Overexpression of \(VCC-1\) gene in human hepatocellular carcinoma cells promotes cell proliferation and invasion

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Vascular endothelial growth factor-correlated chemokine 1 (\(VCC-1\)), a novel chemokine, is hypothesized to be associated with carcinogenesis. \(VCC-1\) is expressed in hepatocellular carcinoma (HCC) cells, but its function remains unknown. To investigate the molecular effects of \(VCC-1\) on HCC cells, the HCC cell line SMMC7721 was stably transfected with the recombinant plasmid \(pcDNA3.1/VCC-1\). Our data demonstrated that overexpression of \(VCC-1\) in SMMC7721 cells significantly enhanced the cellular proliferation, invasive ability, and tumor growth, when compared with both empty vector control cells and parental cells. These results strongly suggest that \(VCC-1\) plays an important role in SMMC7721 invasion and tumor growth, and indicate that \(VCC-1\) may serve as a potential biomarker for anti-HCC therapies.

**Keywords** chemokine; hepatocellular carcinoma; \(VCC-1\); tumorigenesis

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**Introduction**

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. The high frequency of HCC is mainly due to an increasing incidence of alcoholic liver disease, along with hepatitis B and hepatitis C viral infections [1]. As with most solid malignant tumors, hepatocarcinogenesis is considered to be a multi-step process involving uncontrolled cellular growth, detachment from the extracellular matrix and invasion into the surrounding tissue, along with modulation of both the immune system and the blood supply to promote tumor growth [2–4]. The mechanisms of HCC cell invasion and metastasis remain unknown. The prognosis and treatment for the disease need to be improved.

Chemokines represent a large family of about 45–50 proteins in humans, characterized by structural homologies based on conserved cysteine residues as well as binding capacities to particular G protein-coupled receptors [5]. Most chemokines contain four cysteine residues, which are classified based on the spacing of the first two cysteine residues, such as CXC, CC, C, and CX3C [6,7]. Several chemokines are able to bind to the same receptor, while one chemokine can bind to several different receptors, creating multiple combinations. As a result, there are multiple different biologic outcomes. Chemokines associated with tumors are thought to play at least five roles in the biology of primary and metastatic disease: (i) control of leukocyte infiltration into the tumor, in particular during infection and inflammation; (ii) manipulation of tumor immune response; (iii) regulation of angiogenesis; (iv) acting as autocrine or paracrine growth and survival factors; and (v) control of the movement of tumor cells themselves. The expression of many CXC chemokines could influence tumor progression [8]. Several CXC chemokines, especially CXCL1 and CXCL8, were known to function as angiogenic factors to tumors.

\(VCC-1\) (VEGF-correlated chemokine 1) is a novel CXC chemokine with 119 amino acids, which is related to interleukin (IL)-8 and VEGF. The receptor of \(VCC-1\) is still unknown. Expression of \(VCC-1\) mRNA was constitutive in normal lung, skeletal muscle, adult trachea, stomach, and fetal lung, but not in the brain, heart, colon, thymus, spleen, kidney, liver, small intestine, placenta, or peripheral blood lymphocytes [9]. \(VCC-1\) was found to be significantly upregulated in various breast carcinoma and colon tumors [9]. Recent studies demonstrated that overexpression of \(VCC-1\) was able to
promote NIH3T3 cells rapidly developing tumors in nude mice and played a role in tumor progression [9,10]. However, the exact role of VCC-1 in carcinogenesis has not been elucidated. In the present study, we demonstrated that overexpression of VCC-1 in SMMC7721 cells, an HCC cell line, significantly promoted cellular proliferation, tumor growth and invasive ability of these cells. These results strongly suggest that VCC-1 plays an important role in SMMC7721 invasion and tumor growth and that VCC-1 may be an important potential target for anti-HCC therapies.

Materials and Methods

Cell line and animals
The human HCC cell line SMMC7721 was from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institutes for Biological Sciences, and was maintained routinely in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and cultured at 37°C in a humidified incubator containing 5% CO2. Female/male athymic nude mice (6 weeks of age) were purchased from the Central Laboratory of Animal Science at Southern University (Guangzhou, China) and maintained in laminar-flow cabinets under specific pathogen-free conditions.

Stable transfection
To generate the eukaryotic VCC-1 expression vector, a fragment containing the complete VCC-1 open reading frame was released from the recombinant plasmid pMD19T/\text{VCC-1} by restriction digest with XhoI and BamHI, followed by subcloning into the XhoI and BamHI site of pcDNA3.1(−). The recombinant plasmid pcDNA3.1/\text{VCC-1} was confirmed by restriction enzyme analysis and DNA sequencing. Experimental and control SMMC7721 cells ($4 \times 10^5$) were transfected with pcDNA3.1/\text{VCC-1} and pcDNA3.1, respectively, using Lipofectamine™ 2000 (Invitrogen, Carlsbad, USA) according to ratio of 1:2 for various DNA (micrograms) to Lipofectamine™ 2000 (microliters). After 24 h, transfected cells were cultured in selection medium (RPMI 1640 with 10% FBS and 800 g/l of G418) and the medium was changed every 3 days. Selection proceeded for 2 weeks. The G418-resistant clones were harvested and analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot to detect the level of VCC-1 expression.

RT-PCR
Total RNA was isolated from cultured cells using Trizol (Invitrogen) and cDNA was synthesized using RT-PCR kit (TaKaRa, Tokyo, Japan) according to the manufacturer’s instruction. The sequences of the primers were as follows: 5′-CTGAGATGAAATCTCTTCCC-3′ (forward) and 5′-GGATCCCCAGGCAGCAC-AG-3′ (reverse) for \text{VCC-1}; 5′-TGTGACGTGGACATCGCA-AGAGGAGG-3′ (forward), and 5′-GGAAAGCTGCGAC-AGCAGCC-3′ (reverse) for β-actin. PCR analysis was performed under the following conditions: initiated by 4 min incubation at 94°C, and then 30 cycles of denaturation for 20 s at 94°C, annealing for 30 s at 60°C, and extension for 40 s at 72°C, ended by 5 min of extension at 72°C. The amplified products were analyzed by 1.5% agarose gel electrophoresis. The intensity ratio was the relative expression of \text{VCC-1} normalized to β-actin.

Western blot analysis
Cells ($1 \times 10^5$) were washed twice with PBS and proteins were extracted with lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, and 100 μg/ml PMSF) for 45 min on ice. Equal amounts of protein were separated by 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to PVDF membrane using a standard protocol. Membranes were then blocked with TBST (TBS with 0.05% Tween 20) containing 5% fat-free milk for 1 h and incubated with human monoclonal antibody against \text{VCC-1} (R&D Systems, Minneapolis, USA) at a 1:500 dilution for 12 h at 4°C. Membranes were then washed with TBST and incubated with horseradish peroxidase conjugated goat anti-rabbit immunoglobulin G for 1 h. The blots were developed by using the ECL system (Pierce, Chicago, USA). The intensity ratio was the relative expression of \text{VCC-1} normalized to GAPDH.

Cell proliferation assay
Cells were cultured in 96-well plates at a concentration of 1000 cells/well for 1, 2, 3, 4, 5, and 6 days, respectively. At each time point, 20 μl of MTT was added into each well followed by 4 h incubation. Then, the medium was removed and 150 μl of dimethyl sulfoxide was added. The optical density (OD) of each well was measured using a microplate reader set at 570 nm.

Plate clone formation assay
The ability of cells to form macroscopic colonies in soft agar were determined as described previously [11,12].
About 50 cells were added to each well of 24-well plates. After incubation at 37°C for 14 days, colonies containing at least 50 cells were counted under a microscope. Plate clone formation efficiency was calculated using the following formula: plate clone formation efficiency = (number of colonies/number of cells inoculated) × 100%.

**In vitro invasion assay**

Cells were harvested by trypsinization and resuspended in RPMI 1640 containing 0.5% FBS. Cell suspensions containing 15 × 10⁴ cells were seeded into the upper well of a BD Falcon™ 24-Multiwell insert system (BD, Franklin Lakes, USA) and separated from the lower compartment by a porous membrane previously overlaid with Matrigel (to block noninvasive cells from migrating). Each cell group contained three wells. RPMI 1640 (500 μl) containing 10% FBS was added to the lower chamber. After incubation for 16 h, the cells on the upper surface of the filter were removed using a wet cotton swab. The cells that penetrated to the lower surface of the filter were stained with crystal violet, photographed, and quantified by dissolving stained cells in acetic acid and measuring the OD value at the wavelength of 570 nm.

**Cell adhesion assay**

The 96-well plates were coated with 10 μg/well Matrigel. Cells (2 × 10⁶) were blocked in 1% bovine serum albumin (BSA) for 1 h and suspended in 100 μl RPMI 1640 and seeded into coated 96-well plates, incubated in 5% CO₂ at 37°C for 1 h and gently washed for three times with warmed PBS to remove the unattached cells. Attached cells were fixed with 70% ethanol for 15 min at room temperature. The adhesiveness of homogenous cells was counted under a fluorescence microscope from five fields of view.

**In vivo tumor growth assay**

To evaluate in vivo tumor growth, cells from each cell group in the exponential growth phase were harvested and washed with PBS twice before injection. Cell vitality was over 95% as determined by trypan blue dye exclusion assay.

Then, 4 × 10⁶ cells were injected subcutaneously into the flanks of nude mice. Each group contained six mice. After 19 days, the diameter of subcutaneous tumors was measured three times a week using calipers. The mice were sacrificed on the twentieth day and the tumors were excised, weighed, and fixed in 4% paraformaldehyde for pathologic examination. Tumor volumes were calculated according to the following formula: Tumor volume = (width² × length)/2.

**Test of homogenous adhesion**

Cells (2 × 10⁶) were seeded into 6-well plates and grown to a fully confluent monolayer. The same cell type as each well was marked with the fluorescent carboxy cyanine dye DiI (Biyuntian, Nantong, China). Cells in suspension were washed with PBS and incubated with DiI at a concentration of 2.5 mg/ml in PBS for 15 min at 37°C. After three washings with PBS, the cells were resuspended in RPMI 1640 medium and incubated on top of confluent cell monolayer for 1 h. The cells were then gently washed three times with warm PBS to remove unattached cells. Attached cells were fixed with 70% ethanol for 15 min at room temperature. The adhesiveness of homogenous cells was counted under a fluorescence microscope from five fields of view.

**Statistical analysis**

SPSS 10.0 software was used for statistical analysis. All data were expressed as mean ± SD and were analyzed statistically using the one-way analysis of variance. Differences were considered significant at P < 0.05.

**Results**

**VCC-1 expression was upregulated in overexpressed transfectants of SMMC7721 cells**

As expected, the cells transfected with pcDNA3.1/VCC-1 resulted in higher expression of VCC-1 mRNA and protein than those with empty vector or the parental cells (Fig. 1). The three cell groups were named as SMMC7721/VCC-1, SMMC7721/Mock, and SMMC7721, respectively.

**Stable overexpression of VCC-1 gene promoted the proliferation of SMMC7721 cells**

The MTT proliferation assay was used to determine the effect of upregulation of VCC-1 on the growth of the human HCC cell line SMMC7721. As shown in Fig. 2(A), a striking variation in the growth pattern was observed among stable transfectants from the third day to the sixth day. The SMMC7721/VCC-1 cells showed higher growth rate compared with the controls (P < 0.05). The ability of the stably transfected SMMC7721 cells to form colonies in soft agar was also observed [Fig. 2(B)]. The plate clone formation efficiency of
SMMC7721, SMMC7721/Mock, and SMMC7721/VCC-1 were 29 ± 7.4%, 33 ± 7.0%, and 57 ± 6.2%, respectively. The SMMC7721/VCC-1 cells formed significantly more colonies than either the SMMC7721/Mock cells or SMMC7721 cells. These results were consistent with the proliferation assay.

VCC-1 gene overexpression increased cell invasion in vitro

Since VCC-1 is a chemokine and promotes tumor growth and angiogenesis, NIH3T3 cells overexpressing VCC-1 develop rapidly progressing tumors [8,9]. However, the exact mechanism by which VCC-1 upregulation in these cells contributes to the tumor phenotype remains to be elucidated. Therefore, we used the transwell assay to examine migration and invasive ability of our stable HCC cell lines. As shown in Fig. 3(A,B), SMMC7721/VCC-1 cells were more invasive compared with SMMC7721/Mock and SMMC7721 cells. OD values (indicating the number of cells on the underside of the filter) for SMMC7721/VCC-1, SMMC7721/Mock and SMMC7721 were 0.796 ± 0.033, 0.566 ± 0.049 and 0.487 ± 0.057, respectively (P < 0.01). The SMMC7721/VCC-1 cells also showed significant increase in their adhesion ability on Matrigel. OD values of SMMC7721/VCC-1, SMMC7721/Mock and SMMC7721 cells were 0.800 ± 0.109, 0.605 ± 0.102 and 0.523 ± 0.136, respectively [Fig. 3(C)]. In addition, there was an obvious decrease in the homogeneous
adhesion (adhesion to the same cell type) of SMMC7721/VCC-1. Adherent cell number of SMMC7721/VCC-1, SMMC7721/Mock, and SMMC7721 cells were 78.4 ± 14.6, 114 ± 19.5, and 134.8 ± 12.4, respectively [Fig. 3(D,E), P < 0.05].

Increase of tumorigenicity by stable overexpression of VCC1 in vivo
We examined whether overexpression of VCC-1 transfection in SMMC7721 cells would affect tumor growth in nude mice. Tumor growth was determined by measuring the size of the tumor 10 and 20 days after injection. The average size of the SMMC7721/VCC-1 tumor-bearing mice was bigger than those of both the SMMC7721/Mock group and the SMMC7721 group (Fig. 4). The weight of the tumors was 6.0 ± 0.59, 4.1 ± 0.65, and 4.5 ± 0.72 g for the SMMC7721/VCC-1, SMMC7721/Mock, and SMMC7721 group, respectively (P < 0.05).

Discussion
More and more evidence indicates that chemokines play multiple roles in the development and progression of human cancer [13,14]. Chronic or recurrent inflammation is associated with the development of many human tumors, including cancer of the liver, stomach, intestine, bladder and prostate, and so on. It is now becoming clear that chemokines are not only just leukocyte chemotactants, but also have pleiotropic effects on immune modulation, cellular proliferation leading to tumor growth, and cancer cell invasion and homing, which are important for metastasis [15,16]. The different types of chemokines expressed in human HCC cells, and the molecular mechanisms involved, remain to be elucidated.

VCC-1 is also known as VEGF co-regulated chemokine 1, and dendritic cell and monocyte-attracting chemokine-like protein. Human VCC-1 cDNA encodes a 119 amino acid residue precursor protein with a 22
amino acid residue signal peptide, which is cleaved to form a 97 amino acid residue secreted protein with a molecular weight of 11.4 kDa. VCC-1 was co-regulated with three other well-known genes, including IL-8, GRO-1, and VEGF. These genes were also involved in angiogenesis. The expression of both CXCL1/GRO and CXCL8/IL-8 correlated with the expression pattern of VEGF in primary tumors of the lung, breast, and esophagus [15]. Weinstein et al. [9] reported that VCC-1 was overexpressed by 3 fold to 24 fold in breast cancer. Increasing expression of VCC-1 has also been seen in endothelial cells that were induced to form tubes in vitro. Transgenic overexpression of VCC-1 in NIH3T3 cells caused upregulation of proteins such as VEGF, bFGF, and ang-2, and promoted cell growth and tumorigenicity. These studies indicated that VCC-1 played a causative role in promoting tumor growth, and might be a potential target for an anti-angiogenic agent. VCC-1 has also been shown to induce dendritic cell chemotaxis. Based on these data, one could hypothesize that loss of VCC-1 in tumors might also contribute to the regulation of immunologic tumor interactions. Previous studies have shown that VCC-1 played a role in the innate defense against infections, and has been found upregulated in the duodenal mucosa during acute cholera [17].

Recently, we used RT-PCR to show that VCC-1 mRNA was observed in most cancer cell lines including those of the colon, breast, liver, stomach, ovary, lung, and the cervix (data not shown). We hypothesized that upregulation of VCC-1 expression might provide an effective means for promoting tumor formation and metastasis. In the present study, we found that overexpression of VCC-1 could promote not only SMMC7721 HCC cell proliferation and anchorage-independent growth, but also the abilities of the invasion and adhesion, which were considered as important steps in the invasive processes of metastatic tumor cells. Overexpression of VCC-1 resulted in an increase in SMMC7721 cells growth in nude mice. These data suggest that VCC-1 is a tumor-promoting factor in hepatocellular carcinoma tumorigenesis. Taken together, these observations demonstrate that VCC-1 may play a role in cell growth and tumorigenicity. The epigenetic overexpression of VCC-1 probably induces tumor-promoting functions. However, the true molecular mechanisms of this action of VCC-1 remain unclear and require further research.

In conclusion, VCC-1 is a novel chemokine that promotes tumor growth. Our data first demonstrated that upregulation of VCC-1 expression in SMMC7721 cells significantly promoted the invasive ability, cellular proliferation rate, and tumor growth of these cells. These data strongly suggest that VCC-1 plays an important role in SMMC7721 invasion and tumor growth and may be a potential biomarker for anti-HCC therapies.

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