Inhibitory effect of CC chemokine ligand 23 (CCL23)/transcription factor activating enhancer binding protein 4 (TFAP4) on cell proliferation, invasion and angiogenesis in hepatocellular carcinoma

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**ABSTRACT**

Hepatocellular carcinoma (HCC) is a highly vascularized solid tumor with a fast growth rate. According to bioinformatics analysis, CC chemokine ligand 23 (CCL23) has clinical significance for survival and prognosis in HCC. The online databases TCGA and CCLE were used to analyze the expression level of CCL23, and its expression was also measured in HCC cell lines by RT-qPCR and Western blotting. The STRING database and co-immunoprecipitation were employed to evaluate the association between CCL23 and transcription factor activating enhancer binding protein 4 (TFAP4). Overexpression plasmids for CCL23 (Ov-CCL23) and TFAP4 (Ov-TFAP4) were transfected into Huh-7 cells to detect TFAP4 expression. Huh-7 cells injected with Ov-negative control (NC)/Ov-CCL23 or OV-NC/Ov-CCL23 plus Ov-TFAP4 were utilized to study the function of CCL23/TFAP4. Cell proliferation, invasion and human umbilical vein endothelial cell tube formation assays were conducted. The database revealed decreased expression of CCL23 in HCC and that it was commonly downregulated in HCC cell lines. TFAP4 expression was negatively correlated with CCL23. The overexpression of CCL23 inhibited the proliferation and invasion of Huh-7 cells, whereas TFAP4 blocked these effects. Similarly, the supernatant of CCL23-upregulated cells exhibited significantly lower tube formation potential, and low vascular endothelial growth factor A (VEGFA), VEGFRs expression compared with those of non-transfected Huh-7 cells, while TFAP4 plasmid co-transfected markedly increased these. Taken together, the present study suggests that CCL23 is expressed at low levels in HCC; it inhibits HCC cell proliferation, invasion and angiogenesis \textit{in vitro}; and its action is negatively associated with and can be blocked by TFAP4.

**Introduction**

Hepatocellular carcinoma (HCC) is the main type of liver cancer and is considered the most commonly diagnosed cancer type and the leading cause of global cancer-associated mortality [1,2]. In China, patients with HCC account for ~50% of new cases worldwide [3]. Recent surgical treatment of HCC includes liver resection, liver transplantation, local ablation therapy and transarterial chemoembolization as the most successful approaches [4,5]. However, the incidence of postoperative recurrence and metastasis is high, which leads to patient death in the majority of the cases [6]. Therefore, research to identify new factors involved in HCC tumorigenesis is crucial for improving early diagnosis and therapeutic approaches.

CC chemokine ligand 23 (also called myeloid progenitor inhibitory factor 1, macrophage inflammatory protein 3) represents a new member of the CC small chemokine family, which has recently been associated with the pathophysiology of various inflammatory conditions, such as vasculitis, rheumatoid arthritis and atopic dermatitis [7,8]. CCL23 was primarily recognized as a potent hematopoietic suppressor due to its ability to suppress the colony formation of myeloid progenitor cells [9]. Previous studies indicated that CCL23 is a progressive biomarker for the diagnosis of brain lesions such as Alzheimer’s disease and ischemic stroke, and circulating CCL23 levels were reported to function as biomarkers of outcomes in the aforementioned patients, and consequently influence the management and treatment decisions of...
these patients [10–12]. In addition, CCL23 promotes the vascular endothelial growth factor (VEGF)-induced propagation and migration of endothelial cells [13]. Furthermore, CCL23 eliminates the endoplasmic reticulum stress and lipo polysaccharide-induced proliferation reduction in bovine endometrial epithelial cells [14]. In contrast, there are currently few studies indicating the involvement of CCL23 in cancer. One of the studies revealed that CCL23 was necessary and sufficient to promote the migration of ovarian cancer [15]. However, only online databases were used to analyze the expression level of CCL23 in HCC, and its specific role in HCC has not yet been investigated [16].

Transcription factor activating enhancer binding protein 4 (TFAP4) is a member of the basic helix-loop-helix leucine zipper family and is widely involved in the proliferation, differentiation, metastasis, angiogenesis and other biological regulatory functions of human cancer [17]. TFAP4 facilitates HCC invasion and metastasis through activation of the PI3K/AKT signaling pathway [18]. In addition, TFAP4 enhances tumour-forming ability and activates the Wnt/β-catenin pathway in HCC [19].

The present study aimed to determine the expression of CCL23 in HCC cell lines, and to further investigate the influence of CCL23 and TFAP4 on HCC proliferation, invasion and angiogenesis, in order to determine whether CCL23/TFAP4 may serve as a functional prognostic biomarker and a promising therapeutic target for HCC.

Materials and Methods

Bioinformatics analysis

The Gene Expression Profiling Interactive Analysis (GEPIA) database (http://gepia.cancer-pku.cn) based on the data from The Cancer Genome Atlas (TCGA) and The Genotype-Tissue Expression (GTEx) was used to check the expression of CCL23 in HCC patients (n = 369) and normal people (n = 160) [20]. The Cancer Cell Line Encyclopedia (CCLE) database (https://docs.sevenbridges.com/docs/ccle) was applied to assay CCL23 gene expression in different cell lines [21]. STRING database (https://cn.string-db.org/) was used to determine the association between CCL23 and TFAP4 [22]. The data about TFAP4 expression in HCC patients (n = 120) was downloaded from TCGA [18] and represented as a dot graph.

Cell culture

The human HCC cell lines (Li-7, Huh-7 and MHCC97) and the normal liver cell line (HHL5) were purchased from the Chinese Academy of Sciences Cell Bank. Human umbilical vein endothelial cells (HUVECs) were purchased from Cobiolr (Nanjing, China). Cells were all incubated in Dulbecco’s modified Eagle’s medium (DMEM; Gibco; Thermo Fisher Scientific) with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific) at 37°C in a humidified atmosphere with 5% CO₂ [23].

Quantitative reverse transcription PCR (RT-qPCR)

Total RNA was isolated from the Huh-7 cell line using 1 ml TRIzol® (Invitrogen; Thermo Fisher Scientific). Complementary DNA was amplified by PCR under the following thermocycling conditions: 40 cycles at 95°C for 5 min, 95°C for 15 sec, 60°C for 20 sec and 72°C for 40 sec. The relative expression of target genes and internal control gene GAPDH was analyzed using the 2⁻ΔΔCq method [24]. The primer sequences for RT-PCR were as follows: CCL23 forward primer 5’-TAGGAGATCTCAGTGAGGAGG-3’ and reverse primer 5’-CTTGCCACAATGGTCTTG-3’; VEGFA forward primer 5’-TTTCTGCTGCTTACCTCCACCAT-3’ and reverse primer 5’-GGTGATGTTGGACTCCTCAGTG-3’; TFAP4 forward primer 5’-TCACCCACGGACTCACCACA-3’ and reverse primer 5’-CGATGTCCTCAGGCTGATAAA-3’; VEGFR2 forward primer 5’-GGAAGGTTGGCTGCTCTCAT-3’ and reverse primer 5’-CAGGGCAGACAAGTGGGTAT-3’; and GAPDH forward primer 5’-GAGGGAGATCCCTCCTAAAAT-3’ and reverse primer 5’-GGCTGTGTGCAATCTCATGG-3’.
**Western blot analysis**

Total protein from Huh-7 cells was extracted with RIPA lysis buffer (Beyotime Institute of Biotechnology) and detected via the BCA method (Beyotime Institute of Biotechnology). Equal quantities (30 μg) of protein were electrophoresed on 6–12% denaturing SDS gels at 110 V for 90 min, transferred to PVDF membranes (EMD Millipore) and blocked for 1 h at room temperature. The membranes were then incubated with 1% BSA (11021045; Invitrogen; Thermo Fisher Scientific), followed by incubation with antibodies against CCL23 (1:2,000; PA5-100686; Invitrogen; Thermo Fisher Scientific), TFAP4 (1:1,000; HPA001912; Sigma-Aldrich; Merck KGaA), matrix metalloproteinase-2 (MMP2) (1:3,000; sc-13594; Santa Cruz Biotechnology), MMP9 (1:3,000; ABT544; EMD Millipore) and GAPDH (1:5,000; 5174; Cell Signaling Technology) overnight at 4°C. After washing with TBST at room temperature for 30 min, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibodies (1:1,000; 7076, 7074; Cell Signaling Technology) for 2 h at room temperature. The antigen–antibody complexes were visualized with the ECL system (WBUL50500; EMD Millipore). The ImageJ 1.4.3 was used for densitometry analysis of the protein bands [25].

**Cell proliferation assays**

The Cell Counting Kit-8 (CCK-8) kit was purchased from EMD Millipore. According to the manufacturer’s protocols, cells were incubated with 10 μl CCK-8 reagent at 37°C for 2 h, and the absorbance was then read at 450 nm. All assays were performed three times.

The 5-ethyl-2’-deoxyuridine (EdU) assay kit was purchased from Guangzhou RiboBio Co., Ltd. Cells were seeded in 24-well plates (2 × 10⁴ cells/well) and incubated with DMEM containing 10% FBS for 24 h prior to the addition of EdU (50 μmol/l). Cells were incubated for 2 h at room temperature according to the manufacturer’s protocol, followed by fixation with 4% formaldehyde for 30 min and dissolving with 0.5% Triton X-100 (85,111; Invitrogen; Thermo Fisher Scientific) for 10 min at room temperature. After washing with PBS, 1X ApolloR reaction cocktail (400 μl) was added to react with EdU for 30 min. Subsequently, Hoechst 33342 (400 μl) was added for 30 min for visualization of cell nuclei [27]. Photographs of the cells were obtained under a Nikon microscope (Nikon Corporation). Cell proliferation was calculated using the median cell number of three fields of view in each sample.

**Cell migration and invasion assays**

For the invasion assay [28], cells were seeded (5 × 10⁴ cells/well) in 8 μm-well Transwell chambers coated with Matrigel (BD Biosciences) and pre-cooled serum-free medium added for 48 h. After removing the cells from the upper surface of the filter with a cotton swab, the cells were fixed with 10% paraformaldehyde for 1 h and stained with 0.1% hexamethylenetramine for 30 min. The cells that migrated across the membrane and were attached to the submembrane surface were removed, and the number of cells was counted under the microscope. The data were presented as the mean number of cells that migrated through the filter.

For the cell wound healing assay [29], cells were seeded in 6-well plates (1 × 10⁵ cells per well) and incubated in a complete medium. When the cells grew to 80% confluency, a wound was made in the cell layer using a sterile pipette tip. After washing
with PBS several times to remove cell debris, the cells continued to be incubated in a serum-free medium for 48 h. The process of cell migration to the wound surface was referred to as in vitro wound healing and was photographed with an inverted fluorescence microscope. The cell migration rate was assessed by the closure of the gap, and the following formula was applied: Wound healing rate = [(0 h wound width – 48 h wound width)/0 h wound width] × 100%.

Angiogenesis assay

Matrigel (BD Biosciences) was diluted at a 1:1 ratio with cold endothelial cell growth medium (EGM-2), and the mixture was added to 24-well plates. To examine the generation of capillary-like structures in vitro [30], HUVECs were seeded in Matrigel at a density of 8 × 10^4 cells per well. Following cell transformation and attachment to the wall, the culture medium was removed, and different types of supernatant were added, followed by incubation at 37°C for 6 h. The length of the vessels or the number of rings per well formed by HUVECs was calculated. The structure of the capillaries was observed using an Olympus microscope (Olympus Corporation) and statistically analyzed using Image-Pro Plus software.

Statistical analysis

Data are expressed as the mean ± standard deviation and were analyzed with GraphPad Prism 8.0 (GraphPad Software, Inc.). Statistical differences between the two groups were evaluated by Student’s t-test [31], while ANOVA followed by a Tukey’s post hoc test [32] was employed to determine statistical differences between multiple groups. P < 0.05 was considered to indicate a statistically significant difference.

Results

The present article aimed to study the role of CCL23/TFAP4 in HCC. The TCGA and CCLE databases revealed decreased expression of CCL23 in HCC and that it was commonly down-regulated in HCC cell lines. TFAP4 expression was highly expressed in HCC and negatively correlated with CCL23. The overexpression of CCL23 inhibited the proliferation and invasion of Huh-7 cells, whereas TFAP4 blocked these effects. Similarly, the supernatant of CCL23-upregulated cells exhibited significantly lower tube formation potential, and low vascular endothelial growth factor A (VEGFA), VEGFRs expression compared with those of non-transfected Huh-7 cells, while TFAP4 plasmid co-transfected markedly increased these.

CCL23 expression is reduced in HCC tissues and cell lines

To investigate the function of CCL23 in HCC, CCL23 expression was first examined by online databases. The Cancer Genome Atlas database assay showed reduced expression of CCL23 in HCC tissues (Figure 1(a)). In addition, the CCLE database indicated significantly lower mRNA expression of CCL23 in liver cell lines (Figure 1(b)). Next, the protein and mRNA expression of CCL23 was analyzed in human HCC cell lines (Li-7, Huh-7 and MHCC97) and in one normal human hepatic cell line (HHL5). Western blot experiments revealed that the levels of CCL23 were markedly reduced in Li-7, Huh-7 and MHCC97 cells compared with those in HHL5 cells, with the lowest levels being observed in Huh-7 cells (Figure 1(c)). Similarly, RT-qPCR revealed decreased mRNA expression in HCC cell lines (Figure 1(d)). These results indicated that CCL23 is down-regulated in HCC, and Huh-7 cells were selected for subsequent experiments.

CCL23 inhibits TFAP4 expression in HCC

To determine the possible mechanism of CCL23 in HCC, the STRING database was used to predict the proteins to which CCL23 may bind. The results indicated that CCL23 is associated with TFAP4 (Figure 2(a)). A Co-IP assay was used to confirm the connection between CCL23 and TFAP4. Flag and HA were co-precipitated, implying that CCL23 and TFAP4 could interact with each other (Figure 2(b)). Afterward, the expression level of TFAP4 in HCC cell lines and tissues was determined using Western blotting and RT-qPCR (Figure 2(c–e)) along with bioinformatics analysis (Figure 2(f)), respectively. These results suggested that TFAP4 was highly expressed in HCC. To
To further explore the association between CCL23 and TFAP4, the expression of CCL23 and TFAP4 in Huh-7 cells transfected with Ov-NC and CCL23 expression vectors was confirmed via RT-qPCR and Western blotting. As presented in Figure 2 (g–h), CCL23 upregulation in Huh-7 cells significantly reduced TFAP4 protein and mRNA expression. Therefore, CCL23 is negatively associated with TFAP4.

**CCL23/TFAP4 are involved in HCC cell proliferation**

To evaluate the molecular role of CCL23/TFAP4 in HCC, Huh-7 cells were transfected with i) Ov-NC, ii) Ov-CCL23, iii) Ov-CCL23 and Ov-NC and iv) Ov-CCL23 and Ov-TFAP4, respectively. As shown in Figure 3(a,b), treatment with the TFAP4 expression plasmid significantly upregulated TFAP4 protein and mRNA expression, suggesting TFAP4 overexpression was successfully established. The results of CCK-8 assay demonstrated that CCL23 overexpression significantly inhibited Huh-7 cell viability, while co-culture with Ov-CCL23 and Ov-TFAP4 alleviated this effect. No change was observed compared with the control when cells were transfected with Ov-NC, and no change in cells co-cultured Ov-CCL23 and Ov-NC was observed compared with cells transfected with Ov-CCL23 (Figure 3(c)). The results of EdU assay revealed that the number of EdU-positive cells was reduced in Huh-7 cells transfected with Ov-CCL23 compared with that of cells transfected with NC and control cells. Compared with that of the Ov-CCL23 and Ov-NC groups, transfection with Ov-CCL23 and Ov-TFAP4 increased the number of EdU-positive cells in Huh-7 cells.
Taken together, these data demonstrate that overexpression of CCL23 significantly inhibited Huh-7 cell proliferation, while overexpression of TFAP4 blocked this effect.

**CCL23/TFAP4 are involved in HCC cell migration**

To further confirm the effect of CCL23/TFAP4 in HCC, Transwell migration and wound closure assays were performed. A significant increase in the closure of the wound gap was observed after...
CCL23 upregulation mediated by Ov-CCL23 compared with that of control cells, whereas cells transfected with Ov-CCL23 and Ov-TFAP4 exhibited faster gap closure compared with that of cells transfected with Ov-CCL23 and Ov-NC (Figure 4(a,b)). Similarly, the results of the Transwell assay indicated that Ov-CCL23 transfection significantly decreased cell migration compared with that of control cells, while TFAP4 reversed this effect (Figure 4(c,d)).

MMPs are the major secreted proteinases required for extracellular matrix degradation during tumor invasion and metastasis [33]. The overexpression of MMPs in malignant gliomas has been well characterized [34]. Therefore, MMP2 and MMP9 were examined by Western blotting to determine the effect of CCL23/TFAP4 in HCC. The expression of MMP2 and MMP9 was decreased in Huh-7 cells transfected with Ov-CCL23 compared with that of cells transfected with Ov-NC and control cells. On the contrary, overexpression of TFAP4 and CCL23 increased MMP2 and MMP9 levels in Huh-7 cells compared with those observed in cells transfected with Ov-NC and Ov-CCL23 (Figure 4(e)). Collectively, these data suggested that CCL23 could inhibit the invasion and migration of HCC cells and that TFAP4 could reverse this effect.

**CCL23/TFAP4 are involved in HCC cell angiogenesis**

To clarify the role of CCL23/TFAP4 in HCC vascularization, HUVECs were selected because these cells exhibit the ability to grow and form tubular structures, depending on the growth factors...
-contained in the supernatant [35]. HUVECs were treated with the supernatant of NC cultures as a control medium (CM). The supernatant of Huh-7 cells transfected with i) Ov-NC, ii) Ov-CCL23, iii) Ov-CCL23 and Ov-NC and iv) Ov-CCL23 and Ov-TFAP4 supernatant was used as experimental samples. Decreased tube formation was observed in HUVECs treated with the supernatant collected from Ov-CCL23 Huh-7 cells compared with that of Huh-7 cells treated with CM. HUVECs treated with the supernatant of Ov-CCL23 and Ov-TFAP4-transfected cells exhibited better tube formation ability than Ov-CCL23 supernatant-treated cells (Figure 5(a, b)). In addition, the expression of VEGFA, VEGFR1 and VEGFR2 was assessed. The RT-qPCR results suggested that the expression level of VEGFA, VEGFR1 and VEGFR2 was reduced in Ov-CCL23 supernatant-treated cells compared with that of CM-treated cells, whereas TFAP4 upregulation significantly increased the expression of these molecules (Figure 5(c)). The present results demonstrated that CCL23 overexpression reduced Huh-7 cell angiogenesis, while TFAP4 reversed this effect.

Discussion
CCL23 has been reported to be associated with brain injury, immune response, systemic sclerosis, rhinosinusitis and other diseases [11,36,37]. However, to the best of our knowledge, the study of CCL23 and HCC has been limited to network data analysis. Therefore, the present study was undertaken to explore the function of CCL23 in the progression of HCC. Consistent with a previous study, CCL23 expression was decreased in HCC tissues [16]. In addition, the results of
network predictions were validated in different HCC cell lines. These results suggested that CCL23 plays a key role in HCC.

To elucidate the molecular mechanism underlying the function of CCL23 in the regulation of HCC progression, the STRING database was employed to predict the possible binding proteins of CCL23. The results revealed that CCL23 was associated with TFAP4. CCL23 overexpression plasmid was transfected into Huh-7 cells, and it was found that overexpression of CCL23 reduced the expression of TFAP4. Thus, CCL23 inhibits the expression of TFAP4 in HCC. TFAP4 was reported to significantly promote HCC proliferation, invasion and migration [19]. In addition, elevated TFAP4 activates the IncRNA TRERNA1 to facilitate gastric cancer cell migration and invasion [38]. Thus, cell proliferation, migration and invasion assays were performed in the present study to observe the effect of CCL23/TFAP4. The results indicated that elevated CCL23 expression reduces the proliferation, invasion and migration of HCC cells, while upregulation of TFAP4 blocked this effect. Based on the fact that CCL23 is a ligand of CCR1 and has a strong affinity for CCR1 [39], we speculate that following CCL23 binds to CCR1, it regulates the GSK-β signaling pathway [40] and subsequently regulates the expression of TFAP4. Nevertheless, this conjecture needs to be verified in the future.

Vascular supply is crucial for the proliferation and survival of tumor cells [41]. Angiogenesis, the process of developing new blood vessels from existing ones, is the main reason for the growth and expansion of the vascular network, and is one of the important conditions for HCC growth and metastasis [42]. Chemokines are resolvable agents involved in angiogenesis, cell growth control and immune regulation [43]. Superior umbilical artery CCL16 is outstanding in preterm pregnancies with gross growth restriction [44]. It has been reported that CCL23 selectively recruits stationary T lymphocytes and monocytes, suppresses the proliferation of bone marrow progenitor cells and stimulates angiogenesis [39]. Interestingly, the CCL23 was reported to be sufficient to promote ovarian cancer migration [15]. A review states that CCL23 appears to have both anti-cancer and cancer-promoting properties. According to the ‘The Human Protein Atlas’, increased expression of CCL23 is beneficial to the prognosis of liver, prostate and breast cancers, while it has the opposite effect on that of intestine, endometrium cancers and glioma [45]. The present study found that

![Figure 5. Overexpression of CCL23 inhibits angiogenesis in hepatocellular carcinoma. (a and b) Representative images of capillary-like structures formed by human umbilical vein endothelial cells on Matrigel. (c) Detection of VEGFA, VEGFR1 and VEGFR2 mRNA levels in Huh-7 cells. The results are representative of at least three independent experiments. ***P < 0.001 vs. control group; ###P < 0.001 vs. OV-CCL23 group.](image-url)
supernatant collected from Huh-7 cells following CCL23 overexpression exhibited reduced HUVEC angiogenesis, and reduced expression of VEGFA and its receptors VEGFR1 and VEGFR2. TFAP4 contributed to angiogenesis and VEGF action.

**Conclusion**

In conclusion, to the best of our knowledge, the present study is the first to demonstrate that CCL23 is reduced in HCC tissues and cell lines. In addition, CCL23 inhibited HCC proliferation, invasion and angiogenesis. These data provide substantial new evidence that TFAP4 is negatively associated with CCL23 and indicate that CCL23/TFAP4 may serve as a novel prognostic marker and therapeutic target for HCC. However, the association between CCL23 and HCC remains elusive, and further studies are required to verify the role of CCL23 in vivo.

**Authors’ contributions**

KC wrote manuscript and participated in the planning and execution of the experiments; WW along with JW and XD participated in the major experiments and analyzed the results; all authors read and approved final manuscript.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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**Data availability**

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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