Role of CCL5 in invasion, proliferation and proportion of $CD44^+/CD24^-$ phenotype of MCF-7 cells and correlation of CCL5 and CCR5 expression with breast cancer progression

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Abstract. This study was undertaken to observe the effects and possible mechanism of CC chemokine ligand 5 (CCL5) on invasion, proliferation and percentage of $CD44^+/CD24^-$ subpopulation of human breast cancer line MCF-7 and to investigate the correlation of expression levels of CCL5 and its receptors with the progression of breast cancer. We used real-time RT-PCR to detect the expression levels of CCL5 and its receptors CCR5, CCR1 and CCR3 in 36 breast cancer specimens of different TNM stage and their corresponding normal breast tissue. CCL5 expression and invasive ability of four human breast cancer cell lines MCF-7, SK-BR-3, T-47D and MDA-MB-231 were analyzed by real-time RT-PCR and cell invasion assay, respectively. Effects of recombinant human CCL5 (rhCCL5) on cell proliferation and percentage of the $CD44^+/CD24^-$ sub-population in MCF-7 cells were analyzed respectively by MTT assay and flow cytometry. We also used cell invasion assay to detect the invasive ability of both $CD44^+/CD24^-$ and $CD44^+/CD24^+$ subpopulations of MCF-7 cells treated with rhCCL5 and/or CCR5 monoclonal antibody. Our results revealed that CCL5 and CCR5 expression were higher in breast cancer tissue than those in their corresponding normal tissue and breast cancer tissue with higher TNM stage contained more CCL5 mRNA. In addition, CCR5 expression and invasive ability of $CD44^+/CD24^-$ subpopulation were higher than those of $CD44^+/CD24^+$ subpopulation of MCF-7 cells. Moreover, treatment of rhCCL5 increased the proportion of $CD44^+/CD24^-$ cells and the proliferation of MCF-7 cells. Induction of rhCCL5 increased the cell invasive ability of both $CD44^+/CD24^-$ and $CD44^+/CD24^+$ cells, which could be partially antagonized by CCR5 monoclonal antibody. Collectively, our data show that CCL5 increased the proportion of $CD44^+/CD24^-$ subpopulation and induced invasion and proliferation of MCF-7 cells, and expression of CCL5 and CCR5 in breast cancer tissue was positively correlated with breast cancer progression.

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Introduction

Breast cancer is a leading cause of death for women in many countries. The ability of breast cancer cells to metastasize to distal organs makes this disease refractory and incurable and is the vital factor contributing to the therapeutic effect and prognosis of breast cancer (1). The metastasis of cancer consisting of a series of complex steps is influenced and regulated by various factors in the microenvironment (2-5). Chemokines are small soluble molecules that are best known for their potent abilities to induce cellular migration, particularly by leukocytes during inflammation. It is widely accepted that a functional relationship exists between inflammation and cancer. Although inflammatory cells and cytokines may contribute to the host anti-tumor response, inflammation likely promotes tumor growth and progression as well (6). Prolonged inflammation is thought to potentiate carcinogenesis by providing a microenvironment that is ideal for cancer development and growth (6,7). Many cancer cells, including breast cancer cells, express chemokines and chemokine receptors (7). In addition to inducing inflammatory cell infiltration into the tumor, local chemokines may also mediate angiogenesis, serve as growth or survival factors, and regulate tumor cell migration or metastasis (8).

CC chemokine ligand 5 (CCL5), originally identified as a product of activated T cells, is capable of recruiting T cells to inflammatory sites (9). Recent studies have shown that local production of CCL5, a potent chemotactic factor for inflammatory cells, is important in the progression of breast

cancer, but its precise role is complex (8). Niwa *et al* reported that plasma levels of CCL5 were greater in patients with progressive disease compared to those in clinical remission. Also, more advanced stages correlated with higher levels of CCL5 expression (10). Another study showed that 74% of sections from breast cancer specimens exhibited CCL5 expression and the level of expression was greater than that found in normal epithelial cells, ductal epithelial cells and benign sections (11). In addition, a chemokine receptor antagonist of the CCL5 receptors CCR5 and CCR1 was recently shown to inhibit experimental breast tumor growth (12), further implicating CCL5 as an important molecule in breast cancer.

The theory that cancer may be originated and sustained by a small proportion of stem-like, self-renewing cells, which are termed 'cancer stem cells', has gained support in recent years (13-16), and cancer stem cells are thought to be responsible for the growth, progression, and recurrence of a tumor (17-20). In recent years, more and more cancer stem cells in solid tumors have been prospectively identified based on expression of cell surface markers (21-25). Breast cancer stem cells have been identified as $CD44^+/CD24^-$ breast cancer cells and have recently been isolated and propagated *in vitro* (21,26,27). A single breast cancer cell marked by $CD44^+/CD24^-$ could reconstitute the breast tumor (28). Besides the strong tumor-initiating capability, breast cancer cells with $CD44^+/CD24^-$ subpopulation also have highly invasive properties and express higher levels of pro-invasive genes (29,30), and all of these properties contribute to development of the primary and metastatic breast cancer. Meanwhile, activities of breast cancer stem cells are intensively regulated by the microenvironment around tumor, which consists of a variety of chemokines or growth factors (31,32). However, the precise constitution of this microenvironment and how it regulates the activities of breast cancer cells and breast stem cell have been only slightly investigated. In this study, we show that CCL5, an important chemotactic factor in breast cancer microenvironment, increased the proportion of $CD44^+/CD24^-$ subpopulation in breast cancer cell line MCF-7 and stimulated the proliferation of MCF-7 cells in a concentration- and time-dependent manner. At the same time, induction of CCL5 increases the invasive ability of both $CD44^+/CD24^-$ and $CD44^+/CD24^+$ cells, which could be partially antagonized by CCR5 monoclonal antibody. We also discover that CCR5 expression and invasive ability were higher in $CD44^+/CD24^-$ cells than those of $CD44^+/CD24^+$ cells in the breast cancer cell line MCF-7. Besides, we found that CCL5 and CCR5 expression were higher in breast cancer tissue than those in their corresponding normal tissue and breast cancer tissue with higher TNM stage contained more CCL5 mRNA. Therefore, CCL5 and its receptor CCR5 play a significant role in the progress of breast cancer both *in vitro* and *in vivo*.

Materials and methods

Patients. We used snap-frozen breast cancer specimens from 36 patients, each sample consisted of tumor and corresponding normal tissue. All patients underwent breast cancer surgery during the period of March to September in 2006 at the Renmin Hospital of Wuhan University. The age of patients at the time of surgery ranged from 34 to 69 years (average 47 years), and all patients were female. Histopathological evaluation was performed on hematoxylin and eosin-stained sections, and staging and grading were performed according to the TNM classification of the International Union Against Cancer (UICC), which are summarized in Table I.

Table I. The TNM stage characteristics of 36 patients with breast cancer.

TNM stage	No. of patients (%)
I	9 (25.0)
II	12 (33.3)
III	8 (22.2)
IV	7 (19.4)

Cell culture. Human breast cancer cell lines MCF-7, SK-BR-3, T-47D and MDA-MB-231 were purchased from Type Culture Collection Center of Wu Han University, China, and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 50 U/ml penicillin, 50 U/ml streptomycin and 10% calf serum (Hyclone) at 37˚C in a humidified incubator supplemented with 5% carbon dioxide.

Reagents. Recombinant human CCL5 (rhCCL5) was purchased from BioVision (BioVision Corp., USA). CCR5 monoclonal antibody was bought from eBioscience (eBioscience Corp., USA). Fluorochrome-conjugated monoclonal antibodies: FITC anti-human CD44 (cat. #103021) and PE anti-human CD24 (cat. #311105) were obtained from Biolegend (Biolegend Corp., USA). Oligonucleotides were acquired from Shengong Biotechnology (Shanghai, China). The primers were synthesized by Genechem Biotechnology (Shanghai, China). M-MLV retroviridase, Dntp, RNAse Inhibitor were from Promega (Promega Corp., USA). Cell invasion assay kit was brought from Chemicon (#ECM550; Chemicon International, Temecula, CA, USA). Cell culture reagents and other chemical materials were purchased from Sigma (Sigma Corp., USA).

RNA isolation and real-time RT-PCR. Total RNA was isolated from $1x10^7$ breast cancer cells using TRIzol™ reagents. RNA (2 μg) was used for cDNA synthesis by reverse transcription. The RNA sample was incubated at 70˚C for 10 min with 1 μl Oligo dT (0.5 μg/μl) in a final volume of 10 μl, and then chilled on the ice, then mixed with 4 μl 5X buffer, 2 μl dNTPs (10 mM), 0.5 μl RNAse, 1 μl M-MLV-RTase and 3.5 μl DEPC-H_2O. After incubation at 42˚C for 60 min, and at 70˚C for 10 min, the mixture was reversely transcribed into cDNA. Obtained cDNAs were amplified using specific primers respectively. CCL5 sense primer, 5'-CGTGCCCAC ATCAAGGAG-3', and anti-sense primer, 5'-GGACAAGAG CAAGCAGAAAC-3', CCR5 sense primer, 5'-GCAAGGAG ACCACCAACAG-3', and anti-sense primer, 5'-CCCTCAC TTCCAACCCAAATC-3', GAPDH sense primer, 5'-CCAT

CACCATCTTCCAGG-3', and anti-sense primer, 5'-ATGA GTCCTTCCACGATAC-3' were used in the real-time RT-PCR reaction. The PCR reaction system consisted of cDNA1 μl, SYBR premix ex taq 10 μl, sense primer (5 μM) 0.5 μl, anti-sense primer (5 μM) 0.5 μl, and DEPC-H_2O 8 μl. PCR was initiated with initial denaturation step at 95˚C for 15 sec and continued for 45 cycles of denaturation (95˚C, 15 sec), annealing and extension (60˚C for CCL5 and GAPDH; 54˚C for CCR5, 30 sec). At the extension stage of each cycle, the value of threshold cycle (CT) was recorded. A standard curve was generated by plotting the CT. GAPDH expression was assessed as a housekeeping gene to standardize the expression level of CCL5. The comparative expression level of CCL5 = $2^{-\Delta\Delta CT}$.

Cell invasion assay. The invasive abilities of breast cancer cells were detected by the cell invasion assay kit, which allows cells to migrate through an 8-μm pore size polycarbonate membrane with a layer of 25 μg/cm^2 Matrigel. The gels were allowed to polymerize for 2 h at 37˚C and had a minimum thickness of 20 μm. Cells were trypsinized, washed, and resuspended in serum-free DMEM medium (1x10^6 cells/ml). This suspension (100 μl) was added to the upper chamber of the well. Serum-free media or media with 10% serum or media with 100 ng/ml rhCCL5 were placed in the bottom well. After incubation for 48 h at 37˚C in the presence of 5% CO_2, the cells were fixed for 30 min in 4% formaldehyde and stained for 15 min with crystal violet. The filters were then rinsed thoroughly in distilled water and checked by bright-field microscopy to ensure that the cells were adherent and had migrated. The non-migrating cells were then carefully removed from the upper surface (inside) of the well with a wet cotton swab. To quantify cell invasion, cells that had migrated to the bottom surface of the filter were counted. Nine evenly spaced fields of cells were counted in each well, using an inverted phase-contrast microscope at x200 magnification.

Flow cytometry. Cells were washed once with phosphate-buffered saline (PBS) and then harvested with 0.05% trypsin/0.025% EDTA. Detached cells were washed with PBS containing 1% FCS and 1% penicillin/streptomycin (wash buffer), and resuspended in the wash buffer (10^6 cells/100 μl). Fluorochrome-conjugated monoclonal antibodies: FITC anti-human CD44 and PE anti-human CD24 or their respective isotype controls were added to the cell suspension at concentrations recommended by the manufacturer and incubated at 4˚C in the dark for 30 to 40 min. The labeled cells were washed in the wash buffer, then fixed in PBS containing 1% paraformaldehyde, and then analyzed on a FACS-Vantage (BD Biosciences). In addition, cells were sorted into two subpopulations, CD44$^+$/CD24$^-$ subpopulation and CD44$^+$/CD24$^+$ subpopulation, according to their surface markers. Cells were routinely sorted twice, and the cells were re-analyzed for purity, which typically was >95%.

Cell proliferation test by MTT assay. Cells in the exponential phase of growth were harvested and seeded in 96-well plates at a density of 1x10^4 cells per well. After 24 h, rhCCL5 were added to triplicate wells for each concentration (0, 10, 50, 100, 500 ng/ml) and incubation for 24 or 48 h. At 24 or 48 h after rhCCL5 treatment, the cells were washed with PBS. Then 10 μl MTT [3-(4,5-dimethyl thizol-2-yl)-2.5-diphenyl terazolium bromide] was added to each well and the plate was incubated at 37˚C in a CO_2 incubator. After 4-h incubation, the medium was removed, and 100 μl DMSO was added to each well to dissolve the formazan crystals and optical density (OD) was measured at a wavelength of 570 nm using a microplate reader (Multiskan MK3). The absorbance in the treatment groups were calculated relative to the controls (100%).

Statistical analysis. The data are expressed as means ± SEM and were analyzed using one-way analysis of variance (ANOVA) and the Student-Newman-Keul's test for individual comparisons. A probability value of $p<0.05$ was considered statistically significant.

Results

CCL5 expression was higher in breast cancer tissue than that in their corresponding normal tissue and breast cancer tissue with higher TNM stage contained more CCL5 mRNA. In order to investigate the role of CCL5 in breast cancer development, we firstly detected CCL5 mRNA expression in the breast cancer samples and their corresponding normal breast tissue by using real-time RT-PCR. The results showed that the CCL5 expression was significantly higher in cancer tissue than in normal tissues, in all TNM stages (Fig. 1A, $p<0.05$). The data also revealed in stages I and II, CCL5 expression in breast cancer tissue had no significant difference (Fig. 1B, $p>0.05$). CCL5 expression level in stage III and IV were 2.390±0.071 and 3.706±0.209, respectively, and were significantly higher than that in TNM-stage I (Fig. 1B, $p<0.05$). CCL5 level in stage IV was also significantly higher than that of stage III (Fig. 1B, $p<0.05$).

CCR5 expression was higher in breast cancer tissue than that in their corresponding normal tissue. To further observe the role of CCL5 and its receptors CCR5, CCR1 and CCR3 in breast cancer, we also detected the mRNA expression of CCR5, CCR1 and CCR3 in the breast cancer samples and their corresponding normal breast tissue by using real-time RT-PCR. The results revealed that CCR5, CCR1 and CCR3 were expressed in both breast cancer tissue and the normal breast tissue, and the expression of CCR5 in breast cancer tissue was significantly higher than that in the corresponding normal breast tissue, the former is 1.91-fold of the latter (Fig. 1C, $p<0.05$). Meanwhile, there was no significant differences in the expression of both CCR1 and CCR3 between in the breast cancer samples and in their corresponding normal breast tissue (Fig. 1C, $p>0.05$).

Constitutive CCL5 expression levels in different human breast cancer cell lines were not positively related to the invasive ability of cells. To explore the relationship between constitutive CCL5 expression level in different human breast cancer cell lines and the invasive ability of cells, we used real-time RT-PCR to detect the expression of CCL5 mRNA in breast cancer cell lines MCF-7, SK-BR-3, T-47D and

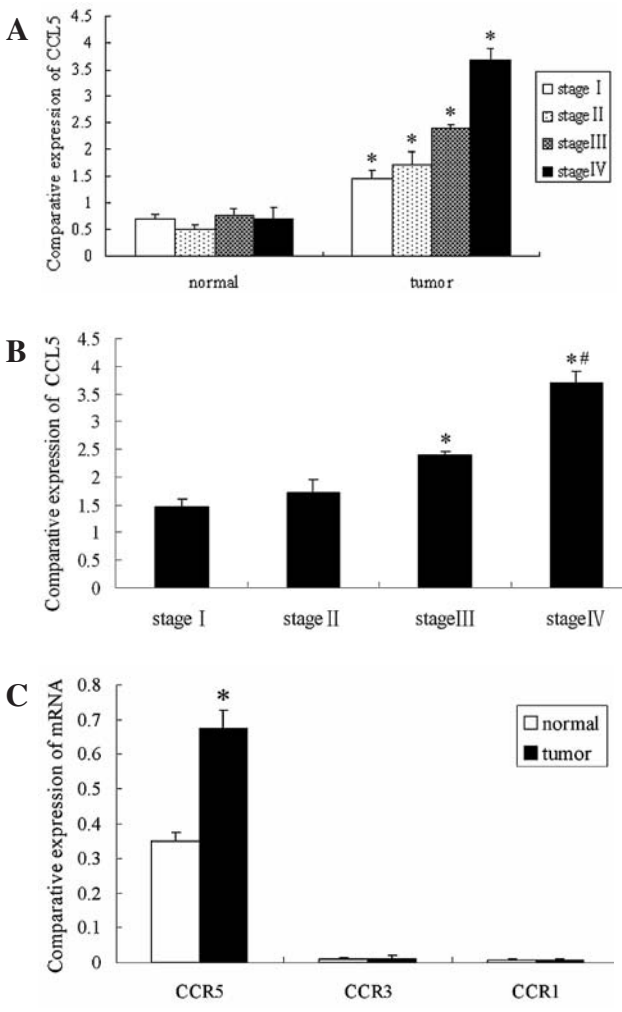

Figure 1. The mRNA expression of CCL5 and its receptors CCR5, CCR1 and CCR3 in the breast cancer specimens and their corresponding normal breast tissue by using real-time RT-PCR. (A) The expression levels of CCL5 mRNA in each specimen were detected by real-time RT-PCR. Three independent experiments were performed. Results are presented as means ± SD of these independent experiments. *p<0.05 vs. normal. (B) CCL5 mRNA expression in breast cancer tissue of different TNM stage was detected by real-time RT-PCR. Three independent experiments were performed. Results are presented as means ± SD of these independent experiments. *p<0.05 vs. stage I, #p<0.05 vs. stage III. (C) Expression of CCR5, CCR1 and CCR3 in the breast cancer specimens and their corresponding normal breast tissue were detected by real-time RT-PCR. Three independent experiments were performed. Results are presented as means ± SD of these independent experiments. *p<0.05 vs. normal.

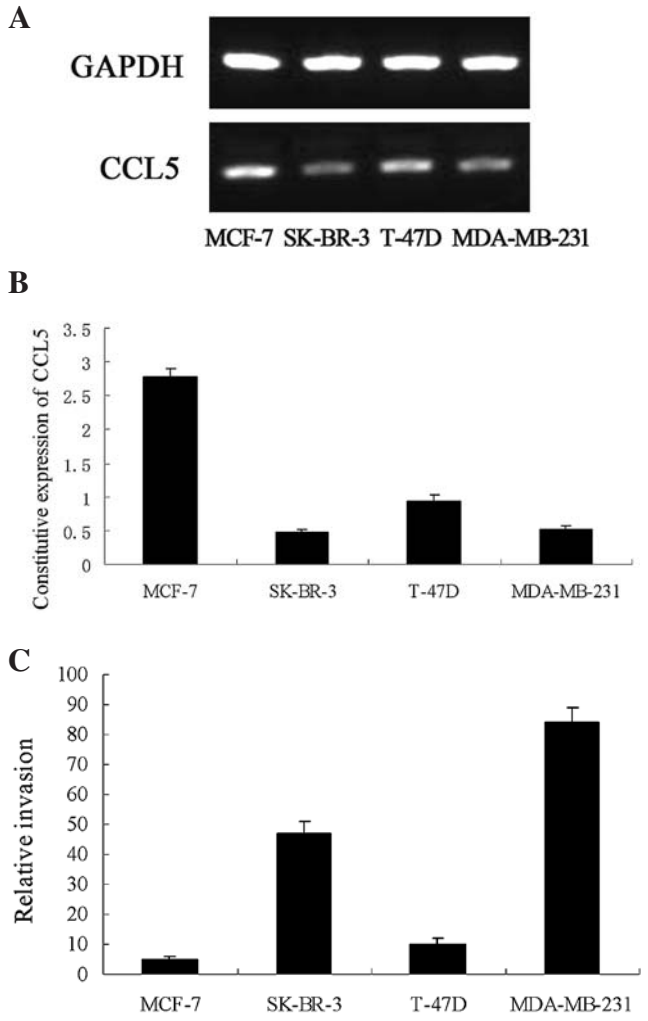

Figure 2. The constitutive expression of CCL5 and the invasive ability of breast cancer cell line MCF-7, SK-BR-3, T-47D and MDA-MB-231. (A) The constitutive expression of CCL5 mRNA in these four breast cancer cell lines was detected by real-time RT-PCR. (B) The mRNA expression levels of CCL5 were standardized by using that of GAPDH in cells. (C) The invasive ability of these four breast cancer cell lines was analyzed by cell invasion assay. Three independent experiments were performed. Results are presented as means ± SD of these independent experiments.

MDA-MB-231 cultured *in vitro* (Fig. 2A). The comparative CCL5 expression levels were, ranked from high to low, MCF-7 (2.78±0.13), T-47D (0.95±0.09), MDA-MB-231 (0.52±0.06) and SK-BR-3 (0.47±0.05) (Fig. 2B).

Meanwhile cell invasion assay was also performed to detect the invasive capacity of these four different breast cancer cells. The results revealed that the ranks of the cell invasive ability were, from high to low, MDA-MB-231, SK-BR-3, MCF-7 and T-47D (Fig. 2C), which were not positively related to the constitutive expression of CCL5 in different cells.

CCL5 increased the proportion of CD44+/CD24- subpopulation in breast cancer cell line MCF-7. To investigate the effect of CCL5 on the proportion of CD44+/CD24- cells in breast cancer cells *in vitro*, MCF-7 cells were treated with or without rhCCL5 of 50, 500 ng/ml previously for 60 h. Then flow cytometry analysis was conducted to characterize breast cancer cells in different groups for surface expression of CD44 and CD24, and the percentage of the CD44+/CD24- subpopulation in each group was analyzed. The data showed that treatment of rhCCL5 increased the percentage of the CD44+/CD24- subpopulation in MCF-7 cells tremendously (Fig. 3A). The proportion of CD44+/CD24- cells was elevated to 5.68-fold of the control cells, after treatment with 50 ng/ml of rhCCL5 (Fig. 3B, $p<0.05$). Meanwhile, when we treated MCF-7 cells with 500 ng/ml of rhCCL5, the proportion of CD44+/CD24- cells increased to 6.05-fold that of control cells (Fig. 3B, $p<0.05$).

CCL5 stimulated the proliferation of MCF-7 cells in a concentration- and time-dependent manner. The effect of CCL5 on the proliferation of breast cancer cells was quantified by

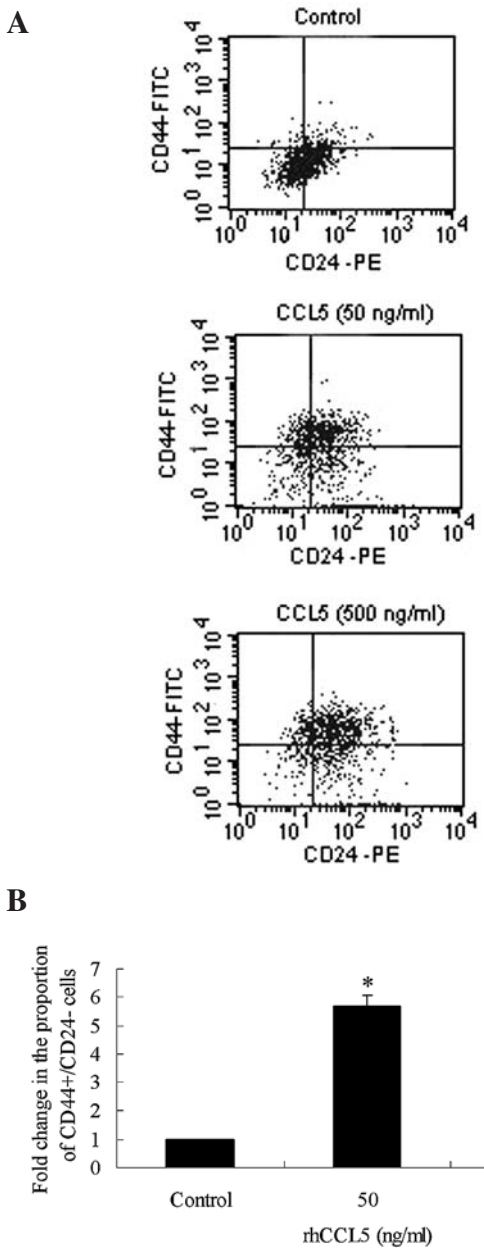

Figure 3. Effect of CCL5 on the proportion of $CD44^+/CD24^-$ cells in breast cancer cell line MCF-7 *in vitro*. (A) MCF-7 cells were treated with or without rhCCL5 of 50, 500 ng/ml previously for 60 h. Then flow cytometry analysis was conducted to characterize breast cancer cells in different group for surface expression of CD44 and CD24. (B) The percentage of the $CD44^+/CD24^-$ subpopulation in each group was analyzed and were shown as the fold change relative to control group, which was coultrued in serum-free medium. Three independent experiments were performed. Results are presented as means ± SD of these independent experiments. $^*p<0.05$ vs. control group.

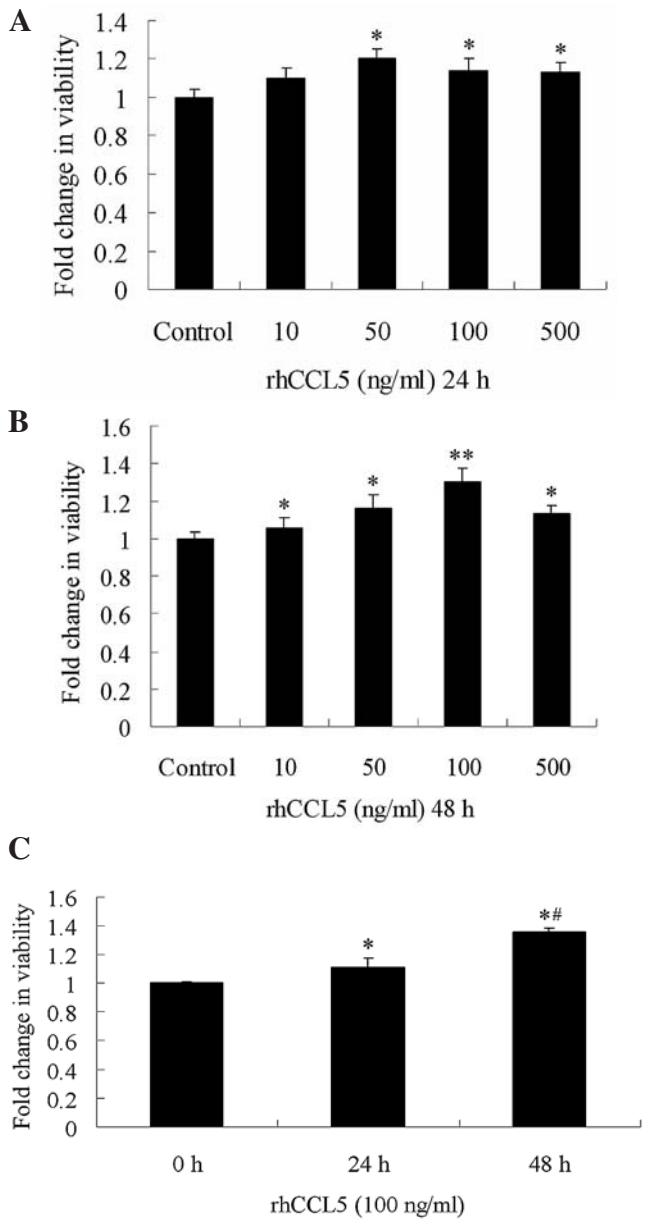

Figure 4. Effect of CCL5 of different concentration on the proliferation of breast cancer cell MCF-7 cultured *in vitro* for 24 and 48 h. (A) and (B) MCF-7 cells in the exponential phase of growth were harvested and seeded in 96-well plates at a density of $1x10^4$ cells per well. Twenty-four hours later, rhCCL5 at the concentration of 0, 10, 50, 100, 500 ng/ml were added to different well and cells were incubated for 24 or 48 h. Then the viability of cells with different treatment was tested by MTT assay. The values of MTT assay were shown as the fold change relative to control group, which was coultrued in serum-free medium. Three independent experiments were performed. Results are presented as means ± SD of these independent experiments. $^*p<0.05$, $^{**}p<0.01$ vs. control group. (C) MCF-7 cells were treated with rhCCL5 of 100 ng/ml, and then cultured *in vitro* for 0, 24 and 48 h. Then the viability of cells with different treatment was tested by MTT assay. The data are shown as the fold change relative to the 0 h group. Three independent experiments were performed. Results are presented as means ± SD of the independent experiments. $^*p<0.05$, vs. 0 h group. $^\#p<0.05$ vs. 24 h group.

MTT assay. MCF-7 cells in the exponential phase of growth were harvested and seeded in 96-well plates at a density of $1x10^4$ cells per well. Twenty-four hours later, rhCCL5 at the concentration of 0, 10, 50, 100, 500 ng/ml were added to different well and cells were incubated for 24 or 48 h. Then the viability of cells with different treatments was tested by MTT assay. The results showed that when MCF-7 cells were treated with rhCCL5 for 24 h, the cell viability was increased and there was a peak at the concentration of 50 ng/ml (1.20-fold of the control cells) (Fig. 4A, $p<0.05$) which then declined while the concentration of rhCCL5 was increasing. When the treatment time of rhCCL5 prolonged to 48 h, the cells viability peak was elevated to 1.31-fold of the control group, which appeared at the concentration of 100 ng/ml (Fig. 4B, $p<0.01$). In addition, the viability of cells treated

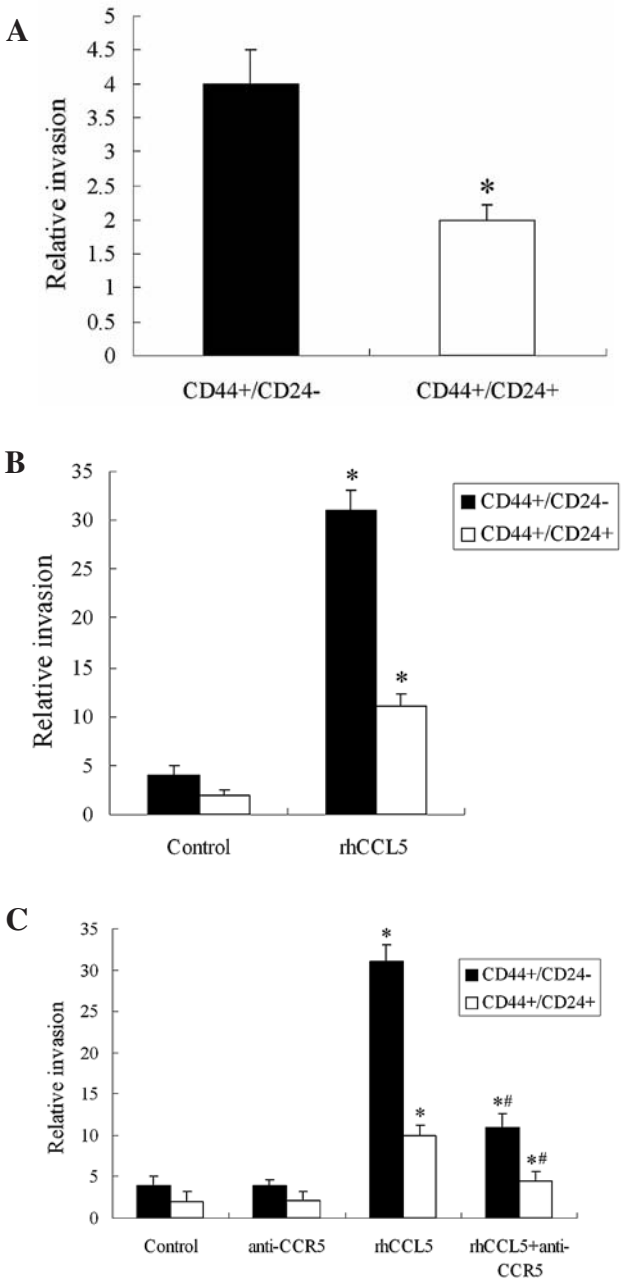

Figure 5. Effect of CCL5 on the invasion of both CD44+/CD24- and CD44+/CD24+ cells in breast cancer cell line MCF-7 and the antagonistic effect of CCR5 monoclonal antibody against the induction of CCL5. (A) MCF-7 cells were initially sorted into two subpopulations, CD44+/CD24- subpopulation and CD44+/CD24+ subpopulation, according to their surface markers by flow cytometry, and then the invasive capacity of these two subpopulations of breast cancer cells were examined by using invasion assay. Three independent experiments were performed. Results are presented as means ± SD of these independent experiments. *p<0.05 vs. CD44+/CD24- cells. (B) MCF-7 cells were sorted into two subpopulations (CD44+/CD24- and CD44+/CD24+) by flow cytometry. Then cell invasion assay was performed on cells of these two phenotypes respectively when medium with 100 ng/ml rhCCL5 or serum-free medium (control) was added into the bottom well. Three independent experiments were performed. Results are presented as means ± SD of these independent experiments. *p<0.05 vs. control group. (C) In the cell invasion assay, CD44+/CD24- or CD44+/CD24+ cells in the upper well were treated with or without 500 ng/ml of CCR5 monoclonal antibody, and medium with 100 ng/ml rhCCL5 or serum-free medium was added into the bottom well. After 48 h, cells that had migrated to the bottom surface of the filter were counted. Three independent experiments were performed. Results are presented as means ± SD of these independent experiments. *p<0.05 vs. control group. #p<0.05 vs. rhCCL5 group.

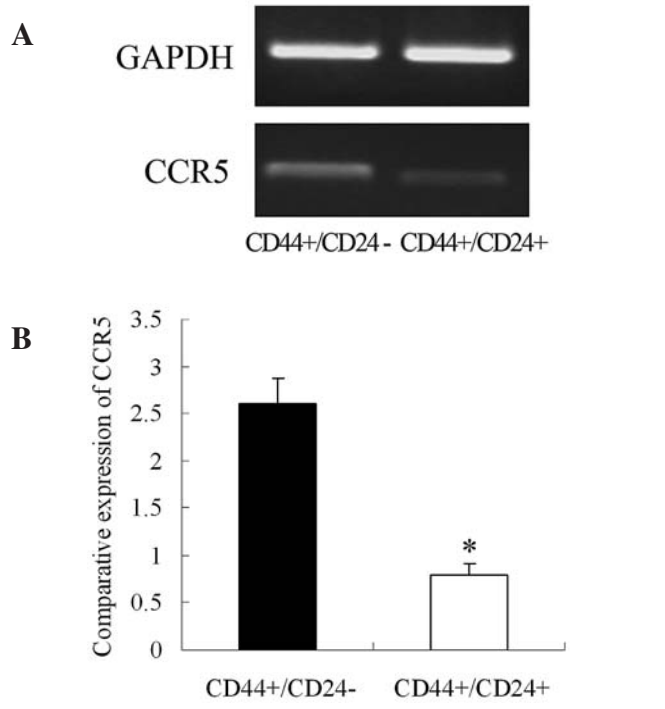

Figure 6. CCR5 mRNA expression in CD44+/CD24- and CD44+/CD24+ cells of breast cancer cell line MCF-7. (A) MCF-7 cells were initially sorted into CD44+/CD24- subpopulation and CD44+/CD24+ subpopulation by flow cytometry, and then expression of CCR5 mRNA in both kinds of cells was detected by real-time RT-PCR. (B) The mRNA expression levels of CCR5 were standardized by using that of GAPDH in cells. Three independent experiments were performed. Results are presented as means ± SD of these independent experiments. *p<0.05 vs. CD44+/CD24- cells.

with 100 ng/ml of rhCCL5 for 48 h was 1.21-fold of the viability of cells treated for 24 h (Fig. 4C, p<0.05).

CD44+/CD24- cells are more invasive than CD44+/CD24+ cells in breast cancer cell line MCF-7. To observe the association between the phenotype of breast cancer cells and the cell invasive ability, MCF-7 cells were initially sorted into two subpopulations, CD44+/CD24- subpopulation and CD44+/CD24+ subpopulation, according to their surface markers by flow cytometry, and then the invasive capacity of these two subpopulations of breast cancer cells were examined by using invasion assay. The results revealed that CD44+/CD24- cells were more invasive than CD44+/CD24+ cells and the invasive ability of the former was 2.03-fold that of the latter (Fig. 5A, p<0.05).

Induction of CCL5 increases the invasive ability of both CD44+/CD24- and CD44+/CD24+ cells in breast cancer cell line MCF-7. To identify the effect of CCL5 on the invasion of breast cancer cells and to compare the influence of CCL5 on cells of different subpopulation, MCF-7 cells were sorted into two subpopulations (CD44+/CD24- and CD44+/CD24+) by flow cytometry. Then cell invasion assay was performed on the cells of these two phenotypes respectively, and medium with 100 ng/ml rhCCL5 or serum-free medium (control) was added into the bottom well. When induced by 100 ng/ml rhCCL5, the invasive ability of CD44+/CD24- cells was elevated to 7.75-fold of the control cells and that of CD44+/

$CD24^+$ cells was also increased to 5.50-fold of the control group (Fig. 5B, $p<0.05$). The data showed that induction of CCL5 increased the invasive ability of both $CD44^+/CD24^-$ and $CD44^+/CD24^+$ cells in breast cancer cell line MCF-7.

CCR5 monoclonal antibody partially antagonizes the inductive effect of CCL5 on the invasion of $CD44^+/CD24^-$ and $CD44^+/CD24^+$ cells in breast cancer cell line MCF-7. Furthermore, in order to investigate the mechanism of the inductive effect of CCL5 on cells invasive capacity, we treated cells with 500 ng/ml of CCR5 monoclonal antibody (anti-CCR5) when they were induced by rhCCL5 in the cell invasion assay. $CD44^+/CD24^-$ or $CD44^+/CD24^+$ cells in the upper well were cultured with or without 500 ng/ml of CCR5 monoclonal antibody, and medium with 100 ng/ml rhCCL5 or serum-free medium was added into the bottom well. The result showed that for both $CD44^+/CD24^-$ and $CD44^+/CD24^+$ cells, treatment of CCR5 monoclonal antibody could effectively, but partially, block the induction of rhCCL5 on cells invasion. The invasive ability of $CD44^+/CD24^-$ cells treated with both rhCCL5 and CCR5 monoclonal antibody was 35.48% of those treated only with rhCCL5 (Fig. 5C, $p<0.05$). As for the $CD44^+/CD24^+$ cells, the inductive effect of rhCCL5 was decreased to 45.00% when cells were treated with CCR5 monoclonal antibody (Fig. 5C, $p<0.05$). However, the invasion of the cells treated with both rhCCL5 and CCR5 monoclonal antibody was still higher than the control cells (Fig. 5C, $p<0.05$), which revealed that the CCR5 monoclonal antibody could not completely block the inductive effect of CCL5 on cells invasive capacity.

CCR5 expression is higher in $CD44^+/CD24^-$ cells than that in $CD44^+/CD24^+$ cells of breast cancer cell line MCF-7. To identify the relationship between the CCR5 expression and the phenotype of breast cancer cells, we used real-time RT-PCR to analyze the CCR5 mRNA expression in both $CD44^+/CD24^-$ and $CD44^+/CD24^+$ MCF-7 cells (Fig. 6A). The result showed that the mRNA expression level of CCR5 was significantly higher in $CD44^+/CD24^-$ cells than that in $CD44^+/CD24^+$ cells in breast cancer cell line MCF-7 (Fig. 6B, $p<0.05$).

Discussion

CCL5, a classical pro-inflammatory chemokine, has been found in several cancers as a promalignant factor (33,34). As for breast cancer, previous studies have showed that local production of CCL5 is important in the progression of breast cancer and that more advanced stages of breast cancer correlated with higher levels of CCL5 expression (8,10), but its precise function and mechanism have not been clearly clarified. CCR5, CCR3 and CCR1 are three known receptors of CCL5, which also play a significant role in the development of breast cancer, especially by interacting with CCL5. A recent study has revealed that an antagonist of CCR5 and CCR1 inhibited experimental breast tumor growth (12).

In this study, in order to identify the role and mechanism of CCL5 in the progression of breast cancer, we firstly detected expression levels of CCL5 and its receptors CCR5, CCR1 and CCR3 in 36 breast cancer specimens of different TNM stage and their corresponding normal breast tissue was tested by using real-time RT-PCR. The results that CCL5 and CCR5 expression were higher in breast cancer tissue than those in their corresponding normal tissue and breast cancer tissue with higher TNM stage containing more CCL5 mRNA infers that expression of CCL5 in breast cancer tissue has a positive correlation with the development of breast cancer, especially at the later periods of breast cancer (the TNM-stage III and IV), and activity of CCL5 is related to the expression of CCR5.

In fact, CCL5 shows quite extensive functions in the microenvironment around breast cancer. CCL5 in the local environment around the tumor induced the infiltration of the immune cells, especially the tumor associated macrophage cell (TAM), which facilitated the development of tumor by secreting the active factors such as angiogenesis factor, matrix metalloproteinase and a variety of growth factors (35,36). The animal experiment had further verified that CCL5 could recruit monocytes to the locus of breast cancer in the animal model (12,37). Moreover, CCL5 was able to trigger angiogenesis in the breast cancer. The CCL5 in breast cancer recruited the monocytes and macrophage cells to the microenvironment around the tumor to secret angiogenesis factors such as matrix metalloproteinase-9 (MMP-9) (12), CXCL8 and vascular endothelial growth factor (VEGF) (38). At the same time, CCL5 also recruited endothelial cells to the tumor forming area, which facilitated angiogenesis (4).

The vast majority of breast cancer fatalities is due to metastasis to distant organs and metastasis of breast cancer is a complicated multiple-step process, which is mainly related to invasion and proliferation of breast cancer cells (1,2), we further clarified the role of CCL5 in the invasion and proliferation of breast cancer cells. We then studied the relationship between the constitutive expression of CCL5 in human breast cancer cell lines and the cell invasive ability. To our surprise, constitutive expression levels of CCL5 in four different human breast cancer cell lines were not positively related to the invasive ability of cells, as predicted based on the study of the breast cancer specimens. The results of *in vitro* research revealed that the breast cancer cell line (MCF-7) expressing the highest CCL5 mRNA level did not have a strong ability to invade in the cell invasion assay, while the expression of CCL5 mRNA in the breast cancer cells which possessed the highest invasive ability was relatively low. We offer a possible explanation for this phenomenon as follows: while previous study showed the constitutive expression of CCL5 by the T47D and MCF-7 breast cancer cells *in vitro* in physiological concentrations (11), an additional study demonstrated the inducible expression of CCL5 by the human breast cancer MDA-MB-435S and BT-20 cells (12). These studies suggested that CCL5 expression in human breast cancer cells might be influenced by certain factors existing around these cells. Since what we detected were the baseline constitutive expression of CCL5 mRNA in these four breast cancer cells without any induction, the result could not reflect the real capacity of different breast cancer cells to express CCL5.

Meanwhile, our results concerning the influence of CCL5 on proliferation of MCF-7 cells revealed that CCL5 could

stimulate the proliferation of MCF-7 cells in a concentration- and time-dependent manner, and its activity was limited within certain range of concentration, which had a functional peak around the concentration of 50-100 ng/ml. This infers that CCL5 could effectively increase the proliferation of MCF-7 cells, but which could just function within certain range of concentration, and if the concentration of CCL5 was beyond this range, such function would decrease.

On the other hand, we have learned from previous research that cancer may be originated and sustained by a small proportion of stem-like, self-renewing cells, which are termed 'cancer stem cells' (13-16). Breast cancer stem cells, characterized by surface markers of $CD44^+/CD24^-$, have been identified and isolated from both solid tumors and breast cancer cell lines (21,26,27), which control not only growth, progression but also invasion of breast cancer (17-20,29,30). The activity of breast cancer stem cells was intensively affected by the chemokines, growth factors and other substances in the microenvironment around breast tumor (31,32).

Thus, we further studied the effect of CCL5, an important chemotactic factor in breast cancer progression (8,10-12), on the proportion of $CD44^+/CD24^-$ phenotype in MCF-7 cells and its influence on the invasion of breast cancer cells. According to the results, CCL5 obviously increased the proportion of $CD44^+/CD24^-$ subpopulation in MCF-7 cells, which means that CCL5 induced the change of phenotype constitution within the breast cancer cell line and elevated the percentage of $CD44^+/CD24^-$ phenotype, a proportion of stem-like, self-renewing cells.

At the same time, the results of cell invasion assay showed that $CD44^+/CD24^-$ cells had higher invasive ability than that of $CD44^+/CD24^+$ cells in breast cancer cell line MCF-7, which conformed to the discovery of Sheridan *et al* (29). Moreover, we found that induction of CCL5 could increase the invasive ability of both $CD44^+/CD24^-$ and $CD44^+/CD24^+$ cells in breast cancer cell line MCF-7, but such inductive effect was partially antagonized by CCR5 monoclonal antibody, which implied that the function of CCL5 on cells invasion was partially, but not completely, depended on the interaction between CCL5 and CCR5.

In summary, our study revealed that CCL5 was able to increase the proportion of $CD44^+/CD24^-$ cells and induce the invasion and proliferation of MCF-7 cells, and these functions of CCL5 in breast cancer cells cultured *in vitro* might provide a reasonable explanation for the positive correlation of CCL5 and CCR5 expression with breast cancer progression. Nevertheless, animal experiments should be conducted to verify the functions of CCL5 in inducing invasion, proliferation and proportion of $CD44^+/CD24^-$ phenotype of breast cancer cells *in vivo* further, and mechanism of interaction between CCL5 and CCR5 as well as the consequent signal pathway should also be clarified.

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