Identification of hub genes associated with hepatitis B virus-related hepatocellular cancer using weighted gene co-expression network analysis and protein-protein interaction network analysis

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ABSTRACT

Background. Chronic hepatitis B virus (HBV) infection is the main pathogen of hepatocellular carcinoma. However, the mechanisms of HBV-related hepatocellular carcinoma (HCC) progression are practically unknown. Materials and Methods. The results of RNA-sequence and clinical data for GSE121248 and GSE17548 were accessed from the Gene Expression Omnibus data library. We screened Sangerbox 3.0 for differentially expressed genes (DEGs). The weighted gene co-expression network analysis (WGCNA) was employed to select core modules and hub genes, and protein-protein interaction network module analysis also played a significant part in it. Validation was performed using RNA-sequence data of cancer and normal tissues of HBV-related HCC patients in the cancer genome atlas-liver hepatocellular cancer database (TCGA-LIHC). Results. 787 DEGs were identified from GSE121248 and 772 DEGs were identified from GSE17548. WGCNA analysis indicated that black modules (99 genes) and grey modules (105 genes) were significantly associated with HBV-related HCC. Gene ontology analysis found that there is a direct correlation between DEGs and the regulation of cell movement and adhesion; the internal components and external packaging structure of plasma membrane; signaling receptor binding, calcium ion binding, *etc*. Kyoto Encyclopedia of Genes and Genomes pathway analysis found out the association between cytokine receptors, cytokine-cytokine receptor interactions, and viral protein interactions with cytokines were important and HBV-related HCC. Finally, we further validated 6 key genes including C7, EGR1, EGR3, FOS, FOSB, and prostaglandin-endoperoxide synthase 2 by using the TCGA-LIHC. Conclusions. We identified 6 hub genes as candidate biomarkers for HBV-related HCC. These hub genes may act as an essential part of HBV-related HCC progression.

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Key words: GEO; TCGA-LIHC; HBV-related HCC; bioinformatics.

Acknowledgments: we would like to show sincere appreciation to the anonymous reviewers for their many useful comments on the early version of the manuscript.

Contributions: WW, FL and ZC contributed equally.

Conflict of interest: the authors declare no potential conflict of interest.

Funding: this work was supported by Changzhou Society Development Funding (CE20205038); Changzhou Applied Basic Research Program (CJ20220231); Natural Science Foundation of Jiangsu Province (BK20190138); Project of Nanjing Municipal Health Commission (ZKX22048); Changzhou Applied Basic Research Program(CJ20210080).

Ethical approval and consent to participate: this study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Changzhou First People's Hospital.

Availability of data and material: data and materials are available by the authors.

Received: 9 July 2023.
Accepted: 10 July 2023.

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Licensee PAGEPress, Italy
Italian Journal of Medicine 2023; 17:1626
doi:10.4081/itjm.2023.1626

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Introduction

As the seventh commonest cancer and the second commonest trigger of cancer-related death all over the world, hepatocellular carcinoma (HCC) makes up 80-90% of primary liver cancer.[1] Due to its huge population base and frequent population movements, China has the highest incidence of hepatocellular carcinoma.[2] And HCC always goes together with chronic infection of hepatitis B virus (HBV) or hepatitis C virus. The rate of patients who are infected with chronic HBV is over 50%.[3,4] Recent research shows that antiviral therapy

could reduce but not obviate the risk of HCC.[5] Sadly, the diagnosis rate of chronic HBV infection in the world is only 10%, and only 25% of those diagnosed are receiving antiviral therapy.[6] Therefore, it is extremely important to block the transmission of HBV and actively treat patients with chronic HBV hepatitis.

HBV induces chronic necroinflammation in hepatocytes, increases the mutation frequency of hepatocytes, and predisposes them to HCC.[7] 70-90% of patients develop cirrhosis during chronic HBV infection.[8] However, cirrhosis is not an inevitable path for the progression of HBV-related HCC, HBV carriers or chronically infected individuals without cirrhosis may also develop HCC.[9] Factors that promote the development of HBV-related HCC mainly include chronic infection, high level of HBV replication, specific HBV mutants, and HBV-encoded oncoproteins. Furthermore, the host immune response in chronic HBV-infected patients can cause recurrent liver inflammation. Afterward, the inflammation will lead to liver fibrosis and cirrhosis, accelerate the rate of cell turnover, and result in the accumulation of oncogenic mutations.[10]

We got the dataset of GSE121248 and GSE17548 from Gene Expression Omnibus (GEO) for analysis. The included tissue samples were all obtained from HBV-related HCC patients. We compared the data of RNA-sequence of paracancerous and cancerous tissues to choose differentially expressed genes (DEGs). We performed weighted gene co-expression network analysis (WGCNA) to screen out key modules and hub genes. The role of DEGs in HBV-related HCC was determined through Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. The protein-protein interaction (PPI) network module was also constructed for core module analysis. Finally, the data of cancer and normal tissue from HBV-related HCC patients in the cancer genome atlas-liver hepatocellular cancer database (TCGA-LIHC) were selected for validation.

Materials and Methods

Microarray data

Two gene expression profiling datasets (GSE121248 and GSE17548) of cancer and adjacent tissues of HBV-related HCC patients were downloaded from the GEO dataset (https://www.ncbi.nlm.nih.gov/geo/).[11-13] GSE121248 contained 37 cancer tissues from HBV-related HCC patients, 37 paracancerous tissues; GSE17548 contained 10 cancer tissues from HBV-related HCC patients, 11 paracancerous tissues. Sangerbox 3.0 (http://vip.sangerbox.com) was used to identify DEGs. Fold change >1.5, and $P<0.05$ were the screening criterion.

Weighted gene co-expression network analysis

Potential functional modules that can characterize the biological function of each subgroup were sought out by WGCNA under subgroup-specific signatures.[14] WGCNA begins with the level of thousands of genes to find out gene modules which clinical staff are interested in and finally uses intra-module connectivity, and gene importance to identify pivotal genes in disease pathways for further validation. Rather than associating thousands of genes with microarray sample features, we focused on the relationship between a few (usually less than 10) modules and sample features, identifying 6 functional modules. The corresponding analysis was performed on the Sangerbox 3.0 website.

Protein-protein interaction

The STRING 11.5 (https://cn.string-db.org/) website was chosen to assess the relationship between the various proteins. Based on the gene map on the STRING website, the interaction relationship is then generated and downloaded. The network was then visualized using Cytoscape 3.6.1 software. The plugin "cytohubba" is operated to determine the centrality parameters of the most important nodes of the screen, and we can use the "MCC" algorithm to discover the top modules in the PPI.

Functional enrichment analysis

Functional enrichment analysis included GO analysis and KEGG pathway analysis, which can cut down the complexity by dividing hundreds of genes, proteins, or other molecules into different pathways. GO analysis consisted of molecular function, cellular components, and biological processes.

The cancer genome atlas database

To further validate the previous results, external validation was performed using cancer tissues and normal tissues from HBV-related HCC patients in TCGA-LIHC.[15] According to the clinical data of HCC patients, a total of 75 cancer tissue samples from patients with HBV-related HCC and 7 corresponding paracancerous tissue samples were screened. These key genes in cancer tissues and normal tissues of HBV-related HCC patients were compared in the expression levels by the Wilcoxon test. $P<0.05$ was regarded as statistically significant.

Results

Identifying differentially expressed genes in GSE121248

Comparing cancer tissues and paracancerous tissues from patients with HBV-related HCC in GSE121248, we screened out 2080 DEGs for further

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study, including 1082 markedly down-regulated DEGs and 998 dramatically up-regulated DEGs. As shown in Figure 1, the DEGs were described in the volcano plot and heatmap. Moreover, these DEGs were subsequently used for WGmoreCNA analysis.

Differentially expressed genes-weighted gene co-expression network analysis in GSE121248

WGCNA was implemented to find stable co-expression modules in HBV-related HCC. By using cluster analysis and a dynamic tree felling algorithm, 6 gene units of yellow-green, magenta, tan, black, blue, and grey were generated. Eventually, our results found that the blue module had the highest negative correlation with HBV-related HCC, while the grey module had the highest positive correlation (Figure 2).

Functional enrichment analyses

Based on the information from DEGs, GO enrichment analysis yielded 3 results on the basis of GO classification. DEGs are distinctly enriched in cell migration, adhesion, duct development, anatomical structure formation, and morphological development during biological processes. For cellular components, DEGs mainly existed in the plasma membrane's intrinsic components, the outer encapsulation structures, the cell surface, and the collagen-containing extracellular matrix. Regarding molecular functions, DEGs were significantly enriched for signaling receptor binding, calcium ion binding, glycosaminoglycan binding, and cytokine receptor binding. Circumstantial results are shown in Figure 3A. KEGG pathway analysis identified 5 marked enriched pathways, such as cytokine-cytokine receptor interaction, viral protein and cytokine-cytokine receptor interaction, chemokine signaling pathway, IL-17 signaling pathway, and TNF signaling pathway. Circumstantial results are shown in Figure 3B. These results may contribute to the further comprehension of the correlation between DEGs and HBV-related HCC.

Identification of differentially expressed genes in GSE17548

By comparing cancer tissues from HBV-related HCC patients with adjacent liver tissues, we screened out 1249 DEGs, consisting of 811 hypo-expressed DEGs and 438 hyper-expressed DEGs. The heat map and volcano map of these DEGs are exhibited in Figure 4. And all these DEGs were used for following PPI.

Protein-protein interaction network analysis of differentially expressed genes in GSE17548

The relationships between PPIs were obtained from the STRING website and consisted of 259 nodes and 3273 edges. Through the plugin "cytohubba", we identified 2 modules consisting of the top 100 genes from the PPI network. The genes in the first module were AURKA, DLGAP5, NCAPG, CCNB1, KIF11, NDC80, BUB1B, RRM2, NEK2, TTK, UBE2C, NUSAP1, CCNA2, MELK, PBK, TPX2, TOP2A, CDK1, ASPM, CEP55, KIF20A, OIP5, ZWINT, PTTG1, CDKN3, CENPF, PRC1, RACGAP1, HMMR, DTL, KIF15, KIF4A, KIF14, HJURP, TRIP13, FOXM1, CENPE, ANLN, CDC20, RAD51AP1, KIAA0101, NUF2, CKS2, CDC6, SHCBP1, SPC25, MCM5, UBE2T, UHRF1, MND1, E2F8, CENPW, FAM83D, EZH2, CENPK, UBE2S,

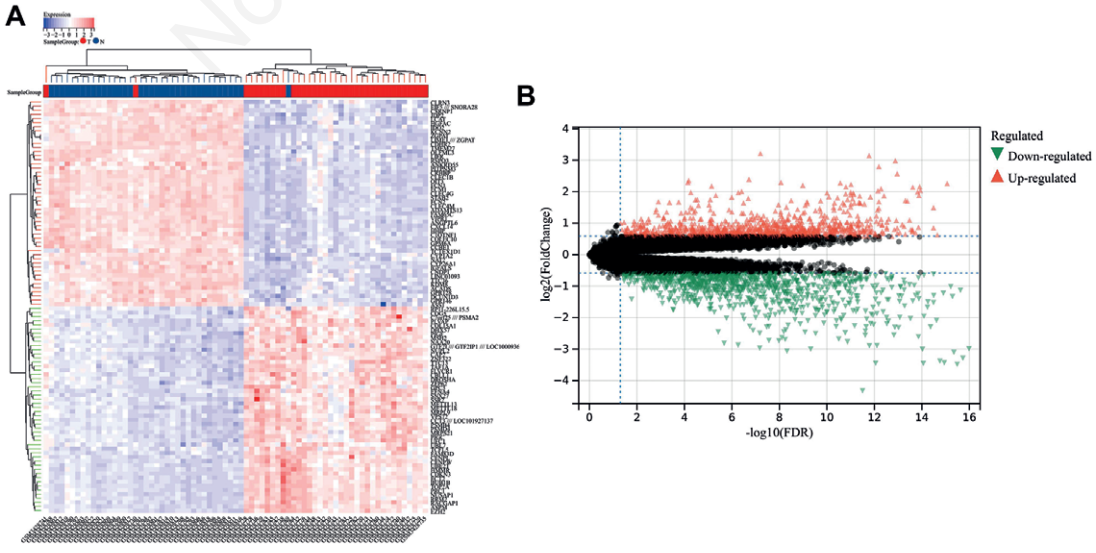

Figure 1. A) Heatmap; B) Volcano plot of differentially expressed genes from GSE121248.

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CYP4A11, CYP2B6, CYP3A4, CYP1A2, CYP2C9, AOX1, CYP3A7, UGT2B15, CCNE2, CYP2C8, CYP29A6, CYP2B18, CYP29A6, CYP2B18, CYP4F2, CYP2C18, GINS1, SGOL2, AKR1C3, HSD17B6, AKR1D1, SRD5A2, FOS, FOSB, EGR1, SLCO1B1, HSD11B1, APOA5, CENPL, EGR3 (Figure 5A). The genes in the second module were PON1, APOA5, LPA, HRG, FGB, PLG, SEPRING, FETUB, C8A, C8B, C8G, C9, C7, C6 (Figure 5B).

Identification of crossover genes

Eventually, we obtained 474 objects from GSE121248 and 100 objects from GSE17548 as candidate genes for HBV-related HCC progression. 6 co-crossed genes including C7, EGR1, EGR3, FOS, FOSB, and prostaglandin-endoperoxide synthase 2 (PTGS2) were taken into consideration (Figure 5C).

Validation of the cancer genome atlas-liver hepatocellular carcinoma

Furthermore, we utilized TCGA-LIHC to confirm the individual expression levels of these intersecting genes between cancer and normal liver tissues of HBV-related HCC patients. Ultimately, a total of 6 genes were validated, consisting of C7, EGR1, EGR3, FOS, FOSB, and PTGS2. Figure 6 shows the expression levels of these hub genes in HBV-related HCC patients from TCGA-LIHC.

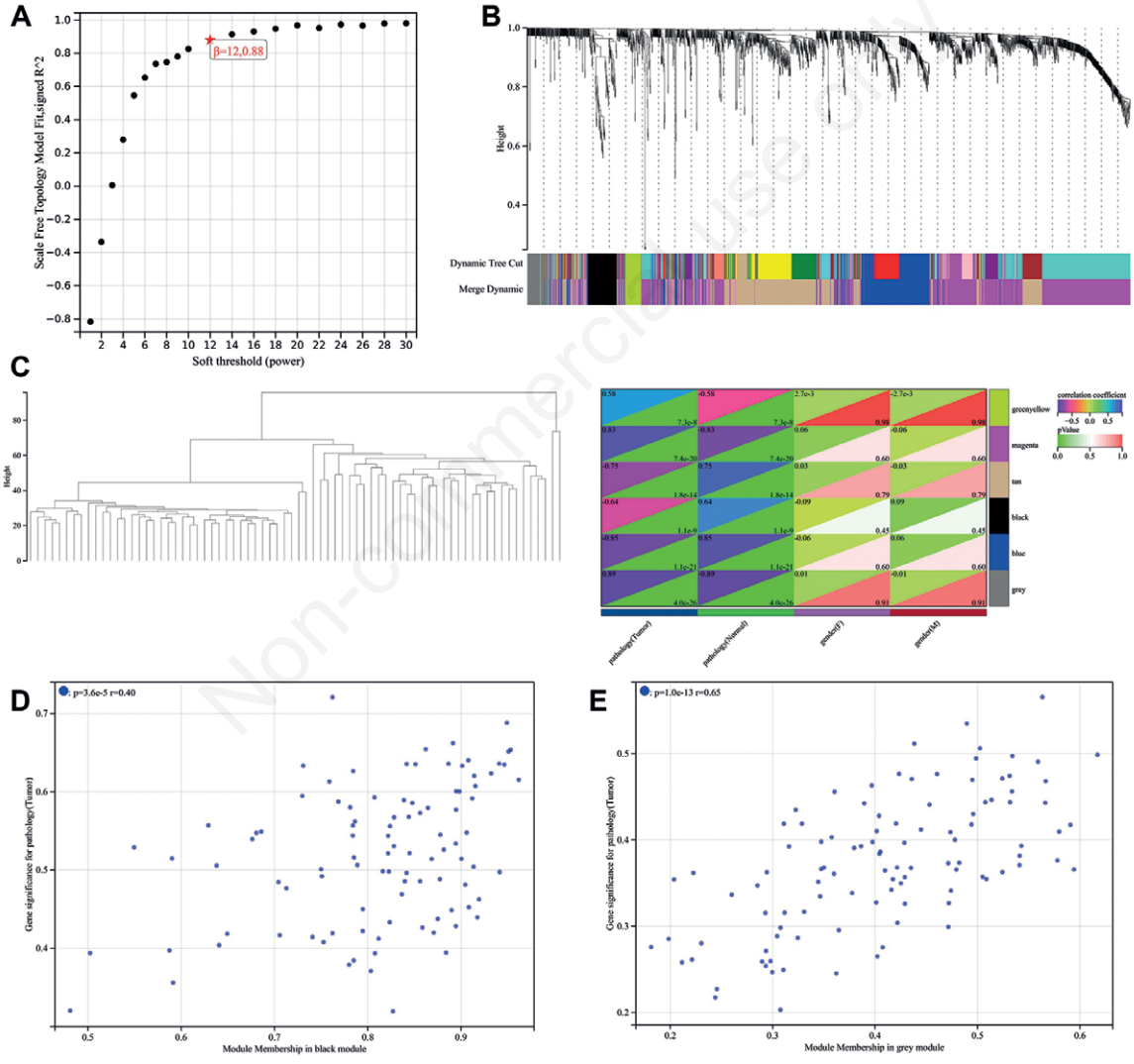

Figure 2. The results of weighted gene co-expression network analysis. A) The calculation diagram of the weight parameter (power) of the adjacency matrix; B) weighted gene co-expression network analysis reveals clustering and modular screening based on gene expression patterns; C) Dendrogram of characteristic genes of consensus modules obtained by weighted gene co-expression network analysis. Each row and each column correspond to a module. The colors in the table indicate the gene counts at the intersection of the corresponding modules; D) The correlation between MEblue membership and gene significance; E) the correlation between MEgrey membership and gene significance.

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Discussion

HCC is one of the commonest aggressive malignant tumors, ranking as the second highest cause of cancer-related mortality in the world.[16] On a worldwide scale, the majority of HCC cases (approximately 85% of cases) occur in underdeveloped countries peoples, especially in Eastern Asia.[17] And the rate of HCC cases which is caused by chronic HBV infection is about 50-80%.[18] Chronic HBV is the largest contributor to the occurrence and development of HCC, particularly in China.[19] Uncontrolled Chronic HBV infection is life-threatening. As it could progress to terminal-stage chronic cirrhosis and HCC.[20] HBV infection-induced HCC plays a vital part in the malignant transformation of HCC through hepatocytes transformations, including core gene mutations, chromosomal aberrations, epigenetic changes, and dysregulation of cell signaling pathways.[21,22]

In this manuscript, an integrated bioinformatics approach was performed to identify genetic variants in the progression of HBV-related HCC. We found hub genes by generating WGCNA and PPI network analysis. GO enrichment analysis suggested that during the progression of HBV-related HCC, cell migration, adhesion, ductal development, intrinsic components of the plasma membrane, outer envelope structure, cell surface, signaling receptor binding, calcium ion binding, glycosaminoglycan plays an important role in sugar binding. KEGG pathway analysis gave out a result that chemokine signaling pathway, cytokine-cytokine receptor interaction, IL-17 signaling pathway viral protein and cytokine-cytokine receptor interaction, and TNF signaling pathway were significant in HBV-related HCC.

We identified 6 hub genes that were included in the development and progression of HBV-related HCC, from GSE121248, GSE17548, and TCGA-

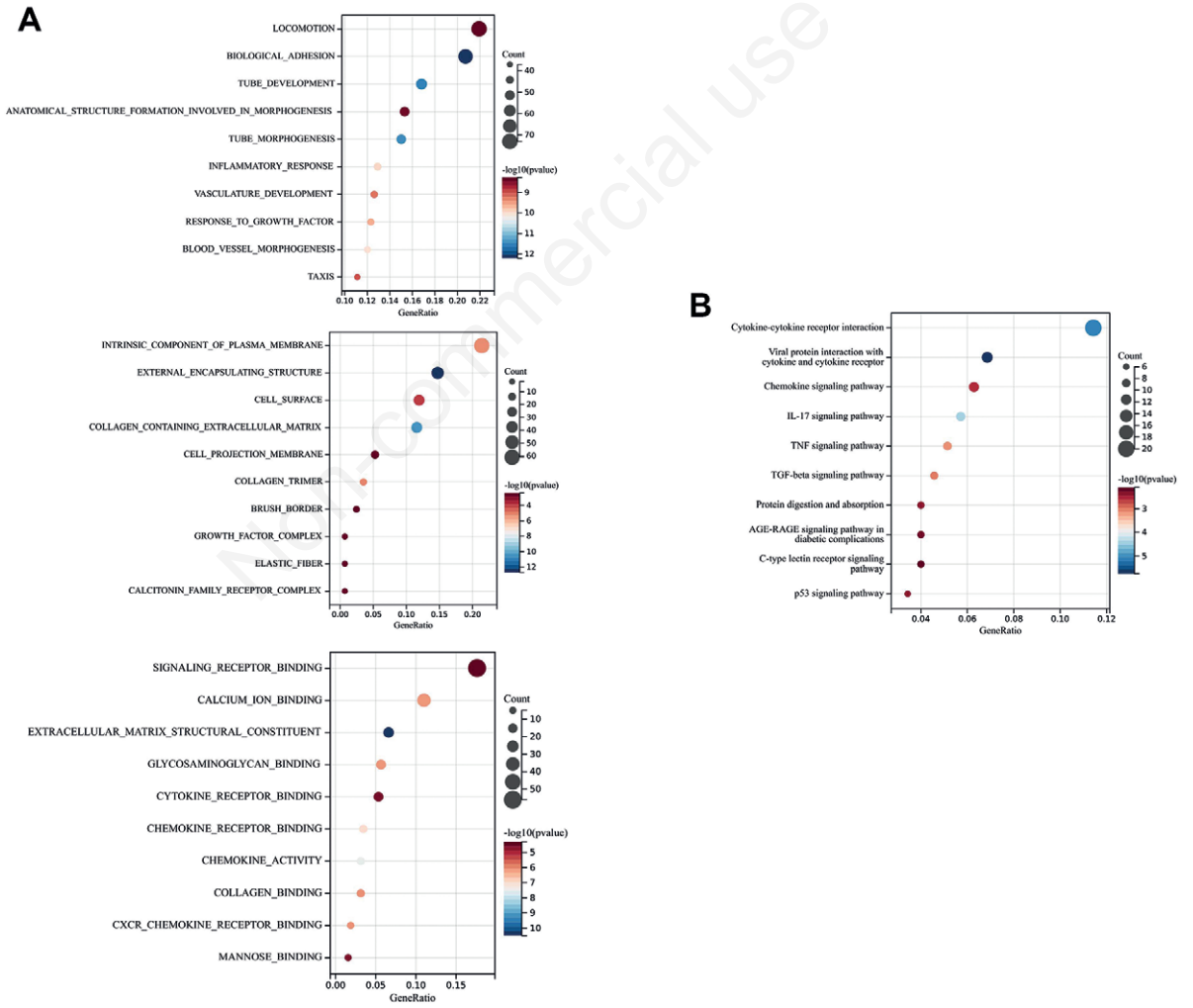

Figure 3. A) Gene ontology; B) Kyoto encyclopedia of genes and genomes enrichment analysis of 474 genes (including 369 genes in the blue module and 105 genes in the turquoise module) identified from weighted gene co-expression network analysis.

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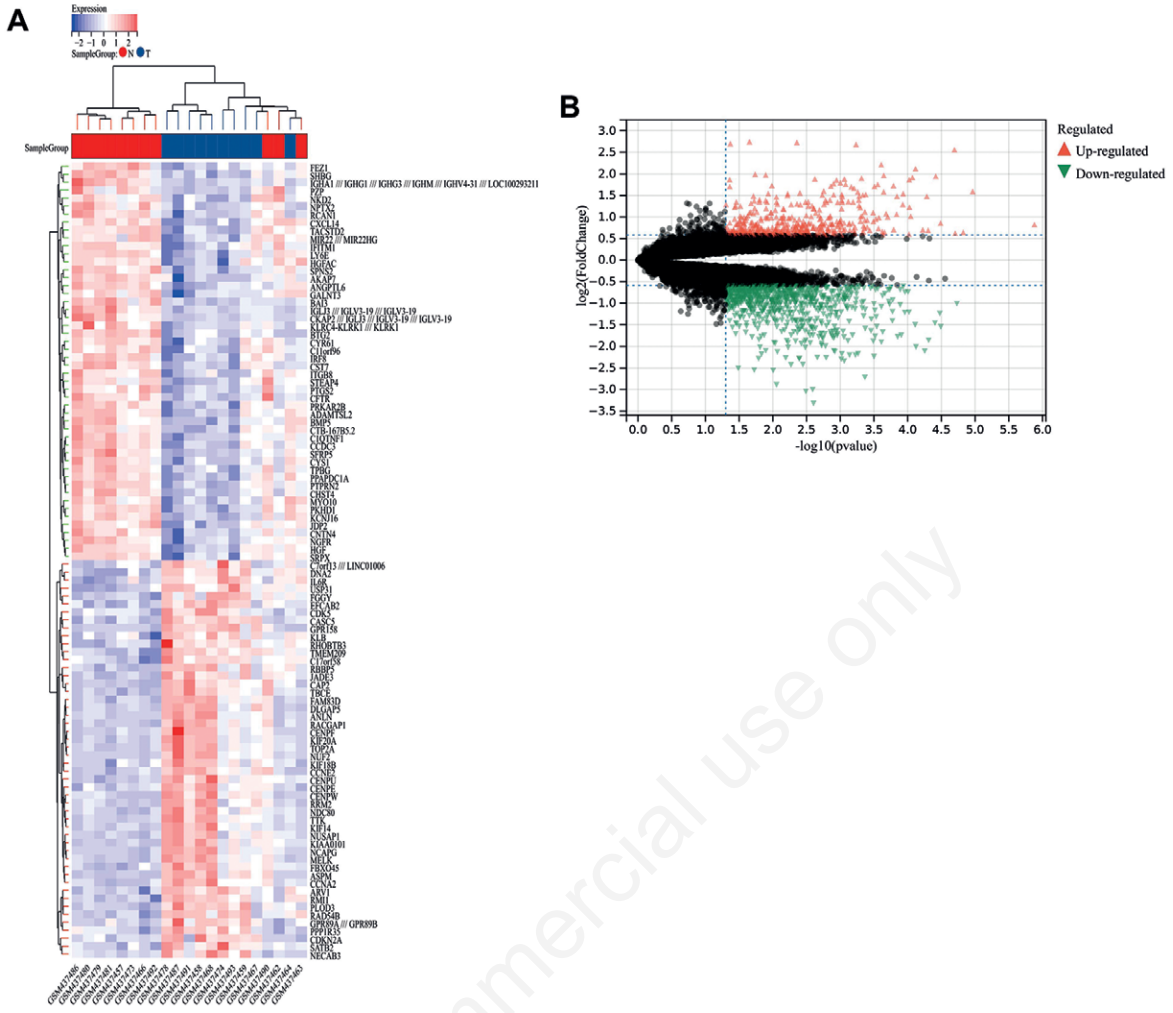

Figure 4. A) Heatmap; B) Volcano plot of differentially expressed genes from GSE17548.

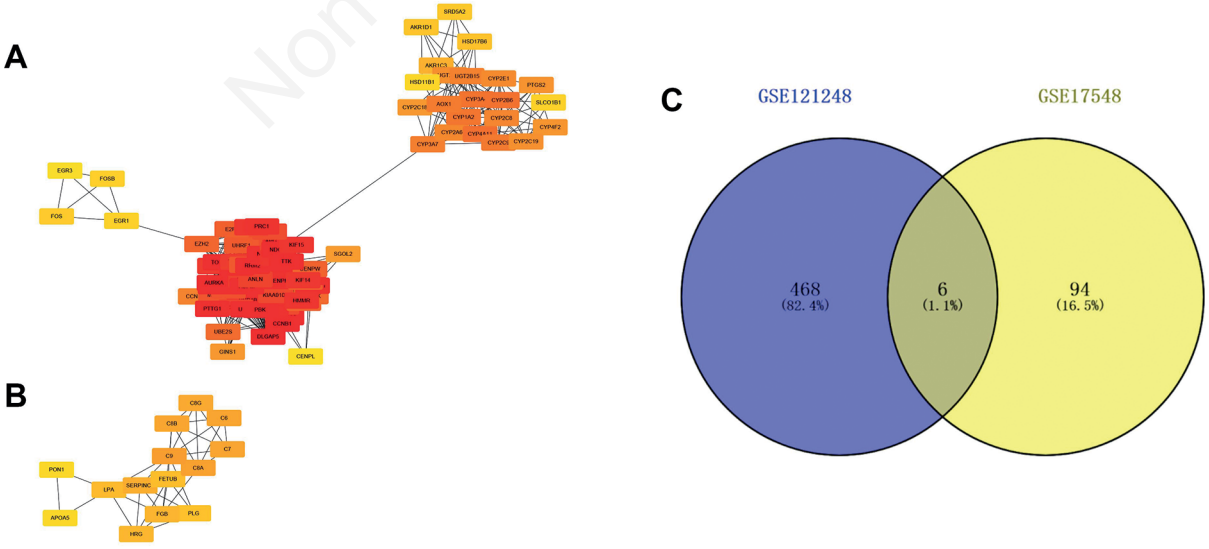

Figure 5. Two most significant modules in the protein-protein interaction network analysis. A) First module in the protein-protein interaction network; B) Second module in the protein-protein interaction network; C) Venn diagram used to identify cross genes.

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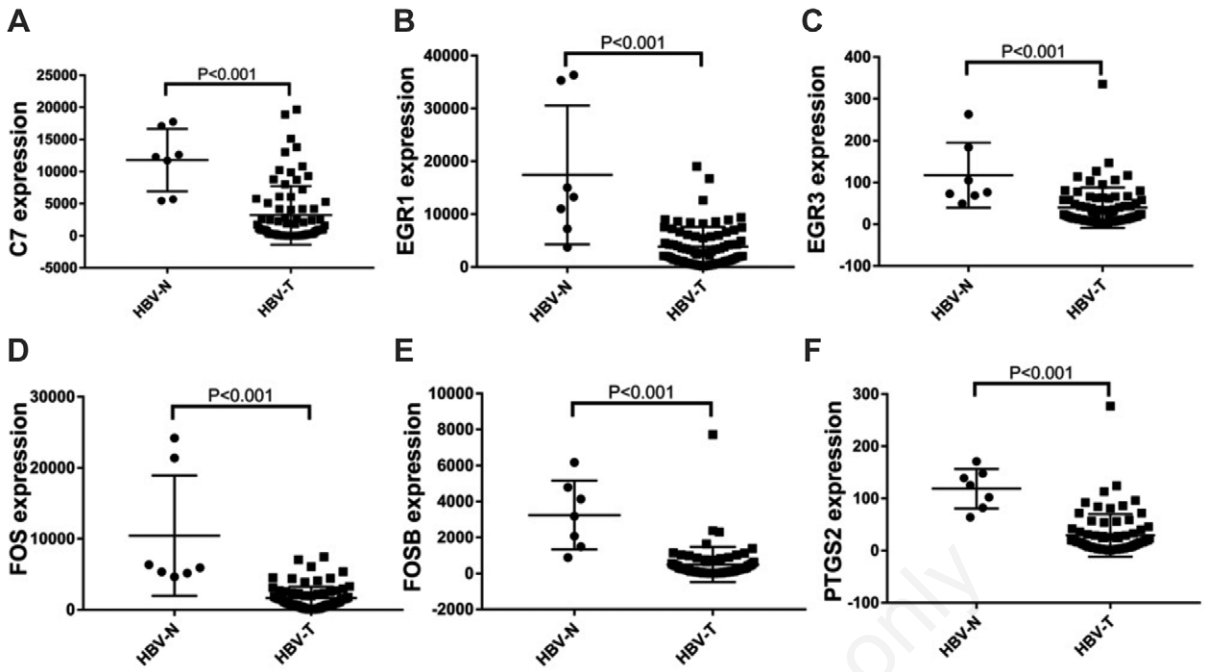

Figure 6. Validation of the expression levels of seven hub genes between hepatitis B virus-related hepatocellular carcinoma tissues and normal tissues in the cancer genome atlas-liver hepatocellular cancer database. A) Complement 7; B) Early growth response 1; C) Early growth response 3; D) FOS; E) FOSB; F) Prostaglandin-endoperoxide synthase 2.

LIHC, including C7, EGR1, EGR3, FOS, FOSB, and PTGS2. Complement 7 (C7) is mainly produced in the liver and works in innate immunity by forming pores in antigen-presenting cells.[23] C7 was significantly upregulated in tumor-initiating cells. Knockdown of C7 and CFH could abolish tumorsphere formation, while overexpression of C7 and CFH could significantly promote stemness factor expression and cell growth *in vivo*.[24] The Early growth response (EGR) family comprises 4 members (EGR1, EGR2, EGR3, and EGR4), which bind to the GC enrichment region and act as a transcriptional regulator.[25] Multiple researches have demonstrated that EGR1 is hyper-regulated in HCC tissues, and promotes drug resistance by enhancing hypoxia-induced autophagy, thus leading to HCC progression; however, data from several laboratories suggest that EGR1 inhibits HCC cell motility and invasion.[26-29] MiR-718 can regulate Early growth response 3 (EGR3) resulting in growth inhibition of HCC cells through upregulation of Fas ligand.[30,31] Studies on human HCC cell lines showed that FOS promoted cell migration *in vitro*, and ectopic expression of FOS increased immortalized human hepatocyte proliferation.[32,33] Not merely necrotic foci, immune cell infiltration, and hepatocyte morphology changes are displayed by FOS-expressing livers. What's more, there is a significant increase in increased proliferation, dedifferentiation, activation of the DNA damage response, and gene signatures of aggressive HCCs.[34] The quantity of studies on FOSB belonging to the FOS family is really little. PTGS2, also called COX-2, is a pro-inflammatory enzyme in T cell function.[35,36] It can be induced by prostaglandins associated with cell proliferation, tumorigenesis, and metastasis.[37]

However, this study also has limitations. First, our sample originates from different institutions in the database, and the sample may be too small to find out some associations. More research on patients at our institution is necessary. Second, experimental evidence remains undiscovered. It may be more convincing if we further validate the findings by cell biology experiments. Overall, we sincerely hope that this manuscript will conduce to finding out new diagnostic and prognostic biomarkers and therapeutic targets for HBV-related HCC.

Conclusions

In summary, we performed a comprehensive analysis of hub genes by bioinformatics methods. Those central genes can be involved in the growth and progression of HBV-related HCC and may serve as potential biomarkers and new therapeutic targets.

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