



Association of glutathione S-transferase polymorphisms with the severity of mustard lung

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Article Info



Article Type:

Original Article

Article History:

Received: 24 May 2017

Revised: 25 Aug. 2017

Accepted: 27 Aug. 2017

ePublished: 30 Aug. 2017

Keywords:

Glutathione S-transferase
 GSTM1 null genotype
 Mustard lung
 Polymorphism
 Pulmonary complications

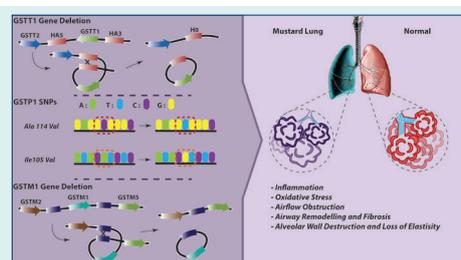
Abstract

Introduction: Glutathione S-transferase (GST) is one of the major detoxifiers in alveoli. Polymorphism in GST genes can influence the ability of individuals to suppress oxidative stress and inflammation. The present study was aimed to explore the hypothesis that the genetic polymorphisms of GST *T1*, *M1* and *P1* are associated with the severity of the mustard lung in the sulfur mustard-exposed individuals.

Methods: Blood samples were taken from 185 sulfur mustard-exposed and 57 unexposed subjects. According to the stage of the mustard lung, sulfur mustard-exposed patients were categorized in the mild/moderate and severe/very severe groups. A multiplex PCR method was conducted to identify *GSTM1* and *GSTT1* null genotypes. To determine the polymorphisms of *GSTP1* in exon 5 (Ile105Val) and exon 6 (Ala114Val), RFLP-PCR method was performed.

Results: The frequency of *GSTM1* homozygous deletion was significantly higher in the severe/very severe patients compared with the mild/moderate subjects (66.3% vs. 48%, $P=0.013$). The *GSTM1* null genotype was associated with the severity of mustard lung (adjusted odds ratio [OR], 2.257; 95% CI, 1.219-4.180). There was no significant association between *GSTT1* and *GSTP1* polymorphisms with the severity of the mustard lung.

Conclusion: The different distribution of *GSTM1* null genotype in severe/very severe and mild/moderate groups indicated that the severity of the mustard lung might be associated with the genetic polymorphism(s).



Introduction

Sulfur mustard or 1-chloro-2-(2-chloroethylsulfanyl) ethane as an alkylating agent can induce a special type of chronic obstructive pulmonary disease (COPD), which is called “mustard lung”.^{1,2} The mustard lung begins with the acute bronchitis and airway hyperreactivity, then continues to form some long-term respiratory complications, which persist throughout the lifetime of most cases. Understanding the molecular mechanism of the mustard lung and the triggers of its severity is thus crucial to restrict the respiratory complications. Individuals exposed to sulfur mustard, develop ongoing lung inflammation and an imbalance in oxidant/antioxidant systems.³⁻⁵ For example, evaluation of

inflammatory chemokines has indicated the elevated level of some acute and chronic inflammatory mediators in these individuals.^{6,7} Inflammation and oxidative stress-related disabilities are associated with genetic variation.⁸⁻¹¹ Thus, the evaluation of genetic variations in patients with different severity of mustard lung may provide helpful evidence to address its underlying molecular mechanism.

Glutathione S-transferases (GSTs) as a detoxifying family of enzymes modulate the addition of glutathione to oxidative stress products. These enzymes are involved in the incidence of oxidative stress and inflammatory-related diseases for various reasons. Firstly, several GST genes are polymorphic and their allelic variations can affect disease susceptibility.¹²⁻¹⁴ Secondly, they detoxify a wide range



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of structurally different substrates produced through oxidative stress.¹⁵ For example, peroxides produced from lipids, DNA, and catecholamines are neutralized by GST enzymes.¹⁶ GSTs are also involved in intracellular signal transduction to regulate inflammation and oxidative stress signaling pathways.^{17,18}

According to their genetic sequences and amino acid content, the mammalian GSTs have been classified into seven distinct classes. These classes and their OMIM accession number are as follows: alpha (e.g., GSTA1; 138359), mu (GSTM1; 138350), kappa (GSK1; 602321), theta (e.g., GSTT1; 600436), pi (GSTP1; 134660), omega (GSTO2; 605482), and zeta (GSTZ1; 603758). The main contribution of this study is to evaluate the association between the severity of mustard lung and some of GST polymorphisms. The studied variations were *GSTM1* null genotype (GenBank accession no. X68676), *GSTT1* null genotype (GenBank accession no. AP000351) and *GSTP1* single nucleotide polymorphisms (SNPs) including Ile105Val (rs1695) and Ala114Val (rs1138272).

Materials and Methods

Materials

All standard chemicals for DNA extraction, including EDTA, NaOH pellets, Tris base, HCl, MgCl₂, Triton X100, sucrose, NaCl, sodium dodecyl sulfate, and sodium perchlorate were purchased from Sigma-Aldrich (Germany). Primers were obtained from Pishgam Biotech Company (Iran). PCR master mix (2X) was provided by Ampliqon (Denmark). GelRed™ Nucleic Acid Gel Stain was purchased from Biotium (USA). Agarose, *Alw26I* and *AciI* restriction enzymes, RFLP reagents, and DNA ladder were obtained from Thermo Scientific (USA).

Study design and participants

The study was performed on 185 male Iranian subjects with long-term respiratory complications 25 years after exposure to sulfur mustard. About 57 unexposed male subjects with mean age of 41.9±9.6 were also used as the control. GOLD spirometric criteria based on FEV1 and FVC was employed to determine the severity of the disease.¹⁹ The sulfur mustard-exposed population was classified in the severe/very severe (83 cases) and the mild/moderate (102 cases) groups based on mustard lung severity. The mean age of the severe/very severe group was 46.2±7.9. There was no significant difference with mild/moderate group (45.6±7.2, *P*=0.59). All participants provided informed consent prior to admission into the study.

Blood collection and DNA extraction

Blood samples were obtained from each participant in fasting state and collected in Na-EDTA coated tubes. DNA isolation was carried out using salting out method.²⁰ The isolated DNA was quantified by measuring absorbance at 260 and 280 nm.

Genetic analysis

GSTM1 and *GSTT1* null genotypes were determined in a single assay by multiplex polymerase chain reaction (PCR) technique.²¹ The forward and reverse primers used were 5'GAACTCCCTGAAAAGCTAAAGC3' and 5' GTTGGGCTCAAATATACGGTGG 3' for *GSTM1* null genotype; 5' TCACCGGATCATGGCCAGCA 3' and 5'TTCCTTACTGGTCCTCACATCTC 3' for *GSTT1* null genotype; 5' GAACTGCCACTTCAGCTGTCT 3' and 5' CAG CTGCATTTGGAAGTGCTC 3' for *CYP1A1* gene. Co-amplification of *CYP1A1* gene was carried out to document successful PCR amplification. PCR was accomplished in a final volume of 20 µL containing 50ng isolated DNA, 10 pmol of each primer, and 10 µL 2X PCR master mix with 1.5 mM MgCl₂. Amplification was conducted as follows: initial denaturation step for 5 minutes at 95°C, followed by 30 cycles at 94°C for 30 seconds, 61.5°C for 30 seconds, 72°C for 30 seconds, and a final extension step at 72°C for 5 minutes. The PCR products from co-amplification of *GSTT1*, *GSTM1* and *CYP1A1* genes were then analyzed by electrophoresis on 2% Agarose gels stained with GelRed™.

In *GSTP1* gene 2 polymorphic sites (Ile105 → Val105; Ala114 → Val114) were assessed. For *GSTP1* 105Val variant, RFLP-PCR was performed according to Harries et al.²² The forward and reverse primers used were 5' ACC CCA GGG CTC TAT GGG AA 3' and 5' TGA GGG CAC AAG AAG CCC CT 3', respectively. In a total volume of 20 µL, isolated DNA (50 ng) was used as an amplification template together with 10pmol of each primer and 10 µL 2X PCR master mix with 1.5 mM MgCl₂. Amplification was carried out with a primary denaturation step at 95°C for 5 minutes, continued with 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and a final extension step at 72°C for 5 minutes. The digestion of PCR product accomplished using 5 units of *Alw26I*, in a total volume of 20 µL. The digested products were analyzed by electrophoresis on 3.5% Agarose gels.

For determining *GSTP1* 114Ala variant, RFLP-PCR was performed according to a method described by Ishii et al.⁹ The forward and reverse primers used were 5' GGGAGCAAGCAGAGGAGAAT 3' and 5' CAGGTTGTAGTCAGCGAAGGAG 3', respectively. In a total volume of 20 µL, isolated DNA (50 ng) was used as an amplification template together with 10pmol of each primer and 10 µL 2X PCR master mix with 1.5 mM MgCl₂. Amplification was carried out with a primary denaturation step at 95°C for 5 minutes, continued with 30 cycles at 95°C for 30 seconds, 62°C for 30 seconds, 72°C for 30 seconds, and a final extension step at 72°C for 5 minutes. The PCR product was digested using 5 units of *AciI* in a total volume of 20 µL. The digested products were analyzed using electrophoresis on 3.5% Agarose gels.

Statistical analysis

The chi-square test was used to explore the distribution

of genotypes in 3 groups. Furthermore, the odds ratio was calculated between mild/moderate and severe/very severe groups to determine the risk of mustard lung severity in different genotypes. PFT parameters were compared between study groups with the *t* test. Data are expressed as the mean \pm standard deviation (SD) and the frequency (percentage). All the statistical analyses were performed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA).

Results

The demographic characteristics of patients with severe/very severe and mild/moderate symptoms are shown in Table 1.

GSTM1 and *GSTT1* gene polymorphisms

GSTM1 and *GSTT1* genotypes were detected by the presence or absence of a band at 480 bp (corresponding to *GSTT1* gene) and a band at 215 bp (corresponding to *GSTM1* gene). A band at 312 bp (corresponding to *CYP1A1* gene) was used as an internal control (Fig. 1).

The distributions of *GST* genotypes were explored to evaluate the probable association between genetic variation in *GST* genes and the severity of pulmonary complications. In the case of *GSTM1*, the frequency of homozygous null genotype in severe/very severe patients was 66.3% in comparison with mild/moderate individuals (48%) and unexposed subjects (57.9%), ($P = 0.044$), (Table 2).

Comparing the 2 sulfur mustard-exposed groups for *GSTM1* polymorphism indicated that the carriers of homozygous null genotype were more likely to develop the severe and very severe form of pulmonary complications ($P = 0.013$; adjusted odds ratio [OR], 2.257; 95% CI, 1.219-4.180), (Table 2). The association between *GSTM1* null genotype and the presence of symptoms like a chronic cough and sputum was also explored. However, there was no statistical association between *GSTM1* null genotype with sputum ($P = 0.793$) and chronic cough ($P = 0.717$), (Table 3).

The frequencies of *GSTT1*-null genotype in severe/very severe, mild/moderate and unexposed groups were 18.1%, 24.5%, and 17.5%, respectively. Although there

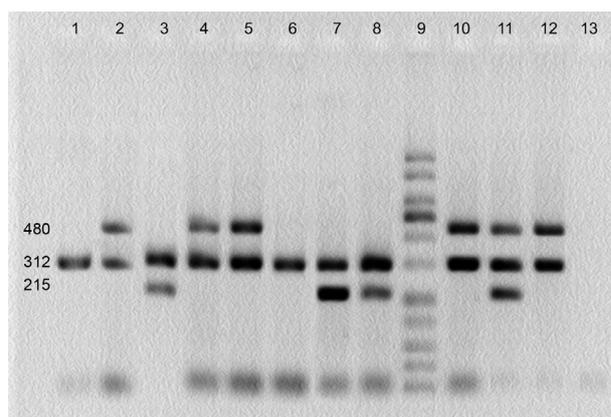


Fig. 1. Multiplex PCR products of *GSTM1* and *GSTT1* gene polymorphisms on agarose gel. Bands at 480, 215 and 312 bp correspond to *GSTT1*, *GSTM1* and *CYP1A1* genes, respectively. Lanes 1 and 6: *GSTM1* and *GSTT1* simultaneous homozygous deletion; lanes 2, 4, 5, 10 and 12: *GSTM1* homozygous deletion; lanes 3, 7 and 8: *GSTT1* homozygous deletion; lane 9: molecular weight ladder; lane 13: negative control.

was a 6.5 percent statistical difference between 2 exposed groups, it was not significant (adjusted OR, 0.676; 95% CI, 0.322-1.419). A combinational study of *GSTM1*- and *GSTT1*-null genotypes was also conducted. Although the association among 3 groups in the combinational study was of borderline statistical significance ($P = 0.069$), the comparison of severe/very severe subjects with mild/moderate patients indicated the elevated risk of pulmonary complications (adjusted OR: 2.058, when 95% CI, 1.099-3.953, $P = 0.033$), (Table 2).

GSTP1 gene polymorphisms

The Ile105Val transition occurs within the exon 5 of *GSTP1* gene. The digestion of PCR product determined 3 different genotypes in this region (Ile/Ile, Ile/Val and Val/Val). The presence of a single 176 bp band indicates the wild-type genotype (Ile/Ile), whereas the conversion of Ile to Val results in 3 bands of 176, 91 and 85 bp in heterozygotes and 2 bands of 91 and 85 bp in homozygotes (Fig. 2). Statistical differences among these genotypes were negligible. For example, the percentage of Ile/Ile

Table 1. Clinical characteristics and age distribution in sulfur mustard-exposed patients

		Pulmonary Assessment (GOLD)				P Value
		Mild/Moderate		Severe/Very Severe		
		No.	%	No.	%	
Chronic cough	No	5	4.9%	3	3.6%	0.669
	Yes	97	95.1%	80	96.4%	
Sputum	No	14	13.7%	12	14.5%	0.887
	Yes	88	86.3%	71	85.5%	
		Mean	SD	Mean	SD	
Age (y)		45.6	7.2	46.2	7.9	0.59
FVC (%)		89.26	6.08	55.9	5.07	<0.001
FEV1 (%)		59.07	4.05	32.67	4.7	<0.001
FEV1/FVC (%)		66.5	6.6	57	9.7	<0.001

Table 2. Distribution of GSTM1, GSTT1 and GSTP1 genotypic frequencies in sulfur mustard-exposed patients and non-exposed individuals

Gene polymorphisms	Unexposed		Mild-moderate		Severe-very severe		P^a	P^b	P^c	P^d	P^e	OR ^f	AOR ^f	95% CI	
	No.	%	No.	%	No.	%									
GSTM1 genotype	-/-	33	57.9	49	48.0	55	66.3	0.044 [§]	0.233	0.314	0.823	0.013 [§]	2.125 [§]	2.257 [§]	1.219-4.180 [§]
	-/+ or +/+	24	42.1	53	52.0	28	33.7								
GSTT1 genotype	-/-	10	17.5	25	24.5	15	18.1	0.450	0.39	0.936	0.506	0.290	0.679	0.676	0.322-1.419
	-/+ or +/+	47	82.5	77	75.5	68	81.9								
GSTP1 Ile105Val	II	24	43.9	42	41.2	39	47	0.896	0.899	0.745	0.871	0.666	0.790	0.695	0.237-2.042
	IV	28	49.1	51	50	36	43.4								
	VV	4	7.0	9	8.8	8	9.6								
GSTP1Ala114Val	AA	50	87.7	86	83.5	68	81.9	0.648	0.474	0.355	0.376	0.778	1.116	0.843	0.388-1.831
	AV	7	12.3	17	16.5	15	18.1								
GSTM1 and GSTT1 combinational genotype	M1-/-and T1-/-	34	59.6	61	59.8	62	74.7	0.069	0.985	0.059	0.344	0.033 [§]	1.98 [§]	2.058 [§]	1.099-3.953 [§]
	other	23	40.4	41	40.2	22	25.3								

Abbreviation: AOR: Adjusted odds ratio adjusted by age.

^a comparison of 3 groups; ^b comparison of mild-moderate individuals with unexposed individuals; ^c comparison of severe-very severe individuals with unexposed individuals; ^d comparison of exposed and unexposed individuals; ^e comparison of severe-very severe individuals with mild-moderate subjects. ^f comparison of severe-very severe individuals with mild-moderate subjects. [§] data show significant differences with $P < 0.05$.

Table 3. Association of GSTM1 genotype with sputum and chronic cough in exposed population

	GSTM1 genotype	Sputum				P value	Chronic Cough				P value
		No		Yes			No		Yes		
		n	%	n	%		n	%	n	%	
-/-	14	53.8	90	56.6	0.793	4	50.0	100	56.5	0.717	
+/- or +/+	12	46.2	69	43.4		4	50.0	77	43.5		

genotype was determined as 47%, 41.2%, and 43.9% for severe/very severe, mild/moderate and unexposed groups, respectively. Consequently, the association between severity of pulmonary complications and the Ile105Val polymorphism was not significant (adjusted OR: 0.695; 95%CI, 0.237- 2.042), (Table 2).

The Ala114Val variation occurs within the exon 6 of GSTP1 gene. The digestion of PCR product revealed 2 genotypes in this region (Ala/Ala and Ala/Val). The wild-type genotype (Ala/Ala) presents 2 recognition sites and creates 3 separated fragments (55, 118 and 247 bp). The conversion of Ala to Val removes one of the recognition sites and consequently, creates 2 fragments (55 and 365 bp) in homozygotes and 4 fragments (55, 118, 247 and 365 bp) in heterozygotes (Fig. 3). Val/Val genotype was absent in this study.

The prevalence of Ala/Ala homozygous genotype in the severe/very severe group was determined 81.9% in comparison with mild/moderate (83.5%) and unexposed groups (87.7%). There was no significant statistical difference between the studied groups (adjusted OR: 0.843; 95% CI, 0.388-1.831, $p = 0.778$), (Table 2).

Discussion

The mustard lung is the main long-term complication in sulfur mustard-exposed individuals. The difference in the

severity of the mustard lung (mild, moderate, severe and very severe symptoms) suggests the involvement of genetic polymorphism in its appearance. To prove such scenario, the evaluation of polymorphism through oxidative stress and inflammation-related genes seems to be necessary. Polymorphisms of GST family play an important role in susceptibility to oxidative stress and inflammation-related disorders such as COPD,⁹ rheumatoid arthritis (RA)¹¹ and multiple sclerosis (MS).¹⁰

GSTM1 and GSTT1 null genotypes were 2 of the selected polymorphisms in this study. Individuals with GSTM1 or GSTT1 deletion represent a complete loss of gene function.^{23, 24} Polymorphisms of these xenobiotic metabolic genes (GSTM1 and GSTT1) induce mutation in several cancers such as breast cancer.²⁵ These genes are also related to detoxification of lipid and DNA hydroperoxides. This is specifically important because sulfur mustard derivatives trigger the formation of lipid peroxidation biomarkers such as malondialdehyde.^{26, 27} In addition, GSTM1 modulates signaling through interaction and inhibition of ASK1 (apoptosis signaling kinase 1).¹⁷ This protective mechanism can preserve cells and is necessary to restrict oxidative stress. ASK1 is a MAP3K and acts through JNK and p38 signaling pathways, which respond to different stresses such as ROS and also regulate inflammation, morphogenesis and apoptosis.¹⁸

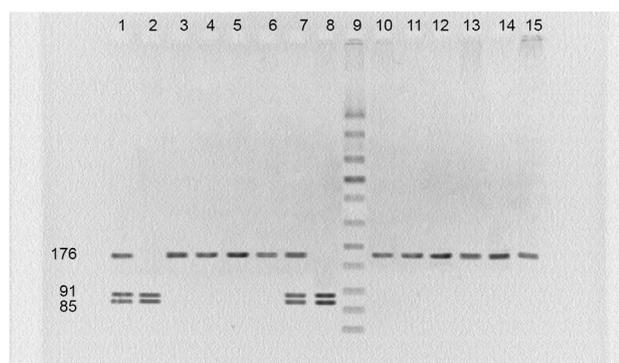


Fig. 2. PCR-RFLP assay for identification of polymorphism at GSTP1 Ile 105 residue. Lanes 3-6 and 10-15: Ile/Ile; lanes 1 and 7: Ile /Val; lanes 2 and 8: Val /Val; lane 9: molecular weight ladder.

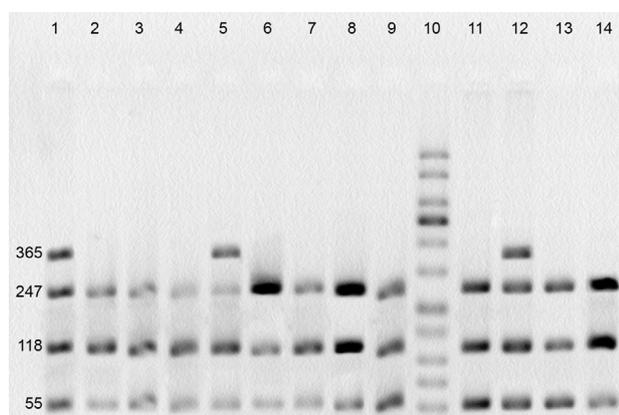


Fig. 3. PCR-RFLP assay for identification of polymorphism at GSTP1 Ala114 position. Lanes 1, 5 and 12: Ala/Val genotype; lanes 2-4, 6-9, 11, 13 and 14: Ala/Ala genotype; lane 10: molecular weight ladder.

Other studied polymorphisms were Ile105Val and Ala114Val within exon 5 and 6 of *GSTP1* gene. Isoleucine to Valine substitution at codon 105 does not change the enzymatic affinity for glutathione.²⁸ However, electrophile substrate and its bulkiness can affect the efficiency of catalytic reaction.²⁹ Thermal stability of the Val 105 variant is also 2.5 fold higher than the Ile 105 variant. The residue 114 (Ala) is located in a position far from the active site, and its variation effects are unclear exactly.

In the current study, obtained results revealed that the *GSTM1* null genotype frequency was 18% higher in severe/very severe patients in comparison with mild/moderate individuals. The frequency of *GSTM1* null genotype was determined 58% in unexposed group, which is consistent with other studies in Iranian population.³⁰ The frequencies of homozygous null genotype in severe/very severe patients (66.3%) and mild/moderate population (48%) indicate the different distribution of genotypes in 2 groups compared with standard population and therefore, imply that *GSTM1* genotype affects the severity of mustard lung. Altogether, present study findings were consistent with the results reported by Cheng et al.³¹ According to their

study *GSTM1* deletion is a risk factor for developing the severe form of COPD while evaluated independently or in combination with other genes such as mEPHX.

In the case of *GSTT1*, there was not a remarkable association between gene deletion and the severity of mustard lung (adjusted OR, 0.676; 95% CI, 0.322-1.419). *GSTT1* null genotype frequency in our studied population was in accordance with other studies in Iranian population.³⁰ The combinatorial communication of *GSTT1* and *GSTM1* with the severity of mustard lung was also examined. This analysis showed that a significant association was established between the genotypes and phenotypes. Although the *P* value was on the border of reliability, the simultaneous association of the disease with *GSTM1* and *GSTT1* was controversial. Accordingly, more studies may be required to determine this association with severity of mustard lung.

Genetic variations of *GSTP1* are associated with COPD.⁹ However, Associations between *GSTP1* polymorphisms and the severity of pulmonary complications were doubtful in this study. Determined frequencies for *GSTP1* polymorphisms in normal people were consistent with other studies in Iranian population.³²

One of the important functions of *GSTM1* is to modulate inflammation-related signaling pathways.¹⁷ When the expression of *GSTM1* increases through ASK1 and c-Jun activation, this factor acts as a negative feedback. This function can protect cells from developing oxidative stress and inflammation. As shown, *GSTM1* homozygous null genotype induces the more severe form of the mustard lung that indicates the importance of ASK1 pathway in the patients. In the other words, high values of *GSTM1* deletion may lead to more activation of ASK1 and consequently, the generation of an inflammatory response. Further investigations are needed to determine the impact of the ASK1 pathway on the severity and molecular mechanism of the mustard lung.

Conclusion

In the present study, we have reported the potential association between genetic polymorphism of *GSTM1* with the severity of the mustard lung because the *GSTM1* null genotype was found frequently in sulfur mustard-exposed patients with severe and very severe stage of lung complications. Although mustard gas is the main cause of the disease initiation, the exploration of genetic polymorphism indicated a complex interplay of genes and environment in the mustard lung patients. The examination of ASK1 over-activation is required to provide more information about the molecular mechanisms of long-term complications of sulfur mustard.

Ethical approval

The study was carried out according to the Declaration of Helsinki and on the basis of good clinical practice. All participants provided written informed consent in their native language.³³

Research Highlights

What is current knowledge?

√ Sulfur mustard derivatives induce long-term complications in respiratory system. Inflammatory response, oxidative stress and airway obstruction are recognized as the main symptoms of mustard lung.

What is new here?

√ The severity of mustard lung is affected by GSTM1 gene deletion, which proposes a complicated interplay among genes after exposure to mustard gas.

Competing interests

Authors declare no conflict of interests.

Acknowledgements

This research was a combined effort of Institute of Biochemistry and Biophysics of Tehran University and Immunoregulation Research Center of Shahed University. We would like to express our appreciation of all the individuals who participated in this investigation.

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