

# A prognostic prediction system for hepatocellular carcinoma based on gene co-expression network

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**Abstract.** In the present study, gene expression data of hepatocellular carcinoma (HCC) were analyzed by using a multi-step Bioinformatics approach to establish a novel prognostic prediction system. Gene expression profiles were downloaded from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) databases. The overlapping differentially expressed genes (DEGs) between these two datasets were identified using the *limma* package in R. Prognostic genes were further identified by Cox regression using the *survival* package. The significantly co-expressed gene pairs were selected using the R function *cor* to construct the co-expression network. Functional and module analyses were also performed. Next, a prognostic prediction system was established by Bayes discriminant analysis using the *discriminant.bayes* function in the *e1071* package, which was further validated in another independent GEO dataset. A total of 177 overlapping DEGs were identified from TCGA and the GEO dataset (GSE36376). Furthermore, 161 prognostic genes were selected and the top six were stanniocalcin 2, carbonic anhydrase 12, cell division cycle (CDC) 20, deoxyribonuclease 1 like 3, glucosylceramidase  $\beta$ 3 and metallothionein 1G. A gene co-expression network involving 41 upregulated and 52 downregulated genes was constructed. SPC24, endothelial cell specific molecule 1, CDC20, CDCA3, cyclin (CCN) E1 and chromatin licensing and DNA replication factor 1 were significantly associated with cell division, mitotic cell cycle and positive regulation of cell proliferation. CCNB1, CCNE1, CCNB2 and stratifin were clearly associated with the p53 signaling pathway. A prognostic prediction system containing 55 signature genes was established and then validated in the GEO dataset GSE20140. In conclusion, the present study

identified a number of prognostic genes and established a prediction system to assess the prognosis of HCC patients.

## Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer type worldwide and the third most common cause of cancer-associated death (1). Viral hepatitis infection is the major cause of HCC (2). The prognosis of HCC is mainly dependent on tumor size and staging (3). Prognosis is typically poor as complete tumor resection only occurs in 10-20% of cases (4).

Recently, considerable efforts have been made to identify prognostic markers for HCC. It has been reported that the expression of survivin mRNA correlates with poor prognosis in patients with HCC (5). In addition, Akt phosphorylation is a risk factor for early disease recurrence and poor prognosis of HCC patients (6). Furthermore, Yes-associated protein was identified as an independent prognostic marker in HCC (7) and the overexpression of pituitary tumor transforming gene 1 (PTTG1) was reported to be associated with angiogenesis and poor prognosis in patients with HCC (8). Furthermore, the downregulation of phosphatidylethanolamine binding protein 1 is associated with aggressive tumor behavior and unfavorable clinical outcomes in HCC patients with hepatitis B infection (9). In addition, HCC patients overexpressing T-cell lymphoma invasion and metastasis 1 displayed a significantly shorter overall survival time (10). Finally, high expression of epidermal growth factor-like repeats and discoidin domains 3 also predicts poor prognosis in HCC patients (11).

Numerous novel prognostic models for HCC have been reported. Yamashita *et al* (12) proposed a classification system defined by epithelial cell adhesion molecule and  $\alpha$ -fetoprotein, revealing novel prognostic subtypes of HCC. Calvisi *et al* (13) indicated that genome-wide hypomethylation and CpG hypermethylation are associated with biological features and the clinical outcome of HCC. The intratumoral balance of regulatory and cytotoxic T cells is a promising independent predictor for recurrence and survival of HCC after resection (14). Budhu *et al* (15) reported that a unique immune response signature (a refined 17-gene signature) of the liver microenvironment may be used to predict venous metastases, recurrence and prognosis in HCC. A Met-regulated expression signature significantly correlated with an increased vascular

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invasion rate, microvessel density and decreased mean survival of HCC patients (16). However, improved prognostic prediction systems are required to guide clinical treatments for HCC patients (17).

In the present study, a multi-step strategy was used to identify prognostic gene signatures in HCC (Fig. 1). Gene expression data from HCC datasets were analyzed and differentially expressed genes (DEGs) were identified. Furthermore, the prognostic genes were used to construct a gene co-expression network and a prognostic prediction system was established. In addition, functional and module analyses were performed for the gene co-expression network. The prediction model was further validated in another independent gene expression dataset. The created prognostic prediction system may be applied to predict the prognosis of HCC.

## Materials and methods

**Raw data and pre-treatment.** The mRNA expression profiles of 421 samples (371 samples from patients with HCC and 50 samples from normal controls), along with their corresponding clinical information (contained in TXT files) were downloaded from The Cancer Genome Atlas (TCGA; gdc-portal.nci.nih.gov). These gene expression profiles had been generated by using the Illumina HiSeq 2000 RNA Sequencing platform (Illumina, Inc., San Diego, CA, USA). The genes in the TCGA dataset were annotated using information retrieved from the Human Genome Organization Gene Nomenclature Committee (HGNC; www.genenames.org), which establishes unique symbols and names for human loci.

The inclusion and exclusion criteria for the selection of microarray datasets were as follows: Human HCC; number of tumor samples, >100; number of control samples, >100; total number of tumor and control samples larger than that of the samples in TCGA dataset. Finally, the mRNA expression dataset GSE36376 was retrieved and raw data (TXT files) were downloaded from the Gene Expression Omnibus repository (GEO; www.ncbi.nlm.nih.gov/geo). This GEO dataset contained gene expression profiles of tumor liver tissues (n=240) and adjacent non-tumorous liver tissues (n=193). Data had been acquired based on the platform GPL10558 Illumina HumanHT-12 V4.0 expression bead chip (Illumina, Inc.). Probes were annotated to genes according to platform annotation profiles. Furthermore, the average expression value of a gene symbol mapped with multiple probes was calculated. The genes with low abundance (expression value <5) were removed and Log 2 conversion was applied for data using the *R* *limma* package (18). The microarray data were normalized by the quantile method (19).

**Identification of DEGs and clustering analysis.** The differences in gene expression levels between tumor and control samples in TCGA and GSE36376 datasets were analyzed using the *limma* package (18) of *R*. The false discovery rate (FDR) was calculated with the *multtest* package (20) of *R*. An FDR <0.05 and a  $\log_2$  (fold change) >0.585 (i.e., absolute fold change >1.5) (21–23) were selected as the cut-off thresholds to identify DEGs. The overlapping DEGs between the TCGA and GSE36376 datasets were used for further analysis. The top 25 downregulated and upregulated DEGs in the

latter subset were used for hierarchical clustering analysis by the *pheatmap* package (version 1.0.8; cran.r-project.org/package=pheatmap) (24) in *R*, based on the Encyclopedia of Distances (25), to intuitively identify the differences in gene expression levels among samples.

**Screening of prognostic genes.** In the TCGA dataset, the mRNA expression profiles and survival information were available for 330 patients. These data were used to screen prognostic genes from the overlapping DEGs using Cox regression (26) from the *survival* package of *R*. The genes with a log-rank P-value of <0.05 were considered to be prognostic. The top 6 prognostic genes ranked by-logRank (P-value) were used for stratification of patients in the Kaplan-Meier (K-M) survival analysis.

**Construction of gene co-expression network.** The expression data of prognostic genes were used to calculate the correlation coefficients (*r*) and the corresponding P-values between pairwise genes were determined using the *cor* function of *R*. The gene pairs with the  $|r| \geq 0.6$  and  $P < 0.05$  were considered to be significantly co-expressed. A gene co-expression network was constructed with these co-expressed gene pairs and visualized by Cytoscape 2.8.0 (www.cytoscape.org) (27).

**Functional annotation and functional module analysis.** Gene Ontology (GO) functional and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed for the prognostic genes in the co-expression network using the *cluster Profiler* package of *R* (28).

After the enrichment analysis, the transcription factors (TFs) significantly associated with genes in the co-expression network were identified using the Database for Annotation and the Visualization and Integrated Discovery platform (DAVID; david.ncifcrf.gov) (29). The identified TFs were also integrated into the co-expression network. Functional modules were unveiled from the co-expression network using GraphWeb (biit.cs.ut.ee/graphweb) (30). Furthermore, GO functional enrichment analyses were performed for the genes in the modules.

**Prognostic prediction system.** The 330 HCC samples with survival information in the TCGA dataset were selected as a training set. These samples were divided into two groups: A favorable prognostic group (status is alive and time of follow-up  $\geq 15$  months; n=139) and a poor prognostic group (status is deceased and the time of survival <15 months; n=191) based on their survival status. Genes in the co-expression network were ranked by logRank (P-value) and then analyzed by Bayes discriminant analysis (31) using the *discriminant bayes* function from package *e1071* (32) of *R*. The optimal combination of genes with the highest discriminative ability between the favorable and poor prognostic groups was defined as prognostic prediction system. The K-M survival curves were plotted for samples from the TCGA dataset to assess the prediction accuracy.

**Validation of the prognostic prediction system.** The predictive value of the abovementioned prognostic prediction system was further assessed using another independent validation

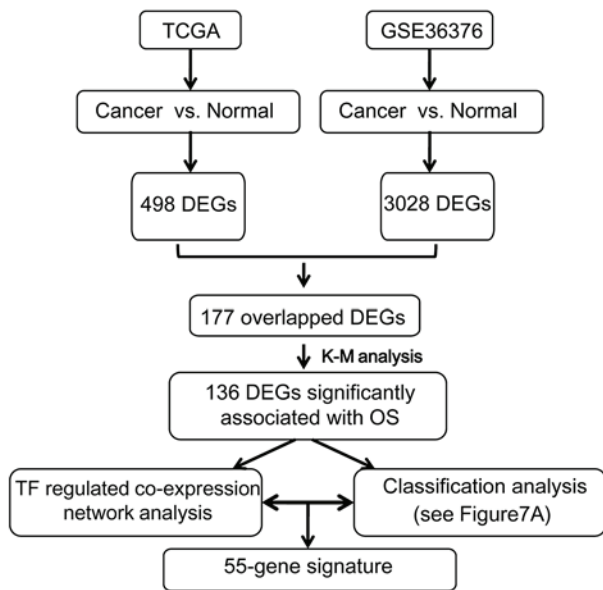


Figure 1. Schematic diagram for a multi-step strategy to identify a gene signature for the prognosis of hepatocellular carcinoma. The results for each step have been summarized. TCGA, The Cancer Genome Atlas; DEGs, differential expressed genes; OS, overall survival; K-M, Kaplan-Meier; TF, transcription factor.

microarray dataset. The independent validation microarray dataset was selected according to the following inclusion and exclusion criteria: Human HCC; containing prognostic information; number of tumor samples, >100; number of tumor samples and control samples larger than that of samples in the TCGA dataset. Finally, the mRNA expression dataset GSE20140 was retrieved for validation and raw data (TXT files) were downloaded from GEO. This independent validation dataset contained 80 HCC samples and 540 hepatitis/cirrhotic liver samples with corresponding survival information. This prognostic prediction system was used to separate the GSE20140 samples into favorable and poor prognostic groups based on the prognostic score. Furthermore, the K-M survival curves were plotted for the GSE20140 samples to examine the predictive accuracy.

## Results

**DEGs.** A total of 17,137 protein-coding mRNAs were annotated in the TCGA dataset based on the information from HGNC. After removal of low-abundance (expression value, <5) mRNAs, 12,370 mRNAs were retained and the expression density was significantly improved (Fig. 2). A total of 498 and 3,028 DEGs were identified from the TCGA and the GSE36376 dataset, respectively, and 177 of these were overlapping DEGs. The hierarchical clustering analysis indicated that, among these genes, the top 25 downregulated and upregulated DEGs were able to discriminate between HCC and control samples (Fig. 3).

**Prognostic genes.** A total of 161 prognostic genes ( $P < 0.05$ ) were identified. The top 6 prognostic mRNAs, ranked by  $-\log_{10}$ Rank in descending order, were stanniocalcin 2 (STC2), carbonic anhydrase 12 (CA12), cell division cycle 20 (CDC20),

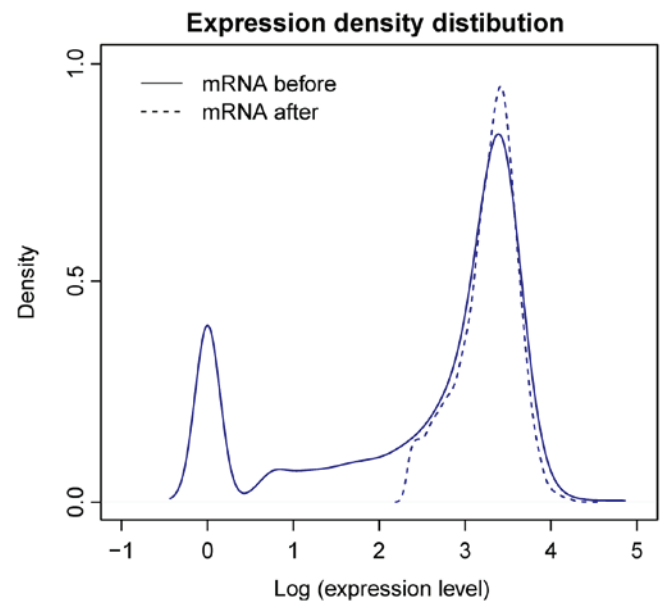


Figure 2. Distribution of mRNA expression density. The solid and dotted line, respectively, indicate the distribution prior to and after the removal of low-abundance mRNAs.

deoxyribonuclease 1 like 3 (DNASE1L3), glucosylceramidase  $\beta$ 3 (GBA3) and metallothionein 1G (MT1G). The K-M survival curves of patients stratified by these 6 prognostic genes individually suggested that these molecular markers may be used to predict the prognosis of HCC patients (Fig. 4). Patients with high expression levels of STC2, CA12, CDC20, DNASE1L3, GBA3 and MT1G had a significantly shorter survival time.

**Gene co-expression network.** The constructed co-expression network for prognostic genes contained 1,017 edges and 93 nodes involving 41 upregulated and 52 downregulated genes (Fig. 5A). A total of 3 TFs, i.e., nuclear transcription factor Y, cooperates with myogenic proteins 1 and signal transducer and activator of transcription 5B were identified and integrated into the gene co-expression network (Fig. 5B). A total of 18 significant GO terms and 7 KEGG pathways were significantly enriched by the prognostic genes in the co-expression network (Fig. 6). The prognostic genes NIMA related kinase 2 (NEK2), cell division cycle 20 (CDC20), aurora kinase A (AURKA), pituitary tumor-transforming 1 (PTTG1), cell division cycle 25A (CDC25A), SPC24 component of NDC80 kinetochore complex (SPC24), family with sequence similarity 83 member D (FAM83D), cyclin B2 (CCNB2), BUB1 mitotic checkpoint serine/threonine kinase (BUB1), centromere protein W (CENPW), cell division cycle associated 5 (CDCA5), cyclin A2 (CCNA2) and cell division cycle associated 3 (CDCA3) were significantly associated with cell division ( $P = 2.29 \times 10^{-13}$ ) and mitotic nuclear division ( $P = 2.76 \times 10^{-12}$ ; Table I). A total of 7 prognostic genes, i.e. CDC7, PRC1, PTH1R, PDGFRA, CDC20, endothelial cell specific molecule 1 (ESM1) and VIPR1, were significantly associated with increased cell proliferation ( $P = 3.56 \times 10^{-2}$ ). Furthermore, CCNB1, FAM83D, CCNE1, PRC1, AURKA, stratifin (SFN), CCNA2, CDC25A and KIF20A were significantly associated with protein kinase binding ( $P = 7.10 \times 10^{-4}$ ). The pathway



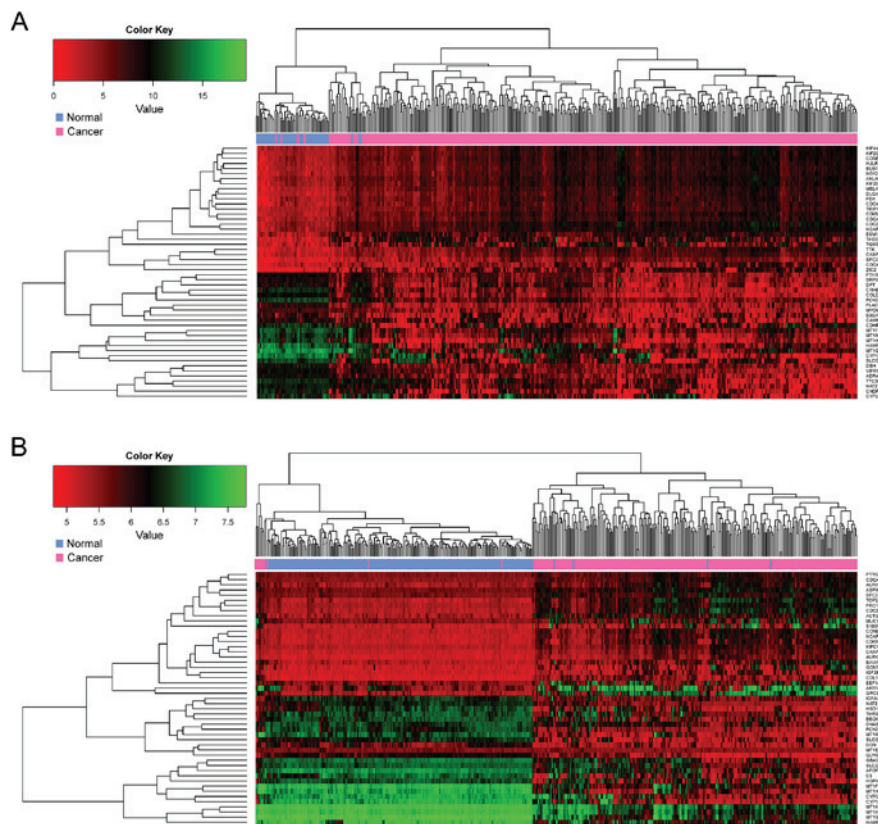


Figure 3. Hierarchical clustering analysis using the top 25 downregulated and upregulated genes among the 177 overlapping differentially expressed genes. (A) for The Cancer Genome Atlas dataset; (B) for the GSE36376 dataset. Pink indicates hepatocellular cancer cases, while blue indicates normal controls.

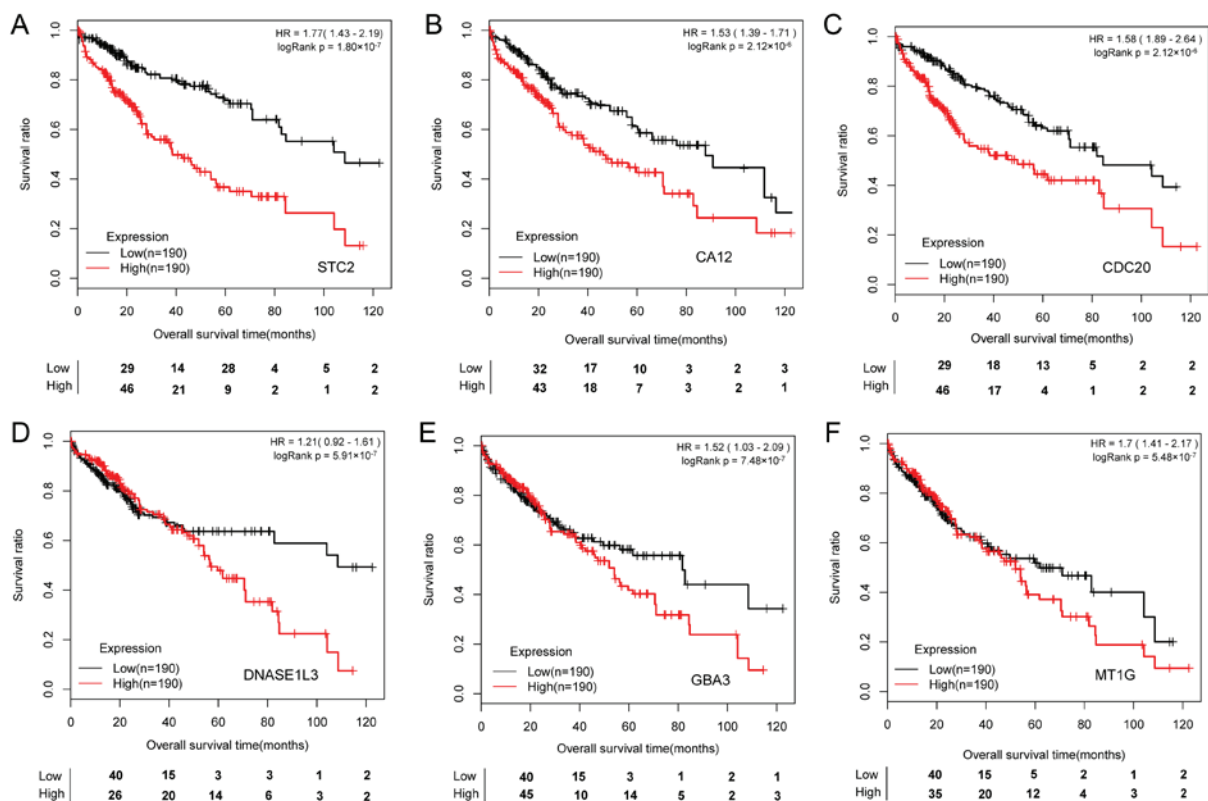


Figure 4. Kaplan-Meier survival curves for the top six prognostic genes. (A) STC2; (B) CA12; (C) CDC20; (D) DNASE1L3; (E) GBA3; (F) MT1G. The red lines indicate cases with high expression (expression value > median), while the black line indicates cases with low expression (expression value < median). 95% confidence intervals are displayed after the HR in brackets. HR, hazard ratio; STC2, stanniocalcin 2; CA12, carbonic anhydrase 12; CDC20, cell division cycle 20; DNASE1L3, deoxyribonuclease 1 like 3; GBA3, glucosylceramidase  $\beta$ 3; MT1G, metallothionein.

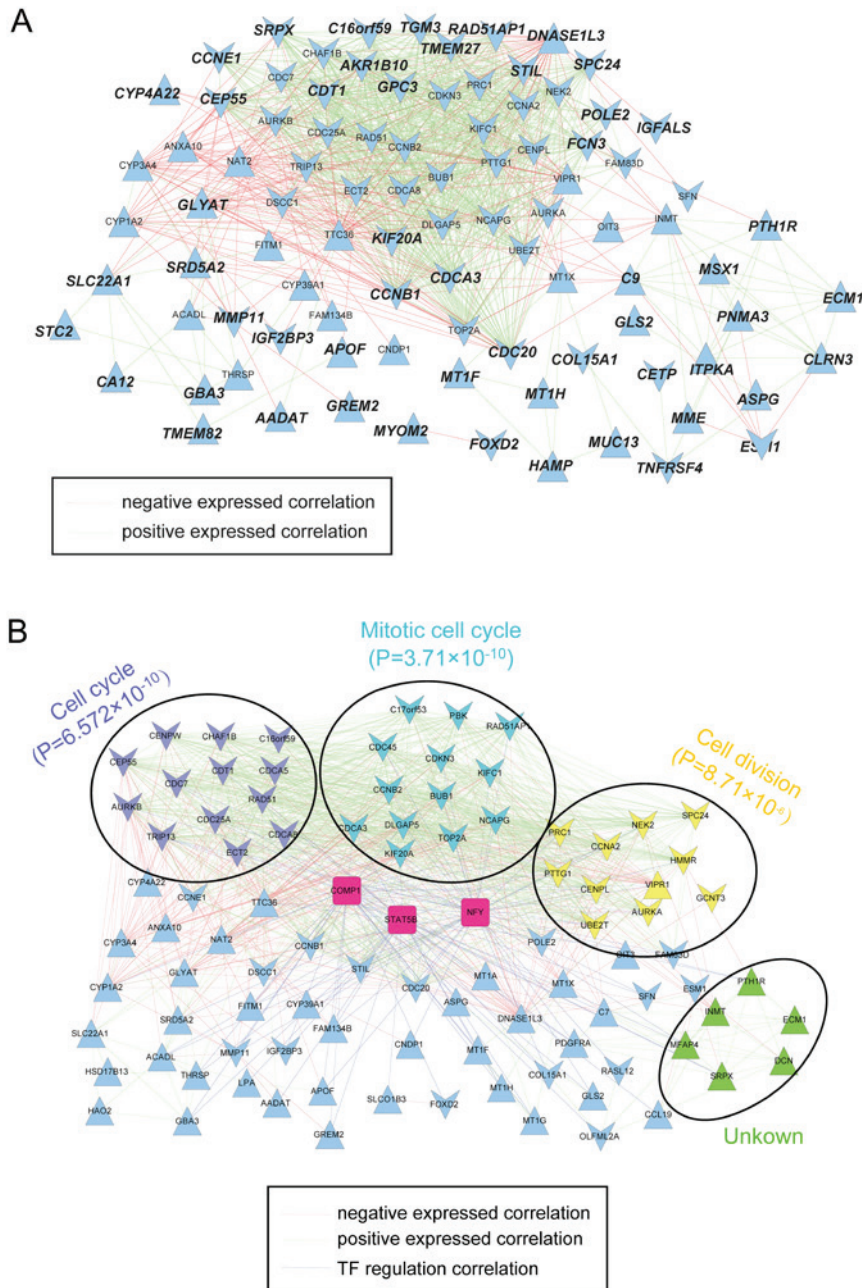


Figure 5. Co-expression network. (A) For prognostic genes; (B) transcription factor-regulated. Red lines indicate negative co-expression pairs, green lines indicate positive co-expression pairs and purple lines indicate transcriptional regulations. Triangles represent upregulated genes, while inverted triangles represent downregulated genes, and pink squares represent transcriptional factors. Functional modules are presented in different colors.

enrichment analysis revealed that most of the prognostic genes in the co-expression network were significantly involved in the cell cycle ( $P=4.51 \times 10^{-9}$ ; Table II), including CCNE1, CDC45, CCNB2, BUB1, CDC20, PTTG1, SFN, CCNA2 and CDC25A. The four prognostic genes CCNE1, CCNE2, CCNB2 and SFN were significantly associated with the p53 signaling pathway ( $P=9.25 \times 10^{-3}$ ).

A total of 4 functional modules were revealed by GraphWeb (Fig. 5B). The prognostic genes in the purple module [chromatin licensing and DNA replication factor 1 (CDT1), CHAF1B, RAD51, CDCA5, CDCA8, CDC7, TRIP13, CDC25A, AURKB and CEP55] were mainly associated with the cell cycle ( $P=6.57 \times 10^{-10}$ ). The prognostic genes in the blue module (CDC20, CCNB2, CDCA3, KIFC1, PBK, NCAPG,

BUB1, DLGAP5 and CDKN3) were significantly associated with the mitotic cell cycle ( $P=3.71 \times 10^{-10}$ ). Furthermore, the prognostic genes in the yellow module (CCNA2, PTTG1, PRC1, NEK2, SPC24 and AURKA) were significantly involved in cell division ( $P=8.71 \times 10^{-6}$ ).

**Prognostic prediction system.** The prognostic prediction system was established based on Bayes discriminant analysis (Fig. 7A). Finally, a prognostic prediction system consisting of 55 signature genes and represented by 29 upregulated genes (including SPC24, TGM3, KIF20A, ESM1, CDC20, CDCA3, CCNE1, TNFRSF4, COL15A1, and CELSR3) and 26 downregulated genes (including CYP4A22, TMEM82, GLYAT, GBA3, APOF, SLC22A1, DNASE1L3, ECM1, CETP,

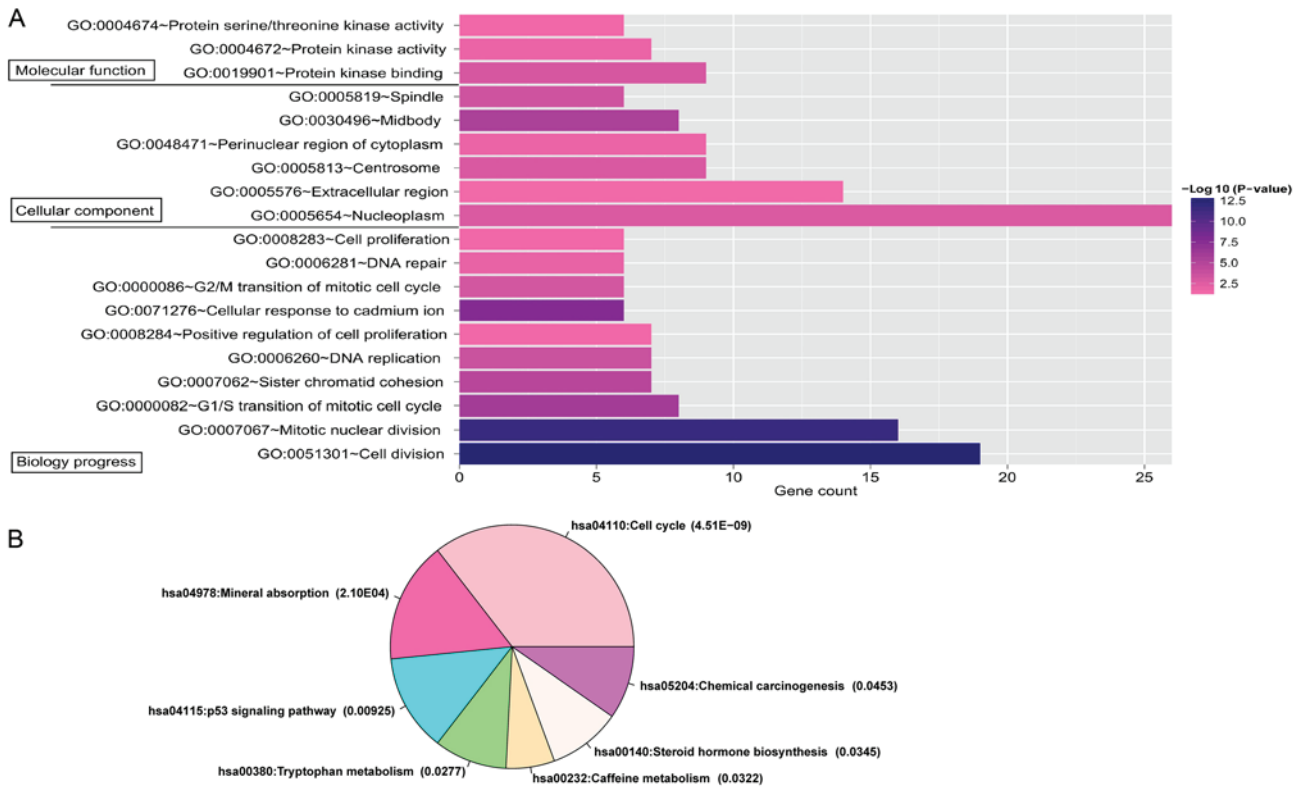


Figure 6. Significantly enriched (A) GO terms and (B) Kyoto Encyclopedia of Genes and Genomes pathways for the 93 prognostic genes from the gene co-expression network. GO, gene ontology; Hsa, *Homo sapiens*.

and GLS2; Table III) was obtained. This system exhibited the highest discriminative ability to predict the survival of 330 samples in the TCGA dataset. The prognostic scoring system was established following the Bayes discriminant analysis method as previously described (33).

The samples were stratified into favorable and poor prognostic groups based on the score. If the prognostic score was between 0 and 3, the patient was defined as having a favorable prognosis, whereas a prognostic score between -3 and 0 was indicative of a poor prognosis. The distribution of the prognostic scores indicated that the prognostic scores of samples in the favorable and poor prognostic groups were obviously different (Fig. 7B). The survival ratio of patients in the favorable prognostic group was significantly higher than that of patients in the poor prognostic group, according to the K-M survival curves (Fig. 7C,  $P=8.16 \times 10^{-8}$ ).

**Validation of the prognostic prediction system.** The predictive value of the identified prognostic scoring system was further validated using another independent GEO dataset, GSE20140. The samples in GSE20140 were divided into favorable and poor prognostic groups based on the prognostic score. The K-M survival curve indicated that patients in the favorable prognostic group survived significantly longer than those in the poor prognostic group (Fig. 7D;  $P=1.73 \times 10^{-3}$ ).

## Discussion

In the present study, 177 overlapping DEGs were identified from the gene expression data from the TCGA and GEO

dataset (GSE36376). Of these, 161 genes were identified as being prognostic based on the analysis of survival. Of note, according to the K-M survival curves, the top 6 prognostic genes (STC2, CA12, CDC20, DNASE1L3, GBA3 and MT1G) were able to predict the prognosis of the HCC patients from the TCGA dataset. It was revealed that patients with high expression levels of STC2, CA12, CDC20, DNASE1L3, GBA3 and MT1G have a significantly shorter survival time. The STC2 protein, encoded by the STC2 gene, is an extracellular matrix protein involved in a number of physiological processes, including bone development, wound healing, angiogenesis and modulation of the inflammatory response (34). Previous studies have reported that HCC patients with expression of STC2 had a poorer prognosis according to the K-M survival curves, suggesting that STC2 expression may be a useful indicator of poor prognosis in HCC patients (35,36). CDC20 is a regulatory protein interacting with the anaphase-promoting complex cyclosome in the cell cycle and has important roles in the progression of multiple tumors (37,38). It has also been demonstrated that increased expression of CDC20 is associated with the development and progression of HCC (39). The expression of MT1G was reported to be negatively associated with aberrant promoter hypermethylation, and the data from TCGA suggested that hypermethylation of MT1G is linked with favorable survival of HCC patients (40). Therefore, the prognostic predictive value of STC2, CDC20 and MT1G in the present study was consistent with the results of previous studies. CA12 is a transmembrane enzyme that hydrates extracellular  $\text{CO}_2$ , leading to the generation of membrane-impermeable  $\text{H}^+$  and  $\text{HCO}_3^-$  (41). It has been



Table I. GO functional terms enriched by the prognostic genes in the co-expression network.

Category/term	Count	P-value	Genes
Biological process			
GO:0051301~cell division	19	2.29x10 <sup>-13</sup>	CDC7, KIFC1, NEK2, AURKA, CDC20, PTTG1, CDC25A, SPC24, FAM83D, CCNB1, CCNE1, CDCA8, CCNB2, NCAPG, BUB1, CENPW, CDCA5, CCNA2, CDCA3
GO:0007067~mitotic nuclear division	16	2.760x10 <sup>-12</sup>	NEK2, CDC20, AURKA, PBK, AURKB, CEP55, PTTG1, CDC25A, SPC24, FAM83D, CCNB2, BUB1, CENPW, CDCA5, CCNA2, CDCA3
GO:0000082~G1/S transition of mitotic cell cycle	8	1.05x10 <sup>-6</sup>	CDC7, CCNE1, CDC45, POLE2, CDKN3, CDCA5, CDC25A, CDT1
GO:0007062~sister chromatid cohesion	7	1.66x10 <sup>-5</sup>	SPC24, CDCA8, CENPL, BUB1, CDC20, AURKB, CDCA5
GO:0006260~DNA replication	7	1.63x10 <sup>-4</sup>	CDC7, CDC45, POLE2, CHAF1B, CDC25A, DSCC1, CDT1
GO:0008284~positive regulation of cell proliferation	7	3.56x10 <sup>-2</sup>	CDC7, PRC1, PTH1R, PDGFRA, CDC20, ESM1, VIPR1
GO:0071276~cellular response to cadmium ion	6	2.07x10 <sup>-8</sup>	MT1A, CYP1A2, MT1H, MT1X, MT1G, MT1F
GO:0000086~G2/M transition of mitotic cell cycle	6	7.65x10 <sup>-4</sup>	CCNB1, CCNB2, NEK2, AURKA, CDC25A, HMMR
GO:0006281~DNA repair	6	7.87x10 <sup>-3</sup>	RAD51AP1, POLE2, PTTG1, CHAF1B, UBE2T, RAD51
GO:0008283~cell proliferation	6	4.32x10 <sup>-2</sup>	FAM83D, STIL, DLGAP5, BUB1, AURKB, CDC25A
Cellular component			
GO:0005654~nucleoplasm	26	1.40x10 <sup>-3</sup>	PRC1, AURKA, AURKB, CDT1, CCNE1, CDC45, CDCA8, POLE2, BUB1, THRSP, CCNA2, CDCA5, TOP2A, CDC7, RAD51AP1, CENPL, CDC20, CDC25A, RAD51, CCNB1, CCNB2, CENPW, CHAF1B, UBE2T, DSCC1, KIF20A
GO:0005576~extracellular region	14	4.86x10 <sup>-2</sup>	C7, CNDP1, HSD17B13, COL15A1, CCL19, DCN, ESM1, ECM1, GREM2, DNASE1L3, MMP11, LPA, APOF, MFAP4
GO:0005813~centrosome	9	1.13x10 <sup>-3</sup>	CCNB1, STIL, CDC45, CCNB2, NCAPG, NEK2, AURKA, CDC20, CEP55
GO:0048471~perinuclear region of cytoplasm	9	1.09x10 <sup>-2</sup>	MT1A, AURKA, CDC20, CDKN3, MT1H, MT1X, MT1G, RAD51, MT1F
GO:0030496~midbody	8	3.22x10 <sup>-6</sup>	CDCA8, PRC1, NEK2, AURKA, CEP55, AURKB, ECT2, KIF20A
GO:0005819~spindle	6	3.16x10 <sup>-4</sup>	KIFC1, PRC1, AURKA, CDC20, AURKB, KIF20A
Molecular function			
GO:0019901~protein kinase binding	9	7.10x10 <sup>-4</sup>	CCNB1, FAM83D, CCNE1, PRC1, AURKA, SFN, CCNA2, CDC25A, KIF20A
GO:0004672~protein kinase activity	7	1.06x10 <sup>-2</sup>	CDC7, NEK2, PDGFRA, BUB1, AURKA, PBK, AURKB
GO:0004674~protein serine/threonine kinase activity	6	4.48x10 <sup>-2</sup>	CDC7, NEK2, BUB1, AURKA, PBK, AURKB

GO, Gene Ontology; APOF, apolipoprotein F; AURKA, aurora kinase A; AURKB, aurora kinase B; BUB1, BUB1 mitotic checkpoint serine/threonine kinase; C7, complement C7; CCL19, C-C motif chemokine ligand 19; CCNA2, cyclin A2; CCNB1, cyclin B1; CCNB2, cyclin B2; CCNE1, cyclin E1; CDC7, cell division cycle 7; CDC20, cell division cycle 20; CDC25A, cell division cycle 25A; CDC45, cell division cycle 45; CDCA3, cell division cycle associated 3; CDCA5, cell division cycle associated 5; CDCA8, cell division cycle associated 8; CDKN3, cyclin dependent kinase inhibitor 3; CDT1, chromatin licensing and DNA replication factor 1; CENPL, centromere protein L; CENPW, centromere protein W; CEP55, centrosomal protein 55; CHAF1B, chromatin assembly factor 1 subunit B; CNDP1, carnosine dipeptidase 1; COL15A1, collagen type XV alpha 1 chain; CYP1A2, cytochrome P450 family 1 subfamily A member 2; DCN, decorin; DLGAP5, DLG associated protein 5; DNASE1L3, deoxyribonuclease 1 like 3; DSCC1, DNA replication and sister chromatid cohesion 1; ECM1, extracellular matrix protein 1; ECT2, epithelial cell transforming 2; ESM1, endothelial cell specific molecule 1; FAM83D, family with sequence similarity 83 member D; GREM2, gremlin 2, DAN family BMP antagonist; HMMR, hyaluronan mediated motility receptor; HSD17B13, hydroxysteroid 17-beta dehydrogenase 13; KIF20A, kinesin family member 20A; KIFC1, kinesin family member C1; LPA, lipoprotein(a); MFAP4, microfibril associated protein 4; MMP11, matrix metalloproteinase 11; MT1A, metallothionein 1A; MT1F, metallothionein 1F; MT1G, metallothionein 1G; MT1H, metallothionein 1H; MT1X, metallothionein 1X; NCAPG, non-SMC condensin I complex subunit G; NEK2, NIMA related kinase 2; PBK, PDZ binding kinase; PDGFRA, platelet derived growth factor receptor alpha; POLE2, DNA polymerase epsilon 2, accessory subunit; PRC1, protein regulator of cytokinesis 1; PTH1R, parathyroid hormone 1 receptor; PTTG1, pituitary tumor-transforming 1; RAD51, RAD51 recombinase; RAD51AP1, RAD51 associated protein 1; SFN, stratifin; SPC24, kinetochore-associated Ndc80 complex subunit SPC24; STIL, STIL centriolar assembly protein; THRSP, thyroid hormone responsive; TOP2A, DNA topoisomerase II alpha; UBE2T, ubiquitin conjugating enzyme E2 T; VIPR1, vasoactive intestinal peptide receptor 1.

Table II. Significantly enriched pathways for the prognostic genes in the co-expression network.

Term	Count	P-value	Genes
hsa04110:Cell cycle	11	$4.51 \times 10^{-9}$	CCNB1, CDC7, CCNE1, CDC45, CCNB2, BUB1, CDC20, PTTG1, SFN, CCNA2, CDC25A
hsa04978:Mineral absorption	5	$2.10 \times 10^{-4}$	MT1A, MT1H, MT1X, MT1G, MT1F
hsa04115:p53 signaling pathway	4	$9.25 \times 10^{-3}$	CCNB1, CCNE1, CCNB2, SFN
hsa00380:Tryptophan metabolism	3	$2.77 \times 10^{-2}$	AADAT, CYP1A2, INMT
hsa00232:Caffeine metabolism	2	$3.22 \times 10^{-2}$	NAT2, CYP1A2
hsa00140:Steroid hormone biosynthesis	3	$3.45 \times 10^{-2}$	CYP3A4, SRD5A2, CYP1A2
hsa05204:Chemical carcinogenesis	3	$4.53 \times 10^{-2}$	CYP3A4, NAT2, CYP1A2

Hsa, *Homo sapiens*; AADAT, aminoadipate aminotransferase; BUB1, BUB1 mitotic checkpoint serine/threonine kinase; CCNA2, cyclin A2; CCNB1, cyclin B1; CCNB2, cyclin B2; CCNE1, cyclin E1; CDC20, cell division cycle 20; CDC25A, cell division cycle 25A; CDC45, cell division cycle 45; CDC7, cell division cycle 7; CYP1A2, cytochrome P450 family 1 subfamily A member 2; CYP3A4, cytochrome P450 family 3 subfamily A member 4; INMT, indolethylamine N-methyltransferase; MT1A, metallothionein 1A; MT1F, metallothionein 1F; MT1G, metallothionein 1G; MT1H, metallothionein 1H; MT1X, metallothionein 1X; NAT2, N-acetyltransferase 2; PTTG1, pituitary tumor-transforming 1; SFN, stratifin; SRD5A2, steroid 5 alpha-reductase 2.

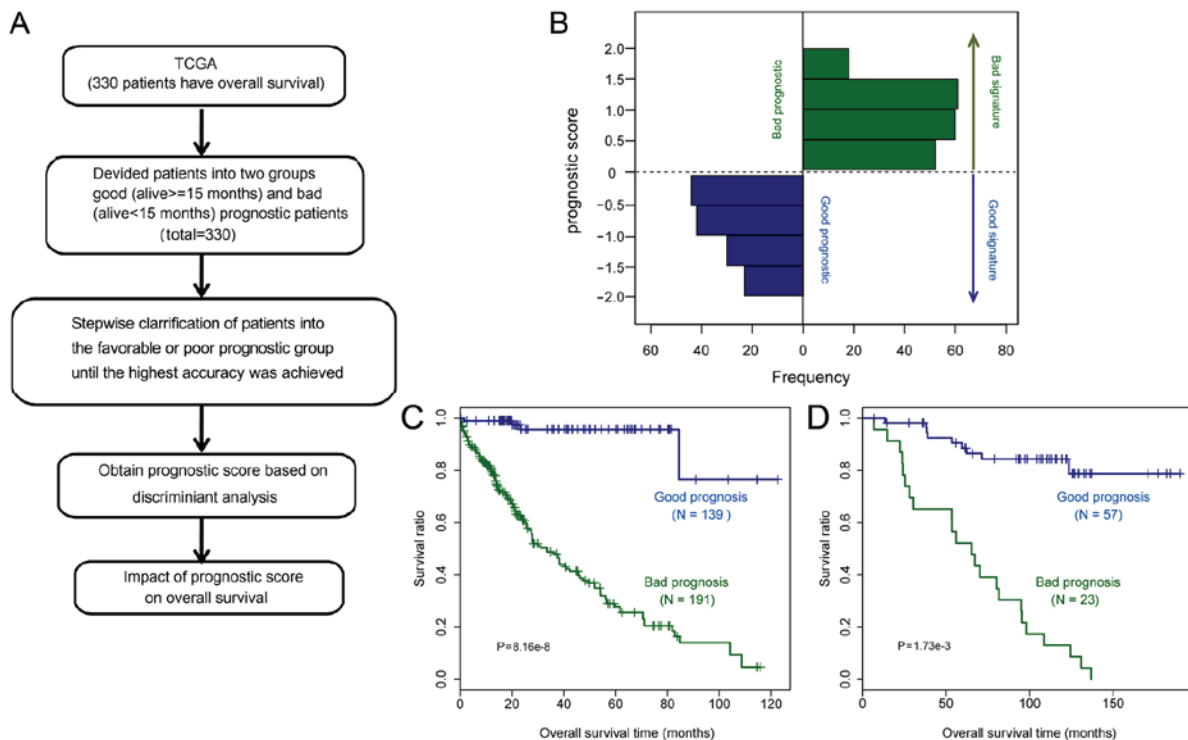


Figure 7. (A) Workflow for the established prognostic prediction system. (B) Distribution of the prognostic score. (C) Kaplan-Meier survival curves for cases from the TCGA dataset. The blue line represents the favorable prognostic group and the green line represents the poor prognostic group. (D) Kaplan-Meier survival curves for cases from the GSE20140 dataset. The blue line represents the favorable prognostic group and the green line represents the poor prognostic group, as identified by the prognostic prediction system. TCGA, The Cancer Genome Atlas.

revealed that inhibition of CA12 with sulphonamide- or coumarin-based small-molecule inhibitors reversed the effects of tumor acidification, thereby inhibiting primary or metastatic tumor cell growth (42). CA12 may affect the capability of invasion and migration of breast cancer cells through the p38/mitogen-activated protein kinase pathway (43). DNase1l3 is an endonuclease encoded by DNASE1L3, and is necessary for cytokine secretion following inflammasome activity (44). GBA3 is a cytosolic  $\beta$ -glycosidase produced by mammals and has broad substrate specificity (45). However, few studies have reported on the roles of CA12, DNASE1L3

and GBA3 in the prognosis of HCC patients. Therefore, the associations of CA12, DNASE1L3 and GBA3 with HCC and their prognostic value require to be further studied.

Next, significantly co-expressed pairs were selected for the prognostic genes and a gene co-expression network involving 93 prognostic genes was constructed. From these, a prognostic prediction system consisting of 55 signature genes was established based on Bayes discriminant analysis. These 55 signature genes comprised 29 upregulated genes (including SPC24, TGM3, KIF20A, ESM1, CDC20, CDCA3, CCNE1, TNFRSF4, COL15A1, CELSR3 and CDT1) and



Table III. The 55 signature genes involved in the prognostic prediction system.

A, Upregulated genes

Gene	logFC	P-value	FDR
SPC24	1.777	8.57x10 <sup>-33</sup>	2.66x10 <sup>-30</sup>
TGM3	1.396	4.38x10 <sup>-35</sup>	1.96x10 <sup>-32</sup>
KIF20A	1.173	2.42x10 <sup>-30</sup>	5.74x10 <sup>-28</sup>
ESM1	1.162	6.50x10 <sup>-30</sup>	1.40x10 <sup>-27</sup>
CDC20	1.106	1.51x10 <sup>-30</sup>	3.88x10 <sup>-28</sup>
CDCA3	1.018	3.84x10 <sup>-23</sup>	4.22x10 <sup>-21</sup>
CCNE1	0.964	4.97x10 <sup>-19</sup>	3.47x10 <sup>-17</sup>
TNFRSF4	0.949	8.28x10 <sup>-19</sup>	5.59x10 <sup>-17</sup>
COL15A1	0.942	2.28x10 <sup>-25</sup>	3.00x10 <sup>-23</sup>
CELSR3	0.921	1.59x10 <sup>-19</sup>	1.16x10 <sup>-17</sup>
CEP55	0.920	1.99x10 <sup>-17</sup>	1.19x10 <sup>-15</sup>
CDT1	0.860	4.43x10 <sup>-20</sup>	3.41x10 <sup>-18</sup>
C16orf59	0.846	1.13x10 <sup>-16</sup>	6.18x10 <sup>-15</sup>
MUC13	0.800	1.07x10 <sup>-19</sup>	7.93x10 <sup>-18</sup>
GPC3	0.778	9.91x10 <sup>-26</sup>	1.36x10 <sup>-23</sup>
MMP11	0.723	2.34x10 <sup>-16</sup>	1.24x10 <sup>-14</sup>
IGF2BP3	0.702	2.73x10 <sup>-12</sup>	1.04x10 <sup>-10</sup>
STC2	0.687	1.29x10 <sup>-13</sup>	5.67x10 <sup>-12</sup>
RAD51AP1	0.672	3.06x10 <sup>-11</sup>	1.05x10 <sup>-9</sup>
CCNB1	0.653	1.50x10 <sup>-14</sup>	7.08x10 <sup>-13</sup>
STIL	0.622	1.95x10 <sup>-10</sup>	6.11x10 <sup>-9</sup>
CA12	0.615	9.69x10 <sup>-11</sup>	3.15x10 <sup>-9</sup>
PNMA3	0.550	8.68x10 <sup>-7</sup>	1.55x10 <sup>-5</sup>
POLE2	0.540	4.99x10 <sup>-8</sup>	1.07x10 <sup>-6</sup>
FOXD2	0.534	3.59x10 <sup>-7</sup>	6.83x10 <sup>-6</sup>
MSX1	0.531	4.53x10 <sup>-7</sup>	8.45x10 <sup>-6</sup>
ITPKA	0.525	1.77x10 <sup>-8</sup>	4.16x10 <sup>-7</sup>
ACTG2	0.525	3.01x10 <sup>-8</sup>	6.78x10 <sup>-7</sup>
AKR1B10	0.514	7.73x10 <sup>-13</sup>	3.14x10 <sup>-11</sup>

B, Downregulated genes

Gene	logFC	P-value	FDR
CYP4A22	-0.492	2.25x10 <sup>-12</sup>	8.69x10 <sup>-11</sup>
TMEM82	-0.519	5.69x10 <sup>-13</sup>	2.36x10 <sup>-11</sup>
GLYAT	-0.536	1.39x10 <sup>-16</sup>	7.57x10 <sup>-15</sup>
GBA3	-0.546	1.40x10 <sup>-15</sup>	7.17x10 <sup>-14</sup>
APOF	-0.555	1.52x10 <sup>-18</sup>	1.00x10 <sup>-16</sup>
SLC22A1	-0.565	1.50x10 <sup>-20</sup>	1.23x10 <sup>-18</sup>
DNASE1L3	-0.580	4.07x10 <sup>-17</sup>	2.31x10 <sup>-15</sup>
ECM1	-0.591	7.68x10 <sup>-17</sup>	4.26x10 <sup>-15</sup>
CETP	-0.602	1.68x10 <sup>-15</sup>	8.57x10 <sup>-14</sup>
GLS2	-0.603	6.99x10 <sup>-20</sup>	5.35x10 <sup>-18</sup>
GREM2	-0.614	1.99x10 <sup>-16</sup>	1.06x10 <sup>-14</sup>
AADAT	-0.615	2.46x10 <sup>-17</sup>	1.42x10 <sup>-15</sup>
MME	-0.616	6.41x10 <sup>-17</sup>	3.57x10 <sup>-15</sup>
SRD5A2	-0.650	5.30x10 <sup>-19</sup>	3.64x10 <sup>-17</sup>
C9	-0.655	7.70x10 <sup>-28</sup>	1.33x10 <sup>-25</sup>
CLRN3	-0.701	2.09x10 <sup>-20</sup>	1.68x10 <sup>-18</sup>
IGFALS	-0.716	7.36x10 <sup>-26</sup>	1.03x10 <sup>-23</sup>
TMEM27	-0.730	5.13x10 <sup>-21</sup>	4.37x10 <sup>-19</sup>
ASPG	-0.735	1.68x10 <sup>-25</sup>	2.26x10 <sup>-23</sup>
SRPX	-0.763	7.97x10 <sup>-21</sup>	6.69x10 <sup>-19</sup>

Table III. Continued.

B, Downregulated genes

Gene	logFC	P-value	FDR
MYOM2	-0.784	3.33x10 <sup>-18</sup>	2.13x10 <sup>-16</sup>
MT1F	-0.846	1.21x10 <sup>-34</sup>	5.04x10 <sup>-32</sup>
PTH1R	-0.866	3.18x10 <sup>-28</sup>	5.82x10 <sup>-26</sup>
FCN3	-0.978	1.24x10 <sup>-42</sup>	9.37x10 <sup>-40</sup>
HAMP	-1.052	2.53x10 <sup>-55</sup>	2.78x10 <sup>-52</sup>
MT1H	-1.425	5.74x10 <sup>-76</sup>	6.94x10 <sup>-72</sup>

logFC, log<sub>2</sub>(fold change); FDR, false discovery rate; SPC24, SPC24 component of NDC80 kinetochore complex; TGM3, transglutaminase 3; KIF20A, kinesin family member 20A; ESM1, endothelial cell specific molecule 1; CDC20, cell division cycle 20; CDCA3, cell division cycle associated 3; CCNE1, cyclin E1; TNFRSF4, tumor necrosis factor receptor superfamily, member 4; COL15A1, collagen type XV alpha 1 chain; CELSR3, cadherin EGF LAG seven-pass G-type receptor 3; CEP55, centrosomal protein 55; CDT1, chromatin licensing and DNA replication factor 1; C16orf59/ TEDC2, tubulin epsilon and delta complex 2; MUC13, mucin 13, cell surface associated; GPC3, glypican 3; MMP11, matrix metalloproteinase 11; IGF2BP3, insulin like growth factor 2 mRNA binding protein 3; STC2, stanniocalcin 2; RAD51AP1, RAD51 associated protein 1; CCNB1, cyclin B1; STIL, STIL centriolar assembly protein; CA12, carbonic anhydrase 12; PNMA3, PNMA family member 3; POLE2, DNA polymerase epsilon 2, accessory subunit; FOXD2, forkhead box D2; MSX1, msh homeobox 1; ITPKA, inositol-trisphosphate 3-kinase A; ACTG2, actin gamma 2, smooth muscle; AKR1B10, aldo-keto reductase family 1 member B10; CYP4A22, cytochrome P450 family 4 subfamily A member 22; TMEM82, transmembrane protein 82; GLYAT, glycine-N-acyltransferase; GBA3, glucosylceramidase beta 3; APOF, apolipoprotein F; SLC22A1, solute carrier family 22 member 1; DNASE1L3, deoxyribonuclease 1 like 3; ECM1, extracellular matrix protein 1; CETP, cholesteryl ester transfer protein; GLS2, glutaminase 2; GREM2, gremlin 2, DAN family BMP antagonist; AADAT, aminoadipate aminotransferase; MME, membrane metalloendopeptidase; SRD5A2, steroid 5 alpha-reductase 2; C9, complement C9; CLRN3, clarin 3; IGFALS, insulin like growth factor binding protein acid labile subunit; TMEM27/CLTRN, collectrin, amino acid transport regulator; ASPG, asparaginase; SRPX, sushi repeat containing protein X-linked; MYOM2, myomesin 2; MT1F, metallothionein 1F; PTH1R, parathyroid hormone 1 receptor; FCN3, ficolin 3; HAMP, hepcidin antimicrobial peptide; MT1H, metallothionein 1H.

26 downregulated genes (including CYP4A22, TMEM82, GLYAT, GBA3, APOF, SLC22A1, DNASE1L3, ECM1, CETP and GLS2). The functional and module analysis of the co-expression network indicated that SPC24, ESM1, CDC20, CDCA3, CCNE1 and CDT1 were significantly associated with cell division, mitotic cell cycle and positive regulation of cell proliferation. SPC24 is an important component of the nuclear division cycle 80 kinetochore complexes. It has been reported that SPC24 is significantly upregulated in HCC and may be a prognostic biomarker for patients with HCC (46). ESM1 is a secreted protein that is mainly expressed in endothelial cells. The expression of ESM1 in HCC tissues is positively correlated with venous invasion (47). Upregulation of CDC20 is associated with the progression of HCC and may be a promising therapeutic target (39). Suppression of oncogene CCNE1, an important mediator in the G1/S-phase transition, exerts tumor-suppressive effects in HCC (48). CDT1 is involved in the formation of the pre-replication complex that is necessary for DNA replication. It has been reported that the expression of CDT1 in HCC tissue is clearly attenuated as compared with

that in normal hepatic tissue (49). Taking all this into consideration, it may be speculated that SPC24, ESM1, CDC20, CDCA3, CCNE1 and CDT1 may have important roles in HCC by regulating functions associated with the cell cycle and cell proliferation.

Kinesin family member 20A (KIF20A) is significantly associated with protein kinase binding; it is a downstream target of glioma-associated oncogene 2, which is important for HCC proliferation and tumor growth. KIF20A has been reported to be upregulated in HCC and to be a predictor of poor prognosis (50). Therefore, KIF20A may promote HCC progression by protein kinase binding. A total of 4 prognostic genes, i.e., CCNB1, CCNE1, CCNB2 and SFN, were associated with p53 signaling. p53 is mostly known for its tumor suppressor properties and is also a major regulator of cell metabolism (51). Of note, the p53 signaling pathway was reported to be significantly dysregulated in HCC (52). Thus, CCNB1, CCNE1, CCNB2 and SFN may participate in the molecular mechanisms of HCC by regulating the p53 signaling pathway.

The predictive value of the prognostic prediction system was also validated in another independent GEO dataset, GSE20140. The K-M survival analysis suggested that the survival ratio of patients in the favorable prognostic group, based on the prognostic prediction score, was significantly larger than that of the patients in the poor prognostic group. Therefore, this prognostic prediction system may be applied to predict the prognosis of HCC. As a limitation of the present study, the expression levels of the important signature genes in the clinical samples were not detected by any experimental methods.

In conclusion, a predictive gene signature for HCC prognosis was identified via a multi-step strategy. The significant functions and pathways enriched by the genes in the co-expression network of the prognostic genes were also determined. A novel prognostic prediction system, consisting of 55 signature genes, that was able to predict the prognosis of HCC patients was established. In future studies, the expression levels of the important signature genes will be validated in clinical samples by experimental methods.

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## Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

## Authors' contributions

HL conceived and designed the study. LG performed data analyses and prepared the manuscript. QL and NL contributed significantly to the data analyses and manuscript revision. All authors read and approved the final manuscript.

## Ethics approval and patient consent

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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