Inhibition of CCR7 promotes NF-κB-dependent apoptosis and suppresses epithelial-mesenchymal transition in non-small cell lung cancer

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Received August 12, 2016; Accepted September 12, 2016

DOI: 10.3892/or.2017.5524

Abstract. Activation of C-C chemokine receptor type 7 (CCR7) has been demonstrated to mediate the occurrence and progression of non-small cell lung cancer (NSCLC). However, the potential therapeutic role of CCR7 inhibition in NSCLC is still obscure. Thus, the present study was conducted to investigate the molecular mechanism underlying the inhibition of CCR7 on cell apoptosis and epithelial-mesenchymal transition (EMT) in NSCLC A549 cells. Chemokine ligand 21 (CCL21) was used to activate CCR7 and the results revealed that CCR7 upregulation inhibited cell apoptosis and affected apoptosis-related protein levels. However, CCR7-siRNA treatment markedly promoted apoptosis and suppressed inflammatory response and transforming growth factor β1 (TGF-β1)-induced EMT. In addition, CCR7-siRNA inactivated the NF-κB signaling pathway and inhibition of NF-κB via its specific antagonist, pyrrolidine dithiocarbamate, indicated that NF-κB was involved in the CCR7-mediated apoptosis. In conclusion, upregulation of CCR7 promoted cell proliferation and inflammation in A549 cells. In conclusion, inhibition of CCR7 via siRNA treatment promoted cell apoptosis and suppressed the inflammatory response and TGF-β1-induced EMT, which may be associated with NF-κB signaling.

Introduction

Lung cancer is the second most common lung tumor in humans and is characterized by uncontrolled cell growth in tissues of the lung and has been reported to be the major cause of cancer-related mortality in China (1). Non-small cell lung cancer (NSCLC) is a type of epithelial lung cancer and accounts for approximately 85% of all lung cancers (2). NSCLC patients undergoing complete resection have a 40-70% 5-year overall survival and chemotherapy administered after complete resection improves overall survival at 5 years by approximately 5% (3). Therefore, studies concerning the molecular mechanisms underlying the occurrence and progression of NSCLC may have a significant impact on the systematic treatment of this disease.

C-C chemokine receptor type 7 (CCR7), a G protein-coupled receptor, is mainly expressed on immune cells and mediates leukocyte adhesion and chemotaxis from peripheral sites of inflammation through lymphatic channels to secondary lymphoid organs (4,5). Recently, the role of CCR7 in tumorigenesis has attracted attention in oncology research, as aberrant CCR7 expression has been identified in certain tumor types and has been linked to pro-survival and invasive pathways. Hong *et al* reported that CCR7 is highly expressed in gallbladder cancer and mediates the TNF-α-induced lymphatic metastasis of gallbladder cancer (6). In NSCLC, CCR7 activation by its specific ligand, exogenous chemokine ligand 21 (CCL21), prevented apoptosis by upregulating the expression of Bcl-2 and inhibiting the expression of Bax and caspase-3 in NSCLC A549 and H460 cells (7). Thus, CCR7 may serve as a novel prognostic biomarker and therapeutic target for NSCLC. However the effect of CCR7 downregulation in NSCLC has not been well characterized. In this study, we investigated the effects of the upregulation and silencing of CCR7 on cell apoptosis and the potential signaling mechanism in human lung cancer cell line A549. In addition, transforming growth factor β1 (TGF-β1) was used to induce epithelial-mesenchymal transition (EMT) in A549 cells and the function of CCR7 in EMT was also investigated.

Materials and methods

Reagents. Anti-GAPDH antibody (ab8245), anti-PCNA antibody (ab29), anti-Akt antibody (ab8805), anti-p-Akt

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Key words: CCR7, NF-κB, apoptosis, EMT, NSCLC

Table I. Primers used in this study.

Gene	Accession no.	Nucleotide sequence of primers (5'-3')	Size (bp)
β-actin	NM_007393.3	F: GTCCACCTTCCAGCAGATGT R: GAAAGGGTGTAAAACGCAGC	117
IL-1β	NM_008361.3	F: CTGTGACTCGTGGGATGATG R: GGGATTTTGTCGTTGCTTGT	213
IL-6	NM_031168.1	F: TGCAAGAGACTTCCATCCAGT R: GTGAAGTAGGGAAGGCCG	116
IL-10	NM_010548.2	F: ACAGCCGGGAAGACAATAAC R: CAGCTGGTCCTTTGTTTGAAAG	116
IL-17	NM_010552.3	F: TACCTCAACCGTTCCACGTC R: TTTCCCTCCGCATTGACAC	119
TNF-α	NM_013693.2	F: AGGCACTCCCCCAAAAGAT R: TGAGGGTCTGGGCCATAGAA	192

F, forward primers; R, reverse primers.

antibody (ab131443), anti-IκBα antibody (ab32518), anti-NF-κB p65 antibody (ab86299), anti-CCR7 antibody (ab32527), anti-p53 antibody [DO-1] (ab1101), anti-Bax antibody [E63] (ab32503), anti-Bcl-2 antibody [E17] (ab32124), anti-caspase-3 antibody (ab13847), anti-vimentin antibody [RV202] (ab8978), anti-N-cadherin antibody (ab18203), anti-E-cadherin antibody [HECD-1] (ab1416), and anti-keratin antibody [C-11] (ab118817) were obtained from Abcam (Cambridge, UK). Pyrrolidine dithiocarbamate (PDTC) was purchased from Calbiochem (San Diego, CA, USA).

Cell culture. Human lung cancer A549 cells were cultured in DMEM-F12 supplemented with 10% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD, USA) and 1% penicillin in a humidified 5% CO_2 atmosphere at 37˚C. Cells were seeded at $1x10^4$ cells/well into 96-well plates and allowed to attach overnight. Cell viability was assessed by the CKK-8 assay (Sigma-Aldrich, St. Louis, MO, USA). Briefly, cells were treated with 1, 10, 20, 50, 100 and 200 nM CCL21 (Peprotech, Rocky Hill, NJ, USA) for 24 h and then assayed.

CCR7-siRNA tranfection. Human CCR7-siRNA was obtained from Guangzhou RiboBio Co. Ltd., (Guangzhou, China) and the sequences were in accordance with a previous study (7). Cells were cultured in 6-well plates and grown to 30-50% confluence before transfection. The duplexes were diluted to give a final concentration of 30 nM. The siRNA was transfected into cells using Lipofectamine RNAiMAX reagent according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA, USA).

Western blotting. Total proteins from 10-cm dishes (10^6-10^7 cells) were extracted using protein extraction reagents (Thermo Fisher Scientific Inc., Waltham, MA, USA). The nuclear proteins from 10-cm dishes (10^6-10^7 cells) were extracted using a CelLytic™ NuCLEAR™ extraction kit (Sigma-Aldrich). Proteins samples were quantified using a BCA protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, China) and denaturated with SDS-PAGE sample loading buffer (Beyotime). Proteins (30-50 μg) were separated by reducing SDS-PAGE electrophoresis. Then the proteins were transferred onto a PVDF membrane (Millipore, Billerica, MA, USA) and blocked with 5% non-fat milk in Tris-Tween buffered saline buffer for 1.5 h. The primary antibody was incubated overnight at 4˚C and the HRP-conjugated secondary antibodies were subsequently incubated for 2 h at room temperature. Then the blots were developed on the membrane using Alpha Imager 2200 software (Alpha Innotech Corporation, San Leandro, CA, USA). We digitally quantified the resultant signals and normalized the data to GAPDH or PCNA abundance.

Real-time PCR. Inflammatory cytokines were quantitatively detected by real-time PCR. Approximately $1x10^6$ cells/ml from each group were collected from 6-well plates for real-time PCR detection. The gene sequences were used to design primers and were synthesized by Invitrogen Life Technologies (Table I). Double-distilled water was used instead of a template as a negative control. The number of β-actin transcripts was used as a reference of endogenous RNA, and the quantification of test genes for each sample was standardized relative to the number of β-actin transcripts. The $2^{-\Delta\Delta Ct}$ cycle threshold formula was used to calculate the relative abundance of the transcripts.

Statistical analysis. All data were analyzed by SPSS 17.0 software. Differences were assessed by Ducan's multiple comparison test. Data are expressed as the mean ± SEM. Values in the same row with different superscripts are significant ($P<0.05$).

Results

Effects of CCR7 on cell apoptosis in A549 cells. CCL21 is a ligand of CCR7 and has been suggested to upregulate CCR7

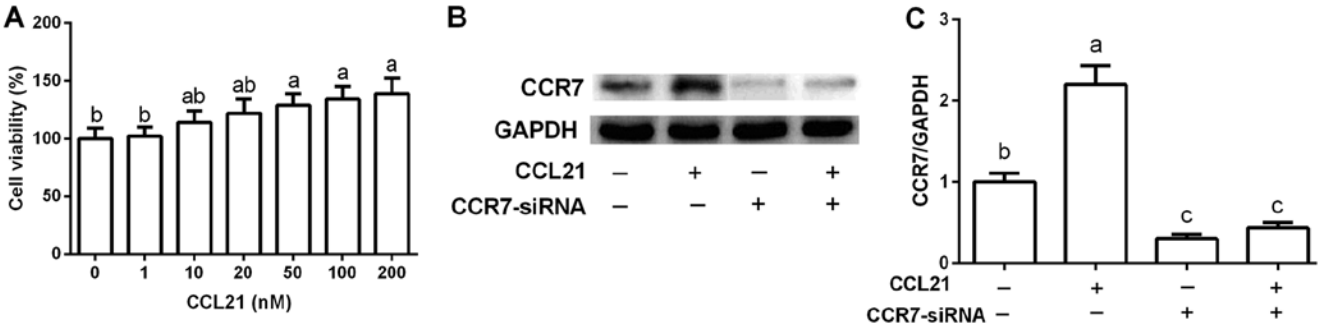

Figure 1. Effects of CCR7 on cell apoptosis. (A) Cell viability after exposure to CCL21. (B and C) CCR7 expression after exposure to CCL21 and CCR7-siRNA transfection via western blotting. Data are presented as the mean ± SEM. The values with different superscripted letters are significantly different (P<0.05; n=3 or 6). CCR7, C-C chemokine receptor type 7; CCL21, chemokine ligand 21.

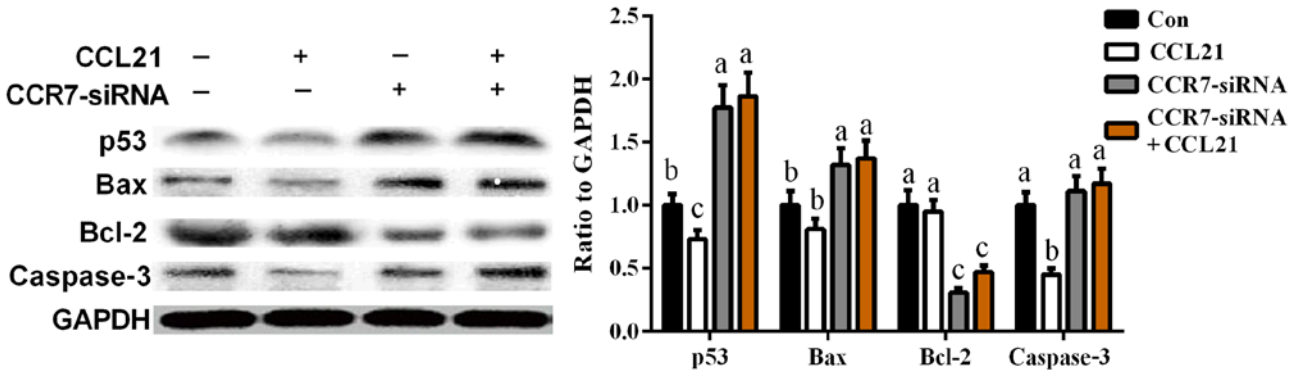

Figure 2. Effects of CCR7 on cell apoptosis-related proteins via western blotting. Data are presented as the mean ± SEM. The values with different superscripted letters are significantly different (P<0.05; n=3). CCR7, C-C chemokine receptor type 7.

expression (7). As shown in Fig. 1A, CCL21 increased cell viability in a dose-dependent manner. After exposure to 50, 100 and 200 nM of CCL21, cell proliferation was markedly promoted compared with that noted in the control group (P<0.05). A significant difference was observed at 50 nM, and was thus used to upregulate CCR7 expression (P<0.05) for the following analysis (Fig. 1B and C). A549 cells were transfected with CCR7-siRNA to inhibit CCR7 expression. The results revealed that CCL21 treatment markedly enhanced CCR7 expression, while CCR7-siRNA suppressed its expression (Fig. 1B and C).

Effects of CCR7 on apoptosis-related proteins in A549 cells. To further investigate the mechanism of CCR7-mediated cell apoptosis, four apoptosis-related proteins (p53, Bax, Bcl-2 and caspase-3) were analyzed after CCR7 overexpression and inhibition in A549 cells. As shown in Fig. 2, CCR7 overexpression markedly inhibited p53 and caspase-3 expression (P<0.05), while CCR7-siRNA enhanced cellular abundance of p53, Bax, and caspase-3 (P<0.05). Meanwhile, the expression of Bcl-2, an anti-apoptotic protein, was significantly decreased after CCR7-siRNA transfection when compared with that in the control group (P<0.05).

Effects of CCR7 on Akt and NF-κB signals in A549 cells. Akt and NF-κB are widely associated with apoptosis. In this study, we found that CCR7 upregulation markedly activated Akt signaling as evidenced by the increased Akt phosphorylation (p<0.05) (Fig. 3A and B), while CCR7-siRNA failed to influence Akt (P>0.05).

Furthermore, CCR7 upregulation enhanced IκBα expression while CCR7-siRNA inhibited the expression of IκBα (P<0.05) (Fig. 3A and C), an inhibitory protein of the NF-κB pathway. Thus, we further determined nuclear NF-κB p65 abundance and the results revealed that CCR7-siRNA markedly increased the expression level of nuclear p65 (P<0.05) (Fig. 3A and D).

Effect of NF-κB signaling on CCR7-mediated apoptosis in A549 cells. PDTC was used to inhibit NF-κB signaling in A549 cells. CCR7-siRNA markedly inhibited CCR7 expression and enhanced nuclear p65 abundance (P<0.05), while PDTC, a special antagonist of NF-κB, suppressed NF-κB activation induced by CCR7 inhibition (P<0.05) (Fig. 4A-C).

Inhibition of NF-κB via PDTC treatment markedly downregulated p53 and Bax when compared with the levels noted following CCR7-siRNA treatment (P<0.05) (Fig. 4D and E). Meanwhile, Bcl-2 abundance was significantly higher after PTDC exposure than that in the CCR7-siRNA group (P<0.05) (Fig. 4D and E).

Effects of CCR7 on inflammatory cytokines in A549 cells. NF-κB signaling has been widely demonstrated to regulate inflammatory cytokines. Thus cellular expression of IL-1β,

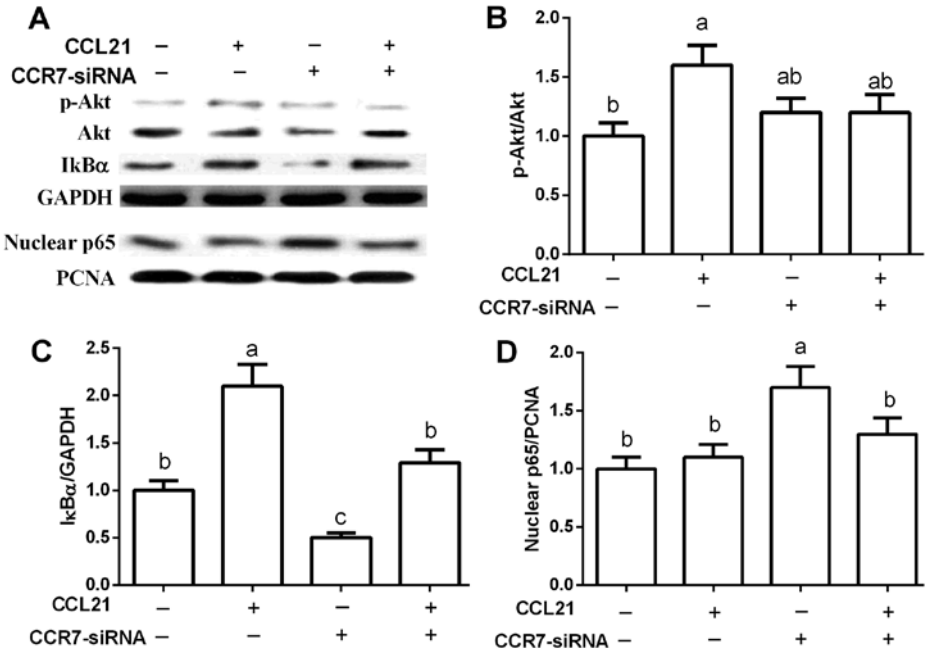

Figure 3. Effects of CCR7 on Akt and NF-κB signals via western blotting. (A) Results from western blotting analyses; (B) Expressions of p-Akt and Akt; (C) IκB abundance; (D) Nuclear p65 abundance. Data are presented as the mean ± SEM. The values with different superscripted letters are significantly different (P<0.05; n=3). CCR7, C-C chemokine receptor type 7.

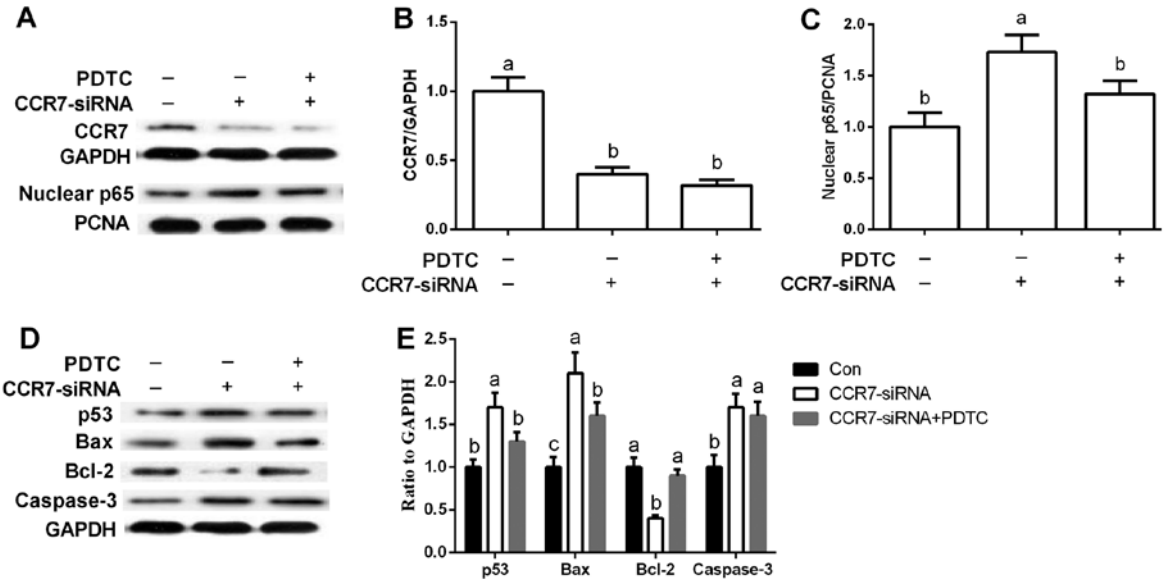

Figure 4. Effects of NF-κB on CCR7-mediated apoptosis. (A and B) CCR7 abundance via western blotting; (A and C) Nuclear p65 abundance via western blotting; (D and E) Expressions of apoptosis-relative proteins via western blotting. Data are presented as the mean ± SEM. The values with different superscripted letters are significantly different (P<0.05; n=3). CCR7, C-C chemokine receptor type 7.

IL-6, IL-10, IL-17 and TNF-α was determined via RT-PCR. The results revealed that CCL21 treatment markedly upregulated IL-1β and IL-10 expression (P<0.05) (Table II), suggesting that overexpression of CCR7 promoted the inflammatory response. However, CCR7-siRNA treatment significantly suppressed IL-1β and IL-10 expression (P<0.05) (Table II).

Effects of CCR7 on TGF-β1-induced EMT in A549 cells. TGF-β1 (20 ng/ml) was used to induce EMT in A549 cells according to a previous study (8). Vimentin (a mesenchymal cell marker), CK (an epithelial cell marker), N-cadherin, and E-cadherin have been widely used to evaluate EMT (8,9). The results showed that TGF-β1 markedly induced cell EMT as evidenced by the increased vimentin and N-cadherin and decreased CK (P<0.05) (Fig. 5). Meanwhile, CCR7-siRNA treatment significantly alleviated TGF-β1-induced EMT in A549 cells via mediating vimentin and CK expression (P<0.05) (Fig. 5).

Inflammatory response in TGF-β1-induced EMT in A549 cells. TGF-β1 treatment induced a cell inflammatory response via upregulation of IL-1β, IL-17, and TNF-α (P<0.05) (Table III),

Table II. Effects of CCR7 on the expression of proinflammatory cytokines via RT-PCR.

Item	Con	CCL21	CCR7-siRNA	CCR7-siRNA+CCL21
IL-1β	1.00±0.12[b]	1.62±0.17[a]	0.86±0.09[b]	1.04±0.13[b]
IL-6	1.00±0.15	1.20±0.16	1.34±0.25	1.23±0.13
IL-10	1.00±0.11[b]	1.40±0.14[a]	1.09±0.09[b]	1.12±0.15[b]
IL-17	1.00±0.19	1.31±0.23	0.93±0.12	1.12±0.21
TNF-α	1.00±0.23	0.95±0.21	1.12±0.16	1.17±0.24

Data are presented as mean ± SEM. The values having different superscripted letters are significantly different (P<0.05; n=3). CCR7, C-C chemokine receptor type 7; CCL21, chemokine ligand 21.

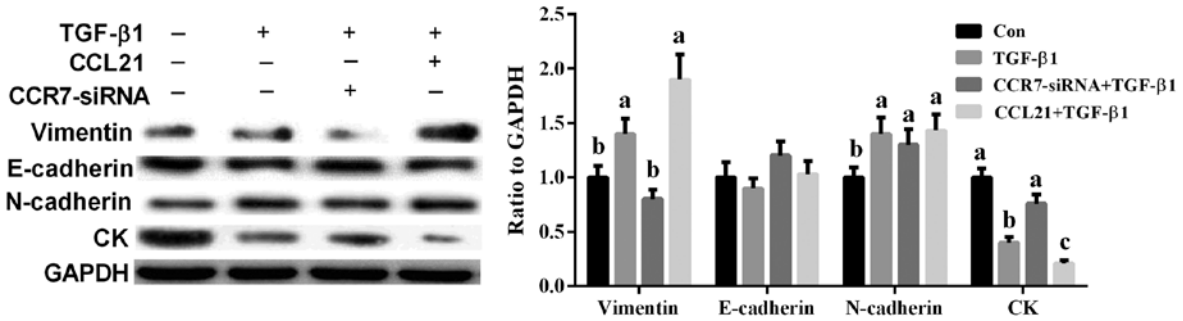

Figure 5. Effects of CCR7 on TGF-β1-induced EMT. Data are presented as the mean ± SEM. The values with different superscripted letters are significantly different (P<0.05; n=3). CCR7, C-C chemokine receptor type 7; TGF-β1, transforming growth factor β1; EMT, epithelial-mesenchymal transition; CCL21, chemokine ligand 21.

Table III. Effects of TGF-β1 and CCR7 on the expression of proinflammatory cytokines via RT-PCR.

Item	Con	TGF-β1	TGF-β1+CCR7-siRNA	TGF-β1+CCL21
IL-1β	1.00±0.11[b]	1.93±0.21[a]	1.44±0.16[b]	2.33±0.25[a]
IL-6	1.00±0.17	1.33±0.21	1.23±0.17	1.38±0.25
IL-10	1.00±0.14	1.13±0.16	1.17±0.21	1.32±0.19
IL-17	1.00±0.12[b]	1.56±0.19[a]	1.23±0.16[a,b]	1.67±0.22[a]
TNF-α	1.00±0.14[b]	1.46±0.14[a]	0.96±0.09[b]	1.77±0.28[a]

Data are presented as the mean ± SEM. The values with different superscripted letters are significantly different (P<0.05; n=3). CCR7, C-C chemokine receptor type 7; TGF-β1, transforming growth factor β1; CCL21, chemokine ligand 21.

while CCR7-siRNA treatment significantly alleviated IL-1β and TNF-α expression after TGF-β1 exposure (P<0.05) (Table III).

Discussion

Although previous studies suggest that CCR7 is involved in carcinogenesis (10,11), the functions and underlying mechanisms of CCR7 in NSCLC are still largely obscure. In this study, we investigated the effects of CCR7 activation and silencing on apoptosis and the signaling mechanism in A549 cells. The results revealed that CCR7 upregulation by CCL21 promoted cell proliferation, while CCR7 inhibition through siRNA treatment accelerated cell apoptosis and the signaling mechanism may be associated with the NF-κB pathway. To our knowledge, this is the first study demonstrating the effect of CCR7 on apoptosis in an NSCLC cell model, which may serve as a potential tumor marker or a therapeutic target for NSCLC.

CCR7 has been widely investigated in the immune response and previous studies suggest that CCR7 is involved in T-cell homeostasis, activation and polarization (12,13). Recent studies indicate an effect of CCR7 in carcinogenesis (10,11). In this study, we confirmed that CCR7 is involved in cell apoptosis in A549 cells. CCL21 is a ligand of CCR7 and has been suggested to upregulate CCR7 expression (7,14). CCL21 treatment markedly enhanced CCR7 expression, which further inhibits cell apoptosis. Mo *et al* reported that

CCL21-mediated CCR7 expression exhibited an antiapoptotic activity in human bladder cancer T24 cells via regulation of Bcl-2 and Bax proteins (15), while inhibition of CCR7 via RNA interference led to a significant inhibition of prostate cancer cell proliferation, migration and invasion (16). In NSCLC, CCR7 activation promoted G2/M phase progression and upregulated vascular endothelial growth factor-D expression via the ERK pathway (17-19). However, little is known concerning the effect of CCR7 inhibition on NSCLC. In this study, A549 cells transfected with CCR7-siRNA showed marked cell apoptosis and CCR7-siRNA transfection influenced apoptosis-related genes, such as p53, Bax, Bcl-2 and caspase-3.

Akt has been demonstrated to be involved in cell growth, proliferation, apoptosis and neoangiogenesis (20,21). In this study, we found that upregulation of CCR7 activated Akt signaling as evidenced by the increased Akt phosphorylation, which is similar with previous studies which reported that CCL21/CCR7 is associated with Akt signaling (22,23). However, CCR7-siRNA treatment failed to influence Akt, thus we speculated that inhibition of CCR7-mediated cell apoptosis may not be associated with Akt signaling.

To elucidate the underlying mechanisms involved in CCR7-mediated cell apoptosis, NF-κB signaling was further investigated after CCR7 upregulation and silencing. The results revealed that CCR7-siRNA markedly activated the NF-κB pathway. Thus, PDTC, a specific antagonist of NF-κB, was used to inhibit NF-κB after CCR7-siRNA treatment, which suggested that CCR7-mediated cell apoptosis in A549 cells may be associated with NF-κB signaling. Similarly, Kuwabara *et al* reported that CCR7 ligands upregulate IL-23 via the NF-κB pathway in dendritic cells (24). NF-κB signaling has been widely demonstrated to regulate inflammatory cytokines (25,26) and the present data indicated that NF-κB was involved in CCR7-mediated apoptosis. Thus, inflammatory cytokines were determined after CCR7 upregulation and inhibition and the results revealed that inhibition of CCR7 suppressed CCL21 and TGF-β1-induced inflammation, indicating that CCR7 mediates inflammation-associated tumor progression. These results are similar with previous studies (4,27).

EMT has been considered to be a key process promoting tumor metastasis in epithelial cancers (28,29). In this study, TGF-β1 was used to induce EMT and inhibition of CCR7 suppressed TGF-β1-induced EMT in A549 cells via the mediation of vimentin and CK expression. Similarly, Li *et al* reported that the CCL21/CCR7 axis is involved in the EMT process during chemotaxis of breast carcinoma cells and knockdown of CCR7 by shRNA suppressed tumor cell invasion, migration and the EMT phenotype (30). In gastric cancer, CCR7 promoted Snail expression to induce EMT, resulting in cell cycle progression, migration, and invasion in gastric cancer (11). Thus, the CCR7-EMT pathway may provide a potential regimen for cancer therapy, especially in NSCLC.

In conclusion, upregulation of CCR7 promotes cell proliferation and inflammation in A549 cells. However, silencing of CCR7 via siRNA treatment promoted cell apoptosis and suppressed the inflammatory response and TGF-β1-induced EMT, which may be associated with NF-κB signaling.

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