Research Paper

Expression of miR-106a-5p, miR-106b-5p, and TGFβ1I1 in Peripheral Blood Mononuclear Cells (PBMCs) of Chemical Veterans Exposed to Sulfur Mustard With Long-term Pulmonary Complications

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ABSTRACT

Background: Sulfur mustard as a chemical warfare agent causes short and long-term pulmonary complications in its victims. MicroRNAs are known to act as remarkable regulators of biological pathways, monitoring, and treatment of diseases including respiratory problems. In this study, we investigated the expression of miR-106a-5p and miR-106b-5p, two regulators of TGF-β signaling, as well as their target molecule, TGFβ1I1, in peripheral blood mononuclear cells from SM-exposed individuals.

Materials and Methods: A total of 70 veterans with SM-induced pulmonary complications were examined and compared to 35 gender and age-matched healthy controls. After clinical examination and pulmonary function tests, the severity of pulmonary complications was classified. Total RNA was extracted from PBMCs and the purity of extracted RNA samples was evaluated by a NanoDrop 2000. The miR-106a-5p, miR-106b-5p, and TGFβ1I1 expression levels were measured by real-time RT-PCR.

Results: The miR-106a-5p expression levels were significantly increased in both mild (P=0.015) and severe groups compared with the control group. The miR-106b-5p expression levels were considerably elevated in the severe group TGFβ1I1 expression levels were notably reduced in the severe group compared with the control group. Although, a slight decrease in TGFβ1I1 expression levels was observed in the mild group compared with the control.

Conclusion: Our results indicate that exposure to sulfur mustard affects the expression of miR-106a-5p, miR-106b-5p, and their target gene, TGFβ1I1, in peripheral blood mononuclear cells. Considering the role of TGFβ1I1 in the regulation of TGF-β signaling, the mentioned changes might point to a potential mechanism by which SM exposure causes chronic pulmonary complications. In a ROC analysis, miR-106a-5p and miR-106b-5p potentially turned out to be a suitable diagnostic biomarker in the mild and severe categories of patients. Although, miR-106a-5p could be considered a better biomarker than miR-106b-5p.

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1. Introduction

Sulfur mustard (SM) is an alkylating chemical warfare agent with carcinogenic, mutagenic, and cytotoxic properties that was used during the Iran-Iraq war between 1983 and 1988 [1-3]. The pulmonary system, skin, and eyes are the major targets for the early and late toxic effects of SM [4]. The dosage and duration of mustard gas exposure are directly related to the severity of pulmonary complications [5, 6]. Respiratory complications are the most common delay problem, while skin and eye lesions diminish or remain constant over time [6, 7]. The most common delayed pulmonary complications following exposure to SM include asthma, chronic obstructive pulmonary disease (COPD), chronic bronchitis, bronchiolitis obliterans, obstructive bronchitis, and pulmonary fibrosis [8-10]. Although the role of chronic inflammatory factors, changes in cytokines, free radicals, destructive effects on enzymes and mitochondria of cells, as well as the cessation of cell proliferation have been evaluated, the exact immunopathological mechanisms of acute and chronic effects of mustard gas are still unknown [11, 12].

Transforming growth factor β (TGF-β) is a cytokine that is widely involved in many biological processes such as differentiation, cell proliferation, inflammation, tissue homeostasis, apoptosis, and angiogenesis [13, 14]. It is clear that cells such as neutrophils, alveolar epithelial cells, alveolar macrophages, endothelial cells, NKT, fibroblasts, myofibroblasts, and Tregs are responsible for the production of TGF-β in the lung and also Tregs and TGF-β (three TGF-β isoforms include TGFβ1, TGFβ2, and TGFβ3) have an important character in the development of lung-related immune disorders like asthma, allergies, and COPD [15].

TGF-β signaling is carried out by TGFβR1 and TGFβR2, which are its membrane receptors and are part of serine/threonine kinase receptors. The altered expression of proteins involved in the TGF-β signaling pathway is associated with some main diseases including cancer, inflammation, and fibrosis [16]. TGF-β intracellular signaling pathways are regulated by several intracellular mediators and regulators such as SMADs and TGFβ1I1 [17]. The TGFβ1I1 protein, which is also known as HIC-5 or ARA55 , has four LIM motifs and is expressed in more organs such as the lung and spleen. This protein induces an inhibitory signal by binding its LIM3 domain to the MH2 domain in Smad3. TGFβ1I1 also acts as a TGF-β signaling pathway activator through physical interaction and inhibitor of Smad7 [18, 19].

MicroRNAs (miRNAs) are small, non-coding RNAs that control gene expression by destroying target mRNAs or suppressing protein translation [20]. These RNAs are involved in regulating various physiological and pathological processes including cell cycle, aging, proliferation, and apoptosis [21, 22]. Due to the close relationship between their aberrant expression and various diseases, as well as their high preservation and stability in clinical specimens, miRNAs are considered novel and remarkable biomarkers for the diagnosis and treatment of various diseases [22].

The miR-106a-5p and miR-106b-5p are located on the long arm of chromosome X and chromosome 7, respectively. The miR-106 plays key roles in a wide range of diseases including various types of cancer especially lung cancer, COPD, asthma, fibrosis, and cardiovascular [23-27]. As well, post-transcriptional regulation of miR-106 is performed by several factors including TGF-β; based on evidence from mirdb and targetscan databases, the miR-106a-5p and miR-106b-5p target the 3'UTR region of the TGFβ1I1 gene and, by suppressing its expression, affect the function of the TGF-β signaling pathway [28-30]. Some papers found the essential roles of miR-106a-5p and miR-106b-5p in development of the asthma, COPD as well as respiratory complications [31, 32].

This study aimed to evaluate expression levels of miR-106a-5p, miR-106b-5p, and TGFβ1I1 in PBMCs from chemical warfare victims who have developed chronic pulmonary complications following exposure to SM. The expression analyses, potential correlations between miRNAs, and TGFβ1I1 levels were evaluated.

2. Materials and Methods

Sample collection

In this case-control study, 70 SM-exposed individuals based on the medical documents verified by the Medical Committee of the Foundation of Martyr and Veterans Affairs as well as GOLD 2018 classification, and 35 healthy individuals without any history of SM exposure, were recruited. All patient samples were obtained from people who were injured in the chemical bombing of Sardasht on June 28, 1987. Individuals in the patient group were classified into mild and severe groups based on the initial symptoms of SM. All groups were male, 30 to 60 years old, and non-smokers with no history of alcoholism who had no underlying or inflammatory lung disease interfering with the study. According to documents, all patients suffered from long-term pulmonary complications. Chronic cough, sputum, hemoptysis, and dyspnea were considered common pulmonary symptoms of SM exposure.

The mild group (n=35) included exposed individuals who had no early symptoms at the time of exposure. The severe group (n=35) included individuals who experienced early symptoms immediately after the exposure and were hospitalized accordingly [33].

Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were purified using the Ficoll-Paque gradient method; 10 mL of peripheral blood was collected from patients and healthy individuals and stored at 4°C to minimize cell activation. Briefly, 4 mL of ficoll-Paque gradient was pipetted into two 15 mL Falcon tubes. Then K2EDTA-blood samples were layered over the Ficoll-Paque gradient (10 mL/tube) carefully. The tubes were centrifuged for 20 min at 2800 rpm at 7°C, then the layer containing PBMCs was carefully harvested and then washed twice with PBS, after that the cells were resuspended in PBS and were counted manually using a Neubauer chamber (Ningbo Finer Medical Instruments Co., Ltd.) and light microscope (magnification×100) [34].

RNAe extraction and cDNA synthesis

Total RNA was extracted from PBMC samples using Hybrid-RTM miRNA Kit (Gene All, South Korea) following the manufacturer's protocol. The purity and concentration of extracted RNA samples were evaluated by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). First-strand cDNA synthesis was performed from 1 µg total RNA using BON high sensitivity microRNA first strand cDNA synthesis Kit (Bon-yakhteh) for microRNA analyses and TAKARA kit for gene expression analyses, according to the manufacturer's instructions.

Real-time RT-PCR

miRNAs (miR-160a-5p and miR-160b-5p) and their predicted target levels were measured by real-time reverse transcription–PCR using BON- high-specificity microRNA QPCR master mix (Bon-yakhteh, IRAN) on applied biosystems StepOnePlus system. MicroRNA expression data were normalized against SNORD 47 expression levels, and also the expression of the other genes was normalized against β-actin mRNA levels (Table 1).

Statistical analysis

The normality of the data was assessed by the Kolmogorov-Smirnov and D'Agostino-Pearson tests. Due to the abnormal distribution of the data, the Mann-Whitney and Kruskal–Wallis tests were used to compare the expression level of the genes. Furthermore, the Spearman correlation coefficients (r) were used to determine a potential correlation between TGFβ1I1 expression levels and those of miR-106a-5p and miR-106b-5p. The $P<0.05$ was presumed as statistically significant. As well, GraphPad Prism software version 9 was used for all of the statistical analyses and graph creation.

The receiver-operating characteristic (ROC) curve analysis was employed to determine whether the expression of miR-106a-5p and miR-106b-5p has the sensitivity and specificity to discriminate between cases and controls.

3. Results

In the current study, 35 healthy individuals were studied in a control group, 35 in a mild group, and 35 in a severe group. The average age of the groups was 46.71±7.36, 44.40±9.14, and 47.77±8.2, respectively, and there was no significant difference between them.

Table 1. β-actin and TGFβ1I1 genes designed primers sequences to evaluate mRNA expression of TGFβ1I1 in sulfur mustard –exposed lung

Primers		Sequence (5`→3`)
TGFβ1I1‡	Forward	CCCTTCGGAGATGAGGGTTTC
	Reverse	AAGTGGTTCTCGCACAACGG
Product length		236
β-actin	Forward	AGATCAAGATCATTGCTCCTCCTG
	Reverse	TGTCACCTTCACCGTTCCAG
Product length		318

‡ TGFβ1I1: Transforming growth factor Beta 1 induced transcript 1.

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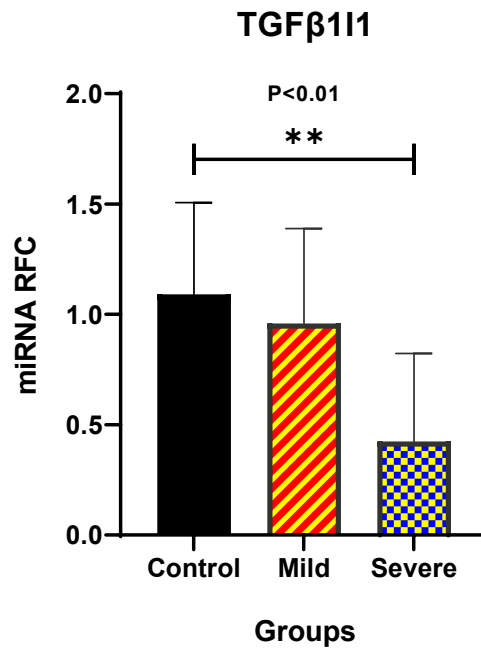
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TGFβ1I1

Figure 1. TGFβ1I1 mRNA expression level in SM-exposed and healthy controls. The expression of the TGFβ1l1 gene was measured in PBMCs by Real-time RT-PCR. The level of TGFβ1I1 was decreased in both mild and severe groups respectively. Data are shown as Mean±SD. Number of samples in each group=35, **P<0.01, Kruskal–Wallis tests.

Real-time PCR was used to determine TGFβ1I1 gene expression levels. Data were normalized to β-actin as the housekeeping gene. After statistical evaluation, it was demonstrated that TGFβ1I1 expression levels decreased in both mild and severe groups and were significant in the severe group (P<0.01) (Figure 1) (Table 2).

The expression level of miR-106a-5p was considerably increased in the mild (P<0.05) and severe groups (P<0.01) compared to the control one (Figure 2) (Table 3).

Also the miR-106b-5p decreased in the mild group and increased significantly in the severe one (P<0.05) (Figure 2) (Table 3). Data were normalized to SNORD47 levels.

There was a significant correlation between the expression of TGFβ1I1 and miR-106a-5p and also miR-106b-5p in either SM-exposed individuals or control groups (Figure 3). Details are shown in Table 4.

Association of miR-106a-5p and miR-106b-5p expression with patients' clinical features

ROC curve analysis was used to evaluate the sensitivity and specificity of miR-106a-5p and miR-106b-5p expression levels in discriminating subjects with mustard lung from the control group. According to ROC curve analysis, miR-106a-5p was in the mild group; area under the curve (AUC)±SE 0.918±0.033, P<0.0001, and in the severe group (AUC±SE 0.995±0.005, P<0.0001), as well as miR-106b-5p was in the mild group (AUC±SE

Table 2. Differential expression of TGFβ1I1 mRNA gene in control, mild and severe groups

Gene		Control	Mild	Severe
TGFβ1I1[†]	Median	1.12	0.905	0.28
	Q1	0.795	0.55	0.155
	Q3	1.415	1.183	0.44
	Mean±SD	1.092±0.418	0.925±0.425	0.463±0.4646
	P	-	-	<0.01

[†] TGFβ1I1: Transforming growth factor Beta 1 induced transcript 1.
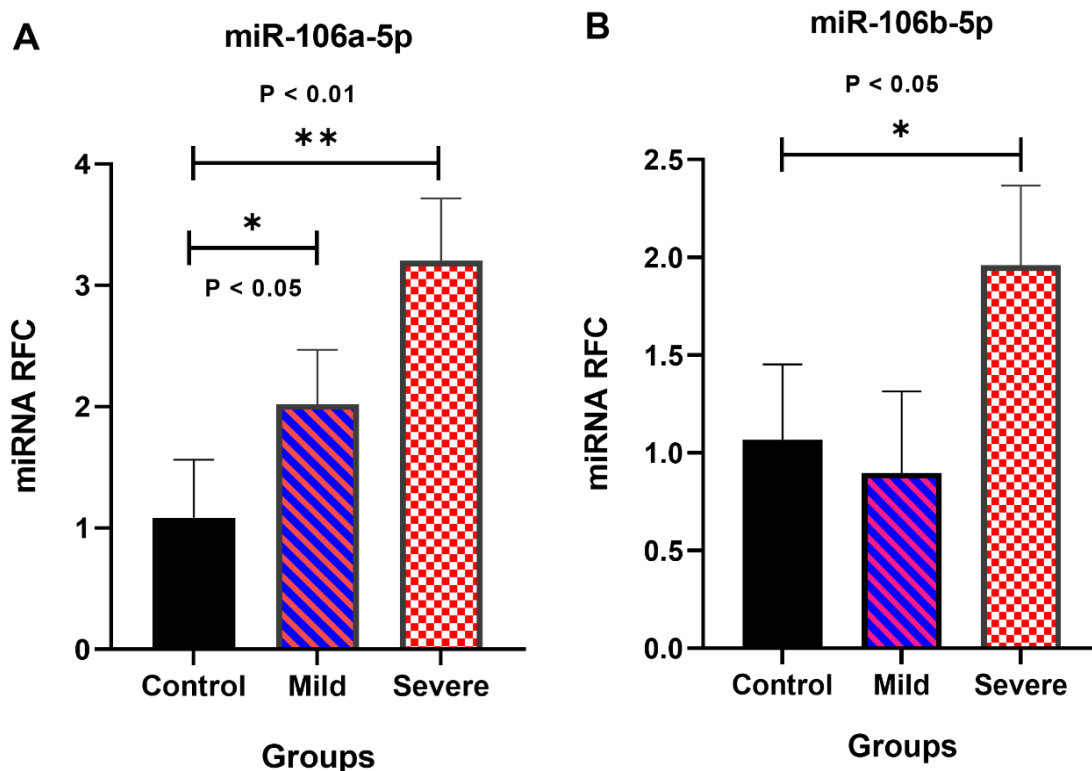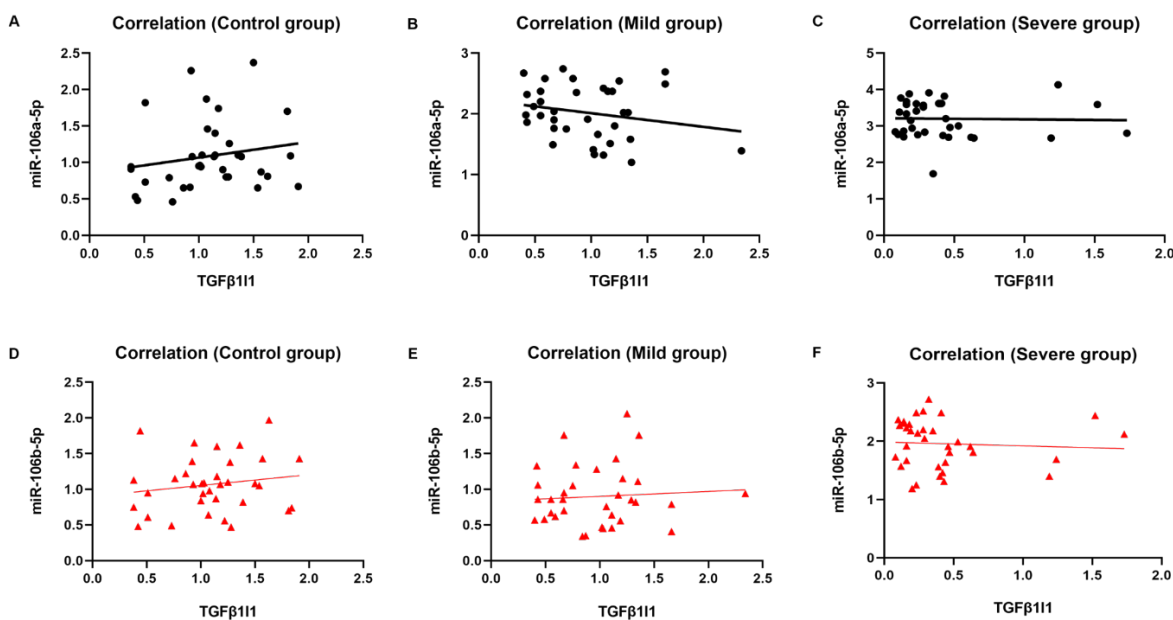
A   miR-106a-5p

B   miR-106b-5p

Figure 2. miR-106a-5p and miR-106b-5p mRNA expression levels in SM-exposed and healthy controls. Expression of miR-106a-5p and miR-106b-5p genes were measured in PBMCs by Real-time RT-PCR. (A) The level of miR-106a-5p was increased in both mild and severe groups. (B) the expression level of miR-106b-5p was decreased in the mild and increased in the severe group. Data are shown as Mean±SD. Number of samples in each group=35, *P<0.05, **P<0.01, Kruskal–Wallis tests.

Table 3. Differential expression of miR-106a-5p and miR-106b-5p genes in control, mild, and severe groups

Gene		Control	Mild	Severe
	Median	0.95	2.02	3.2
	Q1	0.79	1.66	2.77
miR-106a-5p	Q3	1.26	2.37	3.62
	Mean±SD	1.086±0.475	2.02±0.447	3.2±0.5147
	P	-	<0.05	<0.01
	Median	1.07	0.85	1.99
	Q1	0.75	0.58	1.64
miR-106b-5p	Q3	1.38	1.11	2.29
	Mean±SD	1.067±0.385	0.898±0.416	1.959±0.409
	P	-	-	<0.05

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Figure 3. Correlation between the expression TGFβ1I1 and miR-106a-5p and also miR-106b-5p. The expression of miR- 106a-5p has more effect on inhibiting and reducing the expression of the TGFβ1I1 gene.

Table 4. Correlation between miRNAs and TGFβ1I1 genes expression

		Correlation		P
miR-106a-5p	Control	Spearman r	0.2510	>0.05
	Mild	Pearson r	-0.2157	>0.05
	Severe	Spearman r	-0.1311	>0.05
miR-106b-5p	Control	Pearson r	0.1635	>0.05
	Mild	Spearman r	0.07614	>0.05
	Severe	Pearson r	-0.06382	>0.05

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0.645±0.066, P<0.05) and in the severe group (AUC±SE 0.940±0.025, P<0.0001). A high AUC indicates that the studied test could distinguish between the absence and presence of the disease in question (Figure 4).

4. Discussion

One of the most commonly reported complications is respiratory problems in SM-exposed individuals [35]. SM can lead to pulmonary issues such as bronchiolitis obliterans, COPD, asthma, and pulmonary fibrosis.

Recently, altered expression of TGF-β in the tissue of subjects exposed to SM has been investigated in several studies. However, some other studies reported that TGF-β expression was increased in tissue samples exposed to SM [36, 37]. TGF-β, as a remarkable character in causing chronic pulmonary complications in SM-exposed veterans, is mediated in the signaling pathway by TGFβ1I1. Actually, by inhibiting SMAD7, TGFβ1I1 increases the activity of the TGF-β signaling pathway [19]. Also, another study by Zandvoort et al. reported that SMAD7 gene expression is increased in COPD patients after stimulation with TGF-β [38]. Despite extensive research, we have not found any studies on the expression of TGFβ1I1 in SM-exposed individuals with COPD as well as asthma. Another research team, Cui et al., reported that hic-5 is essential for myofibroblast differentiation [35, 39] and Lei et al. showed that hic-5 expression was increased in hepatic fibrosis in mice and humans [40].
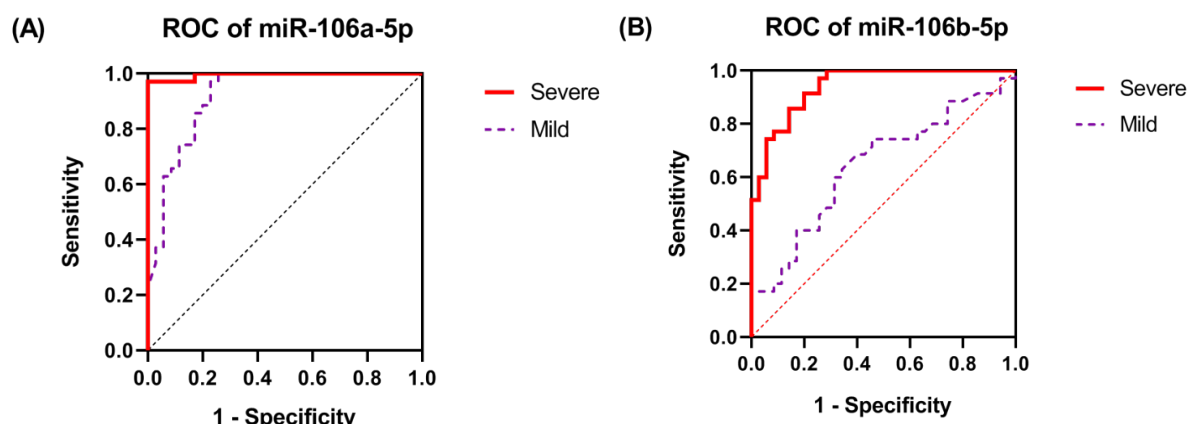
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Figure 4. Receiver operating characteristic (ROC) curve analysis determined the good sensitivity and specificity for miR-106a-5p and miR-106b-5p expression levels in discriminating cases from controls. (A) In the mild group the calculated area under the curve (AUC) was 0.918 and in the severe group was 0.995 for miR-106a-5p. (B) AUC for miR-106b-5p in the mild group was 0.645 and in the severe group was 0.940 demonstrating the suitability of miR-106a-5p and miR-106b-5p to correctly classify cases and controls. However, miR-106a-5p has a more acceptable performance and could be considered a better potential biomarker. The x and y axis denote the values of the performance parameters 'specificity' and 'sensitivity' over the full range from 0 to 1.

The data of this study showed that the expression level of the TGFβ1I1 gene was significantly decreased in the group of patients with severe symptoms compared to the control group.

TGF-β signaling pathway plays a key role in cell proliferation, differentiation, development, senescence, apoptosis, and, inflammation. Probably, the main cause of inflammation in patients with pulmonary complications exposed to SM is due to the downregulation of TGF-β, which is considered the main anti-inflammatory cytokine released during the wound healing process [41, 42]. The result of the present study can justify the suppressed TGF-β signaling pathway in war veterans exposed to SM who have severe inflammation with skin lesions. Also, the obtained result is in complete agreement with the previously published works in animal models [42-44].

Our gene expression studies showed significantly increased levels of miR-106a-5p in both mild and severe groups and miR-106b-5p isoform in the severe group. In another study, Sharma et al. reported that the knockdown of mmu-miR-106a in an allergic murine model reduces the respiratory complications of asthma. In other words, in asthma, the expression of miR-106a increases with the severity of the disease and can be a marker for assessing its disease status [26].

Consistent with our study, Wang et al showed the expression of miR-106b-5p in peripheral blood leukocytes was impressively increased. It seems that miR-106b-5p is a possible marker for COPD severity [32]. In this part, unlike our findings, miR-106b-5p was significantly down-regulated in the plasma of COPD patients compared with healthy individuals [25].

In addition, Kung et al. perceived miR-106a and miR-106b elevate in LUAD, and the inhibition of one or both of these miRNAs may provide a strategy for the treatment of advanced disease [45].

Previous studies have denoted that TGF-β gene expression changes in various lung diseases and SM-exposed patients [8].

As our result showed, a significant increase of miR-106a-5p and miR-106b-5p was significantly related to the down-regulation of TGFβ1I1 in PBMC exposed to SM. So, it seems that miR-106a-5p and miR-106b-5p play an important role in inhibiting the TGF-β signaling pathway leading to exacerbation of the inflammatory response and chronic pulmonary complications.

In this study, the correlation between TGFβ1I1 expression levels with miR-106a-5p and miR-106b-5p expression was evaluated and results revealed a significant correlation between TGFβ1I1 with miR-106-a-5p and miR-106b-5p. It should be noted that miR-106a-5p had a more significant association with TGFβ1I1 than miR-106b-5p. It could be said that the increase in the expression of the miR-106a-5p and miR-106b-5p by inhibiting the TGFβ1I1, suppresses the TGF-β signaling pathway and exacerbate the chronic pulmonary complications in SM-exposed individuals. Both miR-106a-5p

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and miR-106b-5p are considered potentially acceptable biomarkers for distinguishing between the patient and control groups. With the description, it can be said that miR-106a-5p is a more discriminating and outstanding biomarker.

Data from this gene expression analysis point to the potential involvement of miR-106a-5p and miR-106b-5p in the inhibition of the TGFβ1I1 molecule in pulmonary complications that occur after exposure to SM.

It is suggested to use people who have suffered from chronic pulmonary complications for reasons other than exposure to mustard gas in future studies, and compare it with the results of studies of COPD complications caused by exposure to mustard gas in chemical victims.

5. Conclusion

The results of this study show that miR-106 isoforms are up-regulated in SM-exposed PBMC patients. Increased expression of these miRNAs may play a role in inflammation by interfering with the TGF-β signaling pathway. The miRNAs can be used as a new approach in the diagnosis and treatment of patients exposed to SM due to their high stability in biological samples as well as their successful use in clinical trials.

Ethical Considerations

Compliance with ethical guidelines

This study was performed following a large research project, which has been approved by the Research Ethics Committees of Shahed University (Code: IR.SHAHED. REC.1399.066), and approved by the Research Ethics Committees of Shahed University (Code: IR.SHAHED. REC.1400.125).

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Authors' contributions

Study design and statistics analysis: Tooba Ghazanfari and Farideh Talebi; Investigation, laboratory tests and manuscript draft: Saeed Sepehrnia and Farideh Talebi; Writing, review, and editing: Ali Mohammad Mohseni Majd.

Conflicts of interest

The authors declared no conflict of interest.

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