IL-37 mediates the anti-tumor activity in non-small cell lung cancer through IL-6/STAT3 pathway

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Abstract

The occurrence and development of lung cancer is closely related to inflammation. Thus, we conducted the present study to investigate the effects of IL-37 (Interleukin 37), a newly identified anti-inflammatory factor, on non-small cell lung cancer (NSCLC), which accounts for about 85% of all lung cancers. To address the function of IL-37 in NSCLC, we first evaluated IL-37 expression in the human NSCLC tissues; then the IL-37 function was assessed in vitro and in vivo in a xenografted lung tumor model. IL-37 was barely expressed in the NSCLC tissue but highly expressed in the adjacent normal tissue. This expression profile was validated by ELISA (Enzyme-linked immunoassay), western blot and immunohistochemical staining. Recombinant IL-37 could suppress cell migration, invasion and proliferation and promote cell apoptosis in NSCLC cell line A549 and SK-MES-1. IL-37 inhibited the IL-6/STAT3 pathway and also the downstream targets Bcl-2, NEDD9 and Cyclin D1. Overexpressing IL-6 or constitutive active STAT3 eliminated the anti-tumor effects of IL-37. Furthermore, IL-37 expression in vivo could inhibit the cancer development. Our results showed that IL-37 plays an inhibitory role in lung cancer development, possibly through IL-6/STAT3 pathway.

Keywords:
Interleukin-37
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Introduction

Lung cancer is currently the most common cause of tumor related mortality in both men and women around the world (DeSantis et al., 2016; Miller et al., 2016; Siegel et al., 2016; Torre et al., 2016). Over half of patients diagnosed with lung cancer die within one year of diagnosis and the 5-year survivals are around 17.8% (DeSantis et al., 2016; Miller et al., 2016; Siegel et al., 2016; Torre et al., 2016). There are two main subtypes of lung cancer, small-cell lung carcinoma and non-small-cell lung carcinoma (NSCLC), accounting for 15% and 85% of all lung cancer, respectively (DeSantis et al., 2016; Miller et al., 2016; Siegel et al., 2016; Torre et al., 2016). NSCLC is further classified into three types: squamous-cell carcinoma, adenocarcinoma, and large-cell carcinoma.

Despite advances in early detection, radical surgical resection, and multimodal therapeutic modalities over the recent decades, the long-term survival remains poor due to the high rate of recurrence and metastasis (Miller et al., 2016). Therefore, there is an urgent need to identify novel biomarkers that will help select the patients with high chance of lung cancer recurrence and uncover the underlying mechanisms which would provide better targets for NSCLC treatment.

Dysregulated inflammatory response is related to an increased risk of chronic disease and cancers. And pro-inflammatory cytokines play an important role in many tumor related processes, including growth, metastasis, apoptosis and angiogenesis (Zhong et al., 2016). Interleukin-37 (IL-37) is a newly discovered member of the interleukin family with anti-inflammatory and immune inhibitory effects (Chen and Fujita, 2015; Dinarello et al., 2016; Nold et al., 2010). Recently, it has been demonstrated that IL-37 plays a protective role in tumor progression, including fibrosarcoma (Gao et al., 2003), hepatocellular carcinoma (Zhao et al., 2014), cervical cancer (Wang et al., 2015a), breast cancer (Wang et al., 2015b), renal cell carcinoma (Jiang et al., 2015) and lung cancer (Chen et al., 2016; Ge et al., 2016). The underlying mechanisms were proposed as CD57+ NK recruitment (Zhao et al., 2014).
IL-6/STAT3 signaling suppression (Jiang et al., 2015; Wang et al., 2015a), angiogenesis and epithelial-mesenchymal transition inhibition (Chen et al., 2016; Ge et al., 2016). Although it had been demonstrated that the angiogenesis and epithelial-mesenchymal transition inhibition were involved in the anti-tumor effects of IL-37 in NSCLC, the other functions of IL-37 in NSCLC remains largely unknown (Chen et al., 2016; Ge et al., 2016).

The occurrence and development of lung cancer is closely related to inflammation (Dougan et al., 2011), and few studies have been performed concerning the role and function of IL-37 in NSCLC. Thus, we conducted the present study to investigate the effects of IL-37 on NSCLC. To address the function of IL-37 in NSCLC, we first evaluated IL-37 expression in the human NSCLC tissues; then the IL-37 function was assessed in vitro and in vivo in a xenografted lung tumor model. Our results showed that IL-37 plays an inhibitory role in lung cancer development, possibly through IL-6/STAT3 pathway.

Materials and methods

Patients

A total of 182 patients who underwent surgery for histologically verified NSCLC at the First People’s Hospital of Foshan between 2004 and 2014 were enrolled in this study. The median age of the patients was 54.7 years (range 27–76 years). None of them received any preoperative anticancer treatment prior to sample collection. This study was approved by the local ethics committee and written informed consent was obtained from each patient. All 182 specimens were reevaluated with respect to their histological types, differentiation status, smoking status, and tumor TNM stages. Tumor stages were determined by TNM classification according to the 2002 International Union against Cancer guidelines. The histological diagnosis and grade of differentiation of the tumors were defined by evaluation of the hematoxylin and eosin-stained tissue sections, according to the World Health Organization guidelines of classification (2004). Every patient specimen included two matched pairs, namely, NSCLC tissues and adjacent normal lung tissues (>5 cm away from the tumor). Tissues were processed within 1 h after surgery. For each specimen, half were immediately flash-frozen in liquid nitrogen and then frozen at −80°C until RNA and protein extraction was performed. The remainder was fixed with formalin for immunohistochemistry.

Enzyme-linked immunoassay (ELISA)

The protein level of IL-37 and IL-6 was detected in culture supernatants and tumor homogenate using IL-6 ELISA Kit (R&D systems) and human IL-37 ELISA kit (Adipogen AG, Liestal, Switzerland) according to the manufacturer’s instructions. All samples were assayed in triplicate.

Immunohistochemical staining for IL-37

Surgically excised tumor specimens were fixed with 10% neutral formalin and embedded in paraffin, and 4-μm-thick sections were cut for immunohistochemical analysis. Sections were dewaxed in xylene and rehydrated through graded alcohols. For IL-37 immunostaining, a microwave-based antigen retrieval process was employed with EDTA buffer, pH8.0, for 30 min. After the sections had been cooled, endogenous peroxidase was inhibited with 3% hydrogen peroxide for 10 min at room temperature. Non-specific binding was blocked with fetal calf serum for 15 min before incubation of the sections with mouse anti-human IL-37 antibody (ab57187, 1:1000 dilution, Abcam, Cambridge, MA, USA) at 4°C overnight. As a negative control, sections were incubated with normal mouse IgG. After being incubated with the primary antibodies, the sections were then incubated with horseradish peroxidase (HRP)-labeled anti-mouse IgG at 37°C for 30 min, followed by visualization with 3, 3-diaminobenzidine (DAB) and counterstaining with Mayer’s hematoxylin. Desired color reaction was observed when monitored with the microscope.

Western blotting

Total protein from tumor tissues and cultured cells were lysed in RIPA buffer with protease inhibitor (Beyotime, Shanghai, China). The protein was quantified using a BCA assay kit (Beyotime, Shanghai, China). A total of 20 μg of total protein were separated by 10% SDS-PAGE, transferred onto polyvinylidene fluoride membranes, and then reacted with primary antibodies against IL-37, pSTAT3, STAT3, Cyclin D1, Bcl-2, NEED9 and β-actin (all from Abcam, Cambridge, UK). After being extensively washed with PBS containing 0.1% Triton X-100, the membranes were incubated with alkaline phosphatase-conjugated goat anti-rabbit antibody for 30 min at room temperature. The bands were visualized using 1-step TM NBT/BCIP reagents (Thermo Fisher Scientific, Rockford, IL) and detected by an Alpha Imager (Alpha Innotech, San Leandro, CA).

Recombinant human IL-37 protein cloning

Interleukin-37 gene (homo species) was amplified from cDNA of peripheral blood mononuclear cell using the primer pair 5’-CGGATCCATGGTTCAACAGTCCA-3’ and 5’-CCGAATTCCTTAATGCTGACTC-3’. The PCR fragments were double digested with restriction endonucleases and ligated into the prokaryotic expression vector. The fusion protein was expressed in E. coli in a stable prokaryotic expression system. The plasmids of positive clones were then sequenced by Sangen method with 100% identity with the published sequence (GenBank: AF167368). The induced and un-induced cultures were analyzed by SDS-PAGE to identify the expression of recombinant protein. The harvested cells were resuspended in NaCl-Tris-HCl buffer, sonicated in an ice bath, 12000 rpm centrifuged for 30 min, and then the supernatant were collected. The supernatant were added to His Trap HP, 1 mL column (GE) that had been equilibrated with NaCl-Tris-HCl buffer. Different concentrations of imidazole buffer were used to elute the recombinant protein. Collected target protein peaks were examined by SDS-PAGE electrophoresis and immunoblot analysis using anti-human IL-37 antibody (Abcam, UK). The eluted recombinant protein was dialyzed in PBS at 4°C for overnight. The concentration was detected by Bradford methods, and the recombinant protein was stored at −20°C.

Cell culture

The A549 and SK-MES-1 human NSCLC cell lines were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) and cultured in DMEM ( Gibco, Shanghai, China) supplemented with 10% FBS. Plasmids pIL-6 (Addgene Plasmid #67061) and pSTAT3-C (Addgene Plasmid #8722) were subcloned into pcDNA3.1 vector. The plasmids were transfected into cells using Lipofectamine™ 2000. 48 h after transfection, transfectants were selected in culture medium supplemented with 600 μg/mL G418. G418-resistant monolayers were picked and expanded in the selection medium. Recombinant human IL-37 (rhIL-37) protein, with a concentration ranging from 0 to 100 ng/mL (0, 1, 10, 100 ng/mL), was added to the medium of A549 and SK-MES-1 cells after cultured for 24 h, according to previous reports (Li et al., 2015).
RNA extraction and real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Then the quantity and purity of RNA was determined by absorbance on a FilterMax F5 Multi-Mode Microplate Reader (Sunnyvale, California, USA) at 260 nm and 280 nm. Samples with ratios from 1.8 to 2.0 were accepted for next reverse transcription reaction. cDNA was prepared by using the iScript™ cDNA Synthesis kit (Bio-Rad, USA). PCR primers (Generay, Shanghai, China) used for RT-PCR were as follows: for IL-37, sense: 5'-AGTGTGCTTGAAGAC CCGG-3' and anti-sense: 5'-AGAATGCGAAGGAGC-3'; IL-6, sense: 5'-AGCAGACCTCCTCTGGAAC-3' and anti-sense: 5'- AATGTCACCTCCTCTAGGCC -3'; β-actin, sense: 5'-GACGTCACCTCAACTCTGAAG-3' and anti-sense: 5'-GACGTCACCTCAACTCTGAAG-3'. RT-PCR amplification reaction was prepared with the SYBR Green PCR kit (Bio-rad, USA) and performed using the 7500 fast Real-Time PCR system (Applied Biosystems, USA). PCR products were verified by melting curve analysis. Relative mRNA levels of target genes were calculated by the 2^−ΔΔct method.

Cell viability assay

Cell viability was evaluated using CCK-8 (Beyotime, Shagnhai, China) according to manufacturer's instructions. Briefly, cells were seeded into 96-well plates at 5 × 10^3 cells per well and cultured for 48 h. Then the cells were detached by trypsinization, washed twice in PBS (2000 rpm, 5 min; Allegra X-12R centrifuge; Beckman Coulter, USA), and resuspended in 500 µl binding buffer. A volume of 5 µl Annexin V-FITC and 5 µl propidium iodide was added and mixed gently, and the cells were stained in the dark for 10 min at room temperature. The cells were analyzed immediately by flow cytometry (BD FACSCalibur, BD Bioscience, San Diego, CA, USA) and analyzed using Flowjo software (Flowjo, Ashland, OR, USA). The experiment was repeated three times.

Cell migration assay

The migration of cells was detected by wound-healing assay. Cells were cultured in 6-well plates. When the cells grew to 80–90% confluence, a wound in a line across the well was made by a plastic pipette tip. The area of cell-free wound was recorded 24 h after incubation with rhIL-37 protein using an inverted microscope and analyzed by the NIH Image 1.55 software. % Wound healing = (100 × (1-the remaining cell-free area/the area of the initial wound). All tests were performed in triplicate.
Transwell invasion assay

Invasive ability of cells was determined within a transwell system. 6.0 × 10⁴ cells were seeded onto the upper surface of the transwell membrane and cultured at 37 °C in 5% CO₂ for 24 h, 48 h and 72 h. The number of cells that migrated to the lower surface of the membrane was counted under a microscope (200×).

Animal study

Female 6–8 weeks old BALB/c nu/nu mice (Charles River Laboratories, Beijing, China) were housed in specific pathogen-free conditions. The study was approved by the Research Ethics Committee of The First People’s Hospital of Foshan. For evaluation of the tumor growth in vivo, 5 × 10⁶ cells were suspended in 200 μl PBS and injected subcutaneously into the flank region of nude mice. Tumor growth was monitored every 3 days and tumors were measured with fine digital calipers and tumor volume was calculated by the following formula: tumor volume = 0.5 × width² × length. Tumor-bearing mice were sacrificed 4 weeks after tumor inoculation and the tumors were removed and weighed. The fatality of mice was recorded every 3 days.

Statistical analysis

Data were expressed as mean (±SE) o and analyzed by Graphpad Prism V.5.00 software (GraphPad Software, San Diego CA, USA). Comparisons between groups were made using nonparametric Mann-Whitney U test. p values under 0.05 were considered statistically significant.

Results

Decreased IL-37 expression in NSCLC patients

It has been reported that IL-37 mRNA expression has been found in diverse human tissues, including lung (Kumar et al., 2000). We first analyzed IL-37 expression in 182 NSCLC specimens. The data showed that the protein levels of IL-37 in NSCLC tissues were lower than corresponding normal tissues (Fig. 1A), and this was further confirmed by western blot analysis (Fig. 1B). Moreover, immunohistochemical staining showed that the presence of IL-37 positive cells was mainly observed in the normal lung tissues, and IL-37 expression was significantly lower in NSCLC tissues compared with normal lung tissues (Fig. 1C). Taken together,

Fig. 2. Migration, invasion, proliferation, and apoptosis of A549 and SK-MES-1 cells after treated with different concentrations of rhIL-37 for 48 h. (A) Wound healing assay of A549 and SK-MES-1 cells with different concentrations of rhIL-37 protein (0, 1, 10, 100 ng/ml). n = 3. *P < 0.05. (B) Cell invasion assay of A549 and SK-MES-1 cells with different concentrations of rhIL-37 protein (0, 1, 10, 100 ng/ml). n = 3. *P < 0.05. (C) Analysis of NSCLC cell apoptosis following treatment of rhIL-37. A549 and SK-MES-1 cells were treated at the indicated doses, harvested, and stained with Annexin V-FITC and 7-AAD. Annexin V-FITC-positive apoptotic cells were determined by flow cytometry. n = 3. *P < 0.05. (D) The survival rate of A549 and SK-MES-1 cells treated with different concentrations of rhIL-37 (0, 10, 100 ng/ml) were analyzed. n = 3. *P < 0.05.
these results suggested that the decrease in intratumoral IL-37 expression might be associated with NSCLC progression.

**IL-37 suppresses NSCLC migration, invasion, proliferation and promotes apoptosis in a dose-dependent manner**

To further investigate the role of IL-37 in NSCLC, cell migration, invasion, apoptosis, and proliferation were analyzed in two human NSCLC cell lines, A594 and SK-MES-1, after the administration of various concentrations of rhIL-37. IL-37 inhibited the migration and invasion of A594 and SK-MES-1 cells in a dose-dependent manner (Fig. 2A, B). Additionally, IL-37 promoted the apoptosis of A594 and SK-MES-1 cells in a dose-dependent manner (Fig. 2C). Moreover, IL-37 dampened the proliferation of A594 and SK-MES-1 cells (Fig. 2D). These results indicated that an appropriate concentration of IL-37 was efficient to inhibit the migration,
invasion and proliferation, and accelerate the apoptosis of NSCLC cells. Therefore, the dose of 100 ng/mL rhIL-37 was used for the following study.

**IL-37 modulates IL-6/STAT3 signaling pathway in NSCLC cells**

We further investigated the underlying mechanism of anti-tumor effects of IL-37 in NSCLC cells. The expressions of IL-6, pSTAT3 (Y705), Bcl-2, NEDD9 and cyclin D1 in A594 and SK-MES-1 cells were analyzed after treatment with 100 ng/ml rhIL-37 for 24 h. The mRNA and protein levels of IL-6 were suppressed by IL-37 in these two cell lines (Fig. 3A, B). Activation of signal transducer and activator of transcription 3 (STAT3), which was phosphorylated at tyrosine 705, is an important event through which IL-6 influences cancer progression (Cuadros et al., 2014). Accordingly, pSTAT3 (Y705) level was reduced by IL-37 (Fig. 3C). Meanwhile, the expressions of NEDD9, Bcl-2 and cyclin D1 were also inhibited by IL-37 in these cells (Fig. 3C). To further confirm the correlation between IL-37 and IL-6/STAT3 signaling pathway, we transfected the A594 and SK-MES-1 cells with IL-6 or STAT3-C (constitutive active form of STAT3) plasmids. Compared with the control group, the effects of IL-37 on cell migration, invasion, apoptosis, and proliferation were abolished in IL-6 or STAT3-C overexpressing cells (Fig. 3D-G). Taken together, these results suggest that IL-37 may modulate the migration, invasion, proliferation, and apoptosis of NSCLC cells via IL-6/STAT3 signaling pathway.

**Overexpression of IL-37 suppresses NSCLC tumorigenesis in vivo**

To investigate the role of IL-37 in tumorigenesis, we stably transfected lung cancer cell line A549 with gene IL-37 or empty vector as control (mock transfectant). We then used a xenograft NSCLC model to elucidate the effects of IL-37 on NSCLC tumorigenesis in vivo. The results showed that IL-37-transfected A549 cells grew significantly slower than mock-transfected cells when transplanted in nude mice (Fig. 4A, B). However, the survival rate was not significantly different among the two groups (data not shown). These results suggest that IL-37 plays a vital role in suppressing tumorigenicity in vivo.

**Discussion**

Lung cancer is one of the most common malignancies worldwide, and it remains the leading cause of cancer related death with low early stage diagnosis rate. Due to the complexity and heterogeneity of this disease, the therapeutic strategies of patients with lung cancer differ from histological subtypes (Larrayoz et al., 2014). However, the knowledge of biological genotypes and underlying molecular mechanisms of each histological subtype is still limited.

IL-37 has been identified as a natural suppressor of innate inflammatory and immune responses (Dinarello et al., 2016; Nold et al., 2010; Nold-Petry et al., 2015). It is highly expressed in inflammatory tissues to inhibit the excessive inflammatory response. Recent studies indicate that IL-37 plays a protective role in tumor progression in mouse fibrosarcoma, human hepatocellular carcinoma and cervical cancer cells (Gao et al., 2003; Wang et al., 2015a; Zhao et al., 2014). However, there is little information about how IL-37 influences the pathogenesis of NSCLC development, progression, and prognosis (Chen et al., 2016; Ge et al., 2016). The function of IL-37 in tumor is largely unknown. Inflammation is the seventh hallmark of tumors (Hanahan and Weinberg, 2011), inferring that IL-37 might influence inflammation related tumors. Here, we provided the evidences that IL-37 suppresses the NSCLC progression through IL-6/STAT3 pathway.

In the current study, we first investigated the expression pattern of IL-37 protein in NSCLC patients. Using immunohistochemical staining, we found that IL-37 was expressed in non-tumor tissues and it was down-regulated in lung cancer tissues. Zhao et al. (2014) reported that the IL-37 expression level was significantly negatively associated with tumor size in hepatocellular carcinoma, indicating that IL-37 might inhibit tumor growth in the tumor microenvironment. To confirm our findings of clinical, we treated lung cancer cell line A549 and SK-MES-1 with different concentration of IL-37. We found that IL-37 suppressed migration, invasion, and cell growth of NSCLC in vitro. In the meantime, IL-37 also promoted NSCLC apoptosis.

IL-37 could suppress the IL-6 expression (Dinarello et al., 2016). And IL-6 exerts its biological roles through binding to the ligand receptor gp80 and immediately to the transducing receptor gp130. Phosphorylated tyrosines in gp130 can activate STAT3 through phosphorylation at tyrosine 705 (Heinrich et al., 1998). Aberrant activation of STAT3 contributes to cancer progression (Yu and Jove, 2004). To gain further insight into the role of IL-37 in NSCLC, we examined the expression of IL-6, the phosphorylation of STAT3 and the downstream genes of STAT3. We found that IL-37 down-regulated the expression of Bcl-2, cyclin D1, and NEDD9. Bcl-2, a proto-oncogene, is closely related to the regulation of cell death by inhibiting apoptosis (Vogler et al., 2009). Cyclin D1, a cell cycle regulator which promotes DNA synthesis and S phase entry, is overexpressed in NSCLC and may contribute to cell proliferation (Gautschi et al., 2007). NEDD9 is one of the cell adhesion proteins.

**Fig. 4.** IL-37 suppresses NSCLC tumorigenesis in vivo. The tumor volume (A) and weight (B) were analyzed 4 weeks after A549 cell injection. The cells were overexpressing IL-37 or empty vector. n = 12. * P < 0.05.
involved in cell mobility (Xu et al., 2010). In our study, the reduced expression of Bcl-2, cyclin D1 and NEDD9 may explain the antitumor and anti-proliferation activity of IL-37. Furthermore, we confirmed the anti-tumor effects of IL-37 in xenograft NSCLC mice model.

In conclusion, our data showed that IL-37 expression is lower in the tumor of NSCLC patients. By inhibiting IL-6 expression, IL-37 impeded the activation of STAT3 and reduced the expression of Bcl-2, cyclin D1, and NEDD9. Thus, IL-37 could be a potential candidate for immunotherapy in NSCLC. However, the manner in which IL-37 diminished the expression of IL-6 in NSCLC is not clear. It has been reported that NF-κB is a master regulator of many genes modulating tumor development and could mediate the secretion of IL-6 in all kinds of cancer (Karim et al., 2002). Therefore, further studies are needed to determine the exact mechanism of action of IL-37 in NSCLC.

Conclusions

Our results indicate that IL-37 was decreased in human NSCLC and IL-37 is capable of exerting anti-tumor activity by modulating the IL-6/STAT3 pathway. Our findings suggest that IL-37 might have clinical potential as a novel therapeutic target in the treatment of NSCLC.

Conflict of interests

The authors declare that they have no competing interests.

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