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Original article

The role of CCL20/CCR6 axis in recruiting Treg cells to tumor sites of NSCLC patients

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ABSTRACT

Inflammatory chemokine CCL20 and its receptor CCR6 have been reported to correlate with colorectal cancer patients' metastasis. However, the role of CCL20 in patients with NSCLC is not well defined. In this study, we detected the expression of CCL20 in tumor samples and corresponding adjacent ones ($n = 71$) from patients with NSCLC using RT-PCR and observed that CCL20 showed higher expression in tumor samples (0.28 ± 0.17) than in adjacent ones (0.20 ± 0.13) ($n = 71$, $P = 0.0056$), which was also verified in protein level using IHC. Analysis results showed that CCL20 expression was positively associated with CD4 ($n = 80$, $P = 0.0046$), Foxp3 ($n = 80$, $P = 0.0020$) and IL-10 ($n = 61$, $P = 0.0003$) in tumor samples. And the flow data showed that Treg cells accumulated in TIL (MFI: 961 ± 760) compared with PBMC (MFI: 683 ± 460) ($n = 40$, $P = 0.0046$); and the percentage of CCR6 – the sole receptor of CCL20 – on Treg cells was higher in TIL (MFI: 1311 ± 1268) than in PBMC (MFI: 976 ± 780) ($n = 40$, $P = 0.0219$). It was interesting to find that the expression of CCL20 in tumor sites was almost 1.5-fold higher in samples from high-stage patients (III–IV stage, 0.34 ± 0.17) compared with those from low-stage patients (I–II stage, 0.22 ± 0.11) ($P = 0.0056$). Furthermore, the higher expression of CCL20 was associated with a lower overall survival ($P = 0.0198$). The IHC data showed that tumor cells were the main source of CCL20, and after treated cell line A549 with docetaxel, we found that the secretion of CCL20 was decreased heavily ($n = 3$, $P = 0.0046$). Our results demonstrated that CCL20 cooperated with CCR6 could recruit Treg cells to tumor sites, and chemotherapy medicine docetaxel could decrease the expression of CCL20.

1. Introduction

As one of the most common human cancers worldwide, lung cancer is characterized as chronic inflammatory response [1]. Among all lung cancer cases, non-small cell lung cancer (NSCLC) as a primary carcinoma of lung, occupies almost 85% and its 5-year survival still less than 5% [2] [3] [4]. Clinically, chemotherapy plays an important role in curing patients with NSCLC [5]. Recently it has been reported that besides inducing death of tumor cells directly, chemotherapy medicines could also change the profiling of chemokines expression by tumor cells [6], which may in turn have effects on T cells distribution.

Chemokines (CKs) are a superfamily of small (8–14 kDa), secreted proteins that mainly regulate leukocyte trafficking via interacting with their corresponding receptors [7–11]. Until now, more than 50 chemokines and 20 chemokine receptors have been identified in human genes [12]. According to the number and position of Cysteine residues in *N*-terminal, chemokines are classified into 4 subfamilies (CXC, CX3C, CC, and C) [13]. Many studies have shown that chemokines could recruit immune cells in a dose-dependent manner [14–16].

The CC chemokine CCL20, also named as macrophage infiltrating factor protein-3α (MIP-3 α) or liver activation regulated chemokine (LARC) [17], has been revealed to involve in the advancement of many types of cancers via cooperating with its sole receptor CCR6 [18,19]. However, the role of CCL20 in patients with NSCLC was still unrevealed. The aim of this study was to determine the role of CCL20-CCR6 interactions in patients with NSCLC and its clinical significance.

2. Materials and methods

2.1. Collection of samples

Tumor tissues ($n = 80$) and corresponding adjacent ones ($n = 71$) were obtained from patients with NSCLC who had

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Table 1
Clinical characteristics of patients with NSCLC.

Patients characteristics ($n = 80$)	Number	Percent (%)
Age at diagnosis		
< 65 years old	48	60.00
≥ 65 years old	32	40.00
Gender		
Male	42	52.50
Female	38	47.50
Clinical stage number		
I–II	55	68.75
III–IV	25	31.25
Histological grade		
G1	10	12.50
G2	40	50.00
G3	30	37.50
Lymph node metastasis		
Positive	33	41.25
Negative	47	58.75

undergone operation in the First Affiliated Hospital of Zhengzhou University. Clinico-pathological features of the 80 NSCLC patients were listed in Table 1. Also, paraffin-embedded samples were collected for detection of CCL20 in protein level and analyze the significance of CCL20 on patients' survival. All patients gave written informed consent, and the First Affiliated Hospital of Zhengzhou University's ethics committee approved the study protocol.

2.2. Preparation of peripheral blood monocyte cells (PBMCs) and tumor-infiltrating cells (TILs)

As described in the paper, about 20 mL of peripheral blood were taken into Na–heparin anti-coagulated tubes from patients with NSCLC. Then, PBMCs were obtained from blood samples by performing Ficoll-Hypaque (d = 1077 g/mL; Sigma) and centrifuged at 2500 rpm for 25 min. The PBMC layer was washed twice using RPMI 1640 medium.

TILs were isolated using methods described previously [20]. Briefly, tumor samples were cut into pieces in 2 h after collection, washing the tissues twice with RPMI 1640 medium and then cultured in the 24-well plate for 2 weeks with IL-2 stimulation. Following centrifuged the medium at 1800 rpm for 10 min to obtain the cells.

Approximately 10^6 PBMCs or TILs were re-suspended in 1 mL of solution for freezing [50% RPMI 1640 medium, 40% inactivated FBS (Gibco, Invitrogen Co.) plus 10% DMSO (Sigma)] and stored initially at both 4° C and –20° C for 1 h before being introduced into liquid nitrogen, and aliquots were cryopreserved for later research.

2.3. RNA extraction and quantitative reverse transcription-PCR analysis

Using Trizol reagent, total RNA was extracted from fresh tumor and adjacent tissues according to the manufacture's protocol (Invitrogen, Carlsbad, CA, USA). After the concentration was detected by Nano drop2000, 2 μg extracted RNA was reverse-transcribed to cDNA according to the manufacture's protocol (TaKaRa Bio Inc., Dalian, China).

We chose specific primers for PCR amplification (Table 2). The expression of CCL20/CD4/Foxp3/IL-10 was determined by PCR (Agilent Technologies) using Premix according to manufacturer's instruction (TaKaRa Bio Inc., Dalian, China). House-keeping gene GAPDH was examined under identical conditions.

The products were subjected to electrophoresis on 1.5% agarose gels and the electrophoresis images were scanned by UV spectrophotometry (Beckman Coulter Inc., Brea, CA, United States). Results were quantified using the Image J analysis software and normalized to the expression of GAPDH.

2.4. Immunohistochemistry for CCL20

Formalin-fixed and paraffin-embedded tissues were cut as 3 μm thickness. The slides were deparaffinised and rehydrated. After antigen retrieval, antigenicity was performed by heating the tissue in citrate buffer; tissues were then blocked with 3% H_2O_2 for half an hour at room temperature followed by incubation with goat serum for another 30 min at room temperature. Samples were then incubated with human CCL20-specific IgG antibody (1:50, abcam) in dark box at 4 °C overnight, followed by treatment with secondary antibody (ZSGB-BIO). For negative control, samples were incubated with PBS instead of specific antibody. After washing 4 times with PBS, the sections were incubated with biotinylated HRP labeled streptavidin (ZSGB-BIO). Reaction was revealed with 3,3-diaminobenzidine (DAB, ZSGB-BIO) as a peroxidase substrate and the sections were counter-stained with Meyer's hematoxylin. Negative controls were performed in the same conditions. Slides were evaluated using a Nikon ECLIPSE E600 microscope with the Cool SNAP-Procf Color camera. In each case, 5 images were randomly acquired by Image Pro software, and the percentage of positive cells was quantified (number of positive cells/5 fields). The results were evaluated and expressed as IHC scores, where IHC score = percentage of positive cells × staining intensity.

2.5. Flow cytometry

PBMC were separated from blood samples by performing Ficoll-Hypaque (Invitrogen, Carlsbad, CA, USA) and washed twice in PBS for detection. Fresh tissues were rinsed with RPMI 1640 (Life Technologies) to remove traces of blood. TILs were obtained as described [21,22]. Six-colored BD FACS CaliburTM flow cytometer (BD Biosciences) was used to analyze cell surface immunostaining. 2×10^5 cells were re-suspended in PBS and stained with the following antibodies: PE-Cy7 conjugated anti-CD3, APC-Cy7-conjugated anti-CD4, APC-conjugated anti-CCR6, and PerCp-Cy5-conjugated anti-7-AAD antibodies (antibodies purchased from BD Bioscience), for 15 min in the dark at 4 °C; then PE-conjugated anti-Foxp3 was conducted using the human Regulatory T cell staining kit from eBioscience according to the manufacturer's protocol. The cells were washed once for detection. Data was analyzed using Diva and Flowjo software.

Table 2
Primers of RT-PCR analysis.

Gene	Froward primer (5′–3′)	Reverse primer (5′–3′)	Amplication (bp)
GAPDH	GGAGCCAAAAGGGTCATCATCTC	GAGGGGCCATCCACAGTCTTCT	233
CCL20	ATTGTGCGTCTCCTCAGTAAAAA	TGTGATGCTTAAACAAAGCAAAC	225
CD4	AACCGGGGAGTCCCTTTTAG	GAGTCAGCGCGATCATTCAG	242
FoxP3	TCCAGGACAGGCCACATTTC	GGGATTTGGGAAGGTGCAGA	236
IL-10	ACATCAAGGCGCATGTGAAC	GCCACCCTGATGTCTCAGTT	241