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Expression of miR-15b-5p, miR-21-5p, and SMAD7 in Lung Tissue of Sulfur Mustard-exposedIndividuals with Long-term Pulmonary Complications

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ABSTRACT

Sulfur mustard (SM)-exposed individuals develop late pulmonary complications, which are associated with chronic inflammation and fibrotic changes in the lung tissue. MicroRNAs are known to act as important regulators of inflammatory responses, including inflammation and fibrosis-related cytokine signaling. In this study, we investigated the expression miR-15b-5p and miR-21-5p, two regulators of TGF-β signaling, as well as their target molecule, SMAD7, in lung tissues from SM-exposed and control individuals.

Total RNA was extracted from formalin-fixed paraffin-embedded (FFPE) lung tissue biopsies obtained during surgery from SM-exposed (n=20) or control (n=20) cases. Quality of the extracted RNA was evaluated by an Agilent Bioanalyzer and RNA was quantified using a NanoDrop. MiR-21-5p, miR-15b-5p and SMAD7 expression levels were measured by real-time RT-PCR.

miR-21-5p expression levels were significantly decreased (2.7 fold) in the lung tissues from SM-exposed individuals compared with tissues obtained from the control group(p=0.02). There were no significant differences in miR-15b-5p expression levels between the two groups(p=0.94). Interestingly, SMAD7 expression levels were significantly higher (5.8 fold) in SM-exposed individuals' lung tissues compared with the control group(p=0.045).

Our data indicate that exposure to sulfur mustard affects the expression of miR-21-5p as well as its target, SMAD7, in lung tissues many years after exposure. Considering the role of SMAD7 in the regulation of TGF-β signaling, these changes might point to a potential mechanism by which SM-exposure regulates inflammatory/fibrotic alterations in lung tissue.

Keywords: miR-15b-5p; miR-21-5p; SMAD7; Sulfur mustard; Transforming growth factor β

INTRODUCTION

Sulfur mustard (SM) is an alkylating agent with cytotoxic, mutagenic and carcinogenic properties.[1,2] Early and long-term complications occur after exposure to SM and the most commonly affected organs are lungs, eyes, and skin.[3,4] Pulmonary complications in SM-exposed individuals are dependent on the dose and duration of exposure and these complications

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generally progress with time.[5,6] Chronic bronchitis, asthma, bronchiectasis, lung fibrosis, chronic obstructive pulmonary disease (COPD) and bronchiolitis obliterans are the most common long-term pulmonary complications of SM exposure.[7,8] Inflammation and fibrosis are two major pathological features of SM-induced tissue injury and different inflammation and fibrosis-related molecules are known to be involved in SM-mediated diseases in various organs. Nonetheless, precise immunological/pathogenic mechanisms underlying this compound's pulmonary complications are not yet fully recognized.[9,10]

TGF-β is a cytokine that has been extensively studied in the context of chronic inflammation and tissue fibrosis. In the lungs, TGF-β is produced by many cell types including alveolar macrophages, neutrophils, activated alveolar epithelial cells, endothelial cells, fibroblasts, and myofibroblasts.SMAD proteins are intracellular mediators and regulators of TGF-β signaling; these molecules are activated following cytokine binding to the receptor, translocate to the nucleus and regulate gene expression.[11]One of SMAD molecules, SMAD7, has been shown to play key roles in regulating TGF-β signal transduction. TGF-β induces SMAD7 transcription and SMAD7 negatively regulates TGF-β signaling, thus establishing important negative feedback loop.[11,12]

MicroRNAs (miRNAs) are non-coding small RNAs which modulate various physiological and pathological processes in different tissues. They regulate gene expression by the degradation of target mRNAs or suppression of protein translation.[13]

Human miR-21 (has-mir-21) is a broadly conserved microRNA species, which is transcribed from a locus within the 3' UTR region of VMP1 gene, on the long arm of chromosome 17. This miRNA generates two mature isoforms; miR-21-5p and miR-21-3p. MiR-21-5p, which is the dominant isoform, plays pivotal roles in many diseases including cardiovascular diseases, inflammation, and cancer.[14-16] Recently, additional roles of miR-21 in pulmonary diseases, including pulmonary fibrosis, have been reported.[17]Post-transcriptional regulation of miR-21 is performed by several factors including TGF-β and bone morphogenetic protein (BMP). TGF-β increases pre-miR-21 levels 30 minutes after exposure of cells to the cytokine.[18,19] miR-21-5p targets SMAD7 and suppresses its expression levels.[17,20] The role of miR-21-5p in organ fibrosis has been reported by several groups.[21,22]

MiR-15b is a member of the miR-15/miR-107 family of microRNAs.[23] miR-15b locus is placed inside one of the introns of SMC4 gene, on the long arm of chromosome 3. This miRNA generates 5p and 3p isoforms, with miR-15b-5p being the dominant isoform. Similar to miR-21-5p, miR-15b-5p has a known role in the regulation of TGF-β signaling, through targeting SMAD7 molecules.[24]

In the present study, expression levels of miR-21-5p, miR-15b-5p, and SMAD7 were evaluated in lung tissues from individuals who had developed chronic pulmonary complications following exposure to SM. Following expression analyses, potential correlations between miRNAs and SMAD7 levels were evaluated.

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.1396.81).

Sample Collection

In this study, archived lung formalin-fixed, paraffin-embedded (FFPE) tissues were used. Biopsies had been performed during surgery at general hospitals in Tehran, Iran in the period between 2005 and 2011. All samples were used with a code without any name or other patient information. Twenty samples of patients with a history of SM exposure in the Iraq–Iran war (25-30 years ago) and 20 samples of patients without any background of SM exposure as a control group were used. The SM-exposed patient suffered from long-term pulmonary complications. Prior to the study, three trained pathologist examined hematoxylin and eosin (H&E) stained slides. The pathology of pulmonary complications was reported in SM-exposed and normal historical blocks of control samples were selected. Exclusion criteria were systemic or local diseases such as acute or chronic infections and autoimmune disorders, and history of other toxic gas exposures. Both groups were male, 30–60 years old, none smoker and had no history of addiction to alcohol. As samples were randomly collected, there was a vast diversity of

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histopathologic features in the microscopic specimens; in both SM-exposed and control groups (Table 1; Figure 1).

RNA Extraction

Total RNA was extracted from FFPE samples using Recover All Total Nucleic Acid Isolation Kit (Ambion Ltd., Cambridgeshire, UK) following the manufacturer's protocol. Initially, four 10 µm sections were cut from each block and deparaffinized by xylene (Merck, Germany) at 56°C for 30 min with 400 rpm agitation, twice. The residual xylene was washed by ethanol 96% (Merck,-Germany) and the sections were air dried. Protocol steps followed by proteinase K digestion for 3 hours at 56°C, DNase digestion on column and elution in nuclease-free water as described in the protocol.

Table1. Histopathological characteristics of sulfur mustar-exposed and control groups

	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

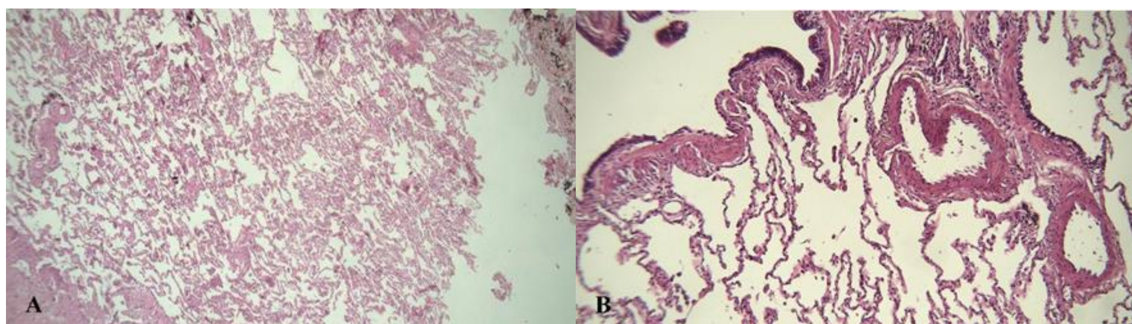

Figure 1. A; Photomicrograph of a specimen from the control group in a study on the expression of miR-15b-5p, miR-21-5p, and SMAD7 in lung tissue of sulfur mustard-exposed individuals, showing an intact region with mild emphysema and normal morphology. The patient was diagnosed to have a pneumothorax. Magnification: X 40. B; A lung specimen from exposed group obtained by wedge biopsy. Patchy areas of fibrosis especially around bronchioles infiltrated bronchiolar walls by inflammatory cells and other morphological deviations are consistent with constrictive bronchiolitis, Magnification: X 100.

Evaluation ofQuantity and Quality of RNA

The concentration and purity of extracted RNA samples were examined by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The purity of RNA samples was assessed according to the OD A260/A280 ratio. Samples with an A260/A280 ratio of less than 1.8 were discarded. The RNA integrity was evaluated using Agilent RNA 6000 Nano Kit on an Agilent 2100 Bioanalyser (Agilent technologies, Waldbronn, Germany). RIN (RNA Integrity Number) and DV200 (percentage of RNA fragments with more than 200 nucleotides) were measured for determining the quality of RNA. Samples with DV200 upper than 30% were used.

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Real-time PCR microRNA assay

Total RNA was reverse transcribed using TaqMan MicroRNA Reverse Transcription Kit and looped RT primers (Applied Biosystems, USA). Each RT reaction consisted of 7 µLof the master mix, 3 µLof primer, and 10 ng (~5 µL) of the RNA sample. The RT reaction was performed using Thermal cycler T100 (Bio-Rad, USA) with the following steps: 16°C for 30 min, 42°C for 30 min and 85°C for 5 min.

Real-time PCR was carried out triplicate using TaqMan 2x Universal PCR Master Mix, TaqMan Real-time PCR microRNA assay (Applied Biosystems, USA) and 1.33 µLof 1:15 diluted cDNA. An amplification program was applied with the following steps: 10 min of denaturation at 95°C,40 cycles of real-time PCR with 2-step amplification including 15 s at 95°C for denaturation, 60 s at 60 °C for annealing and polymerase elongation. Data was normalized to small RNA U6 levels.

Quantitative RT-PCR for SMAD7 Expression

Total RNA was reverse transcribed by a high capacity cDNA reverse transcription kit (Applied Biosystems, USA). Following manufacturer's protocol to prepare 2X reverse transcription master mix, we mixed 2 µL 10X RT buffer, 0.8 µL 25X dNTP Mix (100 mM), 2 µL 10X RT random primers and 1 µL MultiScribe™ Reverse Transcriptase. For each sample, 10 µL2X reverse transcription master mix were mixed with 400 ng of extracted RNA (~10 µL).

For evaluation of SMAD7 expression levels, primers were designed using NCBI mRNA sequence. One of the primers was designed to span an exon-exon junction. Thermodynamic state and secondary structure of primers were examined by Gene Runner 5.0.1 software and Oligo Analyzer software.Primers specification are shown in Table 2.The amplification was carried out on the Applied Biosystems StepOnePlus Real-Time PCR System. The 20 µl reaction included Real Q Plus 2X Master Mix Green (Ampliqon, Denmark), 7 µLRNase free H2O, 10 pmol of each primer, and 2 µL1:2 diluted cDNA. 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 stage at the end of the run with slow heating started at 60°C that increased to 95°C with a rate of 0.3°C per second with measurement of fluorescence continuously. Data was normalized to PGK level as the reference gene.[24]

All PCRs were performed in triplicate. The real-time PCR efficiency of the primers was evaluated using LinReg PCR software, version 11.0. The relative expression of each miRNA and SMAD7 mRNA were calculated on the ΔCT method (where ΔCt is the value obtained by subtracting the Ct value of each mRNA or miRNA from mean Ct value of them) withthe formulas below:

$$\text{Ratio}=\frac{(E_{target})^{\Delta CT,\ target)(calibrator\text{-}test)}}{(E_{Ref})^{\Delta CT,\ target)(calibrator\text{-}test)}}$$

Table2. PGK1 and SMAD7 genes designed primers sequences to evaluate mRNA expression of SMAD7 in sulfur mustard-exposed lung

Gen number	Forward (3>5)	Reverse (3>5) length (bops)	Amplicon	Efficiency (%) Accession	GenBank
SMAD7	CCTTCCTCCGCTG AAACAG	CCCACTCTCGTCT TCTCCT	134	90.1	AH011391.2
Phosphoglycerate kinase 1 (PGK1)	GGCATACCTGCTG GCTGGATG	ACAGGACCATTCC ACACAATCTGC	104	88.03	NM_000291.3

Statistical Analysis

Mann-Whitney U test was used for mean comparison between groups. Correlation between expression levels was analyzed by Spearman's rho test. *p* values below 0.05 were considered as statistically significant. SPSS version 16 used for all of the statistical analyses. Graph-creation was performed using Prism V5 (GraphPadSoftware).

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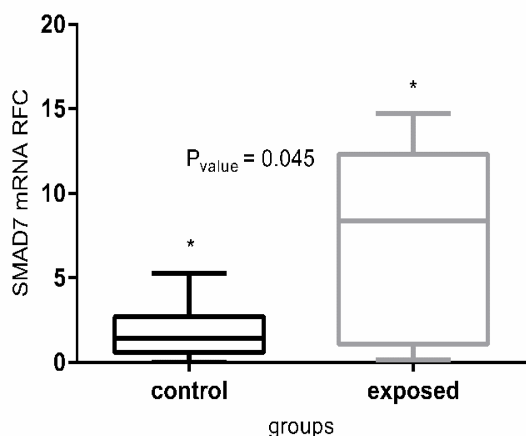

Figure2. SMAD7mRNA expression of 40 samples (20 sulfur mustard-exposed and 20 control) was evaluated by RT-qPCR. The test was performed in triplicateand data was analyzed withMann-Whitney Whitney U test. The results show that SMAD7 relative fold change was upregulated in lung tissues from SM-exposed patients(*p*=0.045).

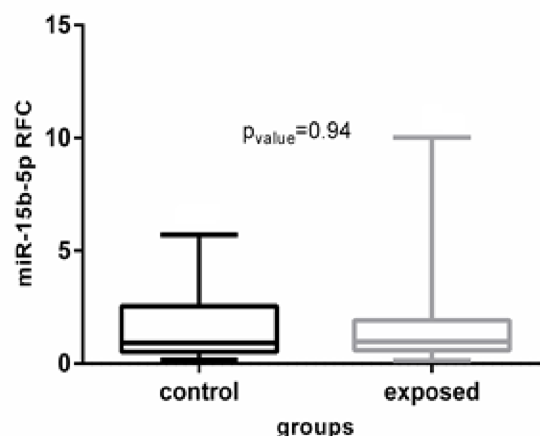

Figure 3. MiR-15b-5p mRNA expression of 40 samples (20 sulfur mustard-exposed and 20 control) was evaluated by RT-qPCR. The test was performed in triplicate and data was analyzed with Mann-Whitney Whitney U test. The results show that miR-15b-5p relative fold change was unchanged in SM-exposed patients compared with the control group (p^*=0.94)

RESULTS

In the present study, 20 lung tissue samples from SM-exposed patients and 20 samples from control unexposed people 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.

QRT-PCR was used to determine SMAD7 gene expression levels. Data were normalized to PGK as the housekeeping gene. After statistical evaluation, we found that SMAD7 expression levels in SM-exposed individuals was significantly higher than the control, unexposed group (*p*=0.045) (Figure 2)(Table 3).

The expression level of miR-15b-5p was unchanged between SM-exposed and control groups (*p*=0.94) (Figure 3). But, miR-21-5p was down-regulated significantly in exposed individuals as compared to the control group (*p*=0.02) (Figure 4)(Table 4). Data were normalized to U6 snRNA.

There was no correlation between the expression level of SMAD7 and miR-21-5p and also miR-15b-5p in either SM-exposed individuals or control groups. Details have been shown in Table 5.

Table3. Differential expression of SMAD7 mRNA gene in SM-exposed and control groups lung tissues

	Exposed				Control				
Gene	Median	Q1	Q3	Mean±SD	Median	Q1	Q3	Mean±SD	*p*-value
SMAD7	8.383	0.184	3.576	1.880±1.696	1.433	1.272	13.028	7.150±5.878	0.045

Table 4. Differential expression of miR-15b-5p and miR-21-5p genes in SM-exposed and control groups lung tissues

	Exposed				Control				
Gene	Median	Q1	Q3	Mean±SD	Median	Q1	Q3	Mean±SD	*p*-value
MiR-15b-5p	0.10	0.06	0.28	0.17±0.16	0.10	0.07	0.20	0.13±0.09	0.94
MiR-21-5p	0.34	0.24	0.55	0.49±0.46	0.93	0.62	1.06	0.89±0.56	0.02

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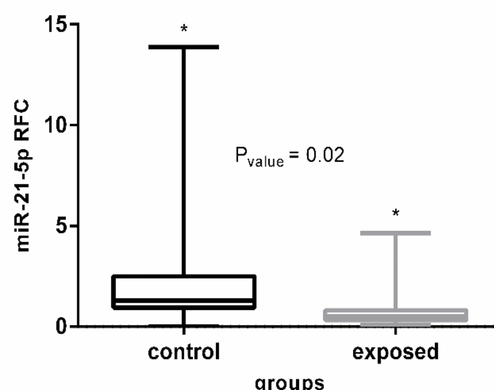

Figure4. MiR-21-5pmRNA expression of 40 samples (20 SM-exposed and 20 control) was evaluated by RT-qPCR. The test was performed in triplicate and data was analyzed with Mann-Whitney Whitney U test. The results show that miR-21-5p relative fold change was down-regulated in SM-exposed patients(*p*=0.02).

Table5. Correlation between miRNAs and SMAD7 genes expression in SM-exposed subject

	Exposed		Control	
Gene	**Correlation**	***p*-value**	**Correlation**	***p*-value**
MiR-15b-5p	0.350	0.356	0.079	0.781
MiR-21-5p	-0.017	0.966	0.100	0.798

DISCUSSION

Pulmonary disorders are one of the most common complications in SM-exposed individuals.[3] SM can lead to pulmonary issues such as COPD, asthma, bronchiolitis obliterans and IPF. Several studies have indicated that TGF-β gene expression might be altered in lung tissues of SM-exposed individuals.[7] TGF-β plays an important role in fibrotic conditions and its signaling pathway is mediated/regulated by SMAD molecules including SMAD7. Indeed, SMAD7 is able to diminish excessive activation of the TGF-β signaling pathway.[24] TGF-β induces the expression of miR-21-5p which can target SMAD7 transcripts and inhibit its expression.[17] As a consequence, activation of TGF-β signaling could exacerbate fibrotic conditions through SMAD7 suppression. SMAD7 also targeted and regulated by miR-15b-5p.[24]This study was aimed to investigate the expression of miR-15b-5p and miR-21-5p, two regulator of TGF-β signaling pathwayand their targeted molecule, SMAD7, in lung tissue of SM-exposed individuals.

Consistent with our data,Zandvoort et al have reported that SMAD7 gene expression is increased in individuals with COPD after stimulation with TGF-β.[25]

In a study on SM-exposed individuals, Adelipour et al have shown that SMAD7 expression levels did not change in airway wall biopsies following SM exposure.[26]This discrepancy between our results in comparison with that study might be due to differences in samples and analyzing method. Although lung tissue parenchyma was used and analyzed by real-time PCR method in the present study,Adelipour's study examined SMAD7 in air wall biopsy by PCR method.

According to some studies, TGF-β gene expression in SM-exposed individuals with pulmonary complications was increased.[8,27] It seems that SMAD7 up-regulation is able to act as negative feedback to suppress TGF-β signaling pathway and fibrotic disorders.

Valizadeh et al evaluated miR-21 and TGF-β in skin samples of SM-exposed individuals. Similar to our data, they showed that miR-21 down-regulated and it is related to increased TGF-β2 gene expression.[28]TGF-β is one of miR-21 mRNA target molecules and it could induce miR-21 expression. It seems that reduced expression of miR-21 is followed by TGF-β increased expression.

In patients with Non-Small Cell Lung Cancer (NSCLC) overexpression of miR-21 with a positive

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regulatory effect on epidermal growth factor receptor (EGFR) pathway, has an anti-apoptotic effect on cancer cells. Inhibition of miR-21 leads to an increase in apoptosis in cancer cells.[29] Based on the study earlier performed on our samples, apoptosis increased in lung tissue of SM-exposed individuals with long term pulmonary complications.[30]It seems that reduced expression of miR-21 increases apoptosis in lung tissues of SM-exposed patients.

mir-21 gene expression is increased in inflammatory diseases such as a solid tumor, heart, and tissue injury that could be considered as a negative regulator of inflammatory responses. Down-regulation of miR-21 in leucocytes is associated with elevated inflammatory immune responses.[31] Also, down-regulation of miR-21 leads to activate NF-κB signaling pathway.[32]AS the result show, miR-21 suppresses the TGF-β expression and this may promote inflammation. On the other hand, it is a negative regulator of inflammation, therefore, it proposes that miR-21 has a dual role in inflammation.

In a study by Pinelo et al researchers examined miR-15b-5p expression in the lung of individuals with COPD and they found that miR-15b-5p gene expression was increased compared with the control group.[33] MiR-15b-5p is a negative regulator of SMAD7 and down-regulates its expression. Therefore, with the suppression of SMAD7expression, miR-15b-5p could potentiate the progression of fibrosis.[34]In our study, it seems that miR-15b-5p is not a key player in the pathogenesis of pulmonary fibrosis in SM-exposed individuals.

We also evaluated the correlation between SMAD7 expression levels with miR-15b-5p and miR-21-5p expression. Herein,results did not show any significant correlations between SMAD7 with either miR-15b-5p or miR-21-5p gene expression. However, it should be noted that miRNAs might affect their target genes protein levels, without affecting their transcript abundance. Examining SMAD7 protein levels in lung tissues could be helpful in this context.

According to the results of this study, It seems that the regulatory mechanism of the negative control is activated by increasing the SMAD7 expression and decreased expression of miR-21-5p and no change in the expression of miR-15-5p, to modify the amount of TGF-β in this group of patients after years of SM-exposure. However, the number of samples is a limitation of this study and it is recommended to carry out further studies with more samples.

Data from this gene expression analyses point to the potential involvement of miR-21-5p and SMAD7 molecules in pulmonary complications that occur after exposure to sulfur mustard.

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