Research Paper:
The Association Between the rs1805329 of Rad23B Polymorphism and Severity of Lung Complications of Patients Exposed to Sulfur Mustard in Long Term

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ABSTRACT

Background: During the Iran-Iraq war, the Iraqi regime frequently used sulfur mustard gas as a weapon in various residential areas, including Sardasht, causing multiple and severe long-term destructive effects on all living organisms, including humans. The pulmonary system is among the most essential organs with varying severity involved in sulfur mustard chemotoxicity in the long term. This study aimed to evaluate the effects of Rad23B (rs1805329) polymorphism on the severity of lung complications in patients exposed to SM gas 30 years ago.

Materials and Methods: The study explored 106 severe and 105 mild PBMC patients exposed to SM gas. Furthermore, 47 PBMC samples were added to the study as a non-exposed group (control). Genotyping of rs1805329 was performed using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP).

Results: The results revealed no significant differences in genotype frequencies between the mild, severe, and the unexposed group. Moreover, no statistically significant difference was observed in genotypic frequency in all co-dominant, dominant, recessive, and over-dominant models.

Conclusion: The obtained data indicated that the rs1805329 of Rad23B was not associated with the severity of the lung complications of patients exposed to sulfur mustard 30 years ago.

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1. Introduction

Sulfur Mustard (SM) or bis(2-chloroethyl) Sulfide (HD) is among the chemotoxic agents that cause Biologically destructive effects and is highly persistent in the environment [1]. In World War I, this substance was first used in chemical warfare [2]. Seventy years later, on June 28, 1987, during the Iran-Iraq conflict, one of the border cities of Iran with Iraq called Sardasht was bombarded by sulfur mustard. Until today, the destructive effects of these chemotoxic agents on all living beings in that area continue [3-5].

Examining patients and studies revealed that sulfur mustard causes short-term and long-term complications by affecting the skin, eyes, respiratory system, and other organs [4, 6-8]. The respiratory system is among the most influenced organs in exposure to sulfur mustard patients, and its long-term difficulties threaten patients' health [2]. The altered severity of respiratory system complications in individuals exposed to sulfur mustard gas indicated that genetic variation plays a significant role in these patients [9]. Lately, Single Nucleotide Polymorphisms (SNPs) are genetic variations many researchers have focused on [10, 11].

Besides, sulfur mustard is among the alkylation agents which cause the alkylation of numerous macromolecules, including DNA molecules [12]. Therefore, the DNA repair system is critical in dealing with sulfur mustard cytotoxicity. An essential DNA repair mechanism related to sulfur mustard is Nucleotide Excision Repair (NER) [13]. The NER mechanism consists of three step:1- recognition of base damage, 2- incision of DNA, 3-excision of oligonucleotide fragments [14]. The recognition of the base damage step is the responsibility of XPC along with UV excision repair protein Rad23B (HHR23B) and Centrin 2 (CETN2). In vitro studies highlighted that XPC, in the absence of Rad23B, can proceed with the NER mechanism; however, its presence improves the efficiency of the in vitro reaction. Yet other studies revealed the deletion of HHR23A And HHR23B genes in mice resulted in embryonic lethality [14, 15]. These studies signified that Rad23B is most likely involved in other critical cellular mechanisms besides NER.

The current study aimed to genotype rs1805329 SNP located at Rad23B gene in patients exposed to sulfur mustard 30 years ago with varying degrees of long-term respiratory complication in the Sardasht population.

2. Materials and Methods

This study consists of 211 confirmed sulfur mustard exposed patients with varying degrees of long-term respiratory complications. Thirty-eight unexposed male subjects were also used as the control.

Samples were collected from Sardasht, Iran's border town with Iraq, chemically attacked on June 28, 1987. Whole blood sampling was performed between September and October 2014. The Iranian Foundation of Martyrs and Veterans Affairs approved the history of sulfur mustard exposure.

The pulmonary function of the participants was assessed by spirometry test (Chest 801 Spirometry), according to the American Thoracic Society criteria. The parameters including forced expiratory volume in one second (FEV1%), Forced Vital Capacity (FVC%), and pulmonary specialists recorded FEV1/FVC% ratio, pulmonary auscultation, and symptoms. According to the diagnostic protocol adopted by the Iranian Medical Committee of Veterans and Martyrs Affair Foundation (VMAF) [16], the classification of pulmonary complication severity was performed. VMAF assessment considers abnormal lung sounds like the first entrance gate to this classification. Patients with 65% FEV1 or FVC <80% were predicted to have mild, 50%≤ FEV1 or FVC <65% were predicted to have moderate, and 40%≤ FEV1 or FVC <50% were predicted to have severe lung lesions (Table 1).

Sampling and genomic DNA extraction

A 5mL of whole blood sample was obtained from each study subject and collected in vacuum blood collection tubes coated with EDTA. The whole blood samples were stored at 4C. Genomic DNA was isolated from the whole blood using the salting-out method. The salting-out DNA extraction method was setup and optimized by the Immunoregulation Research Center of Shahed University [11, 17]. The concentration (ng/uL) and purity (260/280 ratio) of the genomic DNA were evaluated by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

Genotyping of Rad23B gene (C/T, rs1805329) was operated by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). The PCR of genomic DNA was performed using the following primer pairs: 5′- GCACCCAGGTCATTTCTAATC -3′ (Forward) and 5′- TTCCGCTTTACCTCCAGAAC -3′ (Reverse). PCR Reaction was Performed in a total of

20 µL as follows: 10 µL TEMPase Hot Start 2x Master Mix (Cat#: A230301, Ampliqon, DENMARK) together with 10pmol of each primer and one µL (~100 ng) of genomic DNA as a DNA template. The thermal cycling conditions were: initial denaturation at 95 °C for 15 min followed by 35 cycles, including denaturation at 95 °C for 30 s, annealing at 63 °C for 30 s and extension at 72 °C for 60 s, with a final extension step at 72 °C for 5 min. The PCR product had 337 bp fragments in length. PCR product was then verified by electrophoresis on 2.5% agarose gels, stained with Safe stain (Cat#: YT0001, Yekta Tajhiz Azma, IR), and visualized using Gel Doc System (Uvitec silver, UK). Then The verified PCR product was digested for 2 hours at 37 °C with 10 units of the restriction enzyme. Eventually, the digestion products were electrophoresed on a 2.5% agarose gel, stained with Safe stain visualized using Gel Doc System. All the PCR reaction and digestion assays were performed on a T100™ Thermal Cycler (Cat#: 1861096, Bio-Rad, USA). For double-checking the obtained results, 10% of randomly selected samples were hecked and confirmed by Sanger sequencing analysis.

Descriptive analysis was conducted in SPSS. The Chi-squared test analyzed categorical data. Hardy-Weinberg Equilibrium (HWE) and Association between genotypes and severity of lung problems were estimated in different genetic models (co-dominant, dominant, recessive, over-dominant, and log-additive) SNPStats website software http://bioinfo.iconcologia.net/snpstats/start.html. Statistical significance was set at $P<0.05$

3. Results

Characteristics of subjects:

Demographic and clinical data obtained from all study participants are listed in Table 2. The research participants consisted of 249 subjects, of whom 105 were mild, 106 were severe, and 38 were controls. The Mean±SD age of the study subjects was 53.31±11.03, 48.62±10.06, and 40.97±9.89 years for mild, severe, and control groups, respectively. Most patients who participated in the study were nonsmokers (80% for the mild & 95.3% for the severe group). Statistical analysis of Respiratory Function Tests results indicated a significant difference ($P<0.02$) between the mild and severe groups. The BMI status and Respiratory Function Tests of each group were well demonstrated in Table 2. The control groups were not questioned about their smoking, Respiratory Function Tests, and BMI status.

The genotypes of rs1805329 SNP were examined using PCR-RFLP and subsequent gel electrophoresis. All Genotypes are identified by band size. Homozygous (C/C) genotype (cut) and homozygous (T/T) genotypes (uncut) were identified at sizes of 256+ 81 bp and 337 bp, respectively; however, the heterozygous genotypes (C/T) were determined with both forms (cut and uncut) (three bands with 256, 81 and 337 bp size). Furthermore, Sequencing of 10% of all samples was performed to confirm gel electrophoresis results. Figure 1 shows the sequencing results of all three different genotypes.

The Allele and genotype distributions of rs1805329 are presented in Table 3. The collected results indicated a similar frequencies frequency of alleles T and C in all groups. The genotype frequencies showed no significant differences between the mid, severe, and unexposed groups, with a P of 0.36, 0.71, 0.73, respectively.

Additionally, no significant difference was observed in genotypic frequency in all Co-dominant, Dominant, Recessive, and over Dominant models.

The Odds Ratios (ORs) and 95% Confidence Intervals (CIs) in all genotypic models showed no significant variation between all study groups (Table 3).

Table 1. The classification criteria according to the diagnostic protocol adopted by the Iranian Medical Committee of VMAF

Diagnostic Category (Lesional Involvement of Lungs)	Spirometry	Physical Exam Findings
Mild	65≤ FEV1 <80 or 65≤ FVC <80	Abnormal lung sounds
Severe	40≤ FEV1 <65 or 40≤ FVC <65	Abnormal lung sounds. May include cyanosis and intercostal retraction; or tracheal stenosis in bronchoscopy

Table 2. Frequency distribution for demographic and clinical characteristics of the study population by divided into mild, severe, and control groups

Variables		No. (%) or Mean±SD		
		Mild Group	Severe Group	Control Group
Smoking	No	84(80.0)	101(95.3)	0(0.0)
	Yes	21(20.0)	5(4.7)	0(0.0)
	Missing	0(0.0)	0(0.0)	38(100.0)
Age Categories	Under 25	0(0.0)	0(0.0)	3(7.9)
	25-35	5(4.8)	3(2.8)	8(21.1)
	35-45	23(21.9)	51(48.1)	11(28.9)
	45-55	30(28.6)	24(22.6)	16(42.1)
	55-65	32(30.5)	21(19.8)	0(0.0)
	Bigger then 65	15(14.3)	6(5.7)	0(0.0)
	Missing	0(0.0)	1(0.9)	0(0.0)
BMI Categories	Underweight	0(0.0)	2(1.9)	0(0.0)
	Normal weight	22(21.0)	9(8.5)	0(0.0)
	Pre-obesity	32(30.5)	10(9.4)	0(0.0)
	Obesity class I	24(22.9)	6(5.7)	0(0.0)
	Obesity class II	3(2.9)	0(0.0)	0(0.0)
	Obesity class III	24(22.9)	0(0.0)	0(0.0)
	Missing	0(0.0)	79(74.5)	38(100.0)
Respiratory Function Test	FVC %	91.09±16.26	59.56±44.10	NA
	FEV1 %	89.94±17.06	40.56±17.08	NA
	FEV1 / FVC %	101.87±15.35	68.71±19.21	NA
Age (y)		53.31±11.03	48.62±10.06	40.97±9.89
BMI (Kg/m^2)		27.85±3.98	26.21±4.85	NA

BMI: Body Mass Index; FVC: Forced Vital Capacity; FEV1: Forced Expiratory Volume in one second.

4. Discussion

Evaluating the long-term complications of patients exposed to sulfur mustard gas 30 years ago shows that Various organs are affected by the toxicity of SM, including the respiratory system, skin, eyes, and so on. The respiratory system is one of the most affected organs in exposure to sulfur mustard gas, and its conditions are directly related to the health status of patients. Furthermore, the cellular and molecular responses of patients exposed to sulfur mustard are varied and genetic-dependent [18, 19].

The current study genotyped rs1805329 SNP located at Rad23B gene (a gene from the NER pathway) in 211 confirmed sulfur mustard exposed patients with varying degrees of long-term respiratory complications (mild & severe) and 38 control individuals. We also investigated the correlation between genotypes and available demographic data. Overall, we observed no significant differences between the mid, severe, and control groups. Furthermore, no correlation was found between available demographic and genotypes data.

This is the first study to evaluate rs1805329 and the severity of lung complications in patients exposed to sulfur mustard. However, similar studies have been performed on the effects of rs1805329 in various diseases, including cancers. Accordingly, a general overview of a comprehensive meta-analysis revealed that the RAD23B 249Val/Val polymorphism might unlikely contribute to the individual susceptibility to cancer risk [20]. However, controversial results are reported in the literature. Shen et al. reported Chinese subjects with lung cancer who possessed one or two copies of the T allele of rs1805329 had a 2-fold increased risk of lung cancer (OR: 5-1.91, 95%CI: 1.12-3.24) [21].

Another study led by Tomoda et al. among the Japanese population on hepatocellular carcinoma in patients with hepatitis C virus suggested that subjects with 2 copies of the C allele of rs1805329 had a 1.79 fold, which increased the risk of cancer hepatocellular carcinoma [22]. Conversely, studies on various diseases in different populations indicated that no C and T alleles are significantly different in the case and control groups [23]. The previ-

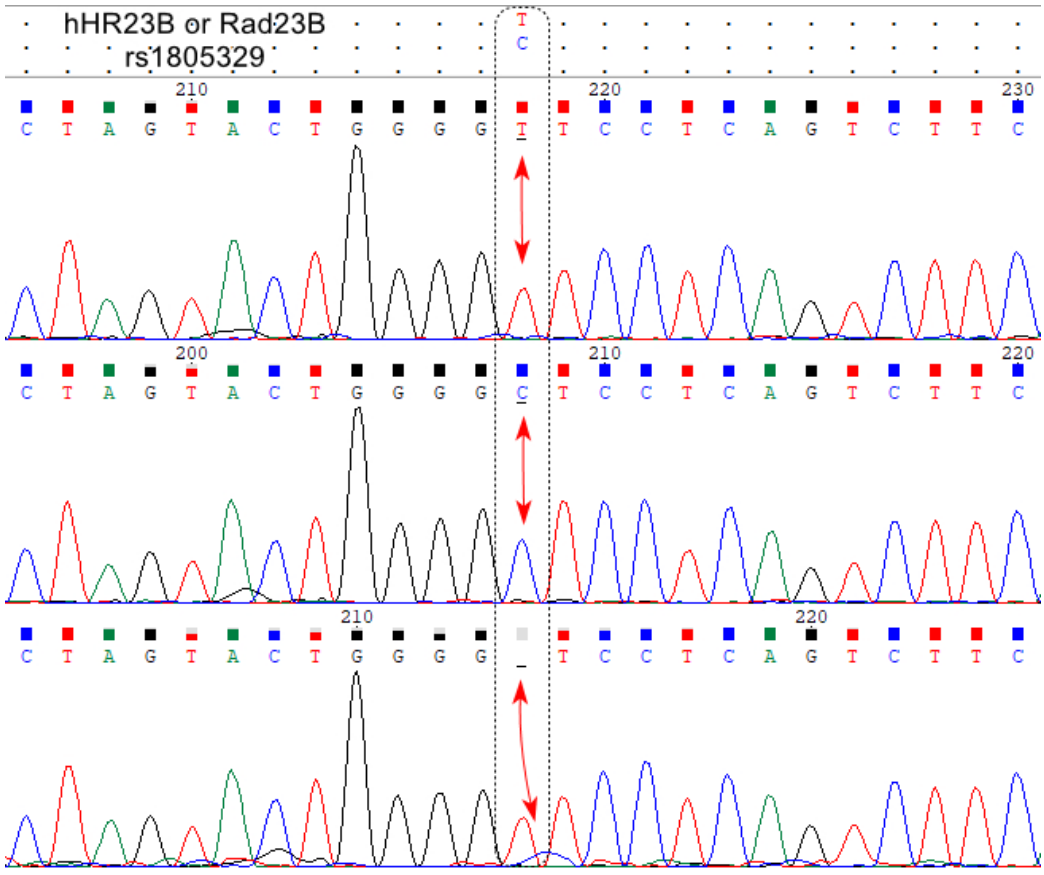

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Figure 1. Sequencing results of all three different genotypes of rs1805329 SNP

Validation of the RFLP for detection of the variant alleles was performed by sequencing. Variant allele T is shown in the first row. Variant allele C is shown in scound row, and the hetero variant (T/C) is shown in the third row.

Table 3. Rad23b allele frequencies

Variables		No. (%)			P^A	OR^A	P^B	OR^B	P^C	OR^C
		Mild	Severe	Unexposed						
Allele	T	106(50)	110(52)	39(51)	-	-	-	-	-	-
	C	104(50)	102(48)	37(49)	-	-	-	-	-	-
Codominant (Genotype)	T/T	2(1.9)	4(3.8)	1(2.6)	0.36	1.00	0.71	1.00	0.73	1.00
	C/T	102(97.1)	102(96.2)	37(97.4)		0.50		1.38		0.69
	C/C	1(1)	0(0)	0(0.0)		0.00		NA		-
Dominant	T/T	2(1.9)	4(3.8)	1(2.6)	0.41	1.00	0.79	1.00	-	-
	C/T-C/C	103(98.1)	102(96.2)	37(97.4)		0.50		1.39	-	-
Recessive	T/T- C/T	104(99)	106(100)	38(100)	0.24	1.00	0.43	1.00	-	-
	C/C	1(1)	0(0)	0(0)		0.50		NA	-	-
Over dominant	T/T- C/C	3(2.9)	4(3.8)	1(2.6)	0.71	1.00	0.94	1.00	-	-
	C/T	102(97.1)	102(96.2)	37(97.4)		0.75		0.92	-	-

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[A]Comparison between mild and severe group; [B]Comparison between mild and unexposed group; [C]Comparison between severe and unexposed group; OR: Odd ratio.

ous studies reflect that the risk allele of rs1805329 can depend on the type of disease and the population studied.

5. Conclusion

Our findings indicated that rs1805329 SNP of Rad23B gene is not associated with the severity of the lung complications of patients exposed to sulfur mustard 30 years ago. Some limitations of this study should be addressed. First, sample sizes were relatively small. Second, more SNPs related to DNA repair genes must be examined. Third, it was challenging to classify patients exposed to sulfur mustard 30 years ago based on pulmonary problems.

Ethical Considerations

Compliance with ethical guidelines

The Bioethics board approved the present study of the Science and Research Branch of the Islamic Azad University (The Bioethics Board Reference Number: IR.IAU.SRB.REC.1398.005). Informed consent was provided before the collection of samples.

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Authors' contributions

All authors equally contributed to preparing this article.

Conflicts of interest

The authors declared no conflict of interest.

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