FULL LENGTH	Iranian Biomedical Journal 29 (5): 344-351 September 2025

Alterations in the Expression of *miR-148a-5p*, *TGF-β1*, and *TGF-βR2* in Skin Samples Exposed to Sulfur Mustard

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OPEN ACCESS

Article type: Research Article
Received: June 30, 2025
Revised: August 18, 2025
Accepted: August 26, 2025
Published online: August 27, 2025

How to cite:
Tavakoli A, Heidari Keshel S, Valizadeh M. Alterations in the Expression of *miR-148a-5p*, *TGF-β1*, and *TGF-βR2* in Skin Samples Exposed to Sulfur Mustard. *Iran. Biomed. J.* 2025; 29(5): 344-351.

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ABSTRACT

Background: Sulfur mustard exposure causes chronic cutaneous injuries characterized by inflammation, fibrosis, and delayed wound healing. MiRNAs, such as *miR-148a-5p*, have been implicated in regulating the TGF-β signaling pathways involved in these processes. This study aimed to evaluate whether alterations in the expression of *miR-148a-5p*, *TGF-β1*, and *TGF-βR2* are associated with long-term SM-induced skin damage.
Methods: Skin biopsy samples were collected from 20 SM-exposed veterans and 20 healthy controls. Total RNA was extracted, and quantitative real-time PCR was performed to assess the expression levels of *miR-148a-5p*, *TGF-β1*, and *TGF-βR2*. Group differences were analyzed using a t-test, and ROC curves were generated to evaluate diagnostic performance.
Results: Expression levels of *miR-148a-5p*, *TGF-β1*, and *TGF-βR2* were significantly lower in SM-exposed skin compared with controls (*miR-148a-5p*: $p = 0.0010$; *TGF-β1*: $p < 0.0001$; *TGF-βR2*: $p < 0.0001$). Based on ROC analysis, *miR-148a-5p* and *TGF-βR2* indicated promising discriminative potential, whereas *TGF-β1* did not reach statistical significance (AUC = 0.65; $p = 0.0877$).
Conclusion: Our findings suggest that reduced expression of *miR-148a-5p* and *TGF-βR2* may contribute to SM-related skin injury. Both markers demonstrated potential diagnostic utility and could aid in risk stratification and monitoring in SM-induced skin disease, pending further validation in larger cohorts. ***DOI: 10.61882/ibj.5198***

Keywords: Biomarkers, Gene expression, Mustard gas, Skin, Transforming growth factor beta

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INTRODUCTION

Sulfur mustard is a vesicant chemical warfare agent that has been utilized in many conflicts, most prominently during World War I (1914-1918) and the Iran-Iraq War (1980-1988). Its first recorded use as a chemical weapon occurred on July 12, 1917, when German forces deployed it against French troops in Belgium, resulting in 15,000 injures within three weeks. During World War I, approximately 77% of British chemical warfare attacks involved SM[1]. In the Iran-Iraq War, nearly 100,000 Iranian troops and civilians were exposed to SM, with many continuing to suffer long-term effects decades later[2].

The severity of injuries among surviving veterans varies based on multiple factors, such as the time and dose of exposure, the presence of other chemical agents,

List of Abbreviations:
AUC: area under the curve; **cDNA:** complementary DNA; **miRNA:** microRNA; **ROC:** receiver operating characteristic; **R-Smads:** receptor-regulated Smads; **SD:** standard deviation; **SM:** sulfur mustard

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[DOI: 10.61882/ibj.5198]

and individual characteristics such as age and gender. These factors influence the severity of tissue damage, the onset of symptoms, treatment requirements, and the duration of hospitalization.

SM causes extensive cellular damage through interactions with DNA and other cellular components, leading to apoptosis, necrosis, inflammation, and impaired wound healing[3-5]. Aalkylation and cross-linking of DNA significantly disrupt vital cellular functions, likely resulting in cell death or cell growth inhibition[4,6,7]. The severity of skin damage induced by SM exposure depends on several factors, including the dose of exposure, duration of exposure, and the area of skin involved[8-10]. SM exposure typically causes skin toxicity characterized by initial inflammation, blistering, and vesicle formation. These blisters mostly rupture, giving rise to necrotic tissue and subsequent scar formation[7,11]. Healing from SM-induced cutaneous injury is a prolonged process and often results in residual pigmentation changes and scar tissue development[12]. Skin lesions caused by SM are generally characterized by erythema, blistering, necrosis, and fibrosis[13].

A major clinical challenge in SM-exposed veterans is the impaired wound healing. Beyond the direct alkylating injury caused by SM, the specific molecular drivers of this impairment remain incompletely defined[14]. Research has highlighted a key role for miRNAs in the wound-healing process[15]. These small non-coding RNAs are transcribed as precursors and processed in the nucleus and cytoplasm to generate mature miRNAs that regulate diverse cellular pathways[16,17]. Notably, the miR-148 family—including *miR-148a-5p*—has been linked to the modulation of the TGF-β/Smad signaling pathway. When activated, TGF-β receptors phosphorylate R-Smads, which translocate to the nucleus and influence gene expression[18-21]. Studies have indicated that *miR-148a-5p* regulates TGF-β signaling and is implicated in fibrotic and inflammatory processes across multiple tissues, including skin[22,23]. *miR-148a-5p* has been reported to modulate profibrotic factors (e.g., collagen) and influence inflammatory cytokines, thereby contributing to fibrosis and inflammation[24]. Moreover, TGF-β is a central mediator of cutaneous fibrosis and inflammation, and dysregulation of this pathway has been associated with chronic skin disease as well as SM-induced cutaneous lesions[20,25,26]. These literature references provide biological context only and do not imply measurement of these pathways beyond the markers examined in the present study.

Currently, no specific antidotal therapy exists for SM exposure; available treatments are mainly symptom-directed, including anti-inflammatory approaches. Advancing our understanding of the molecular effects of SM may enable the development of gene-based and targeted therapies. In this study, we examined the skin expression of *miR-148a-5p*, *TGF-β1*, and *TGF-βR2* in individuals exposed to SM. We hypothesize that chronic cutaneous lesions in SM-exposed veterans may involve dysregulation of *miR-148a-5p* and *TGF-β* signaling, contributing to impaired wound healing, excessive fibrosis, and persistent inflammation. This hypothesis is supported by prior literature linking altered miRNA expression—particularly *miR-148a-5p*—to the TGF-β/Smad pathway in chronic skin conditions.

By identifying the molecular mechanisms underlying compromised wound healing following SM exposure, this study aimed to identify possible biomarkers and therapeutic targets that could enhance treatment strategies for affected individuals. Furthermore, understanding the interactions between these miRNAs and TGF-β signaling pathways may provide insights into the chronic impacts of SM on skin regeneration, contributing to the design of novel gene-based therapeutic strategies.

MATERIALS AND METHODS

Study population

Twenty skin biopsy specimens were obtained from the chemical warfare-exposed veterans hospitalized in Baqiyatallah Hospital in Tehran, along with 20 control skin tissue samples sourced from the biobank of the hospital (Table 1). Biopsy samples were collected from active lesion sites characterized by erythematous plaques and pruritus, including both the epidermal and dermal layers. All the specimens were stored at -80 °C before further processing.

Site selection

Biopsies were performed only on active skin lesions, characterized by erythema (erythematous plaques) and pruritus. Lesions with necrosis or scarring (i.e., healed wounds) were not sampled. As patients presented with multiple skin lesions in different regions of the body (e.g., trunk, forearms, and legs), the biopsy site varied among individuals. In each patient, a visible, accessible, and active lesion was selected. Standardization was achieved by sampling clinically similar lesions (active and inflammatory) across all patients to ensure consistency in clinical status. A 3-mm punch biopsy was used to obtain tissue from both the epidermal and dermal layers.

Timing of biopsy

Biopsies were performed during the first clinical visit, immediately after the suitability of the lesion for sampling was confirmed by clinical examination. At the time of biopsy, no new treatments had been

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Table 1. Clinical specifications of samples

Normal volunteers		SM-exposed patients	
Type	Age	Type	Age
N1	53	C1	57
N2	56	C2	53
N3	60	C3	60
N4	62	C4	62
N5	55	C5	58
N6	58	C6	63
N7	58	C7	59
N8	51	C8	58
N9	49	C9	60
N10	61	C10	49
N11	64	C11	60
N12	58	C12	61
N13	55	C13	48
N14	58	C14	55
N15	62	C15	58
N16	58	C16	60
N17	61	C17	64
N18	49	C18	61
N19	58	C19	56
N20	60	C20	54

administered, and the lesions were in their active inflammatory phase. The time between the initial SM exposure and biopsy collection spanned several years, indicating the chronic nature of the lesions. Thus, all biopsies were obtained during the chronic active phase of the disease, excluding those taken during the acute phase.

Lesion stage

All biopsies were collected from clinically active lesions, characterized as erythematous, pruritic plaques with persistent inflammation. These represented chronic active lesions—long-standing, non-healed sites—demonstrating clear inflammatory activity in the absence of necrosis, blistering, or scarring. Healed or inactive lesions were not included. Inclusion criteria for participants required a confirmed history of SM exposure, an age range similar to that of the study group, and male participants. The control group was age-matched to the exposed group, with no history of military service and residence in urban areas affected by war or chemical attacks. Exclusion criteria included individuals with occupational or environmental exposure to other chemical substances or those with pre-existing skin conditions before SM exposure.

RNA extraction and cDNA synthesis

A proteinase K treatment step was performed to eliminate the inhibitory effects of proteins in skin samples. Materials and tools required for this experiment included skin samples, diethyl pyrocarbonate-treated microtubes containing proteinase K at a concentration of 20 mg/ml (Takara, Japan), and a thermal block set at 56 °C for one hour. RNA extraction was performed using TRIzol (Invitrogen Corp., Carlsbad, CA) following the manufacturer's protocol. The concentration of RNA was measured with a NanoDrop ND-1000 instrument, and its quality was assessed through gel electrophoresis using an ultraviolet-visible spectrophotometer. The extracted RNA was then treated with DNase I enzyme to remove residual genomic DNA. Subsequently, cDNA was synthesized using 20 ng of purified total RNA treated - DNase I and the Universal cDNA Synthesis Kit (Exiqon, Denmark). The incubation was carried out at 42 °C for one hour, followed by inactivation at 95 °C for 5 minutes to deactivate the reverse transcriptase enzyme. For the selected genes, specific primers were used, including custom primers for *miR-148a-5p* and *5S rRNA* (Exiqon). Unfortunately, the exact catalog numbers for these primers were not provided. The primers for *TGF-β1*, *TGF-βR2*, and *GAPDH* were synthesized by Gene Fanavaran Teb Azma (Tehran, Iran. The sequences for the primers included *TGF-β1* forward: TGGCGATACCTCAGCAAC, *TGF-β1* reverse: ACCCGTTGATGTCCACTTG; *TGF-β1* receptor 2 forward: CCTATGAGCCAAGCCTATTG; *TGF-β1* receptor 2 reverse: AATAAGTTCTGGGAAG GGAAG, *GAPDH* forward: GTGAACCATGAGAA GTATGACAAC, and *GAPDH* reverse: CATGAGTC CTTCCACGATACC.

Real-time PCR

For normalization of miRNA and mRNA expression, *5S rRNA* and *GAPDH* were used as internal reference genes, respectively. These genes were selected based on previous validation studies in skin and wound-healing models[27-31]. Real-time PCR was performed using SYBR Green Master Mix (Takara) on an ABI 7500 instrument (Applied Biosystems, Foster City, CA, USA) under the following conditions: initial denaturation at 95 °C for 10 minutes, followed by 45 cycles consisting of denaturation at 95 °C for 10 seconds, annealing, and extension at 60 °C for 60 seconds. Primer efficiency was assessed using LinRegPCR software (2021.2; AMC, Amsterdam, The Netherlands, http://LinRegPCR.nl; Table 2). Standard curve analysis was performed using serial dilutions of the template to assess the amplification efficiency of each assay. The expression levels of the candidate genes were calculated using the $CT^{\Delta\Delta 2-}$ method, normalized to reference genes: *5S rRNA* for *miR-148a-5p* and *GAPDH* for *TGF-β1* and *TGF-βR2*. All experiments were conducted in triplicate. The data were presented as mean ± SD and analyzed using a t-test. ROC curves and AUC were used to evaluate the sensitivity and specificity of the selected miRNA in distinguishing patients exposed to SM from normal controls.

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Table 2. Primer amplification efficiency values (E) calculated by both serial dilution standard curve and LinRegPCR software.

Genes	Gene Efficiency based on serial dilution (E)	Efficiency determined by LinRegPCR (E)
miR-148a-5p	0.923	1.923
5s rRNA	0.912	1.912
TGF-β1	0.934	1.934
TGF-βR2	0.956	1.956
GAPDH	0.947	1.947

An efficiency value of E = 2.0 represents 100% amplification efficiency.

Statistical analysis

All statistical analyses were performed using GraphPad Prism v.6.07 (GraphPad Software Inc., CA, USA). The normality of data was assessed using Kolmogorov-Smirnov tests, and an independent t-test was used for comparisons among the groups. A p value less than 0.05 was considered statistically significant.

RESULTS

Expression of *miR-148a-5p*, *TGF-β1*, and *TGF-βR2* in SM-exposed samples

Given the use of fluorescence in this study, melt curve analysis was performed using the StepOnePlus™ Real-Time PCR System to confirm the correct primer binding, ensure the amplification of specific fragments and check for the absence of non-specific products and primer dimers in the PCR product (Fig. 1). Serial dilutions were used to generate standard curves for the assessment of primer efficiency, as summarized in Table 2. The expression levels of *miR-148a-5p*, *TGF-β1*, and *TGF-βR2* were assessed in the patients exposed to SM and then compared to healthy controls using real-time PCR. The reference genes used for normalization in this analysis were *5S rRNA* and *GAPDH* (Fig. 2). Quantitative analysis revealed significant differences in the expression of these markers between the SM-exposed patients and control skin samples ($p < 0.0001$). Specifically, the expression levels of *miR-148a-5p* (Fig. 2A; $p = 0.0010$), *TGF-β1* (Fig. 2B; $p < 0.0001$), and *TGF-βR2* (Fig. 2C; $p < 0.0001$) significantly reduced in the exposed samples compared to the controls. This reduction in expression was further analyzed using the real-time PCR analysis program (ABI; Fig. 3). Real-time PCR analysis confirmed the downregulation of these biomarkers in the SM-exposed group.

ROC curve analysis of *miR-148a-5p, TGF-β-1*, and *TGF-βR2*

ROC curve analysis was conducted to evaluate the diagnostic potential of *miR-148a-5p*, *TGF-β1*, and *TGF-βR2* in distinguishing SM-exposed patients from healthy controls. For *miR-148a-5p*, the ROC analysis showed a sensitivity of 100%, specificity of 65%, AUC of 0.81, and $p = 0.0008$. For *TGF-β1*, the sensitivity was 90%, specificity 60%, AUC 0.65, and $p = 0.0877$. For *TGF-βR2*, the sensitivity was 80%, specificity 80%, AUC 0.87, and $p = 0.0001$ (Fig. 4). These findings indicate that *miR-148a-5p* and *TGF-βR2* could have a strong diagnostic potential and serve as reliable biomarkers for distinguishing SM-exposed patients from healthy ones. In contrast, *TGF-β1* did not reach statistical significance in ROC analysis ($p = 0.0877$), suggesting limited diagnostic utility in this context. Supplementary Figure S1 represents a conceptual diagram based on the findings from a previous study[25], in which they propose a molecular pathway that *TGF-β* induces the expression of *miR-21* and *miR-20a*. These miRNAs subsequently target and inhibit SMAD7 expression. Overall, *miR-148a-5p* and *TGF-βR2* demonstrated strong diagnostic performance, whereas *TGF-β1* showed limited discriminatory value.

DISCUSSION

This study demonstrates a significant reduction in the expression of *miR-148a-5p*, *TGF-β1*, and *TGF-βR2* in skin biopsy samples from individuals exposed to SM compared to healthy controls. These findings suggest a disruption of the cutaneous TGF-β signaling axis and provide a plausible molecular context for the chronic inflammatory phenotype and delayed wound healing observed in SM-related skin lesions. Interpretation of these results was confined to the markers directly measured in this study; therefore, we limited our discussion to the potential relationship between these expression changes and impaired inflammatory resolution and matrix remodeling in the skin.

Our analysis focused specifically on the expression of *miR-148a-5p*, *TGF-β1*, and *TGF-βR2*. Other molecules mentioned, such as *miR-21*, *miR-20a*, and *SMAD7*, are cited only to provide biological context from previous literature and were not evaluated in our dataset[19,20,21].

In our cohort of SM-exposed veterans, expression levels of *miR-148a-5p*, *TGF-β1*, and *TGF-βR2* were quantified in skin biopsies, and a significant downregulation was identified relative to healthy controls. These findings indicate a reduction in the expression of key components of the TGF-β signaling

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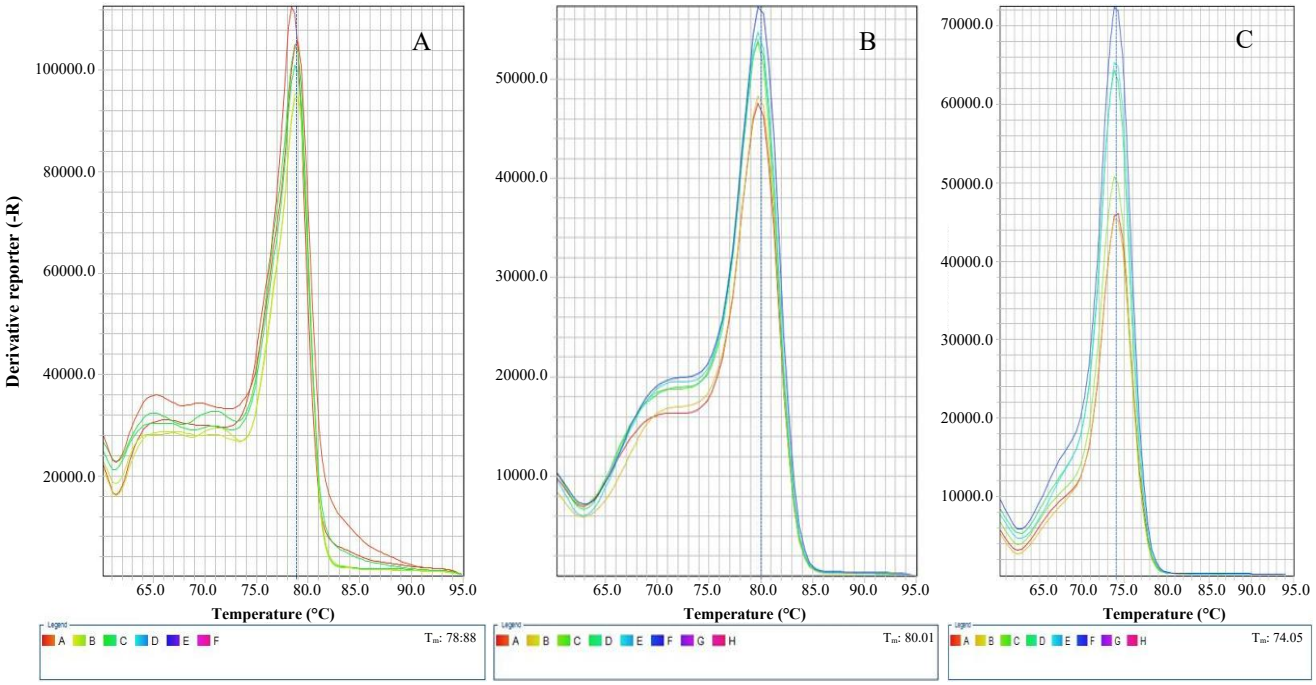

Fig 1. Melt curve analysis of (A) *miR-148a-5p*, (B) *TGF-β1*, and *TGF-βR2* in skin tissue samples from patients with chemical injuries and healthy controls. Each curve represents a melting profile automatically generated by the real-time PCR software, where different colors and letter codes (A-F) correspond to individual wells/samples rather than distinct genes or conditions. All reactions were performed using SYBR Green, and each target displayed a single, sharp peak, confirming amplification specificity. The injury group refers to skin biopsies obtained from MS-exposed veterans, whereas the control group includes healthy individuals with no history of chemical exposure.

pathway, a mechanism that could contribute to persistent inflammation and delayed wound repair in this population. DNA damage and additional cytokine pathways were not addressed; therefore, our interpretations were limited to the observed expression changes in these three markers and their established roles in cutaneous homeostasis and healing.

Studies have linked SM exposure to broad inflammatory responses in the skin[32,33]. Because our data are restricted to gene expression measurements, we did not draw conclusions about pathways or molecular mechanisms that were not directly assessed in this study. Anti-inflammatory roles for *TGF-β* and IL-10 in skin biology and SM-related contexts have been indicated in earlier studies[34,35]. In our study, reduced expression of *TGF-β1* and *TGF-βR2* was identified in skin tissue. This reduction may contribute to incomplete inflammatory resolution, which is consistent with prior reports describing impaired TGF-β-mediated regulation in chronic cutaneous injury. Findings from transgenic

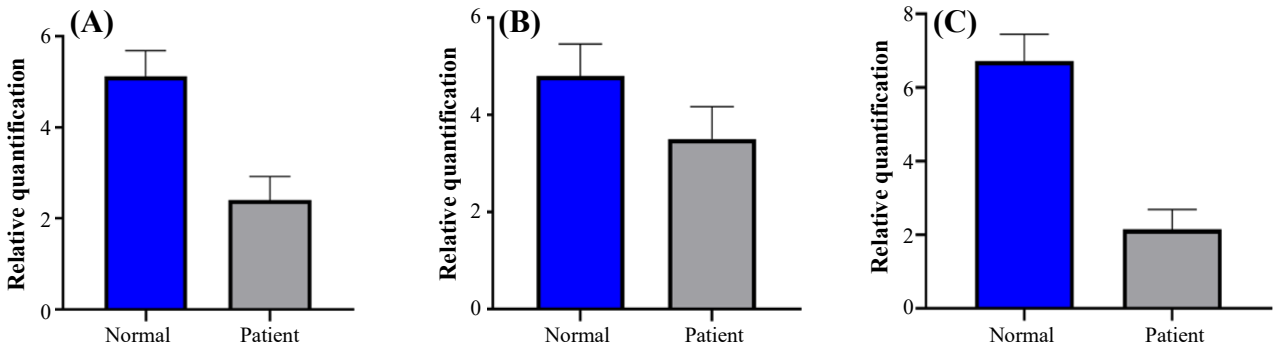

Fig. 2. Expression analysis of (A) *miR-148a-5p*, (B) *TGF-β1*, and (C) *TGF-βR2* in skin tissues from patients with chemical injuries and healthy controls.

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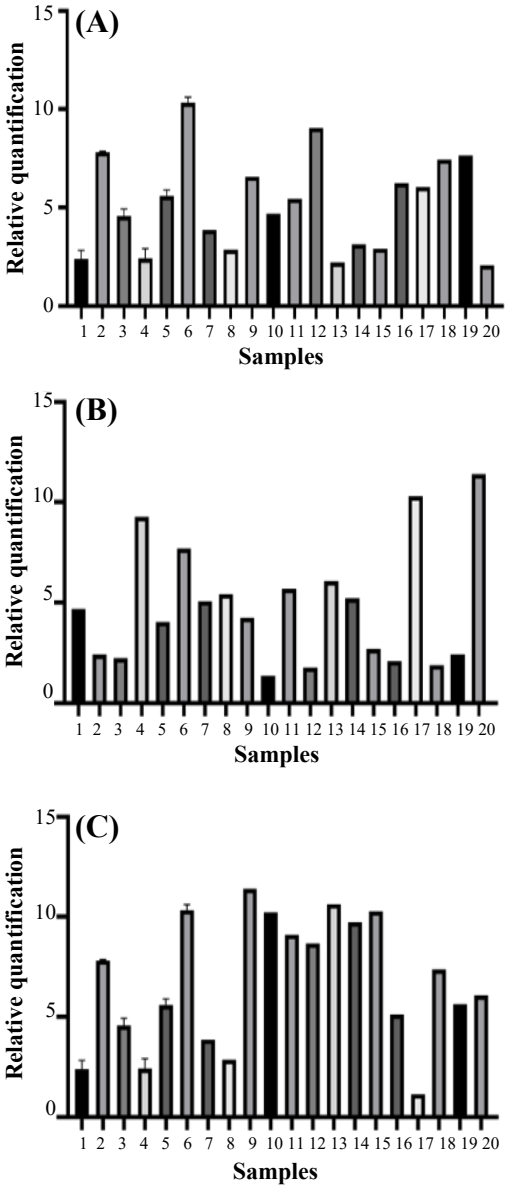

Fig. 3. Expression levels of (A) *miR-148a-5p*, (B) *TGF-β1*, and (C) *TGF-βR2* in skin tissue samples from SM-exposed patients, assessed by relative quantification. Each bar represents an individual patient sample. Control samples are not shown in the Figure, as comparisons with healthy controls were performed statistically and are reported in the Results Section. Numbers below the bars indicate sample identifiers.

mouse models have demonstrated that disruption of TGF-β signaling can lead to inflammatory skin lesions[36,37], though direct causal inferences in humans require functional validation. The central role of *TGF-β* signaling pathway in maintaining cutaneous homeostasis and facilitating wound repair has been well established. Accordingly, the reduced expression of *TGF-β1* and *TGF-βR2* suggests a disruption this pathway, potentially contributing to persistent inflammation and delayed healing in this cohort [15,24].

This study included a modest sample size (n = 40; 20 patients and 20 controls), which may limit statistical power.. The cohort consisted of a highly specific population—Iranian veterans with chronic SM-related skin lesions from defined exposure regions (e.g., Oramanat: Paveh, Javanroud, Thalas Babajani, Ravansar) during the Iran-Iraq war. Therefore, the findings may not generalize to broader populations. Although this specificity provides rare and clinically valuable insight into SM-associated skin pathology, the findings should be interpreted with caution. Larger and independent studies are needed to confirm these observations and evaluate their reproducibility.

The use of *5S rRNA* and *GAPDH* as reference genes represents a methodological consideration in this study. Both genes have been validated in previous skin-related studies[27-31]. While the stability of housekeeping genes can differ between tissues, the identical processing of all samples in this study minimizes potential bias. Future studies will include validation of multiple reference genes using the geNorm and NormFinder algorithms to ensure optimal normalization accuracy.

CONCLUSION

This study shows a significant reduction in the expression levels of *miR-148a-5p*, *TGF-β1*, and *TGF-βR2* in the skin tissues of patients exposed to SM compared to healthy controls. Our findings underscore the critical role of TGF-β signaling pathways in regulating inflammation and wound-healing processes associated with chemical burn injuries. Dysregulation of these pathways may contribute to the chronic inflammatory state and impaired healing typically observed in dermatological conditions resulting from SM exposure. Further investigation into these pathways could provide valuable insights for developing therapeutic strategies targeting TGF-β signaling, with the potential to alleviate the long-term effects of SM exposure on skin health.

DECLARATIONS

Acknowledgments

We would like to express our sincere gratitude to Dr. Mojtaba Amiri, a dermatology specialist at Baqiyatallah Hospital, for his collaboration in sample diagnosis and his support throughout the course of this study. No artificial intelligence-assisted tools were used in the production of the submitted work.

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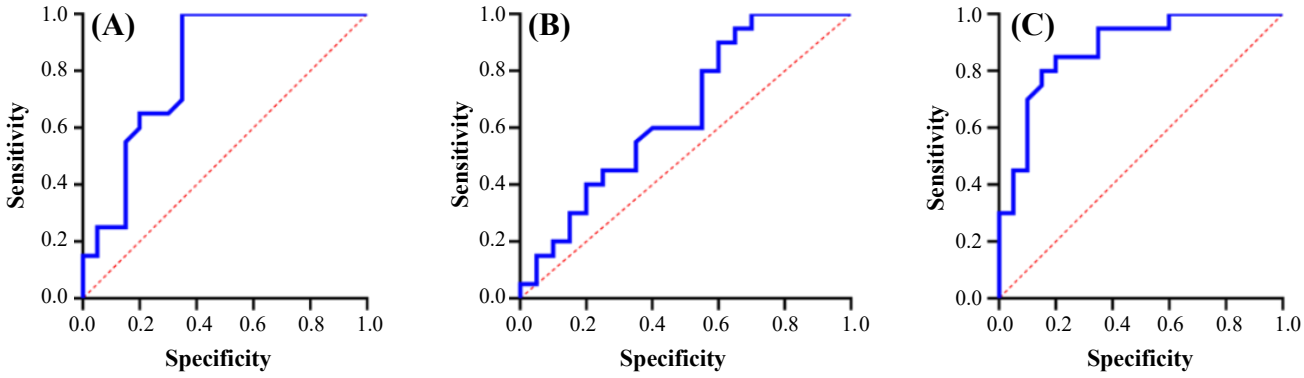

Fig. 4. ROC curve analysis of (A) *miR-148a-5p*, (B) *TGF-β1*, and (C) *TGF-βR2* in skin tissue samples from chemical injury patients and healthy controls. The analysis demonstrates the diagnostic potential of these biomarkers in distinguishing SM-exposed patients from healthy controls.

Ethical approval

The study was approved by the Ethics Committee for Biomedical Research, Vice-Chancellor for Medical Affairs (no. 177), Shahid Beheshti University of Medical Sciences, Tehran, Iran (ethical code: IR.SBMU.RETECH.REC.1404.139).

Consent to participate

All participants voluntarily agreed to take part in this study. Written informed consent was obtained from each individual enrolled in the research.

Consent for publication

All authors reviewed the results and approved the final version of the manuscript.

Authors' contributions

AT: performed experiments and collected data; SHK: conducted data analysis and contributed to scientific editing; MV: designed the study, supervised the research, and drafted the manuscript.

Data availability

All relevant data can be found within the manuscript.

Competing interests

The authors declare that they have no competing interests.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Supplementary information

The online version contains supplementary material.

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[DOI: 10.61882/ibj.5198]