High CCR6/CCR7 expression and Foxp3$^+$ Treg cell number are positively related to the progression of laryngeal squamous cell carcinoma

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Received March 12, 2013; Accepted May 13, 2013

DOI: 10.3892/or.2013.2603

Abstract. Chemokine receptors CCR6 and CCR7 have been reported to play important roles in T cell migration and organ-specific metastasis of various tumors. In the present study, we evaluated the expression and clinical significance of CCR6, CCR7, their ligands and CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells in laryngeal squamous cell carcinoma (LSCC) and metastatic lymph nodes (LNs). The expression of CCR6, CCR7 and their ligands mRNA (CCL20, CCL19/CCL21) as well as the CCR6 and CCR7 proteins were detected by real-time RT-PCR and immunohistochemistry (IHC), respectively. Flow cytometry was used to investigate the percentage of CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells (Tregs) in peripheral blood mononuclear cells (PBMCs). Furthermore, a number of cytokines, including interleukin (IL)-2, IL-4, IL-10, IL-12p70, interferon (IFN)-γ and transforming growth factor (TGF)-β1 were detected by ELISA. The results showed that CCR6 and CCR7 were expressed in tumors *in situ*, metastatic LNs and CD4$^+$CD25$^+$Foxp3$^+$ Tregs. It was hypothesized that the expression profile of CCR6, CCR7 and the proliferation of CD4$^+$CD25$^+$Foxp3$^+$ Tregs affected the process of LN metastasis in LSCC patients. Therefore, the increased percentage of the Foxp3$^+$ Tregs and the upregulation of Foxp3 expression on CCR6$^+$ Tregs in LSCC patients may have accounted for the downregulation of antitumor immunity in these patients, which could be valuable for assessment of prognosis in LSCC treatment.

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Key words: CCR6, CCR7, forkhead box P3, regulatory T cell, laryngeal squamous cell carcinoma

Introduction

Laryngeal squamous cell carcinoma (LSCC), a type of head and neck cancer (HNSCC), is the 11th most common cancer in men worldwide (1). It is also the only cancer with a decreased survival rate in the USA (2). Once it metastasizes, the 5-year survival rate of HNC patients is reduced by 50% (3). The lack of progress has been mainly attributed to local and regional recurrences particularly in patients with stages III and IV disease (4). Therefore, further efforts must be made to improve our understanding of LSCC pathogenesis and prognosis. Similar to most types of tumor, LSCC can result in a suppressed immune system with an altered serum cytokine profile and immune cells that function aberrantly (5). In recent years, a concept has emerged that peripheral tolerance to tumors is maintained and enhanced by T cells with their immunoregulatory function (6).

Regulatory T cells (Tregs) are a subgroup of CD4$^+$ T cells characterized by expression of CD25 and forkhead box P3 (Foxp3) (6). To date, there are 3 main types of CD4$^+$ Treg cells partly characterized in humans: i) CD4$^+$CD25$^-$IL-10$^+$Foxp3^{low} type 1 T regulatory (Tr1) cells, which arise in the periphery in an IL-10-dependent manner (7); ii) naturally occurring CD4$^+$CD25$^+$Foxp3$^+$ T cells (nTregs), which arise directly in the thymus and have the ability to suppress responses of both CD4$^+$ and CD8$^+$ T cells in a contact-dependent, cytokine-independent and antigen non-specific manner (8-10); and iii) Th3 cells, which are defined by their production of large amounts of transforming growth factor (TGF)-β (11).

Tregs can suppress the activation, proliferation and effector functions of various immune cells *in vitro* and *in vivo* (12), which could play an important role in the maintenance of immune tolerance. However, Tregs can also suppress anticancer immune responses, which is in favor of tumor progression (13). The underlying mechanism of the enrichment of the Treg subset in tumor mass remains to be fully elucidated, but may aid in understanding the mechanisms of distinct Treg subsets in immunosuppression and in improving patient treatment and quality of life (14). However, the Treg migration and accumulation in local tissue is the precondition

for its full functionality. Increasing evidence has shown that Tregs express chemokine receptors, which take part in their migration through interaction with specific ligands (15,16).

This chemokine system is a superfamily composed of ~50 ligands and 20 receptors, which are directly involved in trafficking along with lymphocyte activation and homing. Furthermore, it participates and plays a key role in inflammatory reactions (17-19). It has been demonstrated that $CD4^+CD25^+$ regulatory T cells can express a number of chemokine receptors, including CCR6 and CCR7 (20), which are also expressed by several cancer cells (21). It has been demonstrated that other chemokine receptors were expressed on cancer cells and acted at all stages of tumor development and progression, including neoplastic transformation of cells, promotion of angiogenesis, clonal expansion and growth (22). Chemokines have been shown to have quite a multifaceted role in cancer development and progression (23). Previous reports suggested that chemokines contribute to protection mechanisms that enable malignant cancer cells to resist chemotherapy and radiation therapy (24,25).

Several cancer cells overexpress chemokine receptors and numerous metastasis sites express the corresponding chemokines. For example, CXCR4-CXCL12 signaling has been shown to play a role in breast cancer metastasis to bone, brain and liver (26). Meanwhile, Treg can also migrate to specific locations (such as tumor sites) via this mechanism. It has been proved that CCR7 expressing Treg can be chemoattractant to draining lymph nodes (LNs) where CCR7 ligands (CCL19 and CCL21) are expressed (27,28). The CCR6 also plays a role in organ selective liver metastasis of colorectal cancer (29). Therefore, in the present study, we investigated whether chemokines and their receptors favor the Treg migration, LSCC cell metastasis and the subsequent LSCC progression. This study was conducted to analyze the possible role of CCR6, CCR7 and their ligands CCL20 (also known as MIP-3α, LARC), CCL19 (also called MIP-3β, ELC) and CCL21 (also called 6Ckine, SLC) and to explore the possible association between their expression levels and clinical/pathological characteristics of LSCC.

Materials and methods

Patients and healthy donors. A total of 88 LSCC cases were enrolled from patients who were diagnosed and underwent surgery in the Otolaryngology Head and Neck Surgery Department of the Eye, Ear, Nose and Throat Hospital, Fudan University, between November 2008 and 2009. A total of 50 tumor specimens and paired adjacent pathologically confirmed normal mucosa (at least 1 cm from the tumor margin) were collected from patients undergoing total or partial laryngectomy for LSCC. These samples included 1, 16, 21 and 12 patients in stages I, II, III and IV. The detailed clinicopathological characteristics of these patients are summarized in Table I. Peripheral blood samples were obtained from another 38 untreated LSCC patients. None of these patients received chemotherapy or radiotherapy prior to specimen collection. Tumor stage was determined according to the 2002 International Union Against Cancer TNM classification system (30). Blood samples were also obtained from 20 healthy volunteers. All specimens were collected under study protocols approved by the Ethics Committee of Fudan University and all subjects provided written informed consent prior to their inclusion in the study (KJ2007-01).

Table I. Clinicopathological characteristics of LSCC patients.

Variable	Patients (fresh tissue) (N=50) n (%)	Patients (blood) (N=38) n (%)
Age/years		
Mean (range)	60.82 (37-81)	60.92 (41-82)
Gender		
Male	47 (94.0)	37 (97.4)
Female	3 (6.0)	1 (2.6)
Location		
Supraglottic	23 (46.0)	14 (36.8)
Glottic	23 (46.0)	22 (57.9)
Subglottic	4 (8.0)	2 (5.3)
cT stage		
T1+T2	18 (36.0)	26 (68.5)
T3+T4	32 (64.0)	12 (31.6)
pN stage		
N0	34 (68.0)	34 (89.5)
N1+N2	16 (32.0)	4 (10.5)
Clinical grade		
I+II	17 (34.0)	24 (63.2)
III+IV	33 (66.0)	14 (36.8)

LSCC, laryngeal squamous cell carcinoma.

Reagents and kits. The following reagents were used in this study: TRIzol® (15596-018; Life Technologies, USA), PrimeScript™ RT-PCR kit (Perfect Real Time) (DRR063A; Takara, Japan), mouse anti-human CD4-FITC, CD25-PE-Cy5, Foxp3-PE (11-0049, 15-0259, 12-4777; eBioscience, USA), CCR6- and CCR7-Alexa Fluor 647 (BioLegend, USA) monoclonal antibodies (mAb) and their respective isotypes, anti-Human Foxp3 Staining Set PE (72-5774; eBioscience), mouse anti-human CCR6 monoclonal antibody (MAB195; R&D Systems, USA), mouse anti-human CCR7 monoclonal antibody (550937; BD Pharmingen, USA), EnVision™+ Single Reagents (GK400115; Dako, Denmark) and human IL-2/IL-4/IL-10/IL-12p70/interferon (IFN)-γ/TGF-β1 ELISA Ready-SET-Go (eBioscience).

RNA isolation and reverse transcription. Total RNA was extracted from patient frozen tissues using TRIzol reagent according to the manufacturer's instructions. A total of 1 μg of total RNA was reverse transcribed to cDNA in a 20-μl reaction system using PrimeScript RT reagent kit (Perfect Real Time) to prepare the template cDNA, which was then diluted with sterile water and stored at -20˚C. The reverse transcription procedure was performed according to the manufacturer's instructions.

Table II. Primer sequences used for real-time PCR.

Primer name	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')	Product (bp)
CCR6	TGCTCTACGCTTTTATTGGG	TTGTCGTTATCTGCGGTCTC	163
CCR7	GATTACATCGGAGACAACACCA	AGTACATGATAGGGAGGAACCAG	106
CCL19	GGCACCAATGATGCTGAAGAC	GCAGCCATCCTTGATGAGAAG	102
CCL20	CAACTTTGACTGCTGTCTTGGAT	ACTTTTTTACTGAGGAGACGCAC	195
CCL21	CAGCTATCCTGTTCTTGCCC	TTGGAGCCCTTTCCTTTCTT	181
IL-2	AACTCCTGTCTTGCATTGCAC	TGCTCCAGTTGTAGCTGTGTTT	94
IL-10	CTTTAAGGGTTACCTGGGTTG	CACATGCGCCTTGATGTCT	109
IL-12p40	TGGACCTTGGACCAGAGC	CTCGCCTCCTTTGTGACAG	108
TGF-β1	CCCACAACGAAATCTATGACA	AGCAACACGGGTTCAGGT	103
IFN-γ	TCGGTAACTGACTTGAATGTCCA	TCCTTTTTCGCTTCCCTGTT	100
Foxp3	TCCCAGAGTTCCTCCACAAC	ATTGAGTGTCCGCTGCTTCT	122
GAPDH	GAAGGTCGGAGTCAACGGAT	CCTGGAAGATGGTGATGGG	224

CCR, chemokine receptor; IL, interleukin; TGF-β1, transforming growth factor-β1; IFN-γ, interferon-γ; Foxp3, forkhead box P3.

Semi-quantitative real-time PCR. Semi-quantitative real-time PCR for chemokine ligands, receptors and cytokines was performed on an Applied Biosystems 7500 Fast Real-Time PCR System and the data was analyzed using the 7500 software. In brief, 2 μl of cDNA was added in a 20-μl reaction mixture containing 10 μl of 2X SYBR Premix Ex Taq, 0.4 μl forward primer (10 μM), 0.4 μl reverse primer (10 μM), 0.4 μl ROX reference dye and 6.8 μl sterile water. All primers were designed by Primer Premier 5 software, with their specificity confirmed by BLAST on the NCBI webpage (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Detailed information of these primers is listed in Table II. The PCR conditions were: 95˚C for 30 sec, followed by 40 cycles at 95˚C for 5 sec and 60˚C for 25 sec. The expression level of target gene mRNA was normalized by GAPDH and was represented as $100{,}000 \times 2^{-\Delta Ct}$, in which the ΔCt represented the difference between the Ct value of the target gene and GAPDH ($Ct_{target\ gene}$-Ct_{GAPDH}). The real-time PCR products were subjected to 2% (w/v) agarose gel electrophoresis and were stained with ethidium bromide.

Flow cytometry (FCM). The peripheral blood mononuclear cells (PBMCs) were isolated from 5 ml of heparinized blood from the patients with LSCC as well as healthy individuals using Ficoll. Half of these cells were suspended in TRIzol and were stored at -20˚C for future use. The other half was extracellularly stained with specific antibodies against human CD4, CD25, CCR6 or CCR7 for 30 min, fixed, permeabilized with Fixation/Permeabilization solution and intracellularly stained with anti-Foxp3 according to the manufacturer's protocol. Flow cytometry was performed on a BD FACSCalibur and the results were analyzed by CellQuest Pro software. To determine the percentage of $CD4^+CD25^+Foxp3^+$ Tregs, lymphocytes were gated by plotting forward vs. side scatter followed by gating on $CD4^+$ T cells. The gated cells were then analyzed for CD25, Foxp3, CCR6 or CCR7 expression.

Immunohistochemistry (IHC). Paraffin-embedded tissue sections of human LSCC were used for immunostaining followed by standard procedures for the avidin-biotin-peroxidase method. The color reaction was developed in diaminobenzidine solution and the cells were counterstained with hematoxylin solution. Tissue sections were stained using mouse anti-human CCR6 mAb (1:200) and CCR7 mAb (1:200), followed by incubations with secondary Abs. Histopathological evaluation was independently carried out by 2 pathologists. As previously described (31,32), the evaluation of staining was performed based on its intensity and the percentage of stained cells. The staining was ranked as no staining, weak staining, medium staining and strong staining with the values of 0, 100, 200 and 300 assigned to each staining intensity, respectively. The final scores were determined by multiplying the staining values by the percentage of positively stained cells.

Enzyme-linked immunosorbent assay (ELISA). The expression level of IL-2, IL-4, IL-10, IL-12p70, IFN-γ and TGF-β1 was determined with ELISA Ready-SET-Go in the plasma of LSCC patients and healthy volunteers according to the manufacturer's instructions.

Statistical analysis. Statistical analysis was performed with SPSS 13.0 for Windows. The data are reported as mean ± SD or mean ± SE. Statistical significance of the data was assessed using paired or unpaired t-tests, Mann-Whitney U test and one-way ANOVA, where appropriate. $P<0.05$ was considered to indicate a statistically significant difference.

Results

The mRNA expression of CCR6, CCR7 and their ligands in the LSCC group and the paired adjacent normal tissue (ANT) were measured. The CCR6, CCR7, CCL19 and CCL21 mRNA were downregulated in the LSCC tissue, while the CCL20 mRNA (the sole ligand of CCR6) was significantly upregulated as compared to the ANT (Fig. 1A). The CCR6,CCR7 and CCL19 mRNA expression was downregulated in LN(+)

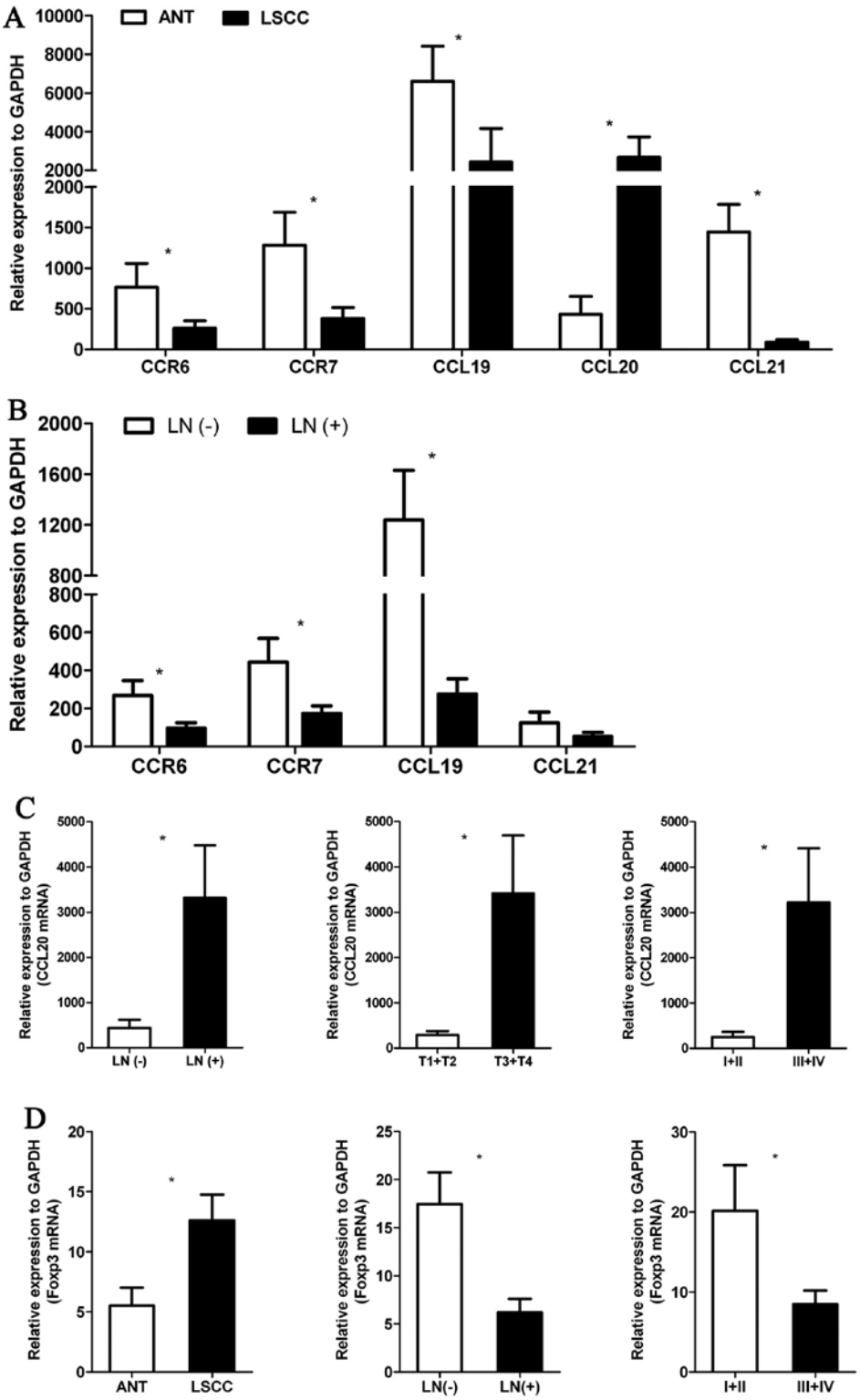

Figure 1. Target gene mRNA expression in adjacent normal tissue (ANT) and laryngeal squamous cell carcinoma (LSCC) tissue. (A) The CCR6, CCR7 and their paired ligand expression level in ANT and LSCC tissue. (B) The CCR6, CCR7, CCL19 and CCL21 expression in lymph node (LN)(-) and LN(+) LSCC tissue. (C) The CCL20 (ligand of CCR6) expression of LSCC tissue in different TNM stage and clinical grade. (D) Expression level of transcription factor forkhead box P3 (Foxp3) in ANT and LSCC tissues grouped by different classification criteria. Results represent mean ± SE (*P<0.05).

subjects (Fig. 1B) in LSCC tissue, while the CCL20 mRNA was increased in LN(+) samples (Table III and Fig. 1C). The CCL20 mRNA expression was higher in T3+T4 and III+IV groups as compared to that of the T1+T2 and I+II groups, respectively (Fig. 1C). The expression of CCL21 mRNA in LSCC tissue showed no significant difference within various pT stages, cN stages and clinical groups (Table III and Fig. 1B). Our data further indicated that the age and tumor localization had no correlation with the expression of CCR6, CCR7 and their ligands.

Table III. Correlation between mRNA expression in cancer and clinical characteristics.

		P- or F-value				
Variable	N	CCR6	CCR7	CCL19	CCL20	CCL21
Age/years						
≤60	25	0.57	0.66	0.32	0.33	0.58
>60	25					
Location						
Supraglottic	23	0.16	0.11	0.39	0.21	0.35
Glottic	23					
Subglottic	4					
cT stage						
T1+T2	18	0.26	0.63	0.04	0.03	0.17
T3+T4	32					
pN stage						
N0	34	0.04	0.04	0.04	0.01	0.08
N1+N2	16					
Clinical grade						
I+II	17	0.26	0.63	0.04	0.01	0.09
III+IV	33					

CCR, chemokine receptor.

Table IV. Correlation between tumor CCR6, CCR7 IHC scores and clinical characteristics.

Variable	N	CCR6 score (mean ± SD)	P-value (mean ± SD)	CCR7 score (mean ± SD)	P-value
Age/years					
≤60	25	155.8±28.2	0.38	178.3±32.1	0.41
>60	25	163.2±29.3		190.4±24.8	
T stage					
T1+T2	18	131.8±23.4	0.03	147.5±23.2	0.02
T3+T4	32	175.1±19.6		205.1±27.6	
N stage					
N0	34	152.5±19.7	0.02	173.7±15.7	0.03
N1+N2	16	174.4±12.8		207.0±12.8	
Clinical grade					
I+II	17	143.7±16.7	0.02	158.3±15.6	0.03
III+IV	33	167.6±20.2		197.8±17.3	

CCR, chemokine receptor; IHC, immunohistochemistry.

Immunostaining of CCR6 and CCR7 in LSCC and LN. To study the chemokine receptor expression *in vivo*, the paraffin-embedded tissue sections were stained for CCR6 and CCR7 by IHC (Fig. 2). Differential CCR6 and CCR7 expressions in LSCC and metastatic LN were confirmed. The results indicated that the primary and metastatic cancer cells expressed both CCR6 and CCR7. Co-expression of CCR6 and CCR7 was found in 40/50 primary and 15/16 lymphatic metastatic cancer samples. Using semi-quantitative histopathological evaluation, our results indicated that CCR6 and CCR7 were upregulated significantly once the metastasis occurred. Similarly, both CCR6 and CCR7 expression levels were higher in T3 + T4 stage and III + IV group as compared to those of the T1 + T2 stage and I + II group, respectively (Table IV). Notably, the data from 16 LN(+) LSCC patients showed that CCR6 and CCR7 scores of primary cancer were higher (stronger staining)

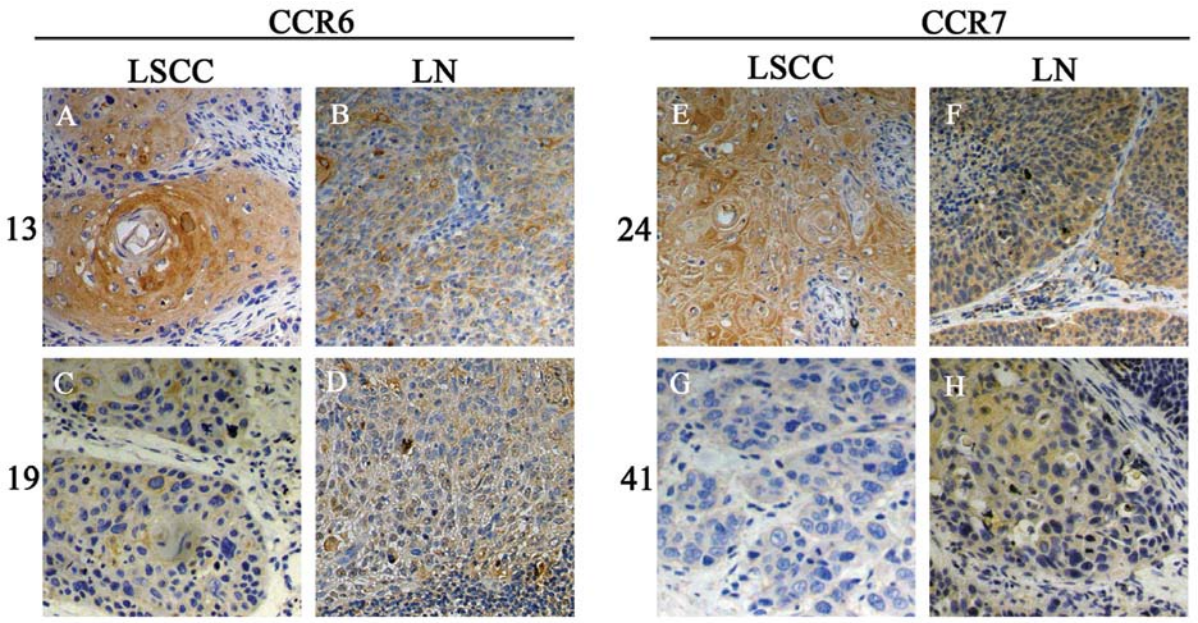

Figure 2. (A-D) CCR6 and (E-H) CCR7 expressions in laryngeal squamous cell carcinoma (LSCC) tissue and lymph node (LN) by immunohistochemistry; (A, C, E and G) were from LSCC, while (B, D, F and H) were from LN tissues. The metastatic LN tissue showed stronger positive staining when the paired LSCC showed stronger staining. Data are representative of 4 LSCC patients (no. 13, 19, 24 and 41).

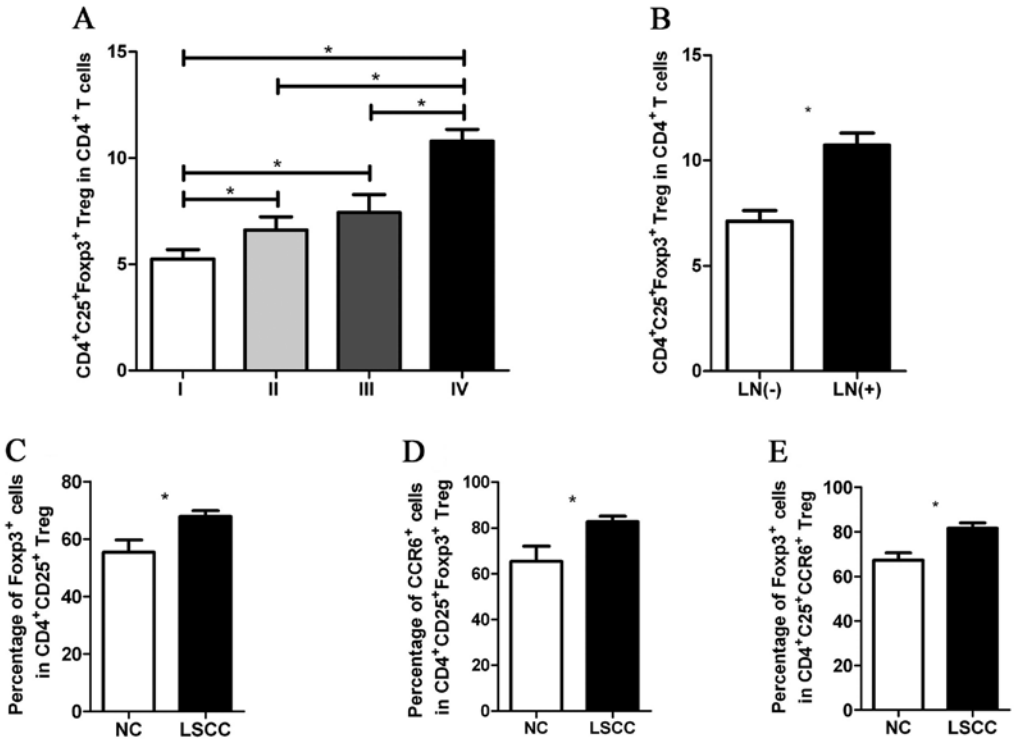

Figure 3. The $CD4^+CD25^+Foxp3^+$ Treg proportion and their CCR6 positive components were detected by FACS. (A) The percentage of $CD4^+CD25^+Foxp3^+$ Treg in $CD4^+$ T cells increased with the progress of clinical stage. (B) The percentage of $CD4^+CD25^+Foxp3^+$ Treg was higher in lymph node (LN) positive laryngeal squamous cell carcinoma (LSCC) patients. (C) The percentage of forkhead box $P3^+$ ($Foxp3^+$) cells in $CD4^+CD25^+$ T cells of normal control (NC) and LSCC patients. (D) The LSCC patients had a higher frequency of $CCR6^+$ cells in $CD4^+CD25^+Foxp3^+$ Treg. (E) More $CD4^+CD25^+CCR6^+$ Tregs expressed Foxp3 in LSCC patients. Results are represented as mean ± SE ($^*P<0.05$).

in patients with a higher score (stronger staining) of CCR6 or CCR7 in their metastatic LN (Fig. 2).

We divided the 16 LN(+) LSCC patients into 2 groups according to the IHC score of tumor CCR6 or CCR7 respectively, CCR6 high expression group (tumor CCR6 score >174.4) and CCR6 low expression group (tumor CCR6 score <174.4); CCR7 high expression group (tumor CCR7 score >207.0) and CCR7 low expression group (tumor CCR7 score <207.0).

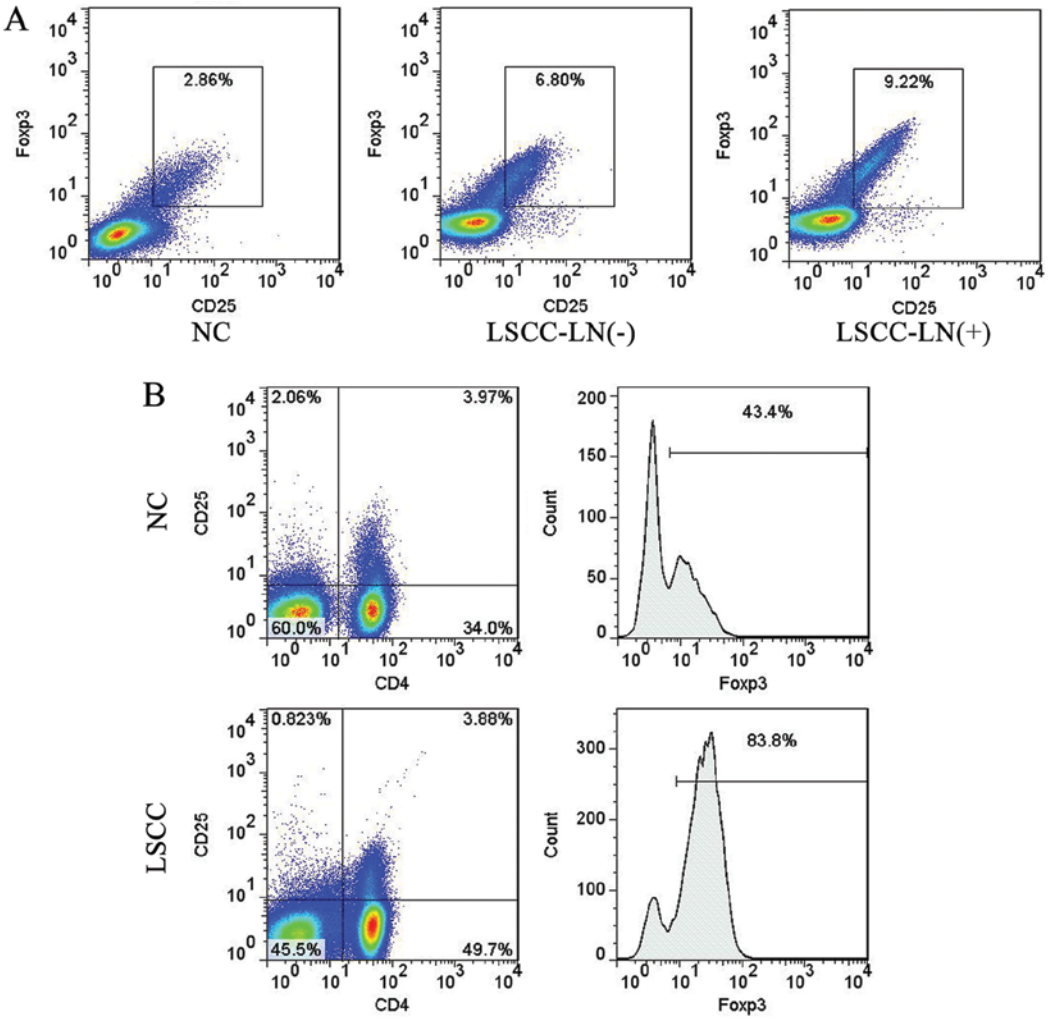

Figure 4. The frequency of $CD4^+CD25^+Foxp3^+$ Tregs in normal control (NC), lymph node (LN) negative laryngeal squamous cell carcinoma (LSCC) and LN positive LSCC. (A) The LN (+) LSCC had the highest frequency of $CD4^+CD25^+Foxp3^+$ Treg, while the NC had the lowest. Data are representative of 3 subjects. (B) The proportion of $Foxp3^+$ Treg increased in the LSCC patients. Data are representative of two subjects. Foxp3, forkhead box P3.

CCR6 or CCR7 high expression group had a higher score of their expression in LN, compared with their low expression group, respectively (Fig. 2).

$CD4^+CD25^+Foxp3^+$ Treg analysis and CCR6, CCR7 expression patterns detected by FACS. In order to understand the role of $CD4^+CD25^+Foxp3^+$ Tregs and the potential function of CCR6 and CCR7 on Tregs, flow cytometry was used to investigate the proportion of $CD4^+CD25^+Foxp3^+$ Tregs in peripheral blood and the CCR6, CCR7 expression pattern in them in LSCC patients. The frequency of circulating $CD4^+CD25^+Foxp3^+$ Tregs in the LSCC patients (7.55±2.82% of the $CD4^+$ T population) was significantly increased as compared to the normal controls (NCs) (3.91±1.81% of the $CD4^+$ T population). Furthermore, the frequency of $CD4^+CD25^+Foxp3^+$ Tregs was compared among different groups. The distribution of $CD4^+CD25^+Foxp3^+$ Tregs was calculated in 4 clinical groups and is presented in Fig. 3A. The frequency of the Tregs was increased with the clinical group progression (Fig. 3A). Furthermore, the frequency of Tregs increased in LN(+) LSCC patients (10.73±0.81% of the $CD4^+$ T population) as compared to LN(-) LSCC patients (7.13±2.32% of the $CD4^+$ T population) (Figs. 3B and 4A). Further analysis of the data showed that the ratio of Foxp3 in the $CD4^+CD25^+CCR6^+$ and $CD4^+CD25^+$ T-cell subpopulation was higher in LSCC patients than in LNs (Figs. 3C, E and 4B). Meanwhile, the $CD4^+CD25^+Foxp3^+$ Tregs of LSCC patients had a higher CCR6 expression ratio (Fig. 3D).

The Foxp3 gene expression pattern in ANT and LSCC tissues. Foxp3, a valid marker of regulatory T cells, was thoroughly investigated by real-time RT-PCR. Our results showed a significant increase of Foxp3 expression in LSCC tissue (Fig. 1D). As Tregs are known to be able to suppress the induction of effective antitumor immunity, this result was consistent with our expectations. However, further analysis showed that Foxp3 expression of LSCC tissue from LN(+) patients was downregulated as compared to that of the LN(-) patients. The same trend was also observed between the early and advanced LSCC (Fig. 1D).

Cytokine profiles in ANT and LSCC tissues and plasma. A series of cytokines, including IL-2, IL-10, IL-12, IFN-γ and TGF-β1, were detected by real-time RT-PCR and ELISA.

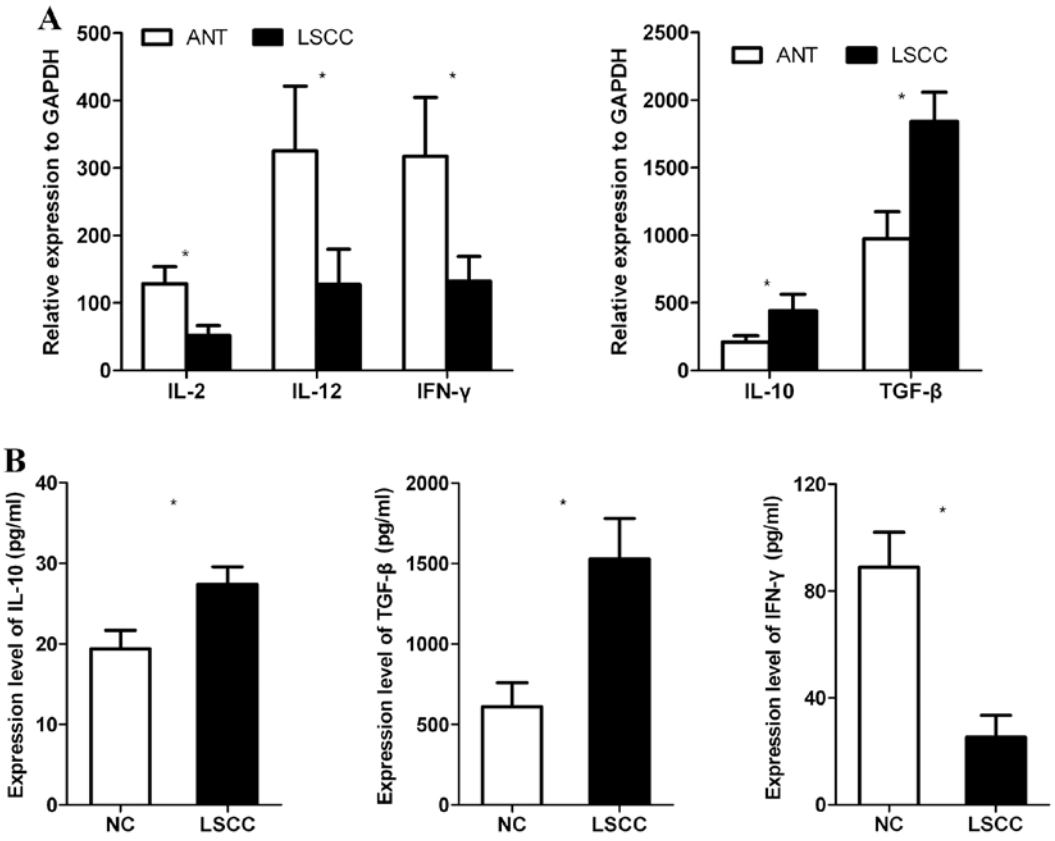

Figure 5. Cytokine profiles in adjacent normal tissue (ANT), laryngeal squamous cell carcinoma (LSCC) and plasma. (A) Relative expression levels of interleukin (IL)-2, IL-12p40, interferon (IFN)-γ, IL-10 and transforming growth factor (TGF)-β1 were detected by real-time RT-PCR. The IL-10 and TGF-β1 mRNA expression level of LSCC tissue increased, while IL-2, IL-12p40 and IFN-γ decreased as compared to the matched ANT tissue. (B) Cytokine expression pattern in plasma of normal control (NC) and LSCC patients was determined by ELISA. The LSCC patients had a higher level of IL-10 and TGF-β1, and a lower level of IFN-γ in plasma (*P<0.05).

Real-time RT-PCR showed that the immune suppressive cytokine IL-10 and TGF-β1 of LSCC tissue were upregulated, while IL-2, IL-12 and IFN-γ were downregulated, as compared to those of the ANT (Fig. 5A). ELISA was used to detect the cytokine expression levels at the protein level. Our results also showed that IL-10 and TGF-β1 expression was increased in the plasma of LSCC patients, while the IFN-γ expression was decreased (Fig. 5B).

Discussion

Until the 1980s, total laryngectomy was considered the most appropriate therapy for patients with locally advanced laryngeal and hypopharyngeal cancer. Although this strategy did help to achieve a better disease control, it had a significant negative impact on patient quality of life due to the presence of a permanent tracheostomy and the loss of natural voice (33). Therefore, non-surgical treatment of LSCC became a hot topic in head and neck cancer (34-38). Cervical LN is the first stop of metastatic laryngeal cancer cells, which plays a leading role in its prognosis. Therefore, it is reasonable to identify LN metastases at an early stage in LSCC.

Our data showed that CCR6 and CCR7 mRNA expression levels were not increased and they were even significantly decreased in LN(+) patients. Although the CCR6 and CCR7 proteins were both expressed in ANT (data not shown), their expressions were shown to have some significance in the development of this cancer in LSCC patients. The immunostaining results showed that the score of advanced stage samples (T3 + T4 or III + IV or LN positive) was higher than that of the early stage samples (T1 + T2 or I + II or LN negative). Moreover, the strong staining of CCR6 and CCR7 in LN indicated a more advanced stage of LSCC. This could be used as a potential marker to assess the condition and prognosis, and may help in choosing the most suitable treatment for LSCC patients. Considering the downregulation of CCR6 and CCR7 mRNA expression levels, we speculated that the expression of CCR6 and CCR7 was more sensitive in LSCC tissue for the assessment of LSCC. Unlike Wang *et al* (21), we found a heavier staining of CCR7 in metastatic LN as compared to that of the primary LSCC tissue. However, we did not find a decrease in CCR6 expression at the protein level. To some extent, this finding could be the result of the difference in composition of the subjects.

Real-time RT-PCR showed an increased expression of CCR6, CCR7, CCL19 and CCL21 and a decreased expression of CCL20 in ANT, which may have indicated that CCR7 played a more significant role in local infiltration and metastasis as compared to CCR6. The distinction of CCR7 was reinforced by its expression pattern in LN(+) and LN(-) LSCC tissue. Our results further showed that the CCL20 expression was elevated in cancer tissues with a higher level in metastatic and advanced

cancer patients. It has been reported that CCL20 stimulates the cell proliferation and their adhesion to collagen in various tumor cells. Furthermore, overexpression of CCL20 in tumor cells promoted the growth and adhesion *in vitro* and increased tumor growth and invasiveness *in vivo*. Moreover, neutralizing antibodies to CCL20 inhibited the *in vivo* growth of tumors that either overexpressed CCL20 or naturally expressed CCL20 (39,40). Previous studies have showed that LN, spleen, tonsil T zone and lymphatic endothelial cells, which expressed CCL19 and CCL21 attracted the $CCR7^+$ cells (17). Increasing evidence has demonstrated the role of CCR7 in LN metastasis, in oral, gastric, esophageal and lung cancer (41-43). Although CCR6 has been reported to be involved in hepatocellular carcinoma metastasis (44), its role in LSCC was not the same. Therefore, we speculated that CCR6 and CCR7 may have a different effect on the progression and metastasis of LSCC, where CCR6 could conduct the proliferation of LSCC cells, while CCR7 could mediate the migration and metastasis.

Previous studies indicated that CCR2, CCR4, CCR5, CCR6, CCR7, CCR8 and CXCR4 are expressed in $CD4^+CD25^+$ Treg (20,45-47), and they may participate in the process of $CD4^+CD25^+$ Treg migration, homing and selective immune response. The frequency of Treg cells in this study was significantly elevated in $CD4^+$ T cells in LSCC patients as compared to the healthy controls, and was positively correlated with the disease progression or the tumor burden. Further analysis demonstrated that the percentage of Foxp3 positive $CD4^+CD25^+$ Treg and $CD4^+CD25^+CCR6^+$ Treg was elevated in LSCC, which indicated that $CD4^+CD25^+Foxp3^+$ Tregs may be induced and expanded in LSCC patients. The $CD4^+CD25^+Foxp3^+$ Tregs suppressed the activation, proliferation and effector functions such as cytokine production in a wide range of immune cells, including $CD4^+$ and $CD8^+$ T cells, natural killer (NK) and NKT cells, B cells and antigen-presenting cells (APCs) *in vitro* and *in vivo* (12). These results clearly showed that $CD4^+CD25^+Foxp3^+$ Tregs were involved in the LSCC progression and metastasis. Continuous proliferation of Tregs gradually strengthened the suppression of immune system and induced an immune tolerance status, which favored the LSCC progression and metastasis. Similar results were demonstrated in several other types of cancer (48-50). Furthermore, 82.70±15.08% of the $CD4^+CD25^+Foxp3^+$ Tregs expressed CCR6 in LSCC, while this percentage was 65.43±22.71% in NCs. There was no difference in CCR7 expression on $CD4^+CD25^+Foxp3^+$ Tregs between the LSCC and NC groups, although >50% of the $CD4^+CD25^+Foxp3^+$ Tregs expressed CCR7 (data not shown). Xu *et al* (14) demonstrated, in a mouse breast cancer model, that dendritic cells (DCs) in the tumor masses induced the proliferation of $CCR6^+$ Tregs through TGF-β. This finding was in line with our results reporting a high percentage of CCR6 expression on $CD4^+CD25^+Foxp3^+$ Tregs in LSCC patients. As CCL20 mRNA was highly expressed in the LSCC tissue, the $CCR6^+$ Tregs may have been attracted and cumulated in the center of LSCC, which may have formed a more suppressive microenvironment. However, this requires further investigation to confirm whether the CCL20 had an effect on the proliferation of $CCR6^+$ Tregs.

Several mechanisms of $CD4^+CD25^+Foxp3^+$ Treg-mediated suppression have been proposed. It is believed that there are 2 main types of mechanisms for contact-dependent suppression including i) downregulation of APC co-stimulatory function, interaction with CD80 and CD86 on conventional T cells and conventional T cell lysis, and ii) cytokine-mediated suppression including attenuation of DC function, conversion of conventional T cells to Tr1 cells, cell cycle arrest and apoptosis in conventional T cells (13). In this study, IL-2, IL-12 and IFN-γ mRNA levels were decreased in cancer tissue, while IL-10 and TGF-β1 mRNA levels were increased as compared to those of the ANT. The ELISA assays showed that IFN-γ protein level was reduced to 25.32 pg/ml in the plasma of LSCC patients, while the IL-10 and TGF-β1 protein levels were increased to 27.38 and 1,527.00 pg/ml, respectively, and IL-2, IL-4 and IL-12p70 were not detectable. The results of this study showed that the Th1/Th2 cytokine responses were skewed toward a Th2 bias in the plasma of patients with LSCC as compared to the healthy controls. Furthermore, suppressive cytokines (IL-10 and TGF-β1) played a key role in forming an immunosuppressive status for LSCC patients. These results were consistent with Strauss *et al* (51).

Transcription factor Foxp3 was thought to be a special marker of Tregs (52); however, recent studies demonstrated that Foxp3 was also expressed in cancer cells (53,54). Our study showed a high expression level of Foxp3 in LSCC tissue at the genomic level, but a decreased expression in LN(+) and advanced LSCC patients. Although protein expression level and cellular localization of Foxp3 in LSCC remain to be identified, Ladoire *et al* (55) demonstrated that the Foxp3 upregulation was closely related to a better prognosis in breast cancer, which indicated a high predictive value for Foxp3. We speculated that the Foxp3 gene may be involved in certain tumor-suppressing mechanisms in association with tumor metastasis.

Although the chemokine system-based research of tumor immunology has made some progress, the results from different tumors are not fully compatible (21,56-58). This illustrated not only the complexity of the chemokine system, but also the unique characteristics of different types of cancer. In this study, several methods were used to detect the expression of CCR6, CCR7 and their ligands, the expression patterns of CCR6 and CCR7 on $CD4^+CD25^+Foxp3^+$ Tregs, as well as the cytokine profiles of LSCC patients. The present study revealed that CCR6 and CCR7 may directly mediate the migration of cancer cells and induce immune tolerance by recruiting $CD4^+CD25^+Foxp3^+$ Tregs to cancer sites in order to form a particular cancer microenvironment in favor of LSCC initiation, invasion and metastasis. These results could function as a foundation to further explore a chemokine system-based cancer intervention strategy.

Since Müller *et al* (26) reported the involvement of chemokine receptor in tumor growth and progression in 2001 and the identification of regulatory T cells by Sakaguchi *et al* in 1995 (59), significant progress has been made in the field of cancer pathogenesis, prevention and treatment (60). The CCR6, CCR7, their paired ligands and the ligand-receptor interaction bridged the gap between LSCC cells and $CD4^+CD25^+Foxp3^+$ Tregs. Therefore, they may directly or indirectly be involved in tumor progression and should be evaluated as novel candidate target molecules for specific treatment interventions as well as prognosis assessments in LSCC treatment.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (30801283, 30972691), the Shanghai Science and Technology Development Funds (09QA1401000, 10QA1405900), the Training Program of the Excellent Young Talents of the Shanghai Municipal Health System (XYQ2011055, XYQ2011015) and the Shanghai Municipal Science and Technology Foundation (11JC1410802).

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