



***CDKN2A* as a prognostic and diagnostic marker is a possible key contributor in hepatocellular carcinoma**

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Background: Hepatocellular carcinoma (HCC) represents one of the deadliest cancers with a rising incidence worldwide. Although the treatment of HCC has made some breakthroughs, the incidence and mortality of HCC are still increasing. The major cause for this is the lack of early diagnostic markers and effective therapeutic targets. This study aimed at identifying new diagnostic and therapeutic candidates, and determining the expression characteristic, clinical relevance, prognostic significance, diagnostic value and expression regulation of cyclin-dependent kinase inhibitor 2A (*CDKN2A*) in HCC.

Methods: Whole transcriptome sequencing data of normal and HCC tissues were used to screen and identify the diagnostic and therapeutic candidates. Tumor Immune Estimation Resource (TIMER) 2.0, University of California Santa Cruz (UCSC) Xena, Kaplan-Meier, immunohistochemical (IHC) analysis of tissue microarray, ESTIMATE, LinkedOmics, STRING and GeneMANIA were used to analyze the associations of *CDKN2A* expression with clinicopathological indices and tumor immune microenvironment, and determine the prognostic significance, diagnostic value, and possible expression regulation of *CDKN2A*.

Results: *CDKN2A* expression was significantly increased in HCC tissues, and closely correlated with patients' tumor size and clinical stage. High expression of *CDKN2A* was closely associated with poor prognosis and tumor microenvironment of HCC patients. *CDKN2A* expression has a clear diagnostic value for HCC. The up-regulation of *CDKN2A* in HCC may be related to the methylation of its promoter region. The enrichment analyses of *CDKN2A* co-expressed genes and interacting proteins revealed that *CDKN2A* likely promoted HCC progression through involvement of cell cycle regulation.

Conclusions: *CDKN2A* may serve as a new and promising prognostic and diagnostic marker, and an important therapeutic target in HCC.

Keywords: Hepatocellular carcinoma (HCC); prognosis; diagnosis; immune microenvironment; expression regulation

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Introduction

Liver cancer mainly includes hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC), of which HCC accounts for 85–90% (1). Early diagnosis of HCC is difficult, and most patients with HCC (about 90%) are already in the middle and late stages when diagnosed, resulting in the survival of HCC patients being generally less than 6 months and the survival rate of patients with more than 5 years being less than 10% (2). Over the years, some breakthroughs have been made in the treatment of HCC, such as surgery, liver transplantation, targeting and immunotherapy, which have improved the treatment effect of HCC and extended the survival time of patients. However, the incidence and mortality of HCC are not decreased, but still increased year by year. The main cause for this issue is the lack of early diagnostic markers and effective therapeutic targets. Therefore, there is an urgent need to identify new molecular markers for early diagnosis, pinpoint key regulatory factors, and elucidate the specific molecular mechanisms underlying HCC pathogenesis. Developing effective diagnostic and treatment methods based on these findings is crucial for the prevention and ultimate cure of HCC. This approach represents the most promising strategy to comprehensively address and combat the disease. In general, the molecules with potential to

serve as diagnostics and key players in HCC are bound to undergo the most pronounced changes. Following extensive screening and analysis using big data genomics and comprehensive literature reviews, cyclin-dependent kinase inhibitor 2A (*CDKN2A*) has emerged as one of the most aberrantly expressed yet under-investigated molecules in HCC. Human *CDKN2A* is localized on chromosome 9 (9p21.3), and has been reported to be aberrantly expressed in a variety of tumors, including lung, oesophageal, gastric, colorectal, ovarian, leukaemia and renal cancers (3–6). Previous study has revealed that *CDKN2A* plays a key role in the cell cycle as an oncogene and prognostic marker in acute lymphoblastic leukemia (4). *CDKN2A* specifically binds to cyclin-dependent kinase 4/6 (*CDK4/6*), inhibiting the formation of the *CDK4/6-Cyclin D* complex. This leads to the suppression of the *Rb/E2F* transcriptional complex activity, thereby inducing G1 phase cell cycle arrest (3). Recent studies have identified *CDKN2A* as an important tumor suppressor in cervical and lung cancers (5,6). These findings suggest that *CDKN2A* may act an important role and has a potential diagnostic value in HCC. But the expression regulation, clinical values, main function, and possible mechanism of *CDKN2A* in HCC, are still largely unclear.

In this study, we found that *CDKN2A* may be an important candidate for (early) clinical diagnosis of HCC and may play key roles in the process of HCC development and metastasis. We present this article in accordance with the REMARK reporting checklist (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-24-1845/rc>).

Highlight box

Key findings

- Cyclin-dependent kinase inhibitor 2A (*CDKN2A*) has emerged as a promising prognostic and diagnostic marker for hepatocellular carcinoma (HCC) and may play crucial roles in HCC development and metastasis by regulating the tumor cell cycle.

What is known and what is new?

- Despite ongoing efforts, the incidence and mortality rates of HCC continue to rise annually. A primary contributor to this trend is the lack of effective early diagnostic markers and therapeutic targets.
- This study suggests that *CDKN2A* may serve as a novel and promising prognostic and diagnostic marker, as well as an important therapeutic target in HCC.

What is the implication, and what should change now?

- This study offers a promising potential intervention target for the (early) diagnosis and effective treatment of HCC patients. However, some of the current findings require large-scale validation using expanded experimental samples. Additionally, the proposed mechanisms, which have been predicted using computer algorithms, will need to be confirmed through a series of subsequent functional experiments.

Methods

Patient samples

Tissue microarray (TMA) containing cancerous and paracancerous tissues from 89 HCC patients who underwent surgical resection between 2007 and 2008 were purchased from Shanghai Outdo Biotech Co., Ltd. (Shanghai, China). The clinicopathological information was retrieved from the patient's electronic medical record, including age, gender, lymph nodes (negative or positive), tumor size, histological grade and clinical stage defined according to the 7th edition of the American Joint Committee on Cancer (AJCC) and the new tumor node metastasis (TNM) classification, as well as follow-up information (5–8 years) for overall survival (OS) rate and disease-free survival (DFS) rate. The patients were followed for a maximum of 104 months and a

median follow-up of 49 months. OS was defined as the time from surgery to death or last observation. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Ethics Committee of Shanghai Outdo Biotech Company (No. YB M-05-02) and the Ethics Committee of Third Affiliated Hospital of Chongqing Medical University (CQMU, No. 2021-95). All patients signed an informed consent form.

TMA generation

The samples of 89 HCC patients were histologically examined using hematoxylin and eosin staining. To construct TMA sections, two cores were taken from each representative tumor and adjacent non-cancerous tissue (within distance of 20 mm). Adjacent non-cancerous tissue was compared with normal tissue, stained with hematoxylin-eosin and examined histologically by at least two pathologists.

Immunohistochemical (IHC) analysis

IHC analysis was performed using CDKN2A antibody (specifically recognizes p16/INK4A, GB111143-100, Servicebio, Wuhan, China). IHC staining results were assessed, evaluated, and quantified under a microscope by two pathologists. The percentage of positive cells was calculated based on the ratio of positive cells to the total number of cells. Staining was considered positive when the percentage of tumor cells staining was $\geq 10\%$. The percentage of positive cells was scored into five categories: 0 ($<10\%$), 1 (10–25%), 2 (26–50%), 3 (51–75%), and 4 ($>75\%$). Staining intensity was scored in four categories: 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). Protein expression levels were determined by multiplying the scores of positive cell percentage and staining intensity (the range of CDKN2A protein expression: 0–12). Samples were then grouped into high and low expression categories based on the average value.

Analysis of available datasets

Screening for important changed molecules in HCC using the Gene Expression Profiling Interactive Analysis (GEPIA) 2.0 website

Whole transcriptome sequencing data from tumours and normal tissues in the HCC dataset ($|\log_2$ fold change| ≥ 1 , $P \leq 0.01$) were analyzed via the GEPIA 2.0 (<http://gepia2.cancer-pku.cn/>) website to identify molecules that underwent significant changes. The GEPIA 2.0

website was further used to analyze the differences of the top 10 expression changed molecules, and determine the correlations of the changed molecules with clinical stage, OS, and DFS (using median grouping). TIMER2.0 database (<http://timer.cistrome.org/>) and Oncomine database (<https://www.oncomine.org>) were used to further confirm *CDKN2A* expression in HCC.

HCC clinical sample data sources and processing

The University of California Santa Cruz (UCSC) Xena database (<http://xena.ucsc.edu/>) was used to download the *CDKN2A* gene expression matrix, clinical correlation data, gene promoter region methylation data, and copy number variant data from The Cancer Genome Atlas (TCGA) cohort. Integration of clinical and pathological information via the VLOOKUP function identified 374 HCC tissue samples and 50 para-cancerous tissues for which expression and clinical parameter data were available. These data were used for differential expression analysis of *CDKN2A*, correlation analysis of clinicopathological indicators, receiver operating characteristic (ROC) curve analysis and expression regulation analysis. The high and low expression groups of *CDKN2A* were classified according to the median expression.

Analysis of the correlation between CDKN2A expression and clinicopathological indicators of HCC

The downloaded *CDKN2A* expression matrix data were matched with clinical data using VLOOKUP function. Patients were grouped using clinical parameters to further compare the differences in *CDKN2A* expression among different patients, and the results were visualised using Graphpad 8.0, and the P value was calculated using the Mann-Whitney test. The P values less than 0.05 were considered statistically significant.

Kaplan-Meier survival analysis

The Kaplan-Meier plotter (<https://kmplot.com/analysis/>) database was used to analyze the prognostic value of *CDKN2A* in HCC patients, and the same analysis was performed for the patients with different clinical stages. The boundaries of high and low expression of *CDKN2A* were determined using the median of expression, and the P value was calculated using the Logrank test. A P value that less than 0.05 considered to be statistically significant.

ROC curve analysis

The above data were analyzed by ROC curve using

Graphpad 8.0 software, with the horizontal X-axis as specificity (also known as false positive rate) and the vertical Y-axis as sensitivity (also known as true positive rate), and the area under the ROC curve was calculated to assess the diagnostic value of *CDKN2A* expression for HCC.

Analysis of copy number variation (CNV) for *CDKN2A* gene

The UCSC Xena database was used to download the CNV data of the *CDKN2A* gene from the TCGA cohort of HCC patients, and the VLOOKUP function was used to match the CNV and clinical parameter data of 364 HCC tissue samples. CNV was determined by the copy number threshold, with -1 or -2 defined as a copy number deletion (del), 0 as a copy number normal [wild type (wt)], and 1 or 2 as a copy number amplification (amp). The distribution of copy number variants in the patients with different clinical parameters was assessed by chi-square test. The Mann-Whitney test was used for analysis of variance (ANOVA) and P values were calculated. All results were visualised using Graphpad 8.0, and P values less than 0.05 were considered statistically significant.

Methylation analysis of the promoter region of *CDKN2A* gene

The UCSC Xena website was used to download *CDKN2A* methylation data from the TCGA cohort of HCC patients. Gene methylation β -value and gene expression of 370 HCC samples were obtained after matching from UCSC Xena website. The promoter region of *CDKN2A* gene (the promoter region is generally located 2,000 bp before the transcription start site) was located through the National Center for Biotechnology Information (NCBI) database (<https://www.ncbi.nlm.nih.gov/>), and the promoter methylation β -value was obtained by calculating the average of methylation β -values of all probes located at the promoter region. The median was used for the boundaries of the high and low methylation groups, the differences were analyzed using the Mann-Whitney test, and all results were visualised using Graphpad 8.0. The P values less than 0.05 were considered statistically significant.

Correlation analyses of *CDKN2A* expression with tumour microenvironment

The stromal score, immune score and ESTIMATE score in HCC were calculated by the ESTIMATE algorithm and used to predict the infiltration of non-tumour cells.

To analyze whether the above three metrics differed in the patients with different *CDKN2A* expression levels, the data were obtained from the ESTIMATE data (<https://bioinformatics.mdanderson.org/estimate/>). The TIMER2.0 database provides six types of immune cell infiltration (B cells, CD4⁺ T cells, CD8⁺ T cells, neutrophils, macrophages, and dendritic cells), which was used to further validate the correlation between *CDKN2A* expression and immune cell infiltration.

***CDKN2A*-related gene enrichment analysis**

Genes co-expressed with *CDKN2A* in HCC were obtained from the LinkedOmics database (<http://www.linkedomics.org/>) and entered into the DAVID database for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichments and functional annotation.

***CDKN2A* interaction protein network and enrichment analysis**

STRING (<https://cn.string-db.org/>) and GeneMANIA (<http://genemania.org/>) were used to construct the *CDKN2A* protein-protein interaction (PPI) network, and the interacting proteins were subjected to GO and KEGG enrichments and functional annotation.

Statistical analysis

Statistical analyses were performed using SPSS 26.0 software (SPSS, Inc., Chicago, IL, USA). Spearman and Pearson tests were used for correlation analysis, *t*-test was selected for comparison between two groups, and one-way ANOVA test was used for comparison between multiple groups. The OS and DFS were calculated using the Kaplan-Meier method and evaluated using the log-rank test. *CDKN2A* expression was classified as high and low groups using the median score. The P values less than 0.05 were considered statistically significant difference.

Results

The screening for candidate molecules of diagnosis and key participant in HCC

By analyzing the transcriptome sequencing data of HCC and normal tissues, the expression levels of 2,207 genes were significantly changed ($|\text{Log}_2 \text{ fold change}| \geq 1$, $P \leq 0.01$) in HCC compared with normal tissues. Among these altered genes, 1,482 were up-regulated and 725 were down-

regulated (Figure 1A). The distribution of these altered genes on chromosomes revealed that the up-regulated genes were preferentially located on chromosomes 1, 6, 11, 17, 19 and 20, while the down-regulated genes were preferentially located on chromosomes 1, 11, 16 and 19 (Figure 1A). The screened 2,207 molecules were subjected to GO and KEGG enrichment analyses. The analyzed GO processes were sorted according to the number of aggregated molecules, and the top 20 GO processes were displayed (Figure S1). These altered genes mainly involved in ribosome structure and protein localization from the top 20 GO processes (Figure S1). The KEGG enrichments were also analyzed, and the top 10 positive and negative signaling pathways were displayed (Figure S1). The positive genes primarily involved in disease-related pathways, and the negative genes majorly involved in metabolic pathways from the top 10 signaling pathways (Figure S1).

To search for the candidate molecules of diagnosis and key participant in HCC, the most altered molecules (top 20) were selected and were then confirmed in independent HCC samples. The most significantly altered candidates among the expression-changed genes included *RP11-40C6.2*, glypican 3 (*GPC3*), aldo-keto reductase family 1 member B10 (*AKR1B10*), ubiquitin D (*UBD*), midkine (*MDK*), phosphatidylinositol glycan anchor biosynthesis class Y (*PIGY*), C-C motif chemokine ligand 20 (*CCL20*), *CDKN2A*, ubiquitin conjugating enzyme E2 C (*UBE2C*), and cyclin B1 (*CCNB1*) (Figure 1B). The results demonstrate that *RP11-40C6.2*, *GPC3*, *AKR1B10*, *UBD*, *MDK*, *PIGY*, *CCL20*, *CDKN2A*, *UBE2C* and *CCNB1* are likely the candidates of diagnosis and key participant in HCC.

The clinical relevance of the candidates with patient's stage and prognosis

The expression differences of these candidates (*RP11-40C6.2*, *GPC3*, *AKR1B10*, *UBD*, *MDK*, *PIGY*, *CCL20*, *CDKN2A*, *UBE2C*, and *CCNB1*) were then analyzed in the HCC patients at different clinical stages. The expression of *CDKN2A*, *UBE2C*, and *CCNB1* was closely associated with clinical stages, whereas the expression of other candidates was not correlated with clinical stages of HCC patients (Figure 2A; Figure S2). For prognostic values of these candidates, the expression of *CDKN2A* and *CCNB1* was strongly associated with the OS and DFS of HCC patients, the expression of *AKR1B10* was associated with OS of HCC patients, and the expression of *UBE2C* was only associated with DFS of HCC patients (Figure 2B). However, the

expression of other candidates was not associated with OS and DFS of HCC patients (Figure S3). These data suggest that *CDKN2A* and *CCNB1* probably acts important roles in diagnosis, prognosis, and metastasis of HCC. After a literature searching, the roles and potential clinical significance of *CCNB1* had been well studied in many types of tumors, including HCC. However, *CDKN2A* has been less studied in cancers specially in HCC and deserves further in-depth research.

The validation of CDKN2A at different expression levels in HCC

To further demonstrate the expression of *CDKN2A* in cancers, a pan-cancer analysis was performed using multiple datasets from different databases. The transcriptional expression level of *CDKN2A* were clearly up-regulated in the most tumor types especially HCC (Figure 3A,3B). To further determine the changed expression of *CDKN2A* at protein level in HCC, the IHC using *CDKN2A* antibody was performed in HCC tissues on TMA. The protein expression of *CDKN2A* was significantly higher in HCC tissues than that in adjacent normal tissues (Figure 3C). In addition, *CDKN2A* protein appeared to be primarily expressed at membrane, cytoplasm and nucleus of tumor cells (Figure 3C).

The associations of CDKN2A with clinical indexes and survival prognosis

To further explore the relationship between *CDKN2A* expression and clinical data, as well as the prognostic value of HCC, we analyzed the associations of *CDKN2A* expression with clinical indexes and survival prognosis in HCC using the TCGA data downloaded from UCSC database. The up-regulation of *CDKN2A* mRNA was confirmed in HCC compared to normal tissues ($P < 0.001$, Figure 4A). To assess the clinical significance of this changes in *CDKN2A* expression, we used the VLOOKUP function to match *CDKN2A* expression with clinical data of HCC patients, and compared the expression levels of *CDKN2A* mRNA in patients with different clinical indicators. The expression of *CDKN2A* mRNA was closely associated with tumor sizes (T) and clinical stages of HCC patients ($P < 0.001$, Figure 4A). These results suggest that *CDKN2A* may be an important molecule in promoting HCC growth and progression. To analyze the prognostic value of *CDKN2A* expression changes in HCC, survival analysis

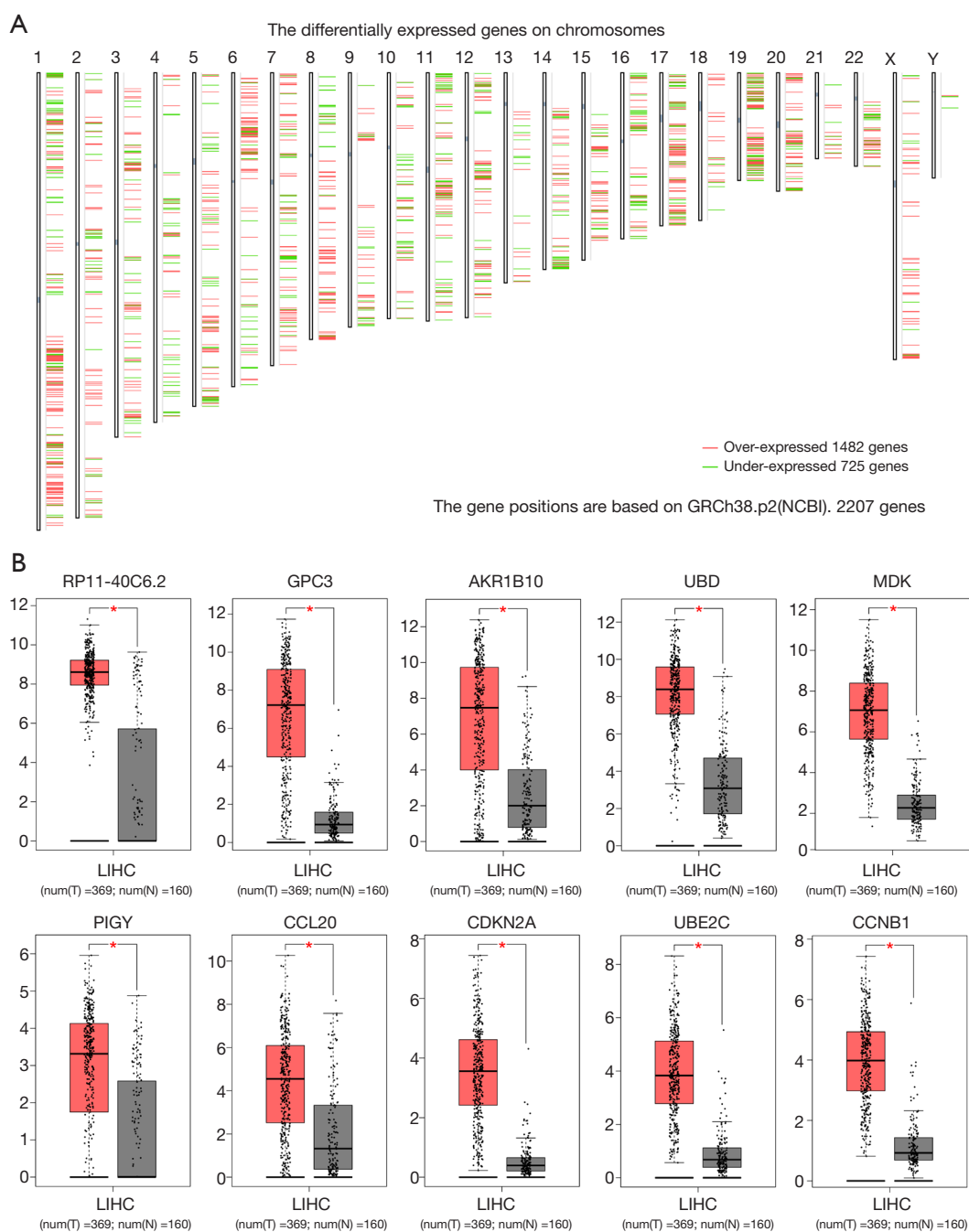


Figure 1 The screening for candidate molecules of diagnosis and key participant in HCC. (A) The differential expression genes with significant changes in expression levels in HCC tissues and their distribution on different chromosomes using the GEPIA 2.0 online website analyses. $|\log_2FC|$ cut-off value ≥ 1 ; P value cut-off ≤ 0.01 ; differential expression analysis method is ANOVA. (B) The top 10 most changed molecules were further validated in HCC. The “*” represents P value < 0.05 , the “num” represents number of samples, the “T” represents tumour, the “N” represents normal, the “LIHC” represents HCC. NCBI, National Center for Biotechnology Information; *GPC3*, glypican 3; *AKR1B10*, aldo-keto reductase family 1 member B10; *UBD*, ubiquitin D; *MDK*, midkine; *PIGY*, phosphatidylinositol glycan anchor biosynthesis class Y; *CCL20*, C-C motif chemokine ligand 20; *CDKN2A*, cyclin-dependent kinase inhibitor 2A; *UBE2C*, ubiquitin conjugating enzyme E2 C; *CCNB1*, cyclin B1; HCC, hepatocellular carcinoma; FC, fold change; ANOVA, analysis of variance; GEPIA, Gene Expression Profiling Interactive Analysis; LIHC, Liver hepatocellular carcinoma.

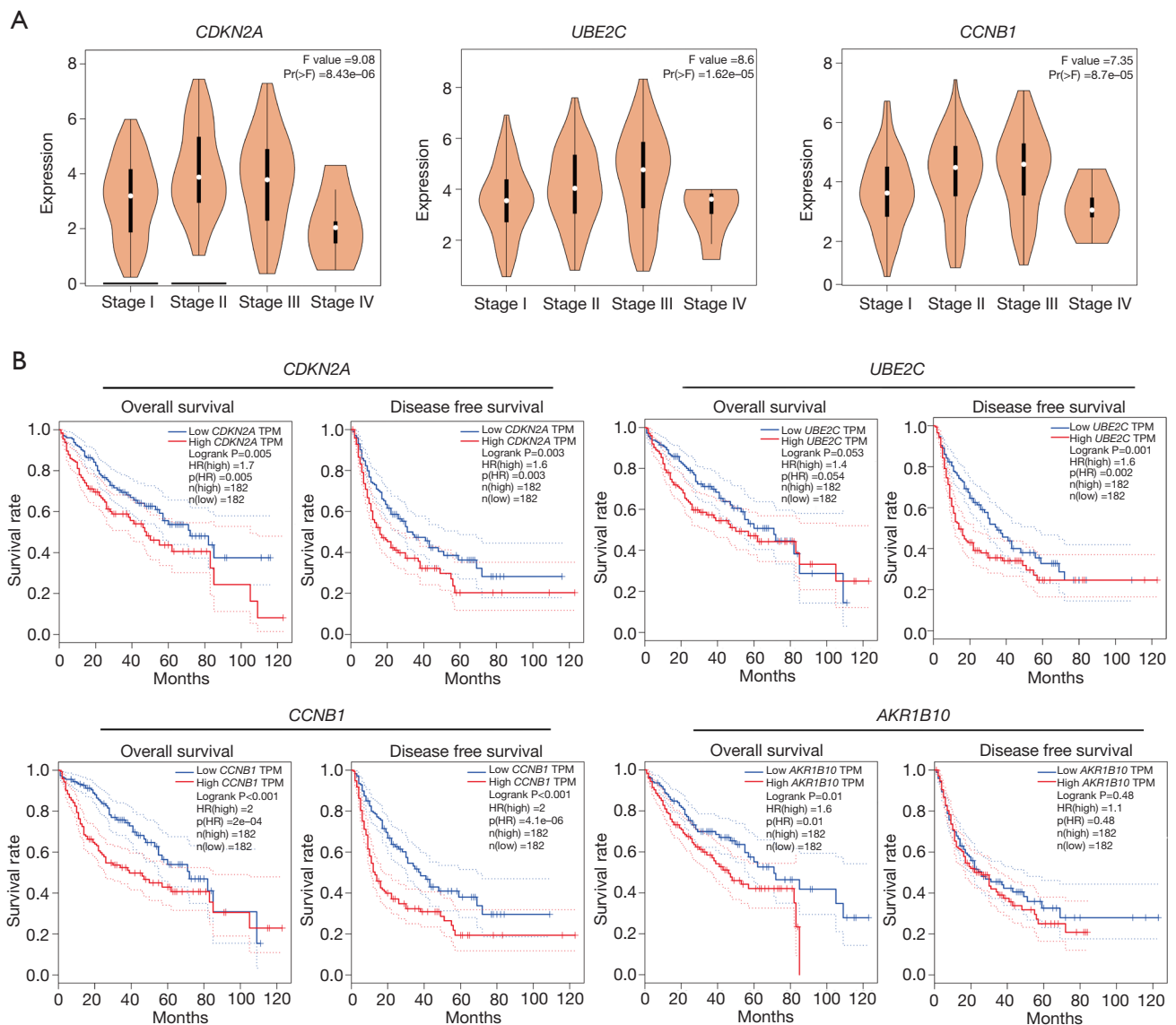


Figure 2 Associations of the expression of candidate molecules with clinical stage and prognosis of HCC patients. (A) The correlation of the expression of candidate molecules with clinical stage of HCC patients. Analyses were performed using the F test, and differences between the two groups were considered significant when $\text{Pr}(> F)$ was less than 0.05. (B) The relationship between candidate molecule expression and prognosis of HCC patients. The median expression values were used for group cutoff. The survival was analyzed using the log-rank test. The P values less than 0.05 indicate significant differences. The “n” represents number of samples. *CDKN2A*, cyclin-dependent kinase inhibitor 2A; *UBE2C*, ubiquitin conjugating enzyme E2 C; *CCNB1*, cyclin B1; *AKR1B10*, aldo-keto reductase family 1 member B10; TPM, transcripts per kilobase million; HR, hazard ratio; HCC, hepatocellular carcinoma.

was performed using Kaplan-Meier Plotter database. The HCC patients with high *CDKN2A* expression had a poorer prognosis compared to those with low *CDKN2A* expression (Logrank P=0.005, Figure 4B). Further analysis revealed that high *CDKN2A* expression was associated with poorer prognosis of the patients at stage II (Logrank P=0.045,

Figure 4C), whereas *CDKN2A* expression was not associated with prognosis of the patients at stage I (Logrank P=0.052) and III (Logrank P=0.21, Figure 4C). The results suggest that *CDKN2A* may be a prognostic marker for HCC patients.

To further investigate the clinical value of *CDKN2A*

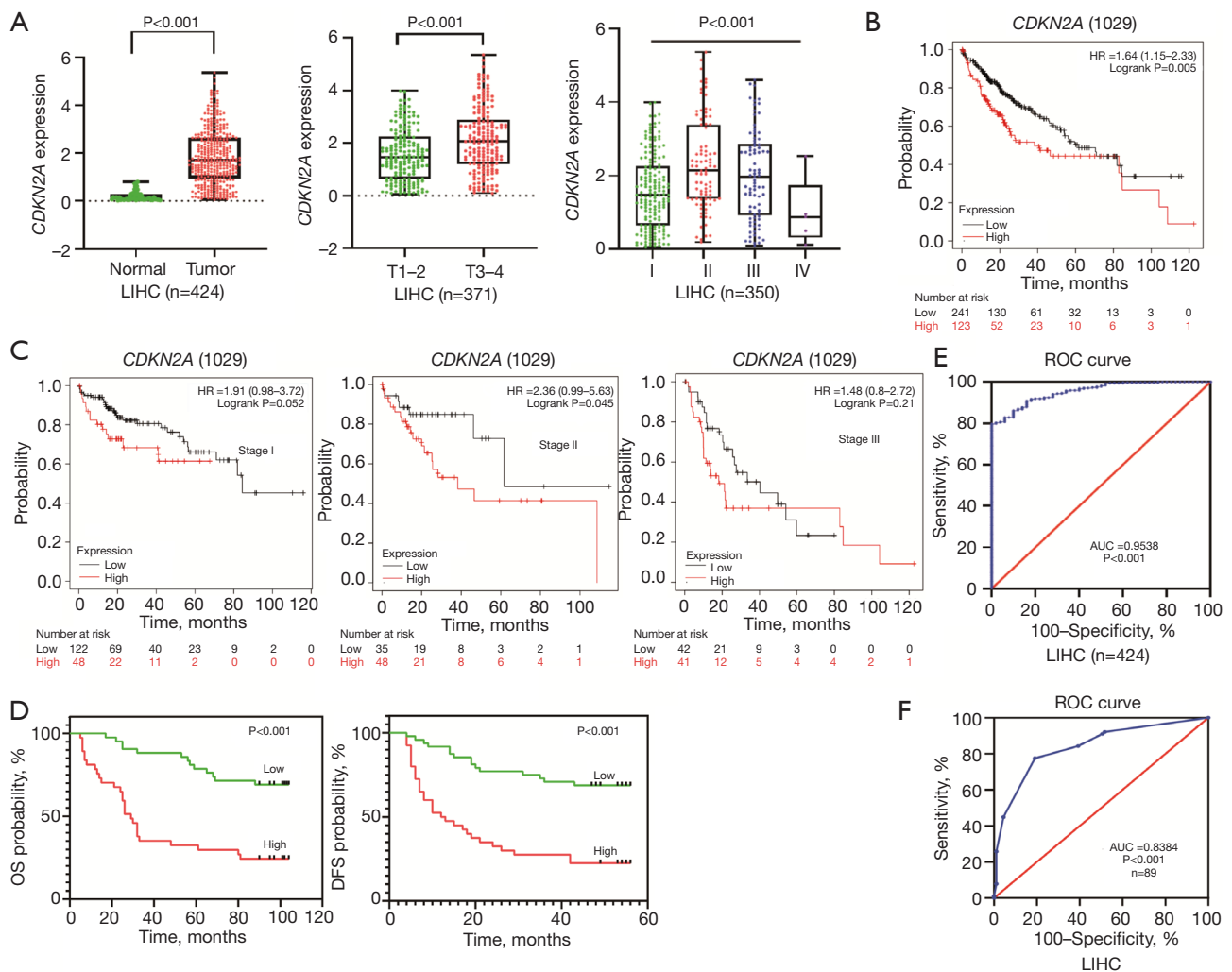


Figure 4 The expression verification, prognostic and diagnostic value of *CDKN2A* in HCC patients. (A) *CDKN2A* mRNA expression levels in HCC from the TCGA database and differences in *CDKN2A* expression in HCC tumour size and clinical stage. Analyses were performed using the Mann-Whitney *U* test, with *P* values less than 0.05 considered statistically different. (B,C) Kaplan-Meier survival curves to assess the prognostic value of *CDKN2A* expression in HCC patients, and the patients at clinical stages I, II, and III. Survival was analyzed using the log-rank test. HR used 95% confidence interval. The *P* value less than 0.05 indicates significant difference. (D) The OS and DFS of HCC patients with different *CDKN2A* expression at protein level. Analyses were performed using the Wilcoxon test, and the *P* values less than 0.05 were considered statistically significant. (E) ROC curve analysis of *CDKN2A* expression in HCC patients. The *P* values less than 0.05 were considered statistically significant. (F) ROC curve analysis of *CDKN2A* protein expression in HCC patients. “LIHC” represents HCC. *CDKN2A*, cyclin-dependent kinase inhibitor 2A; HR, hazard ratio; ROC, receiver operating characteristic; AUC, area under the curve; OS, overall survival; DFS, disease-free survival; HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas.

expression at protein level in HCC, IHC staining of *CDKN2A* antibody was performed on TMAs containing 89 HCC cases. The expression score was the product of staining intensity and positivity of *CDKN2A*, and the patients were divided into two groups based on the average of the score: low expression group (score ≤ 4) and high

expression group ($4 < \text{score} \leq 12$). Analyses of the relationship between *CDKN2A* protein expression and the prognosis of HCC patients showed that the OS of the patients with high *CDKN2A* protein expression was significantly shorter than that of the patients with low *CDKN2A* protein expression, and the DFS of the patients with high *CDKN2A* expression

was also clearly shorter than that of the patients with low expression ($P < 0.001$, *Figure 4D*).

The diagnostic value of altered CDKN2A expression in HCC

To evaluate the diagnostic value of *CDKN2A* expression in HCC, ROC curve analysis was performed using *CDKN2A* expression and patient data of HCC from the TCGA database. The area under the curve (AUC) of *CDKN2A* in HCC was 0.9538 [95% confidence interval (CI): 0.9332–0.9744; $P < 0.001$, *Figure 4E*], suggesting that the altered *CDKN2A* expression possess a good diagnostic value. To further confirm the diagnostic value of *CDKN2A*, the changed protein expression was analyzed to determine the diagnostic significance in the HCC cases. ROC curve analysis showed that the AUC of *CDKN2A* was 0.8384 (95% CI: 0.7793–0.8975; $P < 0.001$, *Figure 4F*). These data indicate that the altered *CDKN2A* expression is important for disease diagnosis of HCC both at mRNA and protein levels, suggesting that *CDKN2A* may be a promising marker for diagnosis of HCC.

CDKN2A promoter methylation is associated with its expression with clear diagnostic value

To further explore the cause of *CDKN2A* changed expression in HCC, we analyzed the associations of *CDKN2A* expression with its CNV and DNA methylation using the HCC data from public TCGA database. The deletions (del) of *CDKN2A* predominantly existed in HCC patients accounting for 40.93%, whereas there was no significant difference of deletions and amplifications (amp) with different tumor sizes ($P = 0.26$, *Figure 5A*) and clinical stages ($P = 0.06$, *Figure 5A*) in the HCC patients. The relationship between *CDKN2A* expression and its CNV was next analyzed in HCC. *CDKN2A* expression was lower in the patients with *CDKN2A* deletions ($P = 0.009$, *Figure 5B*). Meanwhile, the methylation β -value of *CDKN2A* was positively correlated with its expression, and the methylation degree of *CDKN2A* promoter region was significantly higher in HCC than normal tissues ($P < 0.001$, *Figure 5B*). ROC analysis showed that the AUC of the methylation β value of *CDKN2A* promoter region was 0.9538 (95% CI: 0.9342–0.9734; $P < 0.001$) in HCC (*Figure 5C*), suggesting the methylation of *CDKN2A* promoter as a valuable diagnostic marker for HCC. These results demonstrate that the up-regulation of *CDKN2A*

expression in HCC may contribute to the methylation of its promoter region, and this methylation pattern has a great diagnostic value for HCC.

CDKN2A expression is associated with tumor microenvironment

The stromal score, immune score, and ESTIMATE score were calculated using the ESTIMATE algorithm. These scores were then analyzed to determine whether they differ in patients with varying levels of *CDKN2A* expression. The HCC patients with high *CDKN2A* expression had lower stromal scores compared to the patients with low *CDKN2A* expression ($P < 0.001$), whereas there was no significant difference in immune score and ESTIMATE score between the patients with high *CDKN2A* expression and that with low *CDKN2A* expression (*Figure 6A*). Immune cells are non-tumor components that play important roles in the body's fight against tumors. To analyze the correlation of *CDKN2A* expression with immune cell infiltration, the immune cell infiltration data and expression data of six types of immune cells from the TIMER database were utilized. The *CDKN2A* expression levels were positively correlated with B cells, CD8⁺ T cells, neutrophils, macrophages and dendritic cell (*Figure 6B*). These data reveal that *CDKN2A* expression is closely associated with tumor microenvironment in HCC.

CDKN2A likely involves in HCC progression by major regulating cell cycle

To determine the major potential function and the possible mechanism of *CDKN2A* in HCC, the co-expressed genes of *CDKN2A* in HCC were downloaded from the LinkedOmics database. There are 3,946 genes clearly associated with *CDKN2A* expression in HCC [false discovery rate (FDR) < 0.01], of which 2,259 were positively associated genes and 1,687 were negatively associated genes (FDR < 0.01 , *Figure 7A*). Enrichment analyses were conducted on the top 500 genes most positively and negatively associated with *CDKN2A* in HCC. GO enrichment analysis showed that the total 1,000 co-expressed genes of *CDKN2A* in HCC were mainly involved in the biological processes (BPs) such as cell division, mitotic spindle organization, DNA repair, and DNA replication; cellular components (CCs) such as nucleoplasm, nucleus, cytosol, and spindle; molecular functions (MFs) such as protein binding, adenosine triphosphate (ATP) binding, microtubule binding, and DNA helicase activity (*Figure 7B*, *Figure S4A*).

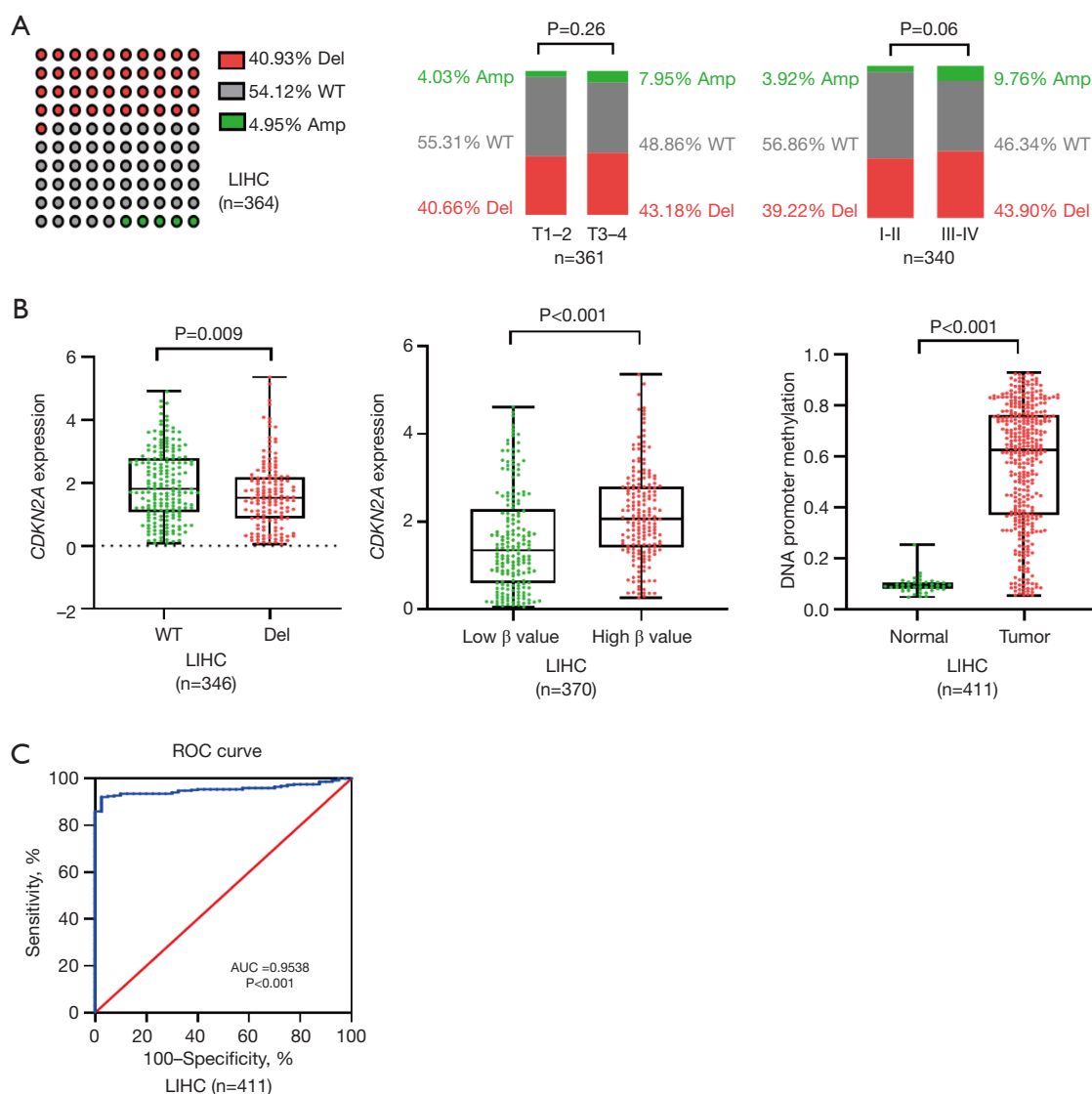


Figure 5 Analysis of copy number variation and promoter region methylation of *CDKN2A* in HCC. (A) The *CDKN2A* copy number and their distribution in different tumour size and clinical stage of HCC patients. Analyses were performed using the chi-square test, and the P values less than 0.05 were considered statistically significant. (B) The correlation of *CDKN2A* expression with its deletion and total methylation in HCC patients, and the promoter region methylation in the normal and tumour groups. The P values were calculated using the Mann-Whitney test, and less than 0.05 were considered statistically significant. (C) The diagnostic value of methylation β value of the promoter region of *CDKN2A* in HCC patients. The P value less than 0.05 was considered statistically significant. “LIHC” represents HCC. Del, deletion; WT, wild type; Amp, amplification; *CDKN2A*, cyclin-dependent kinase inhibitor 2A; ROC, receiver operating characteristic; AUC, area under the curve; HCC, hepatocellular carcinoma.

The top 500 positively co-expressed genes were primarily involved in the BPs such as cell division, DNA repair, DNA replication, and cell cycle; CCs such as nucleoplasm, nucleus, kinetochore, and cytosol; MFs such as protein binding, DNA binding, DNA helicase activity, and

microtubule binding (Figure 7B, Figure S4B). The top 500 negatively co-expressed genes were mainly involved in the BPs such as cell adhesion, actin cytoskeleton organization, vasculature development, and vasculogenesis; CCs such as extracellular matrix, mitochondrial matrix, mitochondrion,

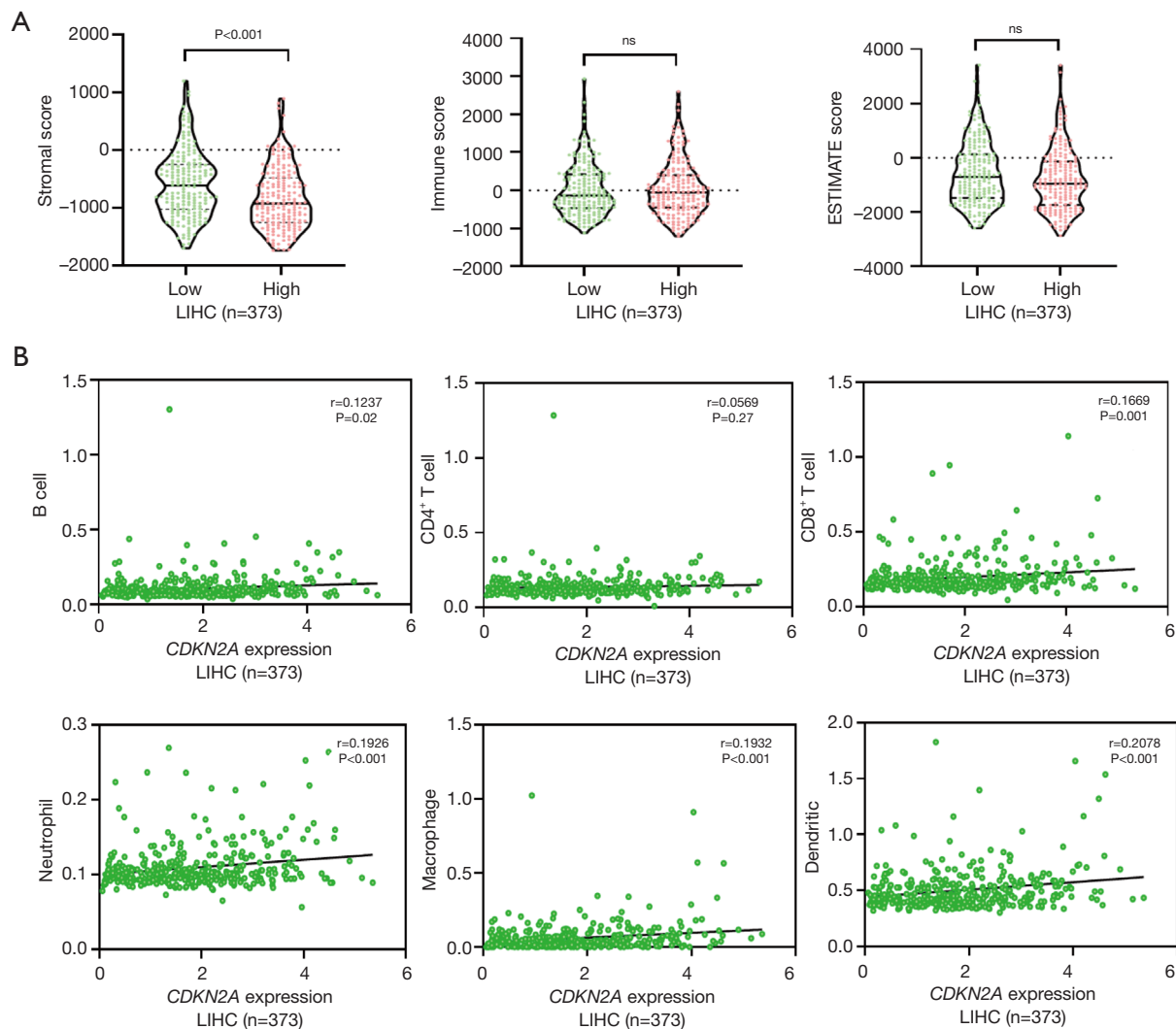
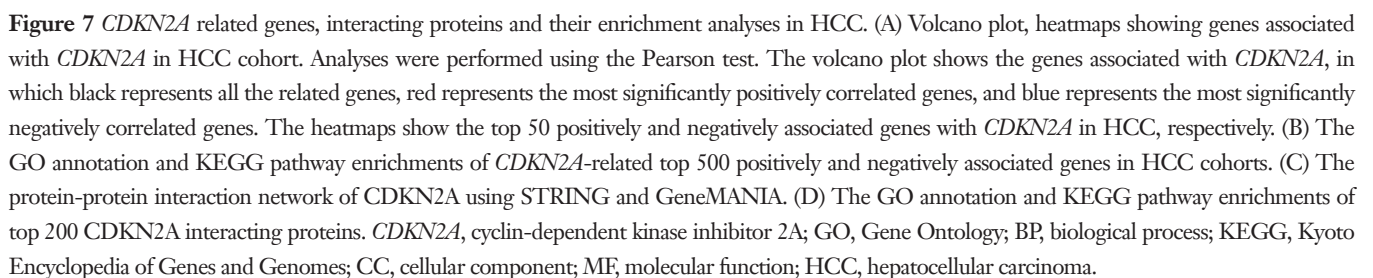


Figure 6 The correlation of *CDKN2A* expression with tumour microenvironment. (A) The relationship between *CDKN2A* expression levels and the stromal scores, immune scores and ESTIMATE scores in HCC patients. Analyses were performed using the Mann-Whitney test, and the P values less than 0.05 were considered statistically different. The “ns” represents no statistical significance. (B) The correlation of *CDKN2A* expression with B cell, CD4⁺ T cell, CD8⁺ T cell, neutrophil, macrophage and dendritic cell infiltration in HCC. The “r” is Spearman’s correlation coefficient. The P values less than 0.05 were considered significant correlation between the two variables. “LIHC” represents HCC. *CDKN2A*, cyclin-dependent kinase inhibitor 2A; HCC, hepatocellular carcinoma.

and endosome; MFs such as extracellular matrix structural constituent, calcium ion binding, extracellular matrix constituent conferring elasticity, and growth factor binding (Figure 7B, Figure S4C). KEGG enrichment analysis showed that the total 1,000 associated genes of *CDKN2A* were primarily involved in the signaling pathways such as cell cycle, DNA replication, Fanconi anemia pathway, and cellular senescence; the top 500 positively co-expressed genes were mainly involved in the signaling pathways such

as cell cycle, DNA replication, Fanconi anemia pathway, and homologous recombination; the top 500 negatively co-expressed genes were primarily involved in the signaling pathways such as citrate cycle, valine/leucine/isoleucine degradation, and proximal tubule bicarbonate reclamation in HCC (Figure 7B).

Next, PPI network and interacting proteins of *CDKN2A* were analyzed using STRING and Gene MINIA databases. The PPI network results showed that the main proteins



interacting with *CDKN2A* were regulatory proteins of cell cycle, such as *CDK2*, *CDK4*, *CDK6*, *TP53*, *CCND1*, *CCND2* and *MDM2* (Figure 7C). A medium confidence level of 0.400 was used as the minimum required interaction score, and a total of 200 interacting proteins of *CDKN2A* were selected for enrichment analysis using the STRING database. The GO enrichment analysis of these interacting proteins showed that *CDKN2A* interacting proteins were involved in the BPs such as cell cycle, cyclin-dependent protein serine/threonine kinase activity, G1/S transition of mitotic cell cycle, and cell cycle; CCs such as nucleoplasm, nucleus, chromatin, and cytosol; MFs such as protein kinase binding, protein binding, DNA binding, and cyclin-dependent protein serine/threonine kinase regulator activity (Figure 7D). KEGG enrichment analysis of these interacting proteins revealed that *CDKN2A* interacting proteins were involved in cell cycle, cancer-related pathways, cellular senescence, and P53 signaling pathway (Figure 7D). These findings suggest that *CDKN2A* may play a significant role in promoting the development of HCC.

Discussion

Previous studies have shown that *CDKN2A* is aberrantly expressed in a variety of tumor types including lung, oesophageal, gastric, colorectal, ovarian, leukaemia and renal cancers (3-6). However, little is known about its clinical relevance and value in these cancers. In this study, we comprehensively investigate the clinical relevance and significance of *CDKN2A* in HCC. Our findings indicate that *CDKN2A* expression is generally elevated in HCC and exhibits a strong correlation with tumor size and clinical stage in patients. The patients with low *CDKN2A* expression had correspondingly prolonged OS and DFS compared to the patients with high *CDKN2A* expression. Moreover, ROC curve analysis shows a good diagnostic value of *CDKN2A* expression specially the methylation of *CDKN2A* promoter for HCC. These data suggest that *CDKN2A* is a promising biomarker for HCC prognosis and diagnosis.

Previous evidence suggests that *CDKN2A* is a susceptibility gene for melanoma and pancreatic cancer (7). Recent studies have identified *CDKN2A* as an important tumor suppressor in cervical and lung cancers (5,6). However, there are few studies on *CDKN2A* in HCC. In the present study, *CDKN2A* expression is significantly elevated and closely associated with tumor size and clinical stage of HCC patients, suggesting *CDKN2A* likely an important participant in HCC growth and progression.

The GO enrichments of co-expressed genes and interacting proteins of *CDKN2A* in HCC reveal that *CDKN2A* may act crucial roles on regulation of tumor cell cycle, which further confirm the important roles of *CDKN2A* in HCC growth and progression.

Few studies have demonstrated the mechanisms of *CDKN2A* expression regulation and tumor progression. A recent study has revealed that *CDKN2A* inhibits the proliferation and invasion of cervical cancer cells through the *LDHA*-mediated *AKT-mTOR* pathway (5). In the present study, KEGG enrichments of co-expressed genes and interacting proteins of *CDKN2A* indicate that *CDKN2A* likely acts important roles in HCC by regulation of the signaling pathways related to cell cycle, cellular senescence and apoptosis (e.g., *P53* signaling pathway). These data suggest that *CDKN2A* probably participates in HCC progression by primarily regulating tumor cell cycle. Moreover, *CDKN2A* as a cyclin-dependent kinase inhibitor should play key roles in cell cycle regulation. We noted that the cell cycle, cellular senescence, and *P53* signaling pathway were enriched in the interacting proteins with *CDKN2A*. We speculate that *CDKN2A* may promote HCC progression by regulating the *P53* signaling pathway. However, further experiments are required to confirm this speculation, and more studies are needed to understand how *CDKN2A* regulates *P53*, which induces changes in downstream molecules.

The immune cells are non-tumor cells in tumor microenvironment. The relationship between immune cells and tumor development is well established. Immune cells are able to sense various signals in the microenvironment, and initiate specific immune functions in response. There is growing evidence that the immune response is associated with dramatic changes in tissue metabolism, including nutrient depletion, increased oxygen consumption, and the production of reactive nitrogen and oxygen intermediates (8-10). Similarly, many metabolites in the tumor microenvironment can in turn affect immune cell differentiation and effector functions (11,12). Emerging evidences suggest that cancer cells are able to suppress anti-tumor immune responses by competing for and consuming essential nutrients or otherwise reducing the metabolic capacity of tumour-infiltrating immune cells (13,14). In this study, we analyzed the correlation between *CDKN2A* and immune cell infiltration, and found that in HCC, *CDKN2A* expression was closely correlated with the infiltration of various immune cells. These data suggest that *CDKN2A* may play a role in HCC by altering the tumour

microenvironment. Further studies are required to address this issue.

CDKN2A expression is clearly elevated in HCC, to clarify the possible regulatory mechanism of this elevated expression, we analyzed the contributions of CNV and methylation of *CDKN2A* gene to its expression. The association analysis shows that *CDKN2A* expression in HCC may be mainly regulated by the methylation of its promoter region. Moreover, the methylation of *CDKN2A* promoter region possesses a good diagnostic value for HCC, suggesting that detecting *CDKN2A* methylation can be used as an important mean for diagnosis of HCC.

Conclusions

CDKN2A has been identified as a promising prognostic and diagnostic candidate for HCC, and may have crucial roles on the process of HCC development and metastasis by regulating tumor cell cycle, providing an important therapeutic target in HCC. However, there are shortcomings in this study. Some of the current results need to be validated on a large scale in our experimental sample. The possible mechanisms are predicted by computer algorithms and will require a series of subsequent functional experiments for further validation.

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Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at <https://tcr.amegroups.com/article/view/10.21037/tcr-24-1845/rc>

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-24-1845/coif>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Ethics Committee of Shanghai Outdo Biotech Company (No. YB M-05-02) and the Ethics Committee of Third Affiliated Hospital of Chongqing Medical University (CQMU, No. 2021-95). All patients signed an informed consent form.

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