Cyclin-dependent kinase inhibitor 3 is overexpressed in hepatocellular carcinoma and promotes tumor cell proliferation

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ABSTRACT

CDKN3 belongs to the protein phosphatases family and has a dual function in cell cycling. The expression of this gene has been studied in several kinds of cancers, but its role in human hepatocellular carcinoma (HCC) remains to be elucidated. In this study, we found that CDKN3 was frequently overexpressed in both HCC cell lines and clinical samples, and this overexpression was correlated with poor tumor differentiation and advanced tumor stage. Functional studies showed that overexpression of CDKN3 could promote cell proliferation by stimulating G1-S transition but has no impact on cell apoptosis and invasion. Microarray-based co-expression analysis identified a total of 61 genes co-expressed with CDKN3, with most of them involved in cell proliferation, and BIRC5 was located at the center of CDKN3 co-expression network. These results suggest that CDKN3 acts as an oncogene in human hepatocellular carcinoma and antagonism of CDKN3 may be of interest for the treatment of HCC.

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers worldwide, with an estimate of 748,300 new cases and 695,900 deaths annually, half of these cases and deaths occur in China [1]. Despite the recent advancement in diagnosis and treatment modalities, the prognosis of HCC patients remains dismal. Because current therapies are insufficient for most patients to achieve complete tumor eradication, it is essential to define the molecular mechanisms of HCC and identify novel targets to develop effective therapeutic approaches that will improve the clinical outcome of HCC patients.

Cyclin-dependent kinase inhibitor 3 (CDKN3) belongs to the protein phosphatases family and is involved in regulating the cell cycle [2,3]. It has a dual function in cell cycling. First, CDKN3 acts as a cyclin-dependent kinase inhibitor that selectively binds to CDK2 kinase [4,5] and reduces its capacity to phosphorylate the retinoblastoma protein (Rb) [6]. Non-phosphorylated Rb binds to transcription factor E2F1 and prevents the generation of proteins required for G1/S transition, thereby inhibiting G1/S transition [7–9]. Second, CDKN3 is also a Mdm2-binding protein. It could form a complex with Mdm2 and p53, therefore abolishing the induction of p21, a product of the p53 target gene, thus facilitates cell cycle progression [10]. Furthermore, this gene was reported to be deleted or overexpressed in several kinds of cancers [11–13], but the expression pattern and biological functions of CDKN3 in human hepatocellular carcinoma remain to be elucidated.

In this study, we aimed to assess the functional characteristics and molecular mechanisms of CDKN3 in HCC. We found that CDKN3 was frequently overexpressed in HCC, and its expression was correlated with poor clinical outcome. Overexpression of CDKN3 could stimulate the proliferation of HCC cell lines. Using microarray meta-analysis, a number of important cell cycle regulating molecules were found to be co-expressed with CDKN3, and BIRC5 appeared as the central node of the co-expression network.

2. Materials and methods

2.1. Patients

Fifty-six pairs of primary HCC and adjacent noncancerous liver tissues were collected from patients who underwent hepatic resection between 2005 and 2010 in our hospital (First Affiliated Hospital, Zhejiang University School of Medicine, Zhejiang, China). These patients included 41 males and 15 females with a mean age of 50.4 ± 11.0 y (range: 24–75). Written informed consent was obtained from all patients, and the study was approved by the local ethics committee.
2.2. Cell culture

Ten Human HCC cell lines (HepG2, Hep3B, Huh-7, Bel-7402, SK-Hep-1, PLC/PRF/5, SMMC-7721, MHCC-97L, MHCC-97H and MHCC-LM3) and two immortalized liver cell lines (L-02 and Chang liver) were cultured in Dulbecco’s modified Eagle medium (DMEM, Gibco-Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (SAFC Biosciences, Lenexa, KS), 100 units/mL penicillin and 100 mg/mL streptomycin (Sigma–Aldrich, St. Louis, MO).

2.3. Establishment of stable CDKN3-expressing cell lines

A human Full-length CDKN3 cDNA was cloned into XhoI and EcoRI sites of pEGFP-C1 (Clontech, Palo Alto, CA). The pEGFP-CDKN3 construct was verified by sequencing. HCC cells were transfected with pEGFP-CDKN3 and pEGFP-C1 as a negative control using Lipofectamine 2000. Transfected cells were selected by 400 μg/mL G418 (Gibco-Invitrogen) for 3 weeks.

2.4. Quantitative Real-time PCR analysis

Total RNA was extracted from whole cells using Trizol reagent (Invitrogen) and reverse-transcribed with TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative Real-time PCR (qRT-PCR) was carried out using SYBR Premix Ex Taq (TAKARA, Kusatsu, Japan) on an ABI 7500 Real Time PCR System (Applied Biosystems). All qRT-PCRs were performed in triplicate. The relative RNA expression was calculated using the delta-delta threshold cycle (ΔΔCT) method and normalized to GAPDH expression. PCR was performed with the following primers:

- 5’-CTCTCTGCTCCTCCTGTTCGAC-3’ and 5’-TGAGCGATGTGGCTCGGCT-3’ for GAPDH;
- 5’-CAGCGATGAAGCCGCCCAGT-3’ and 5’-TGACAGTTCCCCTCTGGTCAG -3’ for CDKN3.

2.5. Western blotting analysis

Western blotting analysis was performed as previously described [14] with the following primary antibodies: monoclonal anti-CDKN3 (1:1000 dilution, Sigma–Aldrich, St. Louis, MO), monoclonal anti-p21 (1:1000 dilution, Epitomics, Burlingame, CA) and monoclonal anti-GAPDH (1:2000 dilution, Sigma–Aldrich, Saint Louis, MO).

2.6. Cell viability assay

Cell growth was determined by Cell Counting Kit-8 (CCK-8) cell viability assay (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer’s instructions as previously described [15].

2.7. Cell proliferation analysis

Cell proliferation was measured using the EdU assay kit (Ribobio, Guangzhou, China) according to the manufacturers’ instructions as previously described [16].

2.8. Cell cycle analysis

Cell cycle analysis was carried out as previously described [15].
2.9. Cell apoptosis analysis

Cell apoptosis analysis was carried out as previously described [17].

2.10. Transwell invasion experiment

For transwell assay, $1 \times 10^5$ HepG2 cells or $5 \times 10^5$ MHCC-LM3 cells in serum-free DMEM were seeded into the upper chambers of each well (24-well insert, 8-mm pore size, Millipore, Billerica, MA) coated with Matrigel (BD Bioscience, San Jose, CA). DMEM containing 10% FBS was placed in the lower chambers as a chemoattractant. After 16 h of incubation, cells on the upper membrane surface were wiped off, and the cells that invaded across the Matrigel membrane were fixed with 100% methanol and stained with 0.2% crystal violet. The number of invasive cells was then counted (five randomly chosen high-power fields for each membrane) under a microscope.

2.11. Meta-analysis

The co-expression analysis of microarray data sets was performed with Oncomine database co-expression analysis tool (http://www.oncomine.org). The top 200 genes of each data set were extracted according to the co-expression score. The genes that appeared in three of these four data sets were defined as CDKN3 co-expressed genes. The Expression Analysis Systematic Explorer (EASE) software (http://david.abcc.ncifcrf.gov/ease-ease.jsp) was applied for Gene Ontology (GO) biological process enrichment analysis. Gene interaction network was constructed using the Genomatix Pathway System (GePS, http://www.genomatix.de/).

2.12. Statistical analysis

Pearson’s chi-square test was applied to assess the correlation of CDKN3 expression with different clinicopathological parameters. Student’s $t$ test was used to analyze the differences between two groups. Statistical significance was accepted if $p < 0.05$. Statistical analysis was performed using SPSS 16.0 software (SPSS, Chicago, IL). Data are presented as mean ± SD.

3. Results

Overexpression of CDKN3 is frequent in HCC and is associated with poor clinical outcome.

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**Table 1**

Correlations between CDKN3 expression and clinicopathological features.

<table>
<thead>
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<th>N</th>
<th>CDKN3 expression</th>
<th>p value(^a)</th>
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<td></td>
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<td>High expression(^b)</td>
<td>Low expression(^b)</td>
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<tr>
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<tr>
<td>Age (years)</td>
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<tr>
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<tr>
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\(^a\) CDKN3 low expressers ($n = 18$) and CDKN3 high expressers ($n = 38$) according to the mean relative expression of CDKN3 in adjacent nontumorous tissues.

\(^b\) Fisher’s exact test.
To determine the expression pattern of CDKN3 in HCC, Oncomine database [18] was used to analyze the microarray data sets from the studies of Wurmbach [19], Chen [20], Roessler [21] and Mas [22]. As seen in Fig. 1A, all of the four data sets showed a significantly higher level of CDKN3 in HCC compared with normal liver tissues. To validate this observation, qRT-PCR was then performed to evaluate the expression of CDKN3 in two immortalized liver cell lines (L-02 and Chang liver) and ten HCC cell lines (HepG2, Hep3B, Huh-7, Bel-7402, SK-Hep-1, PLC/PRF/5, MHCC-LM3, MHCC-97L, MHCC-97H and MHCC-LM3). Seven of these ten (70%) HCC cell lines, showed at least twofold overexpression of CDKN3 compared with the mean value of L-02 and Chang liver (Fig. 1B). Further analysis of 56 pairs of primary HCC and adjacent normal tissues confirmed this overexpression of CDKN3 (p < 0.001, Fig. 1C). Clinical association study demonstrated that the expression of CDKN3 was associated with poor tumor differentiation (p = 0.009) and advanced tumor stage (p = 0.011, Table 1).

3.1. Overexpression of CDKN3 promotes the proliferation HCC cells

To investigate the biological function of CDKN3 in HCC, a CDKN3 expression vector was established and stably transfected into HepG2 and MHCC-LM3 cells, (Fig. 2A). As shown in Fig. 2B, forced overexpression of CDKN3 resulted in a marked stimulation of cell growth in both HepG2 and MHCC-LM3 cells (up to 39.9% and 38.1%, respectively), which was based on the increase of proliferating cells (Fig. 2C) but had no effect on cell apoptosis (Fig. 2F). Cell cycle analysis revealed a major reduction of G1 cells by CDKN3 overexpression, along with a concomitant increase of cell population in S and G2 phase (Fig. 2D). It has been reported that CDKN3 could prevent the induction of p21 in breast cancer cells, thereby facilitating cell cycle progression [10], this report intrigued us to explore whether CDKN3 promotes cell cycle progression via the p53-p21 dependent pathway in HCC cells. As shown in Fig. 2E, the expression of p21 was lower in CDKN3 transfected cells compared with the control group.

3.2. Overexpression of CDKN3 has no effect on HCC cell invasion

Till now, most of the studies about CDKN3 focus mainly on its function of cell proliferation, and other functions of this gene are largely unknown. To determine whether CDKN3 is involved in cell invasion, transwell assay was then performed. The result showed no obviously changes of invasive ability in CDKN3 overexpressed cells (Fig. 3A and B).
CDKN3 has been studied in many other cancers, its role in HCC remains to be elucidated.

CDKN3 was reported to be upregulated in breast and prostate cancers [11]. Here, we found that CDKN3 was frequently overexpressed in HCC tissues and cell lines, and its expression was correlated with poor tumor differentiation and advanced tumor stage, suggesting that increased expression of CDKN3 is associated with HCC genesis and progression. The mechanisms of CDKN3 high expression may be the hypomethylation of its promoter region [30] and remains to be further studied. To better understand the biological function of CDKN3, we have then investigated whether overexpression of CDKN3 could promote the malignant phenotypes (such as cell proliferation, apoptotic resistance and invasion) in HCC cell lines.

The role of CDKN3 on cell proliferation has been well studied [3,31,32]. In human breast and colon cancer cells, CDKN3 could prevent the induction of p21, a p53 target gene, thereby inhibiting cell cycle progression [10]. It has been reported that p21 could interacted with cyclin/CDK complexes and PCNA to inhibit their kinase activities [33]. Besides, increased p21 could also cause a specific suppression of cell cycle progression genes, such as BUB1B and ORC1 [34]. In the case of HCC, we found that overexpression of CDKN3 could dramatically promote the proliferation of HepG2 and MHCC-LM3 cells through the induction of G1/S transition. In keeping with previous studies, the expression of p21 was inhibited in CDKN3 transfected HCC cells, suggesting that CDKN3 promotes HCC cell cycle progression, at least in part, via the p53-p21 dependent pathway.

Metastasis is a key event in tumor progression and is the most common cause of recurrence in HCC patients undergoing liver resection or transplantation [35,36]. The role of CDKN3 on cell metastasis is rarely reported and prompted us to investigate whether CDKN3 is of relationship with cell invasion. The functional study demonstrated that there were no different changes of cell invasion after CDKN3 transfection. This finding is coincident with our clinical data, as no significant correlation between the metastasis characterizes (such as venous invasion) and CDKN3 expression was observed, indicating CDKN3 could not affect the metastasis potential of HCC cells. To our knowledge, this is the first report of CDKN3 on tumor metastasis other than its proliferation functions.

To further investigate the molecular mechanism of CDKN3, co-expression analysis was used to identify possible partners of CDKN3 in HCC tissues. We identified 61 co-expression genes for CDKN3, most of them are related in the processes of cell proliferation, such as mitotic cell cycle and nuclear division (Fig. 4A).

To obtain insights into the functional connections of CDKN3 co-expressed genes, these 61 genes were uploaded into the Genomatix GePS software for network analysis. The network showed many co-expressed genes, these 61 genes were chosen high-power fields (400×) for each replicate (n = 3).

3.3. Co-expression analysis of CDKN3 in different HCC microarray data sets

Genes that are co-expressed over a certain number of conditions suggests that these genes might be functionally related or even co-regulated [23]. Using the co-expression analysis of four microarray data sets from Oncomine [19–22], we found a total of 61 genes co-expressed with CDKN3 (Supplementary Table S1). Gene ontology analysis of these co-expressed genes showed many of them were involved in biological processes relevant to cell proliferation, such as mitotic cell cycle and nuclear division (Fig. 4A).

To further investigate the molecular mechanism of CDKN3, co-expression analysis was used to identify possible partners of CDKN3 in HCC tissues. We identified 61 co-expression genes for CDKN3, most of them are related in the processes of cell cycling such as BIRC5, BUB1, PTTG1 and TOP2A (Supplementary Table S1). Functional network investigation of these co-expression genes highlighted BIRC5 to be the central node in the CDKN3 co-expression network, which exhibited the most interactions with other genes. BIRC5 is a member of the inhibitor of apoptosis (IAP) gene family, which encodes negative regulatory proteins that prevent apoptotic cell death [37,38]. In term of cell proliferation, BIRC5 acts as a component of the chromosomal passenger complex that facilitates chromosome segregation and cytokinesis [39,40]. Furthermore, BIRC5 could also increase nuclear accumulation of cyclin D and CDK4 thus increases the phosphorylation of Rb and subsequently facilitate G1/S progression [41]. These findings provide a more comprehensive insight into the molecular mechanisms of CDKN3 involved in HCC cell cycling.

In summary, our results have shown that CDKN3 is frequently up-regulated in hepatocellular carcinoma and is related to poor clinical outcome of HCC. The functional data strongly suggest that CDKN3 behaves as an oncogene in HCC, and overexpression of CDKN3 could promote HCC cell proliferation. Furthermore, our study also generates a list of potential partner genes of CDKN3.

![Fig. 3. Overexpression of CDKN3 has no effect on HCC cell invasion. (A) Representative pictures of transwell assay observed under light microscope. (B) The results of transwell assay were quantitated by counting invasive cells in five randomly chosen high-power fields (400×) for each replicate (n = 3).](image-url)
which provides valuable information for further investigation toward a comprehensive understanding of CDKN3.

Acknowledgments

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Appendix A. Supplementary data
