Iranian Journal of Basic Medical Sciences
Vol. 10, No. 2, Summer 2007, 111 - 117
Received: 17 Jun 2007, Accepted: 10 Sep 2007

New p53 Gene Mutation in non-Cancerous Mustard Gas Exposed Lung

*[1]A. Karami, [2] F. Biramijamal, [3] M. Ghanei, [4]S. Arjmand, [5]M. Eshraghi, [6]A. Khalilpoor

Abstract

Objective
Mustard gas (MG) is a poisoning chemical, mutagenic and carcinogenic alkylating agent. It is used during World War I and also Iran-Iraq conflict. The p53 tumor suppressor gene is involved in the pathogenesis of malignant disease. The aim of this study is to determine possible mutation in p53 gene of lung sample from mustard gas exposed patients.
Material and Methods
Twelve lung biopsy samples from 12 Mustard Gas exposed soldiers cases along with control cell line were studied for the presence of mutations in exons 4-9 of the p53 gene by PCR and direct sequencing.
Results
Among examined biopsies most of the samples demonstrated normal polymorphism with no significant defected mutations but in one sample one type of p53 gene alteration at codon 278 (CCT→CCA) on transcribed strand was detected. This Mutation has not been observed in another studies related to mustard gas exposure and p53 mutation databases.
Conclusion
In this study we have reported for the first time new p53 mutation in the lung sample of MG exposed patients. It is concluded that only one silent mutation were scanned with no signs of any type of cancer. This type of mutation was not in IARC p53 gene mutation database. Moreover, surrounding sequences of the mutated p53 gene codons have more 5'-GT and 5-GC sequences which have been found both by our study and only one another study on Japanese exposed to MG.

Keywords: Lung biopsy, Mustard Gas, p53 mutation, PCR, Sequencing.

1, 4, 6 - Research Center of Molecular Biology, Baqiyatallah Medical Sciences University Tehran, Iran
*Corresponding author: Tel: +98-21- 88039883; Fax: +98-21- 88057023; email: Alikarami1@yahoo.com
2- National Institute of Genetic Engineering and Biotechnology, Tehran, Iran
3- Research Center of Chemical Injuries, Baqiyatallah Medical Science University, Tehran, Iran
5- Manitoba Institute of Cell Biology, Cancer Care Manitoba, Winnipeg, MB, Canada

Introduction

Mustard gas (MG) is a poisoning chemical, mutagenic and carcinogenic alkylating agent (1-4). It is used during World War I and Iran-Iraq conflict (5-11). Besides the vast acute casualties, frequent chemical attacks resulted in chronic illness and disabilities among survival people (10, 12). The lung is one of the most exposable organs to MG (13). Sulfur Mustard inhalation leads to different respiratory complications (14, 15). Exposure to sulphur mustard gas may have extensive immediate effects on the respiratory system but, long-term effects are far less known (16). Lung injury is common after inhalation of this gas and leads to chronic bronchitis and interstitial lung diseases. A large number of the victims present with signs of chronic lung disease long after their exposure to mustard gas (10).

It was shown that p53 gene activated in the response to MG exposure in the lung tissue (17). p53, the tumor suppressor gene modulates apoptosis via trans-activating number genes which therefore inhibits the alteration of genetic material (18).

It is showed previously that MG-exposed cases had p53 gene mutations. So, in this study we looked for p53 gene mutations among lung biopsies taken from MG exposed soldiers.

Materials and Methods

Subjects

Twelve Iranian soldiers exposed to Mustard Gas (MG) with mean age of 39.4, range 35 to 52 years old were selected for the study. They had single exposure to MG during Iraq-Iran war 16 years ago. Patients met all three criteria: 1, all of them had documentation of chemical exposure by military health services at the time of exposure. Patients had been transferred to local military hospitals. Chemical reaction kits had been used to confirm the presence of Mustard at the time of exposure. A previously validated questionnaire was used to identify the patients so exposed. 2, respiratory symptoms have begun immediately after exposure to MG and continuation with no symptom-free period. 3, radiographic evidence of expiratory air trapping >25% and mosaic parenchymal attenuation were found. They did not have history of smoking, history of lung disease before exposure to MG, any occupational history of toxic fume exposure or occupational risk factors that could lead to lung disease, any associated chronic disease (such as heart failure or connective tissue disease) with potential pulmonary involvement, and history of treatment with drugs that may cause acute pneumonitis or ILD as a side effect.

Pulmonary function test (PFT)

We evaluated lung function of all patients by spirometry using American Thoracic Society criteria. We measured forced vital capacity (FVC) and forced expiratory volume in 1 second (FEV1) under the direction of physicians. TLC (Total lung capacity) and RV (Resived volume) were measured by the helium dilution method with a Master Screen apparatus (Jaeger, Hochberg, Germany), and diffusing capacity of the lung for carbon monoxide (DL_{CO}) by the single breath-holding helium dilution method, using an infrared analyzer (SensorMedics Corporation, Anaheim, California, USA, Vmax software version 04-4), which utilizes methane as inert tracer gas.

The patients were seated with a nose clip in place and were asked to perform at least three forced expiratory maneuvers. They were told to continue to blow maximally until they felt there was no air to expel. Both the patients and the technician received visual feedback from a monitor during the test, which was repeated until three technically satisfactory curves with reproducible contour were obtained. All the

indices used for the analysis were derived from the same maneuver, which was the one with the largest FVC.

High resolution CT scan

High resolution CT (HRCT) images were performed on an electron beam CT machine. All 1.5 mm sections obtained at full inspiration with the patient in both supine and prone position. In all cases additional expiratory films and prone sections were taken. Images were photographed at window settings appropriate for viewing the lung parenchyma (–500 HU centre, 1500 HU width). The scans, together with plain chest radiographs, were assessed by a pulmonary radiologist. The presence or absence of patchy ground glass pattern, reticular pattern, emphysema, honeycombing, airways distortion, and bronchial wall thickening scans were recorded.

Bronchial Lung Biopsy

All 12 patients underwent bronchoscopy followed by biopsies of the respiratory lung parenchyma. Biopsy specimens were obtained from all three lobes on the right side or from the upper and lower lobes of the left lung. But the site of biopsies could not be exactly matched with chest HRCT findings site. The biopsies were processed, sectioned, and stained with hematoxylin and eosin, Masson's trichrome, and elastic-van Gieson stains.

DNA extraction

Genomic DNA was prepared from lung biopsies by high pure PCR template preparation kit (Roche Diagnostics GmbH Mannheim, Germany).

Polymerase chain reaction and sequencing

Exons 4-9 of the p53 gene were PCR-amplified independently by a single 40-cycle PCR using 27-30-mer primer by Biramijamal *et al.* (19). PCR cycles comprising denaturation, annealing and elongation at 95^{o}C for 1 min and 60^{o}C for 1 min and 72^{o}C for 3 min were carried out. The PCR products were sequenced directly by BigDye fluorescent dye dideoxy sequencing and microcapillary electrophoresis with an ABI 310 Genetic Analyzer. All samples were verified by two independent cycle sequencing PCR reactions and analysis of both DNA strands both produced the same results. They were sequenced directly by BigDye fluorescent dye dideoxy sequencing with an ABI 310 Analyzer. All samples were verified by two independent cycle sequencing reactions and analysis of both DNA strands both produced the same results.

Table 1. Sequence of primers used in this study.

Exon 4:	F	5'-GTCCTCTGACTGCTCTTTTCACCCATCTAC-3'
	R	5'-GGGATACGGCCAGGCATTGAAGTCTC-3'
Exon 5:	F	5'-CTTGTGCCCTGACTTTCAACTCTGTCTC-3'
	R	5'-TGGGCAACCAGCCCTGTCGTCTCTCCA-3'
Exon 6:	F	5'-CCAGGCCTCTGATTCCTCACTGATTGCTC-3'
	R	5'-GCCACTGACAACCACCCTTAACCCCTC-3'
Exon 7:	F	5'-GCCTCATCTTGGGCCTGTGTTATCTCC-3'
	R	5'-GGCCAGTGTGCAGGGTGGCAAGTGGCTC-3'
Exon 8:	F	5'-GTAGACCTGATTTCCTTACTGCCTCTTGC-3'
	R	5'-ATAACTGCACCCTTGGTCTCCTCCACCGC-3'
Exon 9:	F	5'- TTGGGAGTAGATGGAGCCTG-3'
	R	5'-ACTTGATAAGACGTCCCAAG-3'

Results

These patients diagnosed as bronchiolitis obliterans, based on clinical manifestations, PFT, HRCT, and histopathologic findings (20). All procedures were approved by the Research Center of Chemical Injured, Baqiyatallah University, National Institute for Genetic Engineering & Biotechnology (NIGEB) and local Ethical Committee (letter no 458, 21 Feb 2006) on Human Experimentation. Molecular analysis showed only one case modification, in exon 8 of the p53 gene in case Ir-MG11 (Table 2).

Table 2. p53 mutation analysis in lung biopsies from Iranian Mustard gas exposures.

No.	Patient code	Age (yr)	FEV1/ FVC	Pathologic Diagnosis	BALF Cytology	Chest HRCT	p53 mutations	Codon	Base	Type
1	IGM-1	40	80.02%	squamous metaplasia	no inflammation	air trapping	no	-	-	-
2	IGM-2	44	79.98%	mild chronic inflammation	no inflammation	normal	no	-	-	-
3	IGM-3	40	75.27%	mild chronic inflammation	no inflammation	air trapping	no	-	-	-
4	IGM-4	40	79.95%	mild chronic inflammation	no inflammation	air trapping	no	-	-	-
5	IGM-5	44	82.83%	squamous metaplasia	mild inflammation	air trapping	no	-	-	-
6	IGM-6	38	76.92%	mild chronic inflammation	no inflammation	air trapping	no	-	-	-
7	IGM-7	46	84.58%	mild chronic inflammation	mild inflammation	trapping	no	-	-	-
8	IGM-8	40	90.54%	acute chronic inflammation	severe inflammation	normal	no	-	-	-
9	IGM-9	40	88.38%	mild chronic inflammation	mild inflammation	normal	no	-	-	-
10	IGM-10	38	80.1%	mild chronic inflammation	no inflammation	normal	no	-	-	-
11	IGM-11	39	88.55%	mild chronic inflammation	no inflammation	air trapping	yes	278	CCT→CCA	Silent
12	IGM-12	41	57.05%	mild chronic inflammation	severe inflammation	air trapping	no	-	-	-

Patient list and mutation data from analysis of cases from Tehran are given in Table 2. Case Ir-MG11 had T: A to A: T transversion at codon 278 (in exon 8) on the transcribed strand, without amino acid change (Figure 1).

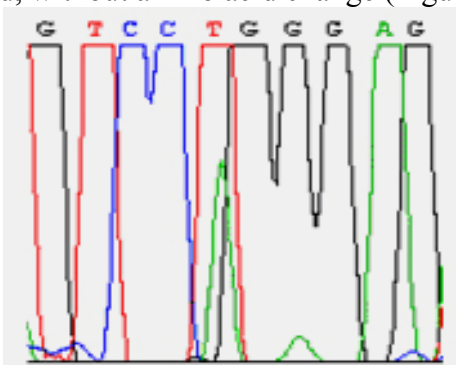

Figure 1. Electropherogram of DNA sequencing (5′ → 3′) showing codon 278 base substitution (CCT→CCA) of p53 gene in MG-exposed lung case.

Consensus nucleotide sequences from PCR products are deposited in the GenBank\EMBL\DDBJ database under accession numbers EF178471, EF178470, EF178469, EF178468, EF178467, EF178466, EF012821, EF012820.

Discussion

Our results showed that MG can modify DNA in one case. Carcinogenicity and mutagenicity of MG have been shown previously. Sulfonium ions are produced by reaction of MG with water, so, these ions can react with DNA and produce DNA adducts in DNA strands. In addition, it is reported that exposure decreases the overall activity of superoxide dismutase (SOD) (21). Also, it was shown that MG analog 2-chloroethyl

ethyl sulphide (CEES) can increase the endogenous production of reactive oxygen species (ROS) in exposed cells (22). So, it is detected that inflammation in lung tissue after exposure to MG are associated with oxidative stress (21). The endogenous production of reactive oxygen species (ROS) increases during oxidative stress which was developed by MG. On the other hand, DNA reactive agents such as ROS can produce different type of DNA alterations including single strand breaks; 8-oxoguanine (8-oxoGua), the most frequently occurring mutagenic base modification, and various other DNA oxidation products. Normally, formation of ROS and the potency of the cellular defense mechanisms lead to basic steady-state level of 8-oxoGua and other oxidation products in DNA. Also, recent studies have shown that the formation of ROS is associated with tumor development in lung tissue which increases the mutation rates of proliferating competent cells in the lung. During presence of excess ROS, p53 tumor suppressor gene can be modified in these cells (23). However, it was reported that p53 is a responsive gene which can also be detected in response to MG exposure in the lung (24). p53 mutations are common missense mutations that exchange amino acids (21). Takeshima and colleagues (1994) showed p53 gene mutations in lung cancer in Japanese workers who exposed to MG. They reported two types of silent p53 gene mutations in exon 7 and also, missense mutations and frame shift mutations among MG-exposed cases. Consistently, our results confirm previous investigation by Takeshima *et al.* too.

In our results, we have reported that there is only one silent mutation for p53 gene in MG shortly exposed soldiers who were scanned after 16 years with no signs of any type of cancer. This type of mutation was not in IARC p53 gene mutation database. Accordingly, the results by Takeshima and colleagues (1994) also is indicative of silent mutations. However the study by Takeshima and colleagues was carried out in Japanese workers who have been exposed to MG for long period and suffered from lung cancer, therefore two types of p53 silent mutations in exon 7, missense mutation and frameshift mutations were seen. Moreover, surrounding sequences of the mutated p53 gene codons have more 5'-GT and 5-GC sequences which have been found both by our study and by Takeshima's results (Table 3).

Table 3. Surrounding squences of cordons with missense mutations including 5'-GG and GT sequences.

a **gt** tcctgca **gt** **ggc** **gg** catgaacc **gg**
Codon 244

tcctga **gt** a **gt** **ggt** aatctact **gg** ga
codon 262

tga **gt** agt **ggt** aat ctact **ggg** ac **gg**
codon 263

tttga **ggt** gc **gt** gtt t **gt** gcct **gt** cct
codon 274

atgac **gg** a **ggt** t **gtg** a **gg** cgctgcccc
codon 173

acc cgc **gt**c cgc gcc at **gg** ccatctac
codon 159

acc atc cac tac aac tacat **gtgt** aac
codon 235

gg catgaacc **gg** agg cccatcctcacc
codon 249

gt tt **gt** gcct **gt** cct **ggg** agagacc **gg**
codon 278

c **gg** a **gg** cccatc ctc accatcatcaca
codon 252

Apparently, this might be due to the preference of solfonium ions to alkylate at 5'-GT and 5-GC sequences. We have suggested that the DNA adducts produced by the MG could occur in TG or GG sequences. This suggestion needs more *in vitro* investigation. Also, we suggest further follow ups for detecting more p53 gene mutations or latent tumor development in lung (8).

Acknowledgements

The author's thanks Dr. P. Tafreshi for her help in this study. This research work was supported by the Baqiyatallah Medical Sciences University and it was as NIGEB project # 244.

References

1. Qabar A, Nelson M, Guzman J, Corun C, Hwang BJ, Steinberg M. Modulation of sulfur mustard induced cell death human epidermal keratinocytes using IL-10 and TNF-alpha. J Biochem Mol Toxicol 2005; 19: 213-25.
2. Kehe K, Szinicz L. Medical aspects of sulphur mustard poisoning. Toxicol 2005; 14: 198-209.
3. Wormser U, Brodsky B, Proscura E, Foley JF, Jones T, Nyska A. Involvement of tumor necrosis factor-alpha in sulfur mustard-induced skin lesion, effect of topical iodine. Arch Toxicol 2005; 79: 660-70.
4. Elsayed NM, Omaye ST. Biochemical changes in mouse lung after subcutaneous injection of the sulfur mustard 2-chloroethyl 4-chlorobuthyl sulfide. Toxicol 2004; 199: 195-206.
5. Mirsadraee M, Attaran D, Boskabady MH, Towhidi M. Airway hyperresponsiveness to methacholine in chemical warfare victims. Respiration 2005; 72: 523 -528.
6. Mahmoudi M, Hefazi M, Rastin M, Balali M. Long-term hematological and immunological complications of sulfur mustard poisoning in Iranian veterans. Int Immunopharmacol 2005**;** 5: 1479-85.
7. Naraghi ZS, Mansouri P, Mortazavi MA. Clinicopathological syudy on acute cutaneous lesions induced by sulfur mustard gas yperite. Eur J Dermatol 2005**;** 15: 140-5.
8. Iyriboz Y. A recent exposure to mustard gas in the United States: clinical findings of a cohort (n=247) 6 years after exposure. Med Gen Med 2004; 6: 4.
9. Ghanei M, Rajaee M, Khateri S, Alaeddini F, Haines D. Assessment of fertility among mustard-exposed residents of Sardasht, Iran: a historical cohort study. Reprod Toxicol 2004; 18: 635-9.
10. Aghanouri R, Ghanei M, Aslani J, Keivani-Amine H, Rastegar F, Karkhane A. Fibrogenic cytokine levels in bronchoalveolar lavage aspirates 15 years after exposure to sulfur mustard. Am J Physiol Lung Cell Mol Physiol 2004; 287: L1160-4.
11. Security Council of the United Nations Document. Reports of Specialists Appointed by the Secretary General to Investigate Allegations by the Islamic Republic of Iran Concerning the Use of Chemical Weapons. New York: 1986; S/16433.
12. Khateri S, Ghanei M, Keshavarz S, Soroush M, Haines D. Incidence of lung, eye, and skin lesions as late complications in 34000 Iranians with wartime exposure to mustard agent. J Occup Environ Med 2003; 45: 1136-43.
13. Balali-Mood M, Hefazi M. The pharmacology, toxicology, and medical treatment of sulphur mustard poisoning. Fundam Clin Pharmacol 2005; 19: 297-315.
14. Ghanei M, Mokhtari M, Mohammad MM, Aslani J. Bronchiolitis obliterans following exposure to sulfur mustard: chest high resolution computed tomography. Eur J Radiol 2004; 52: 164-9.
15. Wormser U, Langenbach R, Peddada S, Sintov A, Brodsky B, Nyska A. Reduced sulfur mustard-induced skin toxicity in cyclooxygenase-2 knockout and celecoxib-treated mice. Toxicol Appl Pharmacol 2004; 200: 40-7.
16. Zang H, Gates KS. Sequence specificity of DNA alkylation by the antitumor natural product leinamycin. Chem Res Toxicol 2003; 16: 1539-46.
17. Ghanei M. Delayed haematological complications of mustard gas. J Appl Toxicol 2004; 24: 493-5.
18. Takeshima Y, Inai K, Bennett WP, Metcalf RA, Welsh JA, Yonehara S, *et al*. P53 mutation in lung cancers from Japanese mustard gas workers. Carcinigenesis 1994; 15: 2075-2079.
19. Biramijamal F, Allameh A, Mirbod P, Groene HJ, Koomagi R, Hollstein M. Unusual profile and high prevalence of p53 mutations in esophageal squamous cell carcinomas from northern Iran. Cancer Research 2001; 61: 3119-3123.

20. Beheshti J, Mark EJ, Akbari H, Aslani J, Ghanei M. Mustard lung secrets long term clinicopathological study following mustard gas exposure. Pathol Res Pract 2006; 202: 739–744.
21. Mukhopadhyay S, Rajaratnam V, Mukherjee S, Smith, M, Das SK. Modulation of the expression of superoxide dismutase gene in lung injury by 2-chloroethyl ethyl sulfide, a mustard analog. J Biochem Mol Toxicol 2006; 2: 142-149.
22. Han S, Espinoza LA, Liao H, Boulares AH, Smulson ME. Protection by antioxidants against toxicity and apoptosis induced by the sulphur mustard analog 2-chloroethylethyl sulphide (CEES) in Jurkat T cells and normal human lymphocytes. Br J Pharmacol 2004; 141: 795-802.
23. Seiler F, Rehn B, Rehn S, Hermann M, Bruch, J. Quartz exposure of the rat lung leads to a linear dose response in inflammation but not in oxidative DNA damage and mutagenicity. Am J Repir Cell Mol Biol 2001; 24: 492-498.
24. Dillman JF, Phillips CS, Dorsch LM, Croxton MD, Hege AI, Sylvester AJ, *et al*. Genome analysis of rodent pulmonary tissue following bis-(2-chloroethyl) sulfide exposure. Chem Res Toxicol 2005; 18: 28-34.