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Critical genes of hepatocellular carcinoma revealed by network and module analysis of RNA-seq data

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Abstract. – OBJECTIVE: RNA-seq data of hepatocellular carcinoma (HCC) was analyzed to identify critical genes related to the pathogenesis and prognosis.

MATERIALS AND METHODS: Three RNA-seq datasets of HCC (GSE69164, GSE63863 and GSE55758) were downloaded from Gene Expression Omnibus (GEO), while another dataset including 54 HCC cases with survival time was obtained from The Cancer Genome Atlas (TCGA). Differentially expressed genes (DEGs) were identified by significant analysis of microarrays (SAM) method using package samr of R. As followed, we constructed a protein-protein interaction (PPI) network based on the information in Human Protein Reference Database (HPRD). Modules in the PPI network were identified with MCODE method using plugin clusterViz of CytoScape. Gene Ontology (GO) enrichment analysis and pathway enrichment analysis were performed with DAVID. The difference in survival curves was analyzed with Kaplan-Meier (K-M) method using package survival.

RESULTS: A total of 2572 DEGs were identified in the 3 datasets from GEO (GSE69164, GSE63863 and GSE55758). The PPI network was constructed including 660 nodes and 1008 edges, and 4 modules were disclosed in the network. Module A (containing 244 DEGs) was found to related to HCC closely, which genes were involved in transcription factor binding, protein metabolism as well as regulation of apoptosis. Nine hub genes were identified in the module A, including PRKCA, YWHAZ, KRT18, NDRG1, HSPA1A, HSP90AA1, HSF1, IKGKB and UBE21. The network provides the protein-protein interaction of these critical genes, which were implicated in the pathogenesis of HCC. Survival analysis showed that there is a significant difference between two groups classified by the genes in module A. Further Univariate Cox regression analysis showed that 72 genes were associated with survival time significantly, such as NPM1, PRKDC, SPARC, HMGA1, COL1A1 and COL1A2.

CONCLUSIONS: Nine critical genes related to the pathogenesis and 72 potential prognostic markers were revealed in HCC by the network and module analysis of RNA-seq data. These findings could improve the understanding of the pathogenesis and provide valuable information to further investigate the prognostic markers of HCC.

Key Words:
Hepatocellular carcinoma, Differentially expressed genes, Protein-protein interaction network, Hub genes, Survival analysis.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common and lethal malignancies worldwide, which has a poor prognosis partly because of the extreme variability of the clinical outcome. Previous studies indicated that several biological pathways attributed to this heterogeneous clinical behavior.

A variety of pathways has been implicated in the pathogenesis HCC or related to the patients' survival time. Some pathways contribute to the progression and metastasis of HCC, such as Wnt/β-catenin pathway[1,2], DNMT1/PTEN/Akt pathway[3], ERK/c-Myc pathway[4]. Other pathways are associated with chemoresistance, such as Akt/PKB survival pathway[5] and IGF-1R signaling pathway[6]. Therapeutic targets are also found in signaling pathways, such as RalA signaling pathway[7] and Rac signaling pathway[8]. Meanwhile, some biomarkers have been revealed. MicroRNA-145 is regarded as a promising biomark-

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er in HCC[9]. Alpha-Methylacyl-CoA racemase (AMACR) can serve as a prognostic biomarker for the early recurrence/metastasis of HCC[10]. Progressive increase of SCCA-IgM immune complexes in cirrhotic patients is associated with the development of hepatocellular carcinoma[11]. NDRG1 is found as a biomarker for metastasis, recurrence and of poor prognosis in HCC[12].

In a word, many researchers have revealed the important pathways and biomarkers in the HCC, some of which have been applied to the clinical stage. However, the improvement of cancer pathogenesis understanding and bioinformatics methods provide the possibility to further study the HCC pathogenesis by novel analysis method. Although many high throughputs sequencing-based transcriptome analysis were carried out to disclose critical genes in HCC[13-17], they focused on metastasis or promoter methylation. In the present study, we try to analyze the data from several RNA-seq datasets with network and module analysis method, to mine valuable information about HCC.

Differentially expressed genes (DEGs) were identified from RNA-seq data of HCC and then protein-protein interaction (PPI) network analysis was performed to unveil critical genes. Besides, genes of prognostic effect were also revealed. These findings could not only supplement the understanding of the pathogenesis of HCC, but also provide potential prognostic markers for HCC.

Materials and Methods

Gene Expression Data

Three RNA-seq datasets (GSE69164, GSE63863 and GSE55758) were downloaded from Gene Expression Omnibus (GEO). The RNA-seq data of all three datasets were acquired using Illumina's platforms. Dataset GSE69164 included 11 paired normal and HCC samples. Dataset GSE63863 contained 12 paired normal and HCC samples. Dataset GSE55758 consisted of 8 paired normal and HCC samples. The survival time of HCC cases was included. Another RNA-seq dataset was obtained from The Cancer Genome Atlas (TCGA) and 54 HCC cases with survival time were selected out as the valid dataset.

Genes with missing value more than 20% were excluded, and others were filled with average value. Log2 transformation was then applied to the following analysis.

Screening of DEGs

DEGs were identified by significant analysis of microarrays (SAM) method[18] using package *samR*[19] of *R*. This method has the advantage in control false discovery rate (FDR) in multiple testing. The relative difference (d) was calculated as following formula:

$$d = \frac{X_1 - X_2'}{S + S_0}$$

Where X_1' and X_2' represent average expression levels of certain gene in two different status, and *s* represents the variance of the gene.

FDR < 0.05 and fold change > 2 were set as the threshold to screen out DEGs.

Construction of Protein-Protein Interaction (PPI) Network

Protein-protein interactions (PPIs) are the basis of biological processes. Investigations on PPIs can provide clues to understand the molecular mechanism of diseases. In the present study, PPI network was constructed based on the information from Human Protein Reference Database (HPRD)[20], which contained the largest number of proteins in the currently available databases.

Identification of modules

Modules were identified with MCODE method[21] using plugin *clusterViz* of CytoScape[22]. The method consisted of three steps: (1) calculate weights for nodes; (2) predict complex; (3) filter complex according to certain criteria. Clustering coefficient (Ci) was chosen as the weight of node:

$$Ci = 2 \times n / Ki \times (Ki-1)$$

Where *Ki* represents the number of nodes directly connected to node *i*; *n* represents number of edges among the Ki nodes.

Node with maximum weight was regarded as seed and then node *j* with weight ratio (W_j/W_{seed}) greater than threshold was included in the module.

Functional Enrichment Analysis

Gene Ontology (GO) enrichment analysis and pathway enrichment analysis were performed with DAVID (Database for Annotation, Visualization and Integration Discovery, http://david.abcc.ncifcrf.gov/)[23], an enrichment test based upon hypergeometric distribution.

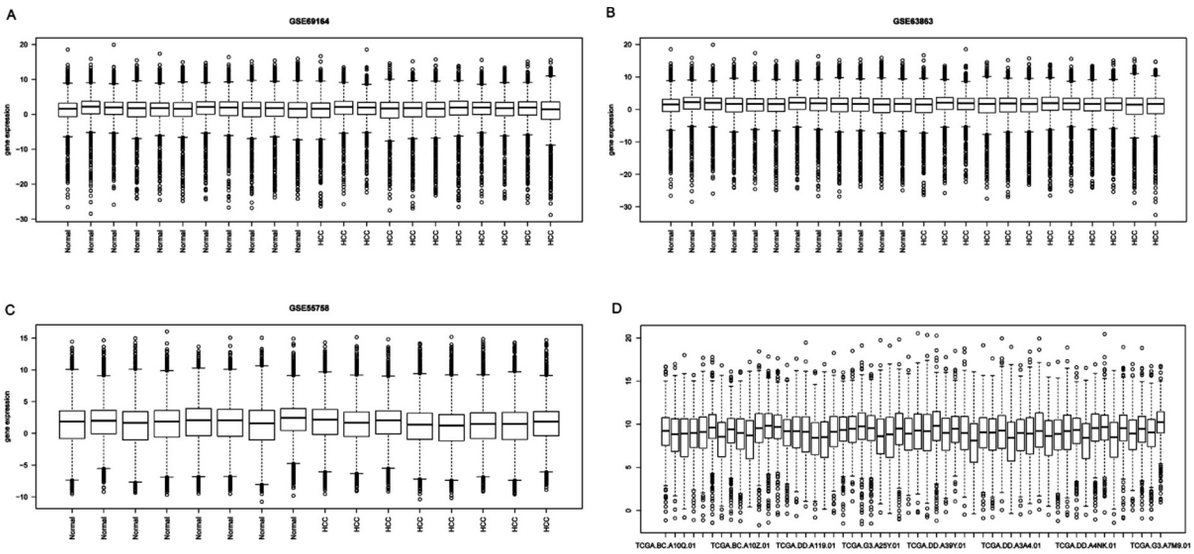

Figure 1. Box plots of pre-treated RNA-seq data. Dataset GSE69164 ***(A)***, Dataset GSE63863 ***(B)***, Dataset GSE55758 ***(C)*** and Dataset from TCGA ***(D)***.

Survival Analysis

Difference in survival curves was analyzed with Kaplan-Meier (K-M) method using package *survival*. Univariate Cox regression analysis was performed to disclose genes associated with survival time. p-value < 0.05 was set as the cut-off.

Results

Pre-treated Gene Expression Data

After pre-treatment, dataset GSE69164, GSE63863and GSE55758 contained 17059, 17110 and 17750 non-redundant genes, respectively (Figure 1), while dataset from TCGA contained 17781 non-redundant genes (Figure 1D). According to the box plots, a good performance of normalization was achieved for all the 4 datasets.

Differentially Expressed Genes

A total of 2439 DEGs were identified in dataset GSE69164, and 2424 DEGs were identified in dataset GSE63863, and 410 DEGs were identified in dataset GSE55857. The 3 sets of DEGs were combined to a union set, which contained 2572 DEGs.

To validate the reliability of the DEGs, principal component analysis (PCA) was performed. As shown in Figure 2, we found that the 3 sets of DGEs have good distinguish effect to separate HCC samples from normal samples, suggesting these DEGs were related to HCC actually.

PPI Network and Modules

A total of 39240 PPI interactions were obtained from HPRD. The 2572 DEGs were mapped into the network and interactions between two DEGs were selected out. Thus, a HCC-related PPI network was constructed, which contained 660 nodes and 1008 pair of interactions.

Four modules were unveiled in the HCC-related PPI network using plugin *clusterViz* of Cytoscape. After careful checking, we found that HCC-related genes were enriched in Module A (including a total of 244 DEGs, Figure 3). The genes were involved in transcription factor binding, protein metabolism as well as regulation of apoptosis (Table I). Therefore, we focus on the genes in module A in the following analysis.

Nine hub genes (top 5% genes ranked by degree) were revealed and 8 of them had been implicated in HCC. They are protein kinase C alpha (PRKCA), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ), keratin 18 (KRT18), N-myc downstream regulated 1 (NDRG1), heat shock protein family A member 1A (HSPA1A), heat shock protein 90kDa alpha family class A member 1 (HSP90AA1), heat shock transcription factor 1 (HSF1), and inhibitor of kappa light polypeptide

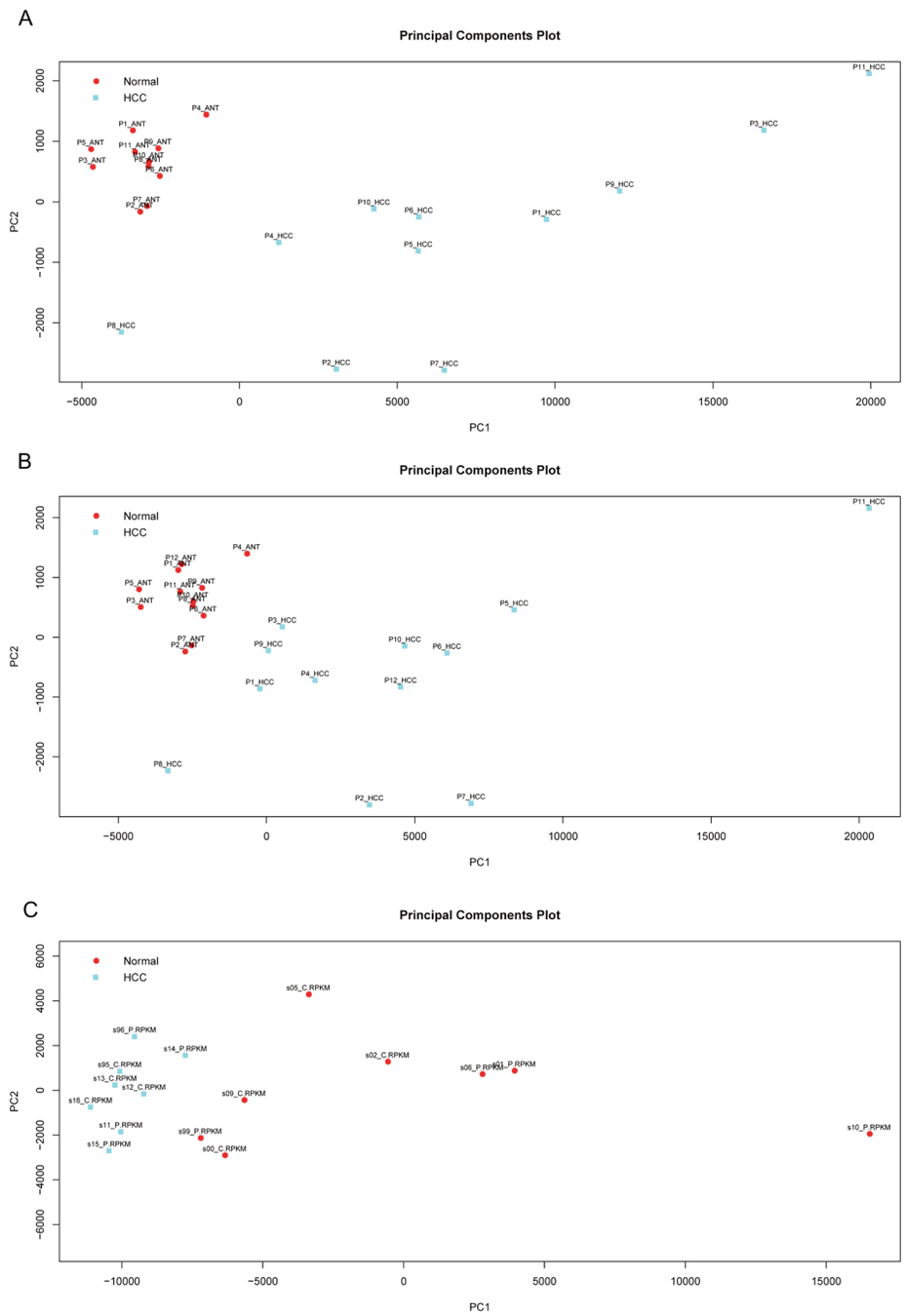

Figure 2. Principle component analysis result using DEGs from datasets. GSE69164 ***(A)***, GSE63868 ***(B)*** and GSE55857 *(C)*. Green circles represent HCC samples and red circles represent normal samples.

gene enhancer in B-cells, kinase beta (IKGKB). Ubiquitin-conjugating enzyme E2I (UBE2I) was identified as a hub gene in module A, which has not yet been reported related to hepatocellular carcinoma. However, it has been reported associated with ovarian cancer[24].

Potential prognostic genes

The 54 cases with survival time obtained from TCGA were clustered into two groups using genes from module A (Figure 4 A). A significant difference was observed between the two groups in survival analysis (Figure 4 B). Univariate Cox

Table I. Top 10 (ranked by FDR) biological processes over-represented in module A.

Category	Term	FDR
GOTERM_MF_FAT	GO:0008134~transcription factor binding	1.09E-04
GOTERM_MF_FAT	GO:0019904~protein domain specific binding	1.58E-04
GOTERM_BP_FAT	GO:0043933~macromolecular complex subunit organization	4.24E-04
GOTERM_BP_FAT	GO:0043067~regulation of programmed cell death	2.77E-03
GOTERM_MF_FAT	GO:0005524~ATP binding	2.81E-03
GOTERM_BP_FAT	GO:0010941~regulation of cell death	3.00E-03
GOTERM_MF_FAT	GO:0032553~ribonucleotide binding	5.18E-03
GOTERM_BP_FAT	GO:0042981~regulation of apoptosis	6.30E-03
GOTERM_BP_FAT	GO:0070271~protein complex biogenesis	2.01E-02
GOTERM_BP_FAT	GO:0006461~protein complex assembly	2.01E-02

FDR: false discovery rate.

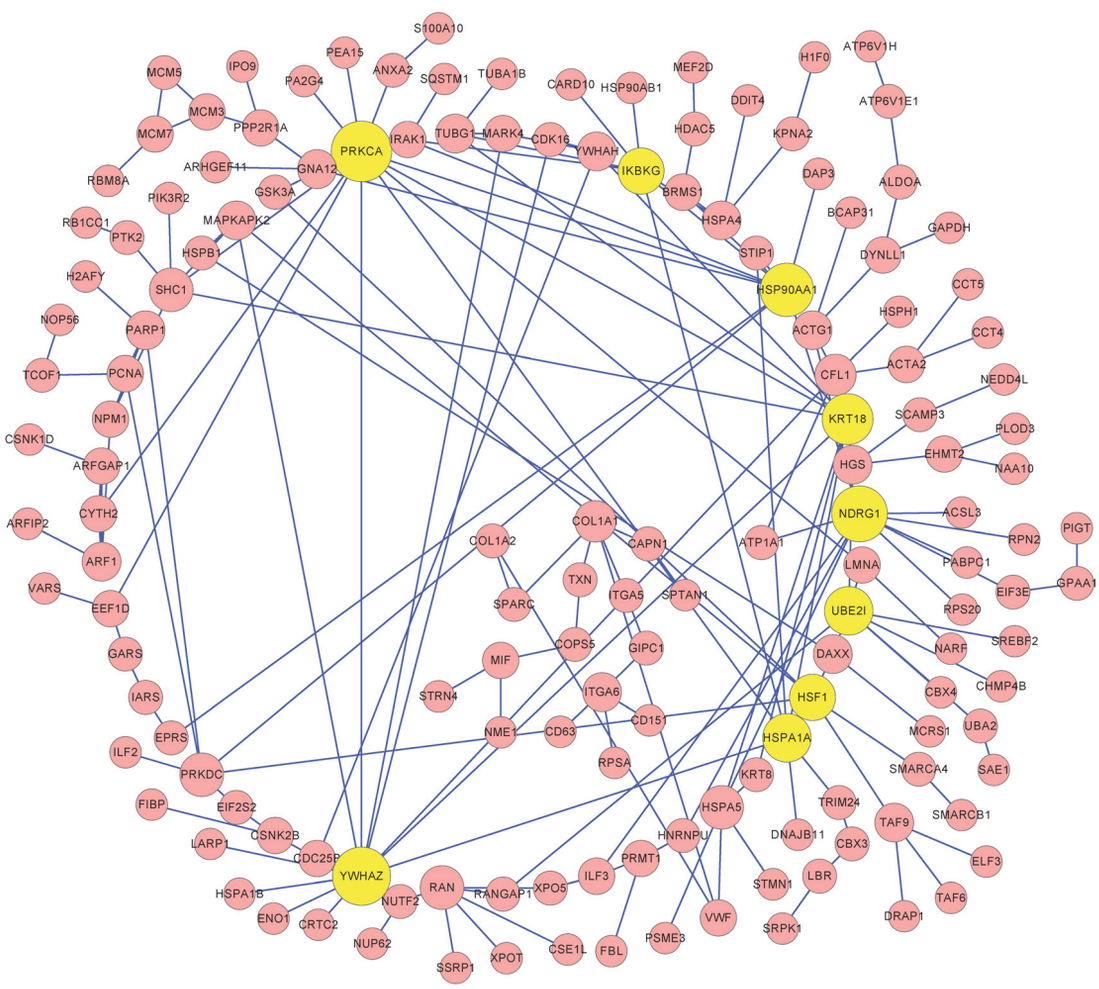

Figure 3. Module A identified from the HCC-related PPI network. Yellow circles represent hub genes.

regression analysis indicated that 72 genes were significantly associated with survival time (Table II). Top 15 genes are listed in Table II, such as nucleophosmin (NPM1), protein kinase, DNA-activated, catalytic polypeptide (PRKDC), secreted protein, acidic, cysteine-rich (SPARC), high mobility group AT-hook 1 (HMGA1), collagen type I alpha 1 (COL1A1) and COL1A2.

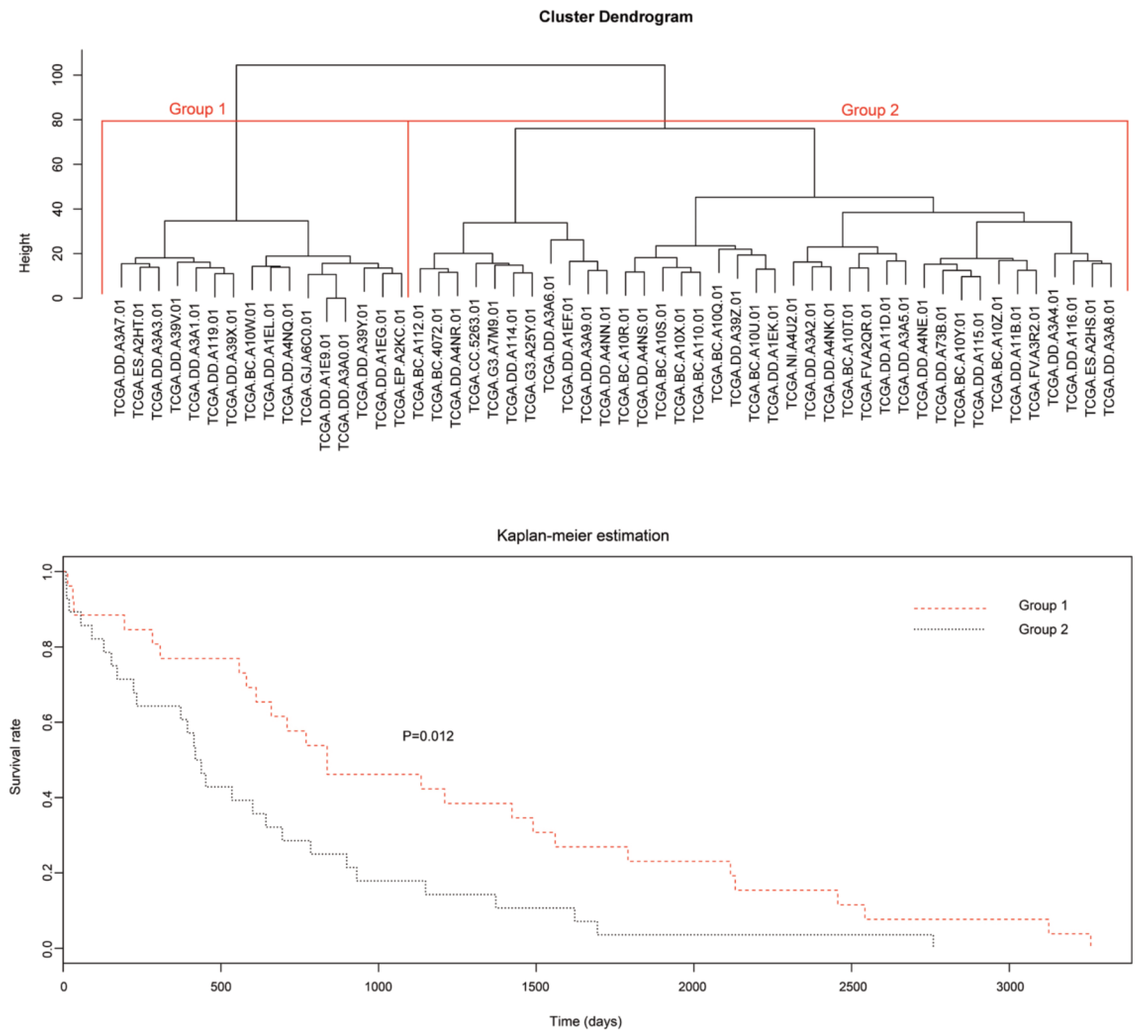

Figure 4. Clustering of the 54 HCC cases ***(A)*** and K-M analysis result of survival curve ***(B)***.

Discussion

In the present study, 2572 DEGs were identified from 3 RNA-seq datasets. A PPI network including 660 nodes and 1008 edges was constructed, from which 4 modules were obtained. Genes from module A were involved in transcription factor binding, protein metabolism as well as regulation of apoptosis, which were closely associated with cancers. Further, 9 hub genes were disclosed and 8 of them have been implicated in HCC.

PRKCA plays a role in many different cellular processes, such as cell adhesion, cell transformation, cell cycle checkpoint, and cell volume control. Reduction of PRKCA decreases cell proliferation, migration, and invasion of HCC cells[25]. YWHAZ

Table II. Top 15 genes significantly correlated to survival time.

Gene Symbol	*p*-value	Coefficient
CACYBP	0.0001	0.7952
IARS	0.0002	0.8284
ACTA2	0.0002	-0.5025
PARP1	0.0003	0.7045
NPM1	0.0004	0.7824
PRKDC	0.0008	0.5632
ACSL3	0.0009	0.7448
ACACA	0.0010	0.6593
AATF	0.0017	0.8597
CBX3	0.0018	0.9965
EPRS	0.0020	0.7482
SPARC	0.0024	-0.4561
COL1A1	0.0027	-0.2546
HMGA1	0.0029	0.3609
COL1A2	0.0030	-0.2886

mediates signal transduction by binding to phosphoserine-containing proteins, which were upregulated in HepG2 with doxorubicin treatment[26]. KRT18 is part of type I intermediate filament chain. Dysregulation of KRT18 is observed in HCC[27]. NDRG1 is involved in stress responses, hormone responses, cell growth, and differentiation. It's necessary for p53-mediated caspase activation and apoptosis. NDRG1 promotes growth of HCC cells by directly interacting with GSK-3β and Nur77 to prevent β-catenin degradation[28]. It's also responsible for doxorubicin and retinoic acid resistance in hepatocellular carcinoma cells[28]. Therefore, it's regarded as a biomarker for metastasis, recurrence and of poor prognosis in HCC[12]. HSPA1A is a member of the heat shock protein 70 family. It is overexpressed in HCC[29] and may be related to aggressiveness and prognosis of HCC. HSP90AA1 aids in the proper folding of specific target protein. It's found to be up-regulated in HCC[30]. HSF1 is a heat-shock transcription factor. It's considered as a key determinant of HCC development by regulating hepatic steatosis and metabolic syndrome[31]. It activates miR-135b expression, consequently enhancing HCC cell motility[32]. It may have prognostic value in patients receiving resection of HCC[33]. IKGKB is a key molecule in signaling to the transcription factor NF-κB[34]. Suppression of IKK-β by miR-451 inhibits HCC cell proliferation[35].

Survival analysis indicated that genes from module A could separate HCC cases with significantly different survival time. A total of 72 survival-related genes were unveiled. Some of them have been regarded as potential prognostic markers in HCC, like HMGA1[36] and COL1A1[37]. Besides, many genes play roles in the development of HCC. NPM1 promotes degradation of activating transcription factor 5 (ATF5) and thus regulates proliferation and survival of HCC[38]. PRKDC is the catalytic subunit of the DNA-dependent protein kinase (DNAPK). The study of Cornell et al[39] indicate that DNAPK is a candidate driver of hepatocarcinogenesis and tissue biomarker that predicts response to treatment and survival. The expression of SPARC correlates with tumor angiogenesis in HCC[40]. COL1A2 is involved in HCC cell migration[41]. More researches on these genes are necessary to uncover their prognostic value.

Conclusions

Overall, network and module analysis of RNA-seq data revealed nine critical genes in HCC. Besides, 72 potential prognostic genes were obtained. These findings could advance the understanding of the pathogenesis and provide valuable information to further investigate the prognostic markers of HCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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