High CCNB2 expression correlates with poor prognosis in hepatocellular carcinoma

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ABSTRACT

Cyclin B2 (CCNB2), a member of the cyclin protein family, has a key role in the progression of G2/M transition. However, the clinical value of CCNB2 in hepatocellular carcinoma (HCC) is still unknown. The aim of our study is to identify the role of CCNB2 in HCC patients. Immunohistochemical analysis using tissue microarray (TMA) was employed to evaluate the expression of CCNB2 in HCC and the correlation between CCNB2 expression and clinicopathological features in HCC patients. The relationship between CCNB2 expression and the prognosis of HCC patients was analyzed using Oncomine and Kaplan-Meier Plotter online resources. High CCNB2 cytoplasmic expression was observed in 77.22% of patients with HCC, which was related to differentiation (P<0.001), tumor diameter (P=0.025), and hepatitis B virus infection (P=0.008). High CCNB2 nuclear expression was seen among 43.43% of cases, which was associated with differentiation (P=0.001). CCNB2 levels were inversely proportional to patient prognosis. The study suggests that CCNB2 expression could be an effective prognostic biomarker for HCC.

Keywords: CCNB2, hepatocellular carcinoma, tissue microarray, immunohistochemistry, prognosis

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most frequent primary liver cancer and therefore presents a significant medical problem. Also, the occurrence of HCC has continued to increase in recent years, and currently HCC is the sixth most common neoplasm and the third leading cause of cancer-related death in the world. HCC has been recognized as a leading cause of death among patients with cirrhosis, and its incidence is expected to increase in the future⁴¹. HCC is a complex disease, which is associated with several risk factors, including chronic hepatitis B, aflatoxin B1 exposure, excessive alcohol intake, hepatitis C, non-alcoholic fatty liver disease and tobacco use⁴²⁻⁴⁵. Surgical resection, transplantation, ablation, transarterial chemoembolization (TACE)⁶⁻⁷, and the tyrosine-kinase inhibitors sorafenib⁸⁻⁹, lenvatinib¹⁰, and regorafenib¹¹ are all treatments with proven survival benefits. However, only a small proportion of pa-

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Conflicts of interests: The authors declared no conflict of interests.
tients with early-stage HCC can benefit from surgical resection and liver transplantation\textsuperscript{[12-13]}. Oral protein kinase inhibitors, such as sorafenib, are usually able to prolong the survival of patients with advanced HCC, however, eventual resistance to sorafenib restricts its application\textsuperscript{[14-15]}. Although previous studies have identified a number of molecular markers involved in the proliferation, invasion, differentiation and metastasis of HCC\textsuperscript{[16]}, the molecular pathogenesis underlying the development and progression of HCC still remains incompletely understood. Therefore, novel biomarkers are urgently needed to improve currently available therapeutic methods.

Cyclin B2 (CCNB2) is a member of the cyclin protein family, which regulate the activities of cyclin dependent kinases (CDKs) and different cyclins’ function, spatially and temporally in specific phases of the cell cycle\textsuperscript{[17]}. CCNB2 triggers the progression of G2-M transition and is involved in the regulation of CDK1\textsuperscript{[18]}. Recently, there has been an increasing number of studies, suggesting that CCNB2 expression was up-regulated in a variety of human cancers, including bladder carcinoma\textsuperscript{[19]}, breast carcinoma\textsuperscript{[20]}, colorectal adenocarcinoma\textsuperscript{[21]}, lung carcinoma\textsuperscript{[22-23]}, and pituitary adenoma\textsuperscript{[24]}. Moreover, CCNB2 mRNA expressed in circulating in the serum has also been found increased in tumor patients and is associated with cancer stage and metastasis status\textsuperscript{[25]}. In HCC, the association between CCNB2 expression and clinical outcome has not been investigated, so the potential of CCNB2 which acts as a candidate for molecular targeted therapy of HCC requires further exploration.

From bioinformatic analysis with Oncomine and Kaplan-Meier Plotter databases, we found differences in CCNB2 mRNA expression and prognosis of HCC. Because of the difficulty of storage and use of fresh samples, the method detecting mRNA is not as convenient as paraffin tissue. In addition, mRNA and protein expression are about 20% consistent. Based on the above, we investigated CCNB2 protein expression in a number of HCC samples by immunohistochemistry, using tissue microarray (TMA) sections. Moreover, we assessed associations between CCNB2 expression and clinicopathological factors, to determine its clinicopathological significance in a selected group of HCC patients. Finally, we evaluated the prognostic significance of CCNB2 protein expression level in HCC.

**MATERIALS AND METHODS**

**Patients and TMA analysis**

We collected a total of 255 liver tissue samples from 215 patients, of whom 198 had HCC. These patients contributed 198 samples of cancer tissue, with 40 having adjacent normal tissues, and 17 samples of hepatic hemangioma. Formalin-fixed paraffin-embedded HCC specimens (\( n = 198 \)) from patients who underwent surgery between 2009 and 2013 were obtained from the Affiliated Hospital of Nantong University. Clinical data (including gender, age, differentiation, TNM stage, tumor diameter, gross classification and other information) were obtained from the medical records of each patient. Prior to surgical therapy, none of the patients had received neoadjuvant chemotherapy, radiation therapy or immunotherapy. Survival time was calculated from the date of surgery until death or last follow-up. TMA was made by Tissue Microarray System (Quick-Ray, UT06, Unitma, Seoul, Korea) according to the method described previously\textsuperscript{[26]}. Ethical approval for this study was obtained from The Human Research Ethics Committee of Nantong University, Jiangsu, China, and informed consent was obtained for the use of all tissue samples before experimentation.

**Immunohistochemical (IHC) analysis**

For IHC analysis, TMA sections were deparaffinized in 100% xylene and rehydrated in graded ethanol solutions. For antigen retrieval, the sections were then boiled under pressure in Tris/EDTA buffer (pH 9.0) for 5 min. TMA sections were incubated for 1 h with a primary mouse anti-CCNB2 antibody (Abcam, UK) diluted 1:100 in TBS containing 1% bovine serum albumin. After washing, the sections were incubated with anti-rabbit horseradish peroxidase–conjugated antibody(Santa Cruz Biotechnology, CA, USA). CCNB2 immunostaining was evaluated independently by two trained pathologists who were unaware of the clinical background of the cases. Positivity of cell staining was recorded as a percentage(0–100%). Staining intensity was graded on a scale of 0(negative) to 3(strong). The final CCNB2 staining score was a product of the intensity grading and percentage of positive cells. The cut-off point for statistically significant CCNB2 expression score in terms of overall survival(OS) was determined using the X-tile software program(Rimm Lab, Yale University, New Haven, CT, USA) as described elsewhere\textsuperscript{[27]}. The degree of staining was quantified using a two-level grading system, and staining scores were defined as follows: for CCNB2 cytoplasmic, 0–100 was regarded as low expression while 101–300 was regarded as high expression; for the CCNB2 nucleus, 0–200 was regarded as low expression while 201–300 was regarded as high expression.
Bioinformatic analysis of the Oncomine database and Kaplan-Meier Plotter curves

The expression pattern of CCNB2 in a large number of HCC tissues was evaluated using Oncomine (https://www.oncomine.org), a database of RNA and DNA sequencing information curated from the Gene Expression Omnibus, The Cancer Genome Atlas(TCGA), and other published literature. "CCNB2", "cancer vs. normal analysis", "hepatocellular carcinoma", and "mRNA" were entered as the search terms to obtain CCNB2 expression data in HCC. We also compared CCNB2 mRNA expression between HCC and other liver tissues using the same approach. The data were provided as log2 median-centered intensity in the Oncomine microarray datasets.

The online database and graphing program Kaplan-Meier Plotter (http://kmplot.com/analysis/) were used to investigate the relationship between CCNB2 mRNA expression and OS of HCC patients. This program can assess the effect of 54,675 genes on the survival of patients with various types of cancer, including HCC, breast cancer, lung cancer and ovarian cancer. We used this program to evaluate the prognostic value of CCNB2 expression in HCC patients.

Statistical analysis

Relationships between clinicopathological factors and CCNB2 expression were examined using chi-square test. For the TMA slides, the following clinical data were assessed: gender, age, differentiation, TNM stage, tumor diameter, gross classification, and other clinicopathological informations. Univariate and multivariate analyses were evaluated using Cox proportional hazards regression models. Survival curves were estimated using the Kaplan-Meier method. For all statistical analyses, P-value less than 0.05 was regarded as statistically significant, and were carried out using SPSS V.20.0 software (SPSS Inc, Chicago, IL, USA). All statistical tests were two-sided.

RESULTS

Clinical features of HCC

The 198 HCC patients’ average age was 51.46 years (range, 31~76 years). There were 43 cases with a tumor diameter ≤ 2 cm and 155 with a tumor diameter > 2 cm. The differentiation of HCC included well (23 cases), moderate (119 cases) and poor (43 cases). All cases were stratified according to TNM (I, 94 cases; II, 69 cases; III, 16 cases; and IV, 0 cases). TNM staging was based on the seventh AJCC TNM staging system [20]. Detailed clinicopathological data are shown in Table 1.

Expression of CCNB2 in HCC by IHC staining

We performed IHC staining to examine CCNB2 expression in HCC. The TMA included 255 archived liver tissue blocks, consisting of 198 HCC, 17 hepatitis B virus infection, 17 hepatitis C virus infection and 40 adjacent normal tissues. Positive staining was localized mainly in the cytoplasm and nucleus of HCC cells. Of the 40 normal adjacent tissues, only 4 tissues detected positive for the expression of CCNB2 (4/40, 10.00%). However, high CCNB2 expression was seen in 86 of 198 cases (43.43%) of HCC, which indicated that the expression of CCNB2 was higher in HCC tissue than in benign tissues (P<0.001; Table 2). Typical IHC staining patterns for CCNB2 in HCC are shown in Fig. 1. These data show that CCNB2 protein expression is higher in HCC tissue than in peritumoral liver tissue.

Association between CCNB2 expression and clinicopathological parameters

The relationship between high CCNB2 expression and clinicopathological features of 198 cases of HCC is shown in Table 1. High CCNB2 cytoplasmic expression was related to differentiation (P<0.001), tumor diameter (P=0.025), and hepatitis B virus infection (P=0.008). And high CCNB2 nuclear expression was only related to differentiation (P=0.001). In contrast, no statistically significant correlation was found between CCNB2 expression and other clinical parameters, including sex, age, TNM stage, gross classification, α-fetoprotein, liver cirrhosis, and portal vein invasion.

Relationship between patient survival, CCNB2 protein expression, and clinicopathological parameters

Several known predictive factors of poor outcome in HCC were assessed, to confirm that our cohort of patients were representative of those with HCC (Table 3). As expected, CCNB2 protein cytoplasmic overexpression (P=0.002) and nuclear overexpression (P<0.001) were significantly associated with 5-year survival by Cox regression univariate analysis. In addition, other prognostic factors such as hepatitis B virus infection (P<0.001) and liver cirrhosis (P<0.001) were also statistically significant. All these factors were included in the multivariable analysis. High CCNB2 nuclear expression (P=0.029), hepatitis B virus infection (P=0.001), and liver cirrhosis (P=0.029) were all identified as independent predictive factors for poor outcome. Kaplan-Meier survival curves demonstrated that patients with low CCNB2 nuclear expression, no HBV infection and no liver cirrhosis had a significantly longer survival time (Fig. 2).
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Correlation between CCNB2 expression and prognosis

To further evaluate the relationship between survival and CCNB2 expression in HCC, we analyzed data from Oncomine. Consistent with our data, CCNB2 mRNA was found to be overexpressed in HCC compared with normal tissues (Fig. 3). We evaluated the correlation between CCNB2 expression and OS of HCC patients using Kaplan-Meier Plotter (Fig. 4) and found that low CCNB2 expression is a favorable prognostic factor for OS (HR = 1.91, 95%CI: 1.28–2.87, P = 0.0013; n = 364).
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DISCUSSION

The development of HCC is a complex multistep process that involves sustained inflammatory damage, including hepatocyte necrosis and regeneration associated with fibrotic deposition. A risk of HCC emerges when cirrhosis is established, increasing in parallel to progressive liver function impairment. HCC is the result of the accumulation of somatic genomic alterations in passenger and driver genes, in addition to epigenetic modifications, which explains its huge molecular heterogeneity. These complex phenomena have been reviewed elsewhere. Even in patients with similar clinical and pathological features, the outcome of HCC varies, highlighting the underlying diversity of this disease. Therefore, efficient anticancer therapies depend on targeting cancer cells at all stages of differentiation. In addition, there is an urgent need for novel biomarkers that relate to the mechanism of the disease for determining prognosis and in guiding therapy.

There are two mammalian B-type cyclins, cyclin

Table 3 Univariable and multivariable analysis of prognostic factors for 5-year survival in HCC

<table>
<thead>
<tr>
<th>Prognostic factors</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
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<tbody>
<tr>
<td></td>
<td>HR</td>
<td>P value</td>
</tr>
<tr>
<td>CCNB2 cytoplasm expression(high vs.low)</td>
<td>1.925</td>
<td>0.002</td>
</tr>
<tr>
<td>CCNB2 nucleus expression(high vs.low)</td>
<td>2.051</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gender(male vs. female)</td>
<td>1.149</td>
<td>0.549</td>
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<tr>
<td>Age (years)(≤ 60 vs.&gt;60)</td>
<td>1.147</td>
<td>0.520</td>
</tr>
<tr>
<td>Differentiation (well vs.moderate vs. poor)</td>
<td>1.225</td>
<td>0.520</td>
</tr>
<tr>
<td>Stage grouping with TNM(stage I vs. stage II vs. stage III~V)</td>
<td>0.809</td>
<td>0.217</td>
</tr>
<tr>
<td>Tumor diameter (cm)(≤ 2 vs.&gt;2)</td>
<td>1.435</td>
<td>0.176</td>
</tr>
<tr>
<td>Gross classification(multifocal vs. unifocal)</td>
<td>1.391</td>
<td>0.420</td>
</tr>
<tr>
<td>Hepatitis B virus infection(yes vs. no)</td>
<td>0.362</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>α-fetoprotein (ng/mL)(&lt;50 vs. 50~900 vs.&gt;900)</td>
<td>1.100</td>
<td>0.448</td>
</tr>
<tr>
<td>Liver cirrhosis(yes vs. no)</td>
<td>0.354</td>
<td>&lt;0.001</td>
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<tr>
<td>Portal vein invasion(yes vs. no)</td>
<td>0.993</td>
<td>0.973</td>
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</tbody>
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Fig.1 Representative immunohistochemistry (IHC) images showing expression of CCNB2 in tissue microarray sections of HCC. A: Poor differentiation HCC. B: Moderate differentiation HCC. C: Well differentiation HCC. D: Liver tissue of hepatic hemangioma. Red arrow represents positive CCNB2 protein expression on HCC and normal tissue, and green arrow represents negative CCNB2 protein expression on normal tissue. Original magnification was ×400.

Fig.2 Survival curves of HCC by the Kaplan-Meier method and the log-rank test. A: Overall survival curves of high CCNB2 nucleus expression (green line, 1) and low CCNB2 nucleus expression (blue line, 0). B: Overall survival curves by hepatitis B virus infection. Yes (blue line, 0) and no (green line, 1). C: Overall survival curves by liver cirrhosis. Yes (blue line, 0) and no (green line, 1).
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Comparison of CCNB2 expression across 5 analyses

<table>
<thead>
<tr>
<th>Median Rank</th>
<th>P-Value</th>
<th>Gene</th>
</tr>
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<tbody>
<tr>
<td>24.0</td>
<td>4.51E-61</td>
<td>CCNB2</td>
</tr>
</tbody>
</table>

Legend

3. Hepatocellular Carcinoma vs. Normal Liver, Cancer Res. 2010
4. Hepatocellular Carcinoma vs. Normal Liver, Cancer Res. 2010

| 1 | 5 | 10 | 25 | 25 | 10 | 5 | 1 | Not measured |

The rank for a gene is the median rank for that gene across each of the analyses. The P-Value for a gene is its P-Value for the median-ranked analysis.

Fig. 3 Five analyses were performed in comparing the RNA expression of CCNB2 between hepatocellular carcinoma and normal tissue. The intensity of color shows the respective levels of CCNB2. The red column shows the CCNB2 mRNA upregulation.

Fig. 4 Prognostic value of CCNB2 expression in patients with HCC. CCNB2 probe number is 9,133. Results were analyzed using the Kaplan-Meier Plotter database, the red line represents the patient with CCNB2 expression above the median, the black line represents the patient with CCNB2 expression below the median.

B1 and cyclin B2, which differ in their NH2 termini but have 57% similarity[31]. The two cyclins are co-expressed in most dividing cells. Cyclin B1 is widely dispersed in cytoplasm, whereas cyclin B2 primarily moves to the Golgi apparatus during the whole cell cycle[31]. Their different intracellular localizations indicate their distinct functions in mitosis[32]. Generally, cell proliferation, differentiation, as well as cell cycle are dysregulated in malignant cells[33-34]. The overexpression of many cyclins has been reported in a variety of malignant tumors[35]. For example, the overexpressions of cyclin D1, B1, A, and E have been often observed in various types of cancers, and are also correlated with prognosis in some of these cancers[36-40]. As far as we know, no research has been previously reported on the correlation between CCNB2 and survival of patients with HCC.

The CCNB2 gene is involved in G2-M transition in eukaryotes, by activating CDC2 kinase and its inhibition induces cell cycle arrest[18, 41]. In accordance with its crucial role in cell growth, numerous studies detected overexpression of CCNB2 in human tumors. Lei et al. found the overexpression of CCNB2 in bladder cancer, while the resultant decrease of CCNB2 inhibited the cells’ invasive and migratory ability[19]. Shubbar et al. reported that cytoplasmic CCNB2 may function as an oncogene, and could serve as a potential biomarker of unfavorable prognosis over short-term follow-up in breast cancer[20]. Qian et al. suggested that the overexpression of CCNB2 serves a significant role in non-small-cell lung cancer (NSCLC) progression through promoting tumor growth and accelerating tumor cell metastasis[23]. In addition, Mo et al. reported that serum circulating CCNB2 mRNA in cancer patients was obviously higher than that in normal controls and the benign disease group[25]. The effect caused by the change of CCNB2 is perhaps related to its binding to the membranous structure in the cytoplasm and its role in the formation of a bipolar spindle at metaphase[32, 42].

In the present investigation, we used TMA-IHC and bioinformatic approaches to investigate the relationship between CCNB2 protein levels and the clinico-pathological features of HCC patients. We primarily analyzed microarray data from tumors and adjacent normal tissue specimens, and found CCNB2 was significantly overexpressed in HCC tissues compared with paired adjacent normal tissue. Our univariate analysis clearly showed that high cytoplasmic and nuclear expression of CCNB2 were significantly associated with tumor differential and poor survival. But the multivariate analysis indicated that only CCNB2 nucleus expression was regarded as an independent prognostic factor for HCC patients. This suggests that nuclear, rather than the cytoplasm expression of CCNB2 is a significant differential site for HCC patients. Further, we used bioinformatic databases to confirm the relationship between higher CCNB2 expression and poor prognosis, and the results were consistent with our analysis of the correlations between CCNB2 protein levels and HCC patients’ clinico-pathological characteristics.

In conclusion, our findings demonstrated a high expression of CCNB2 in HCC specimens, associated with a poor prognosis and CCNB2 might be used as a novel prognostic marker for HCC. Many cytokines, including P53, transforming growth factorβ (TGF–β) , and high mobility group A1 (HMGAl), etc., have been reported as regulating the cell cycle through CCNB2[21, 24, 43-44].
However, we know little about the regulating pathway of CCNB2 in HCC. This is the focus of our following study.

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(Received 17 April 2019, Revised 20 May 2019, Accepted 29 May 2019)