Exploring the Prognostic Value and Biological Function of CPNE1 Gene in Hepatocellular Carcinoma

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Research Article

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Abstract

Background

Liver hepatocellular carcinoma (LIHC), the major histology subtype of primary liver cancer, accounts for 70-80% proportion of total liver cancer cases. CPNE1 (CPNE1), the first discovered CPNE1 family member, participates in the process of carcinogenesis and development of diverse tumors. Our study aimed to investigate the expression and prognostic value of CPNE1 gene in hepatocellular carcinoma (HCC), to explore its functional network in HCC and its effects on biological behaviors such as proliferation, migration and invasion of HCC cells, and to explore its related signaling pathways.

METHODS

HCCDB, CCLE and HPA online databases were used to explore the expression of CPNE1 gene in HCC tissues; LinkedOmics online database was used to analyze the co-expression network of CPNE1 in hepatocellular carcinoma, and gene set enrichment analysis (GSEA) was used for GO functional annotation, KEGG pathway enrichment analysis, kinase target enrichment, miRNA target enrichment and transcription factor target enrichment analysis. The expression levels of CPNE1 in normal hepatocytes and several hepatocellular carcinoma cell lines were detected by RT-qPCR, and finally HepG2 and MHCC-97H cells were selected to construct CPNE1 knockdown cell lines by transfection with siRNA, and the knockdown efficiency was detected by Western Blot and RT-qPCR. The effect of CPNE1 knockdown on the proliferation of hepatocellular carcinoma cells was examined by CCK8 assay and clone formation assay; the effect of CPNE1 knockdown on the migration ability of hepatocellular carcinoma cells was assessed by cell scratch assay and Transwell cell migration assay; finally, the expression of related signaling pathway proteins was examined by Western Blot. The correlation of CPNE1 expression with immune infiltration and immune checkpoint molecules in HCC tissues was analyzed using TIMER online database.

RESULTS

Analysis in several databases showed that CPNE1 was highly expressed in HCC tissues and significantly correlated with sex, age, cancer stage and tumor grade. Overall survival (OS) was significantly lower in patients with high CPNE1 expression than in patients with low CPNE1 expression, and CPNE1 could be used as an independent prognostic indicator for HCC. GSEA analysis showed that co-expressed genes of CPNE1 were mainly involved in biological processes such as establishment of protein localization to membrane, ribonucleoprotein complex biogenesis and lipid localization. Q-PCR showed that CPNE1 expression was upregulated in HCC cells compared with normal hepatocytes, and knockdown of CPNE1 gene inhibited the AKT/P53 pathway, resulting in decreased proliferation, migration and invasion of HCC cells. The level of CPNE1 expression in HCC was significantly and positively correlated with the level of infiltration of B cells, CD8⁺ T cells, CD4⁺ T cells, macrophages, neutrophils, and dendritic cells ($p < 0.001$), and with the expression of immune checkpoint molecules PDCD1, CD274, CTLA4, LAG3, HAVCR2, and TIGIT.

CONCLUSION

The expression of CPNE1 was significantly higher in HCC tissues than in normal liver tissues, and high CPNE1 expression was associated with poor prognosis. Knockdown of CPNE1 inhibited AKT/P53 pathway activation and suppressed HCC cell proliferation and migration. There was a significant correlation between CPNE1 expression and tumor immune infiltration in HCC.

Introduction

Liver cancer, one of the top five deadliest cancers globally, has the high mortality [1, 2]. Liver hepatocellular carcinoma (LIHC), the major histology subtype of primary liver cancer, accounts for 70-80% proportion of total liver cancer cases and is chiefly related to hepatitis C virus (HCV), hepatitis B virus (HBV) and alcoholism [3, 4]. Surgical resection or liver transplantations is the common treatment choice in patients with early hepatocellular carcinoma. However, many cases are usually refractory to treat surgically due to initial diagnosis at an advanced stage. Although advanced LIHC exists multiple treatments, including surgical excision, transarterial embolization, chemotherapy and radiofrequency ablation, the treatments effects are limited and LIHC still has a rate of recurrence as high as 70% [5]. In brief, patients with LIHC have a poor overall survival. Despite the risk factors (HBV, HCV, alcohol-related cirrhosis, smoking, diabetes, fatty liver disease, obesity, iron overload and multiple diet exposure) of LIHC is well known, the precise mechanism underlying development and progression of LIHC remains unclear [6, 7]. Therefore, in-depth studies exploring novel biomarkers and delineating its mechanism are urgently needed.

Copines family, a widely distributed and highly conserved throughout evolution phospholipid-binding protein, shares common structural features: 2 C2 domains in the N terminus, 1 von Willebrand factor A (VWA) domain in the C terminus [8, 9]. C2 domains possess properties of Ca²⁺ dependence and phospholipid-binding and may be associated with signal transduction and cell membranes transport. VWA domain could mediate interactions among extracellular proteins and may be related to recruitment of target proteins and regulating activity of specific proteins [10, 11]. In mammals, it has been identified that Copines family contains 9 members named sequentially as CPNE1-9 in order of discovery [9].

Copine1 (CPNE1), the first discovered CPNE1 family member [8], is located on human chromosome 20q11.21, encodes 537 amino acids and has multiple splice forms [12]. CPNE1 is observed to be upregulated in multiple tumor tissue compared to normal tissues. Studies have highlighted that CPNE1 involves in various cellular biology process, such as apoptosis, growth control, autophagy, mitotic, inflammation, exocytosis and cytoskeletal organization and gene transcription [13]. Meanwhile, CPNE1 participates in the process of carcinogenesis and development of breast cancer [14], non-small cell lung cancer [15], prostate cancer [16], liver cancer [17], thyroid cancer [18] and osteosarcoma [19]. The expression of CPNE1 is associated with TNM staging, lymph node metastasis and distant metastasis of lung adenocarcinoma [15]. The expression of CPNE1 is higher in prostate cancer tissue and castration-resistant prostate cancer tissue than that in normal prostatic tissues and noncastrated-resistant prostate

cancer tissue, respectively. Also, CPNE1 is significantly correlated with the tumor stage, Gleason score and recurrence-free survival of prostate cancer and is positively correlated with expression of TRAF2 as a prognostic marker in prostate cancer [16]. CPNE1 is linked to chromosome deletion of 13q in hepatic carcinoma cells and mediates the process of occurrence and progression by regulating the dedifferentiation, cell cycle and proliferation in liver cancer [17]. CPNE1 can act as potential biomarker to identify well-differentiated thyroid cancer tissue and normal thyroid tissues, which simplifies the process of early thyroid cancer diagnosis[18]. And yet, role of CPNE1 in LIHC is unclear.

Using multiple databases, we detected the expression level of CPNE1 and relationship between CPNE1 and clinicopathologic parameters of LIHC patients. Then, we established CPNE1 knockdown cell lines to explore effects of CPNE1 on malignant phenotypes of LIHC cell. Our results may provide theoretical basis for targeted therapy strategy of LIHC.

Methods

Expression analysis and survival analysis

We searched for the gene symbol 'CPNE1' using the HCCDB database. HCCDB provides visualization of the results of multiple computational analyses, such as differential expression analysis, tissue-specific and tumor-specific expression analysis [20]. Then, the expression of CPNE1 in cancer cell lines was validated using the Cancer Cell Line Encyclopedia (CCLE) dataset (<https://portals.broadinstitute.org/ccle>) [21]. In addition, we validated the protein expression of CPNE1 in the Human Protein Atlas (HPA) database (www.proteinatlas.org) [22].

The UALCAN database (<http://ualcan.path.uab.edu>) [23] was used for subgroup analysis of CPNE1 mRNA expression. The hepatocellular carcinoma of the liver (LIHC) dataset from The Cancer Genome Atlas (TCGA) was selected for analysis. CPNE1 expression levels (gender, age, cancer stage, tumor grade and TP53 mutation status) in different subgroups were analyzed. Then, we analyzed the prognostic significance of CPNE1 in hepatocellular carcinoma using the Kaplan-Meier survival mapping database (<http://kmplot.com>) [24].

Mutation and immune infiltration analysis

The frequency of mutations in CPNE1 in HCC was assessed using cBioPortal (<http://www.cbioportal.org/>) [25]. The mutation types of CPNE1 in HCC were further assessed using the Somatic Mutations in Cancer (COSMIC) database in the catalog (<http://cancer.sanger.ac.uk>) [26]. We assessed the correlation between CPNE1 expression and immune infiltration using the Tumor Immunization Estimation Resource (TIMER) database (<https://cistrome.shinyapps.io/timer/>) [27].

LinkedOmics

LinkedOmics (<http://www.linkedomics.org/login.php>) is a public portal containing multi-omics data from 32 cancers in TCGA [28]. In the "LinkFinder" module, we performed co-expression statistical analysis of

CPNE1 using Spearman's test, and the results are displayed as volcano and heat maps. In the "LinkInterpreter" module, we performed gene ontology (GO), Kyoto Gene and Genome Encyclopedia (KEGG) pathway, kinase-target enrichment, miRNA-target enrichment and transcription factor-target enrichment analysis by gene set enrichment analysis (GSEA). The screening criteria were set as false discovery rate (FDR) < 0.05, and the number of simulations was 1000.

Cell culture

Normal hepatocytes L02 as well as five human hepatoma cell lines MHCC-97H, HepG2, Hep3B, HIF and Huh7 were obtained from the Institute of Liver Diseases (Tongji Hospital, Wuhan, China) and preserved in Dulbecco's modified Eagle medium (DMEM, Hyclone), which contains 10% fetal bovine serum (FBS). Cells were incubated in an incubator containing 5% CO₂ at 37°C.

siRNA transfection

CPNE1-siRNA and control siRNA were synthesized by GeneChem Co, Ltd (Shanghai, China). Suspensions of MHCC-97H and HepG2 cells were prepared and diluted to 6×10^5 /ml. Subsequently, 500 µL of cell suspensions were inoculated into 6-well plates and incubated for 24 hr. The siCPNE1 was transfected into hepatocellular carcinoma cells using Lipofectamine 3000 Transfection Reagent (Invitrogen, USA) according to the manufacturer's protocol.

RNA extraction and real-time PCR assay

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, USA) and the manufacturer's manual was followed. Complementary DNA for reverse transcription was synthesized by the Prime Script RT kit (Takara, Tokyo, Japan). real-time PCR analysis was then performed. The 2- $\Delta\Delta$ Ct method was applied to determine differences between multiple samples. CPNE1 primer sequence: sense strand, 5'-ACCCACTCTGCGTCCTT-3'; antisense strand, 5'-TGGCGTCTTGTGTCTATG-3'.

Protein blotting analysis

The primary antibodies were as follows: anti-GAPDH antibody (Proteintech, 12789-1-AP, 1:1000), anti-CPNE1 antibody (Proteintech, 12789-1-AP, 1:1000), anti-AKT antibody (CST, 4695, 1:1000), anti-phosphoAKT (CST 4370, 1:1000) and anti-P53 antibody (CST, 9252, 1:1000). Anti-rabbit IgG (Promoter, Wuhan, China, 1:5000) and anti-mouse IgG (Promoter, Wuhan, China, 1:5000) were used as secondary antibodies.

CCK8 and clone formation

Cell proliferation capacity was measured by Cell Counting Kit 8 (CCK-8, Promotor, Wuhan, China) according to the instructions. After adding CCK-8 reagent to 96-well plates, the cells were incubated for 2 hours. The absorbance at 450 nm (OD₄₅₀) was recorded. Clone formation assay was used to assess the clonogenic ability of HCC cells. Monolayers (2×10^3 /well) were inoculated into 6-well plates. Afterwards,

cells were continuously cultured in DMEM (promoter, Wuhan, China), which was spiked with fetal bovine serum (10%, Gibco, Grand Island, NY, USA). 2 weeks later, colonies were fixed in an incubator at 37°C for 15 min using methanol and then stained with crystal violet (0.5%, Promoter, Wuhan, China) for 15 min. The number of colonies was counted under an optical microscope.

Transwell migration assay

Hepatocellular carcinoma cells (5×10^4 /ml), digested with 0.25% trypsin and conditioned with serum-free DMEM to a density of 1×10^5 cells/ml, were transferred to the upper chamber. DMEM medium (600 μ l) containing 10% FBS was added to the lower chamber. After incubation in a 5% CO₂, 37°C incubator for 24 h, the cells remaining on top of the Transwell membrane were removed with a cotton swab, and the cells migrating to the lower surface of the cells were fixed with methanol for 10 min and then stained with 0.1% crystal violet staining solution for 20 min. Images of migrating cells were taken by inverted microscopy. Five fields of view were randomly selected and the stained calls were counted. Repeat the experiment three times.

Statistical analysis

All statistical analyses were performed by R 3.6.3 software and GraphPad Prism 8.0 software. the Wilcoxon and Kruskal-Wallis tests were used to compare differences in the expression of CPNE1; logistic regression was used to analyze the relationship between clinicopathological characteristics and CPNE1; Kaplan-Meier survival analysis and log-rank test were used to compare the survival differences between the two groups; correlation analysis was performed according to Spearman's correlation coefficient. Other experimental data were compared between the two groups by t-test, and differences were considered statistically different at $p < 0.05$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Results

High expression of CPNE1 in LIHC

To evaluate the expression level of CPNE1 in HCC tissue and adjacent normal tissue, we analysed 10 HCC cohorts in HCCDB database and found the mRNA level of CPNE1 in HCC tissue was obviously higher than in adjacent normal tissues (Figure 1a). CPNE1 is overexpressed in HCC cell lines compared with most tumor types, which was obtained by the Cancer Cell Line Encyclopedia (CCLE) (Figure 1b). Moreover, we used HPA database to explore the protein expression of CPNE1. Compared to normal liver tissue, HCC tissue exhibited CPNE1 strong positive staining (Figure 1c). Here, we presented the images of HCC liver (Patient IDs: 2556) and normal liver (Patient IDs: 2429). All results suggested the expression level of CPNE1 was significantly upregulated in HCC.

To enhance the credibility of the above results, we evaluated the high expression of CPNE1 in LIHC sample from TCGA through the UALCAN database. Compared with the normal samples ($n = 50$), the mRNA level of CPNE1 was higher in the LIHC samples ($n = 371$) (Figure 2a). By Subgroup analysis, we

found that CPNE1 was also highly expressed in the subgroups of sex and age (Figure 2b, e). In terms of tumour stage and cancer grade, we found CPNE1 was highly expressed in grades 1-4 and stages 1-4 (Figure 2c, d). Furthermore, CPNE1 was evidently linked to TP53 mutation and was markedly upregulated in LIHC patients with TP53 mutations (Figure 2f). Collectively, these data implicated that the overexpression of CPNE1 was strongly linked to LIHC progression.

The prognostic value of CPNE1 in LIHC patients

By using Kaplan-Meier Plotter database, we explored the prognostic valences of CPNE1 in LIHC patients (n = 364). The overexpression of CPNE1 was evidently linked to poor overall survival (OS, HR = 1.73[1.22-2.45], log-rank P = 0.0017), progression-free survival (PFS, HR = 1.41[1.05-1.89], log-rank P = 0.021), relapse-free survival (RFS, HR = 1.34[0.96-1.86], log-rank P = 0.083) and disease-specific survival (DSS, HR = 2.08[1.32-3.26], log-rank P = 0.0011) of LIHC patients (Figure 3a-d). In all, the overexpression of CPNE1 was linked with poor prognosis of LIHC patients.

Using the Kaplan–Meier Plotter database, we investigated the correlations between CPNE1 expression and clinicopathologic features of LIHC patients to better find out the effect of CPNE1 on the survival of LIHC patients (Table 1,2). CPNE1 was linked with poor OS in many patients with LIHC, other than those with Female (HR=1.7, P =0.063), stage 1 (HR =1.66, P =0.15), or grade 3 (HR =1.64 , P =0.11), or hepatitis (HR =1.28 , P = 0.46) (Table 1). In terms of RFS, compared to grade 2/3, the HR values for RFS in patients with grade 1 indicated significant statistic differences (Table 1). In terms of PFS, CPNE1 had no significance in stage 1 (HR = 1.32, P = 0.3) or stage 2 (HR = 0.53, P =0.052) patients, those with grade 3 (HR =0.72, P = 0.21), hepatitis patients (HR =0.63, P = 0.066), or those with alcohol consumption (HR =0.62, P =0.099) (Table 2). In terms of DSS, compared to stage 2/3, the HR values for DSS in patients with stage 1 indicated no statistic difference (Table 2). Furthermore, compared to hepatitis patients, the HR values for PFS and OS in patients without hepatitis indicated significant statistic differences (Table 1). These results showed that overexpression of CPNE1 may decline survival in patients without hepatitis.

Mutations of CPNE1 in LIHC

In the cBioPortal database, we assessed the mutation frequency of CPNE1 in LIHC. In the 366 LIHC patients, CPNE1 gene alteration was identified in only 2 LIHC patients and the somatic mutation rate was 0.5% (Figure 4a). These alterations largely contained amplification and missense mutation (Figure 4a). The somatic mutation rate of CPNE1 was so low that we failed to find a correlation between CPNE1 mutation and the survival of LIHC patients. In addition, we further evaluated the mutation types of CPNE1 in COSMIC database. As shown, approximately 23.33% of the samples were found missense substitutions (Figure 4b). The substitution mutations occurred primarily at C > G (28.57%), followed by A > G (14.29%), A > T (14.29%), C >A (14.29%) , G >T (14.29%) and T > G (14.29%) (Figure c).

Co-expression genes of CPNE1 and enrichment analysis in patients with LIHC

To further elucidate the importance of CPNE1 in LIHC, we explored coexpression patterns of CPNE1 using LinkFinder module in LinkedOmics. The result showed 5,896 genes (dark red dots) were related positively to CPNE1, while 3,780 genes (dark green dots) were related negatively to CPNE1 in LIHC (FDR<0.05) (Figure 5a). Additionally, the top 50 genes clearly related (positively and negatively) to CPNE1 were displayed in Fig 5b and Fig 5c. CPNE1 expression showed a obvious positive link with expression of RALY ($r=0.545$, FDR = $4.06E-26$), SNRPB ($r=0.543$, FDR = $4.86E-26$), TPD52L2 ($r=0.536$, FDR = $2.51E-25$) and PRMT1 ($r=0.536$, FDR = $2.51E-25$). Notably, the top 50 clearly positive genes demonstrated the high possibility of being high risk genes in LIHC, in which 39/50 genes presented high HR (hazard ratio) ($p < 0.05$) (Figure 5f). By contrast, among the top 50 negatively correlated genes, there were 12/50 genes with low HR ($p < 0.05$) (Figure 5g).

Subsequently, we conducted Functional Enrichment Analysis. GO term revealed that CPNE1 and its coexpressed genes were primarily involved in the establishment of protein localization to membrane, ribonucleoprotein complex biogenesis, lipid localization and response to xenobiotic stimulus (Figure 5d). KEGG results showed these genes were mainly enriched in ribosome, spliceosome, metabolic pathways and bile secretion (Figure 5e).

Regulators of CPNE1 networks in LIHC

To further explore the possible regulators of CPNE1 in LIHC, we analysed networks of transcription factor (TF), miRNA or kinase targets of CPNE1 co-expression genes. Kinases ATR, CHEK1, PLK3, CHEK2 and DAPK1 were the top 5 most important targets (Table 3). Interestingly, CPNE1 co-expression genes were not enriched in any significant miRNA targets (Table 3). TF enrichment results revealed CPNE1 co-expression genes were predominantly enriched in E2F transcription factor family, including V\$E2F1DP2_01, V\$E2F_02, V\$E2F1_Q6_01, V\$E2F1DP1_01 and V\$E2F1DP2_01 (Table 3). Results above suggested that CPNE1 had wide-ranging impact on overall transcriptome in LIHC.

The expression level of CPNE1 in LIHC cell lines and the construction of knockdown cell lines

To validate whether CPNE1 was overexpressed in LIHC cell lines, we tested the mRNA expression level of CPNE1 in L02 (a human normal liver cell line) and 5 human hepatoma cell lines (MHCC-97H, HepG2, Hep3B, Huh7 and HLF) by using RT-qPCR. Compared with L02 cell, the expression level of CPNE1 in HepG2, MHCC-97H and huh7 was much higher, which indicated the expression level of CPNE1 in human hepatoma cell lines was higher than that in human normal liver cell line (Figure 6a). The result was consistent with our bioinformatic analysis.

From the results above, we selected HepG2 and MHCC-97H for the subsequent experiments and constructed CPNE1 knockdown cell lines. We chose 3 RNA interference targets (CPNE1-si1, CPNE1-si2, CPNE1-si3) transiently transfected into HepG2 and MHCC-97H cells. The knockdown efficiency of CPNE1 was detected by Western Blot and RT-qPCR. Compared with the negative control group, the expression level of CPNE1 in CPNE1-siRNA transfected cells was significantly decreased (Figure 6b-e). Of these,

CPNE1-si1 and CPNE1-si2 revealed a higher knockdown efficiency in HepG2 and MHCC-97H cells so we selected CPNE1-si1 and CPNE1-si2 for the subsequent experiments.

Effects of CPNE1 on LIHC cell proliferation

CCK8 assay was performed to test the difference of cell viability between negative control group and CPNE1-siRNA transfected group. The result showed the OD value of CPNE1-siRNA transfected cells was much smaller than control group after 48h, which indicated the cell viability of CPNE1 knockdown cell lines was considerably reduced in HepG2 and MHCC-97H cells (Figure 7a, b). In addition, plate clone formation assay revealed the clone numbers of CPNE1-siRNA transfected cells were less than control group in HepG2 and MHCC-97H cells (Figure 7c-e). Above results showed that knockdown of CPNE1 inhibited LIHC cells proliferation.

Effects of CPNE1 on LIHC cell migration

To further explore the correlations between CPNE1 and LIHC cell migration, the scratch assay was performed to test the impact of CPNE1 on LIHC cell migration. The result showed the healing rate of low CPNE1 expression group significantly reduced in comparison to control group in HepG2 and MHCC-97H cells (Figure 8a-d). This suggested that CPNE1 was related with the migration ability of HepG2 and MHCC-97H cells. Meanwhile, Transwell assay was conducted to validate the effect of CPNE1 on LIHC cell lines migration and invasion capabilities. Consistent with the scratch assay results, in HepG2 and MHCC-97H cells, the number of CPNE1 knockdown cells traversing to the lower chamber was less than that in control group (Figure 8e, f). These results indicated that CPNE1 participated in the regulation of migration and invasion capabilities in LIHC cell lines.

Effects of CPNE1 on AKT/P53 pathway in LIHC

Western blot experiment was conducted to detect protein expression levels of AKT and P53 for further exploration in LIHC. Compared to the control group, the protein expression level of p-AKT in CPNE1-siRNA group was markedly decreased and the expression level of TP53 was upregulated, while the expression level of total AKT indicated no significant change in HepG2 and MHCC-97H cells (Figure 9a, b). These results showed that the knockdown of CPNE1 may inhibit AKT/P53 pathway, thus suppressing cell proliferation of LIHC cell lines.

Correlation of CPNE1 expression with immune infiltration and immune markers in LIHC

We explored the relationship of CPNE1 expression and immune infiltration using TIMER. The correlation coefficients between CPNE1 expression and the abundances of multiple immune cells (dendritic cells, neutrophils, CD8 + T cells, macrophages, CD4 + T cells and B cells) were explored using Spearman tests. We found that CPNE1 expression had no correlation with tumour purity (cor =0.051, P = 3.47E-01). Furthermore, CPNE1 expression had significant association with all six immune cells infiltration,

especially B cells (cor =0.398, P = 1.65E-14), macrophages (cor =0.396, P =3.02E-14) and dendritic cells (cor =0.395, P =3.80E-14) (Figure 10a). CPNE1 mutation did not impact immune infiltration (Figure 10b). Additionally, by using Spearman correlation analysis in TIMER database, we assessed the correlation between CPNE1 expression and 7 immune checkpoint molecules (PDCD1-CD274-CTLA4-LAG3-HAVCR2-TIGIT) and found CPNE1 expression was strikingly positively correlated with 7 immune checkpoint molecules (Figure 10c). Then, we analysed the relationship between CPNE1 expression and immune subtypes, which indicated that CPNE1 expression was significantly correlated to immune subtypes in LIHC (P < 0.001) (Figure 10d).

Additionally, we also assessed the association between CPNE1 expression and correlated immune cell gene markers. Consistent with the above results, CPNE1 had significant positive association with almost gene markers of different immune cells. Among these, the top-five gene markers were PU.1(SPI1) (r = 0.407), TGFβ (TGFB1) (r = 0.403), STAT1 (r = 0.382), CD86 (r=0.379) and TNF-α (TNF) (r=0.376) (Table 4). All together, these results suggest that CPNE1 is critically engaged in immune infiltration during the advancement of LIHC.

Discussion

Liver cancer is one of the most frequent and fatal digestive malignancies and leads to over one million deaths every year around the world [29, 30]. LIHC is a highly aggressive disease and its 5-year post-surgical survival rate is 30%-40% [4, 31]. China has the highest incidence of liver cancer across the world [32]. Intrahepatic dissemination, extrahepatic infiltration and metastasis are the leading reason of poor prognosis in LIHC patients [33, 34]. The incidence of LIHC is continually increasing, Nevertheless, there is no successful therapy [35].

CPNE1, a tumor-related gene, plays the role of proto-oncogene to promote tumor development. Similar to other CPNE1 family members, the specialized structures determine the key role of CPNE1 in membranes transport and signal transduction [11]. Via vWA domain, CPNE1 could recruit, modulate transcription factors NF-κB and then activate TNF-α receptor, which in turn regulated TNF-α signaling. Meanwhile, the upregulation of TNF-α influenced the expression of CPNE1 and a positive feedback mechanism existed between CPNE1 and TNF-α. Also, CPNE1 mediated NF-κB signaling by facilitating TNF-α-dependent Inhibitory-κB (IκB) degradation [36]. Via interacting directly with p65, CPNE1 lead to shear of p65 N-terminus and terminated the transcription of NF-κB, which in turn inhibited transcriptional activation of NF-κB [37]. It had been recognized that NF-κB was linked to multiple behaviors of cancer cells, including cell proliferation, apoptosis, migration and invasion [38] and played pivotal functions in initiation and progression of many malignancies (breast cancer, lung cancer, gastric cancer) [39-42]. Study had reported that CPNE1 could promote proliferation and multi-differentiation potency of neuronal stem cells by activating AKT/mTOR signaling [43]. CPNE1 may regulate growth, migration and invasion of lung adenocarcinoma cells through AKT and ERK pathways, which could promote nonsmall-cell lung cancer progression [15]. CPNE1 was a target of miR-335-5 and CPNE1 silencing could effectively improve clinical responses of EGFR-tyrosine kinase inhibitors (TKIs) in non-small cell lung cancer [44]. In osteosarcoma,

downregulation of CPNE1 not only significantly impaired the proliferation and metastasis of Saos-2 cell and enhanced sensitivity to cisplatin and doxorubicin, but also changed the expression of genes related to ECM receptors-associated pathway, MAPK pathway, TGF- β pathway, apoptotic pathway and NOD-like receptor pathway [19]. CPNE1 may promote tumorigenesis and radioresistance of triple-negative breast cancer (TNBC) through AKT pathway activation and so target expression of CPNE1 could be a good strategy to sensitize TNBC to radiotherapy [14]. But the role of CPNE1 in liver cancer is not clear.

The role of a great deal genes is complex in the human body. The development of bioinformatics can markedly improve the accuracy and efficiency of studies target genes and cancer [25, 45]. In our study, we confirmed the expression of CPNE1 was higher in LIHC tissue than that in normal tissues. High expression of CPNE1 showed potential clinical significance and was linked to poor survival of LIHC patients. These results indicated that CPNE1 was a potential target for LIHC treatment. Except for altered gene expression, we also found the mutational signatures of CPNE1 were predominantly missense mutations. However, the mutation frequency of CPNE1 in LIHC was relatively low (only 0.5%) and we failed to find the association between these mutations and prognosis. More analysis are needed to confirm the clinical significance of CPNE1 mutations. To explore the intrinsic mechanisms of CPNE1 in LIHC, the coexpression network of CPNE1 was constructed and gene set enrichment analysis demonstrated CPNE1 and its coexpressed genes were primarily involved in the establishment of protein localization to membrane, ribonucleoprotein complex biogenesis, lipid localization and response to xenobiotic stimulus.

For exploring regulators potentially responsible for CPNE1 dysregulation, we found that CPNE1 is linked with a network of kinases including ATR, CHEK1, PLK3, CHEK2 and DAPK1 in LIHC. These kinases participate in the regulation of mitosis, DNA damage response, cell cycle and genomic stability, and exhibited survival prognosis and differential expression in LIHC. In fact, ATR, a member of phosphatidylinositol-3-kinase-related kinase family, is the major players of DNA damage response, and represents an attractive target for developing antimitotic agents [46]. In addition, activated ATR is critical in the late G2 and S phases to assure appropriate and replication of the whole genome [47, 48]. PLK3 may regulate cell cycle progression, centrosomal functions, mitosis, DNA replication, and Golgi fragmentation [49]. In many human malignancies, PLK3 expression was downregulated, including those in the stomach, kidney, head and neck, lung, colon, liver and rectum. Several studies demonstrated downregulated PLK3 expression may be linked with cancer development [48, 49].

MIR-493 and MIR-381 were the mainly miRNA Targets of CPNE1 in LIHC. MiR-493 has important functions and participates in different oncogenesis, including breast cancer, pancreatic cancer and gastric cancer. MiR-493 plays a key role in the recurrence, metastasis and generation of tumors [50]. MiR-381, one of the most significant miRNAs, regulates radioresistance [51], immune responses [52], epithelial–mesenchymal transition (EMT) [53] and chemotherapeutic resistance [54]. Moreover, miR-381 functions in AKT [55], p53 [56] and Wnt/ β -catenin [57] pathways, involves in tumor metastasis, progression and initiation. Multiple studies have indicated that miR-381 could be recognized as a biomarker [58].

Then, the E2F family account for the main transcription factors for CPNE1 dysregulation. E2F1 is one of the major bonds in the cell cycle regulatory network. In the progression of LIHC, activated E2F signaling was common, and studies have indicated that dosage-dependent copy number gains in E2F3 and E2F1 drive LIHC [59]. Our findings indicate that E2F1 is a critical regulator of CPNE1 and that CPNE1 might function by this factor to modulate the proliferation ability and cell cycle of LIHC.

Here, we revealed that the overexpression of CPNE1 was positively linked to immune infiltration. This finding demonstrates that CPNE1 plays a crucial role in immune infiltration during hepatocarcinogenesis. As far as we know, we are the first to explore the association of CPNE1 and immune infiltration in LIHC.

To validate the effect of CPNE1 on cell proliferation, migration and invasion in LIHC cell lines, we constructed CPNE1 knockdown cell lines and results revealed the capabilities of CPNE1 knockdown cell proliferation, migration and invasion decreased compared to control cells, which suggested that CPNE1 participated in the genesis and progression of LIHC. Furthermore, CPNE1 affected AKT/P53 pathway and might function by this pathway to modulate the malignant transformation of LIHC.

However, this study had some limitations. First, our findings were just confirmed in public databases and not in clinical samples. Second, the precise mechanism of CPNE1 involved in the development of LIHC was not elucidated in our study. Last, some of the findings may need further validation. Nonetheless, CPNE1 is a potential molecular target in the therapy of LIHC.

Conclusions

The expression of CPNE1 was significantly higher in HCC tissues than in normal liver tissues, and high CPNE1 expression was associated with poor prognosis. Knockdown of CPNE1 inhibited AKT/P53 pathway activation and suppressed HCC cell proliferation and migration. There was a significant correlation between CPNE1 expression and tumor immune infiltration in HCC.

Declarations

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Authors' contributions

The study was conceived and designed by HX. JS, YH, and YW performed most statistical analysis and wrote the manuscript. RL, WD, and HZ participated in collecting literature and helped to revise the manuscript. All authors read and approved the manuscript.

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Availability of data and materials

The data used to support the findings of this study are included within the manuscript.

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The authors declare that they have no competing interests.

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References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A: **Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries**. *CA: a cancer journal for clinicians* 2018, **68**(6):394-424.
2. Simard EP, Ward EM, Siegel R, Jemal A: **Cancers with increasing incidence trends in the United States: 1999 through 2008**. *CA: a cancer journal for clinicians* 2012, **62**(2):118-128.
3. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D: **Global cancer statistics**. *CA: a cancer journal for clinicians* 2011, **61**(2):69-90.

4. Altekruse SF, McGlynn KA, Reichman ME: **Hepatocellular carcinoma incidence, mortality, and survival trends in the United States from 1975 to 2005.** *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2009, **27**(9):1485-1491.
5. Bruix J, Colombo M: **Hepatocellular carcinoma: current state of the art in diagnosis and treatment.** *Best practice & research Clinical gastroenterology* 2014, **28**(5):751.
6. Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ: **Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults.** *The New England journal of medicine* 2003, **348**(17):1625-1638.
7. Center MM, Jemal A: **International trends in liver cancer incidence rates.** *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 2011, **20**(11):2362-2368.
8. Creutz CE, Tomsig JL, Snyder SL, Gautier MC, Skouri F, Beisson J, Cohen J: **The copines, a novel class of C2 domain-containing, calcium-dependent, phospholipid-binding proteins conserved from Paramecium to humans.** *The Journal of biological chemistry* 1998, **273**(3):1393-1402.
9. Tomsig JL, Creutz CE: **Biochemical characterization of copine: a ubiquitous Ca²⁺-dependent, phospholipid-binding protein.** *Biochemistry* 2000, **39**(51):16163-16175.
10. Tomsig JL, Creutz CE: **Copines: a ubiquitous family of Ca(2+)-dependent phospholipid-binding proteins.** *Cellular and molecular life sciences : CMLS* 2002, **59**(9):1467-1477.
11. Tomsig JL, Snyder SL, Creutz CE: **Identification of targets for calcium signaling through the copine family of proteins. Characterization of a coiled-coil copine-binding motif.** *The Journal of biological chemistry* 2003, **278**(12):10048-10054.
12. Yang W, Ng P, Zhao M, Wong TK, Yiu SM, Lau YL: **Promoter-sharing by different genes in human genome—CPNE1 and RBM12 gene pair as an example.** *BMC genomics* 2008, **9**:456.
13. Ilacqua AN, Price JE, Graham BN, Buccilli MJ, McKellar DR, Damer CK: **Cyclic AMP signaling in Dictyostelium promotes the translocation of the copine family of calcium-binding proteins to the plasma membrane.** *BMC cell biology* 2018, **19**(1):13.
14. Shao Z, Ma X, Zhang Y, Sun Y, Lv W, He K, Xia R, Wang P, Gao X: **CPNE1 predicts poor prognosis and promotes tumorigenesis and radioresistance via the AKT signaling pathway in triple-negative breast cancer.** *Molecular carcinogenesis* 2020, **59**(5):533-544.
15. Liu S, Tang H, Zhu J, Ding H, Zeng Y, Du W, Ding Z, Song P, Zhang Y, Liu Z *et al*: **High expression of Copine 1 promotes cell growth and metastasis in human lung adenocarcinoma.** *International journal of oncology* 2018, **53**(6):2369-2378.

16. Liang J, Zhang J, Ruan J, Mi Y, Hu Q, Wang Z, Wei B: **CPNE1 Is a Useful Prognostic Marker and Is Associated with TNF Receptor-Associated Factor 2 (TRAF2) Expression in Prostate Cancer.** *Medical science monitor : international medical journal of experimental and clinical research* 2017, **23**:5504-5514.
17. Skawran B, Steinemann D, Becker T, Buurman R, Flik J, Wiese B, Flemming P, Kreipe H, Schlegelberger B, Wilkens L: **Loss of 13q is associated with genes involved in cell cycle and proliferation in dedifferentiated hepatocellular carcinoma.** *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 2008, **21**(12):1479-1489.
18. Paricharttanakul NM, Saharat K, Chokchaichamnankit D, Punyarit P, Srisomsap C, Svasti J: **Unveiling a novel biomarker panel for diagnosis and classification of well-differentiated thyroid carcinomas.** *Oncology reports* 2016, **35**(4):2286-2296.
19. Jiang Z, Jiang J, Zhao B, Yang H, Wang Y, Guo S, Deng Y, Lu D, Ma T, Wang H *et al*: **CPNE1 silencing inhibits the proliferation, invasion and migration of human osteosarcoma cells.** *Oncology reports* 2018, **39**(2):643-650.
20. Lian Q, Wang S, Zhang G, Wang D, Luo G, Tang J, Chen L, Gu J: **HCCDB: A Database of Hepatocellular Carcinoma Expression Atlas.** *Genomics, proteomics & bioinformatics* 2018, **16**(4):269-275.
21. Ghandi M, Huang FW, Jané-Valbuena J, Kryukov GV, Lo CC, McDonald ER, 3rd, Barretina J, Gelfand ET, Bielski CM, Li H *et al*: **Next-generation characterization of the Cancer Cell Line Encyclopedia.** *Nature* 2019, **569**(7757):503-508.
22. Pontén F, Jirström K, Uhlen M: **The Human Protein Atlas—a tool for pathology.** *The Journal of pathology* 2008, **216**(4):387-393.
23. Chandrashekar DS, Bashel B, Balasubramanya SAH, Creighton CJ, Ponce-Rodriguez I, Chakravarthi B, Varambally S: **UALCAN: A Portal for Facilitating Tumor Subgroup Gene Expression and Survival Analyses.** *Neoplasia (New York, NY)* 2017, **19**(8):649-658.
24. Menyhart O, Nagy Á, Györffy B: **Determining consistent prognostic biomarkers of overall survival and vascular invasion in hepatocellular carcinoma.** *Royal Society open science* 2018, **5**(12):181006.
25. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E *et al*: **The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data.** *Cancer discovery* 2012, **2**(5):401-404.
26. Forbes SA, Beare D, Boutselakis H, Bamford S, Bindal N, Tate J, Cole CG, Ward S, Dawson E, Ponting L *et al*: **COSMIC: somatic cancer genetics at high-resolution.** *Nucleic acids research* 2017, **45**(D1):D777-d783.
27. Li T, Fan J, Wang B, Traugh N, Chen Q, Liu JS, Li B, Liu XS: **TIMER: A Web Server for Comprehensive Analysis of Tumor-Infiltrating Immune Cells.** *Cancer research* 2017, **77**(21):e108-e110.

28. Vasaikar SV, Straub P, Wang J, Zhang B: **LinkedOmics: analyzing multi-omics data within and across 32 cancer types**. *Nucleic acids research* 2018, **46**(D1):D956-d963.
29. Siegel RL, Miller KD, Jemal A: **Cancer statistics, 2016**. *CA: a cancer journal for clinicians* 2016, **66**(1):7-30.
30. Pan JJ, Javle M, Thinn MM, Hsueh CT, Hsueh CT: **Critical appraisal of the role of sorafenib in the management of hepatocellular carcinoma**. *Hepatic medicine : evidence and research* 2010, **2**:147-155.
31. Zhu AX, Duda DG, Sahani DV, Jain RK: **HCC and angiogenesis: possible targets and future directions**. *Nature reviews Clinical oncology* 2011, **8**(5):292-301.
32. Wei KR, Yu X, Zheng RS, Peng XB, Zhang SW, Ji MF, Liang ZH, Ou ZX, Chen WQ: **Incidence and mortality of liver cancer in China, 2010**. *Chinese journal of cancer* 2014, **33**(8):388-394.
33. de Lope CR, Tremosini S, Forner A, Reig M, Bruix J: **Management of HCC**. *Journal of hepatology* 2012, **56** Suppl 1:S75-87.
34. Finn RS: **Advanced HCC: emerging molecular therapies**. *Minerva gastroenterologica e dietologica* 2012, **58**(1):25-34.
35. Bruix J, Gores GJ, Mazzaferro V: **Hepatocellular carcinoma: clinical frontiers and perspectives**. *Gut* 2014, **63**(5):844-855.
36. Cheal Yoo J, Park N, Lee B, Nashed A, Lee YS, Hwan Kim T, Yong Lee D, Kim A, Mi Hwang E, Yi GS *et al*: **14-3-3 γ regulates Copine1-mediated neuronal differentiation in HiB5 hippocampal progenitor cells**. *Experimental cell research* 2017, **356**(1):85-92.
37. Yoo JC, Park N, Choi HY, Park JY, Yi GS: **JAB1 regulates CPNE1-related differentiation via direct binding to CPNE1 in HiB5 hippocampal progenitor cells**. *Biochemical and biophysical research communications* 2018, **497**(1):424-429.
38. Manning BD, Toker A: **AKT/PKB Signaling: Navigating the Network**. *Cell* 2017, **169**(3):381-405.
39. Mundi PS, Sachdev J, McCourt C, Kalinsky K: **AKT in cancer: new molecular insights and advances in drug development**. *British journal of clinical pharmacology* 2016, **82**(4):943-956.
40. Cheon DJ, Orsulic S: **Mouse models of cancer**. *Annual review of pathology* 2011, **6**:95-119.
41. Singh M, Johnson L: **Using genetically engineered mouse models of cancer to aid drug development: an industry perspective**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2006, **12**(18):5312-5328.
42. Hay N: **The Akt-mTOR tango and its relevance to cancer**. *Cancer cell* 2005, **8**(3):179-183.

43. Kim TH, Sung SE, Cheal Yoo J, Park JY, Yi GS, Heo JY, Lee JR, Kim NS, Lee DY: **Copine1 regulates neural stem cell functions during brain development.** *Biochemical and biophysical research communications* 2018, **495**(1):168-173.
44. Tang H, Zhu J, Du W, Liu S, Zeng Y, Ding Z, Zhang Y, Wang X, Liu Z, Huang J: **CPNE1 is a target of miR-335-5p and plays an important role in the pathogenesis of non-small cell lung cancer.** *Journal of experimental & clinical cancer research : CR* 2018, **37**(1):131.
45. Tomczak K, Czerwińska P, Wiznerowicz M: **The Cancer Genome Atlas (TCGA): an immeasurable source of knowledge.** *Contemporary oncology (Poznan, Poland)* 2015, **19**(1a):A68-77.
46. Blackford AN, Jackson SP: **ATM, ATR, and DNA-PK: The Trinity at the Heart of the DNA Damage Response.** *Molecular cell* 2017, **66**(6):801-817.
47. Gorecki L, Andrs M, Korabecny J: **Clinical Candidates Targeting the ATR-Chk1-Wee1 Axis in Cancer.** *Cancers* 2021, **13**(4).
48. Brown EJ, Baltimore D: **ATR disruption leads to chromosomal fragmentation and early embryonic lethality.** *Genes & development* 2000, **14**(4):397-402.
49. Wiest J, Clark AM, Dai W: **Intron/exon organization and polymorphisms of the PLK3/PRK gene in human lung carcinoma cell lines.** *Genes, chromosomes & cancer* 2001, **32**(4):384-389.
50. Huang L, Huang L, Li Z, Wei Q: **Molecular Mechanisms and Therapeutic Potential of miR-493 in Cancer.** *Critical reviews in eukaryotic gene expression* 2019, **29**(6):521-528.
51. Zhou S, Cui Y, Yu D, Liang J, Zhang M, Ye W: **MicroRNA-381 enhances radiosensitivity in esophageal squamous cell carcinoma by targeting X-linked inhibitor of apoptosis protein.** *OncoTargets and therapy* 2017, **10**:2527-2538.
52. Wen Q, Zhou C, Xiong W, Su J, He J, Zhang S, Du X, Liu S, Wang J, Ma L: **MiR-381-3p Regulates the Antigen-Presenting Capability of Dendritic Cells and Represses Antituberculosis Cellular Immune Responses by Targeting CD1c.** *Journal of immunology (Baltimore, Md : 1950)* 2016, **197**(2):580-589.
53. Yang X, Ruan H, Hu X, Cao A, Song L: **miR-381-3p suppresses the proliferation of oral squamous cell carcinoma cells by directly targeting FGFR2.** *American journal of cancer research* 2017, **7**(4):913-922.
54. Chan Y, Yu Y, Wang G, Wang C, Zhang D, Wang X, Wang Z, Jian W, Zhang C: **Inhibition of MicroRNA-381 Promotes Tumor Cell Growth and Chemoresistance in Clear-Cell Renal Cell Carcinoma.** *Medical science monitor : international medical journal of experimental and clinical research* 2019, **25**:5181-5190.
55. Qiao G, Li J, Wang J, Wang Z, Bian W: **miR-381 functions as a tumor suppressor by targeting ETS1 in pancreatic cancer.** *International journal of molecular medicine* 2019, **44**(2):593-607.

56. Liang HQ, Wang RJ, Diao CF, Li JW, Su JL, Zhang S: **The PTTG1-targeting miRNAs miR-329, miR-300, miR-381, and miR-655 inhibit pituitary tumor cell tumorigenesis and are involved in a p53/PTTG1 regulation feedback loop.** *Oncotarget* 2015, **6**(30):29413-29427.

57. Xia B, Li H, Yang S, Liu T, Lou G: **MiR-381 inhibits epithelial ovarian cancer malignancy via YY1 suppression.** *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine* 2016, **37**(7):9157-9167.

58. Tian C, Li J, Ren L, Peng R, Chen B, Lin Y: **MicroRNA-381 serves as a prognostic factor and inhibits migration and invasion in non-small cell lung cancer by targeting LRH-1.** *Oncology reports* 2017, **38**(5):3071-3077.

59. Kent LN, Bae S, Tsai SY, Tang X, Srivastava A, Koivisto C, Martin CK, Ridolfi E, Miller GC, Zorko SM *et al.* **Dosage-dependent copy number gains in E2f1 and E2f3 drive hepatocellular carcinoma.** *The Journal of clinical investigation* 2017, **127**(3):830-842.

Tables

Table1. Correlation between CPNE1 mRNA expression and OS and RFS in HCC based on different clinical parameters by Kaplan-Meier plotter.

Clinical characteristics	OS (N=364)			RFS (N=316)		
	N	HR	P-value	N	HR	P-value
Gender						
Female	118	1.7 (0.97-3)	0.063	106	2.02(1.11-3.66)	0.019
Male	246	1.74 (1.12-2.71)	0.013	210	0.66 (0.43-1.02)	0.061
Stage						
I	170	1.66 (0.83-3.32)	0.15	153	0.81 (0.47-1.41)	0.46
II	83	2.24 (1-5.03)	0.045	75	0.48 (0.23-0.98)	0.039
III	83	2.53 (1.37-4.86)	0.0022	70	2.04 (1.08-3.85)	0.026
IV	4	NA	NA	0	NA	NA
Grade						
1	55	3.16 (1.19-8.41)	0.015	45	4.1 (1.48-11.38)	0.0036
2	174	1.77 (1.06-2.96)	0.027	149	1.62 (0.99-2.65)	0.055
3	118	1.64 (0.89-3.05)	0.11	107	0.67 (0.39-1.17)	0.16
4	12	NA	NA	11	NA	NA
Hepatitis virus						
Yes	150	1.28 (0.67-2.45)	0.46	139	0.6 (0.35-1.01)	0.053
None	167	1.85 (1.15-2.96)	0.0097	143	2.22(1.33-3.71)	0.0017
Alcohol consumption						
Yes	115	2.3 (1.18-4.49)	0.012	99	0.65 (0.33-1.29)	0.22
None	202	1.83 (1-3.34)	0.046	183	1.56 (1-2.45)	0.05

OS: Overall Survival, RFS: Relapse Free Survival, HR: Hazard ratio

Table2. Correlation between CPNE1 mRNA expression and PFS and DSS in HCC based on different clinical parameters by Kaplan-Meier plotter.

Clinical characteristics	PFS (N=370)			DSS (N=362)		
	N	HR	P-value	N	HR	P-value
Gender						
Female	121	2.11 (1.26-3.53)	0.038	118	2.06 (0.98-4.33)	0.051
Male	249	0.64 (0.43-0.94)	0.023	244	2.06 (1.16-3.64)	0.011
Stage						
I	171	1.32 (0.78-2.25)	0.3	168	1.71 (0.69-4.22)	0.24
II	85	0.53 (0.28-1.02)	0.052	83	3.37 (1.13-10.06)	0.021
III	85	2.15 (1.2-3.82)	0.0082	83	2.97 (1.41-6.26)	0.0029
IV	5	NA	NA	3	NA	NA
Grade						
1	55	3.83 (1.67-8.75)	0.00069	55	3.24 (0.91-11.53)	0.056
2	177	1.65 (1.05-2.6)	0.03	171	2.31 (1.17-4.54)	0.013
3	121	0.72 (0.43-1.21)	0.21	119	2.07 (0.93-4.61)	0.068
4	12	NA	NA	12	NA	NA
Hepatitis virus						
Yes	153	0.63 (0.39-1.03)	0.066	151	1.85 (0.81-4.23)	0.14
None	169	2.4 (1.54-3.75)	7.7e-05	165	2.52 (1.37-4.65)	0.0022
Alcohol consumption						
Yes	117	0.62 (0.35-1.1)	0.099	117	2.5 (1.16-5.41)	0.016
None	215	1.68 (1.13-2.52)	0.01	199	2.9 (1.48-5.7)	0.0012

PFS: Progression Free Survival, DSS: Disease Specific Survival, HR: Hazard ratio

Table 3 The Kinases, miRNA and transcription factors-target networks of CPNE1 in LIHC (LinkedOmics).

Enriched Category	Geneset	LeadingEdgeNum	FDR
Kinase Target	Kinase_ ATR	29	0
	Kinase_ CHEK1	46	0
	Kinase_ PLK3	12	0.0011024
	Kinase_ CHEK2	11	0.0014699
	Kinase_ DAPK1	8	0.0022048
miRNA Target	ATGTACA,MIR-493	70	0
	CTTGTAT,MIR-381	65	0
	TGCACGA,MIR-517A,MIR-517C	3	0.041935
	CACGTTT,MIR-302A	6	0.048338
	AGTCTTA,MIR-499	19	0.060606
Transcription Factor	SGCGSSAAA_V\$E2F1DP2_01	63	0.0016107
	V\$E2F_02	79	0.0020939
	V\$E2F1_Q6_01	79	0.0021476
	V\$E2F1DP1_01	79	0.0023265
	V\$E2F1DP2_01	79	0.0023265

Figures

Figure 1

The expression of CPNE1 in LIHC. (a) Chart and plot displaying the expression of CPNE1 in cancer tissues and the adjacent normal tissues (HCCDB). (b) The mRNA expression of CPNE1 in multiple cancer cell lines (CCLE). (c) Protein expression of CPNE1 (HPA).

Figure 2

Subgroup expression analysis of CPNE1 in LIHC (UALCAN). (a) The RNA expression of CPNE1 in normal and LIHC patients. (b) The mRNA expression of CPNE1 in normal and LIHC patients with different genders. (c) The mRNA expression of CPNE1 in normal and LIHC patients with different tumour grades. (d) The mRNA expression of CPNE1 in normal and LIHC patients with different tumour stages. (e) The mRNA expression of CPNE1 in normal and LIHC patients with different ages. (f) The mRNA expression of CPNE1 in normal and LIHC patients with different TP-53 mutant status.

Figure 3

High expression of CPNE1 predicts poor prognosis in LIHC (Kaplan-Meier Plotter). (a) The prognostic values of CPNE1 in OS of LIHC patients. (b) The prognostic values of CPNE1 in PFS of LIHC patients. (c) The prognostic values of CPNE1 in RFS of LIHC patients. (d) The prognostic values of CPNE1 in DSS of LIHC patients.

Figure 4

CPNE1 mutations in LIHC. (a) The schematic representation of CPNE1 mutations in LIHC (cBioPortal). (b-c) The mutation types of CPNE1 in LIHC (COSMIC).

Figure 5

Co-expression genes of CPNE1 in LIHC (LinkedOmics). (a) Correlations between CPNE1 and differently expressed genes (Spearman test). (b, c) Heat maps showing top 50 genes positively and negatively correlated with CPNE1 in LIHC. (d) Enrichment GO: Biological process annotations analysis of CPNE1 in LIHC. (e) Enrichment KEGG pathways of CPNE1 in LIHC. (f) Survival map of the top 50 genes positively correlated with CPNE1 in LIHC. (g) Survival map of the top 50 genes negatively correlated with CPNE1 in LIHC.

Figure 6

The expression of CPNE1 in LIHC cell lines and the construction of CPNE1 knockdown cell lines. (a) The mRNA expression level of CPNE1 in LIHC cell lines by RT-qPCR. (b) The mRNA expression level of CPNE1 in HepG2 transfected CPNE1-siRNA by RT-qPCR. (c) The mRNA expression level of CPNE1 in MHCC-97H transfected CPNE1-siRNA by RT-qPCR. (d) The protein expression level of CPNE1 in HepG2 transfected CPNE1-siRNA by Western Blot. (e) The protein expression level of CPNE1 in MHCC-97H transfected CPNE1-siRNA by Western Blot.

Figure 7

Effects of CPNE1 on cell proliferation in LIHC. (a) Effects of CPNE1 on cell viability in HepG2 by CCK8. (b) Effects of CPNE1 on cell viability in MHCC97-H by CCK8. (c-e) Effects of CPNE1 on cell clonal formation ability in HepG2 and MHCC97-H by plate clone formation assay.

Figure 8

Effects of CPNE1 on cell migration in LIHC (a-d) Effects of CPNE1 on cell migration by using scratch assay in HepG2 and MHCC-97H cells. (e, f) Effects of CPNE1 on cell migration by using Transwell assay in HepG2 and MHCC-97H cells.

Figure 9

Effects of CPNE1 on AKT/P53 pathway in LIHC (a) Effects of CPNE1 knockdown on AKT/P53 pathway in HepG2 cell. (b) Effects of CPNE1 knockdown on AKT/P53 pathway in MHCC-97H cell.

Figure 10

Correlations of CPNE1 expression with immune infiltration in LIHC. (a) Correlations of CPNE1 with infiltrating levels of dendritic cells, macrophages, CD8+ T cells, neutrophils, B cells and CD4+ T cells in LIHC. (b) Correlations of CPNE1 mutation with immune infiltration. (c) Correlations of CPNE1 with the expression level of multiple immune checkpoint molecules (d) Correlation of CPNE1 expression and immune subtypes