ORIGINAL ARTICLE
Iran J AllergyAsthmaImmunol
October 2019; 18(5):473-478.

Evaluation of the LTBP1 and Smad6 Genes Expression in Lung Tissue of Sulfur Mustard-exposed Individuals with Long-term Pulmonary Complications

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Received: 17 November 2018; Received in revised form: 30 December 2018; Accepted: 2 January 2019

ABSTRACT

Sulfur mustard (SM) exposure injures different organs such as the lungs and leads toshortand long term complications Transforming growth factor beta (TGF-β) has the main role in altering fibroblast activities linked to airways remodeling. Latency TGF beta binding proteins 1 (LTBP1 facilitates localization of TGF-β in the extracellular matrix. Mothers against decapentaplegic homolog 6 (Smad6) negatively regulates TGF-β signaling, thus establishing a main negative feedback loop. In this study, we investigated the expression of LTBP1 and Smad6 in the lung tissues of SM-exposed and control individuals.

Lung formalin-fixed paraffin-embedded(FFPE) blocks of SM-exposed (20 samples) and control groups (20 samples) were collected from archival pathology department of severalgeneral hospitals. The total mRNA oflung FFPE tissues was extracted. Quality of the extracted mRNA was evaluated by an Agilent Bio analyzer and RNA was quantified using a Nano Drop. LTBP1 and Smad6 expression levels were evaluated by real-time PCR.

LTBP1 expression levels did not change between the two groups (p=0.626), howeverSmad6 expression levels were significantly higher (2.6 fold) in SM-exposed individuals compared to the control group (p=0.001).

Our results revealed that Smad6 may be involved in lung tissue remodeling process in SM-exposedpatients. Smad6 regulatesfibrotic alterations in lung tissue and its function as negative feedback mechanisms in TGF-β.

Keywords: Latency TGF beta binding proteins 1 (LTBP1); Mothers against decapentaplegic homolog 6 (SMAD6); Sulfur mustard;Transforming growth factor β

INTRODUCTION

Sulfur mustard ([bis (2-chloroethyl) sulfide]; SM) isa toxic and mutagenic agent used in World War I (1917) and Iraq-Iran war as a chemical weapon (1980-1988).[1]SM impacts many different organs such as skin, lungs, eye and causes acute and delayed complications.[2,3] The lungs are one of the most injured organs of the SM-exposed individuals.[4] Airway remodeling is one of the main problems in SM-exposed individuals withlong-term complications.[5]Bronchiolitis

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Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

obliterans (BO), chronic obstructive pulmonary complications (COPD), lung fibrosis and asthma are the main long-term pulmonary complications in SM-exposed patients.[6,7] In such chronic repetitive injury, inflammation constantly precedes fibrosis; inflammatory and parenchymal cell types produce cytokines in response to the injury stimulus and direct the adaptive immune and the subsequent fibrotic responses.[8,9]

TGF-β has an essential and central role in fibrogenic tissue responses.[10] TGF-β has three major isoforms in humans, TGF-β1, -β2, and -β3,[11] each of themis ubiquitously expressed by all cell types in the airways and lung parenchyma, but almost entirely in an inactive (latent) form due to the association of the mature TGF-β peptide with the latency-associated peptide (LAP).[12] TGF-β is stored in excess in the extracellular matrix through covalent association with latent TGF-β binding proteins (LTBPs).[13] LTBP1 is expressed in varioustissues, particularly in the lung.[14] LTBP1 facilitates localization of TGF-β in the extracellular matrix.[13] This protein can be released from the latency TGFβ (L TGF-β) by differentproteinases, including plasmin, elastases and matrix metallopeptidases (MMPs) or mechanical changes such as oxidative stress.[15]ActivatedTGF-β canbound to its receptors and the its signaling pathway activated.[16] The excessive activation of signaling TGF-β causes fibrotic disorders.[17]

TGF-β family signaling is regulated through multiple mechanisms and its amplitude is finely tuned by a variety of positive and negative regulators.[18] Inhibitory mothers against decapentaplegic homolog (I-Smads), such as Smad6 and Smad7,[19] are members of the Smad family which inhibit intracellular signaling through interactions with activated type I receptors and the receptor-regulated Smads (R-Smads).[20,21]

Zarrin and colleagues have shown that expression of TGF-β1, -β3 rise in lung tissue of SM-exposed individuals.[22]In addition, an increasing level of TGF-β has been demonstrated in bronchoalveolar lavage (BAL) fluid of SM-exposed individuals with pulmonary fibrosis or bronchiectasis.[23]

Further studies illustrated that expression of LTBP1 gene rise in lung tissue of fibrosis patients compared to the control group.[24] The recent studies have shown that the Smad signaling pathway is involved in pulmonary diseases such as COPD and result in peribronchial fibrosis.[25]

The goal of the present study was to denote the expression of LTBP1 and Smad6 in the lung samples of SM-exposed individuals in comparison with control group.

MATERIALS AND METHODS

Ethical Statement

This study is part of a large research project, which has been approved in Immunoregulation Research Center, Shahed University and Research Ethics Committees of Shahed University (N. IR.Shahed.REC.1396.139).Both groups signed informed consent form.

Sample Collection

In the present study, paraffin-embedded lung tissues were used. All of the samples were collected from archives in the pathology department of several general hospitals of Tehran-Iran. Biopsies had been done during surgery for diagnostic purposesbetween 2005 and 2011 years. In the SM-exposed group, lung biopsy tissues from patients with confirmed sulfur mustard exposure history during Iran-Iraq war (1980-1988) and had long-term pulmonary complications were used (n=20). Lung biopsy samples without any history of SM exposure were selected in the control group (n=20). At first, three tainted pathologists examined hematoxylin and eosin (H&E) stained slides. Then the sections with no pathological changes were used as the control group. Exclusion criteria included infection, systemic and local diseases which could affect the study, such as autoimmune diseases and immunodeficiency, history of exposure to other toxic gases, and drug addiction for both groups. Also, both groups had 30 to 60 years old during sampling.

More information and histological characteristics of the samples are shown in Table 1.

RNA Extraction

Total RNA was extracted from paraffin-embedded lung tissue block using Recover All Total Nucleic Acid Isolation Kit (Ambion - United States) for according to the kit protocol. Four 10 μm sections were cut from each lung tissue block. Then the samples were deparaffinized twice in xylene (Merck-Germany) at 56°C for 30 min with 400 rpm agitation. The residual xylene was washed by ethanol 96% (Merck-sigma). All of the sections were dried in the air. After that, Proteinase K was added and incubated at 56°C for 3 h.

Then proteinase K was inactivated with incubation of the supernatant of prior phase at 80°C for 15 min. Finally, next steps were done according to the manufacturer's protocol of kit.

Evaluation of Quantity and Quality of RNA

Concentration and purity were analyzed by the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). RNA Samples with an A260/A280 ratio of less than 1.8 were discarded. The integrity of RNA samples was evaluated by agilent RNA 6000 Nano Kit (Agilent technologies, Waldbronn, Germany). The samples with DV200 (percentage of RNA fragments with more than 200 nucleotides) more than 30% were accepted.

QRT-PCR for LTBP1 and SMAD6 Expression

RNA (400 ng) samples were transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems, USA). The reactions were incubated for 10 minutes at 25, 120 minutes at 37, and 10 minutes at 85°C. The quality of designed primers was checked by Gene Runner 5.0.1 software and online oligo calculator software.[26] The sequence and characteristics of primers are shown in Table 2. Real-time PCR was carried out triplicate usingRealQ Plus 2x Master Mix Green (Amplicon, Denmark) on the StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) For each reaction, 10 µL of 2X master mix, 7 µL RNase free H2O, 10 pmol from each primers (~ 1 µL), and 2 µL from 1: 2 diluted cDNA were mixed. An amplification program with the following steps was used: 10 min at 95°C for denaturation, two-step amplification, including 15 s at 95°C for denaturation and 60 s at 60°C for annealing/elongation for 40 cycles. This was followed by a melt curve at the end of the run with slow heating started at 60°C, which increased to 95°C with a rate of 0.3°C per second with continuous fluorescence measurement. The data were normalized to phosphoglycerate kinase1 (PGK1) level.[27] Primers efficiency were calculated using LinReg PCR software, version 11.0.

Table 1. Histopathological characteristics of sulfur mustard-exposed and control groups

	SM-Exposed (n=20)	Control (n=20)
Age	48.21±5.07	45.31±13.35
Spontaneous pneumothorax	4	1
Interstitial lung disease (ILD)	4	3
Idiopathic pulmonary fibrosis (IPF)	2	1
Bronchiectasis	2	4
Lung cancer	4	2
Bronchiolitis	2	0
Lung abscess	2	0
Chronic bronchitis	0	1
Pneumonia	0	2
Tuberculosis	0	1
Lung removal	0	1

Table2.Primers sequencesforPGK1,LTBP1, and SMAD6 in a study for evaluation of the LTBP1 and Smad6 in sulfur mustard-injured lungs

Gene	FORWARD (3>5)	Reverse (3>5)	Amplicon length (bops)	Efficiency (%)
LTBP1	CTTGCCTCTGACCGTGAC	TCTTGAAACCTGATGTATCTGG	118	91.4%
SMAD6	CCTACTCTCGGCTGTCTC	AGTGAGGGAGTTGGTAG	101	91.8%
Phosphoglycerate kinase 1 (PGK1)	GGCATACCTGCTGGCTGGATG	ACAGGACCATTCCACACAATCTGC	104	90.7%

PGK1: Phosphoglycerate kinase1; LTBP01: Latency TGF Beta Binding proteins 1

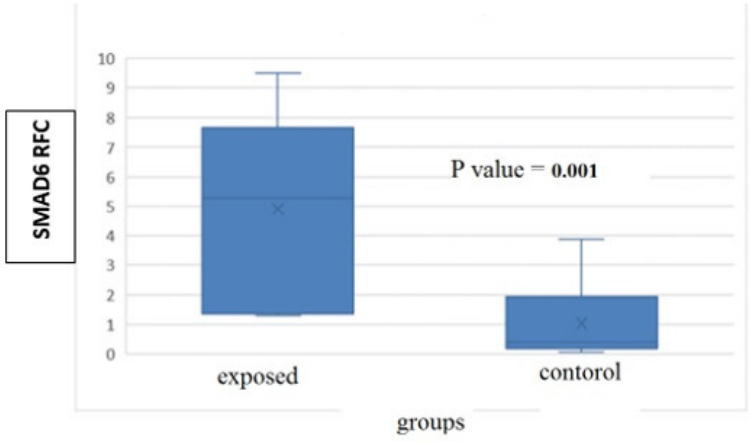

Figure 1. Mothers against decapentaplegic homolog 6 (Smad6) relative fold change (RFC) was upregulated in lung tissues from SM-exposed patients. Gene expression was measured by quantitativereal-time PCR (qRT-PCR)

Statistical Analysis

The threshold cycle (CT) value of each sample was defined by StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA).Pfaffl method was used for calculating relative gene expression.[28]Significant departure from normal distributionin gene expression data was approved with the Kolmogorov-Smirnov test. Mann-Whitney U test was used for mean comparison between groups. *p*-value<0.05 was considered statistically significant. SPSS version 16 used for all of the statistical analyses. Graph-creation was performed using Prism Version 5.0(GraphPad Software Inc., San Diego, CA, USA)).

RESULTS

In this study 20 lung tissue samples from SM-exposed individuals and 20 samples from control individuals were examined. The average age of SM-exposed individuals and control group were 48.21±5.07 and 45.31±13.35 years. The age of the two groups was not statistically different.

The mRNA expression LTBP1 and Smad6 was evaluated by RT-qPCR in samples of SM-exposed and control individuals. Data were normalized to Phosphoglycerate kinase 1 (PGK1) as housekeeping gene.After statistical evaluation, we found that the mean±SD of LTBP1 gene in SM-exposed and control groups was 8.36±10.22 and 3.95±8.31, respectively. There were no significant differences in LTBP1 expression levels between the two groups (p values=0.626). Moreover, the mean±SD of Smad6 gene in SM-exposed and control groups was 3.05±5.29 and 1.17±0.42, respectively. Smad6 expression levels in SM-exposed individuals was significantly higher (2.6 fold) than the control group (*p*=0.01) (Figure 1).

DISCUSSION

The purpose of this study was to evaluate the mRNA expression of LTBP1 and Smad6, two regulators of TGF-β signaling pathway in lung tissue of SM-exposed individuals.

The results of the present study showed that the gene expression of Smad6 was 2.6 folds higher in SM-exposed individuals compared to the control group, Whereas LTBP1 gene expression was not significantly increased from the control group.

Pulmonary damages are one of the long-term complications which observed in SM-exposed individuals.[6,7] The recent reports have suggested that TGF-β may have a main role in pathogenesis of pulmonary complications caused by sulfur mustard.[22,23] The secretion and activation of TGF-β is regulated by their association with LTBP1. LTBP1 adheres and exports Latent TGF-β to the extracellular matrix, where the cytokine is subsequently activated by several different mechanisms.[29] TGF-β signaling pathway is mediated/regulated by SMAD6. SMAD6 is able to reduce excessive activation of TGF-β signaling

pathway.[30]Aghanouri and colleagues showed that TGF-β expression is higher in Broncho alveolar lavage of SM-exposed individuals at delay phase rather than the control group.[31] The high level of TGF-β could be a reason for increasing of SMAD6 expression in the lung of SM-exposed individuals.

It seems that increased expression of Smad6 may involve in the regulation mechanism of fibrotic conditions in the lung tissue of SM-exposed individuals.

In this study, finding biopsies of healthy individuals is very hard, so we used biopsies of pulmonary patients for the control group that was historically normal. There was no other way and this is a limitation of the biopsy sample study. Nevertheless, the obtained findings are valuable.

SM-exposed individuals have been diagnosed with some pulmonary complications like COPD.[32]Springer and colleagues showed that expression of Smad6 and Smad7 genes decrease in lung tissue of COPD patients compared to the control group.[25]The contrast results may reflect differences in molecular level of these two diseases. Ghazanfari et al. reported similar observation. They showed that the serum level of CXCL8/IL-8 was significantly decreased in the SM-exposed cases compared to the control group[33] while the elevated levels of CXCL8/IL-8 in other chronic inflammatory disorders including COPD has been reported.[34]

In agreement with our data, Krafft and colleagues illustrated that the gene expression level of LTBP1 has no significant difference in dogs with lung fibrosis compared to healthy group.[35] However, Lepparanta and colleagues showed that gene expression of LTBP1 and TGF-β rise in of patients with pulmonary fibrosis compared to the control group.[24]Lepparanta and colleagues had used lung tissue from transplantation donors and patients with benign pulmonary tumors as the control group. In our study, finding biopsies of healthy individuals was very difficult, so we used biopsies of pulmonary patients with no significant pathological changes as the control group.

According to the results of this study, it can be suggested that the up-regulation of Smad6 is a key regulating mechanism in order to control chronic fibrosis processes in long-term SM exposure.

In conclusion our study demonstrated thatmaybe Smad6 has been activated by TGF-β in SM-exposed individuals. Smad6 pathway is the most represented signaling mechanism for regulatesinflammatory/fibrotic alterations in lung tissue.

ACKNOLEDGEMENTS

We are thankful to Immunoregulation Research Center in Shahed University.

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