CCL14 serves as a novel prognostic factor and tumor suppressor of HCC by modulating cell cycle and promoting apoptosis

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Abstract
CCL14 is a member of CC chemokines and its role in hepatocellular carcinoma (HCC) is still unknown. In this study, CCL14 expression were analyzed by tissue microarray (TMA) including 171 paired tumor and peritumor tissues of patients from Zhongshan Hospital of Fudan University. We found for the first time that CCL14 was downregulated in HCC tumor tissues compared with peritumor tissues ($P = 0.01$). Meanwhile, CCL14 low expression in HCC tumor tissues is associated with a poor prognosis ($P = 0.035$). CCL14 also displayed its predictive value in high differentiation ($P = 0.026$), liver cirrhosis ($P = 0.003$), and no tumor capsule ($P = 0.024$) subgroups. The underlying mechanisms were further investigated in HCC cell lines by CCL14 overexpression and knock-down in vitro. We found overexpression of CCL14 suppressed proliferation and promoted apoptosis of HCC cells. Finally, the effect was confirmed by animal xenograft tumor models in vivo. The results shown overexpression of CCL14 lead to inhibiting the growth of tumor in nude mice. Interestingly, our data also implied that CCL14 played these effects by inhibiting the activation of Wnt/β-catenin pathway. These findings suggest CCL14 is a novel prognostic factor of HCC and serve as a tumor suppressor.

Introduction
Hepatocellular carcinoma (HCC), one of the most common malignant tumors worldwide, ranks fifth in incidence and second in cancer-related mortality1. The prognosis of HCC remains poor due to its insidious onset and high rate of recurrence after resection. Over the past few years, molecular targeted therapy has been proven to be effective in patients. However, targeted therapy is limited because drug resistance is easily developed. Thus, it is urgent to develop new treatments for HCC.

Chemokines were initially defined as molecular signals to induce leukocyte migration during inflammation, which have been identified and classified into four sub-families (CXC, CC, CX3C, and C)2. Recently, researchers have demonstrated that CCL2 and CCL5 promote cancer cell proliferation, invasion and metastasis in several tumors3–7. Consistent with other reports, we have focused on chemokines for years and previously found that secondary lymphoid tissue chemokine (SLC, also named as CCL21) can attract dendritic cells (DCs) to T cells, serving as a treatment for tumor8–11. Moreover, it was also reported that CC chemokine receptor-like 1 play its function as tumor suppressor, which inhibits the proliferation and invasion of HCC cells12.

In this study, we aim to assess the function of CCL14 in HCC. We found low expression of CCL14 in HCC tumor tissues lead to shorter OS. Overexpression of CCL14 by lentivirus can suppress the proliferation and promote the...
apoptosis of HCC cells, which lead to inhibiting tumor growth in animal xenograft tumor models. Our results also suggest that CCL14 may play these effects by inhibiting the activation of Wnt/β-catenin pathway.

Materials and methods

Clinical samples
Tissue microarray (TMA), including 171 paired tumor and peritumor tissues, was obtained from Zhongshan Hospital, Fudan University (Shanghai, China). All patients were confirmed as HCC by two pathologists independently and underwent complete surgical excision between January 2006 and August 2006. Another 20 pairs of fresh tumor and peritumor tissues were randomly collected from the Liver Cancer Institute at Zhongshan Hospital and analyzed by western blot. This study was approved by the ethics committee of Zhongshan Hospital and informed consent was obtained from each patient.

Immunohistochemistry (IHC)
IHC was performed in TMA as previously described. The immunostaining intensities were semi-quantitatively scored as: 0 for negative; 1 for weak; 2 for moderate; 3 for strong by two observers independently. Groups 0 and 1 were classified as low expression, while groups 2 and 3 were classified as high expression.

Cell culture
The HCC cell lines Huh7, SMMC-7721, and HepG2 were purchased from Chinese Academy of Sciences (Cell Bank, Shanghai Institutes for Biological Sciences). The stepwise metastatic HCC cell lines including MHCC97L, MHCC97H, and HCCLM3 were established by the Liver Cancer Institute of Zhongshan Hospital. All cell lines were routinely cultured.

Western blot assay
Western blot was performed as previously described. ECL system (NCM Biotech) was used to visualize the signals. GAPDH was used as a loading control.

Enzyme-linked immunosorbent assay (ELISA)
To detect the secreted CCL14 levels, cell culture supernatants were harvested after 48 h and analyzed using HCC-1/CCL14 Human ELISA Kit (Raybiotech) according to the manufacturer’s protocols.

CCL14 overexpression and downregulation
Stable over-expression of CCL14 in LM3 was constructed by recombinant Lentivirus encoding CCL14. Blank lentivirus was used as vector control. The knockdown of CCL14 in Huh7 cells was constructed by transfecting specific siRNA with Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions. The siRNA target sequences were as follows: siRNA-1: CCAUCGCCCCUAGGACCAATT; siRNA-2: CCAACA GCC AGUGCUCCCAATT.

CCK-8 colony formation assay and BrdU assay
The CCK8 assay was performed as previously described. BrdU assay was performed according to the manufacturer’s instructions.

Flow cytometric analysis
Flow cytometric analysis was used to detect cell cycle and apoptosis as described previously. Apoptosis was measured using FITC-AnnexinV Apoptosis Detection system (BD Bioscience) according to the manufacturer’s instructions.

Tumor bearing animal models in nude mice
LM3 or Huh7 cells with different CCL14 expression levels were harvested and suspended in serum-free DMEM. Athymic nude mice (4–6 weeks old) were divided into four groups: (1) CCL14 group (n = 5), each mouse injected into the lower back with 2 × 10^6 CCL14 overexpressed LM3 cells; (2) Vector group (n = 5), each mouse injected with 2 × 10^6 blank vector infected LM3 cells; (3) si-NC group (n = 5), each mouse injected into the lower back with 3 × 10^6 Huh7 cells treated with siRNA control; (3) siRNA2 group (n = 5), each mouse injected into the lower back with 3 × 10^6 Huh7 cells threatened with CCL14 siRNA. Every 6 days, tumors were monitored and measured. At 42nd day, the mice were sacrificed and tumors weight were examined.

Statistical analysis
The results were expressed as the means ± SD and were considered to be statistically significant at p < 0.05. The data were analyzed using IBM SPSS Statistics 20 (IBM Corporation, USA). Student’s t test was used for comparisons between groups. Categorical data were analyzed by the chi-square or Fisher’s exact tests. Kaplan–Meier analyses and log-rank tests were used to perform survival analyses.

Results
CCL14 is downregulated in HCC tissues and CCL14 low expression is associated with a poor prognosis in clinical patients
CCL14 expression profile was accessed by IHC in a human TMA containing 171 paired HCC and peritumor tissues and representative images were shown in Fig. 1a. The results indicated that CCL14 were scored as negative or weak expression in 42.69% (73 of 171: negative, n = 5; weak, n = 68) of HCC tissues, as compared with 24.56% of corresponding peritumor tissues (42 of 171: negative,
Twenty pairs of frozen tissues from HCC patients were randomly chosen to examine CCL14 protein expression. Western blot analysis showed that the protein level of CCL14 was remarkably lower in HCC tissues than in adjacent tissues, as shown in representative six pairs (Fig. 1c).

All patients in the TMA were classified into the CCL14\textsuperscript{low} group or CCL14\textsuperscript{high} group according to CCL14 expression. The results showed CCL14 low expression was associated with a poorer prognosis by Kaplan–Meier analysis (Fig. 1d). The predictive value of CCL14 were also observed in high differentiation (\(P = 0.026\)), liver cirrhosis (\(P = 0.003\)) and no tumor capsule (\(P = 0.024\)) subgroups (Fig. 1e). Additionally, as shown in Table 1, low expression of CCL14 was also associated with microvascular invasion (\(P = 0.039\)).
A significant association between the CCL14 expression signature and OS in the univariable Cox regression model was also observed. As shown in Table 2, the HR value of the CCL14 low group vs. the CCL14 high group for OS was 0.631 ($P = 0.037$). At the same time, tumor size (HR = 1.890; $P = 0.004$), microvascular invasion (HR = 1.971; $P = 0.002$), TNM stage (HR = 2.273; $P = 0.004$), and BCLC stage (HR = 1.839; $P = 0.010$) were contributing factors to a shorter OS of patients.

Overexpression of CCL14 inhibits the proliferation of HCC cells in vitro

The expression of CCL14 in different human HCC cell lines were tested by western blot, including SMMC-7721, Huh-7, HepG2, Hep3B, MHCC-97L, MHCC-97H, and HCCLM3. It revealed that CCL14 was highly expressed in Huh-7 cells while lowly expressed in LM3 cells (Fig. 2a). CCL14 expression and secretion were both successfully enhanced by recombinant lentivirus in LM3 cells, whereas knocked down in Huh7 cells by siRNA (Fig. 2b, Supplementary Fig. 1). Because siRNA2 sequences played more specific functions, we choose siRNA2 for the following tests. Overexpression of CCL14 significantly reduced the growth rate of LM3 cells, which was revealed by CCK8 assays (Fig. 2c). On the contrary, down-regulation of CCL14 by siRNA2 significantly increased the growth rate in Huh7 cells (Fig. 2d). At the same time, the overexpression of CCL14 in LM3 cells decreased the colonies, while downregulation of CCL14 in Huh7 cells increased the colonies (Fig. 2e). Additionally, CCL14 overexpression in LM3 decreased the proportion of BrdU-incorporated cells, while CCL14 knockdown in Huh7 increased the proportion of BrdU-incorporated cells (Fig. 2f).

CCL14 modulates cell cycle and promotes apoptosis in vitro

CCL14-mediated changes in cell cycle progression and apoptosis were accessed by flow cytometry analysis. Overexpression of CCL14 in LM3 increased both the percentage of G0/G1 phase cells and the rate of apoptosis cells (Fig. 3a, *$P < 0.05$, **$P < 0.01$). Knock-down of CCL14 in Huh7 decreased the percentage of G0/G1 phase cells and the rate of apoptosis cell (Fig. 3b, *$P < 0.05$, **$P < 0.01$). These data indicated that CCL14 suppressed HCC cell proliferation by inhibiting cell cycle progression and promoting apoptosis in HCC cells.

Overexpression of CCL14 inhibits the tumor growth in nude mice in vivo

In tumor-bearing animal models, we found tumor grew slowly in CCL14-overexpressed group than control group (Fig. 4a). Both tumor volume and weight were decreased at the same time (Fig. 4b, c). Consistently, tumors of siRNA2 group grew faster and bigger than si-NC group (Fig. 4d–f). Moreover, IHC analysis confirmed that CCL14 over-expressed tumors displayed lower PCNA and

| Clinical characteristic | CCL14<sub>low</sub> no. | CCL14<sub>high</sub> no. | $P$-value |
|------------------------|-------------------------|-------------------------|-----------|
| Age (no.)              |                         |                         | 0.478     |
| <60                    | 54                      | 74                      |           |
| ≥60                    | 19                      | 24                      |           |
| Gender                 |                         |                         | 0.268     |
| Male                   | 61                      | 77                      |           |
| Female                 | 12                      | 21                      |           |
| Tumor differentiation  |                         |                         | 0.195     |
| I–II                   | 8                       | 6                       |           |
| III–IV                 | 65                      | 92                      |           |
| Tumor size (cm)        |                         |                         | 0.217     |
| <5                     | 41                      | 62                      |           |
| ≥5                     | 32                      | 36                      |           |
| Tumor number           |                         |                         | 0.201     |
| Single                 | 59                      | 85                      |           |
| Multiple               | 14                      | 13                      |           |
| TNM stage              |                         |                         | 0.172     |
| I–II                   | 62                      | 89                      |           |
| III–IV                 | 11                      | 9                       |           |
| BCLC stage             |                         |                         |           |
| 0–A                    | 26                      | 44                      | 0.144     |
| B–C                    | 47                      | 54                      |           |
| Microvascular invasion |                         |                         | 0.039*    |
| No                     | 41                      | 69                      |           |
| Yes                    | 32                      | 29                      |           |
| Tumor capsule          |                         |                         | 0.276     |
| No                     | 37                      | 44                      |           |
| Yes                    | 36                      | 54                      |           |
| History of cirrhosis   |                         |                         | 0.107     |
| No                     | 12                      | 25                      |           |
| Yes                    | 61                      | 73                      |           |

*Statistically significant

Table 1 Association of CCL14 expression with clinical parameters of 171 HCC patients
Our results indicate that CCL14 suppresses HCC growth in vivo. CCL14 inhibits the proliferation of HCC cells via Wnt/β-catenin-signaling pathway. The expression of several key proteins of Wnt/β-catenin pathway in HCC cells were analyzed. Overexpression of CCL14 decreased the expression of p-GSK3β (S9), β-catenin, cyclin D1, and c-Myc in LM3 cells (Fig. 5a). On the contrary, downregulation of CCL14 by siRNA significantly increased the expression of these proteins in Huh7 cells (Fig. 5b). In HCC samples, CCL14 expression also showed negatively correlated with cyclin D1 levels (as shown in Supplementary Fig. 2). Dickkopf1 (DKK1), the specific inhibitor of Wnt/β-catenin-signaling pathway was used to investigate whether CCL14 inhibits the proliferation of tumor cells via Wnt/β-catenin pathway. In LM3 cells, as shown in Fig. 6a, c, compared with blank-vector control, overexpression of CCL14 decreased the expression of cyclin D1 and c-Myc, at the same time, significantly inhibited the proliferation of LM3 cells (***P < 0.01). The combination of CCL14 and DKK1 caused highest inhibition effect (****P < 0.0001).

On the other hand, in Huh7 cells, as shown in Fig. 6b, d, compared with si-NC control transfection, CCL14 knocking-down by siRNA2 transfection lead to an increase of cyclin D1 and c-Myc, as well as the increased proliferation (***P < 0.001). The combination of DKK1 played an opposite inhibition effect (**P < 0.01).

Additionally, we also explored the potential mechanisms that may lead to CCL14 downregulation in HCC and found that CCL14 levels can be restored by DNA demethylation agent 5-Aza-CdR and histone demethylase inhibitor GSK-467 (Supplementary Fig. 3), indicating the low level of CCL14 is partially the result of epigenetic regulation.

Discussion

CCL14 induces monocytes, macrophages, and THP-1 cells by binding to CCR1, CCR3, and CCR5. Previous reports have shown that CCL14 is also involved in the pathogenesis and progression of various disorders, including allergic airway inflammation and some cancers.

In this study, we examined the expression of CCL14 in paired HCC tumor tissue and peritumor tissues. The results showed that CCL14 low expression in HCC tumor tissues was correlated with poor survival. We also found overexpression of CCL14 inhibited proliferation and promoted the apoptosis of LM3 cells. Knock-down of CCL14 showed opposite effects in Huh7 cells. Moreover, CCL14 suppressed tumor growth in animal models. So CCL14 suppresses the progression and promotes the apoptosis of HCC cells, which contribute to longer OS in HCC patients. In addition, consistent with previous research that reported CCL14

| Variables        | Univariate analysis | Multivariate analysis |
|------------------|---------------------|-----------------------|
|                  | HR | 95%CI | P-value | HR | 95%CI | P-value |
| Age (years)      |    |       |         |    |       |         |
| <60 vs. ≥60      | 1.561 | 0.988–2.465 | 0.056 | NA |       |         |
| Gender           |    |       |         |    |       |         |
| Male vs. female  | 0.921 | 0.540–1.571 | 0.762 | NA |       |         |
| Tumor size (cm)  |    |       |         |    |       |         |
| ≤5 vs. >5        | 1.890 | 1.225–2.915 | 0.004* | 1.749 | 0.891–3.432 | 0.104 |
| Tumor number     |    |       |         |    |       |         |
| Multiple vs. single | 1.682 | 0.986–2.870 | 0.056 | NA |       |         |
| Tumor differentiation |    |       |         |    |       |         |
| I–II vs. III–IV  | 2.109 | 0.772–5.760 | 0.146 | NA |       |         |
| Microvascular invasion |    |       |         |    |       |         |
| Yes vs. no       | 1.971 | 1.276–3.045 | 0.002* | 1.899 | 1.039–3.471 | 0.037* |
| Tumor capsule    |    |       |         |    |       |         |
| Yes vs. no       | 1.208 | 0.783–1.863 | 0.393 | NA |       |         |
| History of cirrhosis |    |       |         |    |       |         |
| Yes vs. no       | 1.155 | 0.698–1.912 | 0.576 | NA |       |         |
| TNM stage        |    |       |         |    |       |         |
| I–II vs. III–IV  | 2.273 | 1.297–3.983 | 0.004* | 1.522 | 0.802–2.890 | 0.199 |
| BCLC stage       |    |       |         |    |       |         |
| 0–A vs. B–C     | 1.839 | 1.154–2.930 | 0.010* | 0.747 | 0.323–1.727 | 0.495 |
| CCL14            |    |       |         |    |       |         |
| Low vs. high     | 0.631 | 0.409–0.973 | 0.037* | 0.703 | 0.452–1.094 | 0.118 |

Note: Univariate and multivariate analyses, Cox proportional hazards regression model
OS overall survival time, HR hazard ratio, CI confidence interval, NA not applicable
*Statistically significant

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expression was suppressed by JARID1B/LSD1/NuRD complex in breast cancer, we found CCL14 expression was upregulated after being treated with DNA demethylating agent 5-Aza-CdR or histone demethylase inhibitor GSK-467. These results suggested that epigenetic regulation plays a critical role in the expression of CCL14 and we will explore more detailed mechanisms subsequently.
The Wnt/β-catenin pathway has been found in various cancer types, including HCC\textsuperscript{22-24}. Previous studies have reported that β-catenin plays an important role in Wnt/β-catenin signaling. When Wnt proteins bind to the receptor, β-catenin will translocate to the nucleus and interact with TCF/LEF transcription factors to regulate downstream gene expression\textsuperscript{25}. In the present study, we showed that knocking-down CCL14 could increase p-GSK3β (S9), β-catenin (S33/S37), and further promote the expression of c-myc and cyclin D1, which are the downstream target genes of Wnt/β-catenin and associated with tumor cell proliferation or apoptosis in various cancers\textsuperscript{26-28}. Our results also suggested that CCL14 exerts its regulatory effects on proliferation of
HCC cells through the Wnt/β-catenin pathway by the treatment of DKK1, the specific inhibitor of Wnt/β-catenin pathways.

These findings suggest CCL14 is a novel prognostic factor of HCC and serve as a tumor suppressor.
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Conflict of interest
The authors declare that they have no conflict of interest.

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