

Received: 2019.04.23

Accepted: 2019.07.30

Published: 2019.11.15

Bioinformatics Analysis Identifies Protein Tyrosine Kinase 7 (PTK7) as a Potential Prognostic and Therapeutic Biomarker in Stages I to IV Hepatocellular Carcinoma

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Statistical Analysis C
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Source of support:

This study was supported by the National Natural Science Foundation of China (No. 81460649 and No. 81360662), the Medical Leading Talent Project of Yunnan Province (No. L-201606), the Joint Project of Science and Technology, Department of Yunnan and Traditional Chinese Medicine (No. 2017FF116-047), and the Doctor Newcomer Award of Yunnan Province, 2017

Background: Worldwide, hepatocellular carcinoma (HCC) accounts for 80–90% of all cases of primary liver cancer, and is one of the ten most common malignancies. This study used bioinformatics analysis to identify genes associated with patient outcome in stages I–IV HCC and the gene pathways that distinguished between normal liver and liver cells and HCC and human HCC cell lines.


Material/Methods: Target genes were defined as those that had marketed drugs or drugs under development targeting a specific gene and acquired from the Clarivate Analytics Integrity Database. Differential expression gene analysis, co-expression network analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, survival analysis and receiver operating characteristic (ROC) curve analysis were used to explore the similarities and differences in gene expression profiles, functional associations, and survival in stage I–IV HCC. Normal liver cells (HL-7702) and HCC cell lines (HepaRG, HepG2, SK-Hep1, and Huh7) were studied using Western blot and quantitative reverse transcription PCR (RT-qPCR).

Results: Hierarchical gene clustering identified target genes that distinguished between HCC and normal liver tissue. For stages I–IV HCC, there were seven commonly upregulated target genes EPHB1, LTK, NTRK2, PTK7, TBK1, TIE1, and TLR3, which were mainly involved in immune and signaling transduction pathways. PTK7 was highly expressed in stage I–IV HCC and was an independent prognostic marker for reduced overall survival (OS).

Conclusions: Bioinformatics analysis, combined with patient survival analysis, identified PTK7 gene expression as a potential therapeutic target and prognostic biomarker for all stages of HCC.

MeSH Keywords: **Gene Expression • Gene Targeting • Hepatocellular Carcinoma**

Full-text PDF: <https://www.medscimonit.com/abstract/index/idArt/917142>

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Background

Worldwide, hepatocellular carcinoma (HCC) accounts for 80–90% of all cases of primary liver cancer and is one of the ten most common malignancies. Chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection are major risk factors for HCC [1]. HCC is more common in China, where it has been a leading cause of cancer death. From 2015, with population growth and an increasingly aging population, the incidence of HCC in China has been increasing [2]. In the US, despite advances in treatments for cancer, the 5-year survival rate for HCC is still only 16%, as HCC can be resistant to conventional chemotherapy and radiotherapy. [3].

Further studies on the pathogenesis and patient outcome for different stages of HCC may help to identify prognostics and therapeutic biomarkers and improve patient outcome. Previous studies have identified several key genes and pathways associated with HCC. The most frequently reported gene mutations involve TP53, CTNNB1, AXIN1, ARID1A, CDKN2A, and NFE2L2, which involve pathways involved in oxidative stress in DNA damage, which may lead to further gene mutations [4]. A recent study identified the MYC-aurora kinase A (AURKA) protein complex as a potential target for the treatment of HCC [5]. Recently published studies by our research group identified PKM2 as an independent predictive marker for prognosis in HCC [6] and showed that PKM2 was an essential metabolic regulatory gene [7]. These findings support that multiple genes are involved in tumorigenesis of HCC.

Patients with HCC present at four main stages, stage I–IV, based on the primary tumor (T), regional lymph nodes (N) and distant metastases (M) [8]. A previous study that included 8,918 cancer patients showed that the staging system was highly consistent with the overall survival (OS) of patients [9]. Recent studies have identified several gene expression signatures at different stages of HCC [10]. Furthermore, different cancer stages also affect treatment response [11,12] and medical expenditure [13]. Because tumor stage is correlated with patient prognosis, this study aimed to use bioinformatics analysis to identify genes associated with patient outcome in stages I–IV HCC and the gene pathways that distinguished between normal liver and liver cells and HCC and human HCC cell lines.

Material and Methods

Data sources

RNA-seq expression and clinical data from patients with stage I to IV hepatocellular carcinoma (HCC) were downloaded from Genomic Data Commons (GDC) Data Portal (<https://portal.gdc.cancer.gov/>). The data contained 371 primary HCC tumor samples

and 50 adjacent normal liver tissue samples, with complete clinical data for 371 patients, including 171, 86, 85, and 5 patients with stage I, stage II, stage III, and stage IV HCC, respectively. We reserved the genes with the 90th percentile of the reads per kilobase of transcript per million (RPKM) mapped reads ≥ 0.1 . Because there were only 5 patients with stage IV HCC, we combined patients in stage III and stage IV (stage III/IV) for subsequent analysis. Target genes for HCC were defined as those that had marketed targeted drugs, including FDA-approved drugs currently available for the treatment of HCC, or drugs in development, including those undergoing preclinical and clinical trials for the treatment of HCC that targeted a specific gene. We retrieved 164 target genes for HCC from the Clarivate Analytics Integrity Database (<https://integrity.clarivate.com/integrity/>).

Bioinformatics analysis

All bioinformatics analysis was performed using R version 3.4.1 software (<https://www.r-project.org/>) and the Bioconductor library (<http://www.bioconductor.org/>). The detailed methods of differential expression gene analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, co-expression network analysis, and survival analysis were performed, as previously described [14]. Differentially expressed genes were calculated for each stage (I, II, III/IV). We used deregulated genes and HCC target genes to construct the co-expression networks. KEGG pathway enrichment analysis was performed using gene lists in each co-expression network. Commonly deregulated target genes in all stages (I, II, III/IV) were used for survival analysis. Each gene was divided into two groups according to the tertile expression value (low, \leq the first tertile; high, \geq the last tertile). Kaplan-Meier survival curves and the Cox proportional hazards model were used to explore the association between genes and patient prognosis. We used the pROC package in R [15] to display and analyze the receiver operating characteristic (ROC) curves of commonly deregulated target genes between HCC and normal adjacent tissue samples. A gene with an area under the curve (AUC) ≥ 0.9 was considered to have high predictive accuracy.

Cell lines

Human HCC cell lines included HepG2, SK-Hep1, and HepaRG cells, which were obtained from the Kunming Institute of Zoology, Chinese Academy of Sciences (Kunming, China), and Huh7 cells, which were obtained from Shanghai BioLeaf Biotech Co., Ltd. (Shanghai, China). Among these four human HCC cells, HepG2 and HepaRG were highly-differentiated cell lines (low-grade), and SK-Hep1 and Huh7 cells were poorly-differentiated cell lines (high-grade). The normal human liver cell line, HL-7702, was obtained from XiangBio Company (Shanghai, China). The cells were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS) (Gibco, ThermoFisher Scientific, Waltham, MA, USA).

Quantitative reverse transcription PCR (RT-qPCR)

Total RNA was extracted from the hepatocellular carcinoma cells by using TRIzol (Invitrogen, Carlsbad, CA, USA). The total RNA was prepared for cDNA synthesis using the PrimeScript RT reagent Kit with gDNA Eraser (Takara, Otsu, Japan). The cDNA was used for real-time PCR to detect the expression level of PTK7 mRNA, using qhomoPTK7 primers with the normalization to the beta-actin (ACTB) gene.

Western blot

The cells were lysed for 30 min on ice using a lysing agent (P0013) and phenylmethyl sulfonyl fluoride (PMSF) (Beyotime, Shanghai, China) and centrifuged at 13,000×g for 10 min at 4°C. The supernatants were transferred to a clean 1.5 mL Eppendorf tube and stored at -80°C. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Burlington, MA, USA). After blocking with dried skimmed milk powder, the blots were probed with a mouse antibody to alpha-tubulin (Proteintech, Manchester, UK) and a rabbit antibody to human protein tyrosine kinase 7 (PTK7) (Proteintech, Manchester, UK). Then, the membranes were incubated with goat anti-mouse or anti-rabbit IgG secondary antibodies, and the blots were examined using by chemiluminescent detection (Merck Millipore, Burlington, MA, USA).

Results

Expression of target genes for different stages (I, II, III/IV) of hepatocellular carcinoma (HCC)

Following bioinformatics gene filtering, an expression matrix of 12149 unique genes was obtained. The number of upregulated genes for each stage of HCC included 702 genes in stage I HCC, 1309 genes in stage II HCC, and 1,454 genes in stage III/IV HCC. The number of down-regulated genes for each stage of HCC included 275 genes in stage I HCC, 396 genes in stage II HCC, and 446 genes in stage III/IV HCC (Figure 1A). There were 924 commonly deregulated genes in all stages (Figure 1B).

We mapped 164 HCC target genes, retrieved from the Clarivate Analytics Integrity Database, to the dataset, and identified 91 unique target genes. There were 8, 13, and 17 deregulated target genes in stage I, stage II, and stage III/IV HCC (Figure 1C). There were 7 commonly upregulated target genes in all stages of HCC that included EPHB1, LTK, NTRK2, PTK7, TBK1, TIE1, and TLR3 (Figure 1D). The expression of the mapped 91 target genes is shown in Figure 1E. Hierarchical clustering indicated that these target genes could distinguish between tumors and adjacent normal tissue samples in all stages of HCC.

The co-expression networks of deregulated genes and correlated target genes

The co-expression networks of deregulated genes and target genes for HCC in each stage are shown in Figure 2. The number of interactions was 38, 109, and 115 for stage I, stage II, and stage III/IV HCC, respectively. There were large differences in the regulatory relationships between deregulated genes and target genes at different stages of HCC. The PTK7 target gene was significantly correlated with multiple deregulated genes in all stages of HCC. The number of genes that correlated with PTK7 were 18 genes for stage I HCC, 55 genes for stage II HCC, and 37 genes for stage III/IV HCC, respectively. Other target genes included EPHA8, PARP1, LTK, and HDAC4, which also correlated with multiple deregulated genes at different stages. However, the number of correlated genes of these target genes was smaller than for PTK7-correlated genes.

Enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and biological functions

We used the genes in the co-expression networks to perform KEGG pathway enrichment analysis for all stages of HCC (Figure 3). There were 8, 39, and 27 significantly enriched KEGG pathways in stage I, stage II, and stage III/IV HCC, respectively. Furthermore, six pathways were significantly enriched in all stages of HCC, including the hepatitis C, the influenza A, the Kaposi sarcoma-associated herpesvirus infection, the regulation of actin cytoskeleton, the signaling pathways regulating pluripotency of stem cells, and the Toll-like receptor signaling pathway. Most of these enriched pathways were immune function and signaling transduction pathways.

The prognostic role of PTK7 expression in HCC

The effect of commonly deregulated target genes on the prognosis of patients with HCC is shown in Figure 4A. Among these target genes, high expression levels of NTRK2, PTK7, and EPHB1 predicted poor overall survival (OS), but other genes were not correlated with patient survival. There was a trend for increased expression of PTK7 with increased stage of HCC, when compared with adjacent normal tissue (Figure 4B). The Kaplan-Meier survival curves showed that patients with high expression of PTK7 showed significantly worse survival when compared with patients with low PTK7 expression (Figure 4C).

The results of univariate Cox proportional hazards regression analysis showed that PTK7 expression, age, and a previous history of cancer were associated with reduced OS (Table 1). Therefore, we further adjusted for age and cancer history and confirmed that PTK7 is an independent risk factor for patient prognosis (Table 2). Receiver operating characteristic (ROC) curve analysis of all the most common deregulated

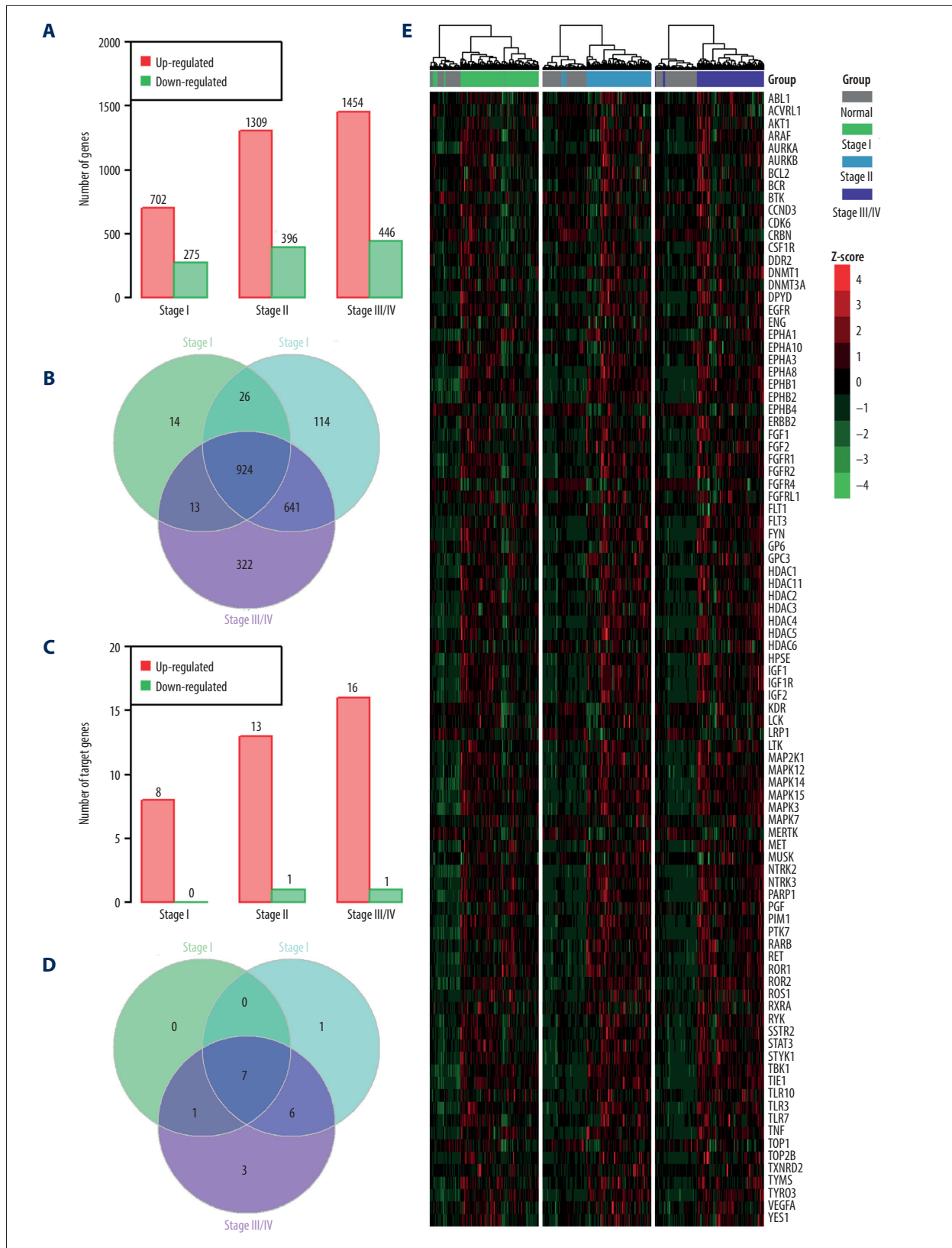


Figure 1. (A-E) Gene expression profiles for stage I to IV hepatocellular carcinoma (HCC). All gene expression values are converted to z-scores.

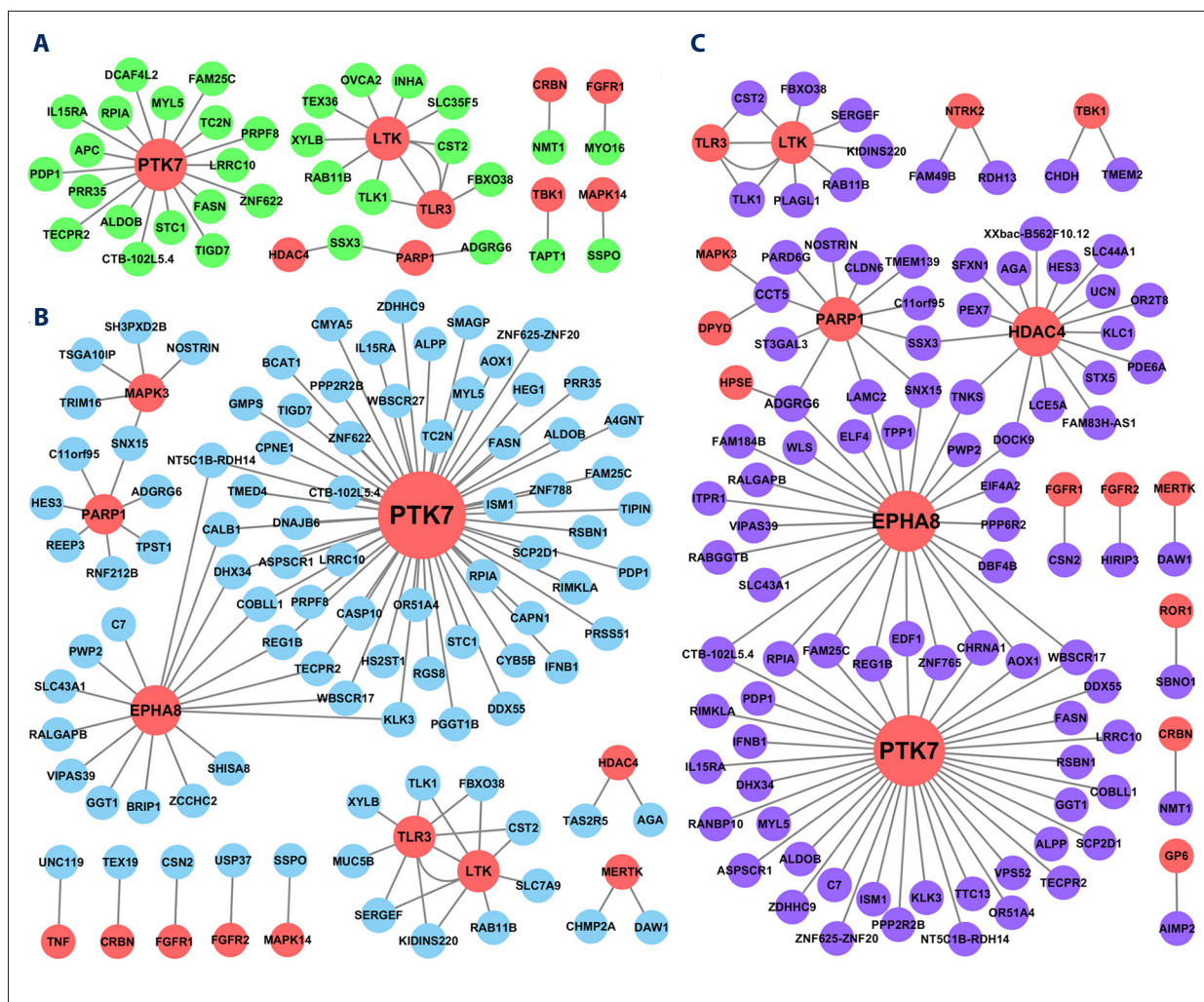


Figure 2. Co-expression networks of hepatocellular carcinoma (HCC) target genes and differentially expressed genes. The red circles represent cancer target genes and the green, blue, and purple circles represent differentially expressed genes in stage I, stage II, and stage III/IV HCC, respectively. The size of the circles represents the number of nodes. (A) The gene co-expression network in stage I HCC. (B) The gene co-expression network in stage II HCC. (C) The gene co-expression network in stage III and stage IV HCC.

target genes, showed that the area under the curve (AUC) of each gene was as follows: NTRK2 (0.977), TBK1 (0.976), PTK7 (0.972, Figure 4D), TIE1 (0.972), EPHB1 (0.966), TLR3 (0.924), and LTK (0.856). An AUC that approximated to 1 indicated a greater difference between the tumor and adjacent normal tissue samples. The findings from this study showed that PTK7 expression had a high AUC of 0.972, which indicated that the gene expression status distinguished HCC from normal tissue. Experimental validation by RT-qPCR (Figure 4E) and Western blot (Figure 4F) showed that PTK7 was highly expressed in HCC cell lines that included well-differentiated and poorly-differentiated cells, compared with normal liver cells. The above findings suggested that PTK7 should be studied further for its potential role as a therapeutic and prognostic biomarker in HCC.

Discussion

This study used bioinformatics analysis to identify genes associated with patient outcome in stages I-IV hepatocellular carcinoma (HCC) and the gene pathways that distinguished between normal liver and liver cells and HCC and human HCC cell lines. Differential expression analysis, co-expression network analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and survival analysis identified PTK7 as a potential therapeutic target and prognostic biomarker for HCC.

Protein tyrosine kinase 7 (PTK7), also known as colon carcinoma kinase 4 (CCK-4), is a highly conserved but catalytically inactive receptor tyrosine kinase and is upregulated in several types of cancer [16]. PTK7 suppresses canonical Wnt signaling

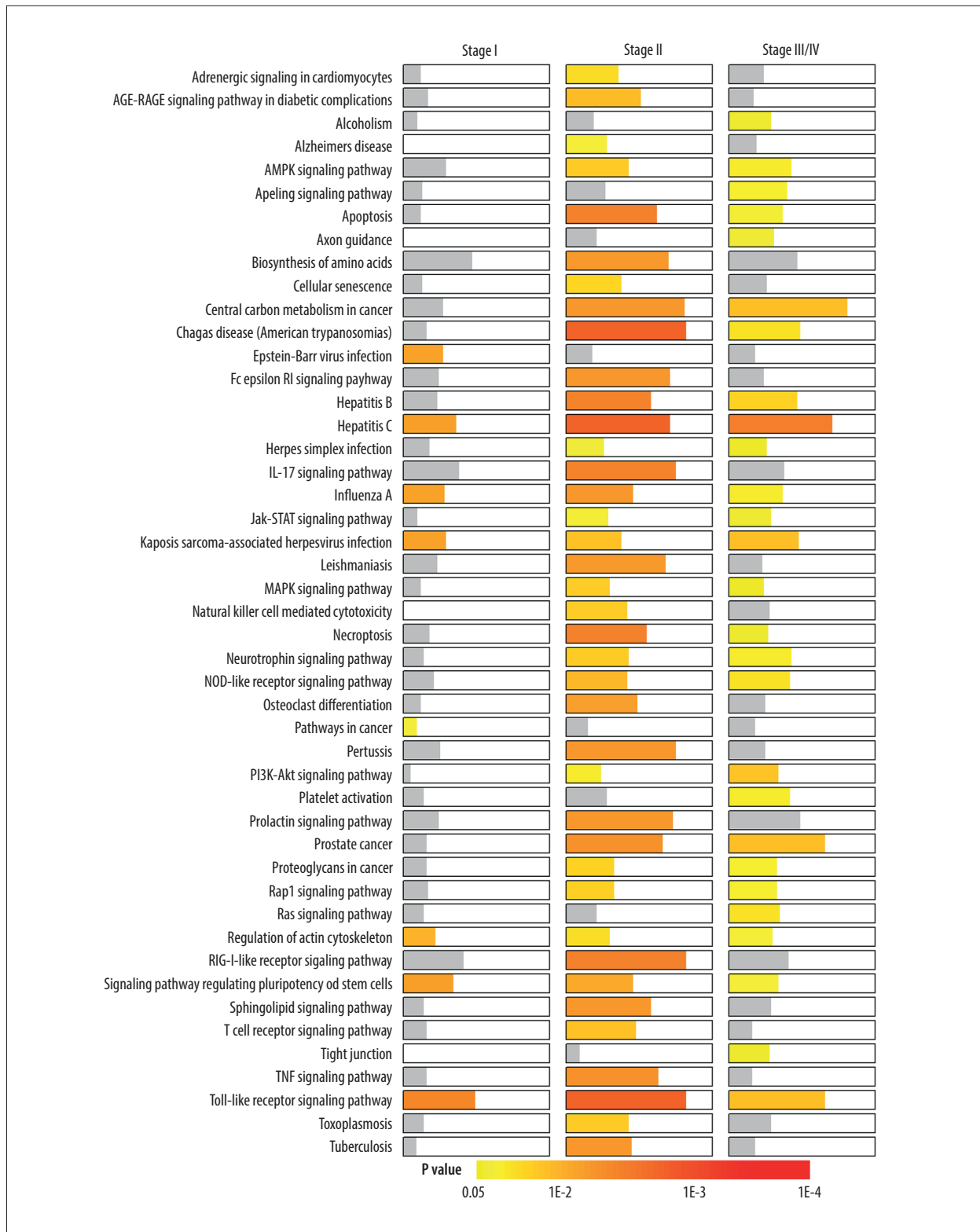


Figure 3. Enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in stage I to IV hepatocellular carcinoma (HCC). The length of the colored box indicates the percentage of enrichment. The enrichment percentage=the number of differentially expressed genes in the pathway/the total number of genes in the pathway. The length of the box shows an enrichment percentage from 10%. Different colors represent the P-values. Gray indicates no significant difference.

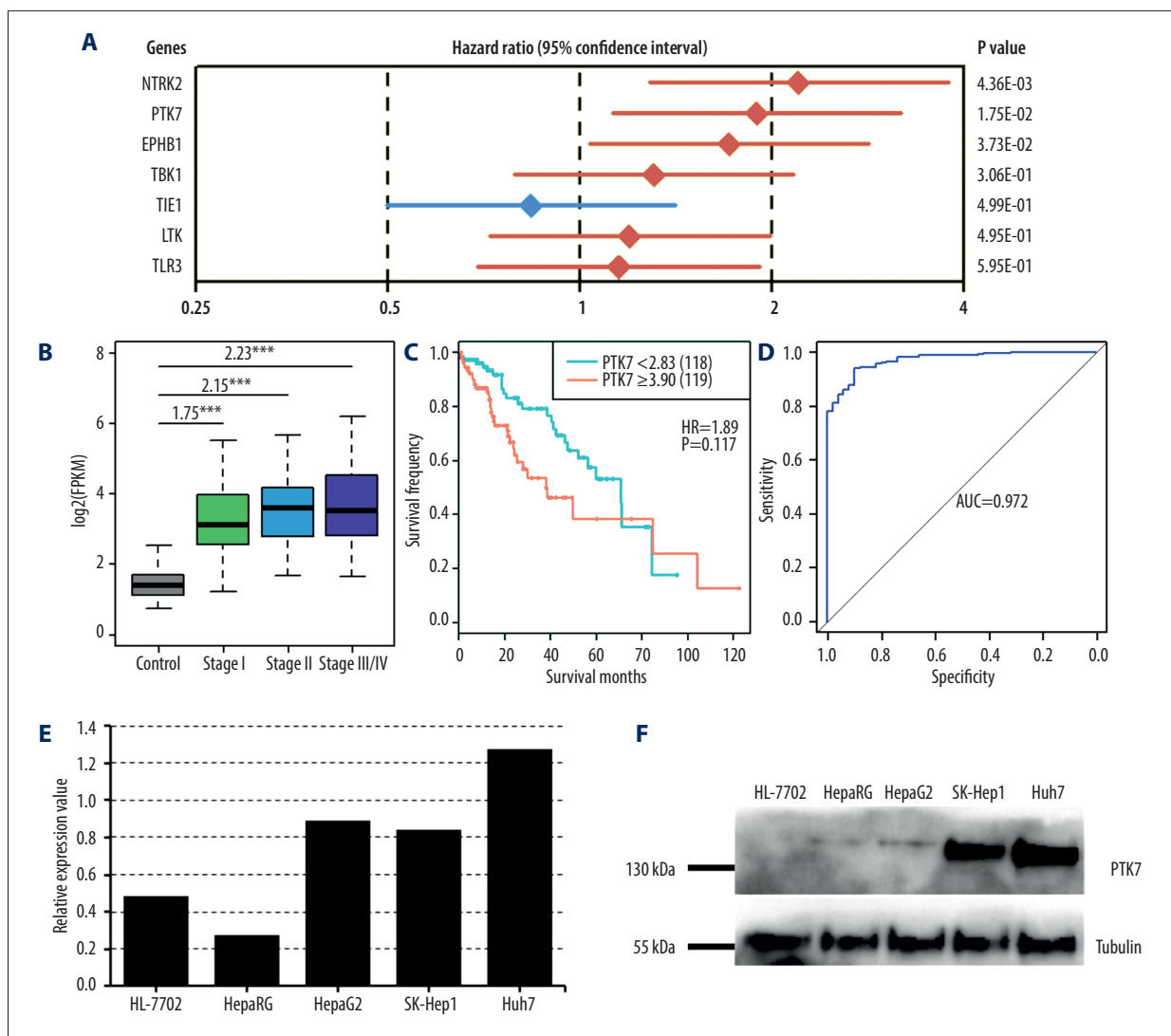


Figure 4. Effect of PTK7 expression in hepatocellular carcinoma (HCC). **(A)** The effect of commonly deregulated HCC target genes on patient overall survival (OS). **(B)** The expression of PTK7 in HCC tumor tissue and adjacent normal tissue samples in cancer at different stages. **(C)** Kaplan-Meier survival curve of PTK7 expression in patients with HCC. **(D)** The receiver operating characteristic (ROC) curve analysis of PTK7 gene expression between HCC and adjacent normal samples. The area under the curve (AUC) that approximates to 1 indicates a greater difference between the tumor and adjacent samples. **(E)** Quantitative reverse transcription PCR (RT-qPCR) of PTK7 in normal liver cells (HL-7702) and HCC cell lines (HepaRG, HepG2, SK-Hep1, and Huh7). **(F)** Western blot of PTK7 in normal liver cells (HL-7702) and HCC cell lines (HepaRG, HepG2, SK-Hep1, and Huh7).

by binding canonical Wnt ligands, preventing their interaction with Wnt receptors [17]. Clinical studies have shown that increased expression of PTK7 was correlated with progression and reduced overall survival (OS) in colorectal cancer (CRC) [18], and the carcinogenic effect of PTK7 was negatively regulated by microRNA-205-5p (miR-205-5p) [19].

Oncomine database expression studies and immunohistochemistry (IHC) studies have shown that PTK7 is overexpressed esophageal squamous cell carcinoma (ESCC) and may interact with p53 and caspases [20]. Also, PTK7-targeted

antibody-drug conjugates reduced the prevalence of tumor-initiating cells and had several anti-tumor effects, including the inhibition of angiogenesis and the stimulation of immune cells [21]. The findings from the present study indicated that PTK7 was highly expressed in HCC and interacted with the largest number of deregulated genes at all stages, from stage I to stage IV. Previous studies have shown that knockdown of PTK7 inhibited cell proliferation, cell migration, and cell invasion and induced apoptosis in cancer cell lines including colon cancer cells [22], oral tongue squamous cell carcinoma cells [23], and malignant glioma cells [24]. Furthermore,

Table 1. Univariate Cox proportional hazards regression analysis of PTK7, clinicopathological parameters, and overall survival (OS) in patients with hepatocellular carcinoma (HCC).

Variable*	N	Median (Q1–Q3)	HR (95% CI)	P-value
PTK7 (categorical)				
PTK7 <2.83	122	393.00 (149.00–1194.25)	Reference	
PTK7 ≥3.90	125	344.00 (107.00–649.00)	1.88 (1.12–3.18)	0.018
Gender				
Male	249	347.00 (129.00–808.00)	Reference	
Female ⁶	121	458.00 (115.00–1005.00)	1.31 (0.86–2.01)	0.205
Age	370		1.03 (1.01–1.04)	0.004
Cancer history				
No	207	347.00 (117.50–877.00)	Reference	
Yes	112	569.50 (226.00–1137.25)	1.95 (1.25–3.04)	0.003
Residual tumor				
R0	323	400.00 (165.50–1018.50)	Reference	
R1	17	455.00 (253.00–837.00)	1.18 (0.48–2.92)	0.722
R2	1	223.00 (223.00–223.00)	–	–
Stage				
Stage I	171	428.00 (175.50–1183.50)	Reference	
Stage II	85	279.00 (63.00–734.00)	1.02 (0.56–1.85)	0.960
Stage III	85	300.00 (101.00–770.00)	1.21 (0.70–2.12)	0.494
Stage IV	5	223.00 (15.00–558.00)	–	–
Albumin	297		0.99 (0.95–1.03)	0.582
Total bilirubin	296		0.96 (0.84–1.10)	0.562
Creatinine	299		1.00 (0.99–1.02)	0.700
Platelet count	304		1.00 (1.00–1.00)	0.885
Alpha-fetoprotein	278		1.00 (1.00–1.00)	0.418

* Data are shown as the median (Q1–Q3 quantiles) survival time. The regression analysis results are shown as hazard ratios (HR), 95% confidence interval (CI), and P-values. N – number of samples.

Table 2. Multivariate Cox proportional hazards regression of PTK7 on overall survival (OS).

Variable*	N	Median (Q1–Q3)	HR (95% CI)	P-value
PTK7 (categorical)				
PTK7 <2.83	105	438.00 (161.00–1271.00)	Reference	
PTK7 ≥3.90	105	364.00 (129.00–660.00)	2.24 (1.27–3.95)	0.006

* Data are shown as the median (Q1–Q3 quantiles) survival time. The regression analysis results are shown as hazard ratios (HR), 95% confidence interval (CI), and P-values. N – number of samples. P-values are adjusted for age and cancer history.

overexpression of PTK7 increased cell migration in colorectal cancer cell lines [25]. However, a study on esophageal squamous cell carcinoma (ESCC) showed that patients with increased PTK7 mRNA levels had an increased OS and lower relative risk than patients with lower PTK7 mRNA levels [26]. Hypermethylated PTK7 in cancer tissue compared with the adjacent noncancerous tissues was also reported in a small cohort of patients with HCC [27]. These previously reported findings support those of the present study and also the need for further studies to investigate the potential role of increased expression of PTK7 in HCC.

In addition to PTK7, other deregulated target genes may also be important for cancer progression. For example, EPHA8 is an Eph receptor in the Eph/ephrin receptor tyrosine kinase (RTK) subfamily and is involved in several processes in tumorigenesis, including angiogenesis, cell adhesion, and cell migration. Increased expression of EPHA8 may be a prognostic marker for epithelial ovarian cancer [28]. The effect of increased expression of PARP1 in HCC has also been previously reported [29,30]. PARP1 has been shown to physically interact with hepatitis B virus X protein and to inhibit the activity of the DNA repair complex at damaged DNA sites, resulting in hepatocarcinogenesis [31]. Leukocyte tyrosine kinase (LTK) is a receptor tyrosine kinase reported to be overexpressed in human leukemia, and the aberrant activation of LTK may contribute to neoplastic cell growth [32]. Studies have shown that the inhibition of LTK may be a potential approach to cancer treatment [33,34]. Histone deacetylase 4 (HDAC4) plays a critical role in transcriptional regulation, cell cycle progression, and developmental events [35]. Increased expression of HDCA4 was found in lung cancer and was negatively regulated by miR-520b [36]. In the present study, PARP1, LTK, and HDCA4 were highly expressed in at least one stage of HCC, and EPHA8 also showed a trend of overexpression in all stages of HCC. However, these genes were shown to have less impact on patient prognosis than PTK7 gene expression.

The co-expression findings and the KEGG pathway enrichment results showed that the deregulated target genes and co-expressed genes were mainly involved in immune function and signaling transduction pathways. This study identified six

commonly enriched pathways in all stages of HCC, which included the Toll-like receptor signaling pathway, regulation of actin cytoskeleton, and signaling pathways regulating pluripotency of stem cells. Abnormal expression of Toll-like receptors (TLRs) and impairment of Toll-like receptor signaling pathways lead to immune dysfunction, increase the probability of hepatitis virus infection, which may increase the risk of HCC [37,38]. Previous studies have shown that the activation of actin cytoskeleton remodeling correlates with increased tumor size, invasion, and metastasis [39]. Increased expression of APLP2 damages the actin cytoskeleton and increases pancreatic cancer growth and metastasis [40]. Also, cancer stem cells (CSCs) are considered to be responsible for tumor initiation, metastasis, relapse, and chemoresistance, although CSC-specific targets remain to be investigated [41].

Conclusions

This study used bioinformatics analysis to identify genes associated with patient outcome in stages I–IV hepatocellular carcinoma (HCC) and the gene pathways that distinguished between normal liver and liver cells and HCC and human HCC cell lines. PTK7 gene expression was identified as a potential therapeutic target and prognostic marker for all stages of HCC. Other genes that were upregulated in HCC were involved in immune function and signaling transduction pathways. The impact of PTK7 expression on tumorigenesis in HCC requires further studies to determine the potential role of PTK7 as a diagnostic and prognostic biomarker in HCC.

Data availability

The hepatocellular carcinoma (HCC) transcriptome and clinical data used to support the findings of this study are available in the GDC Data Portal (<https://portal.gdc.cancer.gov/>). The HCC target genes are available in the Clarivate Analytics Integrity database (<https://integrity.clarivate.com/integrity/>).

Conflict of interest

None.

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