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Differential Expression of miR-21-5p, miR-20a-5p, TGF-β1, and TGF-β Receptor 2 in Skin, Serum, and Lung Samples Exposed to Sulfur Mustard

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ABSTRACT

Sulfur mustard (SM) or mustard gas is a blister chemical agent that causes pulmonary damage by triggering inflammation and oxidative injury. Alterations in microRNA (miR) transcript levels are found in pulmonary diseases and even inflammation. Therefore, we evaluated the expression levels of miR-20a-5p, miR-21-5p, and two target transcripts (transforming growth factor-beta [TGF-β1] and TGF-β receptor 2 [*TGFR2*]) in lung, serum, and skin samples from patients exposed to SM.

Total RNA was extracted from lung, serum, and skin samples of patients with moderate (n=10) and high (n=10) SM exposure, as well as 10 healthy subjects. Following the synthesis of complementary deoxyribonucleic acid using real-time polymerase chain reaction, we determined the expression levels of miR-20a-5p, miR-21-5p, TGF-β1, and *TGFR2* transcripts. Furthermore, we evaluated the sensitivity and specificity of the chosen microRNAs by employing receiver operating characteristic (ROC) curves and calculating the area under the ROC curve.

The results showed that miR-20a-5p and miR-21-5p expressions in the groups with moderate and high SM exposure were significantly lower than the normal controls. The expression analysis demonstrated that *TGFR2* was significantly less expressed in skin samples exposed to SM in both groups of patients compared with healthy controls. Furthermore, the TGF-β1 expression in the skin samples of the group with moderate SM exposure was lower than that of the control group.

Our findings suggest that miR-20a-5p, miR-21-5p, TGF-β1, and *TGFR2* expressions could be used as potential biomarkers for discriminating SM-exposed patients from healthy individuals.

Keywords: Gene expression; MicroRNAs; Mustard gas; Transforming growth factor beta 1

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INTRODUCTION

Sulfur mustard (2,2-dichlorodiethyl sulfide; SM), a blister chemical agent, has been utilized as a chemical warfare weapon in different wars, including the First

World War and the Iran-Iraq war.[1,2] SM can cause various devastating effects on body organs, particularly the lungs (42.5%), eyes (39.3%), and skin (24.5%).[3,4] Pulmonary damage and infection following SM poisoning are the leading reasons for death among chemically injured victims.[5] Lung injury is classified as acute or chronic. Acute lung injury is often associated with inflammation and oxidative injury, while in chronic lung injury, oxidative stress and apoptosis pathways are active, leading to the chronic obstructive pulmonary disease caused by SM.[6]

MicroRNAs (miRs) are small, noncoding RNAs that are comprised of about 22 nucleotides in length. These molecules are involved in regulating various cellular processes, including proliferation, cell cycle, apoptosis, and senescence.[7] microRNAs regulate gene expression at the post-transcriptional level, primarily by binding to the 3' untranslated region and degrading microRNAs of the target genes to control biological processes.[8,9] miR-20a-5p is a tissue- and cell-specific microRNA and one of the members of the miR-17-92 cluster. It exerts anti-inflammatory activities by directly targeting the transforming growth factor beta (TGF-β), its receptor (TGF-β receptor 2 [*TGFR2*]), and mothers against decapentaplegic homolog 4 (SMAD4).[10] miR-20a-5p is also implicated in regulating processes such as cell cycle, apoptosis, hypoxia, and cell proliferation.[11,12] miR-21-5p, another miR involved in inflammation and fibrosis, has been reported as a novel diagnostic biomarker of lung carcinoma.[13,14] In most cancer cells, miR-21-5p upregulation has demonstrated oncogenic functions, which lead to cancer cell growth, invasion, and even metastasis.[1,15]

The accumulation of inflammatory cells, as well as proinflammatory and profibrotic cytokines, in the SM-exposed lungs plays a significant role in the induction of acute and chronic lung injury.[16] In pulmonary diseases, inflammation, and even fibrosis, alteration in the transcript level of microRNAs has captured attention due to their involvement in the molecular pathogenesis of SM.[17,18]

As there is currently no specific therapy for patients exposed to SM, various treatment options, such as anti-inflammatory agents, are used to alleviate clinical symptoms in these patients.[5,19,20] However, evaluating the molecular basis of SM adverse events could result in the discovery of new target therapies for SM-injured patients. Therefore, the present research investigated the relative expression levels of miR-20a-5p, miR-21-5p, TGFβ1, and *TGFR2* genes. Lung, serum, and skin samples were collected from Iranian veterans exposed to different doses of SM.

MATERIALS AND METHODS

Sample Collection

Lung biopsy specimens were obtained from 10 patients with moderate SM exposure, 10 cases with high SM exposure (without a history of exposure to other agents), and 10 healthy subjects. Samples were allocated into two groups: chemically injured individuals with a history of exposure to SM and control subjects. The first group included cases who underwent bronchoscopy due to lung problems caused by previous SM poisoning. This group was classified based on their clinical records at the time of injury, the severity of the lesions of the involved organs (eyes, skin, and lungs), CT scan of the lungs, and pulmonary function test. The classification of both groups of patients was carried out by the Medical Commission of the Martyr Foundation and Veterans Affairs, comprising 50 lung and infectious disease specialists (Table 1). The samples of the control group were obtained from the lung biobank at Baqiyatallah Hospital, Tehran, Iran.

Blood samples (8 mL each) were collected from fasting subjects. The specimens were stored at ambient temperature for 1 hour, allowing the blood to clot, and then centrifuged at 2500*g* for 20 minutes. Following isolation, the serum samples were frozen at −80°C until processed. Skin biopsy samples (weighing approximately 25 mg and having a surface area of 3 mm^2) were collected from pruritic plaque skin lesions or healthy peripheral tissue under topical anesthesia with lidocaine 2% and used as a control. All samples were transferred to sterile RNase-free microtubes containing TriPure. They were maintained at −80°C after being transferred to the laboratory until RNA extraction.

RNA Extraction and Real-time PCR

Total RNA extraction using TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA) was performed in accordance with the protocol recommended by the manufacturer. The concentration of RNA was determined by a NanoDrop instrument (ND-1000 UV-Vis Spectrophotometer, USA), and the quality of RNA was evaluated by gel electrophoresis using the above-mentioned spectrophotometer. For complementary DNA synthesis, the purified total RNA (200 ng) was

reverse transcribed by the miRCURY locked nucleic acid (LNA) universal RT microRNA polymerase chain reaction (PCR) Kit (Exiqon, Copenhagen, Denmark). Specific LNA primers were used for real-time PCR amplification of miR-20a-5p, miR-21-5p (MicroRNA LNA PCR primer set, Exiqon, Denmark), TGF-β1, and *TGFR2*. The internal control GAPDH was designed and manufactured by Gene Runner software version 6 (Hastings Software, Hastings, NY, USA) and TAG Company (Copenhagen A/S, Denmark), respectively. Real-time PCR was carried out using SYBR Green Master Mix (Takara, Japan) and the ABI 7500 real-time quantitative PCR system (Applied Biosystems, Foster City, CA, USA) as follows: 95°C for 10 minutes (polymerase activation and denaturation), 95°C for 10 seconds (denaturation), and 60°C for 1 minute (annealing and extension), for up to 45 cycles. The efficiency of each primer was determined by LinRegPCR (2021.2) software (AMC, Amsterdam, the Netherlands, http://LinRegPCR.nl). The expression level of candidate genes was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to 5S ribosomal RNA (rRNA) as the reference gene.

Table 1. Clinicopathological features of sulfur mustard-exposed patients

Type	Age/Sex	Samples
N1	60/M	Normal volunteer
N2	37/M	Normal volunteer
N3	38/M	Normal volunteer
N4	49/M	Normal volunteer
N5	44/M	Normal volunteer
N6	49/M	Normal volunteer
N7	49/M	Normal volunteer
N8	60/M	Normal volunteer
N9	63/M	Normal volunteer
N10	49/M	Normal volunteer
C1	42/M	Moderately SM-exposed patient
C2	57/M	Moderately SM-exposed patient
C3	46/M	Moderately SM-exposed patient
C4	58/M	Moderately SM-exposed patient
C5	43/M	Moderately SM-exposed patient
C6	57/M	Moderately SM-exposed patient
C7	42/M	Moderately SM-exposed patient
C8	47/M	Moderately SM-exposed patient
C9	40/M	Moderately SM-exposed patient
C10	48/M	Moderately SM-exposed patient
C11	39/M	Highly SM-exposed patient
C12	49/M	Highly SM-exposed patient
C13	47/M	Highly SM-exposed patient
C14	39/M	Highly SM-exposed patient
C15	56/M	Highly SM-exposed patient
C16	41/M	Highly SM-exposed patient
C17	31/M	Highly SM-exposed patient
C18	35/M	Highly SM-exposed patient
C19	41/M	Highly SM-exposed patient
C20	58/M	Highly SM-exposed patient

Statistical Analysis

All experiments were performed in triplicate. Data were analyzed using the Mann-Whitney test and are presented as mean±standard deviation (SD). The sensitivity and specificity of the selected microRNAs were determined using receiver operating characteristic curves (ROC) and the area under the ROC curve (AUC) to distinguish the SM-exposed patients from the normal group. Boxplots of miR-21-5p, miR-20a-5p, TGF-β1, and *TGFR2* Ct values were drawn using the `ggplot2` R package v.3.4.2. The means between the study groups were compared using the t-test analysis, using `t_test()` function of `rstatix`. All the statistical analyses and calculations were performed using `GraphPad Prism` version 6.07 (GraphPad Software Inc., CA, USA) and `rstatix` package in R, respectively. The value of probability was considered statistically significant at $p<0.05$.

RESULTS

Boxplot Generation

The boxplots of miR-20a-5p, miR-21-5p, TGF-β1, and *TGFR2* were generated using the `ggpubr::ggboxplot()` function (Figure 1).

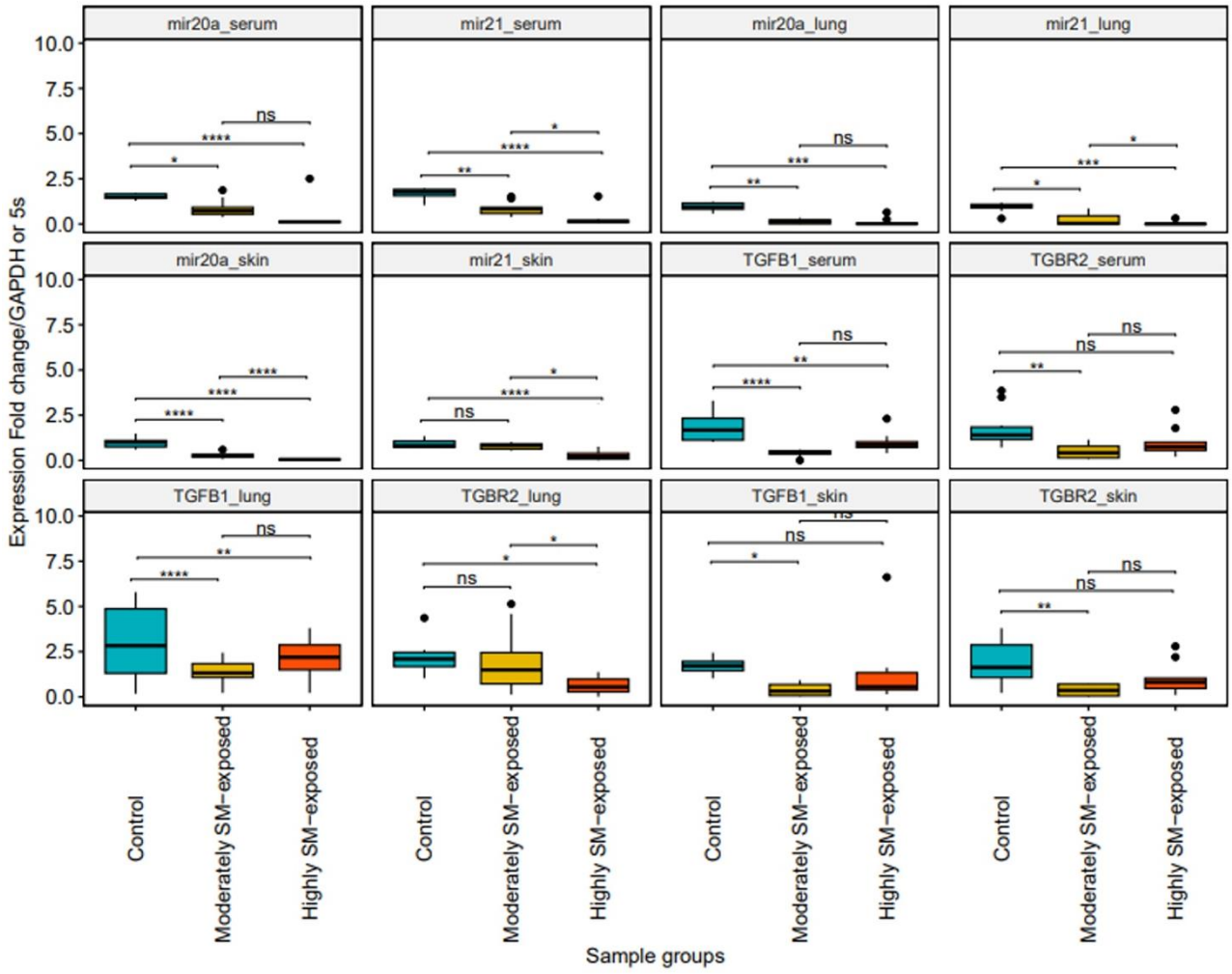
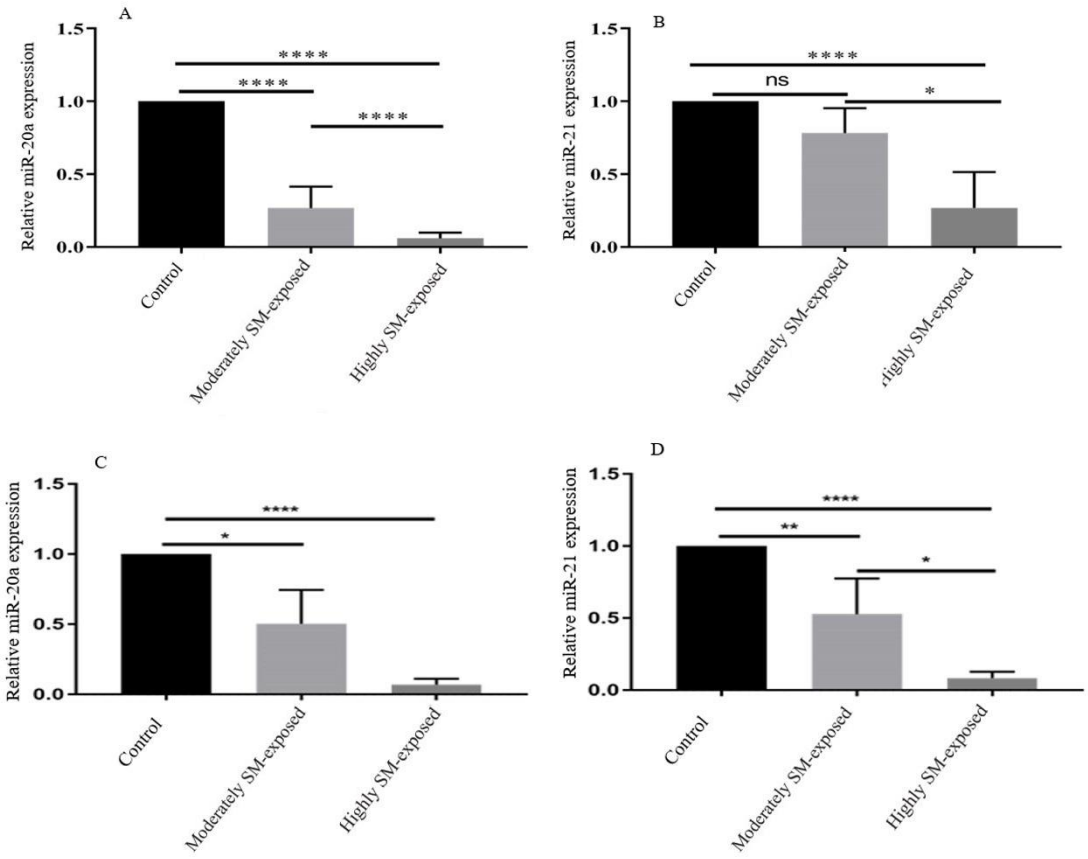


Figure 1. Boxplots of miR-20a-5p, miR-21-5p, transforming growth factor-beta (TGF-β1), and TGF-β receptor 2 (*TGFR2*) expressions (Ct values) in serum, skin, and lung samples from the control group, as well as patients with moderate and high SM exposure. ns: not significant

Downregulation of miR-20a-5p and miR-21-5p in SM-exposed Samples

The relative expression levels of miR-20a-5p and miR-21-5p were evaluated with real-time PCR (normalized to 5S rRNA; Figure 1). Gene expression analysis revealed that the expression levels of miR-20a-5p and miR-21-5p in lung and serum samples of patients exposed to SM were significantly lower than the normal controls (Figure 2). The relative expression level of miR-20a-5p in the lung samples of the group with high SM exposure was moderately lower (Figure 2E) than other exposed groups. Also, miR-20a-5p expression level was lower in the serum samples of the group with high SM exposure than other exposed groups and was insignificant (Figure 2C). However, a significantly decreased miR-21-5p expression level was detected in the lung (Figure 2F) and serum (Figure 2D) samples of the patient group with high SM exposure compared to other groups. The gene expression analysis also showed that miR-20a-5p in skin samples exposed to SM was significantly less expressed than in normal controls (Figure 2A). Also, the miR-20a-5p expression level in the skin samples of the group with high SM exposure was lower than that of the other exposed group and was significant (Figure 2A). In miR-21-5p, we observed a significant relationship between the control samples and skin samples with high SM exposure, as well as between skin samples with high SM exposure and those of moderate SM exposure. However, there was no significant link between the control samples and skin samples with moderate SM exposure (Figure 2B).



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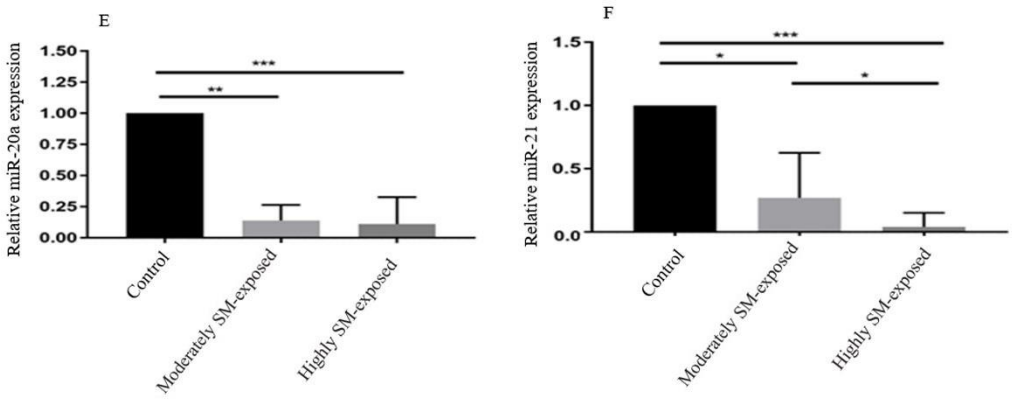


Figure 2. Expression levels of miR-20a-5p and miR-21-5p in skin, serum, and lung samples exposed to sulfur mustard (SM) compared to the normal group. The figure shows the downregulation of miR-20a-5p (A) and miR-21-5p (B) in SM-exposed skin biopsies, miR-20a-5p (C) and miR-21-5p (D) in SM-exposed serum samples, and miR-20a-5p (E) and miR-21-5p (F) in SM-exposed lung biopsies. ns: not significant

ROC Curve Analysis of Differentially Expressed microRNAs

The sensitivity and specificity of miR-20a-5p and those of miR-21-5p in distinguishing the SM-exposed patients from normal individuals were analyzed by the ROC method (Figure 3). The AUCs for miR-20a-5p and miR-21-5p in skin samples were 0.78 (p=0.034) and 0.70 (p=0.13; Figure 3A), and 0.90 (p=0.002) and 0.72 (p=0.096; Figure 3B), in serum samples were 0.8 (p=0.0082) and 0.9 (p=0.0025; Figure 3C) and 0.96 (p=0.0005) and 0.97 (p=0.0003; Figure 3D), and in lung, samples were 0.96 (p=0.0009) and 0.95 (p=0.0007; Figure 3E) and 0.88 (p=0.0058) and 0.94 (p=0.0009); Figure 3F). Moreover, the AUCs for miR-21-5p in skin samples were 0.90 (p=0.002) and 0.72 (p=0.096; Figure 3B), in serum samples were 0.96 (p=0.0005) and 0.97 (p=0.0003; Figure 3D), and in lung samples were 0.88 (p=0.0058) and 0.4 (p=0.0009; Figure 3F). All the aforementioned samples were exposed to moderate and high SM, respectively.

Expression of TGF-β1 and *TGFR2* in SM-exposed Samples

The relative expression levels of TGF-β1 and *TGFR2* were evaluated using real-time PCR (normalized to GAPDH; Figure 4). The gene expression analysis showed that TGF-β1 expression in SM-exposed lung biopsies (Figure 4E) and that of TGF-β1 and *TGFR2* in SM-exposed serum samples (Figure 4) were significantly lower than the normal controls. Also, the *TGFR2* expression in the lung biopsies of the group with moderate SM exposure was higher (Figure 2), but those of TGF-β1 and *TGFR2* in the serum samples of the patient group with moderate SM exposure (Figures 2C and 2D) were respectively lower than in other exposed groups. However, a significantly decreased *TGFR2* expression level was identified in the lung samples of the group with high SM exposure compared to other groups (Figure 2F). The TGF-β1 and *TGFR2* relative expression levels in the skin samples of patients with moderate SM exposure were lower than the other SM-exposed groups (Figures 2A and 2B, respectively). The gene expression analysis also demonstrated that *TGFR2* was significantly less expressed in SM-exposed skin biopsies than in the normal controls. However, the TGF-β1 expression level in the skin samples of the group with moderate SM exposure was lower than the control group (Figure 2).

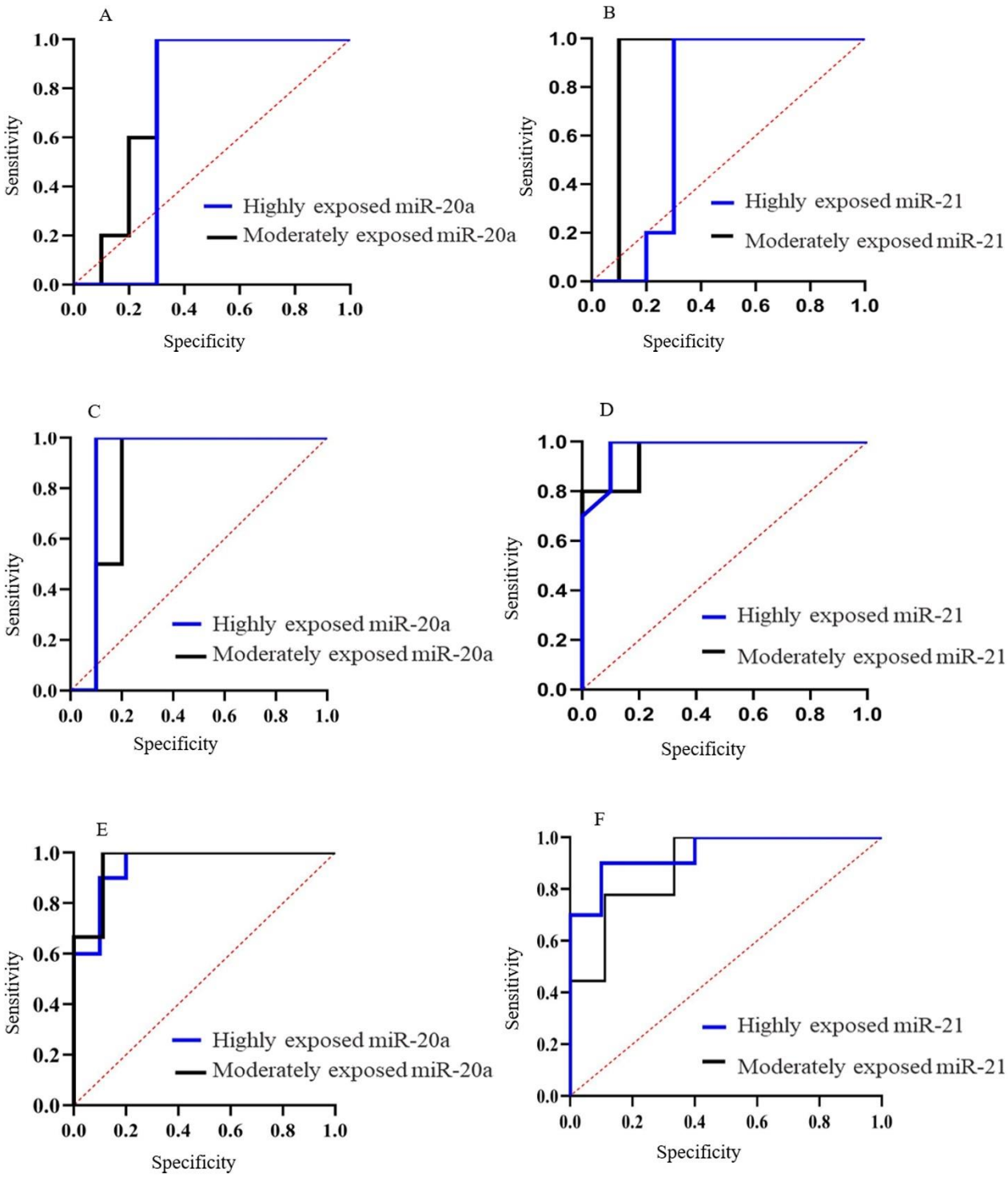
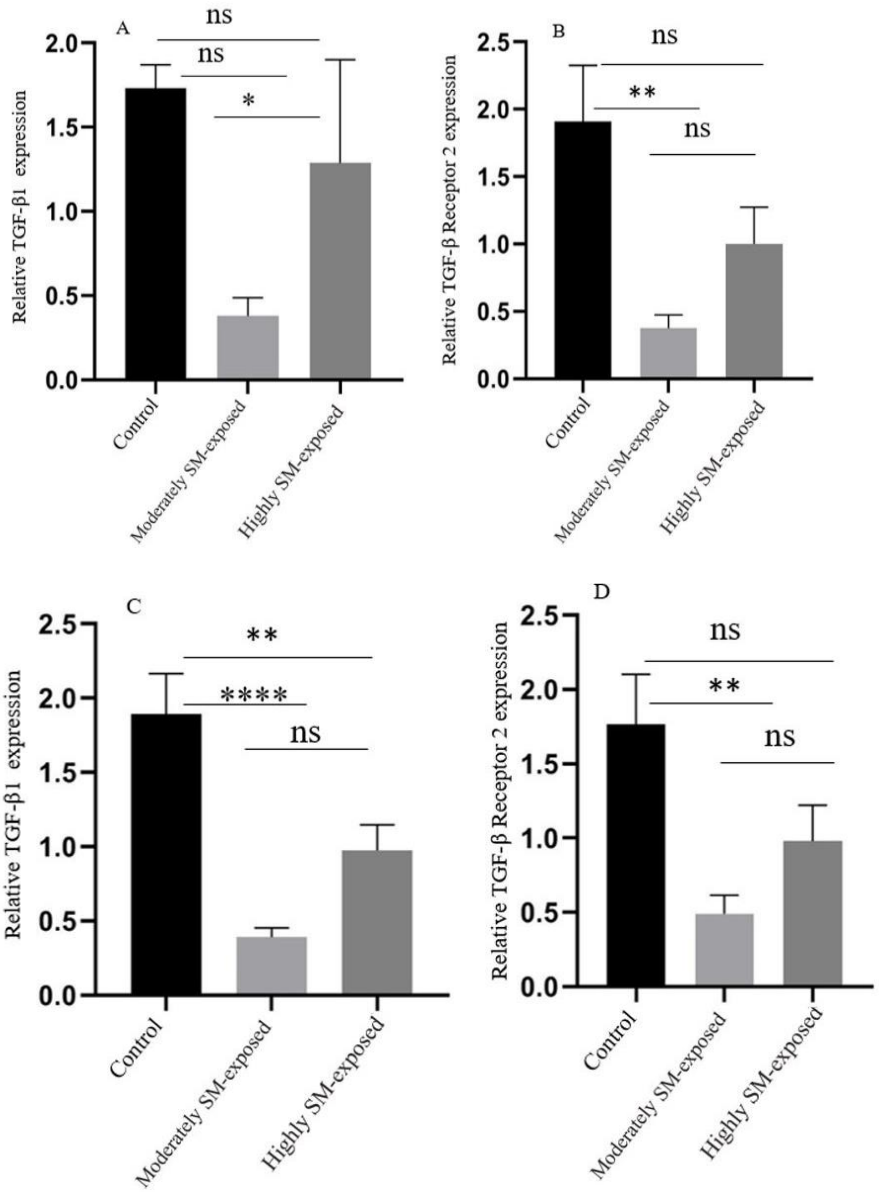


Figure 3. Receiver operating characteristic curve analysis using the expression levels of selected microRNAs in skin (A and B), serum (C and D), and lung (F and G) samples for discriminating sulfur mustard-exposed from healthy controls.

ROC Curve Analysis of TGF-β1 and *TGFR2*

The sensitivity of TGF-β1 and *TGFR2* and their specificity in distinguishing the SM-exposed patients from normal individuals were analyzed by the ROC method (Figure 5). The AUCs for TGF-β1 in skin samples were 0.73 (p=0.0821) and 0.53 (p=0.8206; Figure 5A) and 0.90 (p=0.0025) and 0.75 (p=0.0588); Figure 5B), in serum samples were 0.97 (p=0.0002) and 0.84 (p=0.0102; Figure 5C) and 0.93 (p=0.0012) and 0.80 (p=0.0233; Figure 5D), and in lung samples were 0.87 (p=0.0052) and 0.58 (p=0.545; Figure 5E) and 0.90 (p=0.0025) and 0.62 (p=0.034; Figure 5F). The AUCs for *TGFR2* in skin samples were 0.93 (p=0.0012) and 0.80 (p=0.0233; Figure 5B), in serum samples were 0.90 (p=0.0025) and 0.75 (p=0.0588; Figure 5D), and 0.90 (p=0.0025) and 0.62 (p=0.034; Figure 5F). All the aforementioned samples were exposed to moderate and high SM, respectively. In SM-exposed patients, a significant downregulation was observed in TGF-β and *TGFR2*, which prevented the subsequent maturation of miR-21-5p and miR-20a-5p (Figure 6).



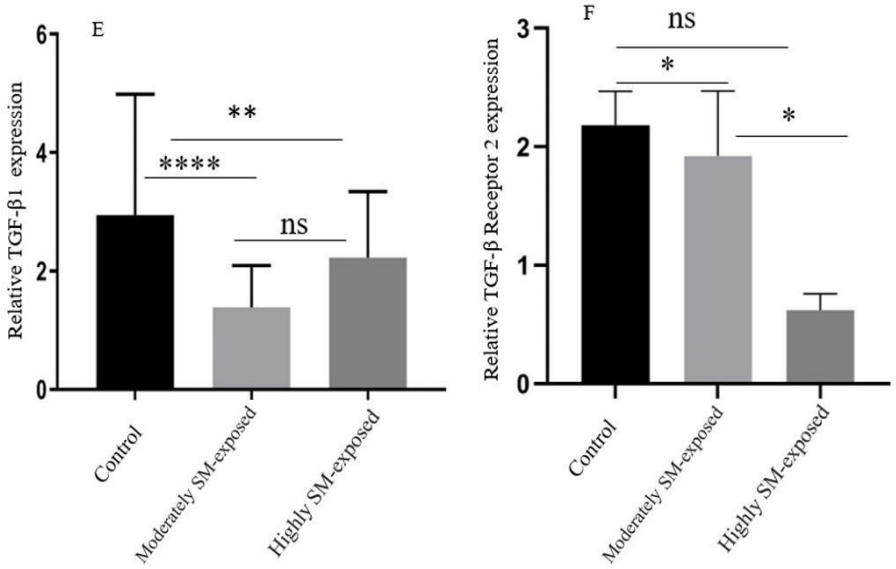
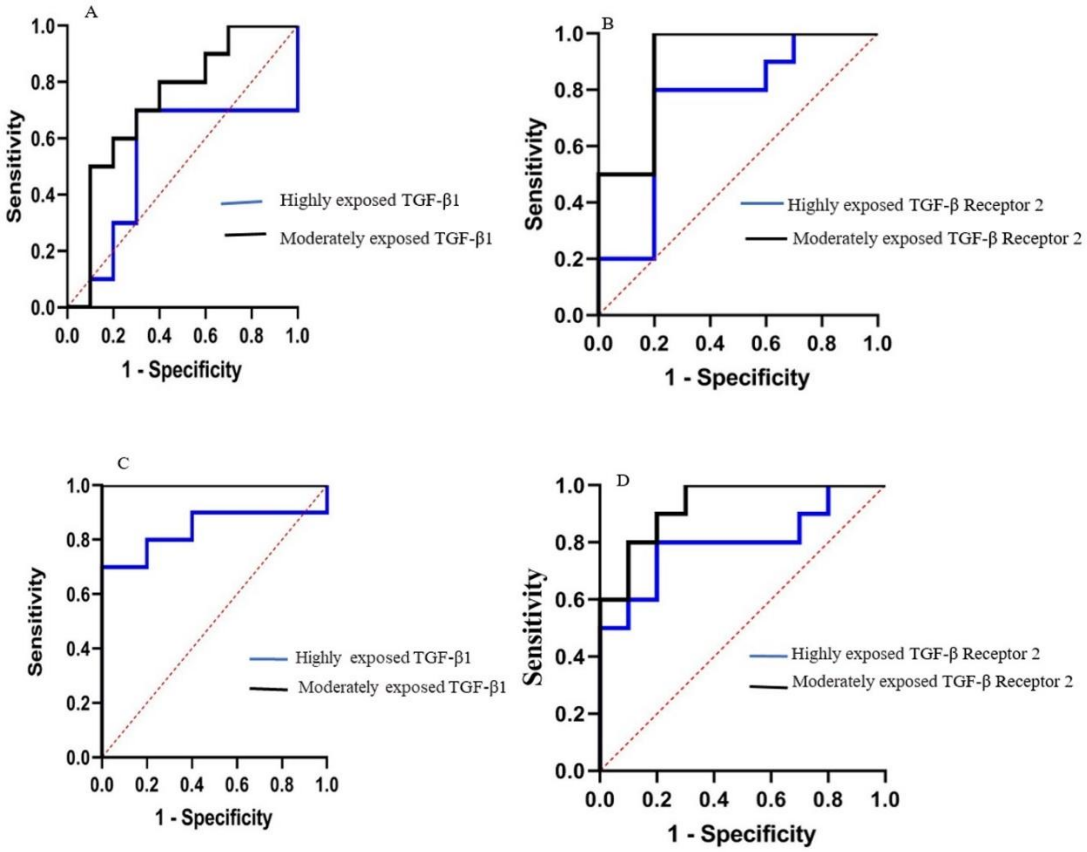


Figure 4. Altered transforming growth factor-beta (TGF-β1) and TGF-β receptor 2 (*TGFR2*) expression levels in sulfur mustard (SM)-exposed skin, serum, and lung samples compared to the normal group. The real-time polymerase chain reaction analysis shows the downregulation of TGF-β1 (A, C, and E) and *TGFR2* (B, D, and F) in skin, serum, and lung samples of the patient group with high SM exposure, respectively. ns: not significant



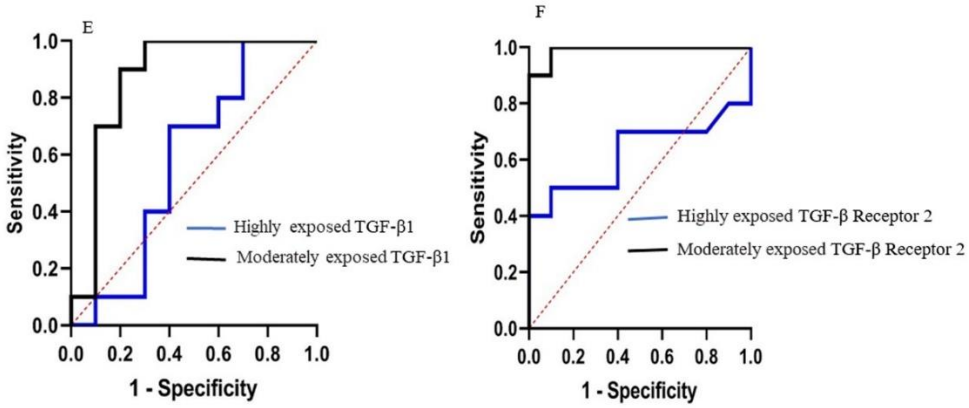


Figure 5. Receiver operating characteristic curve analysis using the expression levels of transforming growth factor-beta (TGF-β1) and TGF-β receptor 2 (*TGFR2*) in skin (A and B), serum (C and D), and lung (E and F) samples to discriminate sulfur mustard-exposed patients from healthy controls.

DISCUSSION

SM exposure can induce significant damage to the lungs, including inflammation and fibrosis, resulting in various respiratory conditions such as bronchiolitis, bronchiectasis, tracheobronchitis, asthma, chronic obstructive pulmonary disease, and large airway narrowing in affected patients.[21] Regarding the unclear underlying pathophysiologic mechanisms of SM, understanding the molecular basis of inflammation and fibrosis in SM-exposed veterans is beneficial. Thus, our study evaluated the relative expression levels of miR-20a-5p, miR-21-5p, TGF-β1, and *TGFR2*, which are associated with TGF-β signaling, in SM-exposed patients, to gain insights into the molecular mechanisms underlying these respiratory conditions.

In the present investigation, we found the downregulation of miR-20a-5p and miR-21-5p transcripts in the lung biopsies of SM-exposed veterans and their serum samples, as formerly reported in SM-exposed skin samples.[7] Based on the current study results, miR-21-5p and miR-20a-5p relative expression is lower in patients with high SM exposure than in cases with moderate SM exposure. This downregulation implies the dose-dependent damage of SM.[5]

ROC curve analysis showed that the miR-20a-5p relative expression in serum and lung samples could discriminate SM-exposed patients from normal individuals.[22] A previous study has reported that miR-21-5p is a potential biomarker for diagnosing non-small cell lung cancer, with sensitivity and specificity of 76.2% and 70%, respectively.[23] In this study, we also explored miR-21-5p as a potential biomarker for the detection of patients with mustard lung with a sensitivity and specificity of 77% and 77% in patients with moderate SM exposure and 0.9% and 0.9% in patients with high SM exposure. Another study has reported the downregulation of TGF-β1, as well as *TGFR1* and *TGFR2*, in skin samples of SM-exposed patients.[7] TGF-β is a cytokine involved in the development of fibrosis, inflammation, and even maturation of miR-21-5p and induces mature miR-21-5p through activating SMAD family member 6 (SMAD6).[1,14,24-26] Therefore, the significant decrease in miR-21-5p could be due to TGF-β downregulation in tissue samples exposed to SM. We found that the *TGFR2* relative expression in lung biopsies of the group with moderate SM exposure is higher than in other exposed groups. The gene expression analysis revealed a significantly low expression of TGF-β1 and *TGFR2* in serum samples and that of *TGFR2* in skin samples exposed to SM, compared to normal controls. In line with our study, Salimi et al. have shown low expression levels of miR-21-5p along with the upregulation of SMAD family member 7 (SMAD7) in lung biopsies of SM-exposed patients.[27] The high level of SMAD7, a negative regulator of TGF-β signaling and miR-21-5p direct target, in SM-exposed lungs is a reason for TGF-β downregulation.[28] Furthermore, the activation of nuclear factor kappa light chain enhancer of activated B cell signaling and elevated inflammatory immune responses are other outcomes of miR-21-5p downregulation.[29] This finding indicates the dual role of miR-21-5p in inflammation.[14] The miR-21-5p upregulation in lung

fibroblasts and myofibroblasts promotes the profibrogenic activity of TGF-β. Kunita et al. have also found that miR-21-5p induces lung fibroblast differentiation to cancer-associated fibroblasts in lung adenocarcinoma and promotes cancer progression.[30] The miR-21-5p upregulation has also been shown to induce tumor cell proliferation and apoptosis inhibition, especially in lung cancers, by targeting phosphatase and tensin homolog (*PTEN*) and B-cell lymphoma 2 (*BCL2*) genes.[31,32]

The results of our study demonstrated the downregulation of miR-20a-5p in the serum and lung samples from SM-exposed patients. Downregulation of miR-20a-5p and miR-miR-92 has also been reported in the serum of SM-exposed patients during the chronic phase of the disease.[22] Similar to miR-21-5p, miR-20a-5p, as a member of the miR-17-92 cluster, has tissue- and cell-specific function with various effects on inflammation.[33,34] miR-20a-5p inhibits fibrotic processes by decreasing the expression of profibrotic genes and enhancing the antifibrotic gene level.[11] Therefore, miR-20a-5p is often regarded as an antifibrotic agent. Owing to different target genes of miR-20a-5p, varied inflammation responses occur in different cell types.[34] MiR-20a-5p acts as an anti-inflammatory agent by suppressing the NLR family pyrin domain containing 3 (NLRP3) inflammasome, inhibiting the production and secretion of inflammatory cytokines such as interleukin-1, and repressing monocyte differentiation into macrophages.[33,35,36] On the other hand, it promotes macrophage inflammatory responses by inhibiting signal regulatory protein α, which is a negative regulator of the alveolar macrophage activity.[37]

Using the analysis of genes and microRNAs, TGF-β showed to induce the expression of miR-21-5p and miR-20a-5p, targeting SMAD7 transcripts and inhibiting its expression in the normal state. The ROC results indicated the capability of miR-20a-5p and miR-21-5p, as well as TGF-β1 and *TGFR2*, in distinguishing SM-exposed patients from healthy individuals.

Our study found that miR-20a-5p and miR-21-5p were downregulated in the lung, serum, and skin samples from SM-exposed patients. The downregulation of these miRs and TGF-β in the lungs of SM-exposed veterans explains the inflammation, fibrosis, and other symptoms of SM toxicity observed in these patients. Additionally, our results suggest that miR-20a-5p, miR-21-5p, TGF-β1, and *TGFR2* relative expressions could be used as potential biomarkers for discriminating SM-exposed patients from healthy individuals. Therefore, utilizing microRNAs as new-generation therapeutics could help reduce the chronic complication of SM poisoning.

STATEMENT OF ETHICS

This study was approved by the Ethics Committee of the Islamic Azad University and Research Branch, Tehran, Iran (Approval ID: IR.IAU.SRB.REC.1399.095). Written informed consent was obtained from each patient.

FUNDING

The authors have received no financial support from any organization for the submitted work.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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