CCL22 and IL-37 inhibit the proliferation and epithelial-mesenchymal transition process of NSCLC A549 cells

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Abstract. In the present study, we aimed to investigate the effects of CC chemokine ligand 22 (CCL22) and interleukin-37 (IL-37) on the proliferation and epithelial-mesenchymal transition (EMT) of non-small cell lung cancer (NSCLC) A549 cells. pDsRed-CCL22 and pEGFP-IL-37 plasmids were constructed. A549 cells were divided into six groups: the control, the pDsRed-N1 blank plasmid, the pEGFP-C1 blank plasmid, the pDsRed-CCL22 plasmid, the pEGFP-IL-37 plasmid and the pDsRed-CCL22 + pEGFP-IL-37 plasmid group. Expression levels and localization of CCL22 and IL-37 in cells were detected by confocal microscopy. Phase-contrast microscopy was applied for observing cellular morphology. Real-time fluorescence quantitative polymerase chain reaction (RT-qPCR) was used for detecting the mRNA levels of vimentin, N-cadherin and E-cadherin, and their protein expression levels were tested using western blotting. Constructed plasmids expressed CCL22 and IL-37, both of which had a co-localization in the cell membrane. MTT assay and cell observation results revealed that CCL22 and IL-37 inhibited the proliferation and EMT process of the A549 cells. The results of RT-qPCR and western blotting revealed that decreased vimentin and N-cadherin mRNA and protein expression levels, and increased E-cadherin mRNA and protein expression levels were found in the pDsRed-CCL22 plasmid, pEGFP-IL-37 plasmid and pDsRed-CCL22 + pEGFP-IL-37 plasmid groups when compared with the control, the pDsRed-N1 blank plasmid and the pEGFP-C1 blank plasmid groups (all P<0.05), and decreased vimentin and N-cadherin mRNA and protein expression levels and increased E-cadherin mRNA and protein expression levels were found in the pDsRed-CCL22 + pEGFP-IL-37 plasmid group when compared with the pDsRed-CCL22 plasmid and the pEGFP-IL-37 plasmid groups (all P<0.05). CCL22 and IL-37 with a co-localization in the A549 cells inhibited the proliferation and EMT process in A549 cells. The antitumor effects of CCL22 and IL37 provide a strategy for the treatment of NSCLC.

Introduction

Lung cancer continues to be the leading cause of cancer-related deaths worldwide due to its high incidence, malignant behavior and lack of major advancements in treatment strategy, accounting for ~26/100,000 patients in China (1,2). Traditionally, lung cancer can be classified into two broad categories, including small cell lung cancer and non-small cell lung cancer (NSCLC) (3). Statistics show that NSCLC accounts for 85-90% of all lung cancers and is mainly comprised of squamous cell carcinoma, adenocarcinoma and large-cell carcinoma (4,5). As a multifactorial cancer, NSCLC can be triggered by many risk factors including tobacco smoking, second-hand or passive smoking, diet, excess alcohol consumption, air pollution, occupational exposure and genetic predisposition (6,7). A combination of radiotherapy and chemotherapy, along with surgical treatment, is the current treatment standard to achieve local tumor control and prolonged survival (8,9). Notably, previous evidence has shown that the long-term survival rates of lung cancer patients remain low and the 5-year overall survival rate of NSCLC is ~15% in the US and <10% in China (1,10,11). Therefore, there is a crucial need for finding prognostic markers to improve the clinical management of NSCLC patients.
To the best of our knowledge, a dysregulated inflammatory response is related to an increased risk of chronic disease and cancers (12). Chemokines, small pro-inflammatory chemo tactic cytokines, play an important role in many tumor-related processes including growth, metastasis and angiogenesis (13). Chemokine (C-C motif) ligand 22 (CCL22), a member of the CC chemokine group, also known as macrophage-derived chemokine (MDC), is expressed by monocytes and dendritic cells (14). CCL22 has been demonstrated to play a homeostatic role in leukocyte trafficking in addition to its well-documented effects in innate immune cell activation and Th2 immunopathology, acting through the combination of CC-chemokine receptor 4 (CCR4) (15). Interleukin-37 (IL-37), also known as IL-1F7, is a newly discovered member of the interleukin family with anti-inflammatory and immune inhibitory effects, and is mainly expressed in the brain, heart, kidney, dendritic and peripheral blood of mononuclear cells (16,17). Previous evidence revealed that IL-37 inhibits inflammation and immune reaction, and is found to be related to many diseases, such as acute myocardial infarction, sepsis and rheumatoid arthritis (18,19). Furthermore, CCL22 also has functional effects on the susceptibility to inflammation, immunological diseases and malignancies (20,21). As far as we know, the occurrence and development of lung cancer is closely related to inflammation (22), and few studies have been performed concerning the role and function of CCL22 and IL-37 in NSCLC. Thus, we conducted the present study to investigate the effects of CCL22 and IL-37 on the proliferation and the epithelial-mesenchymal transition (EMT) of NSCLC A549 cells.

Materials and methods

Plasmid construction. Polymerase chain reaction amplification was used for obtaining cDNA sequences of CCL22 and IL-37. cDNA sequences were recycled and inserted into pDsRed-N1 and pEGFP-C1 plasmids, respectively, via enzyme digestion of XhoI and PstI, to obtain pDsRed-CCL22 and pEGFP-IL-37 expression plasmids. PCR conditions were: pre-denaturing at 95°C for 5 min, 95°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec with 25 cycles, and finally elongation at 72°C for 5 min. The PCR primers were: CCL22 sense, 5'-cgctcgagatgtcctttgtggggag and antisense, 5'-cgctcgagatgtcctttgtggggag and antisense, 5'-cgctcgagatgtcctttgtggggag and antisense, 5'-cgctcgagatgtcctttgtggggag and antisense, 5'-cgctcgagatgtcctttgtggggag and antisense, 5'-cgctcgagatgtcctttgtggggag and antisense, 5'-cgctcgagatgtcctttgtggggag and antisense. Enzyme digestion was conducted at 37°C for 6 h. Fig. 1 shows the plasmid profiles.

Cell culture and grouping. The A549 cells (obtained from Shanghai Institute of Cell Biology of the Chinese Academy of Sciences, Shanghai, China, and retained in our laboratory) were cultured in 90% Dulbecco's modified Eagle's medium (DMEM) + 10% fetal bovine serum (FBS) (both from Thermo Fisher Scientific, Inc., Waltham, MA, USA). The A549 cells were divided into six groups in the present study: the control, the pDsRed-N1 blank plasmid, the pEGFP-C1 blank plasmid, the pDsRed-CCL22 plasmid, the pEGFP-IL-37 plasmid and the pDsRed-CCL22 + pEGFP-IL-37 plasmid groups. After being cultured for 24 h, the cells were transfected with plasmids (total plasmid was 6 µg) via a Lipofectamine 3000 kit (Thermo Fisher Scientific).

Confocal microscopy detection. A coverglass coated with 0.1% gelatin was placed into a 6-well plate, and then the above six groups of the A549 cells were added into the 6-well plate for culturing, respectively. When the cell density reached 80-90% confluency, the medium was emptied and then the cells were washed three times with phosphate-buffered saline (PBS). Next, the cells were fixed with 4% paraformaldehyde for 15 min. Subsequently, 4',6-diamidino-2-phenylindole (DAPI) was also added to mark the nucleus. After being washed three times with PBS, the coverglass was inverted on a glass slide for observation under confocal microscopy (FV1000; Japan Olympus Corporation, Tokyo, Japan). pDsRed-CCL22 and pEGFP-IL-37 were observed after excitation at 558 and 490-nm wavelengths.

Methyl thiazolyl-tetrazolium (MTT) assay. A549 cells in six groups were cultured into a 96-well plate with a density of 5,000 cells in each well (each group has four duplicated wells). After being cultured for 24 h, cell proliferation was tested at four time points (24, 48, 72 and 96 h) via Vybrant® MTT Cell Proliferation Assay kit (Thermo Fisher Scientific).

Real-time fluorescence quantitative polymerase chain reaction (RT-qPCR). After being cultured for 72 h, the cells were washed three times with PBS, and then general RNA extraction...
via the RNAiso Plus kit (Takara, Bao Biological Engineering Co., Ltd., Dalian, China) followed. PrimeScript RT reagent kit (Takara, Bao Biological Engineering Co., Ltd.) and StepOnePlus (ABI) PCR system (Thermo Fisher Scientific) were applied for reverse transcription and RT-qPCR. The PCR system consisted of 1.6 µl cDNA, 5 µl 2X SYBR-Green Taq PCR Mix (Takara, Bao Biological Engineering Co., Ltd.), 0.2 µl forward and 0.2 µl reverse primers (10 µM) and 3 µl deuterium-depleted water (DDW). The reaction system was: pre-denaturing at 95˚C for 5 min, 95˚C for 10 sec, 58˚C for 10 sec, 72˚C for 10 sec, with a total of 60 cycles, and finally 72˚C elongation for 10 min. Vimentin (gene ID 7431), N-cadherin (gene ID 1000) and E-cadherin (gene ID 999) were detected in the present study, while β-actin (gene ID 2597) was regarded as the internal reference. Data analyses were conducted with the 2-ΔΔCt method, 2-ΔΔCt representing the multiple proportions between the test group and its control group. The formula was: ΔΔCt = ΔCt_case_group - ΔCt_control_group, and ΔCt = Ct_miRNA - Ct_β-actin. The primer sequences are listed in Table I.

Statistical analyses. Data analyses were performed using SPSS version 20.0 software (SPSS, Inc., Chicago, IL, USA). Continuous data are presented as the mean ± SD. Comparisons between the two groups were conducted using a t-test, and comparisons among multiple groups by repeated measurements were performed using a one-way analysis of variance (ANOVA). P<0.05 provided evidence of significant differences.

Results

Construction and detection of the pDsRed-CCL22 and pEGFP-IL-37 carrier. Fig. 2 shows the enzyme digestion via the RNAiso Plus kit (Takara, Bao Biological Engineering Co., Ltd., Dalian, China) followed. PrimeScript RT reagent kit (Takara, Bao Biological Engineering Co., Ltd.) and StepOnePlus (ABI) PCR system (Thermo Fisher Scientific) were applied for reverse transcription and RT-qPCR. The PCR system consisted of 1.6 µl cDNA, 5 µl 2X SYBR-Green Taq PCR Mix (Takara, Bao Biological Engineering Co., Ltd.), 0.2 µl forward and 0.2 µl reverse primers (10 µM) and 3 µl deuterium-depleted water (DDW). The reaction system was: pre-denaturing at 95˚C for 5 min, 95˚C for 10 sec, 58˚C for 10 sec, 72˚C for 10 sec, with a total of 60 cycles, and finally 72˚C elongation for 10 min. Vimentin (gene ID 7431), N-cadherin (gene ID 1000) and E-cadherin (gene ID 999) were detected in the present study, while β-actin (gene ID 2597) was regarded as the internal reference. Data analyses were conducted with the 2-ΔΔCt method, 2-ΔΔCt representing the multiple proportions between the test group and its control group. The formula was: ΔΔCt = ΔCt_case_group - ΔCt_control_group, and ΔCt = Ct_miRNA - Ct_β-actin. The primer sequences are listed in Table I.

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Results

Construction and detection of the pDsRed-CCL22 and pEGFP-IL-37 carrier. Fig. 2 shows the enzyme digestion

Table I. Primer sequences of vimentin, E-cadherin, N-cadherin and β-actin by RT-qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense Sequence</th>
<th>Antisense Sequence</th>
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<tbody>
<tr>
<td>Vimentin</td>
<td>AATTTCACGCAGAGCAACAG</td>
<td>CCACTAAGGCAGCAGCTAAA</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>TTAACCTCACAATCTCTTGCT</td>
<td>TAGGCCATTTCCTCCCAATC</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>AACCTAGCTACTGGCCAAA</td>
<td>AACATCGAGTGCTGAAA</td>
</tr>
<tr>
<td>β-actin</td>
<td>GCAGGGGGGGGACACAAAGGGT</td>
<td>TGGTGCGAGTGATGGCCATG</td>
</tr>
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RT-qPCR, real-time fluorescence quantitative polymerase chain reaction.

Figure 2. Enzyme digestion results of the pDsRed-CCL22 and pEGFP-IL-37 carrier (lane A, pDsRed-CCL22; lane B, pEGFP-IL-37).

Figure 3. Expression levels of pDsRed-CCL22 and pEGFP-IL-37 fusion proteins in the A549 cells (lane A, the control; lane B, the pDsRed-N1 blank plasmid; lane C, the pEGFP-C1 blank plasmid; lane D, the pDsRed-CCL22 plasmid; lane E, the pEGFP-IL-37 plasmid; lane F, the pDsRed-CCL22 + pEGFP-IL-37 group).
results of the pDsRed-CCL22 and pEGFP-IL-37 carrier. The fragment lengths of pDsRed-CCL22 and pEGFP-IL-37 were 287 and 664 bp, respectively, after the enzyme digestion by XhoI and PstI, separately.

Expression levels of pDsRed-CCL22 and pEGFP-IL-37 fusion proteins in the A549 cells. Expression levels of pDsRed-CCL22 and pEGFP-IL-37 fusion proteins in the A549 cells were detected by western blotting. The results showed that no expression of CCL22 and IL-37 was found in the control, pDsRed-N1 blank plasmid and pEGFP-C1 blank plasmid groups; however, CCL22 was expressed in the pDsRed-CCL22 plasmid group, IL-37 was expressed in the pEGFP-IL-37 plasmid group, and CCL22 and IL-37 were both found simultaneously expressed in group 6 (Fig. 3).

Result of confocal microscopy detection. The results of confocal microscopy detection (Fig. 4) showed that no fluorescence was found in the control group, and fluorescence of the pDsRed-N1 blank plasmid and pEGFP-C1 blank plasmid groups was distributed in the cytoplasm. Expression of CCL22 and IL-37 proteins in the pDsRed-CCL22 plasmid and pEGFP-IL-37 plasmid groups, respectively, were mainly distributed in the cytoplasm. However, in the pDsRed-CCL22 + pEGFP-IL-37 plasmid group, CCL22 and IL-37 proteins were both found in the cytoplasm. These results revealed that CCL22 and IL-37 had a certain co-localization in the A549 cells.

Proliferation of the six cell groups via MTT assay. After being cultured for 24 h, the activity of the A549 cells was detected every 24 h. The relative activity of the cells in the control group was 1 at 24 h. After 24, 48, 72 and 96 h of cell culture, no significant difference was found in the control, pDsRed-N1 blank plasmid and pEGFP-C1 blank plasmid groups (all P>0.05). When compared with the control, pDsRed-N1 blank plasmid and pEGFP-C1 blank plasmid groups, lower cell proliferation was found in the pDsRed-CCL22 plasmid, pEGFP-IL-37 plasmid and pDsRed-CCL22 + pEGFP-IL-37 plasmid groups (all P<0.05). However, the pDsRed-CCL22 + pEGFP-IL-37 plasmid group had lower cell proliferation when compared with the pDsRed-CCL22 plasmid and pEGFP-IL-37 plasmid groups alone (all P<0.05) (Fig. 5). These results confirmed that CCL22 and IL-37 inhibited the proliferation of the A549 cells.
Cell morphologic observation under EMT. After 72 h of cell culture, the EMT process was observed under a microscope (Fig. 6). The results showed that obvious EMT was found in the control, pDsRed-N1 blank plasmid, and pEGFP-C1 blank plasmid groups. The cell morphologies were altered from pebble shaped cells into fusiform cells with elongated cell appearance. In addition, the cells were separated from peripheral cells, and the cells exhibited loose connections with larger intercellular space (Fig. 6A-C). EMT was also found in a few cells in the pDsRed-CCL22 plasmid and pEGFP-IL-37 plasmid groups. Cells showed fusiform shape and were loosely connected, but most of the cell morphology did not change significantly (still pebble shaped cells) (Fig. 6D and E). While, in the pDsRed-CCL22 plasmid + pEGFP-IL-37 plasmid group, epithelia maintained their pebble shape, tight junctions between cells and no EMT was noted (Fig. 6F). These results revealed that CCL22 and IL-37 inhibited the EMT process of the A549 cells and the inhibitory function was enhanced after the combination of CCL22 and IL-37.

mRNA levels of EMT-related genes. During EMT, the expression levels of vimentin and N-cadherin are increased, while the expression of E-cadherin is decreased. The results of RT-qPCR revealed that no significant difference was found when comparing vimentin, N-cadherin and E-cadherin among the control, the pDsRed-N1 blank plasmid, and the pEGFP-C1 blank plasmid groups (P>0.05). Decreased vimentin and N-cadherin mRNA expression levels, and increased E-cadherin mRNA expression were found in the pDsRed-CCL22 plasmid, the pEGFP-IL-37 plasmid and the pDsRed-CCL22 plasmid + pEGFP-IL-37 plasmid groups when compared with the control, the pDsRed-N1 blank plasmid and the pEGFP-C1 blank plasmid groups (all P<0.05). Decreased vimentin and N-cadherin mRNA expression levels...
and increased E-cadherin mRNA expression were found in the pDsRed-CCL22 plasmid + pEGFP-IL-37 plasmid groups when compared with the pDsRed-CCL22 plasmid and the pEGFP-IL-37 plasmid group (all P<0.05) (Fig. 7). These results showed that CCL22 and IL-37 inhibited the EMT process in the A549 cells.

Protein expression levels of EMT-related genes. The results from western blotting showed that no significant differences were found when comparing vimentin, N-cadherin and E-cadherin protein expression levels among the control, pDsRed-N1 blank plasmid and pEGFP-C1 blank plasmid groups (all P>0.05). Vimentin and N-cadherin expression levels were decreased, and E-cadherin expression was increased in the pDsRed-CCL22 plasmid, pEGFP-IL-37 plasmid and pDsRed-CCL22 plasmid + pEGFP-IL-37 plasmid groups when compared with the control, pDsRed-N1 blank plasmid and pEGFP-C1 blank plasmid groups (all P<0.05), and decreased vimentin and N-cadherin expression levels and increased E-cadherin expression were found in the pDsRed-CCL22 plasmid + pEGFP-IL-37 plasmid groups when compared with the pDsRed-CCL22 plasmid and the pEGFP-IL-37 plasmid group (all P<0.05) (Fig. 8). These results further confirmed that CCL22 and IL-37 inhibited the EMT process in the A549 cells.

Discussion

The present study represents a comprehensive analysis of CCL22 and IL-37 in regards to the proliferation and EMT of A549 cells. Our in-depth analyses concluded that CCL22 and IL-37 with co-localization in the A549 cells inhibited the proliferation and EMT process. The antitumor effects of CCL22 and IL-37 provide a strategy for the treatment of NSCLC.

In the present study, we found that when compared with the control group, the pDsRed-N1 plasmid and the pEGFP-C1 plasmid group, lower cell proliferation was found in the pDsRed-CCL22 plasmid, the pEGFP-IL-37 plasmid and pDsRed-CCL22 plasmid + pEGFP-IL-37 plasmid groups; moreover, the pDsRed-CCL22 plasmid + pEGFP-IL-37 plasmid group had lower cell proliferation when compared with the pDsRed-CCL22 plasmid and the pEGFP-IL-37 plasmid group alone, suggesting that CCL22 and IL-37 can inhibit the proliferation of the A549 cells. Lung cancer is one of the typical inflammation-associated cancers, since smoking can lead to chronic inflammation (such as chronic obstructive pulmonary disease), and bronchioles and lungs are open organs which are more receptive to suffer the stimulation from a variety of physical factors that lead to inflammation (23). Chemokines represent a family of endogenous mediators of inflammation attracting various leukocyte subtypes to sites of infection and have been reported to affect malignancy in various ways, either by promoting or inhibiting tumor growth and metastasis formation via the activation of seven transmembrane domain receptors coupled to heterotrimeric G proteins (12,24). Previous evidence revealed that the binding of chemokines and their receptors can induce aggregation of actin, and regulate cell movement and migration (25). As far as we know, the main source of CCL22 is dendritic cells, which are the most powerful antigen-presenting cells in vivo, and capable of stimulating T cells into Th1 and Th2; and Th1 can secret interferon-γ and inhibit CCL22, thereby inhibiting the proliferation of tumor cells (26). IL-37 is a cytokine which
plays an important role in inflammation, autoimmunity, and other immunological disorders, and is synthesized as a protein which is processed to its mature form after stimulation (27). It has also been reported that IL-37 has the ability to kill microorganisms and increase production of several cytokines for the expansion of the acquired immune function (28). One study also demonstrated that IL-37 downregulated inflammation in a mouse model (29). Mature IL-37 can bind to Smad3 to regulate the key enzymes of multiple signaling pathways, including focal adhesion kinase (FAK), proline-rich tyrosine kinase (Pyk2), MAP kinase p38, signal transducer and activator of transcription (STAT) p53 and mTOR. The expression of these key enzymes is downregulated and these pathways have been confirmed to be related to cell proliferation and migration (17).

Furthermore, we also found that vimentin and N-cadherin expression levels were decreased, and E-cadherin expression was increased in the groups transfected with CCL22, IL-37 or both CCL22 and IL-37, suggesting that CCL22 and IL-37 can inhibit the EMT process in A549 cells. EMT was first described in the 1980’s, and it was reported that EMT is not only a key biological process during embryonic morphogenesis, but also one of the earliest steps in solid tumor progression. EMT is related to tumor growth, metastasis as well as invasion and results in the conversion of tumors from low- to high-grade malignancy (30). E-cadherin, a crucial mediator of EMT, has been proven to result in rapid cell growth and metastasis in most cell types by loss of cell adhesion when E-cadherin loses its function (31). Li and Liu demonstrated that macrophages and activated fibroblasts can release a large number of growth factors, chemokines and matrix proteases, and then activate multiple signaling pathways such as the Smads, the ERK-MAPK and the PI3K-AKT signaling pathways. However, the underlying mechanism of CCL22 and IL-37 inhibition in the EMT process warrants additional confirmation by further study.

Collectively, the present study demonstrated that CCL22 and IL-37 with co-localization in the A549 cells inhibited the proliferation and the EMT process in the A549 cells. The antitumor effects of CCL22 and IL-37 provide a strategy for the treatment of NSCLC. The data from the present study can be explored by studying the effects of cytokines on the invasion and metastasis of NSCLC and by considering different cell lines for further substantiation of the aforementioned results.

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