Persistence of RSV promotes proliferation and epithelial-mesenchymal transition of bronchial epithelial cells through Nodal signaling

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Abstract

Purpose. Nodal may play an important role in the development of cancers. The present study was designed to determine the effects of Nodal induced by respiratory syncytial virus (RSV) infection on the occurrence and development of lung cancer and the underlying mechanisms.

Methodology. After verification of RSV infection by observation of cytopathic effect and indirect immunofluorescence, real-time PCR, Western blot and methylation assays were used to verify the influence of RSV on Nodal expression. Then, a Nodal overexpressed vector was constructed and the effects of Nodal on the proliferation and apoptosis of bronchial epithelial cells (BECs) and epithelial-mesenchymal transition (EMT) were assayed by flow cytometry and Western blot, respectively. Moreover, Lefty and pSmad2/3 were assayed by Western blot and Cyclin D1, CDK4, c-myc and Bcl-2 induced by Nodal overexpression or RSV infection were also assayed by real-time PCR.

Results. The results showed that Nodal overexpression and demethylation of the promoter were observed in BECs after RSV infection. Activation of Nodal promoted proliferation, colony formation and EMT and inhibited apoptosis of BECs. Nodal also promoted malignant change by promoting expression of cyclin D1 and related-dependent kinase and inhibiting apoptosis. Besides, RSV infection inhibited Lefty expression and promoted the activation of pSmad2/3. RSV also promoted Cyclin D1, CDK4, c-myc and Bcl-2 expression through the activation of pSmad2/3.

Conclusions. Our data showed that persistence of RSV promoted the proliferation, epithelial-mesenchymal transition and expression of oncogenes through Nodal signaling, which may be associated with the occurrence and development of lung cancers.

INTRODUCTION

Lung cancer is the most common type of cancer worldwide and many factors are associated with lung tumorigenicity. Viruses can induce tumors, which has been confirmed in many kinds of animal models and great progress has been made in human research. In recent years, a large amount of reports have shown that many kinds of viruses were detected in lung cancer tissues, such as human papillomavirus (HPV) [1], human cytomegalovirus (HCMV) [2], and Epstein-Barr virus (EBV) [3], revealing the new field of lung cancer etiology study. However, many studies have also shown that infection with these viruses is unlikely to play some role in the development of lung carcinomas, because many different kinds of virus such as HPV, EBV, HCMV, human herpesvirus 8 (HHV-8), simian virus 40 (SV40), and respiratory syncytial virus (RSV) were detected in human lung cancer samples and none was identified to be closely related with lung cancer [4, 5].

Human respiratory syncytial virus (RSV) is associated with airway hyperresponsiveness after acute infection in infants and young children [6]. Many data show that persistence of RSV after acute infection was observed in animal models and human samples [7, 8]. Epithelium plays a central role in initiating pulmonary diseases, particularly in the case of RSV, since this virus productively replicates only in the lung epithelium and persists in respiratory epithelium after...
infection [9, 10]. The long persistence of RSV may under
mine the physiological homeostasis of the airway microen
vironment and lead to abnormal expression of host genes
through interaction with host proteins [11]. And RSV may
have an important influence on immunity through inhibiting
the differentiation of Th1, Th2 and Th17 [12].

Epithelial-mesenchymal transition (EMT) is a mechanism
that may account for the accumulation of subepithelial mes
enchymal cells, thereby contributing to abnormal proliferation
and differentiation. Several studies have demonstrated the
capacity of lung epithelial cells to acquire mesenchymal char
acteristics and induce lung cancer [13, 14]. Many mole
ecules have been identified as key contributors to the EMT
process. Among them is Nodal, a kind of secreting protein
belonging to the transforming growth factor (TGF) beta
superfamily, which is closely associated with canceration in
adults [15]. Moreover, Nodal remains at a low level of
expression in adults but high levels of expression are seen in
many kinds of cancers such as prostate cancer, ovarian can
cer, etc. [16, 17]. In our unpublished cDNA chip data, we
observed that RSV promoted the expression of Nodal. RSV
infection was also reported to promote the promoter
demethylation of the Nodal gene [18].

According to the above clues, we speculate that RSV persis
ten infection may have the possibility to promote cell can
ceration through the Nodal gene. To test this hypothesis,
first, an RSV persistent infection cell model was established
by a low level of RSV to infect bronchial epithelial cells
(BECs). Then RT-PCR, Western blot and methylation assay
were used to verify the influence of RSV on Nodal expres
sion. The correlation between Nodal expression and cellular
proliferation, EMT and oncogenes expression were also ex
amined.

METHODS

Cell culture

16HBE14o-cells (derived from normal epithelial cells) and
Hela cells were cultured in DMEM (Hyclone, USA) contain
ning 10 % FBS at 37 °C and 5 % CO2.

Preparation of RSV

RSV (Long strain/A2 type) was obtained from Wuhan Insti
tute of Virology and propagated in Hela cells. 80 % conflu
ent monolayers of Hela cells were infected with 100 µL of
RSV for 2 h at 37 °C. After two washes, fresh DMEM con
taining 3 % FBS was added and the cytopathic effects were
observed daily. The cells were gently blown and subjected to
three successive freeze-thaw cycles after maximum cyto
pathic effects. Supernatant was harvested and tested for
cytotoxicity to remove cell debris. Then the virus was ali
quoted and stored at −80 °C until use. Sham inoculum was
prepared by the same procedure from uninfected Hela cell
lysate.

RSV infection

Confluent monolayer cultures of BECs were infected with RSV at a multiplicity of infection (MOI) of 0.005. The viral
suspension was added to the cells for 2 h. Then BECs were
covered with complete medium and incubated for growth
and passage continuously. Generation 2–4 without obvious
cytopathic effects was used in this study. Non-infected
BECs were used as a control. SB525334 (Selleck Chemicals,
USA), a pSmad2/3 inhibitor was added at the concentration
of 10 µM.

Identification of RSV infection

The virus particles were tested by indirect immunofluores
cence (IFA) by using RSV F protein mouse monoclonal
antibody (Cat.number: sc-57998, Santa Cruz, USA) as a pri
mary antibody and CY3- conjucted antibody as secondary
antibody (Santa Cruz, USA). The results were observed
under confocal microscope.

Construction of Nodal overexpressed vector and tran
sfection

ORF of Nodal was synthesized and ligated to pGM-T vector
by Genscript (Nanjing, China). The Fragments encoding
Nodal were digested from pGM-T with EcoR1and BamH1
and cloned into EcoR I and BamH I of plasmid pcDNA3.1 (+).
The constructed plasmid was transformed into DH-5α
and verified by restriction enzyme mapping and DNA
sequencing (Huada, Shanghai, China). BECs were cultured
in 6-well plate in DMEM/F12 (1 : 1) supplemented with
10 % FBS at 37 °C under 5 % CO2 in humidified air. 90 %
confluent monolayer cultures were cultured in DMEM/F12
without serum at 37 °C under 5 % CO2 for 24 h, then
washed twice by invitrogen optiMEM medium, and added
1.5 ml optiMEM in every well. 5 µg plasmid was added to
250 µl optiMEM medium at room temperature for 10 min
after mixing, then 5 µl plus reagent was added to the pre
pared complex and mixed. At the same time, 15 µl LTX
reagent was added to another 250 µl optiMEM medium at
room temperature for 30 min after mixing. Then the two
ingredients were mixed and the complex was added to cells
don't by drop with gentle mixing, and then incubated at
37 °C and 5 % CO2 for 6 h. Media was replaced by fresh
media 6 h later.

Real-time PCR

RNA was isolated from Nodal overexpressed or RSV
infected BECs using TRIzol reagent (Invitrogen, USA)
according to the instructions of the manufacturer. Each
sample was reverse transcribed into cDNA and analysed by
quantitative real-time PCR. Quantitative real-time PCR was
performed on ‘Applied Bio systems Inc 1900 system’ using
the SYBR Green Real Time PCR Kit (Bio-Rad, America).
The primers for Nodal were 5′-ATCATCCGAGGCTA
CAGGC-3′ and 5′-AATCTGCGCAAGTCTACCTGGA-3′;
for Cyclin D1 were 5′-GAACAGAAGTGCGAGGAGGAGG
and 5′-ACTGGACTACGGACTACGGACTACGGACTAG
GAAGGACAGGATGGCGGAGT-3′; for c-myc were
5′-TGCCAGCGAAACGAATC-3′; for c-myc were
5′-CTCGGTGC
AGCCGTATTGTAC-3' and 5'-CTGGAAGAAGGAACTACGTTACGATAACC-3'; for Bcl-2 were 5'-GGGAGAAGCAGACTACATTAC-3' and 5'-CTAGAAGAGGAACTACGTTACGATAACC-3'. Raw data were normalized to GAPDH (5'-CCACTTCTGAC-3' and 5'-ACCGCTTGCTGTAGCA-3') in each sample. Each cycle included 94 °C for 30 s, 60 °C for 30 s after an initial 94 °C for 4 min.

DNA methylation

The Nodal gene sequences were obtained from GenBank. The MethPrimer primer Express software was used to predict the promoter and CpG islands. DNA was extracted using QIAamp DNA Mini Kit (Qiagen, China). For each sample, 0.5 µg of genomic DNA from each sample was bisulfite-treated using EpiTect Bisulfite kit (Qiagen, China) according to the manufacturer’s instructions. The genomic DNA was treated with sodium bisulfite to convert unmethylated cytosines to uracil. Specific primers for methylation measurement were designed using Methyl Primer Express Software v1.0 as: 5′ GGATTATGTTTTTGGTTTTG 3′ and 5′ AAAACCCAAAATACCAAATT 3′ (417 bp) [18]. PCR was performed in a thermocycler (Eppendorf Mastercycler gradient, Germany). PCR products were ligated to pGM-T vector and sequencing. BiQ_Analyzer was used to BLAST the sequences and the methylation ratios were calculated by methylated CpG sites/total CpG sites.

Western blot assay

BECs were washed by ice-cold PBS twice and resolved in RIPA buffer containing 1 mM PMSF, 5 mM β-glycerophosphate and 1% of a standard protease inhibitor cocktail (Sigma Chemical Co, USA) on ice for 30 min. Insoluble materials were removed by centrifugation for 20 s at 12,000 g at 4 °C. The supernatants were collected and the protein concentrations were measured by Bradford method to be adjusted to a final concentration of 10 mg ml−1. The supernatants were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 3 % BSA in PBS for 2 h and then incubated with monoclonal rabbit anti-Nodal antibody, rabbit anti-human N-Cadherin and E-Cadherin antibodies (Abcam, Hong Kong), rabbit anti-human phosphorylated Smad2/3 antibody (R&D system, USA), polyclonal rabbit anti-Lefty antibody at 4 °C overnight. After being washed, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1 : 5000; ProteinTech Group Inc., Chicago, IL, USA) for 1 h at room temperature. Antibody–antigen complexes were then detected using an ECL chemiluminescent detection system (Gene Co., Ltd., Hong Kong, China). GAPDH was used as a loading control. A densitometry analysis was performed using AlphaEase software version 2200.

Flow cytometry

After various treatments, BECs were fixed in cold 70 % ethanol and stored at −20 °C overnight. The fixed cells were washed twice with PBS, stained in a propidium iodide solution (50 µg ml−1, Sigma, USA) for 1 h, and treated with a ribonuclease A solution (20 µg ml−1, Sigma, USA) for 30 min. Flow cytometry (BD Pharmingen, USA) was then used to examine cell cycle. After various treatments, BECs were labeled with 4 µl of FITC-Annexin V and 8 µl of PI for 5 min in darkness and at room temperature, and the specimens were then detected using one flow cytometer for apoptosis assay. Normal living cells and early apoptotic cells could resist the staining by PI, but necrotic cells could not.

Clonogenic assay

Cells were placed in a 6-well plate at a density of 800 cells for 2 weeks. Thereafter, the cells were then fixed with methanol for 15 min, followed by incubation with 0.5 % crystal violet for 30 min and rinsing with deionized distilled water.

Statistical analysis

Calculations were performed with SPSS software. All values were expressed as Mean ± SE. Other data were compared by use of the Student’s t-test. P value of less than 0.05 was considered significant.

RESULTS

Influence of RSV on Nodal expression

The cytopathic effect of infected cells at Day 5 after infection with RSV at a MOI of 0.1 was seen by microscope (Fig. 1a). IFA also showed that the BECs were infected successfully by RSV after 48 h of incubation (Fig. 1b).

Next, we examined the effects of RSV on Nodal expression of BECs by using real-time PCR and Western blot. Results of real-time PCR showed that the expression of Nodal mRNA increased 6-fold by RSV infection (Fig. 2a). Results of Western blot also showed that RSV infection promoted the expression of Nodal proteins (Fig. 2b). Promoter methylation of Nodal was assayed by bisulfite sequencing PCR (BSP). Agarose gel electrophoresis of BSP amplification products showed the products were 417 bp (from 496 to 912 bp), the same size as the expected target fragments. Sequencing analyses showed the average methylation rates of CpG islands decreased in RSV-infected BECs when compared with BECs (P=0.0019, Fig. 2c). The results showed that RSV promoted mRNA and protein expression and methylation of Nodal.

Nodal promoted proliferation and EMT of BECs

Next, Nodal overexpressed vector was constructed and verified by sequencing. Nodal overexpressed vector can effectively promote the expression of Nodal protein (Fig. 3a). EMT is mainly characterized by the loss of epithelial markers such as E-cadherin and the acquisition of mesenchymal markers such as vimentin and N-cadherin [19]. Proteins were collected from cultured cells in the above groups and evaluated by immunoblotting for the expression of E-cadherin and N-cadherin. The results showed that E-cadherin expression decreased and N-Cadherin expression increased in Nodal overexpressed BECs when compared with control group (Fig. 3a).
**Fig. 1.** RSV infection identified by cytopathic effect (a) and IFA (b). (a) The cytopathic effect of infected cells at Day 5 after RSV infection was seen by microscope. (b) F-protein of RSV in BECs was determined by IFA.

**Fig. 2.** Effects of RSV on Nodal expression. (a) mRNA expression of Nodal in BECs was determined by real time PCR ($n=4$, **$P<0.01$ versus Control). Nodal mRNA increased by 6 folds after RSV infection. (b) Protein expression of Nodal in BECs was determined by Western blot ($n=3$, **$P<0.01$ versus Control). RSV increased the expression of Nodal. (c) Promoter methylation of Nodal was assayed by BSP ($n=4$, **$P<0.01$ versus Control). RSV decreased the average methylation rate of Nodal.
Flow cytometry showed that Nodal promoted G1 phase into S phase of BECs (Fig. 3b). Compared with the control group, the apoptosis rate in the Nodal group was also significantly reduced (Fig. 3c). Long-term effects of Nodal on the growth of BECs were further assessed by colony formation assay. After 14 d of transfection, the colony numbers were increased by 2.2-fold (Fig. 3d).

**Nodal promoted Cyclin D1, CDK4, c-myc and Bcl-2 expression**

Cyclin D1, CDK4, c-myc and Bcl-2 abnormal expression were important biomarkers of cancers. The results of real-time PCR showed that when compared with empty vector control, relative expression levels of cyclin D1 were significantly higher than those in control group (F=10.57, P=0.0008); relative expression level CDK4 (F=9.13, P=0.009), c-myc (F=12.69, P=0.0006) and Bcl-2 (F=17.63, P=0.0003) also increased (Fig. 4). The results showed that overexpression of Nodal may promote malignant change by promoting expression of cyclin D1, CDK4, Bcl-2 and c-myc.

**RSV promoted Cyclin D1, CDK4, c-myc and Bcl-2 expression through activation of pSmad2/3**

To further verify whether RSV infection induced Nodal signaling and expression of Cyclin D1, CDK4, c-myc and Bcl-2, Western blot was used to detect the expression of efty, the natural inhibitor of Nodal and activation of pSmad 2/3. Results showed that Lefty expression decreased and pSmad2/3 expression increased in RSV-infected BECs. p-Smad2/3 inhibitor SB525334 (10 µM) inhibited the activation of pSmad2/3 (Fig. 5a).

Next, Cyclin D1, CDK4, c-myc and Bcl-2 mRNA expression were examined by real-time PCR. The results showed that
Cyclin D1, CDK4, c-myc and Bcl-2 all obviously increased in RSV-infected BECs, which can be inhibited by p-Smad2/3 inhibitor SB525334 (Fig. 5b).

**DISCUSSION**

Lung cancer is a common cancer whose etiology is associated with many factors such as infection, host genetics, and environmental exposures. Although a large number of research report that lung tumors harbor many kinds of virus, the interaction of virus with host genes for lung cancer development is still doubtful.

Nodal is a kind of secreting protein belonging to the transforming growth factor beta (TGFβ) superfamily and is closely associated with cancerous development [15]. In addition, Nodal is also associated with a population of cancer stem cells (CSCs) and facilitates EMT [20]. Based on the previous reports, Nodal dimers are shown to combine to type I and type II activin receptors to form a tetramer, which act through the Smad2/3/4 signaling pathway to the nucleus and promote the transcription of various genes associated with proliferation and differentiation [21]. The Lefty proteins, divergent members of the TGFβ superfamily of proteins, act as extracellular antagonists of Nodal signaling. As the induction of Lefty is dependent upon Nodal expression, Lefty acts a classic feedback inhibitor for Nodal signaling [22]. Nodal signaling is tightly regulated by various microbes in lungs and influences proliferation and T cell differentiation of bronchial epithelial cells through DNA methylation [18]. In previous and present study, we confirmed that RSV promoted the expression and activation of Nodal, which is associated with demethylation of the Nodal promoter.

![Graphs showing changes in gene expression](https://example.com/graphs)

**Fig. 4.** Cyclin D1, CDK4, c-myc and Bcl-2 in Nodal-over expressed BECs assayed by real-time PCR (n=4). Results showed that Cyclin D1, CDK4, c-myc and Bcl-2 mRNA expression all obviously increased (**: P<0.01 vs empty vector control).

**Fig. 5.** Lefty expression and Smad2/3 activation assayed by Western blot (a) and Cyclin D1, CDK4, c-myc and Bcl-2 in RSV-infected BECs assayed by real-time PCR (n=4) (b). Results showed that Lefty expression decreased and pSmad expression increased in RSV-infected BECs. p-Smad2/3 inhibitor SB525334 (10 µM) inhibited the activation of Smad2/3. Cyclin D1, CDK4, c-myc and Bcl-2 mRNA expression all obviously increased in RSV-infected BECs, which can be inhibited by p-Smad2/3 inhibitor SB525334 (**P<0.01 and *P<0.05 vs control; #P<0.05 vs RSV).
It is now believed that one pathogenic mechanism of respiratory viruses is that they either selectively inhibit tumor suppressor or induce aberrant overexpression of oncogenes or allows such cells to persist and eventually transform into cancerous cells in lungs [23]. The main function of cyclin D1 is to promote cell proliferation by combining and activating cell cycle dependence protein kinase CDK4. The main function of c-myc and Bcl-2 is to inhibit cell apoptosis. At present, cyclin D1, c-myc and Bcl-2 have been widely recognized as oncogenes, and excessive expression of them can cause cell proliferation out of control and malignant change. Many reports have shown that overexpression of cyclin D1, c-myc and Bcl-2 was found in a wide variety of tumors such as breast cancer, bladder cancer, parathyroid gland tumor, lymphoma, melanoma, lung cancer and lymphoma [24, 25]. Nodal promoted the expression of cyclin D1, c-myc and Bcl-2 and Bcl-2, indicating that Nodal expression can lead to the occurrence and development of lung cancer.

Although RSV inhibited the proliferation and promoted apoptosis of BECs through many kinds of pathways such as TNF signaling, RSV promoted the expression of Nodal. Therefore, persistence of RSV may play an important role in the occurrence and development of lung cancer through its activation of Nodal and subsequent cyclin D1, c-myc and Bcl-2 overexpression. In conclusion, our data showed that overexpression of cyclin D1, c-myc and Bcl-2 is to promote cell proliferation by combining and activating cell cycle dependence protein kinase CDK4. The main function of c-myc and Bcl-2 is to inhibit cell apoptosis. At present, cyclin D1, c-myc and Bcl-2 have been widely recognized as oncogenes, and excessive expression of them can cause cell proliferation out of control and malignant change. Many reports have shown that overexpression of cyclin D1, c-myc and Bcl-2 was found in a wide variety of tumors such as breast cancer, bladder cancer, parathyroid gland tumor, lymphoma, melanoma, lung cancer and lymphoma [24, 25]. Nodal promoted the expression of cyclin D1, c-myc and Bcl-2, indicating that Nodal expression can lead to the occurrence and development of lung cancer.

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**Conflicts of interest**
The authors declare that there are no conflicts of interest.

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