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The Clinical and Prognostic Significance of CCN3 Expression in Patients with Cervical Cancer

Znaczenie kliniczne i prognostyczne ekspresji CCN3 u pacjentek chorych na raka szyjki macicy

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article; G – other

Abstract

Background. CCN3 plays important roles in growth, differentiation, angiogenesis and adhesion. Recently, the role of CCN3 in human carcinogenesis has become an area of great interest. However, little is known about the function of CCN3 in human cervical cancer.

Objectives. The aim of this study was to investigate the expression profile of CCN3 in cervical cancer and to assess its clinical significance.

Material and Methods. In this study, qRT-PCR, immunohistochemistry and Western blotting analysis were used in the detection of CCN3 mRNA and protein expression, both in cervical cancer and in corresponding normal tissue, respectively. The data was correlated with clinicopathological features. A survival analysis was performed to assess the prognostic significance.

Results. CCN3 mRNA was overexpressed in cervical cancer tissue when compared with corresponding normal tissue, as was CCN3 protein. Upregulation of CCN3 was significantly associated with the stage of the disease \((P = 0.017)\) and with lymph node involvement \((P = 0.006)\). Using the Kaplan-Meier analysis, a comparison of survival curves of low vs. high expressers of CCN3 revealed a highly significant difference in human cervical cancer tissue \((P = 0.021)\), which suggests that overexpression of CCN3 is associated with a poorer prognosis.

Conclusions. The results of the current study suggest that CCN3 may play an important role in cervical carcinogenesis and therefore may have potential as a biomarker for prognosis and as a therapeutic target in cervical cancer (Adv Clin Exp Med 2013, 22, 6, 839–845).

Key words: CCN3; cervical cancer; clinicopathological parameters; prognosis.

Cervical cancer continues to be one of the major causes of cancer-related death in women worldwide. It was reported in Lancet that global cervical cancer incidence has increased 0.6% annually from 1980 to 2010, and the disease killed 200,000 women in 2010 in developing countries [1]. The screening test for cervical cancer is helpful for prevention and early detection, and standard treatments can lead to remission, but clinical outcomes can be very different for different patients, and are not easy to predict. Therefore, an exploration of the molecular pathogenesis of cervical cancer and the identification of potential markers for early detection may play a significant role in treatment and prognosis.

It is well known that various factors are related to cancer metastasis, invasion and tumor angiogenesis. Nephroblastoma overexpressed (NOV or CCN3) is a secreted matrix-associated protein that
belongs to the CCN gene family and is involved in many cellular functions, including growth, differentiation, angiogenesis and adhesion [2]. CCN3 gene expression has been investigated in several tumors [3–5]. Chen et al. have shown that CCN3 increases cell migration and expression of intercellular adhesion molecule-1 (ICAM-1) in prostate cancer cells [2]. A comprehensive study on Ewing’s sarcoma patients has demonstrated that high CCN3 expression was associated with a higher risk of metastasis [6], indicating again that CCN3 is associated with cell migration and invasion. It has also been indicated that CCN3 enhances the migration of chondrosarcoma cells by increasing MMP-13 expression through the avβ3/avβ5 integrin receptor, FAK, PI3K, Akt, p65 and NF-kB signal transduction pathway [7].

However, neither the expression profile nor the clinical significance of CCN3 have been elucidated in cervical cancer thus far. Therefore, in the present study, the authors compared the expression of CCN3 mRNA and protein in normal human cervical tissue and in cervical cancer tissue. Associations between CCN3 and clinicopathological features, as well as the prognosis, were also investigated.

A correlation was found between CCN3 overexpression and poor survival, which suggests that CCN3 may play an important role in cervical carcinogenesis.

Material and Methods

Patients and Tissue Samples

Cervical cancer tissue and adjacent normal tissue were obtained from 56 consecutive patients with cervical cancer that was confirmed by histopathological analysis at the Department of Gynecology at Wuxi and Nanjing Maternity and Child Health Hospital, affiliated with Nanjing Medical University, China, between 2004 and 2006. The study protocol was approved by the Institutional Review Board of Nanjing Medical University and all participants signed an informed consent form.

The clinicopathological patient characteristics are summarized in Table 1.

No patient had received radiotherapy, chemotherapy or other treatment prior to surgery. Following surgical removal, the tissue sample was immediately frozen in liquid nitrogen until used, and was formalin-fixed and paraffin-embedded for histopathologic diagnosis and immunohistochemical examination, respectively.

Quantitative Reverse-Transcriptase Polymerase Chain Reaction (qRT-PCR)

QRT-PCR was performed to detect CCN3 mRNA expression. A power homogenizer in TRizol Reagent (Applied Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer’s protocol to extract total RNA from the frozen tissue by homogenization. The total RNA was then reverse transcribed to generate cDNA (using a PrimeScript RT-PCR kit; Takara Bio). β-actin was used as an internal control. The levels of mRNA encoding were quantified by real-time PCR with the Applied Biosystems 7900 HT Fast Real-Time PCR System using SYBR Premix Ex Taq (Applied Takara Bio). The sequences of the primers were as follows: human CCN3 forward 5’-CACggCggTAgAgggAgATA-3’ and reverse 5’-gggTAAggCCTCCCAgTgAA-3’ (product 251 bp); human β-actin forward 5’-CAAgAgATggCACggCTgCT-3’ and reverse 5’-TCCTTCTgCATCTGTCGGCA-3’ (product 275 bp). The PCR conditions included an initial denaturation step of 95°C for 10 min, followed by 40 cycles of 94°C for 10 s and 60°C for 1 min and a final elongation step of 72°C for 10 min. All qRT-PCRs were performed in duplicate. Relative quantification of CCN3 mRNA expression was calculated using the 2^{ΔΔCT} method.

Immunohistochemistry

Formalin-fixed tissues were embedded in paraffin, sectioned at 5 µm, and mounted on silane-coated slides. The sections were de-waxed and rehydrated through descending grades of alcohol to distilled water, then the endogenous peroxidase was blocked using 3% (v/v) hydrogen peroxidase in PBS. The sections were then subjected to microwave antigen retrieval in 0.02 M EDTA. They were washed in PBS and blocked with rabbit serum (Beijing ZhongShan Biotechnology) for 2 h, then incubated overnight at 4°C with polyclonal CCN3 antibody (diluted 1 : 500, Abcam, Cambridge, MA, USA). Following 3 washes in PBS, the sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (diluted 1 : 1.000; Beijing ZhongShan Biotechnology) for 1 h at room temperature. Immunoreactivity was demonstrated using di-amino benzadine (Sigma, Munich, Germany) for increased sensitivity, which produced a brown insoluble precipitate at the immunopositive sites. The sections were counterstained with hematoxylin and mounted on a cover glass. The negative controls were incubated with a solution that was devoid of any primary antibody.
All the immunostained sections were evaluated blind by two observers. For the assessment of CCN3, 5 high-power fields in each specimen were randomly selected, and brown staining of the cytoplasm was considered positive staining. The distribution of positive cells was graded as follows: 0 = no detectable staining; 1 = staining < 1/3 positive cells; 2 = staining 1/3–2/3 positive cells; 3 = staining > 2/3 positive cells. The intensity of staining was graded as follows: 0 = no staining; 1 = weak; 2 = moderate; and 3 = strong staining. The scores for distribution and intensity were added and graded as follows: 0 = (–); 2, 3 = (±); 4 = (+); 5 = (++); 6 = (+++). Scores < 4 were defined as negative and scores > 4 were defined as positive [8].

Western Blotting

The frozen tissues were homogenized with an Ultra Turrax homogenizer (Ika, Petaling Jaya, Malaysia) in a lysis buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (w/v) DTT, 1% (w/v) Protease Inhibitor Cocktail Kit (Pierce Biotechnology, Rockford, IL, USA), at 11,000 IU/min on ice (10 bursts of 10 s, each interspersed with short pauses). Suspensions were shaken at 4°C for 1 h, and insoluble molecules were removed by centrifugation at 40,000 × g at 4°C for 1 h. The protein concentration in each sample was determined by the Bradford method using BSA as the standard. Samples that contained 50–100 µg protein from 2 groups were electrophoresed on a 12% SDS polyacrylamide gel and transferred to a nitrocellulose membrane (GE Healthcare, San Francisco, CA, USA). The membranes were blocked in tris-buffered saline (TBS) that contained 5% non-fat milk powder for 1 h, then incubated with anti-CCN3 (1 : 500) and anti-GAPDH (1 : 1.00; Abcam) diluted in TBS/5% non-fat milk powder overnight. GAPDH was used as a loading control. Membranes were washed 3 times (10 min each) with TBS and incubated for 1 h with HRP-conjugated goat anti-rabbit IgG (1 : 1.00; Beijing ZhongShan Biotechnology, Beijing, China). Specific proteins were detected using an ECL kit and AlphaImager (FluorChem5500; Alpha Innotech). The protein expression level was analyzed using AlphaEaseFC software (Alpha Innotech).

Statistical Analysis

The statistical analysis was performed using Statistical Program for Social Sciences (SPSS) software, version 16.0 (SPSS Inc., Chicago, IL, USA). The results of CCN3 expression in cervical cancer tissue and in corresponding normal tissue were expressed as the mean ± SE, and the data was analyzed using Student’s t test. The association between CCN3 expression and clinicopathological features was analyzed using χ² test. Kaplan-Meier curves were constructed, and the log-rank test was performed to analyze survival data. A P-value of less than 0.05 was considered statistically significant.

Results

Expression Profile of CCN3 mRNA in Human Cervical Cancer and in Corresponding Normal Cervical Tissue

The qRT-PCR was carried out to identify transcripts that encoded human CCN3 in 15 paired samples of cervical cancer tissue and corresponding normal tissue. The results showed that CCN3 mRNA expression level in cervical cancer tissue was markedly up-regulated (3.46 ± 0.83) when compared with corresponding normal tissue (1.21 ± 0.67); the difference between the 2 groups is statistically significant (P < 0.05).

Expression Profile and Subcellular Location of CCN3 Protein in Human Cervical Cancer and in Corresponding Normal Cervical Tissue

The expression profile and distribution of CCN3 was determined by immunohistochemistry in 56 paired samples of cervical cancer tissue and corresponding normal cervical tissue. Representative examples of reactivity for CCN3 are shown in Fig. 1. There was no or low expression of CCN3 in normal cervical tissue (Fig. 1A). In contrast, cervical cancer samples revealed intensive CCN3 staining, which was scored as positive where strong cytoplasmic staining was present (Fig. 1B). The positive rate of CCN3 expression in cervical cancer is 53.57% (30/56). Western blot analysis was also performed, to confirm the differential expression of CCN3 protein in 6 paired samples of carcinoma and corresponding normal tissue. These results also revealed that CCN3 was dramatically increased in cervical cancer tissue when compared with normal cervical tissue, which showed little or no expression of CCN3 (Fig. 2).
Correlation of CCN3 Expression with Clinicopathological Parameters in Cervical Cancer

Because one aim of the study was to explore the clinical significance of CCN3 expression in the patient samples, the association between CCN3 expression and clinicopathological parameters was evaluated. In all 56 patients, it was found that the FIGO disease stage and lymph node status were significantly correlated with CCN3 expression levels.

Table 1. The correlation of CCN3 expression with clinicopathological parameters in 56 cervical cancer specimens

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Total</th>
<th>CCN3</th>
<th>P-value</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Negative, n (%)</td>
<td>Positive, n (%)</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
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<td></td>
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<tr>
<td>&lt; 40</td>
<td>23</td>
<td>12 (52.17)</td>
<td>11 (47.83)</td>
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<tr>
<td>≥ 40</td>
<td>33</td>
<td>14 (42.42)</td>
<td>19 (57.58)</td>
</tr>
<tr>
<td><strong>Grade</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>19</td>
<td>11 (57.89)</td>
<td>8 (42.11)</td>
</tr>
<tr>
<td>G2</td>
<td>16</td>
<td>7 (43.75)</td>
<td>9 (56.25)</td>
</tr>
<tr>
<td>G3</td>
<td>21</td>
<td>8 (38.10)</td>
<td>13 (61.90)</td>
</tr>
<tr>
<td><strong>Tumor size</strong></td>
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<tr>
<td>&lt; 2.5 cm</td>
<td>25</td>
<td>14 (56.00)</td>
<td>11 (44.00)</td>
</tr>
<tr>
<td>≥ 2.5 cm</td>
<td>31</td>
<td>12 (38.71)</td>
<td>19 (61.29)</td>
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<td><strong>FIGO disease stage</strong></td>
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<td></td>
<td></td>
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<tr>
<td>I–II</td>
<td>27</td>
<td>17 (62.96)</td>
<td>10 (37.04)</td>
</tr>
<tr>
<td>III–IV</td>
<td>29</td>
<td>9 (31.03)</td>
<td>20 (68.97)</td>
</tr>
<tr>
<td><strong>Lymph node</strong></td>
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<td></td>
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<tr>
<td>negative</td>
<td>30</td>
<td>19 (63.33)</td>
<td>11 (36.67)</td>
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<tr>
<td>positive</td>
<td>26</td>
<td>7 (26.92)</td>
<td>19 (73.08)</td>
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<tr>
<td><strong>Menses</strong></td>
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<td></td>
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<td>Pre-menopause</td>
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<td>17 (53.12)</td>
</tr>
<tr>
<td>Post-menopause</td>
<td>24</td>
<td>11 (45.83)</td>
<td>13 (54.17)</td>
</tr>
</tbody>
</table>

Statistical analysis was performed by the χ² test. *P < 0.05 was considered significant.
node metastasis were associated with CCN3 expression (Table 1). Positive staining for CCN3 protein in patients < 40 and ≥ 40 years of age was 47.83% and 57.58%, respectively, which indicated no significant difference between age groups in CCN3 positive staining with cervical cancer (P = 0.472). There were also no significant differences in positive CCN3 protein staining in histological G2 (56.25%) and G3 (61.90%) compared with G1 (42.11%) (P = 0.441). Meanwhile, CCN3 expression was not associated with tumor size (P = 0.197) or menses (P = 0.938). However, there was a higher percentage of positive CCN3 protein staining in disease stages III and IV (68.97%) when compared with stages I and II (37.04%) (P = 0.017). There was also a significant difference in the correlation of positive CCN3 protein staining and lymph node invasion (negative lymph node invasion vs. positive lymph node invasion: 36.67% vs. 73.08%) (P = 0.006). These results indicate that overexpression of CCN3 is related to disease stage and lymph node invasion in cervical cancer.

**CCN3 Protein Expression Associated with Shorter Overall Survival**

The survival analysis of the 56 studied patients was performed using information available from the clinical follow-ups. At the end of follow-up, 26 patients were still alive; 27 had died; and 3 had not reported for follow-up, resulting in a follow-up rate of 94.64%. Of the 53 evaluable patients, only 8 of the 24 (33.33%) in the negative CCN3 staining group had died of the disease, compared with 19 of the 29 (65.52%) in the positive CCN3 staining group. CCN3 overexpression in patients with cervical cancer was related to reduced overall survival time in a log-rank test (P = 0.021) (Fig. 3). These observations suggest that CCN3 overexpression in cervical cancer patients is associated with reduced overall survival.

**Discussion**

Cervical cancer is the 3rd most common malignancy in women and a major cause of morbidity and mortality, particularly in developing countries [9]. Even though radiotherapy, chemotherapy and surgery are used as standard treatment modalities for patients with cervical cancer, and can lead to consequent disease remission, the prognosis of patients remains unsatisfactory. Therefore, characterization of identifiable molecular markers should be of diagnostic, prognostic and therapeutic value in the management of cervical cancer.

CCN3 is a cysteine-rich protein that belongs to the CCN family of matricellular proteins [10, 11], which interact with the extracellular matrix and thereby regulate many cellular functions, including cell division, chemotaxis, apoptosis, adhesion, motility and ion transport [12–15]. In recent years, CCN3 has been shown to play an important role in tumorigenesis, including cancer cell proliferation, survival, adhesion and invasion [6, 7]. Perbal et al. found that elevated CCN3 expression in osteosarcoma significantly correlated with a worse prognosis, which suggests that assessment of CCN3 expression levels at the time of diagnosis may represent a useful molecular tool for the early identification of patients with different prognoses [16]. It has also been demonstrated that CCN3 enhances the migration of chondrosarcoma cells [7]. Vallacchi et al. [17] reported that cells with overexpression of CCN3 by transfection showed increased adhesion to extracellular matrix proteins, whereas inhibition of CCN3 expression by siRNA decreased adhesion to laminin and vitronectin; CCN3 overexpression was associated with melanoma progression, including metastases and relapse by inducing increased expression of laminin and vitronectin integrin receptors α7β1 and αvβ5. Moreover, it has been reported that the most malignant prostate cancer cell line, PC-3, has the highest CCN3 expression [2, 18] and that CCN3 was overexpressed in human prostatic adenocarcinoma samples [18], suggesting that the expression...
of CCN3 is positively correlated with tumorigenesis in prostate cancer. Recent data suggests that CCN3 impairs osteoblast and stimulates osteoclast differentiation to favor breast cancer metastasis to bone [19].

Although many studies have demonstrated the involvement of CCN3 expression in tumor biology, little is known about the expression of CCN3 in human cervical cancer. In this study, the authors initially aimed at analyzing the expression of CCN3 and its clinical significance in cervical cancer, using qRT-PCR, immunohistochemistry and Western blotting analysis to describe the expression and immunolocalization of CCN3 in cervical carcinoma. It was found that CCN3 mRNA was overexpressed in cervical cancer tissue when compared with corresponding normal tissue. Furthermore, it was observed that CCN3 protein overexpression occurred in cervical cancer, whereas adjacent normal tissue showed little or no CCN3 expression. These results were also confirmed by Western blotting analysis. The study suggests that increased CCN3 levels may be closely associated with the pathogenesis of cervical cancer.

In order to investigate the clinical significance of CCN3 overexpression in cervical cancer, the authors evaluated the correlation between CCN3 and clinicopathological parameters, including prognosis. In all 56 patients, there were no significant differences in positive staining for CCN3 protein in patients of different ages (P = 0.472) and with different histological grades (P = 0.441). Meanwhile, the expression of CCN3 was not associated with tumor size (P = 0.197) or with menses (P = 0.938). However, there was a higher percentage of positive CCN3 protein staining in disease stages III–IV (68.97%) when compared with stages I–II (37.04%) (P = 0.017). There was also a significant difference in the correlation of positive CCN3 protein staining with lymph node invasion (negative lymph node invasion vs. positive lymph node invasion: 36.67% vs. 73.08%) (P = 0.006). These results indicate that overexpression of CCN3 is related to the disease stage and to lymph node invasion in cervical cancer.

Using the Kaplan-Meier analysis, a comparison of the survival curves of low vs. high expressers of CCN3 revealed a highly significant difference in human cervical cancer tissue (P = 0.021), which suggests that overexpression of CCN3 in cervical cancer patients is associated with a poorer prognosis and reduced overall survival (Fig. 3). These findings highlight CCN3 overexpression as a cervical cancer-specific event and suggest that CCN3 might be a reliable indicator of prognosis in cervical cancer patients and represents a promising new target for treating cervical cancer.

This study indicates that CCN3 expression is increased in cervical cancer and is positively correlated with a poor prognosis. The results demonstrate the importance of CCN3 in cervical carcinogenesis, which suggests that CCN3 may play an important role as a biomarker for prognosis and as a therapeutic target in cervical cancer. However, further studies are necessary to elucidate the molecular mechanisms of CCN3 in the pathogenesis of cervical cancer.

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