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Detection of SV40 DNA Sequences in Malignant Mesothelioma Specimens from the United States, but not from Turkey

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Abstract The incidence of malignant mesothelioma (MM) shows a strong epidemiological association with exposure to asbestos fibers. Recently, simian virus 40 (SV40) DNA sequences have been reported in MM tumor specimens from the United States and several European countries, and the SV40 tumor virus has been implicated as a potential co-factor in the etiology of this disease. However, several large studies from the US, Finland, and Turkey did not detect SV40 sequences in MM samples. To address this discrepancy, MM specimens from Turkey and the US were analyzed in the same laboratory under identical conditions to detect the presence of SV40 DNA. We detected SV40 sequences in 4 of 11 specimens from the United States, but in none of the 9 Turkish samples examined. These findings suggest that geographical differences exist with regard to the involvement of SV40 in human tumors. J. Cell. Biochem. 84: 455–459, 2002. © 2001 Wiley-Liss, Inc.

Key words: malignant mesothelioma; SV40 virus; tumorigenesis; asbestos; epidemiology

Malignant mesotheliomas (MMs) are highly aggressive tumors that originate from mesothelial cells lining the pleural, peritoneal, and pericardial cavities. More than 2,000 MM cases are diagnosed annually in the US, and the incidence of this disease is expected to increase steadily worldwide until at least the year 2020 [Attanoos and Gibbs, 1997]. Exposure to asbestos is known to be a major contributor to the development of this malignancy [Craighead and Mossman, 1982].

MM is relatively common in Turkey, especially in certain villages in rural Cappadocia, where it accounts for approximately 50% of all deaths [Baris et al., 1987]. In some of these regions, erionite, a form of fibrous zeolite, is used as a building stone in the construction of houses. Like asbestos, erionite is a potent carcinogenic mineral fiber that can cause MM [Baris et al., 1996]. However, since not everyone who develops MM is exposed to asbestos or erionite, other co-factors may contribute to the development of this disease in Turkey and elsewhere in the world.

Simian virus 40 (SV40) was found to be a contaminant of poliovirus vaccines that were administered in the US and certain European countries between 1955 and 1963 [Jasani et al., 2001]. SV40 was shown to be oncogenic in hamsters and capable of transforming human cells in vitro [Girardi et al., 1962; Shein and Enders, 1962; Cicala et al., 1993; Butel and Lednicky, 1999]. This led to conjecture that SV40 might play a role in the etiology of some human tumors. Over the last several years, many different laboratories in the US and Europe have

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reported the presence of SV40 DNA in approximately 50% of human MMs [reviewed in [Jasani et al., 2001]]. However, several large studies from the US [Strickler and Goedert, 1998], Finland [Hirvonen et al., 1999], and Turkey [Emri et al., 2000] did not detect SV40 sequences in MMs. An international, multi-institutional study verified the presence and expression of this DNA tumor virus in MM, and it was postulated that SV40 and asbestos could act as co-carcinogens [Testa et al., 1998]. Another multi-center investigation failed to reproducibly detect SV40 sequences in a series of 25 MMs, and it was suggested that further studies were needed to reconcile these results with previous reports of detection of SV40 DNA in tumor specimens [Strickler, 2001].

To address this discrepancy, we investigated for the presence of SV40 DNA, a series of 20 MM specimens from Turkey and the US. All specimens were analyzed in the same laboratory under identical conditions. We detected SV40 sequences in 4 of 11 samples from the US but in none of the nine Turkish tumors examined. The findings presented here suggest that geographical differences exist with regard to the involvement of SV40 in MM.

MATERIALS AND METHODS

Samples and DNA Extraction

Paraffin embedded pleural biopsy specimens from nine Turkish MM patients were obtained from the Sureyyapasa Center for Chest Diseases and Thoracic Surgery, Istanbul. In addition, 11 frozen, O.C.T.-embedded (Tissue-Tex, Elkhart, IN) MM samples were obtained from the University of Pennsylvania Medical Center, Philadelphia, Pennsylvania.

Tumor tissues were cut in 10 μm sections using a microtome. For each sample, three tissue sections were placed into each of three separate microcentrifuge tubes. Paraffin was eliminated by adding 400 μl xylene to each tube, followed by centrifugation for 10 min at 13,000*g* and removal of the supernatant. This procedure was repeated three times, and the pellet was washed with absolute ethanol, 95% ethanol, and 70% ethanol. O.C.T. was removed by washing with 400 μl PBS three times. All samples were dried for 10 min in a vacuum centrifuge, and then 400 μl of 10 mM Tris-HCl (pH 7.5)/1 mM EDTA (pH 8.0) were added to each tube. After incubation with proteinase K (final concentration, 250 μg/ml) and 5% SDS at 55°C, the DNA was extracted by standard methods and resuspended in distilled sterile water. Non-cellular controls were processed in parallel, including all steps of the extraction procedure.

PCR Analyses

The suitability of DNA samples for PCR analysis was tested using a microsatellite marker, D13S1272, which amplifies a product of ~250 bp. The tumor DNAs were screened for the presence of SV40 sequences using two sets of primers, SV5/SV6 and 2573/2902. Primers SV5/SV6 amplify a 172-bp fragment encoding a portion of the Rb-pocket binding domain of SV40 Tag [Carbone et al., 1997a; De Luca et al., 1997]. Primers 2573/2902 amplify a 330-bp fragment corresponding to the carboxyl terminus of Tag [Stewart et al., 1996]. PCR reactions were performed in a final volume of 25 μl in the presence of 2.5 mM $MgCl_2$, 0.5 μM of each primer, 200 ng of genomic DNA, and 1 U of Amplitaq polymerase (Perkin Elmer, Branchburg, NJ). After 3 min of denaturation at 95°C, 45 amplification cycles were performed. Each cycle consisted of 1 min at 94°C, 1 min at 59°C (primers SV5/SV6), or 65°C (primers 2573/2902), and 1 min at 72°C. All steps in the PCR procedure were conducted in parallel with positive and negative controls (see Fig. 1). The positive control consisted of DNA from

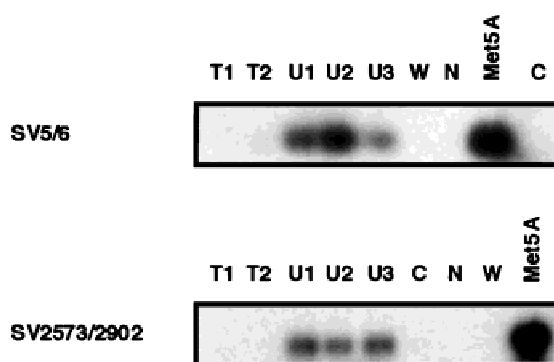

Fig. 1. Hybridization of tumor DNA products amplified by PCR using two different sets of oligonucleotide primers. Data from three of the four SV40-positive cases are shown. T, tumor DNA from Turkish MM; U, tumor DNA from US MM; C, negative control containing no tumor DNA but processed in the same manner as the tumor specimens; N, negative control consisting of DNA from a MM cell line known to be negative for SV40; W, negative PCR control containing water; Met5A, DNA from an SV40-transfected mesothelial cell line used as a positive SV40 control.

SV40-transformed human pleural mesothelial cells (Met5A). The PCR procedures used in our investigation follow those recommended by others as the optimal approach for SV40 detection by PCR [reviewed in Jasani et al., 2001].

Southern Analysis

PCR products were separated on 1% agarose gels and transferred overnight in 0.4 M NaOH (alkaline blotting) to a positively charged nylon membrane (Hybond N+, Amersham). Membranes were then hybridized to ^{32}P-end-labeled oligonucleotides, specific for each of the two SV40 fragments tested [Bergsagel et al., 1992]. Hybridizations were carried out overnight in 5× SSC, 5× Denhardt's solution, 0.5% SDS, and 100 μg/ml of Salmon sperm DNA at 52°C. Filters were washed at a final stringency of 0.5× SSC/ 0.1% SDS and exposed to X-ray film.

RESULTS

A total of 20 MM samples, comprising 9 from Turkey and 11 from the US were investigated for the presence of SV40 by PCR with two different sets of primers. PCR products were transferred to nylon membrane for Southern blot analysis. SV40-positive cases were analyzed further by DNA sequencing to confirm that the amplified sequences corresponded to SV40 and not to other DNA tumor viruses (i.e., BK or JC viruses) which have been found in human tissues [Monini et al., 1995].

None of the Turkish samples was positive for SV40 DNA sequences, whereas 4 of 11 (36%) samples from the US showed PCR products of the expected size with both primer sets in each of the three DNA extractions. The identity of these PCR products was confirmed by Southern blot hybridization (Fig. 1) using SV40 oligoprobes and by DNA sequencing, as described by Rizzo et al. [1998]. Moreover, the DNA sequencing confirmed that the PCR products were identical to the corresponding segment of the wild-type SV40 genome. The carboxyl terminus of Tag in SV40 differs from those found in JC and BK viruses, and our sequence analysis of PCR products corresponding to this region was consistent with SV40 DNA in each case. The incidence of SV40-positive cases in these American and Turkish samples was compared by one-tailed Fisher's exact test. Although the sample sets were small, the results approached statistical significance ($P = 0.068$).

DISCUSSION

Asbestos is a well-documented cause of MM [Craighead and Mossman, 1982; Roggli et al., 1992]. However, approximately 20% of these tumors occur in individuals without a history of asbestos exposure, and less than 10% of people heavily exposed to asbestos will develop this disease [Roggli et al., 1992]. Thus, other factors may contribute to the etiology of some MMs and/ or cause certain individuals to be more susceptible to the carcinogenic effects of asbestos [Roggli et al., 1992; Hirvonen et al., 1996].

One potential co-factor, SV40, induces a very high rate of MM when injected into the pleural space or intracardium of hamsters [Cicala et al., 1993]. The ability of SV40 to transform cells and induce tumor formation is a function of both Tag and a second oncoprotein, small t antigen, which are encoded by the SV40 genome. Laboratory studies have revealed co-immunoprecipitation of SV40 Tag with several tumor suppressor gene products, including p53 and Rb-family proteins, indicating that Tag is biologically active in these tumors and, thus, may contribute to the development of MM [Carbone et al., 1997a; De Luca et al., 1997]. Interestingly, recent investigations have revealed that human mesothelial cells are unusually susceptible to SV40-mediated transformation and asbestos co-carcinogenicity [Bocchetta et al., 2000]. Furthermore, recent studies using a microdissection technique demonstrated that SV40 is present specifically in neoplastic mesothelial cells and not in the surrounding reactive stroma [Shivapurkar et al., 1999].

There have been many reports of SV40 DNA sequences in MMs from the US [reviewed in Butel and Lednicky, 1999] and several European countries [Pepper et al., 1996; Galateau-Salle et al., 1998]. However, several laboratories did not find such DNA sequences in their MM specimens [Strickler and Goedert, 1998; Hirvonen et al., 1999; Emri et al., 2000; Strickler, 2001]. Such interlaboratory variation could reflect undefined technical issues or real geographical differences in the incidence of SV40-associated neoplasms. Interestingly, Hirvonen et al. [1999] did not detect SV40 DNA sequences in Finnish MM specimens. However, the same laboratory observed a high percentage of SV40-positive American cases, consistent with the results obtained by other laboratories participating in a multi-institutional study [Testa

et al., 1998]. These Finnish investigators speculated that the absence of viral sequences in their samples was due to the lack of SV40-contaminated vaccines administered in that country, which may be connected with the relatively low incidence of MM in Finland [Hirvonen et al., 1999]. The observation of a different incidence of SV40 in bone tumors from different regions of the world also suggests geographical differences for SV40 in human neoplasms [Carbone et al., 1997b].

We have demonstrated the presence of SV40 DNA sequences in a subset of MM specimens from the US, but not in those from Turkey. These data are consistent with results of an earlier multi-institutional study of American MM cases [Testa et al., 1998], which found reproducible evidence of SV40 DNA in specimens from the US, and with those from a recent study by Emri et al. [2000], who did not detect SV40 DNA in any of 29 Turkish MMs. The latter investigators indicated that the absence of SV40 sequences in Turkish cases could reflect the fact that polio vaccines were distributed in Turkey after 1970 and, at that time, they were SV40-free.

The incidence of MM is extremely high in some Turkish villages in rural Cappadocia, where it is responsible for approximately 50% of the total deaths. In these villages, erionite exposure is considered to be the main cause of MM, but not all individuals exposed to this carcinogen develop this neoplasm. Pedigree analysis suggests that a genetic factor may be responsible for this unusual susceptibility to the development of MM [Roushdy-Hammady et al., 2001]. In other parts of Turkey, individuals who were thought not to be exposed to asbestos or erionite still developed this malignancy [Baris et al., 1996]. Collectively, the available data indicate that a variety of environmental (asbestos, erionite, and SV40) and genetic factors can contribute to the pathogenesis of MM.

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