Simian virus 40 (SV40) is a polyomavirus that originates from the rhesus macaque and was discovered in 1960 as a contaminant of human polio vaccines produced in monkey cells.1 Recently, there has been considerable interest in the identification of SV40 in human tumors, including malignant mesothelioma (MM) and other rare tumors such as osteosarcoma, epen-
dymoma, and choroid plexus tumors. Subsequently, it has been found that SV40 may play an important role in the etiology of these tumors. However, SV40 was not detected in malignant lymphomas in Korean patients. Oncogenesis driven by SV40 is mediated by the large tumor antigen (T-Ag) oncoprotein, which is capable of transforming different types of cells in the absence of other viral genes. T-Ag induces DNA synthesis in host cells and prolongs the onset of the S-phase through the inhibition of tumor suppressor proteins p53 and Rb.

MM is a rare and extremely aggressive neoplasm that arises from the serosal surfaces of pleural, peritoneal, and pericardial cavities and is very likely associated with amphibole asbestos exposure. In recent years, the incidence of MM has been gradually increasing in concert with industrial development and increased exposure to asbestos. However, only a small number of people who have been exposed to asbestos during their lifetime will develop MM. This suggests that SV40 may act as an independent carcinogenic agent or as a cofactor of asbestos to augment the risk of MM.

The incidence of MM varies according to geographic location, and the highest incidence rates are reported from Australia, Belgium, and Great Britain. In Korea, the incidence of MM is very low compared with other developed countries, with only about one case per million in the last decade. However, the incidence is recently increasing. Korea has a higher proportion of females (33.8%) among MM cases compared with other nations such as Canada (15.4%) or Italy (27.6%), and most of the patients are diagnosed when they are in their 50s, which is younger compared to cases in other countries. Although the majority of MM patients have had occupational (36.8%) and/or environmental (20.4%) asbestos exposure, the remaining patients (42.8%) had no known asbestos exposure.

The role of SV40 in the oncogenesis of MM following asbestos exposure has not yet been studied in a Korean population. The purpose of this study is to identify SV40 DNA and protein in tissues extracted from MM from a Korean population using quantitative polymerase chain reaction (PCR) and immunohistochemical staining.

**MATERIALS AND METHODS**

**Study population**

Using the Korean Malignant Mesothelioma Surveillance (KMMS) tissue bank, 356 cases of MM that occurred between the years 2001 and 2009 were available. Of these, 62 formalin-fixed and paraffin-embedded tissue tumor blocks were selected. The selection was based on the availability of adequate neoplastic tissue within the paraffin blocks. Original diagnoses were based on morphological evaluation in the primary centers and confirmed by three expert pathologists. All cases were analyzed using an immunohistochemical panel (positive marker: calretinin and/or D2-40, WT-1; negative marker: carcinoembryonic antigen). Each tumor slide was re-evaluated to verify the original diagnosis.

**Immunohistochemistry**

Detection of SV40 protein was performed by an automatic staining procedure using the Ventana Benchmark XT (Roche Diagnostics, Basel, Switzerland). The sections were deparaffinized, then pretreated with CC1 (Roche Diagnostics) for 60 minutes at 42°C. The sections were then washed with reaction buffer and incubated with anti-SV40 antibody (Cell Marque Corp., Rocklin, CA, USA) at a 1:100 dilution for 60 minutes at 42°C. Antibody detection was performed using the UltraView Universal DAB kit (Roche Diagnostics) according to the manufacturer’s recommendations. Finally, the slides were counterstained with hematoxylin (Roche Diagnostics) and mounted. As a positive control for SV40 sections from paraffin-embedded tissue, kidney tissue confirmed to contain polyomavirus was used (kindly provided by Professor Young-Mee Cho from the Department of Pathology, Asan Medical Center, Seoul, Korea). The negative controls were performed without the primary antibody.

**DNA extraction from paraffin-embedded tissues**

The paraffin-embedded tissue blocks were de-paraffinized using xylene. Briefly, the paraffin was removed by two extractions with 1 mL of xylene for 30 minutes and two with 0.5 mL of ethanol and subsequently dried at 37°C. DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. Of the paraffin-embedded tissues analyzed, sufficient DNA was only extracted from 36 samples.

**β-actin PCR**

To ensure the quality of the extracted DNA, all samples were analyzed for the presence of the β-actin gene. DNA (200 ng) was amplified using a HotStar Taq Plus master mix kit (Qiagen). After a denaturing step of 15 minutes at 95°C, 30 amplification cycles were performed. Each cycle consisted of 1 minute
at 94˚C, 30 seconds at 56˚C, and 1 minute at 72˚C, followed by a final extension step of 5 minutes at 72˚C using β-actin (forward 5’-CCT TCC TGG GAC TGG AGT CCT-3’ and reverse 5’-GGA GCA ATG ATC TTG ATC TTC-3’) primers.

SV40 quantitative PCR

A real-time PCR method (TaqMan) for SV40 was previously established which uses a primer pair and an oligonucleotide probe with the fluorescein reporter dye FAM attached to the 5’ end and a rhodamine dye (TAMRA) quencher linked to the 3’ end. A threshold cycle value (Ct) was calculated for each sample by determining the point at which the fluorescence exceeded the threshold limit chosen for the specific plate.

The real-time PCR assay used the forward primer 5’-CAC AGC ACT CAA AAA ACT TAG CA-3’ and reverse primer 5’-GAC TCT CAA CAT TCT ACT CCT CCA AAA-3’ and the fluorogenic TaqMan probe 5’-ACC CCA AGG ACT TTC-3’.

Negative controls (water) and positive controls (DNA from the HEK293T cell line) were included on each plate, and a standard curve (SV40 standard solutions) was produced from which the number of genomes in the samples could be calculated. The SV40 quantities used in the standards were 100,000, 10,000, 1,000, 100, and 10 copies per 1 μL (i.e., per well). Thermal cycling, fluorescence detection, and data analysis were performed on an ABI PRISM 7900 Sequence Detector (Applied Biosystems, Foster City, CA, USA) using the software provided by the manufacturer.

The standard stock solution, plasmid pDRIVE, containing the SV40 genome was cultivated in medium, and the culture was purified according to the manufacturer’s instructions (Quagen). The concentration of the plasmid solution was calculated using the absorbance at 260 nm.

Ethics statement

This study was approved by the Institutional Ethics Committee of Yonsei University, Wonju College of Medicine, Wonju, South Korea (IEC No. YWMR-12-4-033) and was in compliance with the Helsinki Declaration.

RESULTS

Clinicopathological characteristics

The study included 38 (61.3%) males and 24 (38.7%) females between the ages of 34 and 79 years, with a mean age of 60.2 years. The majority of patients developed MM when they were in their 50s, followed by patients over 70, patients in their 60s, 40s and finally in their 30s (Fig. 1). Most cases of MM occurred in the pleura (40 cases, 64.5%), followed by the peritoneum (21 cases, 33.9%) and the pericardium (one case, 1.6%). With regard to histologic classification, the epithelioid type was the most common histologic type (29 cases, 46.8%), followed by the biphasic (nine cases, 14.5%), sarcomatoid (six cases, 9.7%), desmoplastic types (four cases, 6.5%), and other undetermined variants (14 cases, 22.6%). The clinicopathological data are summarized in Table 1.

Table 1. Clinicopathologic characteristics of malignant mesothelioma cases

| Parameter                              | n (%) |
|----------------------------------------|-------|
| Mean age (range, yr)                   | 60.2 (34-79) |
| Sex                                    |       |
| Male                                   | 38 (61.3) |
| Female                                 | 24 (38.7) |
| Subtype                                |       |
| Epithelioid                            | 29 (46.8) |
| Biphasic                               | 9 (14.5) |
| Sarcomatous                            | 6 (9.7) |
| Desmoplastic                           | 4 (6.5) |
| Undetermined                           | 14 (22.6) |
| Site                                   |       |
| Pleura                                 | 40 (64.5) |
| Peritoneum                             | 21 (33.9) |
| Pericardium                            | 1 (1.6) |
| Occupation                             |       |
| Asbestos exposure                      | 11 (17.7) |
| None exposure                          | 11 (17.7) |
| Unemployed                             | 1 (1.6) |
| Unknown                                | 39 (62.9) |
| Sampling method                        |       |
| Biopsy                                 | 12 (19.4) |
| Excision                               | 34 (54.8) |
| EPP                                    | 16 (25.8) |

EPP, extended pleuropneumonectomy.

Fig. 1. According to the age distribution, the majority of cases developed malignant mesothelioma when patients are in their 50s, followed by those over 70, those in their 60s, 40s and finally in their 30s.
The occupational history was available for only 23 (37.1%) patients out of 62 patients, and among these, only 11 (17.7%) had a job that placed them at risk for asbestos exposure such as construction, automobile repair and production, shipbuilding and repair, or farming (Table 2).

**Table 2. Occupational history of malignant mesothelioma patients**

| Occupation       | No. of patients | Comments                                      |
|------------------|-----------------|-----------------------------------------------|
| Construction     | 3               | One patient also worked in copper pipe fabrication |
| Automobile repair| 1               |                                               |
| Shipbuilding repair | 2              |                                               |
| Environmental asbestos | 1          |                                               |
| Office worker    | 6               | Administrative, education office worker, Red Cross staff, teacher |
| Farmer           | 3               |                                               |
| Trading          | 1               |                                               |
| Housewife        | 4               |                                               |
| Unemployed       | 1               |                                               |
| Others           | 1               | Production company                            |
| Unknown          | 39              | Occupation history is not available           |
| Total            | 62              |                                               |

**Immunohistochemistry and real-time PCR findings**

None of the 62 MM tissue samples studied showed a positive immunohistochemical staining for SV40, while the positive control kidney tissue for each slide was strongly positive (Fig. 2).

Of the 36 samples that yielded sufficient DNA following extraction and purification, real-time PCR analysis failed to show SV40 DNA amplification in any case. The mean cycle threshold (Ct) value for the 36 available blocks was $34.133 \pm 1.440$ and was not significantly different from the negative control (positive control, $28.413 \pm 0.476$; negative control, $34.437 \pm 0.160$).

**Fig. 2.** Immunohistochemical findings for simian virus 40 (SV40) antibody; (A) positive control tissue from kidney infected with SV40 shows a positive reaction, while tissue sections from malignant mesothelioma show a negative result for SV40 antibody (B, epithelioid type; C, biphasic type; D, sarcomatoid type).
DISCUSSION

The possible association between SV40 and MM has inspired much interest, especially after Carbone et al. reported the presence of SV40 in approximately 60% of mesotheliomas. Several subsequent studies have investigated the relation of mesothelioma with SV40, but reported results have varied considerably. One study which investigated the presence of SV40 T-Ag in 18 autopsied MM patients in Japan involved eight positive samples; however, none of these cases were likely to have received a contaminated polio vaccine due to their age. A recent review showed that the association of SV40 and MM was positive in only three cases out of 12. Another three studies showed that an average of 22% of tumor tissues were positive for SV40.

A strong association between exposure to asbestos and the development of MM has long been established, but there exist cases with unclear etiologies that appear unrelated to asbestos exposure. MM is known to occur in only 10% to 20% of individuals heavily exposed to asbestos, and about 20% of patients lack a history of any exposure. In a case-control study, PCR analysis revealed the presence of SV40 DNA in eight of 19 cases of MM among individuals exposed to asbestos, suggesting that SV40 may increase the risk of MM among patients exposed to asbestos.

In hamsters, intrapleural injection of SV40 virus resulted in the development of MM in 100% of cases within two to six months. Additionally, a strong association between asbestos and SV40 was found among hamsters exposed to low levels of asbestos, as only the SV40-infected animals developed MM. To understand the roles of both T-Ag and t-Ag in the development of MM in hamsters, t-Ag mutant SV40 virus was injected intracardially into newborn hamsters, after which only one animal developed MM, while the majority developed histiocytic lymphomas. While T-Ag binds and deactivates tumor suppressor proteins, t-Ag may play a key role in mesothelial cell transformation and may be necessary to complete inactivation and allow mesothelial cells to enter S phase.

In humans, the role of SV40 in the development of MM is controversial. In total, more than 26 studies have identified SV40 in human mesotheliomas or other human tumors; however, many recent investigators have been unable to detect SV40 in human mesothelioma tissues. In a study by Manfredi et al., of 69 human MM tissues screened, none contained detectable SV40 T-Ag sequences, arguing against the role of SV40 in the development of human MMs.

In this study, no evidence of SV40 was found in any of the MM tissues examined, either by immunohistochemistry or real-time PCR analysis. These results are consistent with those of most recent investigations. The discrepancy found among these different studies may suggest a possible role for geographical differences in the association between SV40 and MM. Alternatively, contamination of laboratory plasmids may have led to false positive results in some cases.

Occupational and environmental exposure to asbestos is the main causes of MM in Korea. Our results do not support an association between MM and SV40 or the proposed function of SV40 as a cofactor to asbestos carcinogenicity. If SV40 is indeed a cofactor for asbestos carcinogenicity, viral transcripts and proteins would have been detectable in the cases examined in our study.

We acknowledge some advantages and limitations of our study. We performed immunohistochemistry and real-time PCR separately to detect SV40 protein and DNA, respectively, in MM tissues. Frozen or fresh tissues are preferred for PCR detection of viral DNA. However, acquiring frozen or fresh MM tissues is impractical due to the infrequency of MM. In addition, our sample size may not have been large enough to detect an association between SV40 and MM in Korea.

In conclusion, no detection of SV40 in MM tissues suggests that the virus does not have a role in development of MM in Korea; therefore, a more active epidemiologic study for asbestos-related occupational and environmental causes in MM patients is needed.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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