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Development of Malignant Mesothelioma During Treatment for Prolymphocytic Leukemia: Is Asbestos or Simian Virus 40 a Link?

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Abstract. *A patient with a history of heavy asbestos exposure presented with B-prolymphocytic leukemia/lymphoma (B-PLL). Soon after, he developed rapidly progressing malignant peritoneal mesothelioma. The concurrent development of both relatively uncommon diseases raised the possibility that a common causative factor might exist. Since asbestos, simian virus 40 (SV40), or both have been associated with lymphoproliferative disease and mesothelioma, we investigated both possible links in our patient. Imaging studies provided evidence for asbestos exposure because bilateral pleural plaques were identified. Tissues from bone marrow (involved with B-PLL) and from a peritoneal nodule (involved with mesothelioma) were examined for SV40 DNA using polymerase chain reaction (PCR): no SV40 DNA was detected. We conclude that asbestos remains the sole possible connection to both malignancies in this patient. It seems possible that fludarabine, an immunosuppressive chemotherapy, accelerated the occurrence and progression of malignant mesothelioma during the therapy for his B-PLL.*

Simian virus 40 (SV40), a rhesus monkey polyomavirus, was discovered in 1960 during the safety testing of poliovirus vaccine (1). A previously unrecognized virus, SV40 contaminated the inactivated (IPV or Salk) and the live attenuated (OPV or Sabin) vaccines produced from 1955 to 1963, since the vaccines were prepared from rhesus monkey kidney cells. Contaminated vaccines were administered to children and adults in many countries, including the United States, the former Union of Soviet Socialist Republics, Japan, England and Wales, Italy, Mexico and several Central American countries, as well as other locations. SV40 can also infect humans who have direct contact with monkeys (zoo-keepers) and it has been suggested that SV40 might spread *via* human-to-human transmission (1).

Since its discovery, SV40 has been associated with various malignancies including brain tumors, bone cancers, malignant mesothelioma and, recently, lymphoma (1-3). The association between SV40 and mesothelioma is evident in both animals and humans. Injection of SV40 can cause both cancers in hamsters and the virus can subsequently be isolated from these tumors (4). In humans, several large case control studies have indicated that SV40 is prevalent in malignant mesothelioma (1,6). A recent metanalysis identified the odd ratios for positive identification of SV40 in mesothelioma and lympho-proliferative disease to be 17 (95% confidence interval 10-28; based on 15 studies with 528 mesothelioma samples and 468 control samples) and 5.4 (95% confidence interval 3.1 to 9.3; based on 3 studies with 301 cases and 579 control samples), respectively (1). The significance of SV40 in lymphoproliferative diseases, however, remains to be determined. It is unclear if SV40 is a pathogen or a passenger in these diseases (2,3,5-9). Recent results suggest that, when SV40 is present in lymphomas, it contributes to the malignant phenotype (6). SV40 DNA was detected in a variable percentage of lymphomas using PCR (0-40%) (2,3,5-9). Some of these discrepancies were caused by differences in interpreting the results. For example, Capello *et al.* (9) reported that SV40 was present in mesothelioma biopsies but not in lymphoma biopsies. Capello *et al.* found that about 5% of the lymphoma tested contained SV40 DNA. However, they considered these samples negative because not all the SV40 primers pairs they used produced positive results (9).

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We recently participated in the care of a patient who initially presented with B-prolymphocytic leukemia/ lymphoma (B-PLL). Five months after therapy for B-PLL, the patient developed malignant mesothelioma. The occurrence of both unusual malignancies in the same patient in close temporal relationship raised the possibility of a common causative factor. We investigated asbestos exposure and SV40 infection as possible causative agents for both malignancies.

Clinical Analysis

The patient, a previously healthy 75-year-old Caucasian man, presented with increased abdominal girth, night sweat and profound weight loss. He was a retired pipe-insulation worker, who reported asbestos exposure for several decades. Physical examination revealed massive hepatosplenomegaly and tense ascites; computerized tomography (CT) scan demonstrated diffuse lymphadenopathy in the thoracic and abdominal cavity. Bilateral multiple pleural plaques, consistent with asbestos exposure, were identified in his thoracic cavity.

His hemoglobin was 12 g/dl; platelet, 75 x 10^3/ml; leukocyte, 3.7 x 10^3/ml: 38% of leukocytes were large lymphoid cells containing abundant cytoplasm and prominent nuclei. A flow cytometry immunophenotyping of his blood revealed a monoclonal B-cell population, positive for CD19, CD20, CD 22, FMC-7, HLA-DR and surface lambda light chain restriction, but negative for CD34, CD10, CD5, CD23 and TdT. Bone marrow aspirates and peritoneal fluid indicated an involvement of a similar lymphocytic clone. In addition, due to gastrointestinal bleeding, the patient underwent esophagoduodenoendoscopy and colonoscopy; the biopsy specimens taken from his antrum and rectum exhibited the same malignant clones. Cytogenetic studies from his bone marrow showed translocation (8;14) and interstitial deletion involving 11q23. Using IgH/c-myc probe set, fluorescence *in situ* hybridization (FISH) assay showed an abnormal signal pattern in 11% of interphase cells; while analysis for Cyclin D1-IgH fusion was negative and Ki-67 was positive in less than 10% of neoplastic cells. A diagnosis of B-PLL was established.

He commenced on therapy with fludarabine 25 mg/m^2 daily for five days, rituximab 375 mg/m^2 and pegfilgrastim 6 mg every 28 days. He tolerated treatment well; his appetite improved and ascites decreased. A CT scan after 5 monthly cycles of therapy showed a marked decrease in the size of his spleen and lymphadenopathy in his chest and abdomen with no lymph node measuring over 1 centimeter; however, multiple new soft tissue masses signifying peritoneal metastases developed. A fine-needle aspiration of one of these masses showed reactive mesothelial cells, without evidence of malignancy; therapy with rituximab alone was continued for one more cycle.

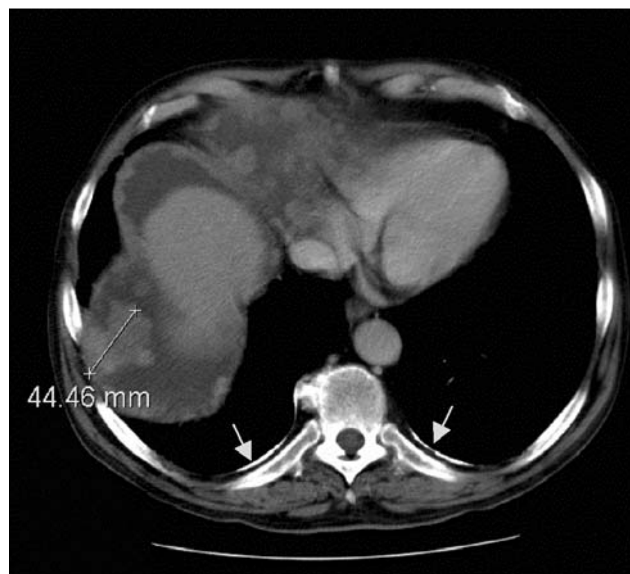

Figure 1. *CT scan of the chest and abdomen demonstrated multiple peritoneal masses (maximal diameter 4.5 cm.) and bilateral calcified pleural plaques in the thoracic cavity (arrows).*

His appetite and his functional status started to decline and his ascites worsened. An interval CT scan demonstrated a marked increase in the size and number of the peritoneal masses, but without residual lymphadenopathy (Figure 1). An exploratory laparotomy revealed multiple tumors in the omentum, falciform ligament and peritoneal surface. The liver appeared cirrhotic with tumors on its surface. At this time, histology and cytology confirmed the diagnosis of biphasic mesothelioma. Pathological examination of the resected peritoneal nodules showed large malignant mesothelial cells which stained positive for cytokeratin 5/6, calretinin, CK7, and epithelial membrane antigen. The same cells were negative for CD45, smooth muscle actin, desmin and S-100. To determine the presence of residual lymphoma, lymph node biopsy was performed during the surgery as well. FISH analysis using 8q24(MYC) and 14q32(IGH) probe was negative; a flow cytometric analysis of one small lymph node taken during surgery, and the peritoneal mass showed no monoclonal B cell populations. The patient underwent chemotherapy with carboplatin area under the concentration-time curve 5 on day 1 and gemcitabine 1000 mg/m^2 on days 1,8 and 15 every 28 days. Despite treatment, his ascites worsened; his performance status also declined rapidly and he died shortly afterwards.

Results and Discussion

We examined the tissue specimens using PCR amplification and Southern blot hybridization with primers and probes

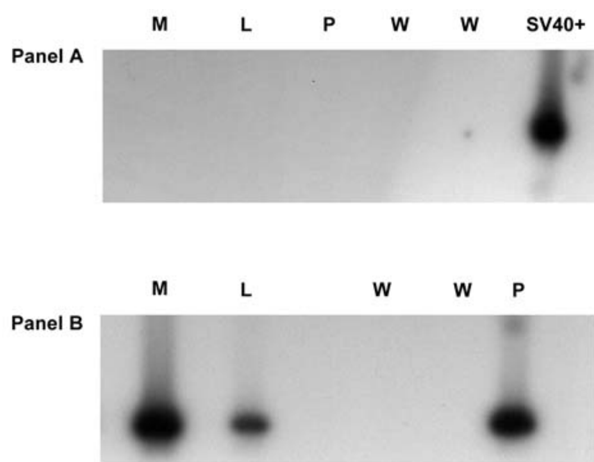

Figure 2. *Panel A: SV40 detection using SV3/SVrev set of primers; panel B: House-keeping gene (ß-globin) detection in the sample tumors; M = patient sample from the peritoneal nodule, L= patient sample from the bone marrow, W= water, P= placental tissue, SV40+= SV40 positive control.*

specific for SV40. Paraffin-embeded tumor samples were obtained from the bone marrow biopsy involved with B-PLL before treatment as well as from a peritoneal nodule involved with mesothelioma, obtained during exploratory laparotomy. DNA was extracted as described and SV40 testing was according to established procedures described in detail in ref. 10 (ref. 10 contains the sequences of all primers and probes used in these studies and mentioned below). Briefly, all of the DNA samples were tested for suitability of PCR amplification with primers specific for a 268-base pair fragment of the ß-globin gene (as described in ref. 10). All DNA the samples could be amplified.

PCR reactions were performed using the hot-start technique (10). The GeneAmp PCR reagent kit containing Amplitaq polymerase (Perkin-Elmer Biosystems; Norwalk, CT, USA) and the Ampliwax PCR gems (Perkin-Elmer Biosystems) were utilized for the analysis. DNA samples from tumors were amplified using the SV3 and SV.rev set of primers, which amplified the retinoblastoma-pocket binding domain of the SV40 tumor antigen (10). The samples were further analyzed with primers R1/Rpara. This set of primers amplified a small fragment of the origin of viral replication. The total volume for each PCR reaction was 100 μL; the concentrations of $MgCl_2$ and that of the primers were 2.5 mM and 0.5 μM, respectively (the concentration of each primer was calculated from the equation 1 OD = 20 μg/mL). One μg of DNA was used per PCR. All other reagents were used according to the recommendations of the manufacturer. Negative controls were included in each PCR experiment to test for PCR contamination. Thermocycling was performed by denaturation for 3 min at 94˚C followed by 45 cycles. For Southern blot analysis, 20 μL of the PCR reaction was loaded onto and run in a 2% agarose gel for 3 h at 100 V. The gel was stained with ethidium bromide to visualize the PCR products. The DNA samples were transferred overnight in 0.4 M NaOH to a nylon membrane (Hybond N+, Amersham Pharmacia Biotech; Uppsala, Sweden). Hybridization was carried out using a SV40-specific ^{32}P-end-labelled internal oligoprobe (10). No evidence of SV40 DNA in either the mesothelioma or the lymphoma tumor specimens was found (Figure 2).

The development of both lymphoproliferative disease and mesothelioma in the same patient is unusual, while a close temporal relationship between both diseases is exceedingly rare (11-16). Many investigators have suggested asbestos exposure as a common causative factor. Although our patient had a history and radiographic stigmata of asbestos exposure, SV40 infection is still a plausible co-carcinogen. In fact, many patients with asbestos-related mesothelioma have evidence of SV40 infection (6). In our experiment, we demonstrated that SV40 was, in fact, not present. The evidence of asbestos exposure was consistent with previous data suggesting asbestos as an etiological factor in mesothelioma and possibly lymphoma, while SV40 did not appear to be a factor for either disease in this patient.

Asbestos, an IARC class I carcinogen, is strongly associated with mesothelioma (17); however, it has not been established that asbestos is a human lymphoid system carcinogen. Some case reports and epidemiological studies during recent decades have associated asbestos exposure to B cell lymphoproliferative diseases, particularly chronic lymphocytic leukemia (CLL) and myeloma (16,18). Mice injected with asbestos and erionite intraperitoneally develop both malignant peritoneal mesothelioma and lymphoma (19) supporting a causal association. Some epidemiological studies supported a causal relationship between asbestos exposure and the development of lymphoproliferative diseases (reviewed in ref. 20). However, metanalyses of case control studies found no causal relationship between asbestos exposure and the development of lymphoproliferative disease (21,22). These studies, however, suffer the limitation that asbestos exposure was determined by patient interview, a subjective measure, rather than by lung content analyses or radiological analyses demonstrating the presence of bilateral pleural plaques, or asbestosis which are objective measures of asbestos exposure. Therefore, it is our opinion that the relationship between asbestos and lymphoma has not been adequately addressed because the epidemiological studies that argued against such an association were limited by the lack of an objective assessment of exposure. B-PLL, with such unusual presentation and cytogenetic abnormalities, has never been associated with asbestosis to date.

The aggressive clinical course of mesothelioma in our patient deserves discussion. Its close interval development

after treatment with fludarabine and rituximab raises the possibility that this therapy may have precipitated the sudden upsurge of malignant mesothelioma. Fludarabine, a purine nucleoside analogue, has been widely used in the treatment of low-grade lymphoproliferative disease such as CLL and PLL (23,24). Rituximab, a monoclonal antibody against CD 20, has been increasingly integrated into the therapy of B cell lymphoproliferative disease, including B-PLL (25). Fludarabine, reduces the number of T suppressor cells, which are essential for tumor surveillance. Although rituximab has never been associated with the development of secondary malignancy, fludarabine has been implicated in the occurrence, recurrence, or worsening of secondary malignancies (26-29). Nevertheless, long-term follow-up data from the National Cancer Institute on 2014 patients, treated with fludarabine for CLL, suggests that the risk of secondary malignancy is, indeed, related to the CLL itself (30).

A limitation of our experiment is the possibility of a false negativity for SV40 (10). Although PCR is a highly sensitive technique, the tissue specimens from the bone marrow and peritoneal nodule were paraffin-embedded. It is possible that SV40 DNA, which is usually present as episomal low-molecular weight DNA inside human mesothelioma cells, could have been leached by formaldehyde during fixation. In addition, the small size of the specimens could also have influenced the test result: the estimated total number of tumor cells in our specimens was less than 100. However, we have extensive experience in detecting SV40 in human tumor biopsies. Based on our experience, we consider the possibility that the SV40 results were falsely negative to be unlikely.

Thus, the only carcinogen possibly associated with the development of this patient's lymphoma was asbestos. It should be noted that asbestos has both local and systemic immunosuppressive effects (reviewed in ref. 31). Therefore, similarly to lymphomas that develop in AIDS or post-transplant patients, asbestos could favor the development of lymphomas through its effects on the immune system.

In conclusion, our B-PLL/mesothelioma patient was not infected with SV40; asbestos appears as the sole known possible contributing factor to both malignancies. The rapid and aggressive clinical course of this mesothelioma, which occurred soon after effective therapy for his B-PLL, suggested that therapy with fludarabine was a possible precipitating factor.

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