Transcription of biological aging markers (ANRIL, P16ink4a, TBX2, and TERRA) and their correlations with severity of sulfur mustard exposure in veterans

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Abstract

Sulfur mustard (SM) exposure has delayed harmful effects, including cell senescence and premature biological aging. This study aimed to evaluate the expression of four main markers of biological aging (i.e., ANRIL, \( P^{16ink4a} \), TBX2, and TERRA) and assess their correlation with the severity of SM exposure in the long term. The study was conducted on two groups of volunteers. 1) the SM-exposed group, exposed to SM once in 1987 during the Iraq-Iran war. This group was divided into three subgroups based on the injury severity, asymptom (exposure to SM without any clinical signs), mild, and severe subgroups; 2) the non-exposed group. ANRIL and TBX2 transcripts were reduced in the total SM-exposed people. Their reduction was more considerable in the SM-exposed subgroups of mild and severe. On the other hand, \( P^{16ink4a} \) and TERRA transcripts were increased due to SM exposure. The increase of TERRA expression was more significant in the asymptom, mild and severe subgroups, while \( P^{16ink4a} \) was only higher in the asymptom subgroup. There was a positive correlation between the TERRA transcript and the severity of injury, while this correlation was negative for the ANRIL transcript. Thus, the delayed toxicity of SM is associated with the dysregulation of aging markers leading to progressive premature cellular senescence that is dependent on the severity of SM exposure.

Introduction

Sulfur mustard (SM) is a type of chemical warfare weapon that contains the chemical structure 1-chloro-2-[(2-chloroethyl)sulfanyl] –ethane (\( C_4H_8Cl_2S \)) (Nair et al. 2021). This compound is extremely toxic and can eliminate chloride ions by intramolecular nucleophilic substitution to form cyclic sulfonium ions in cells. These very reactive intermediates tend to permanently alkylate DNA and RNA strands, proteins, and lipid membranes and interfere with several metabolic, endocrine, and genetic processes. It can damage DNA, cause oxidative stress, prevent cellular division, develop mutation and cancer, induce cellular apoptosis, and death (Ghazanfari et al. 2009a). This weapon was first used in World War I and has been used in several armed conflicts since then, including Iraq–Iran War, resulting in more than 100,000 Iranian casualties and veterans (Kehe and Szinicz 2005). These veterans are suffering from late effects until today (after more than 30 years). It means that people exposed to SM suffer two kinds of acute and chronic complications. Acute complications are observed as ocular and dermal injury, respiratory tract damage, reproductive and developmental toxicity, gastrointestinal effects, and immunological /hematological disturbs (Balalimoud and Hefazi 2005) while the chronic form of complications includes cell senescence and biological aging in addition to many sustained dysfunctions in the mentioned body systems (Ghazanfari et al. 2009b; Nasiri et al. 2021; Nasiri et al. 2023). Recently, many studies confirmed telomere shortening and lipofuscin accumulation in SM-exposed people evidencing biological aging (Ardestani et al. 2022; Behboudi et al. 2018; Behravan et al. 2018; Nasiri et al. 2021; Nasiri et al. 2023). It has also been determined that SM triggers senescence in human dermal fibroblasts and mesenchymal stem cells, leading to wound repair disorder (Horn et al. 2022; Rothmiller et al. 2021). Biological health score (BHS) as another indicator of biological aging was considered in SM-exposed people in our previous studies. In this indicator, eighteen biomarkers from kidney/liver function and four physiological
systems (cardiovascular, endocrine, immune/inflammatory, and metabolic) were involved. The increase of BHS associated with telomere attrition is considered evidence of progressive biological aging in SM-chemical veterans (Nasiri et al. 2021; Nasiri et al. 2022).

Telomeric repeat-containing RNA (TERRA) is a long non-coding ribonucleic acid, including UUAGGG repeats. This RNA is transcribed by RNA polymerase II at the subtelomeric region of a chromosome (Sinha et al. 2015). While several functions have been proposed for TERRA, its biological role remains elusive. The 3' end of the TERRA sequence is complementary to the template region of telomerase RNA that interacts with telomerase in the cell. It has been proposed that TERRA involves in the maintenance of telomere structure and the formation of heterochromatin (Mensà et al. 2019; Sinha et al. 2015). TERRA can also mediate the exchange of single-strand telomere-binding proteins during the cell cycle, promoting telomere capping after DNA replication and protecting it against attrition. Previous studies confirmed that TERRA might involve in aging processing, and its down-regulation may play an essential role in maintaining genomic stability, delaying aging, and treating age-associated diseases such as cancer and neurodegenerative disorders. (Libertini et al. 2020; Sinha et al. 2015).

Long non-coding RNA (lncRNA) ANRIL is known as antisense RNA in the INK4 locus. It is expressed from 9p21 on chromosome locus where P16 INK4a is transcribed (He et al. 2018). The main function of ANRIL is the regulation of gene expression. ANRIL can influence cell proliferation, senescence, apoptosis, extracellular matrix remodeling, and inflammation (Razeghian-Jahromi et al. 2022). In cells, ANRIL can maintain the proliferative state by blocking senescence genes while its silencing decreases cell proliferation and induces senescence (Degirmenci and Lei 2016; He et al. 2018).

T-box transcription factor 2 (TBX2) is a transcriptional repressor that is involved in embryonic development and cell cycle regulation. It can regulate many cycle regulation factors, such as P21 and P14, to make cells resist senescence. It has also been determined that TBX2 knocking down causes a reduction of cell proliferation and induction of apoptosis and senescence. The level of TBX2 is necessary for macromolecular synthesis, energy production, and cell survival. (Li et al. 2023; Wansleben et al. 2013).

P16INK4a, also known as cyclin-dependent kinase inhibitor 2A, is a protein that blunts cell proliferation by slowing the processing of the cell cycle from the G1 phase to the S phase. It is identified as a cell cycle regulator, tumor suppressor, and marker of senescence (Liu et al. 2019; Muss et al. 2020).

The present study was designed to investigate the gene expression of many biological aging markers (ANRIL, P16INK4a, TBX2, and TERRA) and their correlations with lipid peroxidation in SM-chemical veterans with different severities of injury. We hypothesized that changes in these markers might be evidence of progressive biological aging during long-term SM intoxication.

Materials And Methods

Subjects
This study was approved by the ethical committee of Shahed University with the approved code of IR.SHAHED.REC.1399.151. All voluntary contributors wrote informed consent in a project as “Sardasht-Iran cohort study of chemical warfare victims”. The participants were included as 1) the SM-exposed group, 90 male individuals from Sardasht city, exposed to SM once in 1987 during the Iraq-Iran war; 2) the non-exposed group, 55 healthy male individuals, from Rabat city (near Sardasht with similar ethnic living and weather condition) who had no SM exposure. According to the severity of SM exposure and body damage, the participants were divided into three subgroups named as asymptomatic (exposed to SM without any clinical signs), mild, and severe. The blood sample of all contributors was prepared in two weeks, and the following tests were done in one month.

**RNA extraction, cDNA synthesis, and quantitative real-time PCR (RT-qPCR)**

Total RNA of whole blood (100 µL) was extracted using the TRIzol LS reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The quality and integrity of RNA samples were evaluated by spectrophotometry. Only RNA samples representing an A260/A280 ratio of 1.8–2.2 were suitable for cDNA synthesis. The synthesis of cDNA was done using a random hexamer PrimeScriptTM Reverse transcription kit (Takara Bio Inc., Japan). The yielding cDNA was stored at −20°C until RT-qPCR.

To determine the possible changes in the transcriptional levels of ANRIL, p16ink4a, TBX2, and TERRA in two groups of Non- and SM-exposed groups, relative RT-qPCR was performed using a PCR kit (1 HOT FIREPol EvaGreen qPCR Mix, Solis Biodyne, Seoul, South Korea). Phosphoglycerate kinase (PGK) was used as a stable control gene (Eghtedardoost et al. 2020) to normalize the input load of cDNA and quantify the target gene’s expression relatively gene. The specific primers of the target genes are represented in Table 1. The PCR for each sample was performed in three replicates in a real-time thermocycler (Applied Biosystems, ABI Step One, USA), whereby 10 ng cDNA and 400 nM of each specific primer were used in a total volume of 20 µl. The program of PCR amplification was as 95°C for 15 min, then 30–40 cycles of 95°C for 15 s, 50–64°C for 15–30 s, and 72°C for 10–40 s (only for ANRIL, TBX2, and TERRA). The no-template and no-reverse transcriptase controls were used in each PCR reaction. Amplification efficiencies were quantified by using the standard curve method for all target genes. The correlation coefficients ($R^2$) for the standard curves were > 0.997. The relative gene expression (target/PGK) was calculated according to the Livak method (Schmittgen and Livak 2008).
Table 1
The sequence of primers, size of PCR products, and temperature annealing

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequences (5’ - 3’)</th>
<th>Annealing (°C)</th>
<th>PCR Product</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBX2</td>
<td>5’-CAGAAATGACAGATCACACA-3’</td>
<td>50</td>
<td>125 bp</td>
<td>NM_005994.4</td>
</tr>
<tr>
<td></td>
<td>5’-TCGTACAAGCTGAGAGC-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANRIL</td>
<td>5’-GCCTCATTCTGATTCAACAGC-3’</td>
<td>64</td>
<td>204 bp</td>
<td>NR_047543.1</td>
</tr>
<tr>
<td></td>
<td>5’-GATCTCCCGTTCGTTCTTCT-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TERRA</td>
<td>5’-GTTTTTTGAGGGTGAGGGTGAAGGGTG-3’</td>
<td>66</td>
<td>78 bp</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5’-TCCCGACTATCCCTATCCCTATGAGGGTG-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p16INK4a</td>
<td>5’-GGGGGCACCAGAGGCAGT-3’</td>
<td>62</td>
<td>159 bp</td>
<td>NM_000077.5</td>
</tr>
<tr>
<td></td>
<td>5’-GGTTGTGGCCGGGCCCAGT-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGK1</td>
<td>5’-GGCATACCTGCTGGCTGGATG-3’</td>
<td>62</td>
<td>104 bp</td>
<td>NM_000291.4</td>
</tr>
<tr>
<td></td>
<td>5’-ACAGGACCATTCCACACAATCTGC-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Statistical analysis

Data were displayed as mean (SD). Values of each transcript were compared between groups and subgroups. The normality of all data was determined using Kolmogorov–Smirnov test. The non-normally distributed data were compared through Kruskal-Wallis and Mann–Whitney. The Spearman correlation between the transcript of target genes and the severity of SM injury was done. The P values less than 0.05 were noticed as statistically significant. The SPSS software (IBM SPSS Statistics for Windows, version 26.0, IBM Corp., Armonk, N.Y., USA) was used for all mentioned statistical analyses.

Results

Estimation of ANRIL, p16ink4a, TBX2, and TERRA transcripts

Figure 1 shows the comparison of real-time PCR results for the ANRIL between the non-exposed and SM-exposed groups. The relative expression of ANRIL was lower in the total SM-exposed group than in the non-exposed group (a decrease of 59.1%; P = 0.004). The expression of this IncRNA was also lower in the SM-exposed subgroups of mild and severe than in the asymptom subgroup (the decrease of 97.6% and 96.4%, respectively) and the non-exposed group (the decrease of 98.0% and 97.0%, respectively) (P <
0.05). The difference between the asymptom subgroup and non-exposed group, and also between mild and severe subgroups was not significant for ANRIL expression (P > 0.05).

Figure 2 indicates the comparison of RT-qPCR results for the \( P^{16\text{ink}4a} \) gene between the non-exposed and SM-exposed groups. The relative expression of \( P^{16\text{ink}4a} \) gene was higher in the total SM-exposed group than in the non-exposed group (an increase of 21.4%; \( P = 0.048 \)). The expression of this gene was also higher in the asymptom subgroup than in the non-exposed group (an increase of 26.7%; \( P = 0.041 \)). The difference between SM-exposed subgroups was not significant for the expression of this gene (P > 0.05).

Figure 3 shows the comparison of RT-qPCR results for the TBX2 gene between the non-exposed and SM-exposed groups. The relative expression of TBX2 gene was lower in the total SM-exposed group than in the non-exposed group (a decrease of 34.9%; \( P = 0.021 \)). The expression of this gene was also lower in the SM-exposed subgroups of mild and severe than in the asymptom subgroup (a decrease of 64.3% and 66.7%, respectively) and the non-exposed group (a decrease of 65.1% and 67.4%, respectively) (P < 0.05). The difference between the asymptom subgroup and the non-exposed group, and also between mild and severe subgroups, was not significant for the expression of this gene (P > 0.05).

Figure 4 indicates the comparison of RT-qPCR results for the TERRA between the non-exposed and SM-exposed groups. The relative expression of TERRA was higher in the total SM-exposed group than in the non-exposed group (an increase of 82%; \( P = 0.001 \)). Its expression was also higher in the asymptom, mild and severe subgroups than in the non-exposed group (an increase of 71.8%, 87.2%, and 84.5, respectively; P < 0.05). The expression of TERRA was higher in the mild and severe subgroups than asymptom subgroup (an increase of 54.7% and 45.1%, respectively; P < 0.05).

**Correlation of transcription with the severity of injury in SM-chemical veterans**

Table 2 represents the correlation between the relative expression of ANRIL, \( P^{16\text{ink}4a} \), TBX2, and TERRA and the severity of injury in SM-chemical veterans. There was a positive correlation between the relative expression of TERRA and the severity of injury, while this correlation was negative for the expression of ANRIL (P < 0.05). The correlation between TBX2/\( P^{16\text{ink}4a} \) gene expression and severity of injury in the veterans was not significant (P > 0.05).
Table 2
Correlation (spearman) between different transcriptions and severity of injury in SM-chemical veterans

<table>
<thead>
<tr>
<th>Transcription (relative expression)</th>
<th>Correlation coefficient</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANRIL/PGK</td>
<td>-0.204</td>
<td>0.012</td>
</tr>
<tr>
<td>P16/PGK</td>
<td>0.047</td>
<td>0.553</td>
</tr>
<tr>
<td>TBX/PGK</td>
<td>-0.051</td>
<td>0.140</td>
</tr>
<tr>
<td>TERRA/PGK</td>
<td>0.401</td>
<td>0.001</td>
</tr>
</tbody>
</table>

The P < 0.05 was statistically considered significant.

Discussion

This study evaluated the effect and correlation of different severities of sulfur mustard exposure on the gene expression of many biological aging markers (ANRIL, P16ink4a, TBX2, and TERRA).

As mentioned, SM exposure after a long time has still had side effects especially cellular senescence and biological aging. The primary evidence for this pathologic event could be telomere shortening, high accumulation of lipofuscin, and increased BHS in SM-chemical veterans (Abbasi et al. 2012; Nasiri et al. 2021; Nasiri et al. 2022; Nasiri et al. 2023).

P16ink4a, as an aging biomarker, was also assessed in the previous study of SM veterans. It was confirmed that its expression was associated with the shortening of telomere in veterans. This result is in agreement with the present study. We showed a significant increase of P16ink4a in the veterans, but this increase was not associated with progressive injury in SM-exposed people. However, regulation of P16ink4a gene expression is complex. It involves environmental factors and epigenetic controllers, and multiple transcription factors (Rayess et al. 2012), and further studies need to evaluate changes in these factors, especially in the severe form of SM intoxication.

ANRIL, as another senescence marker, was evaluated in this study. We found that the expression of this IncRNA was considerably decreased in the SM-exposed people, especially in mild and severe forms, and it had a negative correlation with the severity of injury. ANRIL modulates typically oxidative stress and inflammation (Shi et al. 2020; Zhou et al. 2016). Then, its downregulation may exacerbate SM-induced oxidative stress and inflammation (Tahmasbpour et al. 2015; Yaraee et al. 2009). ANRIL has been documented to contribute to the protection of DNA damage response through controlling DNA repair. On the other hand, Behboudi et al. (2018) and Khateri et al. (2018) confirmed the DNA damage in the SM victims (Behboudi et al. 2018; Khateri et al. 2018). Therefore, the down-regulation of ANRIL may play a critical role in this effect of SM.
ANRIL is known as an antisense to P16\textsuperscript{ink4a} that suppresses its expression (He et al. 2018). The down-regulation of ANRIL (as observed in our study) may be an main reason for P16\textsuperscript{ink4a} overexpression in the veterans leading to cell senescence (Buj et al. 2021).

Our data showed a TERRA overexpression as a delayed effect of the SM toxicity that was considerably correlated with the severity of injury. It seems this overexpression was simultaneous with the mentioned telomere shortening in the veterans. Previous studies also reported that the shortening of telomeres leads to an upregulation of TERRA (Arnoult et al. 2012; Porro et al. 2014; Rippe and Luke 2015). Of course, this upregulation can occur during oxidative stress, DNA damage (as previously reported in SM-exposed people), and loss of TRF2 (one of the telomere-protecting proteins). In these conditions, TERRA overexpression might represent a signal to trigger telomerase clustering and activity of telomerase molecules to help for repairing telomere length (Cusanelli et al. 2013). It may be a compensatory mechanism to prevent the DNA from progressive damage (Bettin et al. 2019).

TBX2 as an anti-senescence factor was reduced in SM-exposed people with mild and severe injuries, which would be another evidence for biological aging in SM veterans. Li et al. (2023) indicated that TBX2 inhibition causes the accumulation of cellular reactive oxygen species (ROS), increased BAX/BCL2 levels, and the apoptosis rate and mitochondrial disruption (Li et al. 2023). Yi et al. (2017) also determined that TBX2 overexpression decreases caspase-3 cleavage and increases BCL2 upregulation (Yi et al. 2017). However, these harmful effects due to TBX2 dysregulation have already been counted as complications of SM toxicity (i.e., oxidative stress, apoptosis, and mitochondrial dysfunction) (Beigi Harchegani et al. 2019; Ghanei and Harandi 2011; Gould et al. 2009; Ray et al. 2008; Shahin et al. 2001). Probably, SM implies part of its detrimental effects through diminishing of TBX2 gene expression.

As our data showed, the alteration in the mentioned aging markers is dependent on the severity of injury in veterans; Markers such as TERRA and P16\textsuperscript{ink4a} were influenced by even weak severity of SM toxicity (without clinical signs) while other markers (i.e., TBX2 and ANRIL) were only affected by substantial severity of SM toxicity (with clinical symptoms). Therefore, evidence of progressive premature cellular aging exists for all people exposed to SM but with different intensities. Evaluation of these aging markers can be helpful as indicators to indicate the level of susceptibility to age-related diseases in SM-exposed veterans.

**Conclusions**

It is concluded that the delayed toxicity of SM is associated with dysregulation of aging markers such as TERRA, ANRIL, TBX2, and P16\textsuperscript{ink4a}, leading to progressive premature cellular aging that is dependent on the severity of SM injury.

**Declarations**
**Authors’ contributions** L. Nasiri, H. Hassanpour, S. Ghaffarpour and M.S. Zamani performed the experiments. M.R. Vaez-Mahdavi, T. Ghazanfari, S. Kaboudanian Ardestani and N. Askari contributed to experimental planning and design. L. Nasiri and H. Hassanpour, performed data analysis and statistics, and wrote the manuscript in collaboration with the other authors. All authors read and approved the final manuscript and agreed to be accountable for all aspects of the work.

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**Data availability** The authors declare that all data presented are available upon request.

**Ethics approval and consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Conflicts of interest** The authors declare no conflict of interest.

**References**


**Figures**
Figure 1

Comparison of ANRIL relative expression between non-exposed and SM-exposed groups via Kruskal-Wallis and Mann-Whitney tests. The P < 0.05 was statistically considered significant. Data are represented as mean (SD).
Figure 2

Comparison of $P^{16\text{ink}4a}$ relative gene expression between non-exposed and SM-exposed groups via Kruskal-Wallis and Mann-Whitney tests. The $P < 0.05$ was statistically considered significant. Data are represented as mean (SD).
Figure 3

Comparison of TBX2 relative gene expression between non-exposed and SM-exposed groups via Kruskal-Wallis and Mann-Whitney tests. The $P < 0.05$ was statistically considered significant. Data are represented as mean (SD).
Figure 4

Comparison of TERRA relative expression between non-exposed and SM-exposed groups via Kruskal-Wallis and Mann-Whitney tests. The $P < 0.05$ was statistically considered significant. Data are represented as mean (SD).