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No Association Between Simian Virus 40 and Diffuse Malignant Mesothelioma of the Pleura in Iranian Patients: A Molecular and Epidemiologic Case–Control Study of 60 Patients

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Background *Diffuse malignant mesothelioma (DMM) is increasing in incidence on a worldwide basis and is linked to exposure to asbestos. Simian virus 40 (SV40), a DNA virus, was introduced inadvertently to human populations through contaminated polio vaccine during the years 1956–1963. It has been associated with various types of malignancy in animal experiments. There have been suggestions that SV40 might play a role in the pathogenesis of DMM.*

Objective *To evaluate the association between SV40 and DMM in Iranian patients.*

Method *In a case–control study between the years 2007–2008, isolated DNA from 60 paraffin blocks of patients with DMM and 60 controls was assessed to detect three human polyomaviruses (JCV, BKV, and SV40) using three different sets of primers by multiplex nested PCR analysis. We related the patients with diffuse malignant mesotheioma to possible sites of exposure to asbestos.*

Results *None of the DMMs nor any patient in the control group had SV40 genome on polymerase chain reaction (PCR). All of the cases were SV40 T antigen negative.*

Conclusion *This study suggests that DMM is independent of SV40 infection in Iran.*

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KEY WORDS: mesothelioma malignant; PCR; simian virus 40; etiology

INTRODUCTION

Diffuse malignant mesothelioma (DMM) is the most frequent primary malignancy of the pleura [Aoe et al., 2006]. The causative role of asbestos in man has been confirmed, but there is no known exposure to asbestos in some cases. Since only a small proportion of individuals exposed to asbestos develop DMM, and because not all patients with DMM give a history of exposure to asbestos, other factors or co-factors has been suggested as having a role in the etiology of DMM. In recent years there has been speculation about the causative role of Simian virus 40 in human malignancies. This virus entered human populations by contaminated polio virus vaccines in the late 1950s and early 1960s [Kjaerheim et al., 2007], and can infect and transform various types of cells in various animals [Price et al.,

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2007]. Strong oncogenic properties of SV40 were demonstrated in rodents [Simmons, 2000]. Although some studies suggest a contributing role of SV40 to some human cancers, other studies do not. Association between SV40 and brain cancer has been reported in Switzerland in 25–56% of cases [Huang et al., 1999], but cohort studies from other countries have not shown consistent results for an association between SV40 and DMM in humans [Aoe et al., 2006; Jin et al., 2006; Price et al., 2007; Ziegler et al., 2007]. There has been no report on this association from Iran, so we investigated this relation at the National Research Institute for Tuberculosis and Lung Disease, NRITLD, in Tehran, Iran, which sees a large number of patients with DMM.

MATERIALS AND METHODS

Formalin fixed, paraffin-embedded tumoral tissue of 60 patients with a diagnosis of DMM were retrieved from archives of the pathology department of Masih Daneshvari hospital from 1999 to 2008. The diagnosis of DMM was confirmed histologically with invasive tumor in all cases. There was atypical mesothelial proliferation with stromal and fat invasion. Positive immunohistochemical staining with WT1, calretinin, CK 5/6, and HBME-1 and negative staining with CEA, MOC-31, and leu-M1 confirmed the nature of the mesothelial cells. A control group consisted of 60 cases of reactive pleural mesothelial hyperplasia due to empyema or parapneumonic effusion, granulomatous infections, or fibrinous pleuritis, which were matched with cases for age and sex, were studied in a parallel manner. The study protocol was approved by the human subjects review committee of NRITLD. All participants signed written informed consent.

DNA Extraction and PCR

Genomic DNAs from tissue sections were prepared according to the methods described by Impraim et al. [1987]. To avoid contamination of the DNA, care was taken during extraction and PCR (sectioning the blocks to several small groups at different times, using new disposible surgical blades for each sample, and filter tips during extraction and PCR).

The adequacy of the DNA in each specimen for PCR amplification was determined by detection of a 268-bp fragment of the β-globin gene using the GH20/PC04 primer set [Saiki et al., 1986].

For detection of SV40 genome, a sensitive multiplex nested PCR was performed for simultaneous detection and typing of three human polyomaviruses (JCV, BKV, and SV40) [Fedele et al., 1999].

In the first round PCR, the PCR was performed using PM1+ and PM1-primers in a final volume of 50 μl. Each PCR mixture contained 4 mM $MgCl_2$ and 20 pmol of each primers. Amplifications were performed with the following cycling profile: incubation at 95°C for 5 min followed by 40 cycles of 1-min denaturation at 94°C, 1-min annealing at 61°C, and 1 min elongation at 72°C. The last cycle was followed by a final 5-min extension step at 72°C.

In the second round, PCR was performed with BK+, JC+, SV+, and PM2-primers. Two microliters of first PCR product were used as template. Nested PCR was performed in a final volume of 50 μl. Each PCR mixture contained 2 mM $MgCl_2$ and 20 pmol of each inner primers. The cycling conditions were as follows: a 5 min at 95°C that was followed by 30 cycles of 1-min denaturation at 94°C, 1-min annealing at 55°C, and 1-min elongation at 72°C. The last cycle was followed by a final 5-min extension step at 72°C. The positive control; SV40 (135 bp), JCV (189 bp), BKV (353 bp), as well as non-template control (double distilled water) ran in parallel.

Finally, 10 μl of the nested amplificated products were analyzed by electrophoresis on 3% agarose gels with ethidium bromide staining. The PCR products were considered as positive when 353 bp for BKV, 189 bp for JCV, and 135 bp for SV40 were seen.

Statistical Analysis

We used SPSS software version 11.5 for our analyses.

RESULTS

Our patients were 47 males (78.3%) and 13 females (21.7%), with a mean age of 60.6 years (range: 32–80 years). The most common clinical manifestation of the patients was pleural effusion (100%), followed by dyspnea (75%), weight loss (55%), and chest pain (40%).

Fourteen patients (23.3%) had a definite exposure to asbestos when they worked in cement factories. Thirteen cases (21.6), after extensive investigation, were found to have indirect exposure to asbestos [Salehpoor et al., 2010]. The occupational history was obtained by industrial hygiene technician, according to the usual professional standard. We had no evidence of a positive exposure to asbestos in the remaining patients as well as among the control subjects.

Imaging studies, including both chest X-rays and CT scans, showed diffuse pleural thickening and pleural effusion in 78%, localized pleural thickening and effusion in 15%, and pleural effusion in 7% in MM cases.

The methods of obtaining the diagnostic specimens were as follows; needle biopsy (45%), open biopsy (32%), video-assisted thoracoscopy (18%), pleurectomy (2%), and pleuropneumonectomy (3%).

Histologic types of mesotheliomas were as follows: epithelioid, 60%, biphasic, 35%, sarcomatoid 3%, and small cell subtype 2%.

By immunohistochemistry study, 93% of the tumors were positive for calretinin, 80% for HBME1, 77% for WT1, 73% for CK 5/6, and 67% for thrombomodulin. None of the tumors were positive for MOC-31, CEA, or leu-M1.

The control group of 60 cases had 44 males (73.3%) and 16 females (26.7%), with a mean age of 56.2 years (range: 17–80 years). We categorized age groups into $\leq$50 years and >50 years according to the risk of getting vaccination, in which people $\leq$50 years had at least one dose of vaccination, since they are given by the age of starting school (the vaccination policy in Iran has been based on the recommended WHO schedule: 0–2 months, 4 months, 6 months, and booster dose at 18-month and 6 years; Table I).

None of the DMM nor control specimens showed SV40 DNA sequence by multiplex nested PCR analysis (Fig. 1).

DISCUSSION

SV40, a DNA polyomavirus of monkey origin has been shown to have oncogenic activity in animals by binding and inactivating p53 and Rb nuclear proteins. It can produce various tumors including mesothelioma when injected to hamsters. Although some investigators in the United States have reported SV40 DNA present in DMM [Testa et al., 1998], investigators from Switzerland [Ziegler et al., 2007], China [Jin et al., 2006], and Japan [Aoe et al., 2006] have found no relation between virus contamination and DMM.

Some epidemiologic studies have shown an increased rate of DMM in different populations which have received contaminated vaccines [Stratton et al., 2003], while others have not [Engels et al., 2004; Rollison et al., 2004]. Questions have arisen about the accuracy of analytical methods. The primary region of the SV40 genome encodes two oncoproteins, small (tag), and large tumor antigen (Tag) [Manfredi et al., 2005]. The principal role of Tag for tumorigenesis is inactivation of pRb family proteins and tumor suppressor p53. Primers which often are used for identifying SV40 DNA amplify a region of SV40 DNA which is related to Rb binding site on large T antigen and can contain a DNA sequence that exists in many common laboratory plasmid and can cause diagnostic pitfalls [Manfredi et al., 2005]. López-Ríos et al. [2004] used different sets of primer and found that SV40 sequences present in the tumor samples had a deletion specific to plasmid rather than native functional SV40. Using different sets of primer, they found that none of the tumors show a positive reaction with more specific primers. They suggested that differing results might result from a molecular biology laboratory with high risk of plasmid contamination as opposed to a molecular pathology laboratory with little or no plasmid contamination.

A recent report, using a highly sensitive RT-PCR based assay, which is specific for SV40 miRNA and is not found in plasmid, has shown recurrent absence of SV40 miRNAs in DMM [Gee et al., 2010].

In our study, which was performed in a molecular pathology laboratory, we used a sensitive and specific multiplex nested PCR technique that can show one copy of T-Ag sequence in 1,000 cells. We also evaluated BK and JC levels to corroborate the accuracy of our PCR. Accurate analysis of studies based on serological findings showed that positive serum reaction for SV40 in many instances can be caused by cross reactivity with joined viruses like BK and JC [Shah et al., 2004]. By using these two primers in our study in addition to SV40, one can rule out cross reactivity.

Among the different theories for the role of SV40 in DMM, a ''hit and run'' mechanism has been suggested to explain SV40 T antigen in small numbers of tumoral cells. In this theory infected mesothelial cells would be genetically unstable after exposure to SV40 [Mulatero et al.,

TABLE I. Main Characteristic of Cases and Controls, SV40 and Malignant Mesothlioma in Iran

Subject	Cases (n = 60), N (%)	Controls (n = 60), N (%)	*P*-value
Sex			
Male	47 (78.3%)	44 (73.3%)	
Female	13 (21.7%)	16 (26.7%)	0.75
Age (mean ± SD)	60.68 ± 11.5	56.18 ± 13.9	0.056
$\leq$50	11 (18/3%)	17 (28/3%)	
>50	49 (81/7%)	43 (71/7%)	
Diagnostic method			
Needle biopsy	27 (45.0%)	19 (31.7%)	0.13
VATS/open	33 (55.0%)	41 (68.3%)	

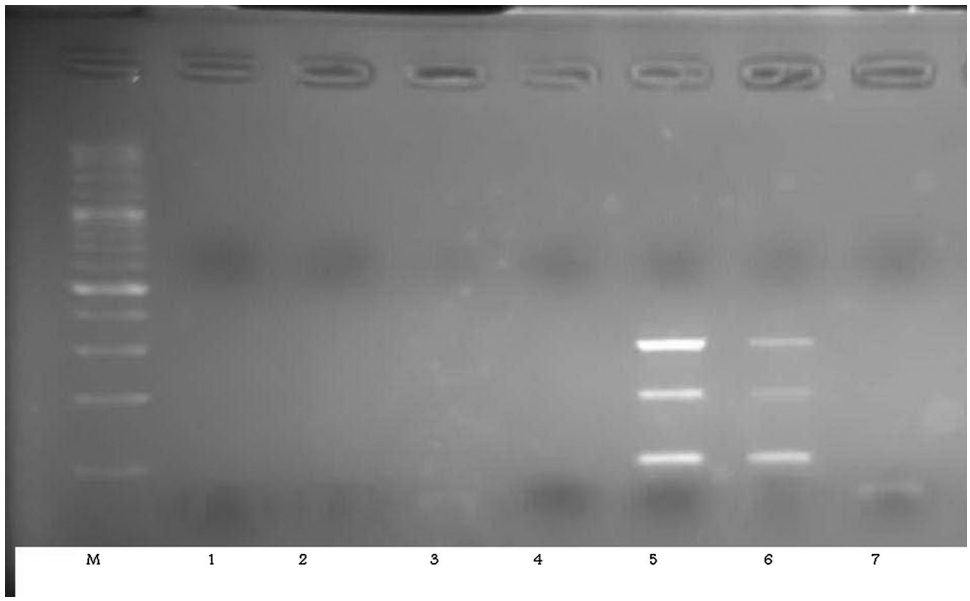

FIGURE 1. Electrophoresis of the multiplex nested PCR products for detection of SV40, BKV, and JCV viruses. Line M: 100 bp ladder marker, Line 1–3: studies samples, Line 4: negative control (human DNA), Line 5 and 6: positive control; SV40 (135 bp), JCV (189 bp), BKV (353 bp), Line 7: nontemplate control (double distilled water).

1999; Rizzo et al., 2001; Hubner and van Marck, 2004]. Other studies have shown a change in phenotype after expression of viral oncoprotein [Ewald et al., 1996; Noble et al., 1996]. Sullivan et al. [2005] reported; miRNA encoded by mutant SV40 is important for this viral infection and they showed that accumulation of miRNA which occur at late time in infection will result in decrease in T antigen without reducing the yield of infectious virus.

The association between DMM and asbestos exposure is established, but not all cases of DMM have a demonstrated history of exposure to asbestos. Fifty-five percent of our cases had no definite or identifiable history of exposure to asbestos even after retrospective investigation [Salehpoor et al., 2010]. The long latency period of the tumor (average 20–40 years) made it difficult to secure information on possible modes of exposure in these patients occurring in prior decades and coming from various part of the country.

Experimental evidence for a co-carcinogenic effect of SV40 was shown in a study where lower amounts of asbestos can produce DMM in animals, in the presence of concomitant SV40 infection [Kroczynska et al., 2006]. It has been suggested that transfected SV40 Tag antigen activates PI3K/AKT signaling and inhibit apoptosis of mesothelial cells when exposed to crocidolite in experimental conditions [Henzi et al., 2009].

In 1973, OPV as a routine vaccination was introduced in the Iranian Expanded Program on Immunization (EPI), and the rate of vaccination reached nearly 100% in 1984. In Iran, for more than 36 years, the MRC-5 diploid cells have been used for propagating and growing the Sabin strains and for producing trivalent OPV vaccine [Mirchamsy et al., 1978; Mirchamsy and Shafyi, 1984]. In comparison with using primary monkey kidney cells (PMKCs) from patas, green monkeys, and rhesus, which are natural inhabitant of various viruses including SV40; it has been shown that OPV vaccines produced in MRC-5 are safer and more effective [Cutrone et al., 2005]. Mass vaccination in Iran began with OPV produced on human diploid cells in which SV40 infection is unlikely. Furthermore, it is noteworthy to mention that vaccine safety manufactured in Iran have been thoroughly investigated by Alirezaie et al. [2011], using phenotypic and genomic analysis of serotype 3 Sabin poliovirus vaccine and the produced vaccine was found to be safe and highly effective. Because our center is a tertiary care center and a national referral center for respiratory disease and thoracic surgery, our patients should be representative of the country. The present study results indicate that our patients were not exposed to contaminated vaccines and that DMM in Iran is independent of SV40 virus in this large controlled human study.

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