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Original Article

Chemokine CCL17 induced by hypoxia promotes the proliferation of cervical cancer cell

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Abstract: Cervical cancer is often associated with hypoxia and many kinds of chemokines. But the relationship and role of hypoxia and Chemokine (C-C motif) ligand 17 (CCL17) in cervical cancer are still unknown. Here, we found that CCL17 was high expressed in cervical cancer. HeLa and SiHa cells could secrete CCL17 in a time-dependent manner. Hypoxia increased expression of CCL17 receptor (CCR4) on HeLa and SiHa cells. Treatment with recombination human CCL17 (rhCCL17) led to an elevation of cell proliferation in HeLa and SiHa cells in a dose-dependent manner. In contrast, blocking CCL17 with anti-human CCL17 neutralizing antibody (α-CCL17) played an oppose effect. However, rhCCL17 had no effect on apoptosis in cervical cancer cells. Further analysis showed that hypoxia promoted the proliferation of HeLa and SiHa cells, and these effects could be reversed by α-CCL17. Stimulation with the inhibitor for c-Jun N-terminal kinase (JNK) or signal transducers and activator of transcription 5 (STAT5) signal pathway not only directly decreased the proliferation of HeLa and SiHa cells, but also abrogated the stimulatory effect of rhCCL17 on the proliferation of HeLa and SiHa cells. These results suggest that a high level of CCL17 in cervical cancer lesions is an important regulator in the proliferation of cervical cancer cells through JNK and STAT5 signaling pathways. In this process, hypoxia magnifies this effect by up-regulating CCR4 expression and strengthening the interaction of CCL17/CCR4.

Keywords: CCL17, hypoxia, cervical cancer cells, proliferation

Introduction

As a gynecological malignancy, cervical cancer is both the fourth most common cause of cancer and the fourth most common cause of death from cancer in women worldwide [1]. However, the pathogenesis of cervical cancer is still almost unclear. Therefore, appropriate cervical cancer treatments, especially individual control strategies are still difficult to achieve.

In most solid tumors, with the enlargement of tumors, the inside cells of tumor tissue undergo necrosis due to hypoxia and reduced blood flow. Cells with a high proliferative level consume more oxygen, which results in a relatively hypoxic status. At the same time, cell proliferation causes the epithelial layer to thicken and nutrients to diffuse over a longer distance. As a result, intraepithelial lesions can become profoundly hypoxic, which further initiates a sequence of critical cellular adaptations [2] and microenvironmental changes, such as the release of a large amount of chemokines [3]. Our previous work has proved that hypoxia stimulates the proliferation of cervical cancer cells through promoting chemokine CXCL8 (also known as IL-8) secretion [4].

Chemokines belong to a superfamily of small molecules that were initially discovered because their interaction with chemokine

receptors was found to regulate trafficking of leukocytes to sites of inflammation and recirculation in secondary lymphatics [5, 6]. More and more evidences indicate that chemokines regulate multiple aspects of tumor cell biology, including survival, proliferation, migration, and angiogenesis [7, 8].

Thymus and activation-regulated chemokine (TARC)/CC chemokine ligand (CCL) 17 is a ligand of CC chemokine receptor (CCR) 4. CCR4 is expressed in type 2 helper T (Th2) lymphocytes, basophils and natural killer cells [9]. CCL17 plays important roles in Th2-type immune responses by selectively recruiting $CCR4^+$ Th2-polarized memory/effector T cells into inflamed tissues. CCR4 is also expressed on several cancers, such as breast cancer, colon cancer, T cell leukemia/lymphoma, Gastric Cancer. It plays important regulation roles in progression of these cancers [10-14]. However, the expression and possible role of CCL17/CCR4 in cervical cancer cells, and the relationship with hypoxia have remained elusive.

Therefore, this study was performed to investigate the expression and role of CCL17 in cervical cancer cells, and whether hypoxia regulates proliferation of cervical cancer cells by modulating CCL17/CCR4 interaction *in vitro*.

Materials and methods

Tissue collection

All tissue samples were collected with informed consent in accordance with the requirements of the Research Ethics Committee in the Obstetrics and Gynecology Hospital, Fudan University. Samples from 10 patients in International Federaton of Gynecology and Obstetrics (FIGO) stages of cervical cancer were obtained from women age 37-57 years. Among them, 4 patients had FIGO Stage Ib2 disease, 2 had Stage IIa disease, 2 had Stage IIb disease, 1 had Stage IIIb disease, and 1 had Stage IVa disease. All the samples were confirmed histologically according to established criteria, and squamous cell carcinoma was found in all patients.

Immunohistochemistry

Immunohistological staining was performed as previously described [15]. Paraffin sections (5 um) of the certivical tissues from cervical cancer (n=10) were dehydrated, and incubated with hydrogen peroxide in 1% bovine serum albumin (BSA)/TBS to block endogenous peroxidase. The samples were then incubated with mouse anti-human CCL17 antibody (25 ug/ml) (R&D Systems, USA), or mouse IgG isotype antibody overnight at 4°C in a humid chamber. After washing three times with TBS, the sections were overlaid with peroxidase-conjugated anti-mouse IgG antibody (Golden Bridge International, Inc., Beijing, China), and the reaction was developed with 3,3-diaminobenzidine (DAB), and counterstained with hematoxylin. The experiments were repeated five times.

Cells and treatments

Cervical epidermoid carcinoma HeLa and SiHa cells were grown in RPMI-1640 medium (Gibco, USA) supplemented with 5% fetal bovine serum (FBS; Hyclone, Logan, UT, USA). The cells were incubated either under normoxic (21% O_2, 5% CO_2, 74% N_2 at 37°C) in a humidified incubator (Heal Force, HF 100, Shanghai, China) or hypoxic conditions (1% O_2, 5% CO_2, 94% N_2 at 37°C) in a humidified incubator (Heal Force, HF 100, China).

Enzyme-linked immunosorbent assay (ELISA) for CCL17 determination

HeLa ($3*10^5$ cells/well) and SiHa cells ($3*10^5$ cells/well) were cultured for 24 h, 48 h, or 72 h. Then culture supernatant was harvested, centrifuged to remove cellular debris, and then stored at -80°C until being assayed by ELISA. And the secretion level of CCL17 in the culture supernatant was analyzed by human CCL17 ELISA kit (R&D Systems, USA) according to standard procedures.

Flow cytometry (FCM)

HeLa ($2*10^5$ cells/well) and SiHa cells ($2*10^5$ cells/well) were cultured under normoxic or hypoxic conditions for 12 h, and then digested with 0.25% trypsin only for 30-50 s, and blown off gently and washed with phosphate-buffered saline (PBS). After blocking with 10% FBS, the recovered cells were mixed with PE-conjugated CCR4 antibody (Biolegend, San Diego, CA, USA) in darkness for 30 min at room temperature. As a negative control, an isotope control was used. After incubation, the cells were washed and

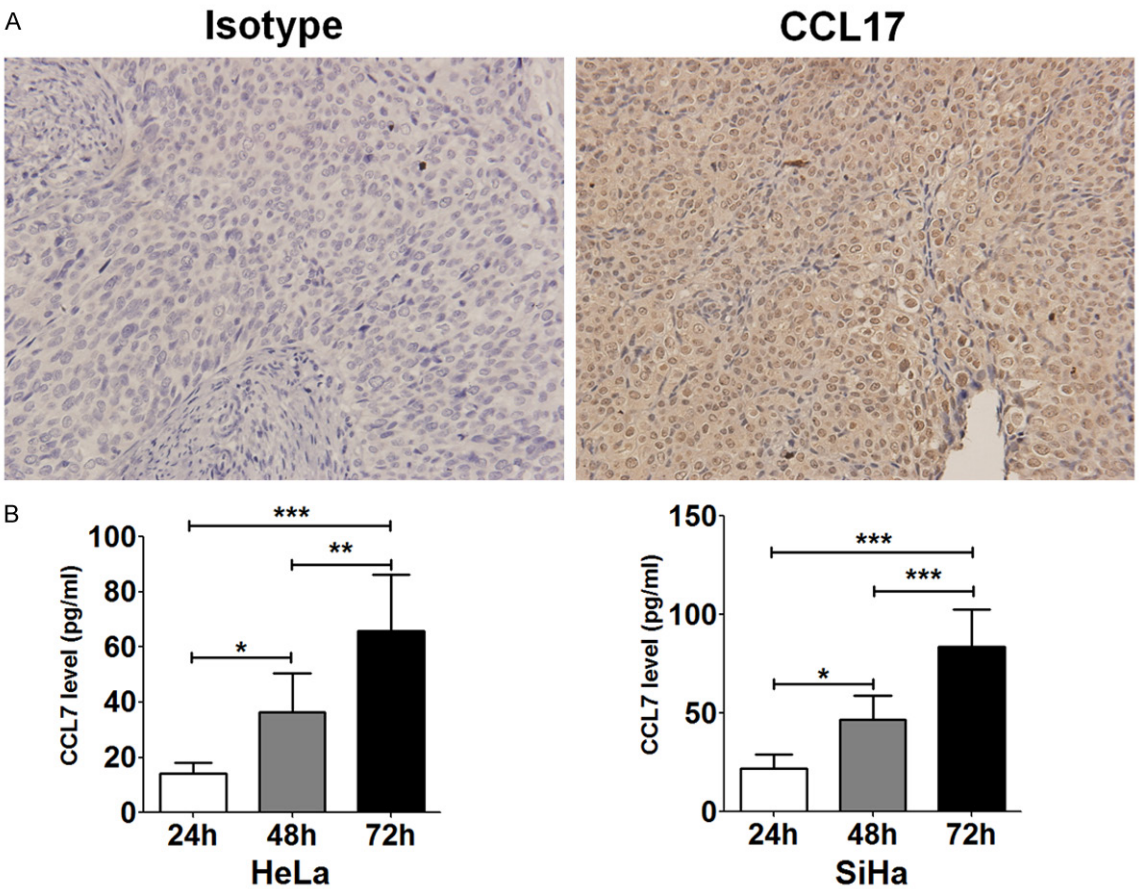


Figure 1. Cervical cancer cells express a high level of CCL17. A. Immunohistochemistry analysis for CCL17 expression in cervical cancer (n=10). Original magnification: ×400. B. The secretion level of CCL17 from HeLa (3×10^5 cells/well) and SiHa cells (3×10^5 cells/well) after culture for 24 h or 48 h. The data are expressed as the mean ± SD. **P*<0.05, ***P*<0.01 and ****P*<0.01 (one-way ANOVA).

analyzed immediately by a flow cytometer (FACS Calibur, BD, USA) and Cellquest software (Becton Dickinson). The statistical analysis was conducted using isotype-matched controls as references. The experiments were repeated six times.

BrdU cell proliferation assay

HeLa and SiHa cells were re-suspended in RPMI-1640 medium supplemented with 10% FBS, and seeded at a density of $5*10^3$ cells/well in 96-well flat-bottom microplates. Thereafter, the cells were starved with RPMI-1640 containing 1% FBS for 12 h before treatment, and then stimulated with recombinant human CCL17 protein (rhCCL17, 0.1, 1, 10, or 100 ng/ml) (R&D Systems, USA), anti-CCL17 neutralizing antibody (α-CCL17, 0.03, 0.3 or 3 ug/ml) (R&D Systems, USA) for 24 or 48 h. In addition, vehicle was added to some wells as a negative control. Then the ability of HeLa and SiHa to proliferate was detected with BrdU cell proliferation assay (Millipore, Darmstadt, Germany) according to the manufacturer's instruction. Each experiment was performed in six parallel wells, and repeated five times.

HeLa ($1*10^4$ cells/well) and SiHa cells ($1*10^4$ cells/well) in 96-well flat-bottom microplates were cultured under normoxic conditions, hypoxic conditions or hypoxic conditions plus α-CCL17 (0.3 ug/ml) for 8 h, and then cultured under normoxic conditions for another 48 h. Subsequently, cells were collected and analyzed as described above.

In addition, HeLa and SiHa cells were stimulated with WP1066 (STAT3 inhibitor, 10 uM, santa cruz biotechnology, inc., USA), N'-((4-Oxo-4H-

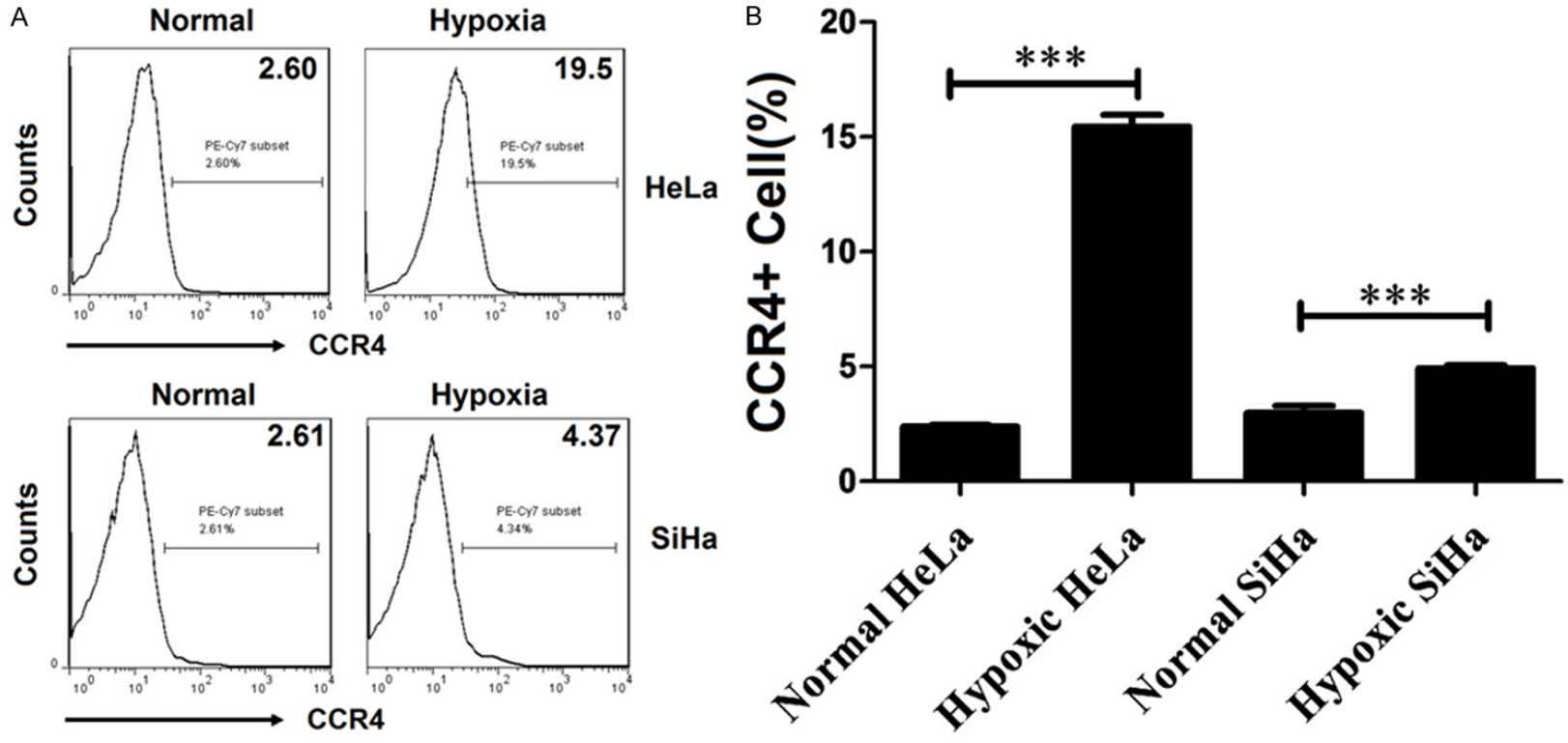


Figure 2. Hypoxia up-regulates CCR4 expression on HeLa and SiHa cells. A, B. After culture under normoxic or the hypoxic conditions for 12 h, the expression of CCR4 on HeLa and SiHa cells was detected by FCM. Normal: cell culture under normoxic conditions; Hypoxia: cell culture under hypoxic conditions. The data are expressed as the mean ± SD. ****P*<0.01 (Student's *t*-test).

chromen-3-yl) methylene) nicotinohydrazide (STAT5 inhibitor, 10 uM, santa cruz biotechnology), LY294002 (AKT signal pathway, 10 uM, santa cruz biotechnology), SP600125 (inhibitor for JNK signal, 10 uM, Santa Cruz Biotechnology), SB203580 (inhibitor for p38/MAPK signal, 10 uM, Santa Cruz Biotechnology), U0126 (inhibitor for ERK1/2 signal, 10 uM, Santa Cruz Biotechnology), BAY (NF-κB inhibitor, 10 uM, Santa Cruz Biotechnology), or pyrrolidine dithiocarbamate (PDTC, an antioxidant and an inhibitor of NF-κB, 10 uM, Santa Cruz Biotechnology) for 6 h, and then treated with or without rhCCL17 (10 ng/ml) for 48 h, with vehicle as the control. Subsequently, cells were collected and analyzed as described above.

Apoptosis assay

After treatment with different concentration of rhCCL17 (10 or 20 ng/ml) for 48 h, HeLa and SiHa cells were collected and analyzed apoptosis level by annexin V-FITC apoptosis assay (Invitrogen, USA), respectively, as described previously [15].

Statistics

All values are shown as the mean±SD. The data were analyzed with GraphPad Prism version 5 by *t*-test or one-way ANOVA. Differences were considered statistically significant at $P<0.05$.

Results

Cervical cancer cells express a high level of CCL17

To investigate whether cervical cancer expresses CCL17, we fist analyzed the expression of CCL17 in cervical cancer tissues by immunohistochemistry. As observed by immunohistochemistry in **Figure 1A**, the cervical cancer cells were positive stain of CCL17. Further analysis showed that HeLa and SiHa cells could secrete CCL17 in a time-dependant manner. These data suggested that a high level of CCL17 from cervical cancer cells might play a regulator role in biological behavior of cervical cancer cells.

Hypoxia up-regulates CCR4 expression on HeLa and SiHa cells

To analyze whether cervical cancer cells express CCL17 receptor CCR4, FCM assay was performed to detect CCR4 on HeLa and SiHa cells. As shown in **Figure 2**, the percentage of CCR4 positive HeLa and SiHa cells was very low (above 2.6%). However, hypoxia stimulation led to a significant elevation of CCR4 level on the cell surface, especially HeLa cells ($P<0.01$) (**Figure 2A** and **2B**). Our previous data also showed that hypoxia promoted the secretion of CCL17 from HeLa and SiHa cells [16]. The results above indicated that CCL17 secreted from HeLa and SiHa cells might combine receptor CCR4 on HeLa and SiHa cells. Hypoxia might further increase the reactivity of HeLa and SiHa cells against CCL17 by up-regulating CCR4 expression.

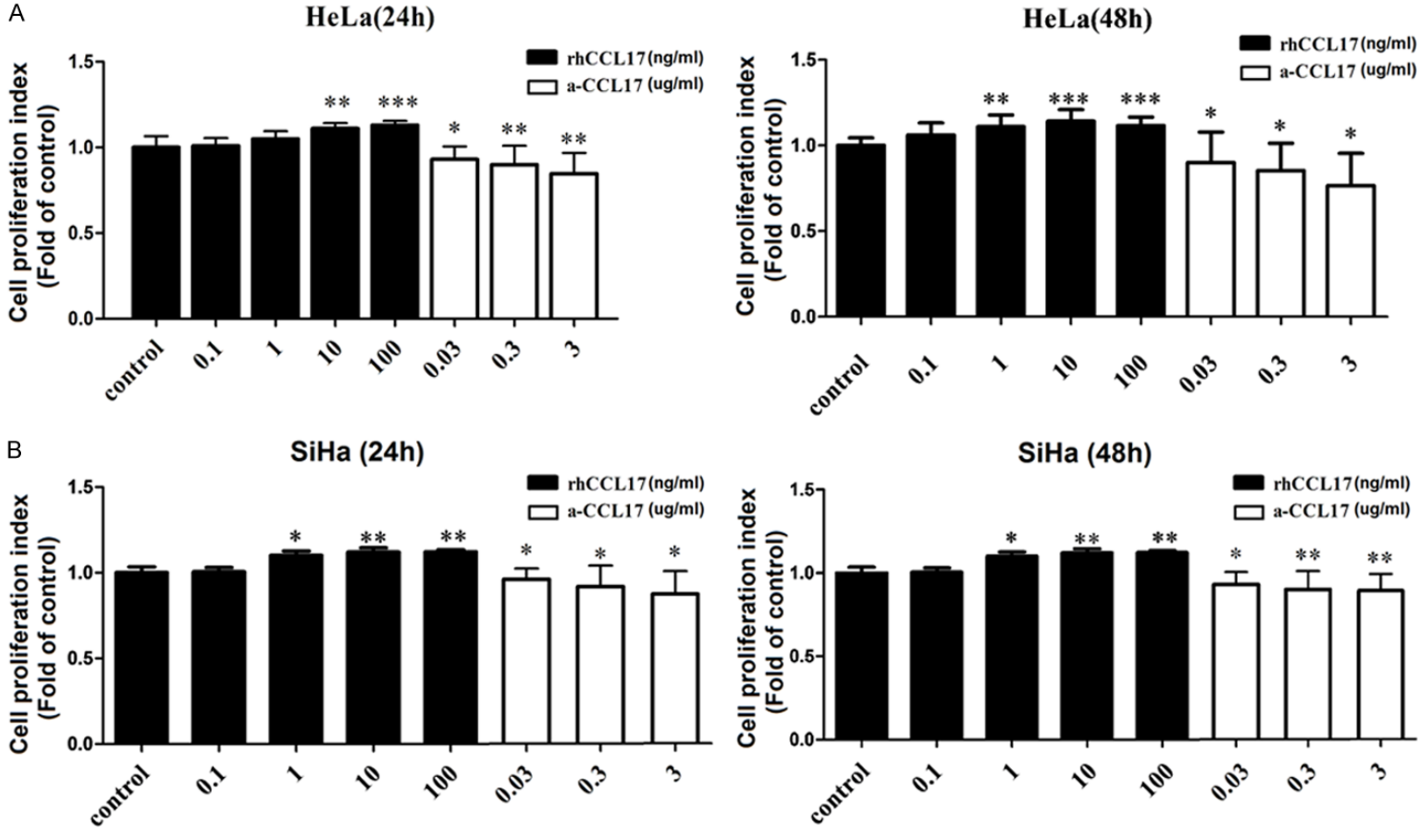
CCL17 promotes proliferation of HeLa and SiHa cells

To investigate the potential effect of CCL17 on cervical cancer cells *in vitro*, HeLa and SiHa cells were incubated with rhCCL17 (0.1, 1, 10 or 100 ng/ml) or α-CCL17 (0.03, 0.3 or 3 ug/ml) for 24 h or 48 h. As shown, treatment with rhCCL17 for either 24 h or 48 h led to a significant increase of cell proliferation in HeLa and SiHa cells ($P<0.05$, $P<0.01$, $P<0.001$) (**Figure 3A** and **3B**). These effects were a dose-dependent manner. However, blocking CCL17 with α-CCL17 decreased proliferation in HeLa and SiHa cells ($P<0.05$, $P<0.01$) (**Figure 3A** and **3B**). The optimum concentration of rhCCL17 and α-CCL17 was 10 ng/ml and 0.3 ug/ml, respectively. Collectively, these results indicated that both exogenous and endogenous CCL17 promoted the proliferation of cervical cancer cells *in vitro*.

Subsequently, we treated HeLa and SiHa cells with different concentration of rhCCL17 (10 or 20 ng/ml) for 48, and found that rhCCL17 did not change the level of apoptosis in Hela and SiHa cells *in vitro* ($P>0.05$) (**Figure 3C** and **3D**).

Hypoxia stimulates proliferation of HeLa and SiHa cells by CCL17/CCR4 interaction

Taking into account the important role of hypoxia in the regulation of cervical cancer cells growth, we first cultured HeLa and SiHa cells under normal or hypoxic conditions for 12 h. Next, the proliferation ability of these cells was evaluated by BrdU proliferation assays. As shown in **Figure 4A**, hypoxia obviously promoted proliferation of HeLa and SiHa cells *in vitro*


A
HeLa(24h)
HeLa(48h)
B
SiHa (24h)
SiHa (48h)
Cell proliferation index
(Fold of control)
rhCCL17(ng/ml)
a-CCL17 (ug/ml)
control
0.1
1
10
100
0.03
0.3
3

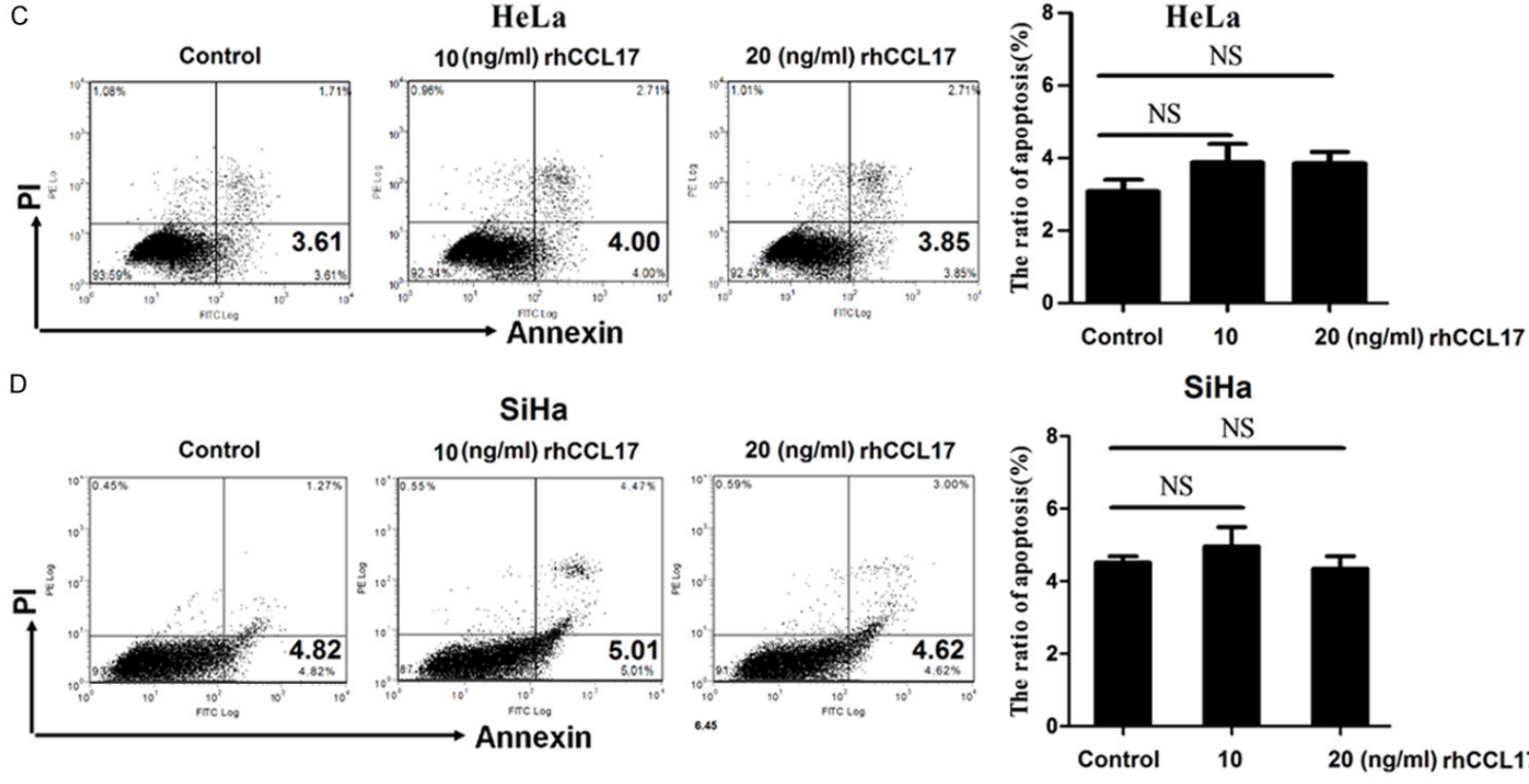


Figure 3. CCL17 promotes proliferation of HeLa and SiHa cells. HeLa (A) and SiHa (B) cells were incubated with rhCCL17 (0.1, 1, 10 or 100 ng/ml) or α-CCL17 (0.03, 0.3 or 3 ug/ml) for 24 h (left) or 48 h (right), then cell proliferation in HeLa and SiHa cells was detected by BrdU proliferation assay. In addition, HeLa (C) and SiHa (D) cells with different concentration of rhCCL17 (10 or 20 ng/ml) for 48, and the level of apoptosis in Hela and SiHa cells was analyzed by apoptosis assay. rhCCL17: recombinant human CCL17 protein; α-CCL17: anti-human CCL17 neutralizing antibody. The data are expressed as the mean ± SD. **P*<0.05, ***P*<0.01 and ****P*<0.01 (one-way ANOVA). NS: no statistically difference.

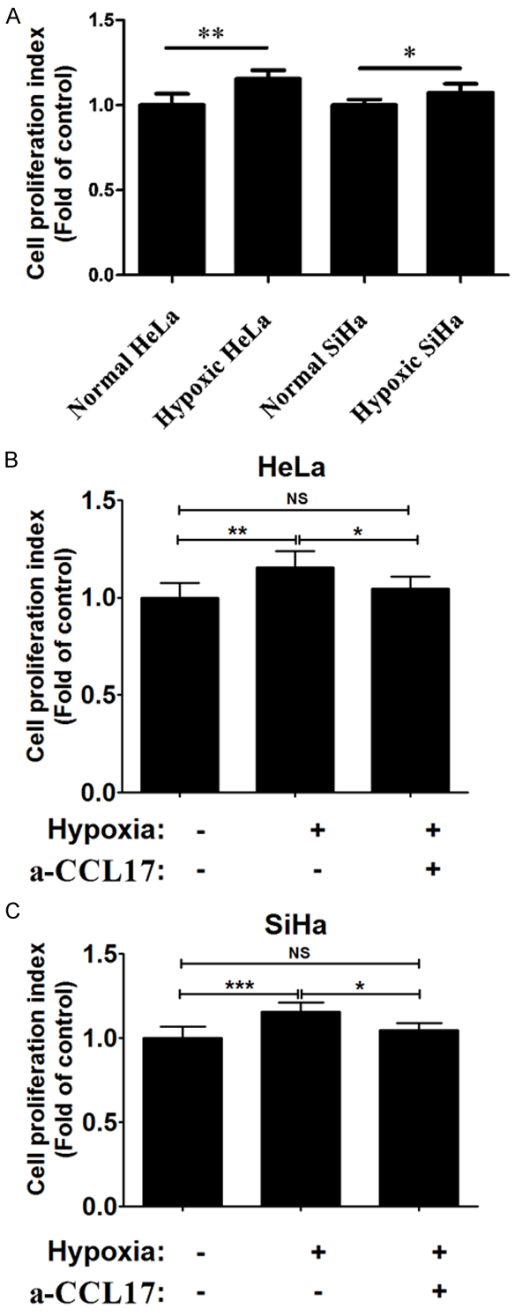


Figure 4. Hypoxia stimulates proliferation of HeLa and SiHa cells by CCL17/CCR4 interaction. (A) We cultured HeLa and SiHa cells under normal or hypoxic conditions for 12 h. Next, the proliferation ability of these cells was evaluated by BrdU proliferation assay. In addition, HeLa (B) and SiHa (C) cells were cultured under normoxic conditions, hypoxic conditions or hypoxic conditions plus α-CCL17 (0.3 ug/ml) for 8 h, and then cultured under normoxic conditions for another 48 h. And BrdU proliferation assay was performed to evaluate the proliferation of these cells. The data are expressed as the mean ± SD. **P*<0.05, ***P*<0.01 and ****P*<0.01 (Student's *t*-test or one-way ANOVA). NS: no statistically difference.

(*P*<0.05, *P*<0.01) (**Figure 4A**). In order to know the role of CCL17 signaling in this effect induced by hypoxia, HeLa and SiHa cells were cultured under normoxic conditions, hypoxic conditions or hypoxic conditions plus α-CCL17 (0.3 ug/ml) for 8 h, and then cultured under normoxic conditions for another 48 h. Of note, we found that the stimulatory effect of hypoxia on proliferation in HeLa (*P*<0.05) (**Figure 4B**) and SiHa cells (*P*<0.05) (**Figure 4B**) could be reversed by treatment with α-CCL17. Taken together, these data suggested that hypoxia promoted CCL17 secretion and CCR4 expression, and further stimulated proliferation of cervical cancer cells *in vitro*.

The stimulatory effect of CCL17 on proliferation of HeLa and SiHa cells was dependent on JNK and STAT5 signaling pathways

In order to explore the down stream signal of CCL17 in regulating cervical cancer cells proliferation, we analyzed proliferation of HeLa and SiHa cells after stimulation with rhCCL17, or rhCCL17 plus different inhibitors for several signal pathways. As shown, compared to the group of rhCCL17 stimulation alone, treatment with rhCCL17 and blocking JNK or STAT5 signaling pathway resulted in a markedly decrease of proliferation in HeLa (*P*<0.01, *P*<0.001) (**Figure 5A**) and SiHa cells (*P*<0.001) (**Figure 5B**). However, blocking AKT, STAT3, p38/MAPK, ERK1/2 or NF-κB signaling pathway with different signal inhibitors had no similar effect (*P*>0.05) (**Figure 5A** and **5B**).

We next found that rhCCL17 significantly promoted proliferation in HeLa and SiHa cells, however, these effects could be partly abrogated after blocking JNK or STAT5 signaling pathway (**Figure 5C** and **5D**). Therefore, these findings provided evidence that rhCCL17 could stimulate cervical cancer cells proliferation partly by JNK and STAT5 signaling pathways.

Discussion

Chemokines have been shown to play pleiotropic roles in promoting tumor invasion, migration, and vascularization [17, 18]. As a member of the CC-motif chemokine family, CCL17 is constitutively expressed in the thymus and by dendritic cells (DC), endothelial cells, bronchial epithelial cells and several tumor cells [19]. CCL17 has been reported to be highly

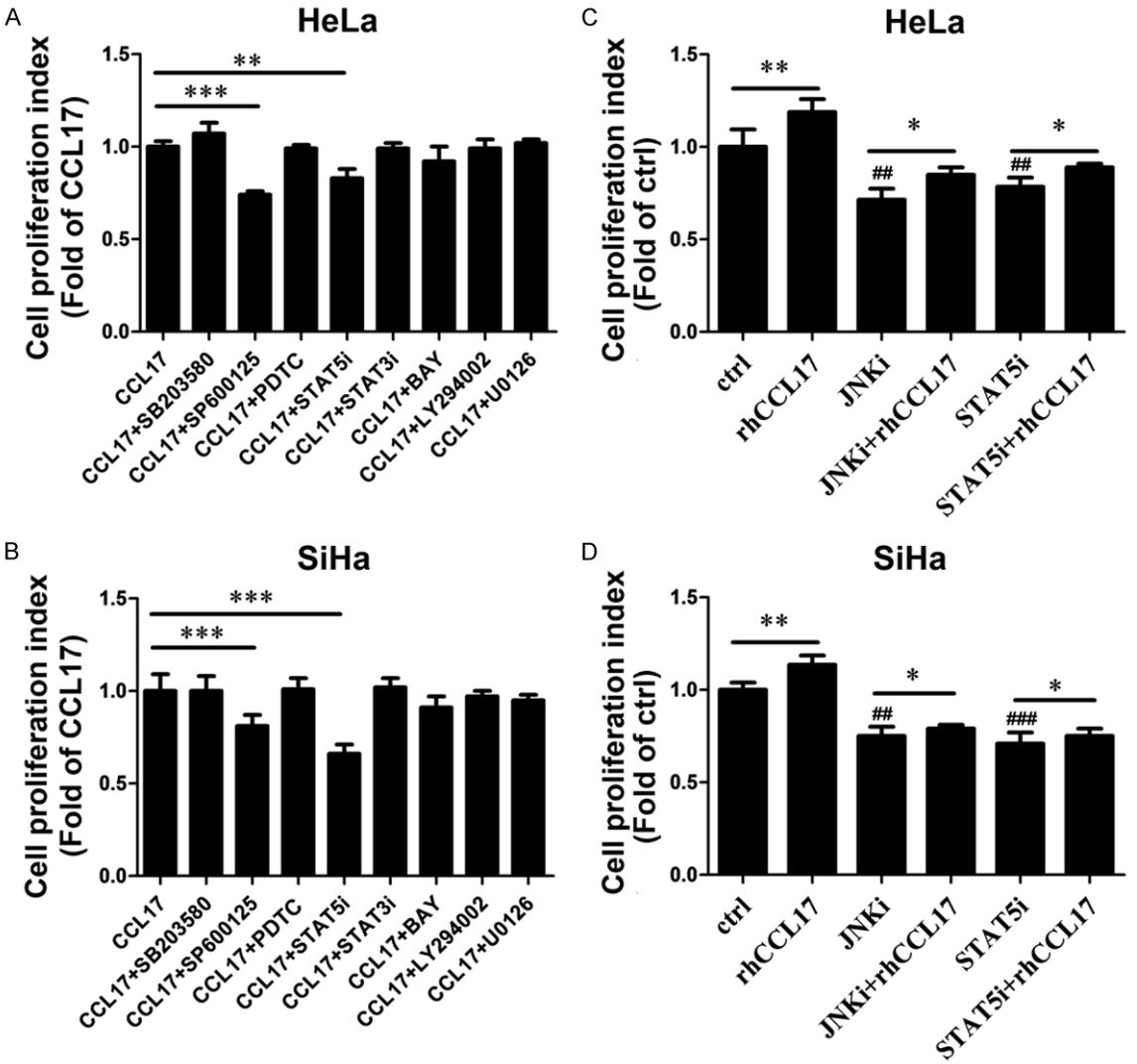


Figure 5. The stimulatory effect of CCL17 on proliferation of HeLa and SiHa cells was dependent on JNK and STAT5 signaling pathway. HeLa (A) and SiHa (B) cells were stimulated with WP1066 (STAT3 inhibitor, 10 uM), N'-((4-Oxo-4H-chromen-3-yl) methylene) nicotinohydrazide (STAT5 inhibitor, 10 uM), LY294002 (AKT signal pathway, 10 uM) SP600125 (inhibitor for JNK signal, 10 uM), SB203580 (inhibitor for p38/MAPK signal, 10 uM), U0126 (inhibitor for ERK1/2 signal, 10 uM), BAY (NF-κB inhibitor, 10 uM), or pyrrolidine dithiocarbamate (PDTC, an antioxidant and an inhibitor of NF-κB, 10 uM) for 6 h, and then treated with rhCCL17 (10 ng/ml) for 48 h, with vehicle as the control. Subsequently, cells were collected and analyzed by BrdU proliferation assay. In addition, HeLa (C) and SiHa (D) cells were stimulated with STAT5 inhibitor (10 uM), inhibitor for JNK signal (10 uM) for 6 h, and then treated with or without rhCCL17 (10 ng/ml) for 48 h. BrdU proliferation assay was used to evaluate the proliferation of these cells. JNKi: treatment with inhibitor for JNK signal pathway; STAT5i: treatment with inhibitor for STAT5 signal pathway. The data are expressed as the mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ (one-way ANOVA). $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ vs. the vehicle control (one-way ANOVA). NS: no statistically difference.

expressed in some hematologic malignancies, such as Hodgkin's and B cell lymphoma [20, 21]. Epigenetic modification is considered to regulate CCL17 expression in Hodgkin's lymphoma [22]. In the current study, we first confirmed that cervical cancer cells highly expressed chemokine CCL17. In addition, cervical cancer cell lines (HeLa and SiHa cells) co-expressed CCL17 and its receptor CCR4. However, the regulatory mechanism of CCL17 expression and the role of CCL17/CCR4 signaling in this process were not understood.

The development of a hypoxic microenvironment stimulates the expression of a variety of genes, such as hypoxia-inducible factor (HIF) in

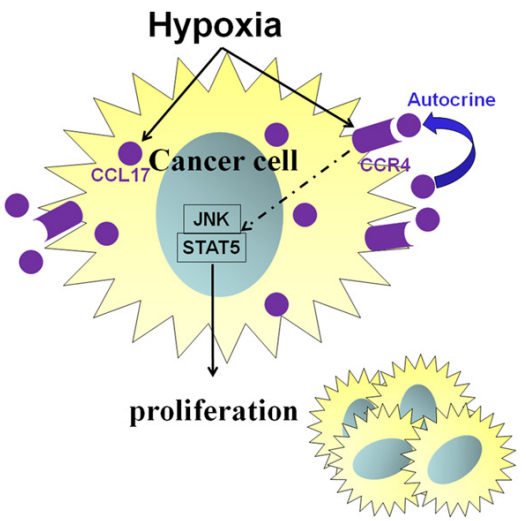


Figure 6. The role of CCL17/CCR4 interaction in the progression of cervical cancer. Accompanied by rapid growth of cervical cancer, hypoxia stimulates the expression of CCR4 on cervical cancer cells. The consequent things should be the higher reactivity of cervical cancer cells against CCL17. These changes in local cancer lesions may directly promote the proliferation and growth of cervical cancer cells through JNK and STAT5 signaling pathways and further stimulate the development of cervical cancer cells.

tumor tissue, that act to promote the growth and survival of tumor cells [23]. Compared to other solid tumors, cervical cancer exhibits some unique differences. Normal cervical stratified epithelia have characteristics of hypoxic tissue. As an essential mediator of cellular response to hypoxia, HIF regulates gene expression for tumor angiogenesis and resistance to oxidative stress. Several studies [24, 25] have demonstrated that HIF-1 level is increased in most solid tumors and correlate with patient prognosis. During the response to hypoxia in cells, HIF-1 plays an important mediating role via the control of its downstream genes, such as chemokine [3, 4, 26-29], members of the vascular endothelial growth factor (VEGF) families [30]. Our study found that hypoxia significantly up-regulated the expression of CCR4, suggesting that hypoxia may increase the reactivity of cervical cancer cells against CCL17 by modulating CCR4 expression. Our recent data [16] also showed that hypoxia promoted the secretion of CCL17 from HeLa and SiHa cells. Further analysis showed that hypoxia stimulated proliferation of HeLa and SiHa cells, and these effects could be abrogated by blocking CCL17 with α-CCL17. But the effect of CCL17 on apoptosis in HeLa and SiHa cells was undetectable. These data above indicated that high levels of CCL17 and CCR4 in cervical cancer cells induced by hypoxia promoted proliferation of cervical cancer cells themselves.

Several signaling pathways (for example, MAPK/ERK1/2, p38, JNK, AKT, STATs, NF-κB) were proved to involve in the regulation of cell proliferation, especially for cancer cells [31-35]. Therefore, we next looked for the possible downstream signaling pathway of CCL17/CCR4, and found that only inhibitor for JNK or STAT5 could partly reverse the stimulatory effect of rhCCL17 on HeLa and SiHa cells. Blocking AKT, MAPK/ERK1/2, p38, STAT3 or NF-κB signaling pathway with different inhibitors had no this effect. Taken together, it could be concluded that the effect of CCL17 on the proliferation of cervical cancer cells was dependent on JNK and STAT5 signaling pathways. But the precise molecular mechanism of CCL17 on these two signaling pathway still needs to further research.

Evidences showed that cancer cells recruit immune cells by producing chemokines, such as CCL17 in peripheral T-cell lymphomas [36-39]. CCL17 might reconstruct tumor immune microenvironment and regulate the development of cervical cancer cells by recruiting regulatory T cells (Tregs) and eosinophils (EOS) to local cancer lesions. The reports about cervical cancer indicate that 25-100% cervical squamous cell carcinoma has EOS infiltration, and 2-26% invasive carcinoma of cervical squamous cell has significant EOS infiltration [40]. Therefore, the big question is whether the abnormal high levels of CCL17 from cervical cancer cells can lead to local EOS and Tregs infiltration, and further reconstruct tumor immune microenvironment and regulate the development of cervical cancer.

Therefore, accompanied by rapid growth of cervical cancer, hypoxia stimulates the secretion of CCL17 and the expression of CCR4 on cervical cancer cells. The consequent things should be the higher reactivity of cervical cancer cells against CCL17. These changes in local cancer lesions, on the one hand, directly promotes the proliferation and growth of cervical cancer cells through JNK and STAT5 signaling pathways

(**Figure 6**); on the other hand, may indirectly modulate the tumor immune microenvironment by recruiting immune cells to cancer lesions and strengthening crosstalk between cervical cancer cells and cancer-related immune cells. These integral effects will stimulate the development of cervical cancer cells. Our data bring new insight into the effective mechanisms of CCL17/CCR4 axis in pathogenesis of cervical cancer. Further research is warranted to explore the functions and significance of CCL17 in the coordination between cervical cancer cells and other $CCR4^+$ cells of local cervical cancer lesions.

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Disclosure of conflict of interest

The authors declare no financial or commercial conflict of interest.

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