 | |
|---|---|---|---|---|
| DE-miRNA | P-value | $log_2FC$ | P-value | $log_2FC$ |
| hsa-miR-125b | 0.03390546 | -1.098388512 | 0.012086138 | -4.843165124 |

DE-miRNA, differentially expressed microRNA.

Table II. Target genes of DE-miRNAs in hepatocellular carcinoma.

A, Upregulated

| DE-miRNA | Number of genes |
|---|---|
| hsa-miRNA-93 | 1,220 |
| hsa-miRNA-106b | 1,091 |
| hsa-miRNA-210 | 124 |
| hsa-miRNA-374b | 234 |

B, Downregulated

| DE-miRNA | Number of genes |
|---|---|
| hsa-miRNA-125b | 432 |

DE-miRNA, differentially expressed microRNA.

GRB2 and CCNE1.The sequencing expression profiles of these genes in the GSE100400 dataset is shown in Fig. 4G. The expression levels of the DE-miRNA target genes were analyzed via UALCAN cancer database (Fig. S4). The present study summarized the potential miRNA-mRNA regulatory association network model in HBV-associated HCC (Fig. 5).

*Verification of candidate miRNAs and potential target genes and exploration of the regulatory association*. To validate the results of the bioinformatics analysis, the expression levels of DE-miRNAs and target genes in HepG2 and HepG2.2.1.5 (a cell line that stably expresses HBV) (41) cells were detected. Compared with those in HepG2 cells, miRNA-93, miRNA-106b, miRNA-210 and miRNA-374b showed significantly higher expression levels in HepG2.2.1.5 cells, while miRNA-125b showed significantly lower expression (Fig. 6A and B). Similarly, compared with HepG2 cells, expression levels of E1F1, E2F3, GRB2, CCND1 and CCNE1 were higher than those in HepG2.2.15 cells, while expression levels of E2F2 and MYC were not significantly different (Fig. 6C).

Base pairing analysis demonstrated that miRNA-93 had binding sites not only in the 3' but also the 5' UTR of GRB2. A total of nine potential binding sites was predicted between miRNA-93 and 5' UTR of GRB2 (Fig. 6D). As aforementioned, in HepG2.2.15 cells with HBV overexpression, the expression levels of miRNA-93 and GRB2 were upregulated. According to the predicted binding site, miRNA-93 has a regulatory effect of activation by targeting the GRB2 5' UTR. Therefore, more attention was paid to the active regulation between miRNA-93 and GRB2.

Transfection was performed to detect the effect of miRNA-93 on GRB2 expression levels *in vitro*. Mimics of miRNA-93 were transfected into HepG2 cells and changes in GRB2 were detected. With the increase in miRNA-93, the expression of GRB2 also increased (Fig. 6E). In addition, transfection of miRNA-93 inhibitors in HepG2.2.15 cells confirmed that the expression of GRB2 decreased with

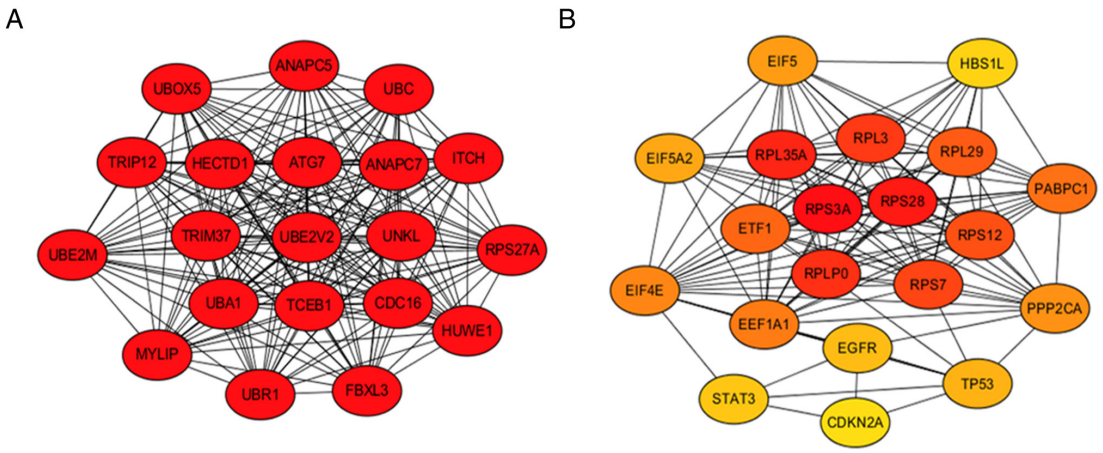


Figure 3. Top 20 hub genes in protein-protein interaction network belong to the target genes of microRNAs. Hub genes in (A) up- and (B) downregulated target genes. According to Cytoscape score, redder color represents higher score.

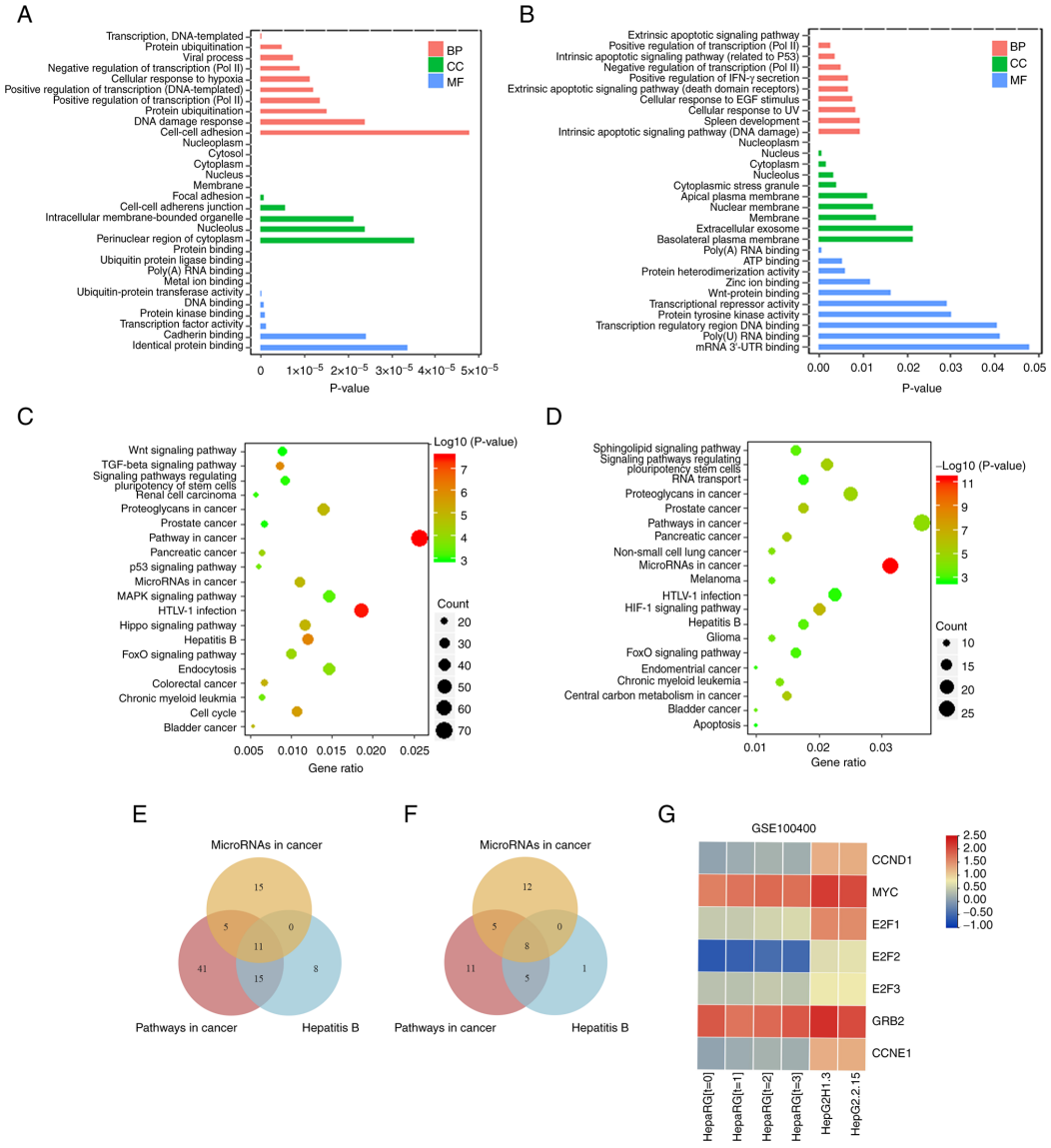


Figure 4. GO and KEGG analysis of function and pathway of potential target genes od DE-miRNAs. GO analysis was performed on the target genes of (A) up- and (B) downregulated miRNAs. KEGG analysis was performed on the target genes of (C) up- and (D) downregulated miRNAs. Intersection analysis of functional terms associated with (E) up- and (F) downregulated miRNAs. (G) Seven genes regulated by HBV were intersected by KEGG analysis and GSE100400 data and the sequencing expression profiles were shown. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; miRNA, microRNA; HBV, hepatitis B virus; BP, biological process; CC, cellular component; MF, molecular function; CCND1, cyclin D1); E2F2 (E2F Transcription Factor 2); GRB2 (Growth factor Receptor Bound Protein 2); CCNE1 (Cyclin E1).

the knockdown of miRNA-93 (Fig. 6E). Thus, GRB2 was a target gene of miRNA-93 and was positively regulated by miRNA-93.

*Prediction of the mechanism of GRB2 promotion of HCC.* TCGA database demonstrated that expression levels of GRB2 in HCC were significantly higher than those in non-tumor tissues (Fig. 7A). Expression of GRB2 was associated with lymph node metastasis in patients with HCC (Fig. 7B). The higher the GRB2 expression, the lower the overall survival rate of patients with HCC (Fig. 7C).

The present study analyzed the role of GRB2 in occurrence and development of HCC. After GSE101728 data normalization, the present study assessed enrichment of the KEGG pathway between the top 25% of the highest and lowest GRB2 expression (Fig. 7D-I). Primary immunodeficiency signaling pathway had the smallest P-value in the low GRB2 expression group.

The association between GRB2 expression and immune infiltration levels in HCC were assessed using the TIMER database. Spearman's correlation was assessed based on microarray expression levels. High GRB2 expression had positive correlations with the infiltration levels of B (R=0.324) and $CD4^+$ T cells (R=0.415), macrophages (R=0.483), neutrophils (R=0.429) and dendritic cells (R=0.386) in HCC (Fig. 7J). GRB2 expression had a weakly positive correlation with the infiltration of $CD8^+$ T cells (R=0.222) and no significant correlation with tumor purity (R=0.026). These findings suggest that GRB2 may serve a specific role in immune infiltration in HCC, especially for $CD4^+$ T cells, macrophages and neutrophils.

## Discussion

Although HBV-related miRNA-mRNA regulatory networks have been considered in previous studies (42,43), the miRNA-mRNA network regulated by HBV needs to be further explored due to continuous updating of databases with different patient samples and technologies. Advanced studies have implied that these factors can predict bioinformatic data more accurately (44,45).

HBV is widely integrated into the host genome and has four open reading frames, primarily encoding four viral proteins: Polymerase, HBx, envelope and core protein. HBx) serves a key role in regulating the replication of HBV and is involved in carcinogenesis associated with HBV (46). HBx can be detected in the nucleus, cytoplasm and plasma (47,48). In the nucleus, HBx directly regulates downstream target genes through gene transcription, while in the cytoplasm, HBx regulates downstream target genes via multiple signaling pathways (49). Similarly, miRNAs are widely distributed in human cells and can be detected in the cytoplasm and nucleus (33). HBx and miRNAs are colocalized in the nucleus, which suggests that HBx is a key molecule that participates in HBV regulation of miRNAs (50,51).

miRNAs are important gene expression regulators that can bind to complementary target mRNAs and inhibit their expression (52,53). They mainly play a role in translation inhibition by targeting the 3' UTR of mRNA and causing its cleavage and degradation (54). Analysis of the interaction sites between

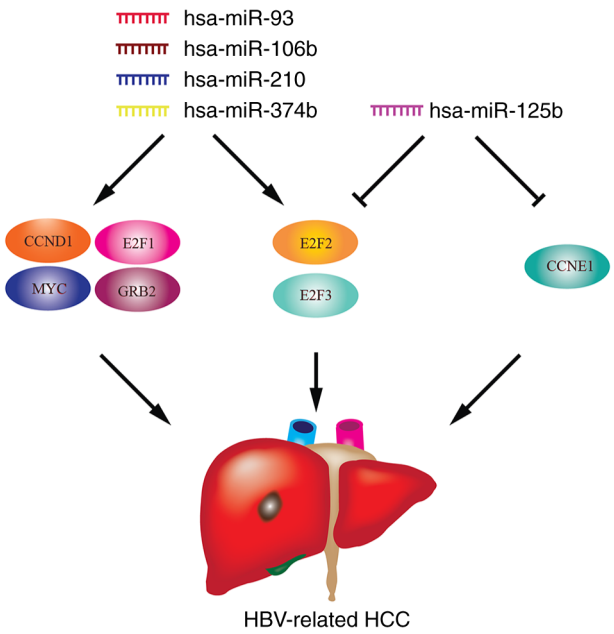


Figure 5. HBV mediates target genes through miRs in HBV-related HCC. HBV, hepatitis B virus; HCC, hepatocellular carcinoma; miR, microRNA; CCND1, cyclin D1); E2F2 (E2F Transcription Factor 2); GRB2 (Growth Factor Receptor Bound Protein 2); CCNE1 (Cyclin E1).

miRNAs and target genes demonstrated that most miRNAs have binding sites in the 3' UTR of the gene and a small number of predicted binding sites are located in the coding sequence region of the gene (54). However, the present study demonstrated that miRNA-93 upregulates the expression of GRB2, while most miRNAs inhibit gene expression (55). A previous study showed that miRNAs located in the nucleus change the chromatin state by binding to enhancers to activate gene transcription (56). Subsequent research confirmed the phenomenon of miRNA activation (57). miRNAs can suppress gene expression in the cytoplasm and activate gene transcription in the nucleus. In breast cancer, miRNA-93 is localized in the nucleus (58). The present study did not clarify the miRNA-93 localization or chromatin state of the GRB2 enhancer region. This would provide understanding of how miRNA-93 is involved in transcriptional regulation in the nucleus.

GRB2, an oncogenic functional gene in HCC, mainly regulates cell proliferation and invasion via the ERK1/2/AKT axis (59). A previous article identified that GRB2-associated binding proteins serve important roles in the regulation of immune response and cancer cell signaling (60). In addition, miRNA-93 regulates FAT atypical cadherin 4 expression, which is associated with prognosis and immune cell infiltration in HCC (61). The aforementioned report suggested that miRNA-93 was indirectly involved in tumor immune regulation. Several studies have reported that tumor-infiltrating lymphocytes are an independent predictor of lymph node status and survival in cancer (62,63).The present bioinformatics analysis indicated that GRB2 expression can be treated as a biomarker to evaluate the prognosis of HCC patients and regulate the tumor immune microenvironment. There was moderate correlation between GRB2 and immune cells, such as $CD4^+$ T cells, macrophages and neutrophils.

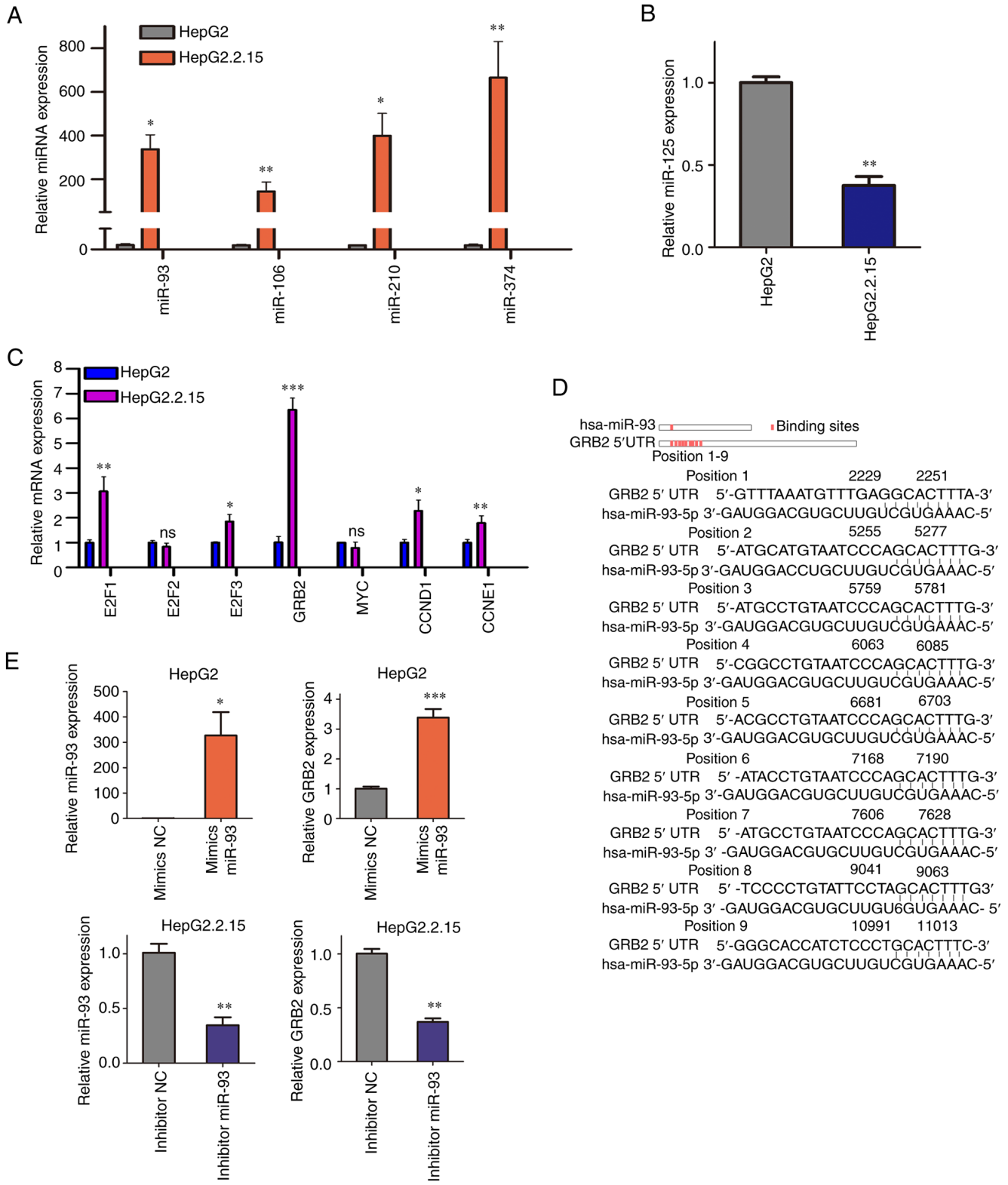


Figure 6. Reverse transcription-quantitative PCR explored the relationship between hepatitis B virus and its target genes. Expression levels of potential (A) up- and (B) downregulated miRs and (C) target genes in HepG2 and HepG2.2.15 cells. $^{*}$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001 vs. HepG2. (D) Predicted binding site of miR-93 and GRB2 gene. (E) Expression levels of GRB2 following transfection. $^{*}$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001 vs. mimics NC or inhibitor NC group. miR, microRNA; GRB2, growth Factor Receptor-bound Protein 2; ns, not significant; UTR, untranslated region; NC, negative control.

The high expression of GRB2 in HCC is accompanied by aggregation of these immune cells. GRB2 may lead to progression of HCC by affecting the tumor immune microenvironment (64). HBV-positive patients have long-term chronic viral infection (65). Liver macrophages are stimulated by HBV-associated inflammatory cytokines and chemokines and can adjust their phenotype based on different signals from the liver microenvironment, such as danger signals, fatty acids,

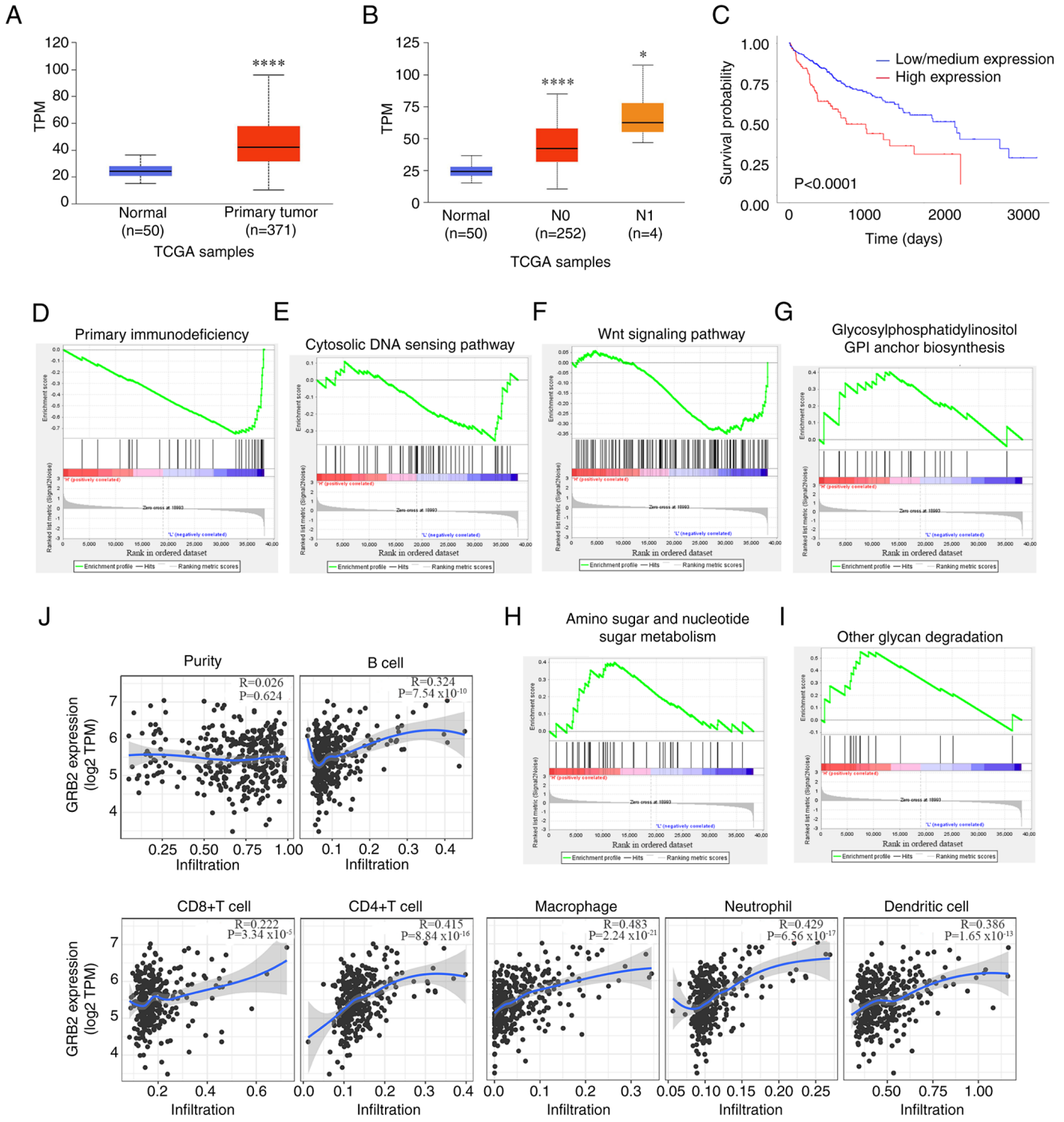


Figure 7. Expression levels and prognosis value of GRB2 were assessed in TCGA database and biological function of GRB2 was predicted. (A) Compared with normal tissues, the expression of GRB2 in primary tumor tissues increased significantly. (B) GRB2 expression levels are positively correlated with lymph node metastasis. (C) High GRB2 expression is associated with worse overall survival rate in patients with HCC. GSEA of (D) primary immunodeficiency, (E) cytosolic DNA sensing, (F) Wnt signaling, (G) glycosylphosphatidylinositol GPI anchor biosynthesis, (H) amino sugar and nucleotide sugar metabolism and (I) other glycan degradation pathway associated with GRB2 expression. (J) Correlation analysis between the expression levels of GRB2 and immune cells. *P<0.05, ****P<0.0001 vs. normal. GRB2 (Growth Factor Receptor Bound Protein 2); TCGA, The Cancer Genome Atlas.

and phagocytosis of cell debris, thereby affecting tumor progression (66). In previous studies, macrophages were not considered to have antigen-specific immune memory (67,68). However, macrophages, as a class of immune cells, can also form antigen-specific immune memory (69). When macrophages encounter a specific antigen again, they stimulate a stronger immune response. This indicates a function of macrophages in HBV-related HCC.

Collectively, the present study summarized a potential miRNA-mRNA regulatory network in HBV-associated HCC and demonstrated miRNA-93 positively regulated immune infiltration-related GRB2 gene expression through the 5'UTR region. This study reports for the first time the correlation between GRB2 expression and immune cell infiltration. Restoring GRB2 levels may be a novel treatment strategy for HCC in future.

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**Availability of data and materials**

The data generated in the present study may be requested from the corresponding author.

**Authors' contributions**

CZ and HF conceived the study. CZ, HS, ML and YQ designed the methodology, performed experiments and analyzed data. CZ wrote the manuscript. CZ, HS, ML and HF edited the manuscript. HF supervised the study. CZ and HS confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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