Research Square

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Analysis of hnRNP K Expression and Relevance for Clinical Outcomes in Hepatocellular carcinoma

Xin Tu
Guangxi Medical University Affiliated Tumor Hospital
Yanming Qin
Guangxi Medical University Affiliated Tumor Hospital
Haiping Zheng
the First Affiliated Hospital of Guangxi Medical University
Caifang Ma
Guangxi Medical University Affiliated Tumor Hospital
Meifang Huang
Guangxi Medical University Affiliated Tumor Hospital
Hong Shu
Guangxi Medical University Affiliated Tumor Hospital
Chao Ou (ouchaocn@126.com)
Guangxi Medical University Affiliated Tumor Hospital

Research Article

Keywords: hnRNP K, hepatocellular carcinoma, prognosis, TMA

Posted Date: March 16th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1424181/v1

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Abstract

Background

Hepatocellular carcinoma (HCC) is the most commonly seen primary liver cancer with poor prognosis and high mortality. Several studies have discovered that the overexpression of Heterogeneous nuclear ribonucleoprotein K (hnRNP K) was related to poorer prognoses of human tumors like colonic and rectal cancer, nasopharyngeal carcinoma, stomach cancer, and oral squamous cell carcinoma (OSCC). Nevertheless, its effects on HCC progression and prognosis remains unclear.

Methods

To evaluate the prognostic value of hnRNP K expression, we extracted data from public databases include The Cancer Genome Atlas (TCGA), Gene Expression Omnibus (GEO) and the Human Protein Atlas (HPA), and analysis of clinical specimens via tissue microarrays (TMA). Furthermore, our team utilized TIMER and GEPIA data bases to explore the association between the expression of hnRNP K and infiltration immunocytes and the relevant genetic biomarker sets. Co-expression analysis of hnRNP K in HCC was revealed using R2 bioinformatics tool. The GO and KEGG pathway analysis was completed via these co-expression genes to explore the possible signaling pathways in HCC.

Result

In the study, we found that the mRNA and protein levels of hnRNP K was increased in HCC. Higher hnRNP K expression is related to unfavorable survival outcomes and poorer clinicopathologic characteristics of HCC sufferers. Kaplan–Meier analyses showed that higher expression of hnRNP K was related to poorer overall survival (OS). Cox regressive analysis verified that hnRNP K was an independent prognostic factor for HCC. Moreover, there was a remarkable positive association existed between hnRNP K and the infiltration level of five types of immunocytes include B cells, CD4 + T cells, CD8 + T cells, macrophages, neutrophilic cells and dendritic cells (DCs). Meanwhile, the levels of the majority of immune sets were related to the expression of hnRNP K in HCC. These findings from pathway analysis might reveal the vital effects of hnRNP K and its co-expressed genes on a variety of carcinogenic processes.

Conclusion

Our findings revealed that hnRNP K have potential prognostic value and might be an underlying prognosis biomarker for HCC sufferers.

Introduction

Primary liver cancer is one of the most prevalent human cancers across the globe, and its incidence and mortality rates are still high. Statistical data show that PLC has become the sixth most commonly diagnosed cancer globally in 2020, ranking the fourth cancer-related mortality over the world[1]. HCC is the most commonly seen type of primary hepatic tumor (approximately 75–85% of cases)[2]. The clinic characteristics of HCC include insidious onset, rapid development and high malignancy and mortality resulting in difficult early diagnoses and poor prognosis in most patients[3]. Despite people has made good progress in diagnosis and treatment management of HCC recently, the 5-year OS of HCC sufferers is still at a low level[4]. Hence, it's imperative to discover reliable prognosis markers for HCC.

hnRNP K mapped to the chromosome 9q21.32 pertains to the sub-family of ubiquitously expressed hnRNPs—They are RNA binding proteins (RBPs) and they complex with hnRNA[5]. Researches have revealed that hnRNP K is mostly expressed in human nucleated cells (located in both nucleus[6]and cytoplasm[7]) and participates in a variety of process like transcriptional process, RNA processing and translational process, etc[8]. Studies have pointed out that hnRNP K is vital for p53 reaction to DNA injury, which acts at the level of transcriptional stimulation and inhibition[9]. There are researches noting that hnRNP K interacts with the CT element (sequences that are C-rich) in the promotor of the c-myc gene resulting in this oncogene overexpression[10, 11]. Besides, hnRNP K was found to interact with c-Src and c-Src is responsible for the mediation of tyrosine phosphonation of hnRNP K and repression of its DICE binding activities[12]. The above studies all indicate hnRNP K is tightly related to the onset and progression of cancers. Further studies suggested that hnRNP K is tightly correlated with the prognosis of multiple tumors like colorectal carcinoma[13], nasopharyngeal cancer[14], stomach carcinoma[15], and OSCC[16]. However, its role in HCC progression and prognosis remains unclear.

Herein, our team systematically studied the expressing of hnRNP K in various cancers and their healthy tissular samples and discovered that the expressing of hnRNP K was remarkably greater in HCC specimens. Then we evaluated the expressing of hnRNP K and its clinical outcome in HCC using online bioinformatics databases and validated the predictive accuracy in tissue specimens via TMA and immune histochemistry (IHC). Finally, the association between hnRNP K expression, clinicopathological features, and patient outcome such as OS was evaluated. Cox regression analysis of prognostic parameters for overall survival in these patients with HCC. Moreover, our team analyzed the association between hnRNP K expression

and immunity infiltration and genes which were co-altered with hnRNP K in HCC. Taken together, these analyses might reveal the potential prognostic value of hnRNP K and provide a realization of the possible underlying mechanism on how high hnRNP K expression leads to poor prognosis in HCC.

Materials And Methods

Analysis of hnRNP K mRNA expression on bioinformatics databases

Our team identified the diversities of the expressing of hnRNP K between the various types of tumors and the adjacent healthy samples via TIMER2.0 database[17] that is a systematic analytical on-line tool for tumor investigators to acquire the cancer immunology, clinic, and genome characteristics. The mRNA content of hnRNP K in HCC and adjacent normal tissues was examined in ONCOMINE (https://www.oncomine.org/resource/main.html)[18], a tumor micro array data base and on-line datamining platform aiming at promoting discoveries from whole genome expression analysis, and validation of hnRNP K expression in the TCGA-Liver Hepatocellular Carcinoma (TCGA-LIHC) database was completed by UALCAN (http://ualcan.path.uab.edu/index.html)[19]. The relation between the clinical parameters and the expressing of hnRNP K was analyzed in these databases. Furthermore, hnRNP K mRNA expression, and survival probability in HCC patient characteristic were examined in TCGA data sets via the GEPIA (http://gepia.cancer-pku.cn//index.html)[20]. The microarray data were obtained from the GEO data base. Two genetic expression data sets (GSE45267 and GSE121248) of HCC were involved herein (GPL570 [(HG-U133_Plus_2) Affymetrix Human Genome U133 Plus 2.0 Array]). The protein expressing levels of hnRNP K in mankind healthy and HCC samples were identified via the HPA, a mankind proteome database which consists of expression and localisation profiles in 48 healthy mankind tissular samples, 20 various tumors and 47 lineage cells.

Patients and tissue samples

A total of 160 cases of tissue specimens including 80 cases of HCC tissue specimens and 80 cases of matched neighboring healthy samples (2 cm from the cancer margin) that were the source of commercially available TMA from Shanghai Outdo Biotech Co.,Ltd (HLivH180Su16) and clinicopathological data on tumor size, number of tumors, Edmondson grades, TNM stages, biochemical markers and outcome data include survival status, survival time, the presence of recrudesce and time to recurrence (TTR). The entire specimens were diagnosed pathologically. The survival duration was computed from the date of operation to last follow-up (February 2012) or death. All the human tissue specimens were approved by the Ethics Committee of Shanghai Outdo Biotech Co.,Ltd (NO:SHYJS-CP-1707005). All experiments complied with relevant laws and regulations and met the criteria of Ethical Review Committee. All patients signed informed consent.

IHC staining and assessment

For IHC dyeing, briefly, Tissue slices were baked under 63°C for 60 min, deparaffinised with dimethylbenzene and subjected to rehydration by a graded alcohol series using a fully automatic IHC staining machine. Sections were the immersed in the EDTA buffering solution and boiled under 120°C in a microwave for 20 min. Subsequently, Commercial blockers were dropped in the slide to reduce nonspecific binding. Next, coverslides were cultivated with rabbit anti-hnRNP K antisubstance (ab52600, 1:7000 dilution, Abcam, Cambridge, UK) under 4°C for one night, cleaned 3 times in PBS cultivated with biotin-labeled second antibody for 20 min under 37°C. Finally, tissue sections incubated with DAB for visualization.

For the sake of quantifying the IHC intensities, the integrated optical density (IOD) was computed via image-Pro Plus program (version 6.0), and the mean optical density (MOD) was computed from IOD/Area. The data in each group are expressed as means±SDs and paired Student's t-test was utilized to contrast data between cancer samples and relevant neighboring samples. Then, tumor tissues were separated into the $expression_{high}$ and $expression_{low}$ groups based on the protein hnRNP K expression that was quantified via MOD, a value ≥ the average was considered high expression, whereas a value < the average was considered low expression in tumors.

Analysis of the association between the expressing of hnRNP K and tumor immune infiltration

TIMER is a web server for comprehensive analysis of cancer-infiltrating immunocytes which includes 10,897 specimens across 32 tumor types from TCGA. We identified the HCC and specific cancer-infiltrating immunocyte subsets (B cells, CD4+ T cells, CD8+ T cells, macrophagus, neutrophilic cells, and DCs) that are associated with the expression of hnRNP K. Next, we use "survival" model in TIMER and univariate survival analysis was performed for different specific cancer-infiltrating immunocytes and Kaplan–Meier curves was drawn to visualize the survival differences in HCC.

Analysis of co-expressed Genes of hnRNP K through GO and KEGG Pathway

Positive and negative coexpression genes of hnRNP K were investigated in TCGA-LIHC data set via R2: Genomics Analysis and Visualisation Platform (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi), with the absolute value of correlation coefficient ($|r|$) was selected as >0.3 and p was set as < 0.01.

For the purpose of investigating pathways and GO shared by hnRNP K-related genes, our team completed GO and KEGG pathway enrichment analyses via the web-based data bases DAVID (https://david.ncifcrf.gov/)[21] and KOBAS (http://kobas.cbi.pku.edu.cn/)[22]. Function and pathway enrichment results were visualized using R language.

Statistical analysis

Statistical analyses including χ2 test, paired Student's t-test, and Kaplan–Meier analyses were completed using SPSS v20.0 for Windows. Differences between HCC tissue specimens and matched adjacent non-tumor tissues are tested using paired Student's t-test. To study the expressing of hnRNP K and its relationship with clinicopathological features in HCC, we used χ2 test or Fisher's exact tests to evaluate. Survival curves were analyzed via the K-M approach, and the log-rank test was employed to identify survival diversities between survival curves. Cox proportion risk regression model was employed to examine univariate and multivariate analyses. $p<0.05$ had significance on statistics.

Results

Overexpression of hnRNP K mRNA in HCC based on bioinformatics databases

In order to study the mRNA expressing level of hnRNP K in various tumors and their healthy samples across all TCGA tumors, we used the TIMER database. From TIMER analysis, the expressing of hnRNP K mRNAs was remarkably greater in HCC specimens (Fig1 A). To identify the expressing of hnRNP K in HCC, our team analyzed datasets from the Oncomine database. In the Oncomine data base, the expressing of hnRNP K mRNA was remarkably greater in HCC in contrast to healthy hepatic samples in the Wurmbach liver (Fig1 B) and Roessler Liver 2 (Fig1 C) datasets. Increased expressing of hnRNP K in HCC was confirmed in the TCGA dataset via the UALCAN (Fig1 D). Our team also explored the relationship between the expressing of hnRNP K and the prognoses of HCC sufferers via GEPIA, the result revealed that the high expressing of hnRNP K mRNA was significantly related to poor clinic outcome (Fig1 E). Meanwhile, similar results were verified in GEO data base as well (Fig2 A-B). Additionally, the expressing of hnRNP K protein was upregulated in HCC samples in contrast to healthy samples in HPA (Fig2 C). Taken together, the data in multiple databases suggested that the expressing of hnRNP K in HCC tumors was remarkably greater in contrast to healthy hepatic samples, and high expressing of hnRNP K may correlate with poor prognosis in HCC.

Association between hnRNP K mRNA expression and clinicopathologic features of HCC

To further assess the relationship between the expressing of hnRNP K mRNAs and clinicopathologic features of HCC via TCGA data via UALCAN web tool. As to cancer gradation, the expressing of hnRNP K was remarkably regulated upward in every cancer gradation, we also discovered that the expressing of hnRNP K was remarkably greater in grade 3 in contrast to grade 1 and 2 (Fig3 A). Interestingly, the expressing of hnRNP K was remarkably elevated in stage 3 compared with stage 1 and 2 and we also observed an increase in hnRNP K expression in stage 1 thru 3 compared to normal samples, but no diversity was observed in the expressing of hnRNP K between stage 4 and normal samples (Fig3 B). Moreover, in contrast to healthy samples, the expressing of hnRNP K was significantly increased irrespective of gender, age, race and histological subtypes. (Fig3 C-F).

Overexpression of hnRNP K protein in HCC

For the sake of exploring the expressing feature of hnRNP K in HCC, the protein expressing of hnRNP K was analyzed in 80 pairs of HCC and neighboring specimens via TMA-based IHC (Fig4 A). The outcomes revealed that hnRNP K expressing level was remarkably greater in HCC samples in contrast to the healthy adjacent liver samples (Table 1, Fig4 B).

Relationship of hnRNP K expression and clinicopathological parameters in HCC tissues

The clinicopathologic parameters of HCC patients are provided (Table 2) to analyze the association between hnRNP K protein level and clinicopathologic parameters. Among the HCC tissue specimens, 40 cases (50%) were identified as having a high hnRNP K protein expression, and the remaining 40 samples had a low hnRNP K protein expression. Higher expressing level of hnRNP K was remarkably related to higher pathologic gradation (p=0.044), and the survival status of all patients (p = 0.011), In addition, our team discovered that higher expressing level of hnRNP K was remarkably related to post-operation recurrence (p = 0.007). However, hnRNP K expression did not correlate with gender, age, tumor size, AFP level, GGT level, TBIL level, TNM stage, tumor number, cirrhotic nodules and HBV infection.

Assessment of prognosis significance of hnRNP K in HCC

The Kaplan–Meier survival analyses were utilized to further assess the prognosis significance of hnRNP K in HCC. Our team observed that patients with high expressing level of hnRNP K was tightly related to shorter OS in contrast to sufferers with lower expressing level (Fig4 C).

A univariate Cox proportion risk model was then performed to investigate which factors affected the overall survival, the results showed that GGT level (p=0.034), Edmondson grade (p=0.015), and hnRNP K expression (p=0.012) correlated with overall survival. while only Edmondson grade (p=0.035) and the expressing level of hnRNP K were remarkable in multivariable analyses, revealing that the expressing of hnRNP K protein was an independent risky factor for poorer prognoses in HCC sufferers (Table 3, Fig4 D).

Association between hnRNP K and tumor immune infiltration

Next, our team assessed the relationship between hnRNP K expressing level and tumor immune infiltration using TCGA LIHC data through TIMER tool. Positive correlations which were statistically significant ($p<0.05$) were found between hnRNP K and the infiltration level of the five immunocytes, include B cells (r=0.321, p=1.13e-09), CD4+T cells (r=0.377, p=4.81e-13), CD8+T cells(r=0.287, p=6.41e-08), macrophagus (r=0.446, p=4.96e-18),

neutrophilic cells (r=0.51, p=3.25e-24), and DCs (r=0.419, p=6.42e-16). In addition, our team discovered that the hnRNPK expressing level was related to cancer purity in a positive way (r=0.128, p=1.17e-02). (Fig5 A)

For the purpose of investigating the underlying association between hnRNPK and infiltration immunocytes, our team identified the association between hnRNPK and multiple immunocyte biomarkers in TIMER and GEPIA. Those biomarkers were extensively considered relevant symbols of diverse immunocytes, like B cells, CD8+ T cells, M1/M2 macrophagus, cancer-related macrophagus, mononuclear cells, NK, neutrophilic cells and DCs in LIHC. The diverse function T cells like Tfh, Th1, Th2, Th9, Th17, Th22, Treg and depleted T cells were studied as well herein. Our team discovered that the contents of the majority of immunity sets marking B cells, diverse T cells, TAMs, M1/ M2 macrophagus, mononuclear cells and DCs were related to the expressing of hnRNP K in HCC (Table 4). These results indicate that hnRNP K might influence the immune cell infiltration level affecting tumor occurrence and development, explaining the poor prognosis in HCC.

GO and KEGG pathway analysis with hnRNP K and correlated genes identifying signaling pathways in HCC

Our team intended to reveal the underlying signaling pathways participating in hnRNP K and correlated genes in LIHC. First, we analyzed transcriptome datasets from TCGA LIHC data sets via the R2: Genomics Analysis and Visualisation Platform. 3314 genes with cutoffs of $|r| > 0.3$ ($r > 0.3$ and $r < -0.3$) and Bonferroni adjusted $p<0.01$ were considered as correlated genes (include 2384 positive correlation and 930 negative correlation). Next, GO and KEGG pathway analysis of hnRNP K and its positively and negatively co-expressed genes were performed though online databases DAVID and KOBAS.

According to the GO analysis results, hnRNP K and the positively associated genes were involved in cell cycle, DNA duplication and repair, p53 binding and hippo signaling etc (Fig6 A). Moreover, we also observed that hnRNP K and its negative correlated genes were relevant to cell autophagy (Fig6 B).

KEGG pathway analyses were also performed on those genes. Results of the KEGG analysis showed these genes were not only related to cell cycle, viral carcinogenesis, Jak-STAT signal path, the expressing of PD-L1 and PD-1 check point pathway in tumor, TGF-beta signal path, and p53 signal path but also related to HCC associated signal paths like MAPK, PI3K-Akt and Hedgehog signal paths (Fig6 C).

Discussion

HCC is one of the most commonly seen cancers of the digestion system whose incidence is continuing to rise. Although with the persistent advancement of surgery resection, antitumor drugs and radiotherapy technology, over 600000 people still die of HCC each year[23] and HCC is still one of the illnesses with the worst prognoses amongst the entire malignancies[24]. Therefore, discover reliable prognostic biomarkers is one of the focal points of current HCC research. Research has shown that hnRNP K is a RBP which plays multiple roles in biological processes. Previous studies have reported that an association between patient survival and hnRNP K expression in colorectal cancer[13], nasopharyngeal cancer[14], stomach carcinoma[15], and OSCC[16], and the prognosis was worse when hnRNP K was highly expressed. However, the association between hnRNP K expressing level and prognoses of HCC sufferers remains unclear. In this study, we systematically analyzed hnRNP K expression using bioinformatics databases including TCGA, GEO and HPA. We demonstrated that the expressing of hnRNP K was remarkably greater higher in various cancer cells, especially in HCC, in contrast to their neighboring normal samples. In addition, we focused on the relationship between hnRNP K and clinicopathological characteristics and OS. We found that hnRNP K with high mRNA expression is related to tumor grade and individual cancer stage, and is negatively correlated with patient survival.

From the TMA, we observed differences in HCC tissue and corresponding adjacent tissue specimens by IHC analysis. These results revealed that the protein expressing of hnRNP K was remarkably greater in HCC samples in contrast to the normal neighboring hepatic samples ($p < 0.0001$). In addition, our team assessed the relationship between hnRNP K protein level and clinicopathologic features. The outcomes herein showed that High hnRNP K protein level was remarkably related to higher pathologic grade ($p = 0.044$), and the survival status ($p = 0.011$) and postoperative recurrence ($p = 0.007$). These results may give a hint that higher expressing of hnRNP K was related to poorer prognoses. Moreover, our team conducted K-M survival analyses. The results unveiled that higher expressing level of hnRNP K was tightly related to a shorter OS and it was also an independent prognosis factor in multivariable analyses. The findings indicated that hnRNP K might exert certain effects on the development of HCC and it can serve as a potential prognosis predictive marker for HCC patients.

However, it is still poorly understood whether hnRNP K participates in the progression of HCC. For the sake of gaining preliminary insights into the biological role of hnRNP K in HCC, we conducted bioinformatics analyses. HCC is regarded as a chronic inflammation-driven cancer and the tumor microenvironment (TME) in HCC is mentioned as having immunosuppressive properties[25]. This is considered to be a vital factor for HCC progression[26]. According to the bioinformatics analysis, there was a significant positive relationship between hnRNP K and the infiltration level of immunocytes include B cells, CD4 + T cells, CD8 + T cells, macrophagus, neutrophilic cells and DCs. Interestingly, the hnRNP K expressing level displayed a remarkable positive relationship with cancer purity, which were indicated its relative enrichment in oncocytes. Deeper analyses of immune cell markers revealed that a significant positive association existed between the expressing of hnRNP K and the markers of Treg cells such as FOXP3, CD25 and CCR8. Treg cells have been perceived as a subset of suppressor T cell, which a significant role in suppressing tumor-associated immunity and promoting immune escape by the tumor[27, 28]. Previous studies have shown that Treg cells have cancer promoting effects and related to poor

prognosis of HCC[29, 30]. Moreover, our team discovered that the expressing of hnRNP K was related to PD-1 and TIM-3, which were considered to be important markers of T cell exhaustion. As reported in the results, T cell depletion is a status of aberrant T cell function in the TME, which is regarded as one of the mechanisms of immune escape[31]. Concurrently, the gene expression analysis of immune cell markers and following KEGG pathway analysis suggested hnRNP K was correlated to PD-1/PD-L1. PD-1 and its ligand PD-L1 are widely reported to be immunosuppressive molecules, which lead to escape being identified by the immune system and promote immune tolerance[32, 33]. In HCC, higher expressing of those molecules was related to the poorer outcome[34, 35]. Taken together, those outcomes revealed that hnRNP K might regulate the immune response affecting cancer onset and progression. Nevertheless, the detailed mechanism of hnRNP K in the regulation of the immune response in HCC still need further research.

Coexpression analysis is a vital method to investigate the underlying functions of genes. To explore hnRNP K-associated changed pathways in HCC, genes which were co-expressed with hnRNP K were studied. Past researches revealed that hnRNP K could jointly work with p53 in the presence of DNA injury, with knockout of hnRNP K inducing flaws in cellular cycle check point arrest[6]. This was in very good agreement with the results of our GO enrichment analysis. Morever, GO analysis may also reveal the vital effect of hnRNP K and its co-changed genes in autophagy which participated in the onset and progression of HCC[36, 37]. KEGG pathway analysis found that hnRNP K was associated with diverse tumor-related signaling pathways including Wnt, JAK-STAT, PI3K, MAPK, PD-1/PD-L1 and so on. A study by Zhang et al[38] reported the interaction of CASC11 and hnRNP K with Wnt pathway to promote colorectal cancer. These studies increased the credibility of enrichment analysis. However, to clarify the effects of hnRNP K on the onset and progression of HCC, further intensive investigations are required.

In conclusion, we used several online bioinformatic databases and tissue microarray to comprehensively study the expression, immune infiltration, associated genes and prognosis significance of hnRNP K in HCC. Our study demonstrates that hnRNP K is regulated upward and is related to clinic results in HCC in a negative way. Additionally, our study also revealed the importance of the expressing of hnRNP K and potential hnRNP K-associated pathways in cancer development. Therefore, this research might offer precious enlightenment pertaining to hnRNP K as a potential prognostic biological marker target for HCC.

Abbreviations

DCs	Dendritic cells
GEO	Gene Expression Omnibus
HCC	Hepatocellular carcinoma
hnRNP K	Heterogeneous nuclear ribonucleoprotein K
HPA	Human Protein Atlas
IOD	Integrated Optical Density
MOD	Mean Optical Density
OSCC	Oral squamous cell carcinoma
OS	Overall Survival
TCGA	The Cancer Genome Atlas
TMA	Tissue Microarrays
TME	Tumor microenvironment

Declarations

Acknowledgements

Not applicable

Authors’contributions

XT, YQ and HZ drafted the main part of the manuscript. HS and CO provided the idea and financial support of the study. CM helped drawing figures in this manuscript. MH was responsible for the statistical analyses. All authors have read and approved the manuscript.

Funding

This study was supported in part by grants from the Key R & D program Natural Science Foundation of Guangxi Province under Grant No. AB19110007. This study was also supported by grants from the “Innovation Project of Guangxi Graduate Education” (grant number: YCSW2021132).

This study was also supported in part by grants from the Natural Science Foundation of Guangxi Province under Grant No.2017GXNSFAA198015, the Technology Development and Promotion Foundation of Guangxi Medical and Health Appropriate under Grant No. S2017104.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Ethics approval and consent to participate

Ethics approval was granted by the Ethics Committee of Shanghai Outdo Biotech Co.,Ltd (NO:SHYJS-CP-1707005). All patients signed informed consent. All experiments complied with relevant laws and regulations and met the criteria of Ethical Review Committee. All methods were carried out in accordance with the Declaration of Helsinki.

Consent for publication

Not applicable

Competing interests

There are no conflicts of interest for all the authors.

Author details

[1]Department of Clinical Laboratory, Guangxi Medical University Affiliated Tumor Hospital, Nanning 530021, China

[2]Department of Oncology, the First Affiliated Hospital of Guangxi Medical University, Nanning 530021, China

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Tables

Table 1 The protein expression of hnRNP K in HCC and adjacent non-tumor tissues

Sample	hnRNP K expression		
	Case	MOD[a]	*p*-value
HCC	80	0.16±0.03	*p*<0.0001
adjacent non-tumor tissues	80	0.14±0.02	

a. MOD, the mean optical density, are expressed as means±SDs.

Table 2 Correlation between hnRNP K expression and clinicopathological characteristics of patients with HCC (n=80)

Parameter	Case	hnRNP K low	hnRNP K high	*p*-value
Sex				
Male	72	35(48.6%)	37(51.4%)	0.709
Female	8	5(62.5%)	3(37.5%)	
Age(years)				
≤ 50	37	19(51.4%)	18(48.6%)	0.823
> 50	43	21(48.8%)	22(51.2%)	
Tumor size (cm)				
≤ 5	57	29(50.9%)	28(49.1%)	0.805
> 5	23	11(47.8%)	12(52.2%)	
AFP(μg/L)				
≤ 400	51	22(43.1%)	29(56.9%)	0.104
> 400	29	18(62.1%)	11(37.9%)	
GGT(U/L)				
≤ 60	49	26(53.1%)	23(46.9%)	0.491
> 60	31	14(45.2%)	17(54.8%)	
ALT(U/L)				
≤ 40	47	23(48.9%)	24(51.1%)	0.820
> 40	33	17(51.5%)	16(48.5%)	
TBIL(μmol/L)				
≤ 21	67	35(52.2%)	32(47.8%)	0.363
> 21	13	5(38.5%)	8(61.5%)	
TNM stage				
I-II	51	27(48.2%)	29(51.8%)	0.626
III-IV	14	13(54.2%)	11(45.8%)	
Edmondson stage				
I-II	39	24(61.5%)	15(38.5%)	0.044
III-IV	41	16(39.0%)	25(61.0%)	
HBV				
HBV +	61	30(49.2%)	31(50.8%)	0.793
HBV -	19	10(52.6%)	9(47.4%)	
Tumor number				
Single	71	37(52.1%)	34(47.9%)	0.479
Multiple	9	3(33.3%)	6(66.7%)	
Recurrence				
Presence	38	25(65.8%)	13(34.2%)	0.007
Absence	42	15(35.7%)	27(64.3%)	

Table 3 Cox proportional hazard model analysis of prognostic factors

Parameters	Univariate analysis			Multivariate analysis		
	HR	95%CI	*p*	HR	95%CI	*p*
Age (≤ 50 vs. > 50 years)	1.23	0.59-2.58	0.583			
Sex (Male vs. Female)	0.28	0.04-2.08	0.214			
Tumor size (≤ 5 vs. > 5 cm)	1.68	0.79-3.56	0.178			
GGT level (≤ 60 vs. > 60 U/L)	2.21	1.06-4.62	0.034	2.08	0.98-4.39	0.056
ALT level (≤ 40 vs. > 40 U/L)	1.45	0.70-3.04	0.318			
TBIL level (≤ 21 vs. > 21 U/L)	0.99	0.93-1.06	0.748			
AFP level (≤ 400 vs. > 400 μg/L)	0.96	0.44-2.06	0.907			
TNM stage (I+II vs. III+IV)	1.71	0.81-3.57	0.158			
Edmondson stage (I+II vs. Ⅲ+IV)	2.66	1.21-5.86	0.015	2.37	1.07-5.25	0.035
HBV (HBV+ vs. HBV-)	0.97	0.41-2.30	0.946			
Tumor number (Single vs.Multiple)	1.83	0.70-4.81	0.220			
hnRNP K level (low vs. high)	2.76	1.25-6.09	0.012	2.47	1.11-5.51	0.027

Table 4 Correlations between HNRNPK and Gene Markers of Immune Cells in TIMER and GEPIA.

Cell type	Gene markers	TIMER				GEPIA			
		None		Purity		Tumor		Normal	
		Cor	*P*	Cor	*P*	R	*P*	R	*P*
B cell	CD19	0.154	*	0.199	**	0.13	0.011	0.24	0.095
	CD20(KRT20)	0.172	**	0.184	**	0.14	*	-0.11	0.45
	CD38	0.207	***	0.301	***	0.15	*	0.39	*
CD8+ T cell	CD8A	0.156	*	0.253	***	0.2	***	0.38	*
	CD8B	0.048	0.358	0.132	0.014	0.16	*	0.35	0.012
Tfh	BCL6	0.526	***	0.523	***	0.47	***	0.39	*
	ICOS	0.215	***	0.323	***	0.26	***	0.34	0.015
	CXCR5	0.178	**	0.267	***	0.11	0.039	-0.07	0.63
Th1	T-bet (TBX21)	0.121	0.020	0.211	***	0.17	*	0.47	**
	IL12RB2	0.375	***	0.39	***	0.16	*	0.41	*
	WSX1(IL27RA)	0.376	***	0.451	***	0.41	***	0.51	**
Th2	GATA3	0.186	**	0.32	***	0.23	***	0.35	0.012
	CCR3	0.276	***	0.333	***	0.18	**	0.25	0.076
	STAT6	0.498	***	0.481	***	0.49	***	0.72	***
Th9	TGFBR2	0.607	***	0.65	***	0.52	***	0.78	***
	IRF4	0.202	***	0.315	***	0.059	0.26	0.083	0.57
	PU.1(SPI1)	0.268	***	0.414	***	0.28	***	0.47	**
Th17	STAT3	0.47	***	0.517	***	0.55	***	0.46	**
	IL-21R	0.249	***	0.375	***	0.26	***	0.37	*
	IL-23R	0.295	***	0.316	***	0.11	0.028	0.24	0.095
Th22	CCR10	0.302	***	0.337	***	0.086	0.1	0.33	0.018
	AHR	0.521	***	0.539	***	0.51	***	0.59	***
Treg	FOXP3	0.289	***	0.33	***	0.11	0.043	0.17	0.25
	CD25(IL2RA)	0.302	***	0.419	***	0.26	***	0.22	0.13
	CCR8	0.431	***	0.529	***	0.31	***	0.29	0.038
T cell exhaustion	PD-1 (PDCD1)	0.135	*	0.203	**	0.13	0.014	0.4	*
	CTLA4	0.14	*	0.228	***	0.19	**	0.28	0.046
	TIM-3 (HAVCR2)	0.248	***	0.389	***	0.17	*	0.51	**
Macrophage	CD68	0.215	***	0.302	***	0.26	***	0.56	***
	CD11b (ITGAM)	0.285	***	0.365	***	0.42	***	0.54	***
M1	INOS (NOS2)	0.171	**	0.186	**	0.081	0.12	0.16	0.26
	IRF5	0.397	***	0.391	***	0.4	***	0.4	*
	COX2(PTGS2)	0.274	***	0.408	***	0.18	**	0.41	*
M2	ARG1	-0.137	**	-0.166	*	-0.082	0.12	0.27	0.057
	MRC1	0.102	0.050	0.156	*	-0.043	0.41	0.39	*
TAM	CCL2	0.149	*	0.252	***	0.16	*	0.35	0.013
	CD80	0.388	***	0.503	***	0.24	***	0.41	*

	CD86	0.282	***	0.244	***	0.35	***	0.5	**
Monocyte	CD14	-0.261	***	-0.243	***	-0.16	*	0.18	0.22
	CD16(FCGR3B)	0.245	***	0.273	***	0.071	0.17	0.47	**
Neutrophil	CD15(FUT4)	0.398	***	0.441	***	0.44	***	0.61	***
	CD11b (ITGAM)	0.285	***	0.365	***	0.42	***	0.54	***
NK	XCL1	0.099	0.056	0.147	*	0.17	*	0.41	*
	CD7	0.026	0.624	0.09	0.0965	0.017	0.75	0.36	0.01
	KIR3DL1	0.176	**	0.213	***	0.086	0.1	0.34	0.015
Dendritic cell	CD1C(BDCA-1)	0.194	**	0.275	***	0.22	***	0.36	0.01
	CD141(THBD)	0.201	***	0.303	***	0.24	***	0.5	**

Tfh, follicular helper T cell; Th, T helper cell; Treg, regulatory T cell; TAM, tumor-associated- macrophage; NK, natural killer cell; None, correlation without adjustment; Purity, correlation adjusted for tumor purity; Cor, R value of Spearman's correlation. *P < 0.01; **P < 0.001; ***P < 0.0001.

Figures

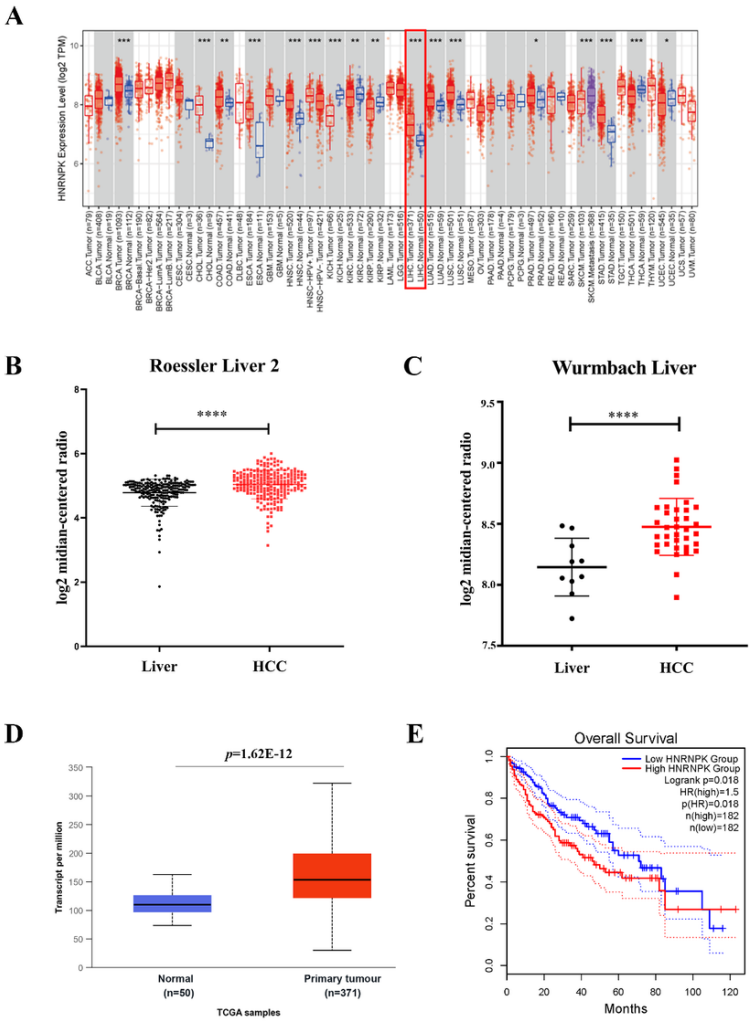

Figure 1

The mRNA expression of hnRNP K is significantly upregulated in Hepatocellular carcinoma (HCC). A. The mRNA expression of hnRNP K in various cancer types were retrieved from the TIMER2.0(http://timer.cistrome.org) database. Boxes represent the median and the 25th and 75th percentiles. Dots represent each individual data. Red boxes represent tumor tissues, and blue boxes represent normal tissues. Red and blue dashed lines represent the average expression value of all tumor and normal tissues. B-C. Scatter plots derived from gene expression data in ONCOMINE(https://www.oncomine.org/resource/main.html) comparing the expression of hnRNP K in normal and HCC tissue. The p value was measured with Student's t-tests. ****p < 0·0001. D. UALCAN(http://ualcan.path.uab.edu/index.html) portal analysis of HCC samples from the TCGA database. Box plots showing the hnRNP K mRNA expression between normal (blue plot) and HCC tumor samples (red plot). E. Overall survival curve showing the significant association between hnRNP K and survival in LIHC (p<0.05) acquired from GEPIA(http://gepia.cancer-pku.cn//index.html).

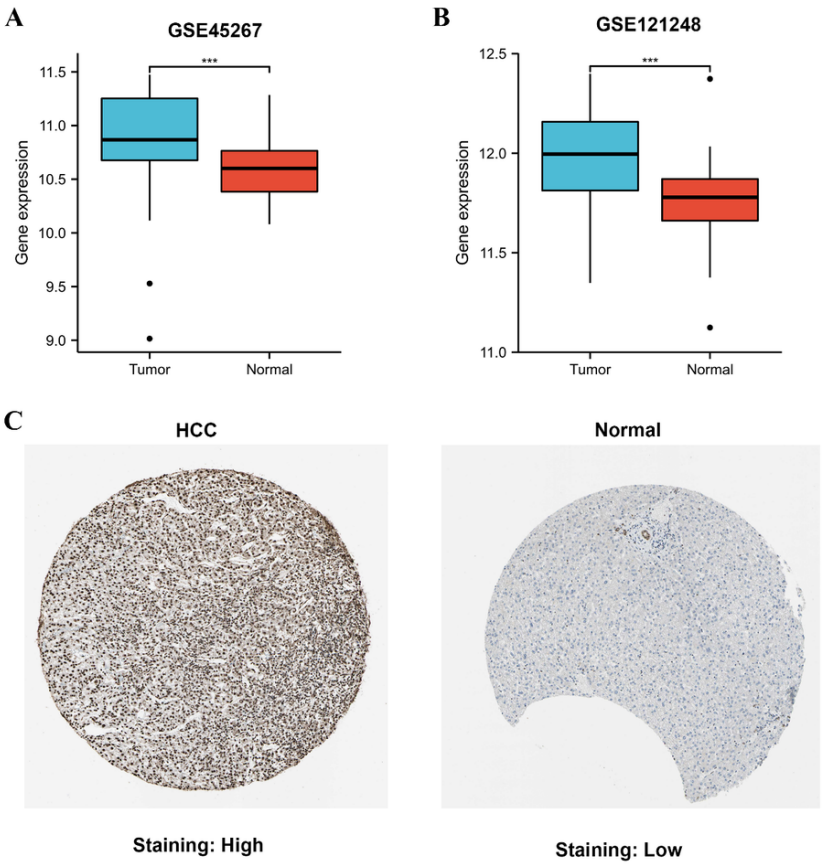

Figure 2

Analysis of the hnRNP K expression in GEO datasets and the human Protein atlas. **A.** the mRNA expression of hnRNP K in HCC and normal tissue in GSE45267 dataset. ***p < 0.001. **B.** the mRNA expression of hnRNP K in HCC and normal tissue in GSE121248 dataset. ***p < 0.001. **C.** Representative IHC images of hnRNP K in HCC and non-cancerous liver tissues derived from the HPA database. (Antibody CAB004435) HCC, hepatocellular carcinoma; HPA, Human Protein Atlas.

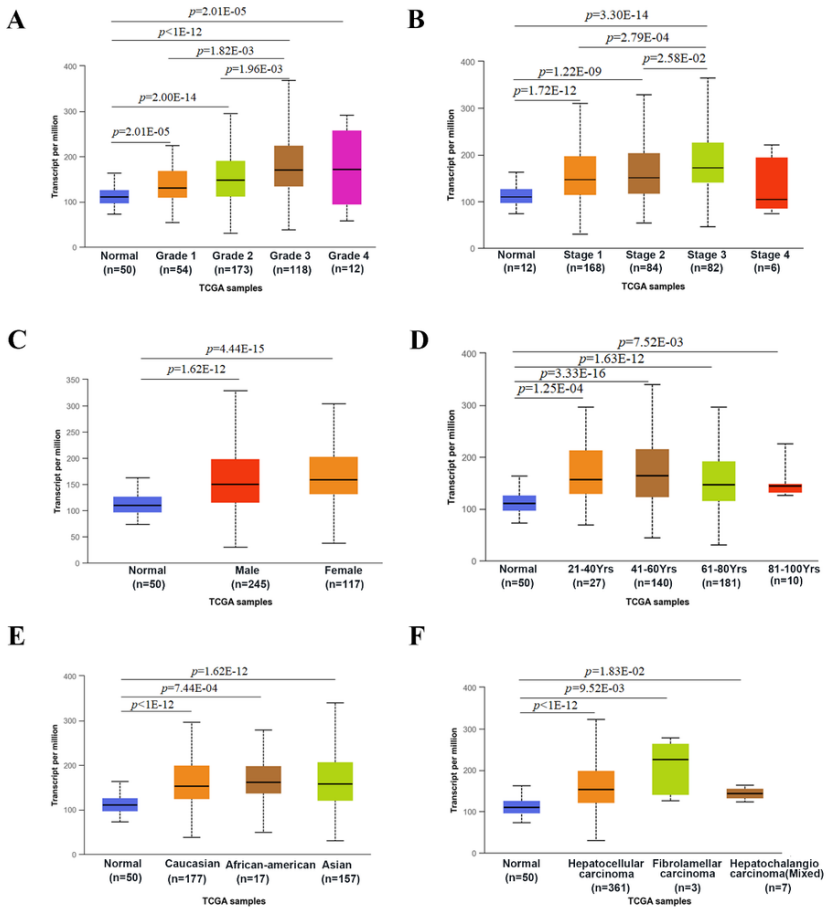

Figure 3

Association between hnRNP K expression and clinical characteristics of HCC patients using UALCAN (http://ualcan.path.uab.edu/index.html) for the patient characteristics of (**A**) tumor grade, (**B**) individual cancer stages, (**C**) patient gender, (**D**) patient age, (**E**) patient race and(**F**) histological subtypes. Box plots showing the hnRNP K mRNA expression level.

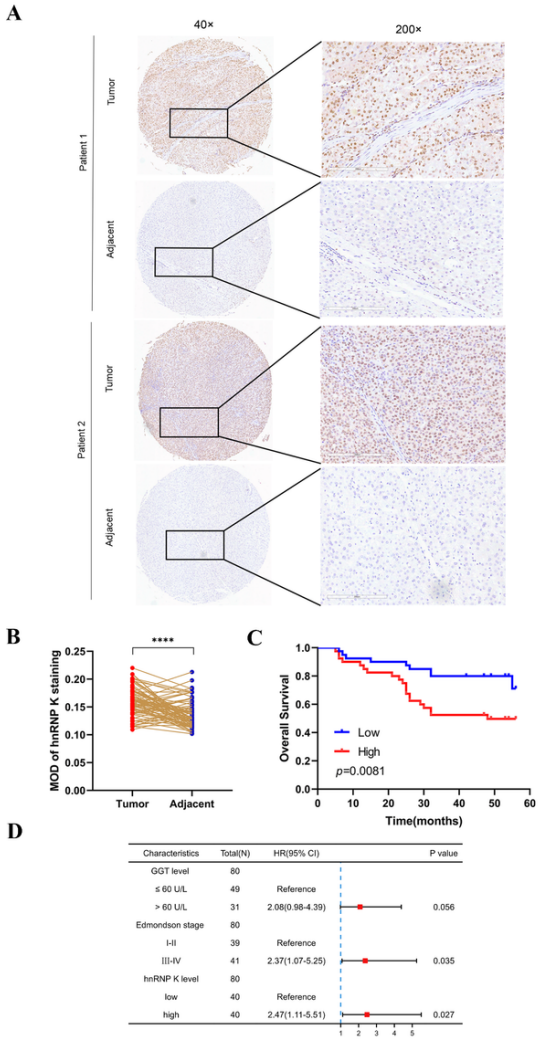

Figure 4

Upregulated hnRNP K expression correlates with poor outcomes of HCC patients. **A**. Representative images of hnRNP K IHC staining in HCC and adjacent normal tissue samples were shown (scale bars, 200 μm; magnification, 40× and 200×). **B**. MOD of matched HCC and normal tissues (n = 80) from HCC patients was evaluated via IHC. ****$p < 0.0001$. **C**. Kaplan-Meier analysis of overall survival of HCC patients based on hnRNP K expression (n = 80). Cutoffs for grouping were determined by the mean of MOD. **D**. Multivariate analysis was employed for HCC patients using COX regression model based on those factors which were statistically significant in univariate analysis. Forest plot of HRs and 95% CIs for overall survival in subgroups, HR, hazard ratio; CI , confidence interval.

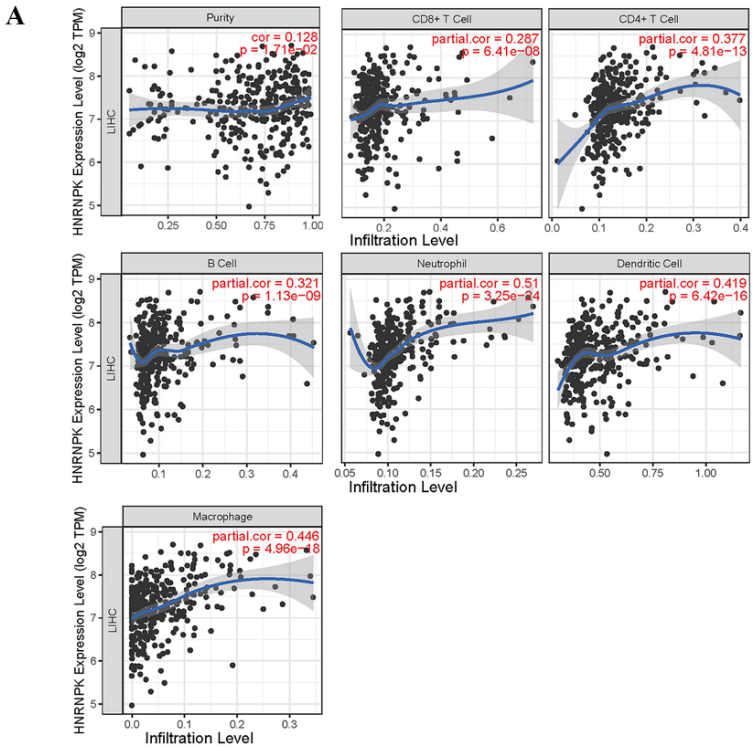

Figure 5

Correlation between hnRNP K expression and tumor immune infiltration levels in LIHC. **A**. hnRNP K expression was positively correlated with tumor purity and

infiltrating levels of B cells, $CD8^+$ T cells, $CD4^+$ T cells, neutrophils, macrophages and dendritic cells. cor, correlation; LIHC, liver hepatocellular carcinoma. $P < 0.05$ is considered as significant.

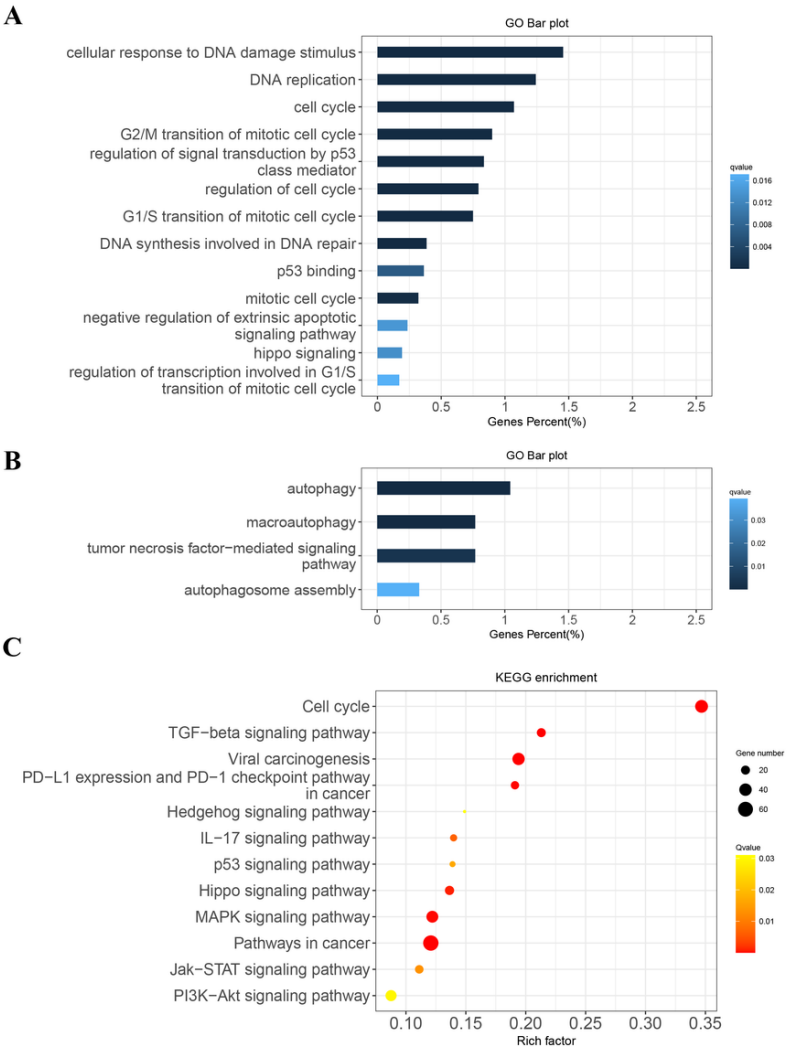

Figure 6

Co-expressed genes profile with the hnRNP K involved in signaling pathways in HCC. **A**. The bar graphs represent genes positively correlated to hnRNP K, showing the GO analysis of hnRNP K and the positively correlated genes in HCC. **B**. The bar graphs represent genes negatively correlated to hnRNP K, showing the GO analysis of hnRNP K and the negatively correlated genes in HCC. **C**. Bubble plot representation of the enriched KEGG pathways of hnRNP K and correlated genes.