

Full Length Research Paper

Changes in transforming growth factor (TGF)- β and mothers against decapentaplegic homolog (Smad) expression in chronic asthmatic rats induced by ovalbumin and aluminum hydroxide

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The aim of this study was to investigate the transforming growth factor (TGF)- β 1/mothers against decapentaplegic homolog (Smad) signaling pathway associated with airway reconstruction in chronic asthmatic rats by studying mRNA and protein expression. Twenty-four (24) Sprague-Dawley rats were randomly divided into two groups: a normal control group treated with 0.9% saline and an OVE+ALU group treated with a mixture of 10% ovalbumin and 10% aluminum hydroxide. Hematoxylin-eosin (HE) staining was used to observe the pathomorphological changes, and the locations of TGF- β and Smad 2, 4 and 7 were determined by immunohistochemistry. The mRNA expression of TGF- β and Smad 2, 4 and 7 was determined by real time-polymerase chain reaction (RT-PCR). The protein expression of TGF- β was determined by enzyme-linked immunosorbent assay (ELISA), whereas that of Smad 2, 4 and 7 was determined by western blotting. HE staining revealed that the OVE+ALU group displayed obvious signs of bleeding and expansion of lung alveolar and inflammatory cells. Immunofluorescence showed that TGF- β and Smad 2, 4 and 7 proteins were located in the bronchial wall or alveolar wall. RT-PCR showed that mRNA expression of Smad 2 was significantly higher in the OVE+ALU group than in the control group ($P < 0.05$) and that mRNA expression of Smad 7 was significantly lower in the OVE+ALU group than in the control group ($P < 0.05$). There was no significant difference in Smad 4 mRNA expression between the two groups ($P > 0.05$). Western blotting showed that TGF- β 1 and Smad 2 protein expression was significantly higher in the OVE+ALU group than in the control group ($P < 0.05$), whereas, in contrast, Smad 4 and 7 protein expression was significantly lower in the OVE+ALU group than in the control group ($P < 0.05$). The process of chronic asthma rats, TGF- β and Smad 2, 4 and 7 expressions were changeable, thus the TGF- β /Smad signaling pathway may play a role in chronic asthma.

Key words: Chronic asthma, Smad 2, 4 and 7, real time-polymerase chain reaction (RT-PCR).

INTRODUCTION

Bronchial asthma is a chronic inflammatory syndrome

that is highly prevalent worldwide, affecting approximately 300 million individuals of all ages (World Health Organization, 2007). The incidence of chronic asthma has increased in recent years due to environmental pollution caused by car exhausts and lifestyle changes. The concept of asthma control includes clinical and functional manifestations, such as symptoms, nocturnal

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Abbreviation: TGF, Transforming growth factor.

awakenings, use of rescue medication, activity limitation, and pulmonary function (Global Initiative for Asthma, 2009). Asthma has multiple negative influences on the patient's quality of life. Although, majority of asthma patients can obtain the targeted level of control, some patients will not achieve control even with the best therapy (Nathan, et al., 2004).

Patients who do not reach an acceptable level of control with the use of reliever medication plus two or more controllers can be considered to have difficult-to-treat asthma (Wenzel, 2005).

Asthma is a chronic complex inflammatory airway disorder characterized by variable degrees of recurring symptoms of airflow obstruction and bronchial hyperresponsiveness (National Institute of Health, 2007), and airway reconstruction is one of the pathophysiological characteristics of chronic asthma. The performances of the airway re-established include thickening of smooth muscle and the airway basal surface, deposition of extracellular matrix, infiltration of inflammatory cells, overgrowth of glandular organs and hypertrophy, which can lead to airway hyper-reactivity, irreversible airflow blockage, etc. Remodeling processes in asthma result from highly complex and poorly defined interactions between inflammatory and resident structural cells (James, 2005). Therefore, the identification of the molecular pathways involved in the crosstalk between these cells is a prerequisite for the development of novel therapy to control airway remodeling.

Previous studies (Chu et al., 1998) have demonstrated that the transforming growth factor (TGF)- β /mothers against decapentaplegic homolog (Smad) signaling pathway is very important in airway re-established in chronic asthma. TGF- β is a founding member of the TGF- β super family, including activins inhibiting growth and differentiation factors, and bone morphogenetic proteins. TGF- β 1 and its isoforms (TGF- β 2 and TGF- β 3) are synthesized by a variety of cells including all cell types, and are secreted as latent precursors (latent TGF- β 1) complexed with latent TGF- β binding proteins (LTBP) (Wang et al., 2005; Roberts, 1998). Active TGF- β then binds its receptors and functions to exert its biological and pathological activities via Smad-dependent and independent signaling pathways (Derynck and Zhang, 2003).

The Smad complexes then accumulate in the nucleus to regulate target gene expression cooperatively with other transcription factors in a cell context-dependent manner (Feng and Derynck, 2005; Siegel and Massague, 2003; Moustakas et al., 2001).

The phosphorylated Smad 2 and 3 then bind to Smad 4 and form the Smad complex, which translocate into the nucleus and regulates the target gene transcription, including Smad 7. Smad 7 is an inhibitory Smad that functions to block Smad 2/3 activation by degrading the T β RI (Hui, 2011).

This study aimed to determine whether airway reconstruction involves changes in the TGF- β /Smad signaling pathway.

MATERIALS AND METHODS

Experimental animals

Specific pathogen-free Sprague-Dawley (SPF SD) male rats, weighing 180 to 200 g were obtained from the Experimental Animal Center of the Chinese Academy of Science (Shanghai, China).

Experimental protocol

Twenty-four (24) healthy SPF SD male rats were randomly divided into two groups: a normal control group (n = 12) and an OVE+ALU experimental group (n = 12). Each rat in the control group received subcutaneous injections of 1 ml of 0.9% saline every day for two weeks, whereas the rats in the OVE+ALU group were injected with 1 ml of a solution of ovalbumin and aluminum hydroxide dissolved in 0.9% saline (ova:alu:saline = 1:1:8, v/v). After two weeks, all the rats were sprayed with 2% ovalbumin aerosol in a sealed container every other day for two weeks to induce asthma. Except for the dead ones, all the rats were anesthetized with ether.

Histological examination

Lung tissues taken from each rat were fixed in 15% buffered paraformaldehyde and dehydrated in a graded alcohol series. The specimens were then embedded in paraffin blocks and cut into 5 μ m-thick sections. The sections were stained with hematoxylin-eosin (HE). Pathological examinations were performed by a pathologist who was blinded to the rats' treatment assignment.

Immunohistochemical analysis

Fresh lung tissues taken from rats anesthetized with ether were embedded in buffer containing 4% paraformaldehyde, 20% sucrose and 30% sucrose. The specimens were then cut into 25 μ m-thick cryostat sections and mounted on Superfrost plus slides or gelatin-coated slides. The slides were stored at -20°C until needed. Before staining, slides were warmed at room temperature for 30 min and fixed in ice-cold acetone for 5 min, followed by air-drying for 30 min. The following antibodies were used: monoclonal anti-TGF- β antibody (1:1000 in phosphate buffered saline (PBS)), monoclonal anti-human Smad 7 antibody (1:1000), monoclonal rabbit anti-Smad 2 antibody and monoclonal rabbit anti-Smad 4 antibody (1:1000). After reacting with the secondary peroxidase-conjugated antibody and washed in PBS, the antibody complex was visualized using 3,3-diaminobenzidine. The specimens were observed under a regular light microscope.

Reverse transcriptase polymerase chain reaction

Total RNA was isolated using a high pure RNA isolation kit according to the manufacturer's instructions. Contaminating DNA was removed by treating the samples with RNase-free DNase. One hundred micrograms of total RNA was reverse-transcribed and subjected to polymerase chain reaction (PCR) with an initial step of 94°C for 2 min followed by 40 cycles of 94°C for 15 s, 58°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 10 min. The following primers were used: rat TGF- β forward, 5'-TACAGGGCTTTCGCTTCAGT-3' and reverse, 5'-TGGTTGTAG-AGGGCAAGGAC-3'; Smad 2 forward, 5'-ATACCCACTCCATTC-CAG-3' and reverse, 5'-CACTATCACTTAGGCACTGG-3'; Smad 4 forward, 5'-AGGTGGCTGGTCGGAAAG-3' and reverse, 5'-

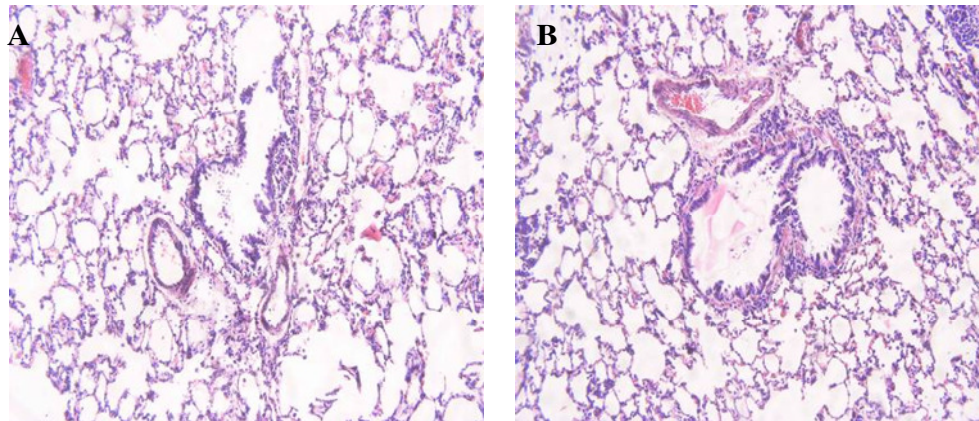


Figure 1. Histological profiles of lung tissue in rats ($\times 200$). A, Control rats; B, OVE+ALU rats.

TTGGTGGATGTTGGATGGTT-3'; Smad 7 forward, 5'-GGCATA-CTGGGAGGAGAAGA-3' and reverse, 5'-CTGTTGAAGATGACCTCTCCAGC-3'; glyceraldehyde 3-phosphate dehydrogenase (GADPH) forward, 5'-CCGTAAAGACCTCTATGCCAACA-3' and reverse, 5'-GGACTCATCGTACTCCTGCT-3'.

All samples were subjected to reverse transcriptase (RT)-PCR along with the housekeeping gene GAPDH as an internal standard, which was amplified using the following primer sequences: GAPDH forward, 5'-CCGTAAAGACCTCTATGCCAACA-3' and reverse, 5'-GGACTCATCGTACTCCTGCT-3'. Reaction specificity was confirmed by gel electrophoresis of products after RT-PCR and melting curve analysis. Ratios for Smad/GAPDH mRNA were calculated for each sample and expressed as the mean \pm standard deviation (SD).

Western blotting

Total protein was extracted from lung tissues and quantified using a bicinchoninic acid protein concentration assay kit. Samples (50 μ g) were fractionated on a 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) separating gel using a Bio-Rad electrophoresis apparatus (Bio-Rad Laboratories, Hercules, CA, USA). After transferring proteins onto nitrocellulose membrane, the blots were probed with a mouse monoclonal antibody to Smad 2 (CST, 1:1000 dilution), a mouse monoclonal antibody to Smad 4 (R&D, 1:1000 dilution) and a mouse monoclonal antibody to Smad 7 (Santa Cruz, 1:1000 dilution). The secondary antibody was goat anti-mouse IgG (1:1000), which was diluted in PBS containing 1% fetal calf serum. GAPDH was used as an internal control.

Statistical analysis

Data were calculated as the mean \pm SD and the groups were compared using one-way analysis of variance (ANOVA). Statistical significance was set at $P < 0.05$.

RESULTS

Histology of lung tissues in chronic asthmatic rats

We examined the effects of OVE+ALU-induced chronic asthma on lung tissue using HE staining. The histology of

the HE-stained tissues showed a marked alteration in the experimental group as compared to the control group (Figure 1).

Immunohistochemistry of lung tissues in chronic asthmatic rats

Figure 2 shows the immunohistochemistry of TGF- β and Smads in the lung tissues of chronic asthmatic rats.

mRNA expression of TGF- β and Smads in chronic asthma

mRNA expression of TGF- β 1 and Smad 2 were significantly increased in the experimental group when compared with the control group ($P < 0.05$). There was no significant difference in Smad 4 mRNA expression between the control group and the experimental group. mRNA expression of Smad 7 decreased significantly in the experimental group when compared with the control group ($P < 0.05$) (Table 1 and Figure 3).

TGF- β and Smad protein expression in chronic asthma

Expression of TGF- β in lung tissue increased significantly in the experimental group, as measured by enzyme-linked immunosorbent assay (ELISA) ($P < 0.05$). Expression of Smad 2, 4 and 7 in lung tissue increased significantly in the experimental group as detected by western blotting (Table 2).

DISCUSSION

The TGF- β family of cytokines encompasses a large number of versatile poly-peptide growth factors regulating

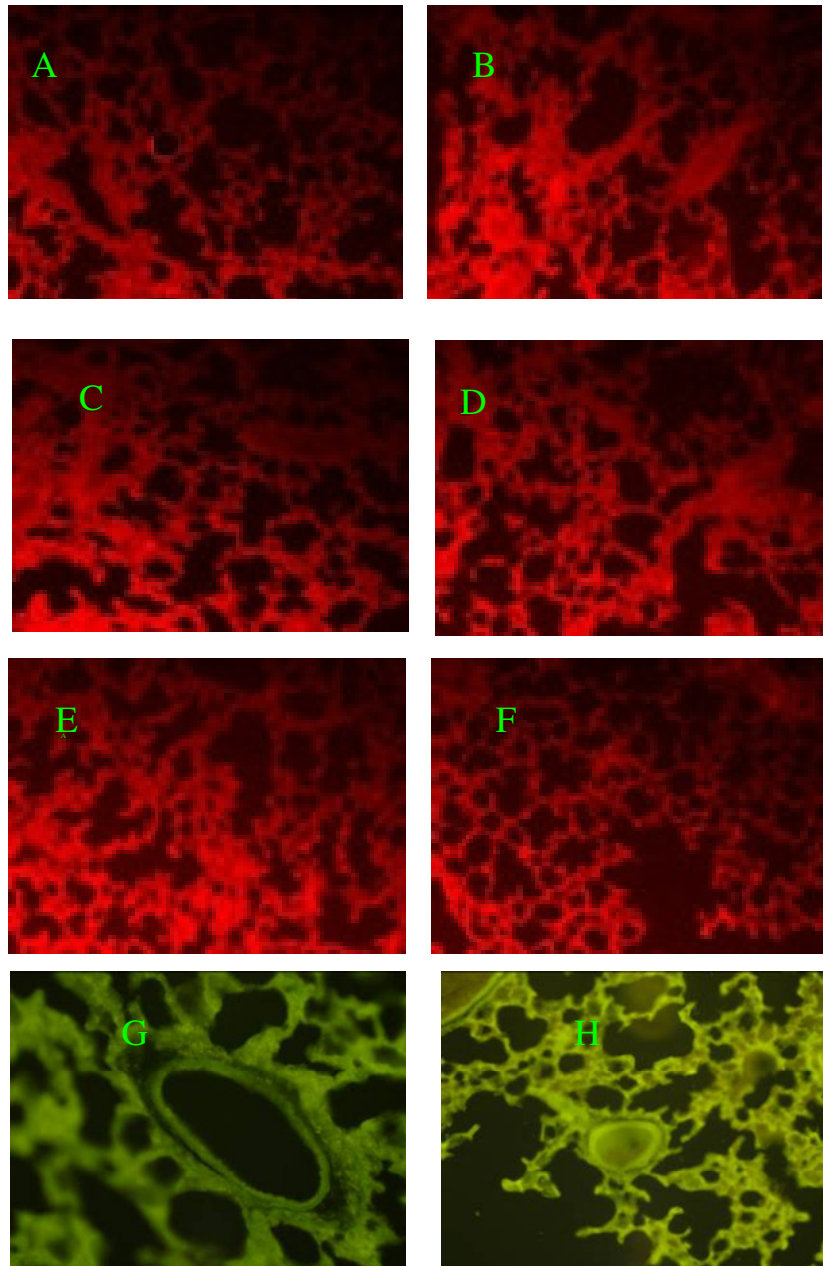


Figure 2. Immunohistochemistry of TGF- β and Smads in the lung tissues of chronic asthmatic rats. A, Smad 2 (control group); B, Smad 2 (OVE+ALU group); C, Smad 4 (control group); D, Smad 4 (OVE+ALU group); E, Smad 7 (control group); F, Smad 7 (OVE+ALU group); G, TGF- β (normal group); H, TGF- β (OVE+ALU group). The results show that TGF- β and Smad 2, 4, and 7 were all expressed on the bronchial or alveolar wall. All groups are different about fluorescence intensity. TGF, Transforming growth factor; Smad, mothers against decapentaplegic homolog.

a multitude of cellular processes in almost every aspect of cellular function. TGF- β is a widely acknowledged multifunctional factor that can regulate cell growth, differentiation, apoptosis and synthesis of extracellular matrix (ECM) (Zhang et al., 2009). TGF- β mediates biological effects through the TGF- β 1/Smad signaling

pathway. Much has been known about the TGF- β signaling pathway since the discovery of Smads 15 years ago; remarkable progress has been made during the past few years in revealing details of different regulatory mechanisms that afford a full range of control of the biological functions of the TGF- β (Ying and Zhang, 2011).

Table 1. Expression of TGF- β 1 and Smad 2, 4 and 7 mRNA (mean \pm SD, n = 6).

Group	TGF- β /GAPDH	Smad 2/GAPDH	Smad 4/GAPDH	Smad 7/GAPDH
Normal control	0.50 \pm 0.31	0.74 \pm 0.22	0.81 \pm 0.22	1.05 \pm 0.09
Experimental	1.09 \pm 0.31*	1.30 \pm 0.13*	0.77 \pm 0.20	0.55 \pm 0.23*

TGF, Transforming growth factor; Smad, mothers against decapentaplegic homolog; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

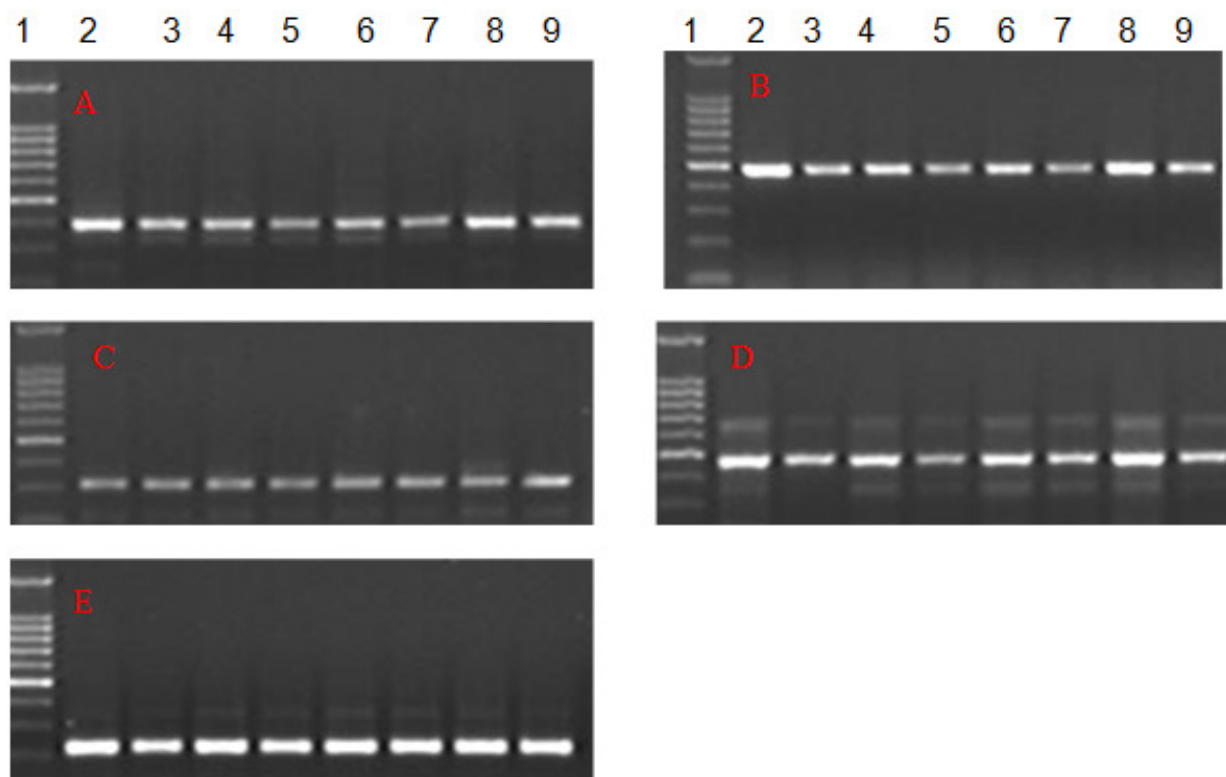


Figure 3. mRNA expression of TGF- β and Smads in chronic asthmalic rats in the lung tissues of chronic asthmalic rats. A, TGF- β 1; B, Smad 2; C, Smad 4 control group; D, Smad 7 OVE+ALU group; E, GAPDH; lanes 1, 3, 5 and 7 (normal control group); 2 lanes, 4, 6, 8, 9 (OVE+ALU group). TGF, Transforming growth factor; Smad, mothers against decapentaplegic homolog; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Cell surface receptors of TGF β signaling are mainly classified into two subtypes: type I (TGF β RI) and type II (TGF β RII). Smad-dependent TGF β signaling from cytoplasm to nucleus are primarily three Smad isoforms in the Smad family, that is, Smad 2, 3 and 4. The binding of ligands to TGF β RII leads (TGF β RI) to phosphorylated Smad 2 and 3 which then bind to Smad 4 forming a trimeric complex and translocate into the nucleus. In the nucleus, the Smad trimeric complex binds the Smad binding element (SBE) of target genes, regulating expression of TGF β response genes directly or through recruiting other co-factors (co-activators or co-repressors) to target genes (Feng and Derynck, 2005; Liu and Feng, 2010).

A third class of Smads, negatively regulate TGF- β and bone morphogenetic protein (BMP) signaling by

competing with R-Smads for binding to T β RI or targeting receptors to proteasomal degradation, therefore, are named the inhibitory Smads (I-Smads) (Imamura et al., 1997; Hayashi et al., 1997; Ebisawa et al., 2001; Kavsak et al., 2000). Previous studies have demonstrated that there are three types of Smads: receptor-regulated (Smad 1, 2, 3, 5 and 8), inhibitory (Smad 6 and 7) and universal (Smad 4) types (Jiang et al., 2008; Massague and Wotton, 2000).

In this study, we examined the effects of OVE+ALU-induced chronic asthma on lung tissue using HE staining, histology of the HE-stained tissues showed a marked alteration in the experimental group as compared to the control group, that it means the model rats are successful which means ovalbumin and aluminum hydroxide can induce the rat's bronchial asthma.

Table 2. Expression of TGF- β 1 and Smad 2, 4 and 7 (mean \pm SD, n = 6).

Group	TGF- β 1 (ng/ml)	Smad 2/NADPH	Smad 4/NADPH	Smad 7/NADPH
Normal control	14.5 \pm 2.2	33.52 \pm 1.05	166.41 \pm 15.29	102.29 \pm 5.0
Experimental	22.0 \pm 2.1*	57.94 \pm 33.02*	125.59 \pm 47.58*	43.87 \pm 5.19*

TGF, Transforming growth factor; Smad, mothers against decapentaplegic homolog; NADPH, Nicotinamide adenine dinucleotide phosphate.

In this study, we determined mRNA expression of TGF- β 1, Smad 2, 4 and 7. The results show that TGF- β 1 and Smad 2 increased significantly in the experimental group when compared with the control group. There was no significant difference in Smad 4 mRNA expression between the control group and the experimental group. mRNA expression of Smad 7 was significantly decreased in the experimental group when compared with the control group. TGF- β and Smad protein expression in chronic asthma were detected by ELISA and western blotting. Protein expression of TGF- β in lung tissue increased significantly in the experimental group. Protein expression of Smad 2, 4 and 7 in lung tissue increased significantly in the experimental group.

We showed that the TGF- β 1/Smad signaling pathway was altered in the airway reconstruction in chronic asthma. Through the investigation of gene and protein expression of TGF- β 1 and Smad 2, 4 and 7, we showed that TGF- β 1 protein and mRNA were both significantly increased, thus indicating that TGF- β 1 was activated.

During TGF- β signal transduction, TGF- β receptor is initially activated. TGF- β and its receptor specifically recognize the Smad subgroup known as receptor-activated Smads [R-Smads (Smad 2 and 3)] (Liu et al., 2010). R-Smads are subsequently activated to form a complex consisting of R-Smads and Smad 4, the latter of which is a co-Smad. The Smads transcriptional complex translocate to the nucleus, where it binds to a certain domain of the target gene and causes gene expression, for example, in the genes it involved collagen production. In chronic asthma, up-regulation of TGF- β may activate the TGF- β receptor, which specifically recognizes the Smad subgroup. The TGF- β receptor then modulates the high expression of Smad 2, which coincides with the receptor-regulate. Excess collagen production can cause alveolar expansion verily, thus, leading to the occurrence of asthma. The role of Smad 7 in the TGF- β /Smad signaling pathway is the inhibition of R-Smads phosphorylation and the blocking of signal transduction, thus, terminating gene expression. In chronic asthma, Smad 7 expression is down-regulated; this causes the balance to be upset in order to promote the TGF- β /Smad, thus, signaling a pathway that can induce proliferation. Smad 4 forms a complex consisting of R-Smads in the TGF- β /Smad signaling pathway. In chronic asthma, Smad 4 gene expression was not altered but protein expression was down-regulated. Further research is needed to investigate this observation.

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