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Presence of Simian Virus 40 Sequences in Malignant Mesotheliomas and Mesothelial Cell Proliferations

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Abstract Malignant mesotheliomas (MMs) are pleural-, pericardial-, or peritoneal-based neoplasms usually associated with asbestos exposure. Mesothelial cells are biphasic and may give rise to epithelial and sarcomatous MMs. In addition, benign or atypical proliferations of mesothelial cells may occur in response to many stimuli. There have been recent reports of simian virus 40 (SV40) DNA large T antigen (Tag) sequences in pleural MMs. To further understand the relationship between SV40, MMs, and mesothelial proliferations, we studied 118 MMs from multiple sites in Germany and North America, including 93 epithelial pleural, 14 sarcomatous or mixed pleural MMs, and 11 peritoneal MMs. In 12 pleural MMs, adjacent noninvasive tumor foci were identified and studied separately. Information about asbestos exposure (detailed history and/or microscopic examination for asbestos bodies) was available from 43 German patients. In addition, 13 examples of reactive mesothelium and 20 lung cancers from the United States were tested. DNA was extracted from frozen tumor and adjacent nontumorous tissues or after microdissection of archival formalin-fixed, paraffin-embedded microslides. Two rounds of PCR were performed with primers SVFor 3 and SVRev, which amplify a 105 bp region specific for SV40 Tag. The specificity of the PCR product was confirmed in some cases by sequencing. Our major findings were: 1) Specific SV40 viral sequences were present in 57% of epithelial invasive MMs, of both pleural and peritoneal origin. No significant geographic differences were found, and frozen and paraffin-embedded tissues were equally suitable for analysis. 2) There was no apparent relationship between the presence of SV40 sequences and asbestos exposure. 3) SV40 sequences were present in the surface (noninvasive) components of epithelial MMs. 4) SV40 sequences were not detected in MMs of sarcomatous or mixed histologies. 5) Viral sequences were present in two of 13 samples (15%) of reactive mesothelium. 6) Lung cancers lacked SV40 sequences, as did non-malignant tissues adjacent to MMs. Our findings demonstrate the presence of SV40 sequences in epithelial MMs of pleural and peritoneal origin and their absence in tumors with a sarcomatous component. Viral sequences may be present in reactive and malignant mesothelial cells, but they are absent in adjacent tissues and lung cancers. J. Cell. Biochem. 76:181–188, 1999.

Key words: malignant mesothelioma; SV40; large T antigen; asbestos; lung cancer

Malignant mesothelioma (MM) is a tumor associated with asbestos exposure that originates from the serosal lining of the pleural, peritoneal, or pericardial cavities [Antman et al., 1997]. Mesothelial cells are biphasic and may give rise to both the lining keratin positive epithelial cells and the paucicellular submesothelial layer consisting of a few fibroblasts, collagen, elastin, and other extracellular proteins. During a variety of reactive processes,

Abbreviations used: SV40, simian virus 40; Tag, large T antigen; MM, malignant mesothelioma.

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the mesothelial and submesothelial cells may undergo markedly proliferative and hyperplastic processes. Such hyperplastic reactions, especially when atypical, may be impossible to distinguish from incipient (noninvasive) well-differentiated epithelial mesotheliomas. The biphasic nature of the mesothelial cell results in three major forms of malignant mesothelioma: epithelial, sarcomatous (fibrous), and mixed (biphasic) [Churg, 1998].

Simian virus 40 (SV40) is a well-studied DNA virus of the Papovaviridae family, whose oncogenic potential is associated with one of its early gene products, large T antigen (Tag) [Butel and Lednicky, 1999; Fanning and Knippers, 1992]. Tag is reported to target and to inactivate growth-suppressive proteins such as the retinoblastoma family and p53 [De Luca et al., 1997], leading to transformation of human cells. There have been recent reports of SV40 DNA sequences in malignant mesotheliomas (MMs) of pleural origin [Carbone et al., 1994, 1997; Cristaudo et al., 1995; De Luca et al., 1997; Gibbs et al., 1998; Pepper et al., 1996]. SV40 sequences have also been found in certain human brain and bone tumors [Bergsagel et al., 1992; Butel et al., 1998; Butel and Lednicky, 1999; Carbone et al., 1996; Huang et al., 1999]. The spectrum of SV40-induced tumors in hamsters is similar [Cicala et al., 1993]. The presence of sequences of a simian virus in human tumors is usually attributed to the widespread contamination by SV40 of both the Salk and Sabin poliovirus vaccines and adenovirus vaccines (prepared in monkey cells), which were administered to millions of subjects in many countries from 1957 until 1965 [Carbone et al., 1997]. However, other explanations including sexual transmission have been hypothesized [Mutti et al., 1998].

Although the majority of investigations found SV40 sequences in varying percentages of the MMs analyzed, some groups [Linnainmaa, 1997; Mulatero et al., 1999; Strickler et al., 1996] failed to find such sequences in their specimens. However, a recent multi-institutional study confirmed the presence and expression of SV40 in human malignant MMs in a small number of cases analyzed independently at four laboratories on two continents [Testa et al., 1998]. These studies also suggested that geographic variations exist in the incidences of SV40 sequences in MMs. While both pleural and peritoneal MMs are strongly asbestos associated [Antman et al., 1997], data regarding the relationship between asbestos exposure in patients with MMs and the presence of SV40 sequences in their tumors are scant [Testa et al., 1998].

Because of uncertainties and disputes about the role of SV40 in the pathogenesis of mesotheliomas, we addressed the following questions:

(1) Are specific SV40 viral sequences present in invasive MMs of different histological types arising in different body sites and are there geographic differences?
(2) Are viral sequences present in early noninvasive stages of MM development, or in benign mesothelial proliferations?
(3) Are frozen and paraffin-embedded tissues equally suitable for analysis?
(4) Is there a relationship between the presence of SV40 sequences and asbestos exposure?
(5) Are SV40 sequences present in tissues adjacent to MMs or in lung cancers?

Some of our findings will be published in abbreviated form elsewhere [Shivapurkar et al., unpublished].

MATERIALS AND METHODS

Subjects and Specimens

Samples of pleural, peritoneal, and preinvasive MMs were obtained from German and pleural MMs, benign proliferations, and lung cancers from North American centers (Dallas; Bethesda, Maryland; Detroit; and Vancouver) (Table 1). However, many of the cases were originally referred to these centers from multiple other sites in North America and Germany. While most samples were formalin-fixed and paraffin-embedded, 15 matched pairs of frozen epithelial pleural MMs and adjacent lung tissues were obtained from North American sources. The MMs, mesothelial proliferations, and related tissues were obtained from large surgical biopsies or resected specimens, while the lung cancers were from patients undergoing curative intent resections.

Pathological Diagnoses

Pathologists familiar with mesothelial and lung cancer pathology (Klaus-Michael Muller, Sara Milchgrub, Adi F. Gazdar, and Andrew Churg, who is chairman of the U.S.-Canadian Mesothelioma Reference Panel) interpreted microslides. Epithelial MMs were mainly charac-

TABLE I. SV40 Sequences in Mesotheliomas, Mesothelial Proliferations, Adjacent Tissues, and Lung Cancers

Type of lesion (Source)	No. SV40 positive/ No. tested (%)
All invasive mesotheliomas (All sources)	57/118 (48%)
Epithelial pleural mesotheliomas (All sources)	50/93 (54%)
Epithelial pleural mesotheliomas (Germany)	33/57 (58%)
Epithelial pleural mesotheliomas (North America)	17/36 (47%)
Sarcomatous or mixed pleural mesotheliomas (North America)	0/14 (0%)
Peritoneal mesotheliomas (Germany)	7/11 (64%)
Noninvasive mesotheliomas (Germany)	4/12 (33%)
Reactive mesothelium (9 benign, 5 atypical) (North America)	2/14 (14%)
Tissues adjacent to MMs (All sources)	1/75 (1%)
Lung cancers (8 SCLC, 12 NSCLC) (North America)	0/20 (0%)

SCLC, small cell lung cancers; NSCLC, non–small cell lung cancers.

terized by papillary and tubular forms of growth, although less differentiated forms also occurred [Churg, 1998]. Sarcomatous (fibrous) MMs were composed largely of densely packed spindled cells in a pattern resembling malignant fibrous histiocytoma or fibrosarcoma [Churg, 1998]. The desmoplastic variant of sarcomatous malignant mesotheliomas was diagnosed on the basis of published criteria [Mangano et al., 1998], namely the presence of a paucicellular pattern of spindled cells in a densely fibrotic stroma, accompanied by bland necrosis, unequivocal tissue invasion, or small overtly sarcomatous foci. Rare tumors had mixed epithelial and sarcomatous components.

Analysis of Mesothelioma Specimens for SV40 Tag Sequences

For archival materials, areas of malignant and other cell types were identified by pathological review of all cases. Depending on the extent of the tumor present in the available slides, the malignant areas were either precisely microdissected under microscopic observation [Hung et al., 1995] or scraped with a razor blade. Corresponding normal lung or stromal tissues from the same slides provided a source of control cells. Approximately 500 to 1,000 cells (tumor and control) were microdissected from each case. The dissected cells were digested using the proteinase K method previously described [Hung et al., 1995], and 5 microliters of the DNA samples were used directly for each multiplex PCR reaction. Frozen tissues were finely minced with a disposable scalpel and DNA was extracted from the samples with a DNA extraction kit (Intergen, NY). DNA from COS-7 cells transformed with SV40 [De Luca et al., 1997] (obtained from Dr. Abigail Sowombo, Dallas) was used as a positive control while sterile double-distilled water was used as a negative control.

Two rounds of PCR [Wistuba et al., 1998] were performed to amplify the 105 bp Tag. Primers SVFor 3 and SVRev, which specifically amplify a 105 bp region of SV40 Tag, and not that of any other virus [Carbone et al., 1997; Cristaudo et al., 1995], were used. To avoid contamination, additions of all the reagents in the PCR reactions were carried out within a laminar flow hood, before PCR amplification. The final PCR product was separated on a 3% agarose gel containing 0.05% ethidium bromide. Ethidium bromide–stained bands were visualized, with a UV viewing box and then photographed. To confirm the identity of the 105 bp amplified product, in representative cases the band was isolated and subjected to direct sequencing by using the dideoxy method [Sanger et al., 1977]. All molecular assays involving SV40 analysis were performed in the laboratory of the senior author.

Determination of Asbestos Exposure

Prior exposure to asbestos was determined in 43 MM cases from Germany. A detailed questionnaire was used to determine occupational and nonoccupational exposure. In addition, the number of asbestos bodies present in lungs was determined according to the method of Eitner and Otto [Eitner and Otto, 1984]. In brief, a cube of 1 cm^3 was cut from formalin-fixed, nonatelectatic, non–inflammatory altered, tumor-free lung tissue. The specimens were incubated in 20 ml sodium hypochlorite solution (12%–15%) until the tissue was completely lysed. The lysate was diluted in water and filtered through a Satorius filter. The entire filter was screened for asbestos bodies by light microscopy using high magnification (200–400 ×). The number of asbestos bodies/cm^3 was calculated. Counts

greater than 22/cm^3 were considered as positive for exposure.

On the basis of the findings, cases were classified into asbestos exposure categories (A–E) (Table 2). Category A contained subjects positive for both exposure history and asbestos body count; category B cases had a positive history, but their asbestos body count either was not performed or was negative; category C cases were positive by microscopy, but a detailed history was unavailable; for category D cases no information was available; and category E cases were negative for exposure by both methods.

Statistical Analyses

Fisher's exact two-tailed test was used for statistical evaluation of the differences between SV40 Tag frequencies from two different groups. Probability values of $P<0.05$ were regarded as statistically significant.

RESULTS

Detection of SV40 Tag Sequences in MMs

The presence of SV40 Tag sequences was detected in 57 (48%) of 118 MMs (Table 1). There were no significant differences in the frequencies between pleural MMs from Germany and North America or between pleural and peritoneal MMs. Representative examples of detection of the 105 bp SV40 Tag PCR product are illustrated in Figure 1. In representative cases, DNA sequencing confirmed that the 105 bp amplified product was the precise SV40 Tag sequence as deposited in GenBank (accession number S79053). For epithelial pleural MMs, there were no significant differences between the frequencies in formalin-fixed, paraffin-embedded tissues (44/78, 56%) and fresh frozen tissues (6/15, 40%).

TABLE II. Relationship Between Asbestos Exposure and SV40 Tag Sequences in Pleural Mesotheliomas From Germany*

	Asbestos exposure		
Category	History	Microscopy	SV40 Tag
A	Positive	Positive	18/34 (53%)
B	Positive	Negative or NA	1/7 (14%)
C	NA	Positive	2/2 (100%)
All			21/43 (49%)

NA, not available.

*Note that there were no cases in category E (negative for asbestos exposure by history and microscopy).

Relationship Between SV40 Sequences and Asbestos Exposure

In 43 cases of MMs from Germany (36 males, 7 females), information about asbestos exposure was available. In all cases, evidence of exposure, usually occupational (obtained either by detailed questionnaire, light microscopy, or both) was present (Table 2). In all of these cases evidence of asbestos exposure was available by one or both methods. In 21 (49%) of these 43 cases the presence of SV40 Tag sequences was detected in tumor tissues (Table 2).

Detection of SV40 Tag Sequences in Noninvasive MMs

In 12 pleural MMs from Germany, a noninvasive component of malignant mesothelial cells could be identified lining the pleural surface adjacent to or near the corresponding invasive components. In some of these cases a reactive, non-malignant component was also present. These components were carefully dissected individually, and lack of cross-contamination with other components was confirmed microscopically. In four of these 12 cases SV40 Tag sequences were present in the invasive component. In all four of these cases SV40 sequences were present in the corresponding noninvasive component (100% concordance).

Absence of SV40 Tag Sequences in Sarcomatous Mesotheliomas

We examined 14 MMs with sarcomatous differentiation (seven sarcomatous, five desmoplastic variants of sarcomatous MM) and two mixed histology tumors having a minor epithelial component. All of these tumors were of pleural origin and all were from North American patients. Follow-up information was obtained on all of these patients, and all were either dead of disease or alive with progressive disease, confirming the malignant nature of their disease process. SV40 sequences were absent in all of these cases, and the frequency differences between epithelial pleural MMs (50/93, 54%) and those with sarcomatous differentiation (0/14) were highly significant ($P = 0.0002$).

Detection of SV40 Tag Sequences in Reactive Mesothelial Cells

We examined reactive mesothelial hyperplasias from 13 cases (eight pleural and five perito-

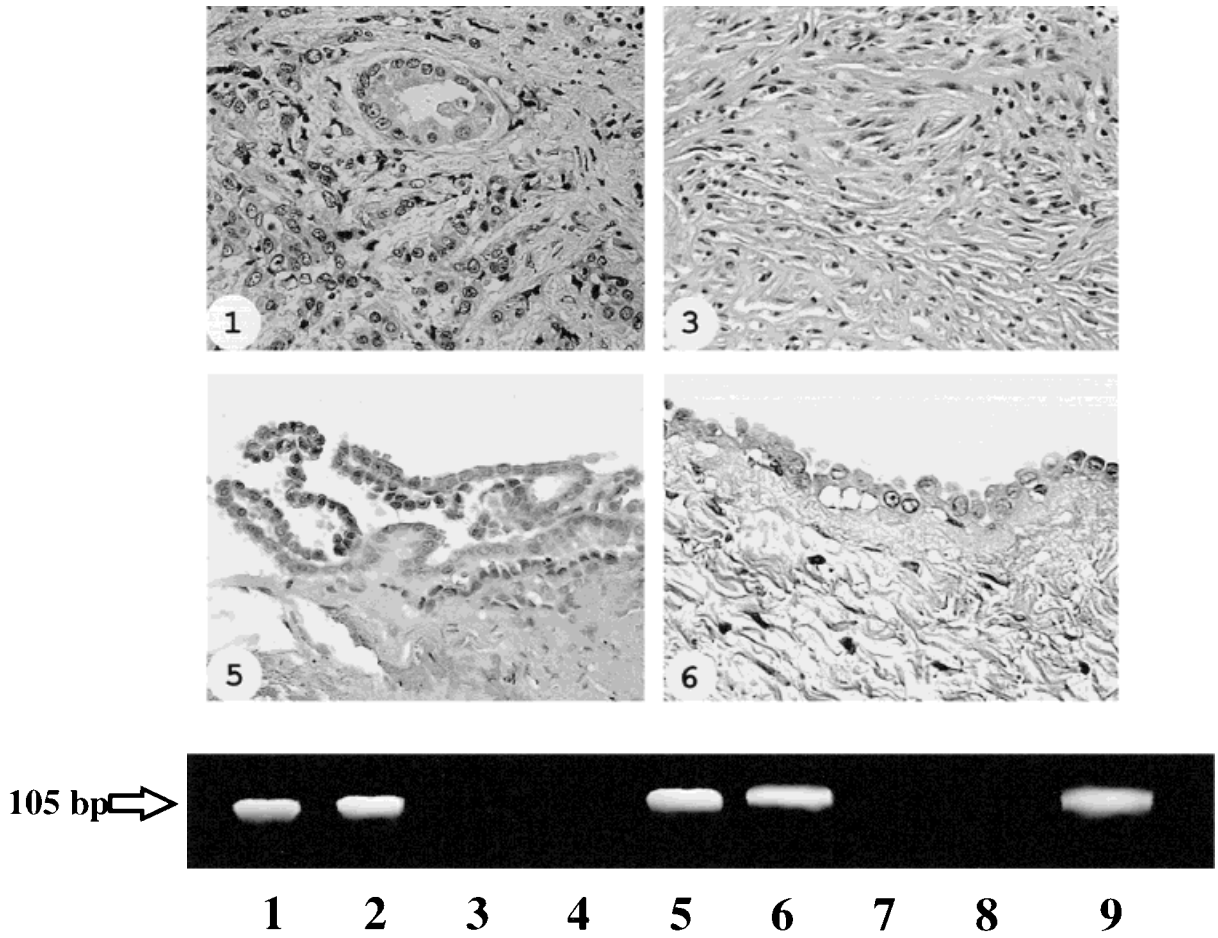

Fig. 1. Detection of SV40 sequences in reactive and malignant mesothelium. The lower panel demonstrates an agarose gel electrophoresis of the 105 bp PCR-amplified SV40 Tag region of representative MMs, mesothelial proliferations, and controls. **Lanes 1** and **2** are examples of positive pleural epithelial MMs; **lanes 3** and **4** are examples of negative pleural sarcomatous MMs; **lanes 5** and **6** are positive examples of reactive mesothelial proliferations; **lane 7** is an example of a negative reactive mesothelial proliferation; **lane 8** is a water blank; **lane 9** is a positive control (COS cells transfected with SV40 virus). The upper panel is a four-photomicrograph composite illustrating the histopathology of some of the samples analyzed in the gel. The numbers on the micrographs correspond to the lane numbers of the gel. No. 1, epithelial MM, SV40 positive; No. 3, sarcomatous mesothelioma, SV40 negative; No. 5 reactive peritoneal mesothelial proliferation associated with inflammation, SV40 positive; No. 6 reactive pleural mesothelial proliferation with atypia, adjacent to metastatic carcinoma, SV40 positive.

neal). Five of these cases were cellular atypia, but none were regarded as malignant (i.e., mesothelioma in situ). The reactive cells were identified and carefully microdissected free of other tissues. In two of these cases SV40 sequences were present (Table 1, Fig. 1). One case represented a pleural reactive mesothelial hyperplasia without atypia associated with chronic inflammation. The other case was a peritoneal reactive mesothelial hyperplasia adjacent to a metastatic carcinoma. The presence of SV40 sequences in these two cases was confirmed by independent analysis after microdissection from a second microslide. The differences in SV40 frequencies in reactive cases (2/13, 15%) were significantly different ($P = 0.01$) from the frequency in epithelial MMs (57/104, 55%).

Absence of SV40 Tag Sequences in Control Tissues

Control tissues (either stromal cells or adjacent nontumorous lung) were available from 75 of cases of MM (Table 1). SV40 Tag sequences were detected in a single case (1%), from the frozen lung tissue adjacent to an invasive epithelial pleural MM (which was also positive). In addition, 20 lung cancers (eight small cell lung cancers and 12 non–small cell carcinomas) from North America lacked SV40 Tag sequences.

DISCUSSION

Most studies reporting the detection of SV40 sequences in human tumors have utilized fresh or frozen tissues. It has been suggested that the use of archival formalin-fixed, paraffin-embedded materials may result in false negative results and reduce the overall numbers of positive cases [Lednicky and Butel, 1997, 1998; Stewart et al., 1998]. However, using highly efficient primers that resulted in a small-sized (105 bp) amplicon [Carbone et al., 1997; Griffiths et al., 1998], our overall detection rate of 57%, in the largest series reported to date, was not significantly different from the rates described in other recent large series. [Pass et al., 1998; Rizzo et al., 1998]. Both Pepper et al. [1996] and we found that fresh-frozen and corresponding formalin-fixed, paraffin-embedded tissue results were in close concordance with each other. SV40 sequences were detected at similar incidences in MMs from Germany and North America, precluding major geographic differences in the incidences of such sequences, at least between these two countries.

Most of the pleural and peritoneal mesotheliomas in men are attributable to exposure to asbestos, although the situation in women is less definitive [Spirtas et al., 1994]. Some MM patients test positive for both asbestos and SV40 [Testa et al., 1998], and it has been suggested that asbestos exposure and SV40 may function synergistically to induce tumors as well as in cellular transformation in vitro [Carbone et al., 1997]. We investigated this association in a subset of 43 German MM patients (mainly males), in whom we determined asbestos exposure by obtaining a detailed history of occupational and nonoccupational asbestos exposure as well as by performing a light microscopic examination of lung tissue for the presence of asbestos bodies. Our data indicate that this group of MM patients showed evidence of asbestos exposure by one or both methods, and that the tumors from about half of these cases had SV40 sequences. However, we failed to identify patients who were negative for asbestos exposure both by history and by microscopic examination. Thus, we were unable to determine whether Tag sequences were present in the minority of MM cases definitely not associated with asbestos exposure. It is interesting that there were no significant differences in the frequencies of viral sequences in MMs of pleural or peritoneal origin.

Of even greater interest is the fact that we did not detect SV40 sequences in MMs that had a sarcomatous component. However, our results will require confirmation from a study with larger numbers. Our findings suggest that either (1) SV40 sequences are selectively found in mesothelial cells demonstrating epithelial cell differentiation or (2) the presence of viral sequences induces epithelial cell differentiation.

A question not studied by others is When do SV40 sequences appear during the pathogenesis of MMs? We found complete concordance between the presence of sequences in invasive MM and their noninvasive components. We also found viral sequences in a small percentage of reactive mesothelial hyperplasias, with or without cellular atypia. These findings suggest that SV40 sequences may lie dormant in the epithelial cell component of the mesothelium for lengthy periods of time prior to the development of invasive MMs.

A recent study from France demonstrated SV40 Tag sequences in 29% of lung carcinomas, 48% of MMs, and 16% of cases with nonneoplastic pleural and pulmonary disease [Galateau-Salle et al., 1998]. However, our study does not confirm their findings regarding the presence of SV40 sequences in benign and malignant lung diseases. All but one of the lung and other non-neoplastic tissues adjacent to MMs in our study as well as 20 lung cancers lacked Tag sequences. These findings help to confirm the specificity of the assay method we used, and exclude the possibility of widespread laboratory contamination. The one sample of adjacent lung tissue, which was positive for viral sequences, was from a frozen sample. Thus, the possibility of invasive MM within or contaminating the sample cannot be excluded. The pathological distinction between MMs and metastatic adenocarcinomas, especially of pulmonary origin, may be difficult to make. Our results suggest that the presence of SV40 sequences will aid in the confirmation that pleural-based tumors are truly MMs. Our data suggest that recombinant vaccine therapies directed against lethal cancers expressing SV40 T antigen [Xie et al., 1999] may be effective in a subset of MMs and that tissues other than mesothelial in origin are unlikely to be damaged by such approaches.

MMs arise from the mesothelial lining cells of pleural and peritoneal cavities. Although the early stages of MM development are rarely available for histological examination, noninvasive malignant cells as well as reactive epithelium may be observed adjacent to or near invasive MMs [Whitaker et al., 1992]. The presence of noninvasive components may precede the development of invasive tumors, supporting the concept of in situ mesothelioma [Whitaker et al., 1992]. We investigated nine cases of noninvasive MMs, and in four of these cases both the noninvasive and adjacent invasive tumor cells contained SV40 Tag sequences. In one of the four positive cases adjacent reactive mesothelium was available for study, and it lacked Tag sequences. Our findings indicate that Tag sequences can be found at the very earliest recognizable tumor cell stage.

Our major findings were as follows:

(1) Specific SV40 viral sequences were present in 48% of invasive MMs of both pleural and peritoneal origin, no significant geographic differences were found, and frozen and paraffin-embedded tissues were equally suitable for analysis.
(2) There was no apparent relationship between the presence of SV40 sequences and asbestos exposure.
(3) SV40 sequences were present in the noninvasive components of epithelial MMs.
(4) SV40 sequences were not detected in MMs of sarcomatous or mixed histologies.
(5) Viral sequences were present in two of 13 samples (15%) of reactive mesothelium.
(6) Lung cancers lacked SV40 sequences, as did non-malignant tissues adjacent to MMs.

Our findings demonstrate the presence of SV40 sequences in epithelial MMs of pleural and peritoneal origin and their absence in tumors with a sarcomatous component. Viral sequences may be present in benign, noninvasive, and invasive mesothelial cell proliferations, but are absent in adjacent tissues and lung cancers.

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