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RESEARCH ARTICLE

Down-regulation of TGF-b1, TGF-b receptor 2, and TGF-b-associated microRNAs, miR-20a and miR-21, in skin lesions of sulfur mustard-exposed Iranian war veterans

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Abstract

Sulfur mustard (SM) affects divergent cellular pathways including cell cycle, apoptosis, necrosis, and inflammatory responses. SM-induced lesions in skin include late-onset hyper-pigmentation, xerosis, and atrophy. It seems that TGF-b signaling pathway is a major player for SM pathogenesis. Here, we have employed a real-time polymerase chain reaction (PCR) approach to evaluate the expression alterations of all TGF-b variants and their receptors in skin biopsies obtained from 10 Iran–Iraq war veterans. Using specific LNA primers, the expression alteration of a TGF-bR2 regulator, miR-20a, and TGF-b downstream target, miR-21, was also assessed in the same samples Our real-time PCR data revealed a significant down-regulation of TGF-b1 and TGF-bR2, the major mediators of TGF-b signaling pathway, in skin biopsies of SM-exposed patients ($p = 0.0015$ and $p = 0.0115$, respectively). Down-regulation of TGF-b signaling pathway seems to contribute in severe inflammation observed in SM-exposed patients' tissues. MiR-20a and miR-21, as two important TGF-b associated microRNAs (miRNAs), were also down-regulated in SM-exposed skin lesions, compared to those of control group ($p = 0.0003$). Based on our findings, these miRNAs could be directly or indirectly involve in the pathogenesis of SM. Altogether, our data suggest the suitability of TGF-b1, TGF-bR2, as well as miR-20a and miR-21 as potential biomarkers for diagnosis and treatment of SM-exposed patients.

Keywords

Gene expression, miR-20a, miR-21, skin, sulfur mustard, TGF-b

History

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Introduction

Sulfur mustard (bis-2-(chloroethyl) sulfide) (SM) is a chemical warfare agent initially used in World War I (1917) and recently during the Iran–Iraq war against Iranian as well as Iraqi–Kurdish citizens (1980–1988) (1). Exposure to SM causes coetaneous, pulmonary, and ocular injury in surviving victims (2). The major chronic skin manifestations of SM are erythema, xerosis, hypo- or hyper-pigmentation, and pruritus (3). Despite a prolonged research, an effective medical treatment for SM-induced maladies is not yet achieved, partly because the molecular mechanism of SM toxicity is not well understood. SM is a bi-functional alkylating agent with a variety of molecular targets and pathways in cells. In the skin, keratinocytes, particularly in the basal layer, are the main target of alkylation induced by SM (4).

Superfamily of transforming growth factor-β (TGF-β) proteins are involved in many biological pathways ranging from cell proliferation, differentiation, recognition, apoptosis, inflammation, tissue homeostasis and angiogenesis (5,6). There are three isoforms of TGF-β: TGF-β1, TGF-β2, and TGF-β3. TGF-β signaling is mediated by its transmembrane receptors, TGFBR1 and TGFBR2, which belongs to serine/threonine kinase receptors (7,8). The intracellular signaling pathways of TGF-β are mediated by Smad proteins, which eventually enter the nucleus and regulate the expression of their targets (9). Altered expression of TGF-β proteins or their signaling pathways have been linked to numerous diseased states including cancer, inflammation and fibrosis (10).

MicroRNAs (miRNAs) are a new class of endogenously made non-coding RNAs with a small size of ~22-nt. MiRNAs are involved in the regulation of various cellular processes including cell cycle, proliferation, senescence, and

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apoptosis (11,12). Due to close link of their aberrant expression to various diseases as well as their high preservation and stability in clinical samples, miRNAs are emerging as potent novel biomarkers for diagnosis and treatment of various diseases (12).

MiR-21, a well-characterized oncomir, is associated with a wide variety of cancers, where it targets several tumor suppressor genes including PTEN, programmed cell death 4 (PDCD4), and tropomyosin 1 (TPM1) (13). Maturation process of miR-21 is greatly amplified by TGF-b signaling pathway through recruitment of specific Smad signal transducers to the complex of RNA helicase p68 and primary transcripts of mir-21 (pri-mir-21) (14). The aforementioned events promotes the processing of pri-miR-21 into its precursor form (pre-mir-21) by the DROSHA complex, and eventually the mature miR-21 production (15).

The members of miR-17-92 cluster (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1) are known to act as oncomirs, where they promote cell proliferation, suppresses cancer cells' apoptosis and induces tumor angiogenesis (16). A series of recent papers establish essential roles for the miR-17-92 cluster of miRNAs in development of the heart, lung, and immune system. Their role in suppressing inflammation through transcriptional regulation of TGF-b signaling pathway is also confirmed (17).

In the present study, we have evaluated the expression alterations of all TGF-b variants, their receptors, as well as TGF-b associated miRNAs, miR-20a and miR-21, in skin biopsies of SM-exposed Iranian war veterans.

Material and methods

Clinical samples

The subjects of this study were 10 SM-exposed patients (Table 1) between the ages of 31 and 58 without any exposure history to any other toxic reagents, and 10 healthy participants between the ages of 35 and 50. Individuals with histories of addiction or topical treatment less than 48 h before taking the biopsy were excluded from the study. Informed consent was obtained from all the patients and the healthy volunteers, and all of them were informed of the probable consequences of a skin biopsy. Biopsy specimens (3 mm^2 in size and about 25 mg in weight) were taken from pruritic plaque skin lesions, or corresponding area of the control group, under topical anesthesia with 2% lidocaine. The samples were put into TRIzol (Invitrogen, Carlsbad, CA) and then stored at −80 °C for further examination. The whole procedure of dealing with clinical samples were initially reviewed and approved by the ethics committee of Baqiatallah Medical Sciences University.

Table 1. The clinico-pathological features of SM-exposed patients.

Type	Age/sex	Sample
Ns1	59/M	Control volunteer
Ns2	55/M	Control volunteer
Ns3	52/M	Control volunteer
Ns4	49/F	Control volunteer
Ns5	47/F	Control volunteer
Ns6	59/F	Control volunteer
Ns7	35/M	Control volunteer
Ns8	50/M	Control volunteer
Ns9	50/M	Control volunteer
Ns10	50/M	Control volunteer
Cs1	39/M	SM-exposed patient
Cs2	49/M	SM-exposed patient
Cs3	47/M	SM-exposed patient
Cs4	39/M	SM-exposed patient
Cs5	56/M	SM-exposed patient
Cs6	41/M	SM-exposed patient
Cs7	31/M	SM-exposed patient
Cs8	35/M	SM-exposed patient
Cs9	41/M	SM-exposed patient
Cs10	58/M	SM-exposed patient

RNA extraction

Total RNA was harvested in conformity with manufacturer's instruction using TRIzol reagent (Invitrogen, Carlsbad, CA). The quality and quantity of the extracted RNA samples were evaluated by gel electrophoresis and NanoDrop instrument (ND-1000 UV – Vis spectrophotometer), respectively.

CDNA synthesize and real-time PCR

One microgram of total RNA samples was reverse transcribed by first-strand cDNA synthesis kit (Bioneer, Daejeon, South Korea) in order to evaluate the expression level of TGF-b variants and their receptors. Reverse transcription (RT) for miRNAs was carried out on 200 ng of total RNA using the miRCURY LNA™ Universal RT microRNA polymerase chain reaction (PCR) kit (Exiqon, Copenhagen, Denmark).

Primer sets for TGF-b1 (*NM_000660*), TGF-b2 (*NM_003238*), TGF-b3 (*NM_003239*), TGF-b receptor 1 (*NM_004612*), TGF-b receptor 2 (*NM_003242*), and GAPDH (*NM_002046*), as an internal control, were designed by Generunner software, version 3.5 (Hastings Software, Hastings, New York, NY; Table 2), and manufactured by TAG Company (Copenhagen A/S, Denmark). The specific LNA primers for real-time PCR amplification of miR-20a and miR-21 were ordered from Exiqon Company (Denmark).

Real-time PCR reactions were performed using SYBR Green (Takara, Japan) master mix for PCR of TGF-b variants and receptors. The PCR reactions for TGF-b and TGF-bR were conducted at: 95 °C for 30 s (initial denaturation), 95 °C for 5 s (denaturation), 60 °C for 1 min (annealing and extension). The following reactions were also used for miR-20a and

Table 2. The sequences and amplicon sizes of the primers used in real-time RT-PCR.

Primer name	Sequence	Amplicon size
TGFβ1	F: 5′-TGGCGATACCTCAGCAAC-3′ R: 5′-ACCCGTTGATGTCCACTTG-3′	181 bp
TGFβ2	F: 5′-AGGAGCGACGAAGAGTACTAC-3′ R: 5′-ACTCTGCTTTCACCAAATTG-3′	169 bp
TGFβ3	F: 5′-TGTCCATGTCACACCTTTCAG-3′ R: 5′-TGTGGTGATCCTTCTGCTTC-3′	145 bp
TGFβ-R1	F: 5′-CGACGATGTTCCATTGGTG-3′ R: 5′-CCATTACTCTCAAGGCTTCACA-3′	165 bp
TGFβ-R2	F: 5′-CATGGCTCTGGTGCTCTG-3′ R: 5′-GCTGGGAATTTCTGGTCG-3′	160 bp
GAPDH	F: 5′-GTGAACCATGAGAAGTATGACAAC-3′ R: 5′-CATGAGTCCTTCCACGATACC-3′	123 bp

F, forward primer; R, reverse primer.

miR-21 amplifications: 95 °C for 10 min (polymerase activation/denaturation), 95 °C for 10 s (denaturation), 60 °C for 1 min (annealing and extension). All PCR reactions carried out for 45 cycles in an ABI 7500 real-time quantitative PCR system (Applied Biosystems, Foster City, CA).

LinRegPCR (12.x) software (AMC, Amsterdam, The Netherlands, http://LinRegPCR.nl) were used to determine the efficiency of each primer pair. Obtained data from real-time PCR were analyzed by Graphpad prism software (version 5.0) (GraphPad Software Inc., San Diego, CA).

Statistical analysis

The normality of the data was assessed by Kolmogorov–Smirnov and Shapiro–Wilk tests. Due to abnormal distribution of the data, Mann–Whitney test was used to compare the expression level of the genes. Moreover, the Spearman correlation coefficients (r) were used to determine a potential correlation between TGF-b variants expression levels and those of miR-20a and miR-21.

Results

TGF-b1 and TGF-bR2 are significantly down-regulated in SM-exposed skin tissue samples

To evaluate the expression level of TGF-b variants and their receptors (TGF-bRs), we used specifically designed primer pairs for each variant and receptor and conducted real-time PCRs for all biopsy samples. After optimizing PCR reactions, the expression alteration of each variant was compared between SM-exposed tissues and the control ones. After calculating the efficiency of the primers, normalized relative expression level of each variant to GAPDH, as the internal control, was obtained for statistical analysis. Our data revealed that the expression level of TGF-b1 and TGF-bR2, as the main variant and the major receptor for this signaling pathway were significantly down-regulated in SM-exposed patients ($p = 0.0015$ and $p = 0.0115$, respectively, Figure 1A and E). However, while the expression level of TGF-b2 and TGF-b3 were slightly higher in patients' samples (Figure 1B and C), in contrast to the control ones, the observed up-regulations were not statistically significant. Similar to down-regulation of TGF-bR2, we had a noticeable down-regulation forTGF-bR1 (Figure 1D). However, the latter observation was not statistically significant ($p > 0.05$).

MiR-20a and miR-21are down-regulated in SM-exposed skin tissues

Using LNA specific primers, we were able to detect and quantify the expression of two miRNAs whose functions are related to TGF-b signaling pathway, i.e. miR-20a and miR-21, in skin biopsies of the patients and control groups. Normalized to the expression level of 5S rRNA, as an internal control, our real-time PCR data revealed a significant down-regulation of miR-20a ($p = 0.0003$) (Figure 2A) and miR-21 ($p = 0.0003$) (Figure 2B) in skin biopsies obtained from the lesion areas of SM-exposed patients, compared to those obtained from apparently normal skin tissues of control group.

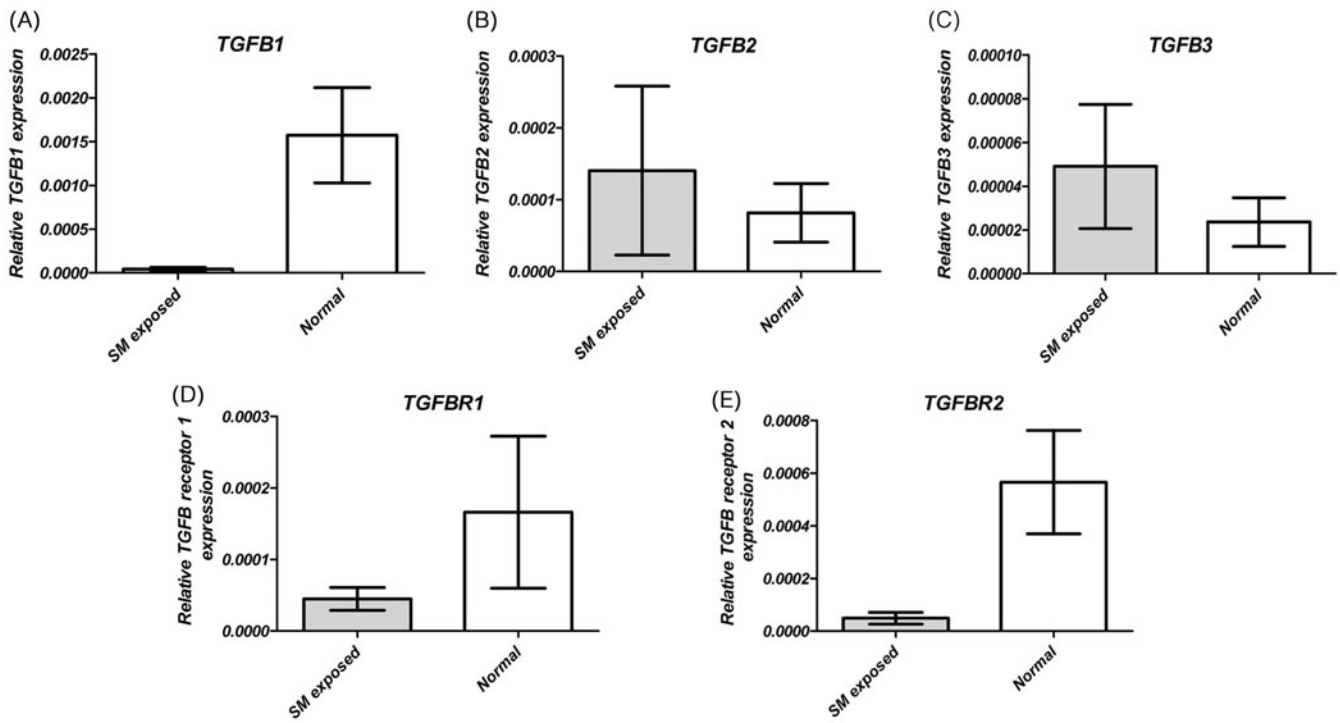

Figure 1. Altered expression of TGF-b variants and TGF-b receptors in SM-exposed skin biopsies. Reversed-transcribed real-time PCR results on relative expression of TGF-b variants and receptors in SM-exposed and unexposed skin biopsies are presented. Note that TGF-b1 (A), ($p = 0.0015$) and TGF-b receptor 2 (E), ($p = 0.0115$) are significantly down-regulated in SM-exposed skin lesion samples, compared to the normal paired samples. In contrast, the expression alteration of TGF-b2 (B), TGF-b3 (C) and TGF-b receptor 1 (D) was not statistically significant ($p > 0.05$).

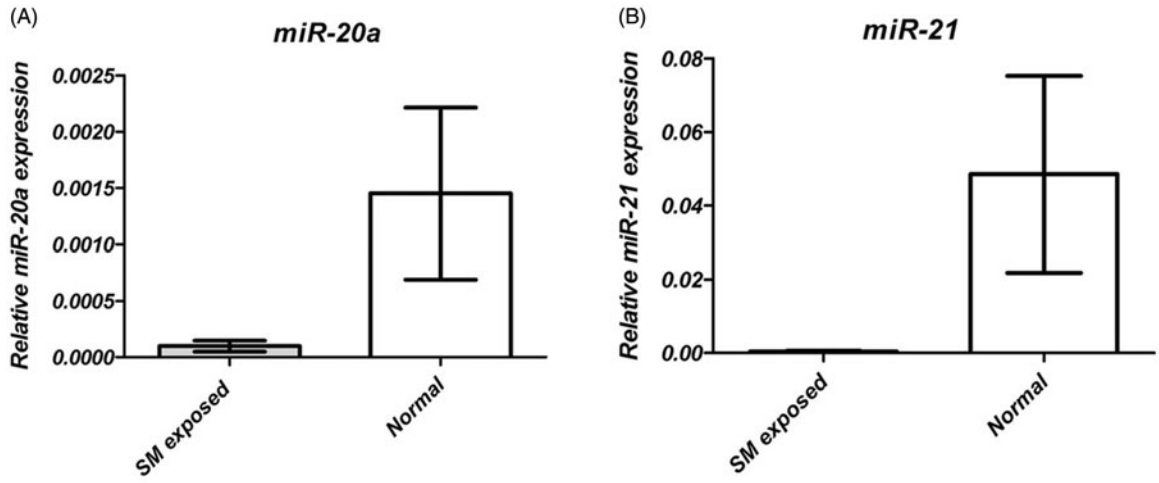

Figure 2. Altered expression of miR-20a and miR-21 in SM-exposed skin biopsies. Using real-time RT-PCR, the expression level of miR-20a and miR-21, normalized to that of 5S rRNA, is compared between SM-exposed and normal skin biopsies. As it is evident, the expression of miR-20a (A), ($p = 0.0003$) and miR-21 (B), (0.0003) is significantly declined in SM-exposed skin lesion specimens compared to the normal tissue.

A positive correlation between the expression alterations of TGF-b1and miR-21 in SM-exposed patients

Primary analysis of our data suggested a potential positive correlation between the expression alteration of miR-20a, miR-21, and TGFβ1 variant, in SM-exposed skin biopsy specimens. To test the hypothesis, we performed the Spearman correlation coefficients (r) statistical analysis. The obtained data demonstrated a strong correlation between the expression level of TGF-b1 and that of miR-21 ($R^2 = 0.6485$, $p = 0.049$). However, despite an apparent correlation between TGF-b1 and miR-20a expression levels, the Spearman test failed to show a statistically significant correlation. Moreover, the same analysis failed to show a significant co-regulation for the other members of TGF-b and miR-20a and miR-21 (Table 3).

Table 3. Spearman correlation analysis among the expression level of TGF-b variants, their receptors, miR-20a and miR-21.

	miR-21	miR-20a
TGF-b1	Spearman R 0.6485 (p value = 0.0425)	Insignificant (p value = 0.1523)
TGF-b2	Undetermined	Undetermined
TGF-b3	Undetermined	Undetermined
TGF-bR1	Undetermined	Undetermined
TGFbR2	Insignificant (p value = 0.3869)	Insignificant (p value = 0.7850)

Note the existence of a strong co-expression only between TGF-b1 and miR-21.

Discussion

Exposure to SM causes severe short- and long-term damages to human body, mostly in lung, skin, eye, and bone marrow (1,18). Cheapness as well as ease of manufacturing and application has made SM a potential deadly chemical weapon in the hands of terrorist groups and regimes. The recent usage of SM by Saddam's regime during Iraq–Iran war, for instance, has left more than 75 000 injured people suffering from several chronic diseases. Treatment of the SM-exposed patients is still a big challenge in clinic, and to find an effective cure requires unraveling the molecular pathways altered by SM.

Recently, the altered expression of TGF-b in SM-exposed tissues is reported by several research groups. However, while some groups reported an up-regulation of TGF-b in SM-exposed tissue samples (19,20), the others showed a down-regulation of the molecule (21,22). The existence of different variants of TGF-b as well as pursuing different technical approaches to detect TGF-b could partly explain the inconsistency observed in the aforementioned papers. Here, we have designed specific primers to discriminately quantify the expression of TGF-b variants and receptors by a real-time RT-PCR approach in skin biopsies of SM-exposed Iranian war veterans. Our data revealed differential expression alteration of TGF-b variants in patients group, compared to the normal control group. In contrast to a very significant down-regulation of TGF-b1, the other variants of TGF-b, i.e. TGF-b2 and TGF-b3, showed a slightly (statistically insignificant) up-regulation in patients group. The finding demonstrated independent regulatory circuits for gene expression regulation of each variant. A significant down-regulation was also observed for TGF-bR2. Nevertheless, while we observed a similar down-regulation for TGF-bR1, the altered expression was not statistically significant. The latter finding could be due to the low number of samples in case and control groups. Altogether, our data revealed a significant suppression of TGF-b signaling pathway in SM-exposed skin of Iranian veteran patients. Our data are in agreement with the findings of Khaheshi et al., in which they reported a significant down-regulation of TGF-b variants and receptors in chronic skin lesions biopsies of SM-exposed patients (21). Our data also support the findings by Panahi et al., in which they reported a significant down-regulation of TGF-b in serum samples of SM-exposed war veterans (22). However, our findings are in disagreement with four previously published works reporting TGF-b elevation in lung and bronchoalveolar tissues as well as lavage aspirates (19,20). These inconsistencies require further clarification; however, a poor technical approach in

some previously published papers (23) could be the cause of this inconsistency.

TGF-b signaling plays key parts in cell proliferation, cell fate determination, development, senescence, and inflammation. It seems the main reason for induced inflammation in SM-exposed tissues is caused by a reduced expression level of TGF-b proteins, as the key anti-inflammatory cytokines released during the wound healing process (24). Our data of suppressed TGF-b signaling pathway in SM-exposed war veterans could explain the observed severe inflammation in the patients' skin lesions. Our data are also in full agreement with the previously published works in animal models (25,26).

Next, we monitored the expression alteration of two miRNAs, which are closely related to the TGF-b signaling pathway, in skin species of SM-exposed patients. MiR-21 is one of the first identified miRNAs with an oncogenic function, i.e. oncomiR, where it is overexpressed in a large number of tumor types (13). MiR-21 regulates many cellular processes including proliferation, differentiation, development, carcinogenesis, aging, inflammation, senescence, and apoptosis. Up-regulation of miR-21 as well as promoting its maturation by TGF-b signaling pathway has been already reported (14,27).

As a direct downstream target of TGF-b signaling pathway, we expected a sharp decline in the expression level of miR-21 in the patients group. Indeed, our real-time RT-PCR data revealed a dramatic decrease in the level of miR-21 in the SM-exposed skin biopsies. Moreover, there was a positive correlation between the reduced expression levels of TGF-b with that of miR-21. A down-regulation of miR-21 could activate the NF-kB signaling pathway, which in turn leads to the inflammation induction in the SM-damaged skin samples (28). In addition, miR-21 indirectly inhibits the expression of TGF-b2. Therefore, down-regulation of miR-21, and hence the release of its inhibitory effects, would lead to up-regulation of TGF-b2 (17). The latter mechanism could explain our observed insignificant up-regulation of TGF-b2 in skin samples.

We also evaluated the expression alteration of miR-20a in SM-exposed skin samples. Similar to its effect on miR-21, TGF-b signaling pathway also promotes the transcription of miR-20a, a member of miR-17-92 cluster (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1) (29). MiR-20a involves in several biological events such as cell proliferation and repression of apoptosis (29,30). Considering the positive transcriptional correlation of miR-20a and TGF-b signaling pathway, we expected a co-suppression of miR-20a in our patients group (24). As our data indicated, a significant down-regulation of miR-20a was paralleled with similar changes in the suppression of TGF-b signaling pathway in SM-exposed skin tissues. Accordingly, suppressed expression of miR-20a, and probably other members of miR-17-92 cluster, could potentially contribute in maladies caused by exposure to SM. A direct inhibition of TGF-bR2 by miR-20a (17) could also explain the more dramatic down-regulation of this receptor in our samples.

The findings could be generalized to other microRNA-receptor regulatory systems, in which the manipulation of a microRNA could affect the expression and activity of its target receptor. A fascinating example is botulinum neurotoxins, which binds to the surface of nerve terminals, and thereby blocks neurotransmitters release. Its effects range from preventing wrinkled skin to causing severe food poisoning and respiratory failure (33). SV2, a conserved membrane protein of synaptic vesicles, has been recently identified as the receptor for botulinum neurotoxin A to mediate its internalization and effects (34). However, the expression and activity of SV2 is regulated by a developed and activity-dependent micoRNA (miR-485). Interestingly, SV2A knockdown mimicked the effects of miR-485 suggesting that overexpression of miR-485 could block the signaling pathways caused by SV2 activation (35).

In conclusion, our data revealed a down-regulation of TGF-b signaling pathway, along with a correlated down-regulation of two important TGF-b associated miRNAs, miR-21 and miR-20a, in SM-exposed skin tissue. A co-downregulation of these molecules has already been linked to inflammation process (15,31–32). For the first time, we have introduced microRNA alterations in SM-exposed tissues. Considering the high stability of miRNAs in clinical samples, and successful utilization of these molecules in clinical trials, we hope these molecules open a new venue in diagnosis and treatment of SM-exposed patients.

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Declaration of interest

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