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MUSTARD GAS EXPOSURE AND CARCINOGENESIS OF LUNG

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Abstract

Sulfur mustard (SM), also known as mustard gas, is an alkylating compound used as a chemical weapon in World War I and by Iraqi forces against Iranians and indigenous Iraqi Kurds during the Iran-Iraq War of the 1980s. Although SM is a proven carcinogen there are conflicting views regarding the carcinogenicity of a single exposure. The present study characterizes lung cancers formed in mustard gas victims from the Iran-Iraq War.

Methods and Materials—Demographic information and tumor specimens were collected from 20 Iranian male lung cancer patients with single high-dose SM exposures during the Iran-Iraq war. Formalin fixed, paraffin-embedded lung cancers were analyzed by immunohistochemistry for p53 protein. In addition, DNA was extracted from the tissues, PCR amplified and sequenced to identify mutations in the p53 and KRAS genes associated with SM exposure.

Results—A relatively early age of lung cancer onset (ranging from 28 to 73 with a mean of 48) in mustard gas victims, particularly those in the non-smoking population (mean age of 40.7), may be an indication of a unique etiology for these cancers. Seven of the 20 patients developed lung cancer

before the age of 40. Five of 16 cancers from which DNA sequence data was obtainable provided information on eight p53 mutations (within exons 5–8). These mutations were predominately G to A transitions; a mutation consistent with the DNA lesion caused by SM. Two of the lung cancers had multiple p53 point mutations, similar to results obtained from factory workers chronically exposed to mustard agent. No mutations were detected in the KRAS gene.

Discussion—The distinguishing characteristics of lung carcinogenesis in these mustard gas victims suggest that a single exposure may increase the risk of lung cancer development in some individuals.

Keywords

Mustard Gas; Lung; Cancer; Iran; P53

INTRODUCTION

The alkylating compound sulfur mustard (SM) has been employed as a chemical warfare agent since its first use in World War I (1). The most recent application of this weapon was during the Iran-Iraq conflict by the Iraqi Baathist government. During this war, which lasted from 1980–88, Iraqi dictator Saddam Hussein made frequent use of SM as a battlefield force multiplier and also extensively targeted unprotected civilians in Iran and within the Kurdish regions of Iraq (2). Over 50,000 survivors of SM attacks remain alive in Iran and as a group suffer from high rates of chronic illnesses, particularly inflammatory conditions of the respiratory system, skin and eye (3–5), which are the major target organs of SM. Latent respiratory syndromes include asthma, chronic bronchitis, bronchiectasis, bronchiolitis, bronchial stenosis, and sinusitis (3,4). The low cost and ease with which SM may be manufactured, transported and deployed, along with negligible odor and ability to cause permanent injuries after a few seconds exposure have made it historically the most frequently used chemical weapon during military operations. A particularly insidious feature of SM has also increased its potential as a combat force multiplier: Tissue damage due to the agent typically does not appear for 16 hours or more after exposure. Hence civilians and Iranian troops with often heavy SM exposure, would assume that absence of symptoms after light, or no decontamination meant that the danger had passed. Such victims typically developed symptoms within a day, often full-body blistering, loss of most skin, deep tissue injury and organ failure (2). These characteristics also make future battlefield and terrorist use of this chemical a real threat. Nevertheless, there are few comprehensive studies on the long-term health consequences of SM exposure, a gap in medical knowledge with potential for significantly adverse impact on public health management of future mass casualties.

The enormous pool of Iranian SM victims, with carefully documented exposure and medical histories, therefore offer an unparalleled resource and opportunity for developing a mechanistic understanding of SM-associated chronic health problems. In the present report we examine selected clinical, pathological and genetic features of Iranian SM victims suffering from lung cancer as an element of a broader investigation to assess the potential carcinogenic risk associated with an acute SM exposure.

The long-term effects of SM exposure may develop as a result of two major processes. One pathway leading to chronic illness in SM victims occurs due to a failure of host immunoregulatory mechanisms to resolve inflammation triggered by the episulfonium ion. Here, high levels of inflammatory cytokines inhibit apoptosis of polymorphonuclear leukocytes, particularly neutrophils, thus prolonging their active state and amplifying damage done by these cells (6,7). A second major pathway leading from SM exposure to disease can occur when the compound causes mutations in tumor suppressor and oncogenes, such as p53

or KRAS. SM is a known alkylating agent, and under conditions of chronic exposure, is a recognized carcinogen (8). The mechanism of SM-induced carcinogensis begins with cyclization of SM in the aqueous environment of a victim, to a highly reactive episulfonium ion which may alkylate DNA. If these are not repaired, these lesions can lead to nucleotide substitutions (9), most commonly the G to A transition (10). This mutation may inactivate tumor suppressor genes such as p53, and greatly increase susceptibility to lung cancer, as was observed in Japanese mustard gas factory workers chronically exposed to SM (11–13). Although chronic SM is a known carcinogen (14–17), there are conflicting views regarding the carcinogenicity of a single exposure (18–21). From a public health perspective, acute exposure is a much more relevant exposure scenario in either past and potential future military conflicts or terrorist attacks.

Studies of cancer incidence in survivor populations with single, high-dose SM exposures have thus far failed to demonstrate strong correlations between exposure and disease occurrence. Kang and Bulman followed the outcome of US veterans exposed to SM in World War II (21). Although analysis of these patients showed no significant increase in lung cancer risk, exposure incidences were relatively low and smoking habits were not considered. There is no study in the medical literature addressing the affect of a single, high-dose SM exposure on the long-term risk of lung cancer. Now, after two decades, Iranians with well-documented exposures to mustard agent in the 1980s can be studied to help clarify the lung cancer risk associated with a battlefield exposure to SM.

Multiple lines of evidence will be required before a causative link between acute mustard agent exposure and lung cancer development can be established. Ongoing epidemiological studies are being pursued in Iran to determine if mustard-exposed individuals are at greater risk for lung cancer development, but these efforts will require an extended period of time before a sufficient number of lung cancer cases develop, and may take years to complete. For the present study, we have collected data from Iranian SM victims with unambiguous, documented exposure histories, who eventually developed lung cancer. Records from the Iran-Iraq war also provide information on the precise exposure timeframes during the eight year conflict. Here, specific personal attributes of victims and molecular/genetic features of their cancers were examined to evaluate carcinogenic effect of SM exposure on lung tissue. These studies include a mutational analysis of two tumor suppressor genes: p53 and KRAS, both of which are known indicators of gene-environment interactions impacting cancer risk. In summary, our data support the view that a single exposure to mustard agent may trigger cancer development in some individuals.

METHOD AND MATERIALS

Patient Population

The present study was conducted using medical record data provided by Janbazan Medical and Engineering Research Center (JMERC) and archived paraffin-embedded lung tumor samples from pathology departments of major Iranian medical centers treating persons exposed to chemical weapons during the Iran-Iraq war. Here, data and samples were drawn from a subject population of 20 Iranian males with single, battlefield SM exposures resulting in acute and chronic symptoms including skin and respiratory injuries. All exposures occurred in the years 1982–1988 and subjects were subsequently diagnosed between the ages of 28 and 73 with three major forms of lung carcinoma (Table 1). Subjects were randomly selected from among deceased individuals for whom complete records and samples existed. Time intervals between exposure to the weapon and onset of disease ranged from 5–20 years. Subjects included 5 current or former smokers, 9 non-smokers and 6 with indeterminate histories of tobacco use (Table 1). We were unable to assemble a significant number of matched tumor samples from patients without a history of SM exposure and smoking that were preserved in a manner that

allowed DNA extraction. We therefore used International Agency for Research on Cancer (IARC) database as our baseline for p53 mutational frequencies in lung tumors (8).

Mustard exposure, inclusion and exclusion criteria

Mustard exposure in this study is defined as any contact with SM in liquid or vapor form, resulting in transient or permanent disability. This definition is based on standards developed in a comprehensive national survey accomplished during the timeframe 1997–2000 that established a uniform convention for designation of Iranian citizens with war-related chemical injuries. Under this convention, mustard exposure is defined as any contact with SM in liquid or vapor form, resulting in transient or permanent disability. Here the minimum threshold for SM-induced disability is defined according to known primary effects of the agent on its major target organs: eyes, skin and lungs. Threshold exposure definitions for each organ are as follows: Eye: edema and visible inflammation of ocular membranes; Skin: redness accompanied by obvious blistering; and Lung: edema accompanied by inflammation and either a productive cough, or hemoptysis in the form of bloody streaks or expectoration of clots. These criteria take into account the highly variable length of time and concentration ranges of SM that personnel are typically subjected to under battlefield conditions and make no attempt to correlate SM dosage with symptoms. Some estimation of SM dosage sustained by subjects of this study may nevertheless be estimated based on reference ranges of the agent known to produce particular outcomes. Acute exposure guideline levels (AEGLs) for SM have been developed by the U.S. National Advisory Committee (NAC). Exposure to SM at 0.60 milligrams/cubic meter (mg/m3) of air for 10 minutes; or 0.013 mg/m3 for 8 hours constitute the threshold level at which edema of the eyes, sensitivity to light, and eye irritation occur (22). These ocular symptoms also define the threshold level for SM exposure established by Janbazan organization. Therefore participants in this study were exposed to at least the level of SM identified by the NAC as needed to produce critical ocular symptoms. Patients participating in this study were selected on the basis of documented exposure to SM based on official certification from the Iranian Veteran's Affairs organization (Janbazan). This documentation included records of medical treatment showing the type and extent of mustard-associated injury and/or disability. Patients with histories of serious major disease other than lung cancer were excluded from this study. This investigation was conducted under the approval of Janbazan organization's ethics committee.

Tissue preparation and DNA Extraction

Lung cancer tissue was obtained from biopsies or surgical sample, fixed with formalin and embedded in paraffin. Neoplastic lesions from representative areas of 4–5 unstained slides containing 10 μm thick tissue slices were scraped into a microcentrifuge tube. After paraffin removal with xylene, tissues were rehydrated and the DNA was isolated using the PicoPure™ DNA extraction Kit (Arcturus Engineering Inc., Mountain View, CA), according to the manufacturer's instructions.

DNA Amplification and Sequencing

The extracted DNA was amplified by the polymerase chain reaction (PCR), using a nested primer approach. The primer sets for P53 exons 5, 6, 7 and 8 are shown in Table 2. Initial amplification reactions yielded the target amplimer with a number of off-target products. The specific product was then selectively amplified using nested p53 primers positioned a few bases downstream of the initial primers. The cycling conditions were as following: denaturation at 95°C for 2 minutes, followed by 35 cycles with denaturation at 95°C for 30 seconds, annealing at 56.2°C (exons 5 and 8) or 64.4°C (exons 6 and 7) for 30 seconds, and then elongation at 74° C for 30 seconds. In the last cycle, the elongation step was extended to 10 minutes. Aliquots of the PCR products were examined by electrophoresis on 1.5% agarose gel containing

ethidium bromide to determine whether a specific product was generated by the amplification. PCR products were prepared for sequencing by treatment of an 8 μl aliquot of the PCR reaction with 3 μL ExoSAP-IT (Amersham Biosciences) at 37°C for 15 min, followed by inactivation at 80°C for 15 min. Primer extension sequencing was performed by GENEWIZ, Inc (South Plainfield, NJ) using Applied Biosystems BigDye version 3.1 (Foster City, CA). The reactions were then run on Applied Biosystem's 3730*xl* DNA Analyzer. All PCR products were sequenced in both directions. A double peak on the sequence chromatograph was considered as a potential point mutation, which was then confirmed by comparison to the opposite strand. Mutations were confirmed by re-amplification and single strand sequencing.

The present study also analyzed the KRAS gene for presence of activating point mutations. The oligonucleotide primers used for the amplification of KRAS at codons 12 and 13 were:

Up-stream: 5′-GACTGAATATAAACTTGTGG-3′;

Down-stream: 5′-CTATTGTTGGATCATATTCG-3′.

The cycling conditions were as performed for p53 gene, except the annealing temperature was 55 °C. As indicated in the Results, none of our samples harbored a KRAS mutation.

Immunohistochemistry

Four-micrometer sections of paraffin-embedded tissue were cut and immunostained using the avidin-biotin-peroxidase complex (ABC) method (23). Sections were deparafinnized by baking at 60°C, rinsed with xylene and re-hydrated. Epitope retrieval was performed by boiling in 10 mM citrate buffer (pH 6.0) plus 0.05% Tween 20. Slides were then incubated in blocking solution (25% goat serum, 1% BSA, 0.1% cold fish gelatin, 0.1% Triton X-100, 0.05% Tween 20, 0.05% sodium azide, 10 mM PBS, ph 7.2) for 30 min and incubated in p53 DO-1 antibody (Santa Cruz Biotechnology) at 1:200 in primary antibody dilution buffer (1% BSA, 0.1% cold fish gelatin, 0.05% sodium azide, 10 mM PBS, pH 7.2) at 4°C overnight. Slides were rinsed in washing buffer (10 mM PBS, pH 7.2, 0.05% Tween 20), followed by peroxidase blocking with 3% H_2O_2 for 10 min. Slides were incubated with biotinylated secondary antibody at a 1:500 dilution in secondary antibody dilution buffer (10 mM PBS, pH 7.2, 0.05% sodium azide) for 30 min. Slides were rinsed in washing buffer followed by incubation with HRP-streptavidin (1:500) in HRP-Streptavidin dilution buffer (10 mM PBS, pH 7.2, 0.05% Thimerosal) for 30 min. Detection was carried out using DAB solution as per the manufacturer's instructions. Slides were counterstained with hematoxylin dehydrated and mounted.

RESULTS

Subjects for the present study were 20 males exposed to a single, high dose of SM during military service in the timeframe 1982–88. All subjects were observed to experience acute and chronic symptoms of mustard toxicity including skin injury and respiratory difficulties and were included in this study based on the criteria described in the Methods and Materials. The distribution of subjects according to age, gender, smoking history, time lapse between exposure and lung cancer diagnosis, tumor pathology, p53 IHC and p53 sequencing data is shown in Table 1.

Of the cancers that could be accurately assessed pathologically, the most frequently occurring type of lung cancer was Adenocarcinoma (9/20), followed by Squamous Cell Carcinoma (SCC) (4/20) and Small Cell Carcinoma (3/20). In addition, a single case of mucoepidermal lung cancer was observed in a 33 year old individual. This type of lung cancer is rare, accounting for less than a few percent of all lung cancers.

Examination of the individuals' age at lung cancer diagnosis shows ages ranging from 28 to 73, with seven of the 20 patients developing lung cancer before the age of 40. This finding was of interest, since lung cancer typically develops after the age of 50 and peaks at approximately 60 years of age (24). Overall, the mean age at diagnosis was 47.4 years. On average, the time lapse between SM exposure and lung cancer development was 13.1 years.

Although DNA extraction from lung tumor samples of SM victims was difficult due to variable tissue fixation procedures used in Iran, we were able to obtain information on the p53 mutational status in 18 of the subjects. Mutations identified and described in these studies were determined by comparison of experimental results with reference data of known normal human p53 sequences published in GenBank

Table 1 shows the location and nature of the base change in these subjects, as well as the predicted consequence on the p53 protein. A total of eight mutations were identified. Five of these were G to A transitions, with single A to G, G to T and A to T changes also observed. The frequent G to A base change in these patients matched that most frequently observed mutation in chronically exposed factory workers (11). Interestingly, two of the cases had multiple point mutations (Table 1). Case 3 showed 3 point mutations: one silent mutation in codon 180 and 2 missense mutations in codons 171 and 238. In this case, all silent and missense mutations were G to A transitions. In Case 14, we observed two missense point mutations: G to A and G to T at codons 151 and 245, respectively. As discussed below, multiple p53 point mutations are relatively rare in lung cancer, although one such case was observed in an individual chronically exposed to SM (11). We also evaluated possible mutations in the KRAS gene of these tumors, but did not find any mutations in codons 12 or 13 of this gene. Finally, IHC for the p53 protein was performed on the lung tumor samples. Just over half (13/20) showed expression of p53, which is similar to the frequency described in other lung cancer studies. In a number of instances p53 reactivity was observed in the absence of a detected mutation. This inconsistency may be due to false-positive IHC staining (25). Alternatively, the p53 mutation may lay outside the region analyzed by sequencing. Representative IHC results from tumors taken from subjects 1–5 are shown in Figure 1, with corresponding demographic and genetic data provided in Table 1. Figure 1 also shows an isotype-matched negative control and a colon carcinoma used as a positive control.

DISCUSSION

All subjects of the present study had sustained single, high-dose SM exposures during the Iran-Iraq war and subsequently developed lung cancer. Analysis of tissue samples in the context of each subject's biodata yielded information relevant to characterization of cancer risk and pathogenesis in mustard-exposed populations. Major significant findings included an overall younger age of cancer onset in SM victims than that typically found for lung cancer (Table 1). In addition, p53 mutations in tumors taken from participants in this study included G to A transitions; a mutation consistent with the DNA lesion caused by SM (Table 1). Additionally the presence of double and triple point mutations in the p53 gene was noted in our subjects; an observation that was also made in factory workers chronically exposed to mustard agent (11). The significance of the sequence data must nevertheless be interpreted with caution. G to A transitions in p53 may indeed be triggered by direct SM-DNA reactivity, however there are other etiologic agents that may induce the same mutation that are also associated with lung cancer (26). For example, chronic pulmonary inflammation suffered by SM victims could contribute to a characteristic mutational spectrum independently of direct SM-DNA reactivity. Nonetheless, this study provides insight as to the spectrum of mutations that may be associated with tumors in persons with single, high-dose SM exposure; along with other features of illness in this population, notably early age of cancer onset.

The relatively early age of onset of lung cancer in SM victims may be an indication of a unique etiology for some of these cancers. The range of lung cancer diagnosis in our subjects was from 28 to 73, with seven of the 20 patients developing lung cancer before the age of 40. Lung cancer typically develops after the age of 50, peaks at approximately 60 years of age, and it is unusual among people under 40 (24). Few studies have been published on lung cancer in Iran, but available data indicates that the mean age of lung cancer is higher than 60, while the mean of age among SM victims was 48 (±12) (27). The causal inference was stronger for those who are in their 20s or 30s at the time of diagnosis.

Many studies have shown that mutation of the p53 gene can contribute to lung cancer development, with mutations in this gene found in over half of all lung cancers (28,29). The protein product of the p53 gene is involved in DNA damage response. Consequently, this gene may be a preferred target for environmental carcinogens, which act as DNA damaging agents. Moreover, carcinogens leave molecular fingerprints on the p53 gene. Thus, the study of the p53 mutational spectrum has been a useful approach for implicating suspected carcinogens to different human cancers (30,31). However, at the time of this writing, only one report has been published characterizing p53 mutations in SM-exposed individuals. This is an article describing p53 mutations in a small Japanese Japanese population chronically exposed to SM through work at a factory producing mustard agent. The p53 analysis performed on 12 tumors isolated from Japanese factory workers showed six out of twelve lung tumors had at least one mutation in p53 (within exons 5–8); and two of the twelve tumor samples had double G to A transitions. It was concluded that these unusual double mutations may be characteristic of lung tumors caused by interaction of SM with DNA and potentially reflects the high mutagenic capacity of mustard agent. Interestingly, we likewise observed multiple point mutations in two of our cases (Table 1). Case 3 showed 3 point mutations: one silent mutation in codon 180 and 2 missense mutations in codons 171 and 238. In this case, all silent and missense mutations were G to A transitions. In Case 14, we observed two missense point mutations: G to A and G to T on codons 151 and 245, respectively. Over 2000 p53 mutations have been reported in human lung cancers, and few double mutations have been documented thus far (11,32,33). In a study of 15 atomic-bomb exposed patients with lung adenocarcinoma or squamous cell carcinoma, two patients were found to have double mutations in p53. Development of lung cancer in these patients was reported in association with radiation exposure (34). Hayes *et al*. also reported that an excess risk of lung cancer is associated with chronic exposure to chromate (35), which were also found to carry multiple mutations in the p53 gene (36). In conclusion, multiple point mutations may be attributed to exposure to strong exogenous carcinogens, such as SM.

It is of interest to note that only one of the observed mutations was among the most common mutations reported for p53. Approximately 30% of p53 mutations in the world are in “hot spot” codons 175, 245, 248, 273, and 282. Among p53 mutations in lung cancers world-wide, GC base-pairs are predominantly attacked (78–86%) (32,37,38). Such mutations have been attributed to spontaneous deamination, or the selective carcinogen targeting, of 5-methycytosine (39,40). However, among the 8 mutations detected in SM exposed lung cancer patients, only one transition at CpG site dinucleotides was found. The under-representation of CpG mutations further suggests a unique etiology of lung cancers formed in SM victims.

Notwithstanding the strengths of this and a previous study of individuals chronically exposed to SM, there are a number of concerns and limitations. For example, analysis of additional p53 mutations in SM victims could generate a mutational spectrum more similar to that found in other lung cancers. In addition, our present study cannot provide an estimate of the increased lung cancer risk associated with an acute exposure to SM in the general population, nor can it substitute for epidemiological studies. Since genetic variation is one of the most important factors contributing to the risk development of cancers in humans, an individual’s sensitivity

to SM is likely depend in part on their innate ability to metabolize and detoxify carcinogens and detect and repair DNA lesions. The individuals studied here may represent a highly susceptible subpopulation that might not be present at a high frequency in the general population. The later concern has been a significant motivator for an on going epidemiological study among SM-exposed Iranian populations in a newly developed program called “Study of Surveillance Health Analysis of Mustard Chemical Exposure” (SHALAMCHE) (41). The program in its present form is configured as a prospective and historical cohort study designed to track the health status of Iranian war veterans and civilians registered in the National Warfare Victims Database in Tehran. Since June 2002, 13441 participants (8500 mustard-exposed men and 4941 controls) were enrolled in SHALAMCHE, with substantially more on a weekly basis. Eventually, this program will provide enormous insight into how SM exposure affects multiple health parameters including epidemiology of lung cancer development SM-exposed Iranian population, including the degree to which an acute SM exposure increases the risk of lung cancer development in a general population. The present report represents an initial contribution to the dynamic database that will result from implementation of the SHALAMCHE program.

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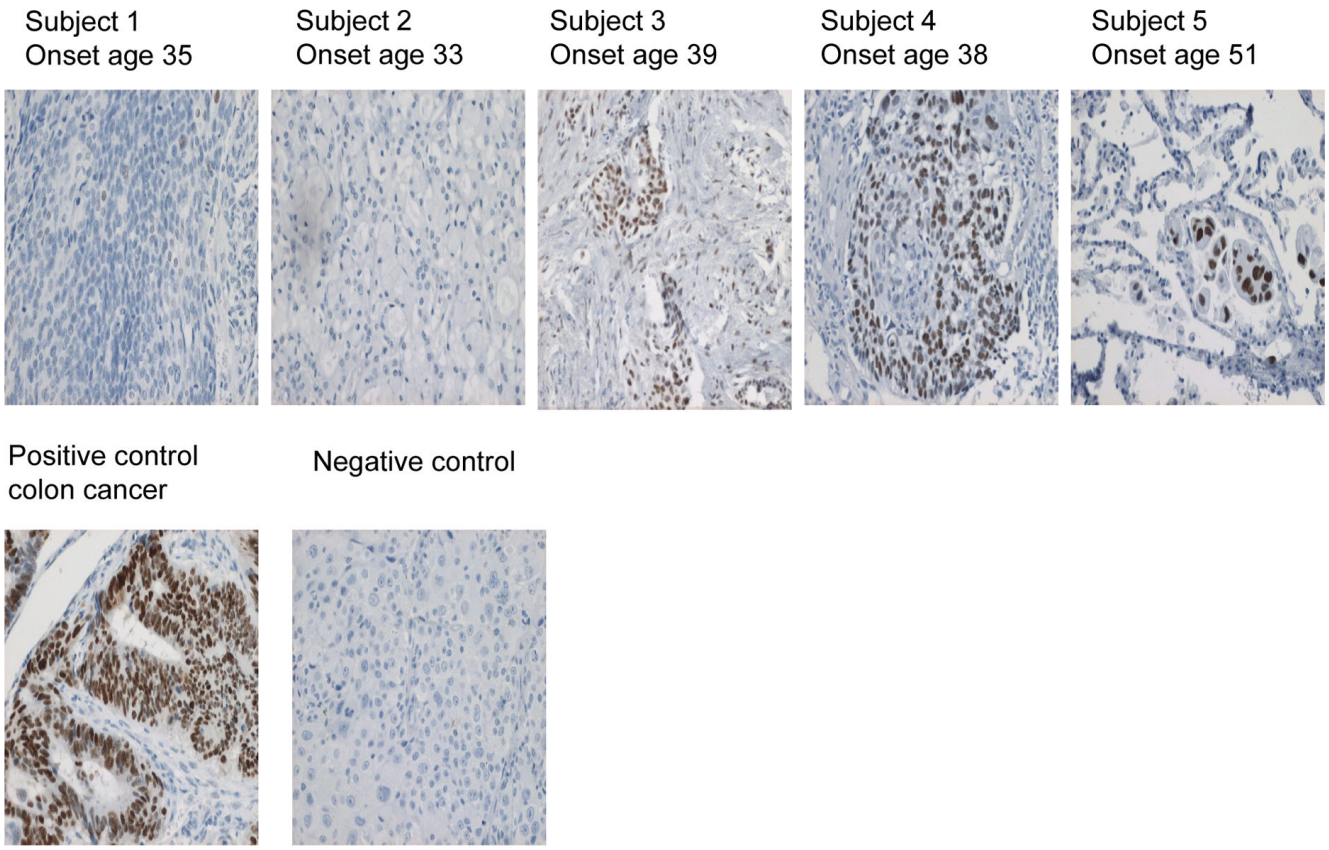

Figure 1.

P53 expression in paraffin-embedded lung tumors. Lung tumor samples were obtained from 5 Iranian males exposed to SM between 1982 and 1988. Tissues were paraffin-embedded, cut into 4 μm sections and analyzed by immunostaining. Visualization of p53 protein expression was accomplished using the p53 DO-1 antibody, with HRP and DAB reagents for detection, and a hematoxylin counterstain. IHC results and age of cancer onset are shown for tumor samples taken from subjects 1–5 shown in Table 1. A section of p53+ colon cancer is included as a positive control; and tissue stained with an isotype-matched control antibody included as a negative control.

Table 1

Biodata, p53 gene expression and mutational spectrum for 20 Iranian males exposed to sulfur mustard during military operations 1982–88.

Subject	Age of onset[a]	Latency interval (years)[b]	Pathology results: tumor classification	Smoking	P53 (IHC)[c]	DNA sequence change[d]	P53 gene exon no.	P53 gene codon change[e]	Predicted phenotypic change[f]
1	35	16	Adeno-carcinoma	-	-	-	-	-	-
2	33	10	Muco-epidermal carcinoma	-	-	GCC→GTC	5	161	Ala→Alasilent)
3	39	9	Small cell carcinoma	-	+	-	-	-	-
4	38	6	Adeno-carcinoma	-	+	GAG→GGG GAG→GAA TGT→TAT	5 5 7	171 180 238	Glu→Gly Glu→Glu (silent) Cys→Tyr
5	51	16	Adeno-carcinoma	-	+	-	-	-	-
6	43	13	Adeno-carcinoma	+	+	-	-	-	-
7	67	11	Adeno-carcinoma	+	_	-	-	-	-
8	55	15	Small cell carcinoma	+	-	-	-	-	-
9	53	5	Small cell carcinoma	Unknown status	+	-			
10	28	7	Small Cell carcinoma	_	+	-	-	-	-
11	73	14	Poorly differentiated. carcinoma	+	+	-	-	-	--
12	47	12	Adeno-carcinoma	Unknown status	_	-	-	-	-
13	40	20	Small cell carcinoma	Unknown status	+	CGC→CAC	5	156	Arg-→His
14	66	17	Small cell carcinoma	+	+	CCC→CTC GGC→GTC	5 7	151 245	Pro→Leu Gly→Val
15	51	14	Adeno-carcinoma	_	+	*			
16	53	18	Poorly dif. Carcinoma	_	+	-	-	-	-
17	54	16	Adenocarcinoma	Unknown status	_	**Exons 5&8***			-
18	38	18	Adenocarcinoma	_	+	**Exons 5&8***			-
19	35	18	Small cell carcinoma	Unknown status	+	GAG→GTG	8	285	Glu→Val
20	49	7	Small cell carcinoma	Unknown status	_	*			-

[a] Age at which cancer diagnosis was made and samples for the present study were collected.

[b] Time interval between mustard exposure and cancer diagnosis (latency).

[c] Detectable expression of p53 protein (immunoreactivity) in tumor samples.

[d] Mutation detected in a particular tumor sample.

[e] Codon number within cDNA p53 sequence used in the present study.

[f] Predicted phenotypic alteration (amino acid change) are shown for formalin-fixed, paraffin-embedded tumor samples taken from each subject.

[*] Samples in which DNA extraction was not possible.

Table 2

Primer sets for PCR amplification of lung tumor tissue p53 exons 5, 6, 7 and 8.

Exon	PCR cycle	Strand	Sequence	Length (bp)	Optimal Annealing Temp (°C)
5	outer	Up-stream	cacttgtgccctgacttt	18	56.2
		Down-Stream	cctggggaccctgggcaa	18	“
	inner	Up-stream	ttgtgccctgactttcaa	18	“
		Down-stream	ggggaccctgggcaacca	18	“
6	outer	Up-stream	cgacagggctggttgcccaggg	22	64.5
		Down-Stream	agggccactgacaaccacc	19	“
	inner	Up-stream	cagggctggttgcccagggtcc	22	“
		Down-Stream	gccactgacaaccaccctta	20	“
7	outer	Up-stream	cttgccacaggtctccccaa	20	64.5
		Down-Stream	aagcagaggctggggcacagcagg	18	“
	inner	Up-stream	gccacaggtctccccaaggc	18	“
		Down-Stream	cagaggctggggcacagcaggcca	22	“
8	outer	Up-stream	taggacctgatttccttactgcct	22	56.2
		Down-Stream	tgaatctgaggcataactgca	19	“
	inner	Up-stream	gacctgatttccttactgcctctt	22	“
		Down-Stream	atctgaggcataactgcaccct	20	“