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Original Article

CCL20 is overexpressed in hepatocellular carcinoma with bile duct tumor thrombus and correlates negatively with surgical outcome

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Abstract: There is not yet a consensus regarding prognosis of hepatocellular carcinoma (HCC) with bile duct tumor thrombus (BDTT) versus without bile duct tumor thrombus. Chemokine (C-C motif) ligand 20 (CCL20) plays critical roles in the progress of many types of tumor. But the clinicopathological and prognostic value of this marker in HCC with BDTT is unceratin. In this study, we reported that the overall survival (OS) and disease-free survival (DFS) in HCC with BDTT were significantly shorter than in those without BDTT ($P<0.05$). CCL20 was expressed at a significantly higher level in bile duct tumor thrombus by real-time PCR, western blot, and immunohistochemistry. Patients with high CCL20 expression levels had a poor prognosis. Multivariate survival analysis indicated that CCL20 was an independent prognostic factor for OS. The presence of bile duct tumor thrombus indicateda poor prognosis in HCC patients, but was not a surgical contraindication. CCL20 was associated with tumor progression and high CCL20 expression was correlated with worse surgical outcomes in HCC with BDTT. Inhibition of CCL20 expression might offer novel promising molecular targets for treatment.

Keywords: Hepatocellular carcinoma, bile duct tumor thrombus, CCL20, prognosis

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in men and the ninth in women worldwide [1]. Bile duct tumor thrombus (BDTT) is uncommon in HCC patients. The reported incidence ranges from 0.53% to 13%, and the presence of BDTT usually implies a poor prognosis [2-4]. In contrast, some studies have found that HCC patients with and without BDTT had comparable OS and comparable DFS [5]. BDTT alone has no impact on survival or recurrence after a hepatectomy if appropriate treatment is given [6]. Therefore, there is still controversy over the treatment and prognosis of HCC with BDTT. It is crucial to understand the behavior and pathology of HCC with BDTT. In this study, we selected 69 cases of HCC with BDTT paired to 69 without BDTT, using matched clinical data, to compare the prognosis.

The specific mechanism underlying the poor prognosis of HCC with BDTT remains unclear. Chemokine (C-C motif) ligand 20 (CCL20) is a small cytokine belonging to the CC chemokine family [7]. It is strongly chemotactic for lymphocytes and weakly attracts neutrophils [8]. CCL20 is implicated in the formation and function of mucosal lymphoid tissues via chemoattraction of lymphocytes and dendritic cells towards the epithelial cells surrounding these tissues [9]. CCL20 elicits its effects on its target cells by binding and activating the chemokine receptor CCR6 [7, 10]. CCL20 has been detected in a variety of primary human epithelial tumors including breast, primary cutaneous melanoma, glioma, pancreas, and liver [11-15]. However, the CCL20 expression level and its significance in HCC with BDTT have not been reported. Using a proteomics approach, we identified elevated CCL20 expression in bile duct tumor thrombus compared with tumour tissues and para-tumour specimens. Therefore,

it is meaningful to investigate the clinical significance and biological function of CCL20 in the development of bile duct tumor thrombus.

This study aimed to investigate the CCL20 expression pattern and determine its contribution to the progression of bile duct tumor thrombus and its clinical prognostic value. In addition, the results would indicate that the expression of CCL20 is significantly correlated with clinical characteristics and is able to predict HCC patients' outcomes after surgical resection. This cytoplasmic transcription factor may also serve as a therapeutic target for the treatment of HCC with BDTT.

Materials and methods

Patients

A prospectively maintained database in our department at Eastern Hepatobiliary Hospital was reviewed between January 2002 and December 2011. Permission from Second Military Medical University's Institutional Review Board was obtained prior to data review. To exclude influence of other prognostic factors, we screened 69 HCC patients with bile duct tumor thrombus and 69 HCC without bile duct tumor thrombus according to tumor size, number of tumor lesions, associated hepatitis and the presence of PVTT.

Tissue microarray screening

Under the premise of the consent of the patient himself or the patient's client, a total of 5 fresh tissue specimens was obtained during surgery. In each case, tumor thrombus, primary cancer lesion, and para-tumour tissues were obtained simultaneously. All patients were given tissue microarray screening and further for real-time PCR verification. Total RNA from liver (liver cancer) tissues was extracted using the Trizol kit from GIBCO BRC and its methods. The RNA was purified using the RNeasy Mini Kit and its manual. After quantification of nucleic acid, using the Whole Human Genome Oligo Microarray from Agilent Inc. and related kits methods, the RNA of the sample was detected by cy3 fluorescent labeling to detect the transcription level of moderate RNA in the primary tumor and tumor thrombus sample. 41,000 human genes were detected on the chip. Housekeeping genes were excluded as internal controls. According to the guidelines for chip analysis, genes that detected positive signals in 80% of the samples were selected as candidate genes for analysis. According to the results of the gene chip, we selected 13 genes with significant differences in cancer and tumor thrombus, such as AQP5, BAX, BCAN, BIRC3, C7, CCL20, CXCl12, CXCL1, DLG5, NDRG2, PTPRS, SERPINE2, and NGFR. Real-time PCR was performed on the specimens of patients to compare the mRNA expression levels of cancer and tumor thrombus. Finally, CCL20 was identified as the target gene.

Immunohistochemical detection of tissue sections

The paraffin blocks of 84 HCC patients between January 2004 and December 2011 were collected from the Department of Pathology, including 42 cases with bile duct tumor thrombus (group A) and 42 cases without bile duct tumor thrombus (group B). In group A, the paraffin blocks of bile duct tumor thrombus were also gathered in 20 cases. In all 84 HCC cases, CCL20 expression was compared between cancer and para-tumour tissues. Among 20 patients with paraffin blocks of tumor thrombus, CCL20 expressions were compared among cancer, para-tumour tissues and tumor thrombus. Paraffin blocks of each tissue were sectioned and then immunohistochemically stained with rabbit anti-human CCL20 polyclonal antibody (Abcam). The areas of positive regions and optical density (OD value) in the immunohistochemical images were calculated by computer. The immunohistochemical index (equal to the positive area multiplied by the OD value) was obtained. The level of the value represented the strength of the positive expression. Grading: OD (0~6 = 0; 7~9 = 1; ≥10 = 2); the areas of positive regions (<1000 = 1; 1000~2000 = 2; >2000 = 3). The immunohistochemical index was (0-1 negative, 2-4 weak positive, 4-6 strong positive).

Analysis of clinical pathological data in Group A based on the results of CCL20 immunohistochemistry

The follow-up period was defined as the interval from the date of surgery to the date of death or the last follow-up. Patients who died from other causes were treated as censored cases.

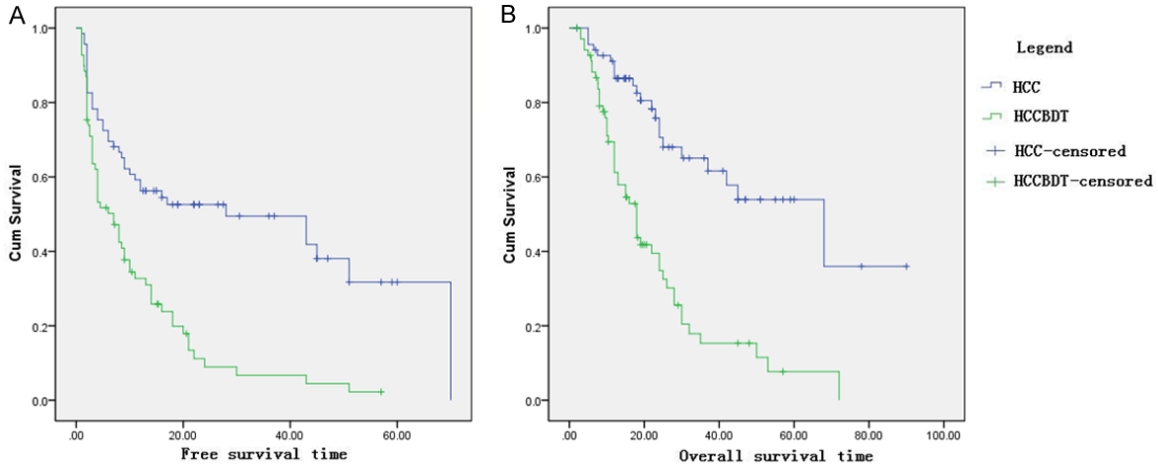

Figure 1. Survival analysis of 69 paired HCC patients with and without BDTT. A: Analysis of Disease-free survival time (DFS). B: Analysis of Overall survival time (OS).

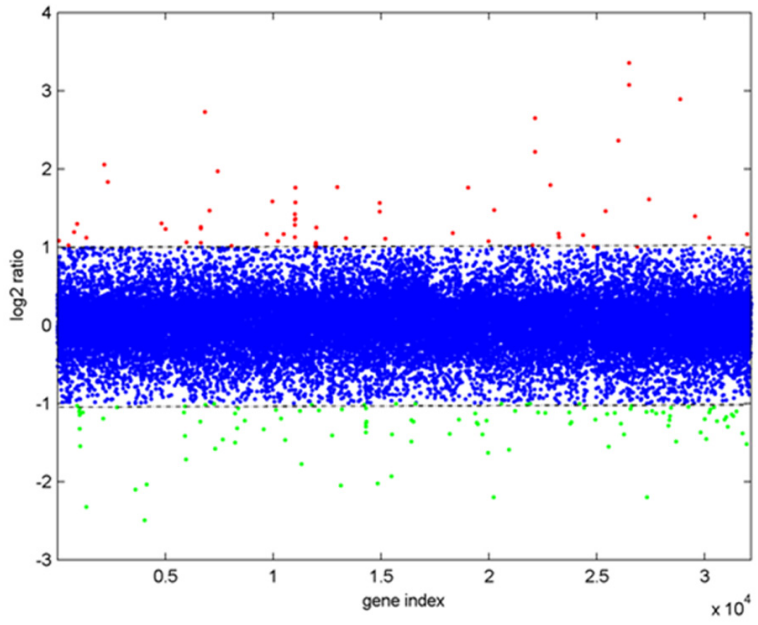

Figure 2. Tissue microarray screening. Red points refer to increased expression of gene. Green points refer to reduced expression of gene.

Overall survival (OS) was defined as the interval between the dates of surgery and death. Disease-free survival (DFS) was defined as the interval between the dates of surgery and recurrence; if recurrence was not diagnosed, patients were censored at the date of death or the last follow-up. The clinical data of 42 patients in group A were analyzed retrospectively. Survival analysis was performed based on the results of CCL20 expression in group A.

Statistical analysis

Comparison between the groups was done using Student's t test for parametric data and the Mann-Whitney U test for non-parametric data. The Chi-square test was used for categorical data. Survival curves were estimated with the Kaplan-Meier method and compared by the log-rank test. Cox regression analysis was carried out to determine which factor was the best prognostic determinant. $P<0.05$ was considered statistically significant. Calculations were done using SPSS Version 17.0 for Windows (SPSS, Inc., Chicago, IL, USA).

Results

Survival analysis of 69 HCC patients with and 69 without bile duct tumor thrombus

To exclude influence of other prognostic factors, we screened 69 HCC patients with BDTT and 69 HCC without BDTT according to tumor size, number of tumor lesions, associated hepatitis and the presence of PVTT. The median disease-free survival time (DFS) in 69 HCC with BDTT and 69 paired HCC without BDTT was 8.87 months and 30.58 months respectively.

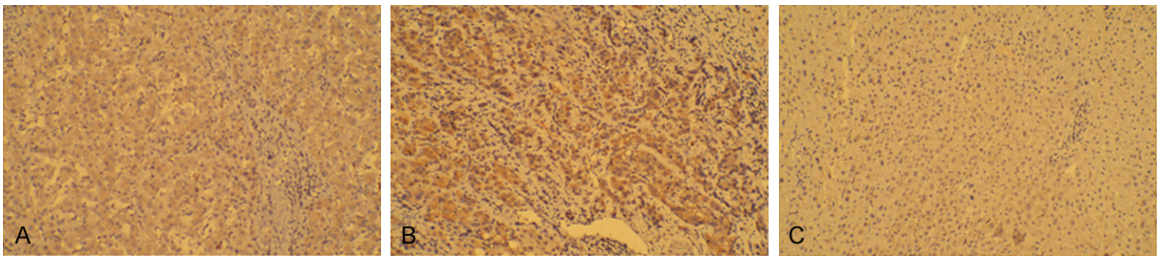

Figure 3. Immunohistochemical results of CCL20.

The cumulative one-, three- and five-year disease-free survival rates in 69 HCC with BDTT were 12%, 5% and 0%, respectively, i.e. significantly worse than those of 69 paired HCC without BDTT (51%, 35% and 8%, P<0.001, **Figure 1A**). The median overall survival time (OS) was 19.52 months and 61.88 months respectively. The cumulative one-, three- and five-year survival rates in 69 HCC with BDTT were 38%, 15%, and 8%, respectively, i.e. significantly worse than those of 69 paired HCC without BDTT (76%, 52% and 37%, P<0.001, **Figure 1B**).

Tissue microarray screening and real-time PCR verification results

As shown in **Figure 2**, a total of 150 significantly differentially expressed genes were detected among primary tumor, para-tumour tissues, and tumor thrombus by tissue microarray screening. 49 genes were up-regulated and 101 genes were down-regulated (P<0.05). The expression level of CCL20 mRNA was significantly increased in tumor thrombus compared toprimary tumor and para-tumor tissues by Real-Time PCR validation in five fresh tissue specimens.

CCL20 is overexpressed in HCC and bile duct tumor thrombus

CCL20 immunohistochemistry results are shown in **Figure 3** (×400 magnification). In 84 HCC tissue sections, the CCL20 expression in hepatocellular carcinoma was significantly upregulated compared to para-tumour tissues (P<0.001) (**Figure 4A**). There was no difference in the expression of CCL20 between group A and B (P = 0.950) (**Figure 4B**). Among 20 patients with paraffin blocks of tumor thrombus, CCL20 expression was significantly higher in tumor thrombus than primary tumor and para-tumour tissues (P<0.001) (**Figure 4C**).

Association of the CCL20 expression with prognosis in group A

Both univariate and multivariate survival analysis suggested that high CCL20 expression levels were significantly associated with a poor prognosis with respect to overall survival (**Figure 5A**). Among 20 patients with paraffin blocks of tumor thrombus, the higher CCL20 expression was in tumor thrombus, a shorter disease-free survival emerged (**Figure 5B**). However, the expression level of CCL20 in tumor thrombus had no significant effect on overall survival (P = 0.780).

Discussion

Hepatocellular carcinoma (HCC) is a common malignancy and cause of death in the developing world. HCC is more prevalent in men than women, and it presents on average in the sixth and seventh decades of life [16]. Jaundice is present in 19-40% of patients with HCC at the time of diagnosis and usually occurs in the advanced stages of the disease. It is caused by diffuse tumor infiltration of the liver parenchyma, progressive liver failure, hepatic hilar invasion, severe cirrhosis or a combination of these factors [1-4]. Obstructive jaundice owing to major bile duct tumor thrombus is an uncommon cause of jaundice in HCC patients. It is important to recognize this condition because with proper treatment, good palliation with prolongation of survival, and occasional cure, is possible [17, 18]. In 1982, Kojiroet al. [19], first described the clinical and pathologic features of HCC with BDTT, and clarified that it was not equal to the portal vein tumor thrombus (PVTT), indicating the possibility of extensive dissemination in terms of severity of the disease. They considered that it was not necessarily a criterion of advanced disease. In the present study, we confirmed that HCC with BDTT had shorter disease-free survival time

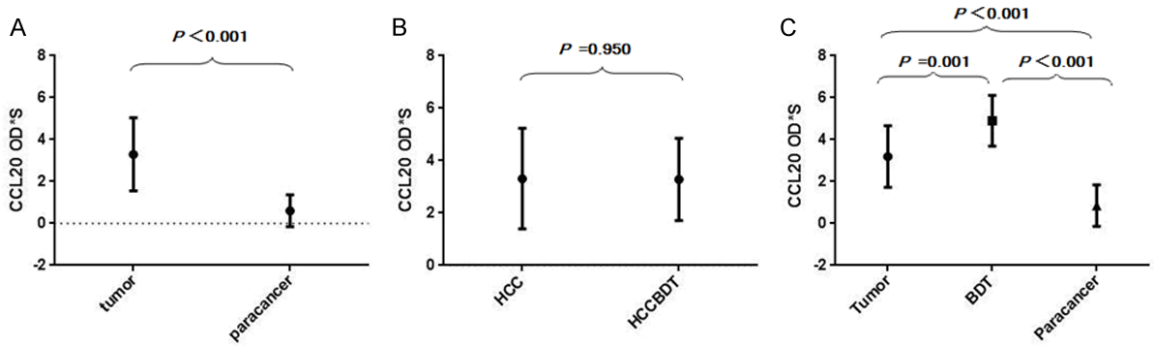

Figure 4. Comparision of CCL20 expressionamong primary tumor, paracancer, and bile duct tumor thrombus.

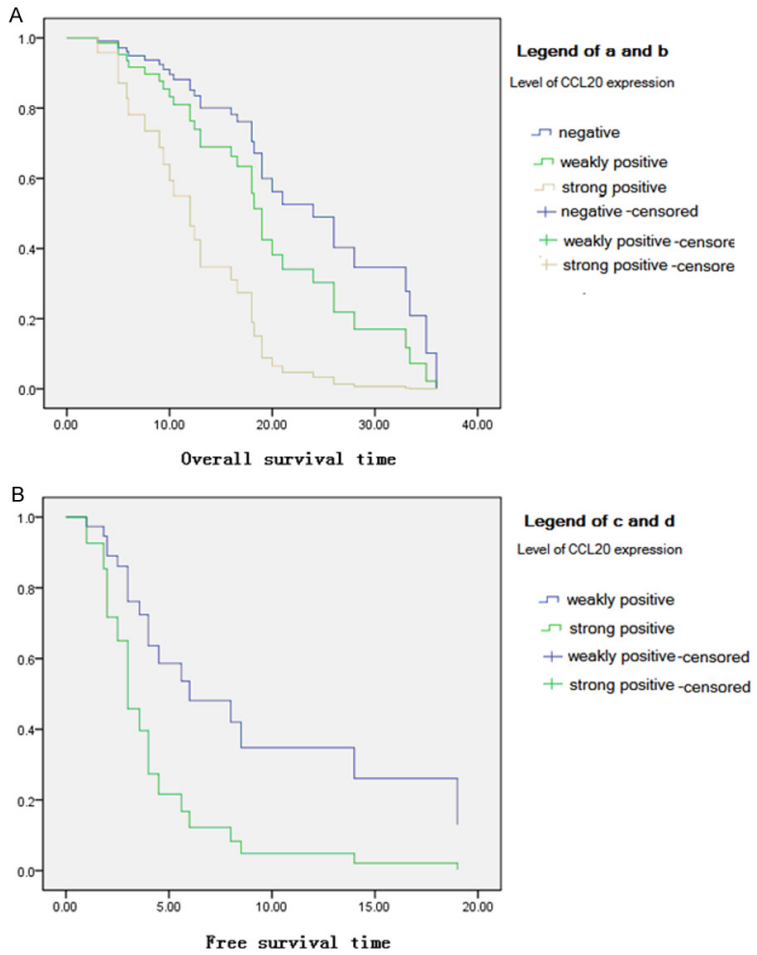

Figure 5. Survival analysis in HCCBDT patients according to CCL20 expression. A: Overall survival time in 42 HCCBDT patients among negative, weak positive and strong positive of CCL20. B: Disease-free survival time in 20 HCCBDT patients between weak positive and strong positive of CCL20.

and poorer 5-year survival than patients without BDTT, which indicated that BDTT was a significant risk factor for poor outcome. In our clinical prognosis assessment, BDTT should be considered when assigning stage.

The occurrence and development of malignant tumors are often accompanied by mutations in certain genes or abnormal expression of specific protein. Based on this, we infer that the formation of bile duct tumor thrombus must be accompanied by a series of genetic changes in cancer cells. Cancer cells with strong invasion and high malignant potential can eventually break through the surrounding matrix, transfer to the bile duct, and survive in the bile environment. We performed tissue microarray screening and real-time PCR verification. Finally, CCL20 was identified as the target gene. We found that the expression level of CCL20 mRNA in the tumor thrombus was significantly more than primary tumor and para-tumour tissues, which was completely consistent with our previous hypothesis. Therefore, we decided to use CCL20 as one of the target genes for further research.

As a chemokine, CCL20 has been found to be related to the invasion and metastasis of hepatocellular carcinoma, colon cancer, breast

cancer, and pancreatic cancer [11, 14, 15, 20], especially closely associated with intrahepatic metastases of HCC [21, 22]. Dellacasagrande et al. [23] found that CCL20 can selectively attract $CCR6^+$ tumor cells to intrahepatic metastases in HCC. Herein, we demonstrated that the CCL20 expression was up-regulated at both mRNA and protein levels in HCC. The association of CCL20 expression with prognosis was further investigated in two dependent cohorts of HCC patients. We found that the expression level of CCL20 was the highest in bile duct tumor thrombus, followed by cancer tissue, and the lowest in para-tumour tissues. The level of CCL20 in tumor thrombus was closely related to the disease-free survival. CCL20 was associated with tumor progression and high CCL20 expression was correlated with worse surgical outcomes in HCC with bile duct tumor thrombus. To explain the above results, we believe that hepatocytes with high CCL20 expression is induced by the specific high expression of CCR6 (CCL20 receptor) in the liver because of its strong invasive ability, and spread to intrahepatic metastases through the bile duct pathway.

In summary, CCL20 is an independent risk factor for the prognosis of HCC with BDTT. CCL20 plays an important role in promoting the development of malignant tumors, especially the spread of tumor cells to the bile duct thrombus. The CCL20 itself is also expected to become a marker for the prognosis of HCC patients with BDT. Furthermore, inhibition of CCL20 expression may offer a novel promising target for the treatment.

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Disclosure of conflict of interest

None.

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