Basic Study

Chemokine ligand 20 enhances progression of hepatocellular carcinoma via epithelial-mesenchymal transition

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AIM: To identify the mechanisms of chemokine ligand 20 (CCL20)-induced hepatocellular carcinoma (HCC) metastasis and evaluate it as a prognostic marker.

METHODS: Expression of CCL20 was evaluated by immunohistochemistry in HCC tissues from 62 patients who underwent curative resection. The relationship between CCL20 expression and clinicopathologic features was analyzed. Univariate and multivariate analyses were performed to evaluate its predictive value for recurrence and survival of HCC patients. The expression levels of epithelial-mesenchymal transition (EMT)-and signaling pathway-related proteins were evaluated by Western blotting and immunocytochemistry. The effects of CCL20 on HCC cell proliferation and migration were analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoltetrazolium bromide (MTT) and Transwell assays.

RESULTS: CCL20 immunoreactivity was detected in all 62 patient specimens. CCL20 expression was associated with preoperative alpha-fetoprotein level (P = 0.043), tumor size (P = 0.000), tumor number (P = 0.008), vascular invasion (P = 0.014), and tumor differentiation (P = 0.007). Patients with high CCL20 expression had poorer recurrence-free and overall survivals compared to those with low CCL20 expression (both P < 0.001). CCL20 induced EMT-like changes in HCC cells and increased their proliferation and migration ability (P < 0.05). Western blotting and immunofluorescence staining showed that CCL20 induced an EMT-like phenotype in HCC cells, and increased expression of phosphorylated AKT, β-catenin and vimentin, and decreased E-cadherin expression (P < 0.05). The correlation analysis revealed that high CCL20 expression in HCC tissue specimens was negatively correlated with E-cadherin expression (13.33%, 4/30), and positively correlated with vimentin (90.0%, 27/30), β-catenin (96.67%, 29/30) and p-AKT (76.67%, 23/30) expression.

CONCLUSION: CCL20 expression is associated with HCC recurrence and patient survival and promotes HCC cell proliferation and migration by inducing EMT-like changes via PI3K/AKT and Wnt/β-catenin pathways.

Key words: Chemokine ligand 20; Phosphoinositide kinase-3/AKT; Prognosis; Wnt/β-catenin; Epithelial-mesenchymal transition; Hepatocellular carcinoma

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Core tip: This study examined the expression and prognostic value of chemokine ligand 20 in hepatocellular carcinoma. The results indicate that increased expression of this protein regulates the growth and migration of hepatocellular carcinoma cells and epithelial-mesenchymal transition via phosphoinositide kinase-3/AKT, and Wnt/β-catenin signaling pathways and is therefore a potential treatment target.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer and third leading cause of cancer death worldwide[1-3]. HCC is associated with a poor outcome and a high rate of mortality due to the high rate of recurrence and metastasis[4-6]. Thus, it is essential to identify novel predictors of recurrence and metastasis. The risk factors for HCC include hepatitis B or C virus infection, alcohol consumption, and non-alcoholic fatty liver disease[7-10]. Moreover, the tumor microenvironment contains various cytokines, chemokines and growth factors that are produced by tumor or stromal cells, which promote tumor initiation, progression or metastatic processes in many malignancies, including breast and colorectal cancer[11,12]. CCL20 is related to tumor formation, progression or metastatic processes in many malignancies, including breast and colorectal cancer[13-15]. The proliferation and migration of tumor cells are considered a foundation of cancer survival and development. Tumor progression and metastasis are associated with the induction of epithelial-mesenchymal transition (EMT)[16,17]. In this study, CCL20 expression was examined in HCC patient samples and the relationships with clinicopathologic features and recurrence and patient survival were examined. Moreover, the effects of CCL20 expression on HCC cell EMT, proliferation and migration and involvement of relevant signaling pathways were investigated.

MATERIALS AND METHODS

Patients and specimens

From January 2002 to October 2008, 62 consecutive patients with primary HCC who underwent radical resection at our hospital were enrolled in the study. The diagnosis was confirmed by histologic examination in all cases. All primary tumor tissues were preserved in paraffin for immunohistochemical analyses. None of the patients received preoperative anticancer treatment. Preoperative liver function was evaluated using the Child-Pugh scoring system. Tumor stage was determined according to the tumor-node-metastasis (TNM) classification system of the American Joint Cancer Committee/Union for International Cancer Control (2002). Tumor differentiation was graded using the Edmondson-Steiner classification system. Data was censored at the last follow-up for patients without recurrence or death. Recurrence-free survival (RFS) and overall survival (OS) was defined as the interval between the time of surgery to that of recurrence or death, respectively. This study was approved by the Ethics Committee of our hospital. Written informed consent was obtained from all patients.

Cell culture and treatment

Human HCC cell lines Hep3B and Huh7 were maintained in Dulbecco’s Modified Eagle’s Medium (Gibco of Thermo Fisher Scientific Inc., Waltham, MA, United States) supplemented with 10% fetal calf serum and 100 IU/mL penicillin and 100 mg/mL streptomycin in a 5% CO2 incubator at 37 ℃. CCL20 (Cat. 360-EP-025; R&D Systems, Minneapolis, MN, United States) was added to the media, which was changed every other day.

Cell proliferation assay

Cell proliferation was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoltetrazolium bromide (MTT) assay. Cells were plated in 96-well culture plates at 5 × 103 cells per well containing 0.2 mL of culture media. After treatment with CCL20 (5 µg/mL) for 24 or 48 h, 0.02 mL of 5 mg/mL MTT was added to each well and incubated for 4 h at 37 ℃. The absorbance was measured at 570 nm. Each assay was performed three times independently.

Invasion assay

Cell invasion was assessed using Transwell chambers (8 µm pore size; EMD Millipore, Billerica, MA, United States) according to the manufacturer’s instructions. ECM (Sigma-Aldrich, St. Louis, MO, United States) was added to the chamber to form a gel layer, and cells (5 × 105) were added to the upper chamber in the presence of CCL20 at a concentration of 5 µg/mL. The cells migrating to the membrane were enumerated with Giemsa staining. The assay was performed three times independently.

Primary antibodies

The following primary antibodies used in the experiments were obtained from Santa Cruz Biotechnologies Inc., Dallas, TX, United States: anti-E-cadherin (sc-21791), anti-vimentin (sc-53464), anti-AKT (sc-5298), anti-phospho(p)-AKT (sc-33437), anti-β-catenin (sc-7963) and anti-GAPDH (sc-25778).
Table 1  Relationship between chemokine ligand 20 expression and clinicopathologic features

| Clinicopathologic features | Number of patients | CCL20 expression | P value |
|---------------------------|--------------------|------------------|--------|
|                           | Low (n = 32)       | High (n = 30)    |        |
| Age (yr)                  |                    |                  |        |
| < 57                      | 35                 | 19               | 16     | 0.632 |
| > 57                      | 27                 | 13               | 14     |       |
| Gender                    |                    |                  |        |
| Male                      | 47                 | 26               | 21     | 0.301 |
| Female                    | 15                 | 6                | 9      | 0.167 |
| Etiology                  |                    |                  |        |
| HBV infection             | 46                 | 26               | 20     | 0.122 |
| HCV infection             | 11                 | 2                | 9      |       |
| Alcohol                   | 5                  | 4                | 1      | 0.043 |
| Background liver pathology|                    |                  |        |
| Normal liver              | 3                  | 2                | 1      |       |
| Chronic hepatitis         | 20                 | 11               | 9      |       |
| Liver cirrhosis           | 39                 | 19               | 20     |       |
| AFP (ng/mL)               |                    |                  |        |
| ≤ 197                     | 33                 | 21               | 12     | 0.043 |
| > 197                     | 29                 | 11               | 18     |       |
| ALT (U/L)                 |                    |                  |        |
| ≤ 66                      | 28                 | 17               | 11     | 0.193 |
| > 66                      | 34                 | 15               | 19     |       |
| Child-Pugh index          |                    |                  |        |
| A                         | 44                 | 21               | 23     | 0.338 |
| B                         | 18                 | 11               | 7      |       |
| Tumor size (cm)           |                    |                  |        |
| ≤ 5                       | 41                 | 29               | 12     | 0.000 |
| > 5                       | 21                 | 3                | 18     |       |
| Tumor number              |                    |                  |        |
| Single                    | 43                 | 27               | 16     | 0.008 |
| Multiple                  | 19                 | 5                | 14     |       |
| Vascular invasion         |                    |                  |        |
| No                        | 51                 | 30               | 21     | 0.014 |
| Yes                       | 11                 | 2                | 9      |       |
| Tumor encapsulation       |                    |                  |        |
| Yes                       | 24                 | 14               | 10     | 0.400 |
| No                        | 38                 | 18               | 20     |       |
| Tumor differentiation     |                    |                  |        |
| I + II                    | 45                 | 28               | 17     | 0.007 |
| III + IV                  | 17                 | 4                | 13     |       |
| TNM stage                 |                    |                  |        |
| I                         | 40                 | 22               | 18     | 0.472 |
| II + III                  | 22                 | 10               | 12     |       |

AFP: Alpha fetoprotein; CCL20: Chemokine ligand 20; HBV: Hepatitis B virus; HCV: Hepatitis C virus; OS: Overall survival; RFS: Recurrence-free survival; TNM: Tumor-node-metastasis; ALT: Alanine aminotransferase.

Western blotting analysis

Lysates were extracted from cells treated with CCL20 at a concentration of 5 mg/mL for 48 h using lysis buffer with phenylmethylsulfonyl fluoride, and the protein concentration was measured with a BCA protein assay kit (Beyotime, Shanghai, China). Total protein extracts (20 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat dried milk in Tris-buffered saline (20 mmol/L Tris-HCl, 150 mmol/L NaCl and 0.1% Tween-20, pH 7.5) for 1 h at room temperature and individually incubated overnight at 4 °C with antibodies against E-cadherin (1:1000), vimentin (1:1000), AKT (1:1000), p-AKT (1:1000), β-catenin (1:1000) or GAPDH (1:5000). Signals were detected by using enhanced ECL chemiluminescence (MultiScience Biotech Co., Shanghai, China) and analyzed using Quantity One (Bio-Rad, Hercules, CA, United States).

Immunohistochemistry

Immunohistochemical detection of E-cadherin (1:100), vimentin (1:500), p-AKT (1:100) and β-catenin (1:100) was performed on 4 µm-thick sections of specimens which had been fixed in formalin, embedded in paraffin and mounted on slides. Replacement of the primary antibody with mouse- or rabbit-isotype control antibody served as a negative control. Counterstaining of the nucleus was performed using hematoxylin. The staining intensity was scored in four levels (0 = negative, 1 = weak, 2 = moderate, 3 = strong), and the percentages of stained cells at each intensity level were counted. The total immunostaining score was calculated as the sum of each intensity score multiplied by the corresponding percentage. The slides were independently evaluated and scored by three pathologists without knowledge of clinical data.

Immunocytochemistry

After reaching confluence, cells plated on coverslips in 6-well dishes were washed twice, fixed with 2% (w/v) formaldehyde and permeabilized with 1% (v/v) Triton X-100. Coverslips were blocked with 10% (w/v) normal goat serum in phosphate-buffered saline at room temperature for 1 h and then incubated in primary antibodies against E-cadherin (1:200), vimentin (1:500), p-AKT (1:200) or β-catenin (1:400) at 4 °C overnight. Cells were washed and incubated with Cy3-labeled secondary antibody (Beyotime) at room temperature for 1 h, and co-stained with DAPI (Sigma-Aldrich) to visualize nuclei. Images were obtained using a fluorescence microscope at magnification ×200.

Statistical analyses

All analyses in the study were performed using SPSS version 16.0 software (SPSS Inc., Chicago, IL, United States). One-way analyses of variance and Student’s t-tests were used for intergroup comparisons. The correlation between CCL20 expression and clinicopathologic features was examined by the χ² test. RFS and OS were calculated by the Kaplan-Meier method. Univariate analyses for factors of recurrence and survival were performed using the χ² test and the log-rank test, respectively. Data are presented as mean ± SD, with P < 0.05 considered as statistically significant.

RESULTS

Clinical data and follow-up

Of the HCC patient samples, 75.8% (47/62) were from men and 24.2% (15/62) were from women (Table 1). The average age of the patients was 57 years (range:
32-79 years), with 91.9% (57/62) of the patients having Hepatitis B or C viral infections, and 95.2% (59/62) presenting with chronic hepatitis history or liver cirrhosis. The average values of alpha-fetoprotein (AFP) and alanine aminotransferase before surgery were 197 ng/ml and 66 U/L, respectively. TNM stages of all HCC samples were divided into a stage I group (40/62; 64.5%) or a stage II and III group (22/62; 35.5%). The average follow-up period was 42.6 ± 19.8 mo (range: 8-65 mo), and 66.1% (41/62) of patients presented recurrence after surgery. The recurrence sites included the liver ($n = 32$), lung ($n = 4$), lymph node ($n = 5$) and bone ($n = 2$). OS and RFS were 88.7% (55/62) and 83.9% (52/62) at 1 year, 74.2% (46/62) and 66.1% (41/62) at 3 years, and 53.2% (33/62) and 33.9% (21/62) at 5 years, respectively.

**Correlation between CCL20 expression and clinicopathologic factors**

The expression of CCL20 was examined in all 62 HCC samples, and was mainly located in the cytoplasm of HCC cells (Figure 1). The average immunohistochemistry score was 165.0 (range: 85-260). All HCC samples were subsequently divided into low CCL20 group ($n = 32$) and high CCL20 group ($n = 30$) by using the average value. The relationships between CCL20 expression and clinicopathologic factors are presented in Table 1. Our findings revealed that CCL20 expression was significantly related to preoperative AFP level ($P = 0.043$), tumor size ($P = 0.000$), tumor number ($P = 0.008$), vascular invasion ($P = 0.014$), and tumor differentiation ($P = 0.007$).

**Correlations between CCL20 expression and HCC recurrence and patient survival**

Univariate analyses indicated that HCC recurrence was related with vascular invasion ($P = 0.042$), TNM stage ($P < 0.001$), and CCL20 expression ($P < 0.001$) (Table 2). RFS was significantly related with preoperative AFP ($P = 0.038$), tumor size ($P = 0.024$), vascular invasion ($P = 0.019$), TNM stage ($P < 0.001$), and expression of CCL20 ($P < 0.001$). OS significantly related with tumor size ($P = 0.014$), vascular invasion ($P = 0.008$), tumor encapsulation ($P = 0.032$), tumor differentiation ($P = 0.047$), TNM stage ($P < 0.001$), and CCL20 expression ($P < 0.001$). There were no significant correlations among the other clinicopathologic factors and recurrence or survivals.

**CCL20 promotes in vitro proliferation and invasion of HCC cells**

The proliferation of Hep3B and Huh7 HCC cells was significantly enhanced by CCL20 after 24 and 48 h (both $P < 0.05$) (Figure 2A). Similarly, results of the invasion assay indicated that CCL20 treatment significantly increased invasion of Hep3B and Huh7 HCC cells (both $P < 0.05$) (Figure 2B).

**CCL20 induces EMT-like phenotype and activates PI3K/ AKT and Wnt/β-catenin pathways in HCC cells**

Western blotting results showed that CCL20 at a concentration of 5 µg/ml for 48 h induced an EMT-like phenotype in Hep3B and Huh7 HCC cells. The expression of the epithelial marker E-cadherin was decreased and the mesenchymal marker vimentin was increased significantly by CCL20 ($P < 0.05$) (Figure 3). Moreover, β-catenin levels and phosphorylation of AKT were also increased compared with controls ($P < 0.05$).

Treatment with CCL20 (5 µg/ml) for 48 h induced an EMT-like phenotype in Hep3B and Huh7 cells (Figure 4A). The detection of β-catenin and p-AKT by

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**Table 2 Univariate analyses for factors associated with recurrence and survivals**

| Factor                  | Recurrence | P value | RFS P value | OS P value |
|-------------------------|------------|---------|-------------|------------|
| Age (yr)                | Yes        | No      | (%)         | (%)        |
| ≤ 57                    | 19         | 16      | 0.184       | 0.211      | 66.7       | 0.135   |
| > 57                    | 18         | 9       | 0.814       | 0.211      | 50.6       |         |
| Gender                  |            |         |             |            |
| Male                    | 26         | 21      | 0.469       | 0.192      | 55.4       | 0.633   |
| Female                  | 9          | 6       | 0.423       |            | 64.8       |         |
| Etiology                |            |         |             |            |
| HBV infection           | 27         | 19      | 0.467       | 0.192      | 56.3       |         |
| HCV infection           | 7          | 4       | 0.392       |            | 54.5       |         |
| Alcohol                 | 2          | 3       | 0.584       |            | 61.7       |         |
| Background liver pathology | 0.953   |         | 0.784      |            | 0.947     |         |
| Normal liver            | 2          | 1       | 0.535       | 0.695      | 63.5       |         |
| Chronic hepatitis       | 13         | 7       | 0.456       | 0.695      | 57.4       |         |
| Liver cirrhosis         | 22         | 17      | 0.409       | 0.695      | 52.2       |         |
| AFP (ng/mL)             |            |         |             |            |
| ≤ 197                   | 15         | 18      | 0.084       | 0.038      | 65.5       | 0.074   |
| > 197                   | 19         | 10      | 0.296       | 0.038      | 42.8       |         |
| ALT (U/L)               |            |         |             |            |
| ≤ 66                    | 15         | 13      | 0.371       | 0.088      | 55.3       | 0.942   |
| > 66                    | 19         | 15      | 0.415       | 0.088      | 52.9       |         |
| Child-Pugh score        |            |         |             |            |
| A                       | 24         | 20      | 0.774       | 0.695      | 54.1       | 0.133   |
| B                       | 11         | 7       | 0.388       | 0.695      | 40.9       |         |
| Tumor size (cm)         |            |         |             |            |
| ≤ 5                     | 21         | 20      | 0.417       | 0.024      | 68.3       | 0.014   |
| > 5                     | 14         | 7       | 0.317       | 0.024      | 44.8       |         |
| Tumor number            |            |         |             |            |
| Single                  | 25         | 18      | 0.217       | 0.052      | 58.3       | 0.233   |
| Multiple                | 13         | 6       | 0.302       | 0.052      | 46.5       |         |
| Vascular invasion       |            |         |             |            |
| No                      | 31         | 20      | 0.042       | 0.019      | 62.8       | 0.008   |
| Yes                     | 8          | 3       | 0.318       | 0.019      | 40.9       |         |
| Tumor encapsulation     |            |         |             |            |
| Yes                     | 14         | 10      | 0.649       | 0.443      | 65.3       | 0.032   |
| No                      | 23         | 15      | 0.382       | 0.443      | 43.1       |         |
| Tumor differentiation   |            |         |             |            |
| I + II                  | 21         | 24      | 0.241       | 0.695      | 59.6       | 0.047   |
| III + IV                | 11         | 6       | 0.418       | 0.695      | 40.1       |         |
| TNM stage               |            |         |             |            |
| I                       | 19         | 21      | <0.001      | 0.001      | 66.4       | <0.001  |
| II + III                | 18         | 20      | 0.204       | 0.001      | 35.7       |         |
| CCL20                   |            |         |             |            |
| Low                     | 13         | 19      | <0.001      | 0.001      | 70.1       | <0.001  |
| High                    | 27         | 3       | 0.214       | 0.001      | 30.6       |         |

AFP: Alpha fetoprotein; ALT: Alanine aminotransferase; CCL20: Chemokine ligand 20; HBV: Hepatitis B virus; HCV: Hepatitis C virus; OS: Overall survival; RFS: Recurrence-free survival; TNM: Tumor-node-metastasis.
Hou KZ et al. CCL20 enhances HCC progression via EMT

Figure 1 Immunohistochemical analysis of hepatocellular carcinoma tissues. Expression of A, B: Chemokine ligand 20 (CCL20); C, D: E-cadherin; E, F: Vimentin; G, H: β-catenin; I, J: Phosphorylated AKT (p-AKT) in HCC and non-cancerous liver tissues (magnification × 200). HCC: Hepatocellular carcinoma.
immunofluorescence was enhanced by CCL20 treatment (Figure 4B).

High expression of CCL20 is associated with the changes of EMT markers in HCC specimens
EMT markers E-cadherin and vimentin, and cell-signaling pathway-related proteins β-catenin and p-AKT were also measured by immunohistochemistry in patient specimens (Figure 1). Results showed that vimentin, β-catenin and p-AKT levels were much higher and E-cadherin level was lower in samples identified as having high CCL20 expression compared to those with low expression and non-cancerous liver tissues. The correlation analysis revealed that high-CCL20 expression was negatively correlated with E-cadherin expression (4/30; 13.33%), and positively correlated with vimentin (27/30; 90.0%),

**Figure 2** Effects of chemokine ligand 20 on proliferation and invasion of hepatocellular carcinoma cells. Hep3B and Huh7 cells were treated with chemokine ligand 20 (CCL20) (5 µg/mL) for 24 h and 48 h. A: Cell proliferation was analyzed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay); B: Invasion was assessed using a Transwell chamber; "P < 0.05 vs Control. Con: Control.

**Figure 3** Effects of chemokine ligand 20 on expression of epithelial-mesenchymal transition-related proteins. A: Representative Western blots for epithelial marker E-cadherin, mesenchymal marker vimentin, total and phosphorylated AKT (t-AKT and p-AKT), and β-catenin in Hep3B and Huh7 cells after treatment with chemokine ligand 20 (CCL20) for 48 h. B: Quantification of Western blotting by gray value analysis; "P < 0.05 vs Control.
Chemokines play a critical role in various biologic events, such as the role of inflammatory responses in regulating the transfer of white blood cells, embryonic development, wound healing, angiogenesis, Th1/Th2 development, leukocyte homeostasis, and lymphatic organ development. Recent studies have suggested that chemokines and their receptors are associated with the pathogenesis, progression and metastasis of many kinds of tumors, including HCC. Li et al.\[18\] found a much higher expression of the CXCL12-CXCR4 axis in HCC specimens than in adjacent, cirrhosis, liver adenocarcinoma, and normal liver tissues. Fujii et al.\[19\] performed in vitro experiments showing that the CCL20-CCR6 axis promotes the growth of Huh7 cells through phosphorylation of mitogen-activated protein kinase. Rubie et al.\[20\] reported that CCL20 was significantly upregulated in HCC specimens. Zheng et al.\[21\] found that CXCR7 expression was increased in HCC tissues and was associated with HCC invasion, adhesion and angiogenesis. Zhou et al.\[22\] found that CXCL5 promotes HCC cell proliferation, invasion and intratumoral neu-

DISCUSSION

Chemokines play a critical role in various biologic events, such as the role of inflammatory responses in regulating the transfer of white blood cells, embryonic development, wound healing, angiogenesis, Th1/Th2 development, leukocyte homeostasis, and lymphatic organ development. Recent studies have suggested that chemokines and their receptors are associated with the pathogenesis, progression and metastasis of many kinds of tumors, including HCC. Li et al.\[18\] found a much higher expression of the CXCL12-CXCR4 axis in HCC specimens than in adjacent, cirrhosis, liver adenocarcinoma, and normal liver tissues. Fujii et al.\[19\] performed in vitro experiments showing that the CCL20-CCR6 axis promotes the growth of Huh7 cells through phosphorylation of mitogen-activated protein kinase. Rubie et al.\[20\] reported that CCL20 was significantly upregulated in HCC specimens. Zheng et al.\[21\] found that CXCR7 expression was increased in HCC tissues and was associated with HCC invasion, adhesion and angiogenesis. Zhou et al.\[22\] found that CXCL5 promotes HCC cell proliferation, invasion and intratumoral neu-

Figure 4  Epithelial-mesenchymal transition-like phenotype and upregulation of related markers in hepatocellular carcinoma cells with chemokine ligand 20. A: Morphologic changes in Hep3B and Huh7 cells after treatment with chemokine ligand 20 (CCL20) for 48 h (most cells exhibited an elongated, spindle-shape mesenchymal morphology with treatment); B: Immunocytochemistry for phosphorylated AKT (p-AKT) and β-catenin in Hep3B and Huh7 cells after treatment with CCL20 for 48 h (magnification × 200).
CCL20: Chemokine ligand 20; p-AKT: phosphorylated AKT.

Table 3 Correlation of chemokine ligand 20 expression with different markers

|       | E-cadherin | Vimentin | p-AKT | β-catenin |
|-------|------------|----------|-------|-----------|
|       | Positive   | Negative | Positive | Negative | Positive | Negative |
| CCL20 | 4          | 26       | 27    | 3         | 23       | 7        | 29      | 1        |
| $\chi^2$ |            |          |       |           |          |          |         |          |
| $P$ value | < 0.001   | < 0.001  | < 0.001 | < 0.001   | < 0.001  |          |         |          |

Trophil infiltration and could be a novel prognostic predictor of HCC.

Our results revealed that high expression of CCL20 in HCC tissues was correlated with poor outcome in HCC patients undergoing resection surgery. Our results also indicated that the expression of CCL20 was associated with tumor size, number and tumor differentiation, and with vascular invasion. Results of in vitro experiments indicated that CCL20 can induce EMT-like morphologic changes and promote the proliferation and invasion in HCC cells. Interestingly, CCL20 induced expression of vimentin and downregulated the expression of E-cadherin. These results extend the role of CCL20 in HCC to the context of chemokine-mediated EMT. Several chemokines have been shown to induce EMT in various tumors. Biswas et al. found that CXCL13-CXCR5 co-expression regulates EMT of breast cancer cells. Ploenes et al. reported that CCL18 induces EMT in lung cancer cells and elevates the invasive potential. Albert et al. found that CXCR4 induces EMT in patients with squamous cells carcinoma. Li et al. found that SDF-1/CXCR4 signaling induces pancreatic cancer cell invasion and EMT in vitro through non-canonical activation of hedgehog pathway. Matsushita et al. showed that CXCL16 plays an important role in liver metastasis of colorectal cancer through the induction of EMT. Our study provides another model of chemokine-mediated EMT, in which CCL20 may play a significant role in HCC progression and metastasis.

A variety of cell signaling pathways have been implicated in the process of EMT. Chang et al. found that EMT is associated with activation of the PI3K/AKT/mTOR pathway in prostate cancer radio-resistance. Tsai et al. reported that downregulation of PI3K/AKT and Wnt/β-catenin signaling cascade reverses EMT and inhibits breast cancer cell invasiveness. Chioi et al. demonstrated that PI3K/AKT, ERK1/2 and Smad3/4 pathways are associated with transforming growth factor β1-induced EMT in human lung carcinoma cells. By examining protein expression in patient specimens and in HCC cells in vitro in our study, we showed that CCL20 expression was negatively associated with E-cadherin and positively associated with vimentin, p-AKT, and β-catenin expression. These results suggest that CCL20 expression is involved with the EMT process in HCC and there might be crosstalk between the PI3K/AKT and Wnt/β-catenin signaling pathways.

In conclusion, the present findings indicate that CCL20 expression is correlated with clinicopathologic factors, recurrence and survival in HCC patients, as well as the proliferation, migration and invasion of HCC cells. CCL20 induces EMT-like changes in HCC cells through activation of PI3K/AKT and Wnt/β-catenin signaling pathways. CCL20 may therefore represent a promising molecular marker for predicting outcomes and treatment of HCC patients undergoing resection surgery.

COMMENTS

Background

Chemokine ligand 20 (CCL20), also known as liver activation regulated chemokine or macrophage inflammatory protein-3, is a small cytokine that is strongly chemotactic for lymphocytes and weakly attracts neutrophils. Increasing evidence suggests that CCL20 is involved with tumor formation, progression and metastatic processes in many malignancies, including breast and colorectal cancer.

Research Frontiers

Recent studies on CCL20 have mostly focused on the expression in hepatocellular carcinoma (HCC) tissues. Thus far, no studies have described the underlying mechanism by which CCL20 regulates the growth and metastasis of HCC cells. The results of this study provide new targets and a theoretical basis for the therapy of HCC.

Innovations and Breakthroughs

This study demonstrates that CCL20 plays an important role in the proliferation and migration of HCC cells. CCL20 expression could induce an epithelial-mesenchymal transition (EMT)-like change in HCC cells via crosstalk between phosphoinositide kinase-3 (PI3K/AKT) and Wnt/β-catenin signaling pathways. CCL20 may be useful as a molecular monitor of metastasis and recurrence in HCC patients.

Applications

CCL20 induces EMT-like changes in HCC cells via crosstalk between PI3K/AKT and Wnt/β-catenin signaling pathways, which may therefore be used as indicators for anti-HCC therapy and prognosis evaluation.

Peer review

The authors detected expression of CCL20 in HCC tissues and analyzed its prognostic significance for HCC patients. They further identified the PI3K/AKT and Wnt pathways as possible mechanisms for the EMT-like phenotype induced by CCL20.

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