

CCL15 overexpression predicts poor prognosis for hepatocellular carcinoma

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Abstract

Objectives The purpose of this study was to study the expression of CCL15 in hepatocellular carcinoma (HCC) and explore its clinicopathological significance, and study relationships between expressions of CCL15 and malignant behaviors of HCC.

Methods The SP immunohistochemical method was used to detect expression of CCL15 in routinely paraffin-embedded sections from 80 cases of HCC, 80 of adjacent cancerous specimens and 50 of normal liver tissue. In these patients with HCC, Kaplan–Meier was used to assess survival outcomes.

Results The positive rates and scores of CCL15 were significantly higher in HCC than adjacent cancerous specimens and normal liver tissue ($p < 0.05$), but not significantly higher between adjacent cancerous specimens and normal liver tissue ($p > 0.05$). The expression of CCL15 was significantly correlated to tumor size, tumor thrombi in portal vein of HCC, capsule and TNM stage ($p < 0.05$), but not to sex, age, liver cirrhosis and the level of AFP so on ($p > 0.05$). Survival time of the patients with

positive CCL15 expression was significantly decreased, and multivariate analysis indicated CCL15 expression was one of the independent predictors of survival ($p = 0.042$). **Conclusion** The expression of CCL15 was significantly correlated with malignant behaviors of HCC, and CCL15 might be important biological markers for reflecting the carcinogenesis, progression, biological behaviors and prognosis of HCC.

Keywords Hepatocellular carcinoma · CCL15 · Immunohistochemistry · Survival time

Introduction

Primary liver carcinoma is one of the most common malignancies in the world. It is the fifth most frequently diagnosed cancer worldwide but the second most frequent cause of cancer death in males globally. Half of these cases and deaths have been estimated to occur in China [1]. It is also the second greatest cause of cancer-related deaths in China [1]. Early metastasis and recurrence have become two main determinants in patient survival. Therefore, the identification of the key molecules and signaling pathways that correlate with tumor invasion, size, stage, and metastasis has the potential to provide new targets and novel clues for diagnosing, treating, preventing, and controlling early recurrence and metastasis of hepatocellular carcinoma (HCC) and improving prognosis.

The poor prognosis of HCC is mainly attributed to recurrence and metastasis [2]. Recent studies have shown that chemokines and their receptors play important roles in the metastatic potential of cancer cells [3]. For example, the CXCL12/CXCR4 pair has been identified to mediate metastasis of breast cancer cells [4].

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Results from our previous study showed that CCL15 was uniquely expressed in serum samples from HCC patients using the SELDI-TOF technique. Biological analysis suggested that CCL15 promoted HCC migration and invasion [5].

This study explores the expression of CCL15 in 80 cases of HCC, and discusses the correlation between changes in CCL15 expression in HCC samples with clinicopathological parameters such as tumor size, vascular tumor thrombus, TNM stage, and prognosis, etc. The aim of this study was to add to the existing knowledge of the mechanisms of HCC progression and development, with the hope of gaining significant insights that can be used to treat liver cancer targeting CCL15.

Materials and methods

Patients with HCC

From June 2006 to June 2008, paraffin-embedded specimens from primary HCC lesions were collected from 80 patients with histologically proven HCC who had undergone hepatectomy at Tianjin Medical University cancer institute and hospital. Fifty patients with normal liver were included in a normal control group. A complete clinical picture of each patient was collected, including sex, age, tumor size, levels of AFP, TNM stages, etc. Other pathological information, such as presence of liver cirrhosis, capsule and vascular thrombosis, was also collected for each patient. None of the patients were pretreated with any kind of adjuvant therapy, such as radiotherapy or chemotherapy.

Immunohistochemistry (IHC) assay

Normal ($n = 50$), adjacent liver tissues ($n = 80$) and HCC tissues ($n = 80$) were processed according to standard approaches. The anti-CCL15 serum (1:1600, Immunechem Pharmaceuticals Inc., China) was applied to the slides of three groups and incubated in a moist chamber at 4 °C overnight. A total of 0.01 ml PBS was used as the negative control in all experiments. Slides cut in parallel to the IHC-treated sections were stained by HE for better identification of the different tissue areas. To avoid interindividual bias of IHC staining differentiations, all slides were determined by an experienced pathologist.

Immunohistochemistry evaluation

Semi-quantitative immunohistochemical detection was used to determine the CCL15 protein levels. Cytoplasm immunoreactivity for the CCL15 protein was scored by

evaluating the sum of positive tumor cells and the staining intensity over the total number of tumor cells. In brief, the percent of positive cells was scored as “0” (0 %), “1” (1–10 %), “2” (11–50 %), “3” (51–80 %), and “4” (81–100 %). Intensity was scored as “0” (no staining), “1” (weakly stained), “2” (moderately stained), and “3” (strongly stained). Both the scores were decided under double-blind conditions by three independent professionals. The final immunoreactive scores were determined by multiplying the intensity scores with the extent of positivity scores of stained cells. The scores were then classified as follows: “–” (score 0), “+” (score 1–4), “++” (score 5–8), and “+++” (score 9–12). For the purpose of statistical analysis, the cohort was grouped into a negative group (“–”, “+”) and a positive group (“++”, “+++”).

Statistical analysis

Statistical analyses were performed with the SPSS 13.0 software (SPSS, Chicago, IL, USA). The correlation between CCL15 expression and various clinical and pathological characteristics were evaluated by Paired *t* test. Kaplan–Meier analyses were used to analyze CCL15 as univariate in prediction of patients’ survival. Comparisons of survival distributions were done with log-rank test. The multivariate survival analyses were performed by a stepwise Cox proportional hazard model using the Wald statistics. *p* values of <0.05 were considered significant.

Results

Clinical and pathological data of 80 hepatocellular carcinoma patients

The age of 80 patients with HCC ranged from 21 to 76 years, of whom 55 patients were younger than 60 years of age, 25 patients 60 years of age or older, and the median age was 67 years. Fifty patients were males and 30 were females; the male to female ratio was 1.67:1. For preoperative blood serum α -fetoprotein (AFP), 28 patients had 20 $\mu\text{g/L}$ or less and 52 had more than 20 $\mu\text{g/L}$. For tumor diameter, 38 patients had 5.0 cm or less and 42 had more than 5.0 cm. In accordance with histological differentiation, 15 cases were well differentiated HCC, 20 were moderately differentiated HCC and 45 poorly differentiated HCC. Twenty-nine patients were found to have vein tumor thrombus and 35 patients with liver cirrhosis. For the TNM stage (American Joint Committee on International Union against Cancer), 25 patients were stage I, 32 were stage II and 27 were stage III.

Expression of CCL15 in hepatocellular carcinoma and normal liver tissues

Among 80 tumor samples, CCL15 was detected in 64 tumor tissues and only in 16 tissues adjacent to tumor (Table 1). Only one out of 50 samples from healthy tissues showed positive staining with CCL15. The immunostaining expressions of CCL15 have been shown in HCC and adjacent cancer tissues using IHC (Fig. 1).

Relationship between clinic pathological characteristics and the CCL15 expression in HCC tissues

The expression of CCL15 was significantly correlated to tumor size, tumor thrombi in portal vein of HCC, capsule and TNM stages ($p < 0.05$), but not to sex, age, liver cirrhosis and the level of AFP, etc. ($p > 0.05$) (Table 2).

Association of CCL15 expression with survival in patients with HCC

Hepatocellular carcinoma with patients' CCL15 expression was associated with a significantly lower overall survival rate than was HCC without CCL15 expression ($p = 0.038$, Fig. 2). In univariate analysis, tumor size ($p = 0.036$), vascular tumor thrombus ($p = 0.047$), tumor capsule ($p = 0.038$), TNM stage ($p = 0.025$) and CCL15

expression ($p = 0.029$) were important predictor for poor prognosis. Subsequently, multivariate analysis indicated CCL15 expression was one of the independent predictors of survival ($p = 0.042$), as were TNM stage ($p = 0.045$) (Table 3).

Discussion

CCL15/Leukotactin-1 (Lkn-1), a member of the CC chemokine family and expressed only in the gut and the liver, has been recently cloned and partially characterized [6–8]. CCL15 was demonstrated to have a chemoattractant role for monocytes, lymphocytes, neutrophils, eosinophils, and dendritic cells [8]. In addition, this molecule plays an important role in the development of inflammation and allergic inflammatory diseases. CCL15 has in vitro and in vivo angiogenic activity [9]. Moreover, desensitization studies have indicated that CCL15 acts mainly via the CC chemokine receptor, CCR1 [7, 10]. Accumulating evidence has suggested that CCL15 may have a crucial role in the progression of tumor cells by promoting leukocyte infiltration and modulating tumor cell motility [10–14].

Data from our previous study showed that CCL15 was uniquely expressed in serum samples from HCC patients using the SELDI-TOF technique. Biological analysis suggested that CCL15 promoted HCC migration and invasion [5]. Itatani et al. [14] found that in human CRC cells, loss of SMAD4 leads to up-regulation of CCL15 expression, and human liver metastases that express CCL15 contain higher numbers of CCR1(+) cells; patients with these metastases have shorter times of disease-free survival, and also showed that reagents designed to block CCL15 recruitment of CCR1(+) cells could prevent metastasis of CRC to liver.

This study shows that positive expression of CCL15 is significantly higher than, and different from, those in the adjacent cancer and normal tissues. CCL15 closely

Table 1 The expression of CCL15 in the liver tissues

Liver tissue	Samples	Positive rate (%)	Score
Normal	50	1 (2.00)	0.12 ± 0.13^a
Adjacent cancer	80	16 (20.00)	0.55 ± 1.23^b
HCC	80	64 (80.00)	2.25 ± 1.29^c

^a Normal vs HCC, $p < 0.05$

^b Adjacent cancer vs normal, $p > 0.05$

^c Adjacent cancer vs HCC, $p < 0.05$

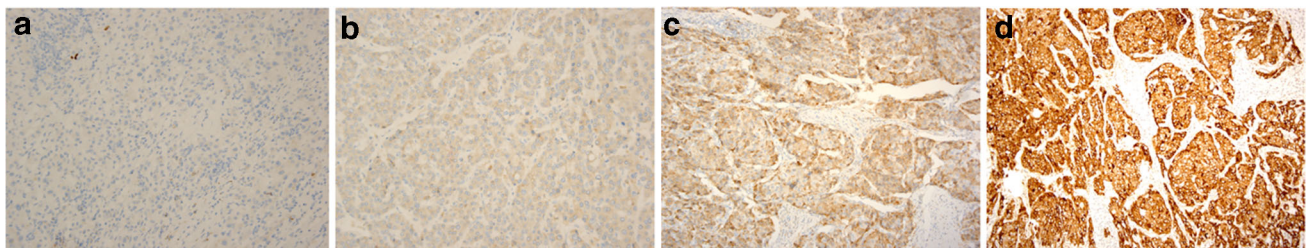


Fig. 1 The immunostaining expression of CCL15 in HCC and adjacent cancer tissues. **a** No staining in adjacent cancer tissues. **b** Weakly stained in HCC. **c** Moderately stained in HCC. **d** Strongly stained in HCC

Table 2 The relationship between clinical pathological data of patients with HCC and the expression of CCL15

Variables	Cases	Positive rates (%)	Scores
Sex			
Males	50	39 (78.0)	2.06 ± 1.14
Females	30	25 (83.3)	2.29 ± 0.96
Age (years)			
≥60	25	19 (76.0)	2.16 ± 1.21
<60	55	45 (81.8)	2.27 ± 1.01
Tumor size (cm)			
≥5	42	38 (90.4)	2.29 ± 0.89*
2–5	30	21 (70.0)	1.28 ± 0.95
≤2	8	5 (62.5)	1.08 ± 0.89
Liver cirrhosis			
Yes	35	29 (82.8)	2.09 ± 1.07
No	45	35 (78.8)	2.11 ± 0.23
Vascular tumor thrombus			
Yes	29	26 (89.6)	2.25 ± 0.19 [#]
No	51	38 (74.5)	1.56 ± 1.08
Capsule			
No	42	38 (90.4)	2.26 ± 1.04 [△]
Yes	38	26 (68.4)	1.71 ± 0.63
Serum AFP level			
≥20 µg/L	52	45 (86.5)	2.16 ± 1.16
<20 µg/L	28	19 (67.9)	2.12 ± 1.24
Histological differentiation			
Well differentiated HCC	15	10 (66.7)	2.07 ± 0.99
Moderately differentiated HCC	20	15 (75)	2.11 ± 1.01
Poorly differentiated HCC	45	39 (86.7)	2.17 ± 1.05
TNM stage			
I	25	19 (76.0)	1.98 ± 1.21
II	32	25 (78.1)	2.06 ± 0.96
III	23	20 (86.9)	2.34 ± 1.06 ^{▲*}

Tumor size ≥5 vs 2–5, ≤2, **p* < 0.05

Vascular tumor thrombus yes vs no, [#]*p* < 0.05

Capsule yes vs no, [△]*p* < 0.05

TNM stage I vs III, [▲]*p* < 0.05, II vs III ^{*}*p* < 0.05

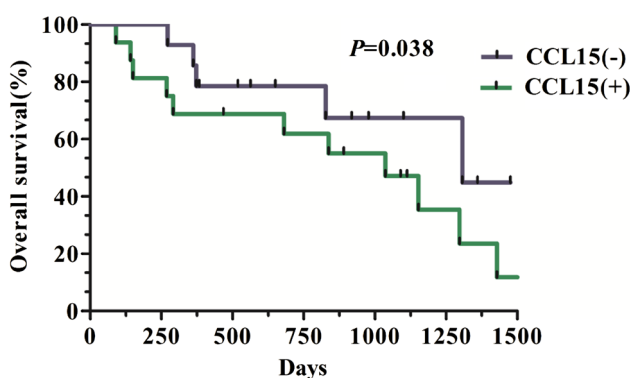


Fig. 2 Kaplan–Meier survival analysis of patients with hepatocellular carcinoma after surgical resection

correlate with HCC tumor size, vascular tumor thrombus, capsule, TNM stage and patient survival time after surgery. Patients with positive CCL15 expression also have shorter survival times after surgery. Furthermore, the multivariable Cox proportional hazards model illustrated that positive expression of CCL15 was an independent prognostic variable, whereas the prognostic values of CCL15 expression in tumors and TNM stage were statistically significantly compromised by other clinical factors.

In conclusion, in HCC tumor samples, CCL15 is overexpressed. The overexpression significantly correlated with tumor size, tumor thrombi in portal veins of HCC, capsule and TNM stages. Postoperative survival time in the patients with CCL15 overexpression was decreased. A more in-

Table 3 Prognostic factors of patients with hepatocellular carcinoma: univariate and multivariate Cox regression analysis

Variable	Univariate analysis			Multivariate analysis		
	HR	95 % CI	<i>p</i> value	HR	95 % CI	<i>p</i> value
Tumor size (cm)						
≥5 vs <5	1.798	1.387–2.857	0.036	1.543	0.986–1.876	0.053
Vascular tumor thrombus						
Yes vs no	1.010	1.001–1.081	0.047	0.976	0.578–1.075	0.069
Tumor capsule						
Yes vs no	1.854	1.231–2.012	0.038	1.757	1.087–1.965	0.058
TNM stage	2.567	1.034–4.321	0.025	2.142	1.134–3.432	0.042
CCL15 expression						
Positive vs negative	1.897	1.324–3.245	0.029	2.011	1.543–2.453	0.045

depth study in CCL15 may offer a new target for biological therapy for malignancy.

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Compliance with ethical standards

Conflict of interest All authors have no conflict of interest. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Ethical standard Human ethics approval was duly obtained from the Institutional Human Ethics Committee of Tianjin Medical University Cancer Institute and Hospital.

Informed consent A written informed consent approved by the ethical committee was obtained from all participants involved in the study. Tissue specimens were obtained by diagnostic or therapeutic procedures from patients after obtaining the patients' consent.

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