Significant Association Between Human Osteosarcoma and Simian Virus 40

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BACKGROUND: Simian virus 40 (SV40) has been considered to be an oncogenic viral agent in the development of osteosarcoma (OS), which to the authors’ knowledge continues to be of unknown etiology. METHODS: In the current study, serum samples from patients with OS were investigated with an indirect enzyme-linked immunosorbent assay (ELISA) to test for the presence of immunoglobulin G antibodies, which react with SV40 antigens. In ELISA, SV40 antigens were represented by 2 synthetic polypeptides that mimic epitopes of the viral capsid proteins 1 to 3. Additional sera from patients with breast cancer and undifferentiated nasopharyngeal carcinoma as well as healthy subjects were the controls. RESULTS: Immunologic results suggested that antibodies that react with SV40 mimotopes were more prevalent (44%) in serum samples from patients with OS compared with healthy subjects (17%). The difference in prevalence between these cohorts was statistically significant \( P < .001 \). It is interesting to note that in the patients with OS, significance indicated the difference between OS versus breast cancer (44% vs 15%; \( P < .001 \)) and OS versus undifferentiated nasopharyngeal carcinoma (44% vs 25%; \( P < .05 \)). CONCLUSIONS: The data from the current study indicate an association between OS and SV40. These data could be transferred to clinical applications for innovative therapies to address SV40-positive OS. Cancer 2015;121:708-15. © 2014 American Cancer Society.

KEYWORDS: osteosarcoma, simian virus 40 (SV40), antibody, viral agent.

INTRODUCTION

Human osteosarcoma (OS) is a rare malignant neoplasm of the bone that for the most part affects children and young adolescents.¹ To the best of our knowledge, little is known regarding the biology and pathology of this bone tumor. Cellular and animal experimental models were used in an attempt to elucidate the mechanism of OS onset.²⁻⁵ OS has multiple genetic risk factors, including groups of genes involved in cell proliferation/cell cycle/DNA damage repair. It has been reported that the incidence of OS is increased significantly in patients with specific hereditary diseases.⁶

The risk factors associated with the onset and progression of OS to our knowledge have not been completely elucidated, but as with other human cancers, carcinogenic chemicals, radiation, and oncogenic viruses most likely are involved.⁷⁻⁸ Simian virus 40 (SV40) footprints have been detected among oncogenic viruses in bone tumors by some investigators,⁹⁻¹⁺⁻¹⁻ whereas other studies have not reported SV40 in tumor samples, including OS.¹⁺⁻¹⁹⁻²⁰ It has been shown that SV40-negative data in human tumor samples, as reported by some teams, could be due to some technical pitfalls.²¹⁻²² SV40 was isolated from monkey kidney cells, which were used between 1955 and 1963 for the production of inactivated (Salk) and live (Sabin) poliomyelitis vaccines.²³⁻²⁴ As a result of this accidental contamination, polio vaccines with SV40 were distributed in several countries and administered to millions of individuals.²⁴⁻²⁵

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Recently, experimental data have indicated that SV40 is able to infect and transform human B cells and T cells. Despite being transformed, these SV40-positive lymphocytes have the potential to spread infectious viral progeny. Therefore, infected B cells and T cells could potentially act as SV40 vectors for other tissues in an infected individual. However, this assumption is based on in vitro data and there is no evidence of this occurring at the community level. Thus, the transmission route is still a matter of active investigation.

Transformation and oncogenic processes are operated by SV40 with 2 main oncoproteins: the large T antigen (Tag) and the small t antigen (tag). Tag and tag bind and abolish the functions of tumor suppressor p53 and pRB gene products. Many investigations, mainly performed using polymerase chain reaction and immunologic methods, have indicated that, at present, SV40 spreads in children and adults by horizontal infection. Thus, it appears that infection occurs independently of earlier SV40-contaminated vaccines.

In some studies, SV40 footprints have been detected in different human cancers, including bone tumors, as well as in healthy individuals. However, to our knowledge, the association between SV40 and human tumors remains to be established. Indeed, both SV40-positive and SV40-negative data have been reported. These conflicting results have provoked a debate within the scientific community. Results of the 2012 World Health Organization/International Agency for Research on Cancer meeting held in Lyon, France and the subsequent monograph indicate that SV40 is not classifiable as a carcinogenic viral agent in humans.

The problems related to SV40 infection in the human population and its contribution to human cancer also have been evaluated by the Immunization Safety Review Committee, which was established by the Institute of Medicine. The committee recommended the development of specific, sensitive immunologic assays for SV40 and the use of standardized methods, which should be accepted and shared by those laboratories involved in SV40 investigations.

The presence of the specific SV40 antibody in human serum samples has been assayed in several studies using different immunologic tests, mainly with virus-like particles. However, the high protein similarity among the 3 main polyomaviruses—SV40, BK virus (BKV), and John Cunningham virus (JCV)—has hampered the results, which are affected by some cross-reactivity. It is possible that these results have influenced the negative statistical data reported in subsequent studies.

Recent investigations performed with an indirect enzyme-linked immunosorbent assay (ELISA) with SV40-specific synthetic peptides from its viral proteins have reported the detection of SV40 antibodies in serum samples from healthy subjects and patients with cancer, without cross-reactivity with BKV and JCV (both wild-type strains and genetic variants).

In the current study, serum samples from patients with OS and other cancers, together with sera from healthy subjects (HS), were analyzed for the presence of SV40 immunoglobulin G (IgG) antibodies by indirect ELISA with specific mimotopes/synthetic peptides from SV40 viral capsid proteins (VPs).

**MATERIALS AND METHODS**

**Samples**

Sera from different institutions were harvested between 2006 and 2013. These were collected from patients with OS (55 patients) with a histologically proven diagnosis of different types of OS localized in distinct areas (Table 1). After analyses, OS sera were collected from discarded samples at the Rizzoli Orthopaedic Institute in Bologna, Italy. Sera from HS (114 individuals) were obtained from the clinical laboratory analysis performed at the University Hospital of Ferrara in Ferrara, Italy and clinical laboratory analysis performed at the State Hospital in the Republic of San Marino. Additional samples were obtained from our serum collections (Table 2). Informed consent was obtained from adult patients/individuals, whereas for children and young adolescents (those aged <18 years), consent was provided by parents. Anonymously collected sera were coded with indications of age, sex, and pathology. The project was approved by the County Ethical Committee of Ferrara, Italy.

**SV40 Antigens/Mimotopes and Control Serum Samples**

Two specific SV40 peptides were selected by computer-assisted analysis, which compared the homology of the 3 VPs (VP1, VP2, and VP3) with amino acids from the human BKV and JCV polyomaviruses. The 2 selected antigens belong to VP1, VP2, and VP3 (ncbi.nlm.nih.gov/nucore). The amino acid similarity analysis of BKV and JCV and other polyomaviruses with SV40 has been previously reported. Preliminary, ELISA data have suggested that 2 SV40 polypeptides did not cross-react with the BKV and JCV hyperimmune sera that were used as controls. The 2 SV40 peptides, known as VP1B and VP2/3C, have the following sequences:
Because of their specific reaction, these SV40 antigens/mimotopes were used in indirect ELISA with rabbit hyperimmune serum that had been experimentally immunized with SV40 (positive control serum). BKV and JCV hyperimmune sera were not found to react with the 2 SV40 polypeptides VPB and VPC (negative control sera). These SV40 mimotopes are reported to demonstrate low similarity with BKV and JCV antigens and their strains, and with other less related human polyomaviruses. The polypeptides were synthesized as previously reported.

The hyperimmune serum samples that reacted with SV40 and BKV were obtained from rabbits immunized with purified viral stocks, as previously reported. The immune serum anti-BKV was titered using the hemagglutination inhibition (HAI) test.

<table>
<thead>
<tr>
<th>Human Sera</th>
<th>No. of Patients/Individuals</th>
<th>Median Age, Years</th>
<th>Male Sex, %</th>
<th>No. of Positive Samples (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OS</td>
<td>55</td>
<td>18</td>
<td>53</td>
<td>24 (44) 25 (46) 24 (44)*</td>
</tr>
<tr>
<td>HS</td>
<td>114</td>
<td>18</td>
<td>58</td>
<td>30 (29) 21 (18) 20 (17)</td>
</tr>
<tr>
<td>BC</td>
<td>78</td>
<td>42</td>
<td>—</td>
<td>14 (18) 13 (17) 12 (15)*</td>
</tr>
<tr>
<td>UNPC</td>
<td>64</td>
<td>57</td>
<td>81</td>
<td>16 (25) 17 (27) 16 (25)</td>
</tr>
</tbody>
</table>

Abbreviations: BC, breast cancer; HS, healthy subjects; IgG, immunoglobulin G; OS, osteosarcoma; SV40, simian virus 40; UNPC, undifferentiated nasopharyngeal carcinoma; VP, viral capsid protein; VPB, viral capsid protein B; VPC, viral capsid protein C.

*The different prevalence of SV40 antibodies noted between the cohorts of patients with OS was statistically significant compared with the cohorts of HS with the same median age (P < 0.001).

*The prevalence of SV40 antibodies in sera from patients with OS differed statistically from that of patients with BC (P < 0.001) and from that of patients with UNPC (P < 0.05). Statistical analysis was performed using the chi-square test.
using human erythrocytes from the 0, Rh factor-positive group. Anti-SV40 serum was titered by neutralization assay.8

Indirect ELISA
Indirect ELISA was developed and standardized to detect specific antibodies against SV40 in human sera using synthetic peptides.8,29

Plate coating
Plates were coated with 5 µg of the selected mimotope and diluted in 100 µL of coating buffer (pH 9.6) (Candor Bioscience, Wangen, Germany).

Blocking reaction
Blocking was made with 200 µL per well of the Blocking Solution (Candor Bioscience) at 37°C for 90 minutes.

Primary antibody
Different wells were covered with 100 µL of the following sera: positive controls, represented by the immune rabbit serum containing anti-SV40 antibodies; negative controls, represented by the immune sera anti-BKV and anti-JCV; and human serum samples under analysis diluted at 1:20 in LowCross-Buffer (pH 7.2) (Candor Bioscience).

Secondary antibody
The secondary antibody solution contained goat antihuman or antirabbit IgG heavy-chain and light-chain specific peroxidase-conjugate (Calbiochem-Merck, Darmstadt, Germany), diluted 1:10,000 in LowCross-Buffer (Candor Bioscience).

Spectrophotometric reading
Samples treated with 100 µL of 2,2’-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid solution (Sigma-Aldrich, Milan, Italy) were read at the spectrophotometer (model Multiskan EX; Thermo Electron Oy Corporation, Vantaa, Finland) at a wavelength of 405 nanometers. This approach detects the color intensity in wells in which the immunocomplexes were formed by optical density (OD).

Automatic ELISA
Indirect ELISA was transferred and repeated on the automatic processing system with the DSX instrument (Dynex Technologies Inc, Chantilly, Va).

Cutoff determination
The cutoff was determined in each assay by an OD reading of 2 negative controls added to the standard deviation (SD) and multiplied 3 times (+3SD). Sera with antibodies against SV40 were considered to be VP positive if they reacted to both peptides of the late region and when sera, which had been analyzed 3 times by indirect ELISA, provided the same positive result.

Cell, Viruses, and Neutralization Assay
Viral stocks were grown in Vero cells infected with the SV40 776 strain or BKV (Gardner strain). CV-1 monkey kidney cell monolayers, which are permissive to SV40 infection, were used in the neutralization assay using the plaque reduction method.28,38 The neutralization assay included the following controls: 1) SV40 only in phosphate-buffered saline; 2) SV40 mixed with rabbit or human nonimmune serum; 3) SV40 mixed with hyperimmune rabbit serum; and 4) cells only in phosphate-buffered saline. Cultures were observed using a light microscope for the presence of a cytopathic effect (CPE) for 3 weeks. BKV and JCV titrations were performed by hemagglutination and HAI assays.

Specificity of SV40 Synthetic Peptides
Earlier studies have reported comparative analyses of SV40 VP peptides B and C and the corresponding amino acid sequences of new human polyomaviruses and hundreds of different BKV and JCV genetic variants28,29 using the Basic Local Alignment Search Tool (BLAST) program. These data indicated low homology for the BKV and JCV prototypes and other polyomaviruses.28,29

Indirect ELISA with the SV40 B and C polypeptides demonstrated no cross-reactivity with the BKV and JCV hyperimmune sera (negative controls). Characterizations and details have been reported in previous reports.28,29

Statistical Analysis
The prevalence of SV40-positive sera from patients with OS was compared with that in HS and patients with breast cancer (BC) and undifferentiated nasopharyngeal carcinoma (UNPC). Data are presented as a percent. The statistical significance between the 2 groups was determined using a 2-sided chi-square test with Yates correction. The statistical analysis was performed using Prism 4.0 statistical software (GraphPad software, La Jolla, Calif). The serologic profile was statistically analyzed using a Student t test for unpaired data. A P value <.05 was considered to be statistically significant.

RESULTS
Prevalence of Antibodies That React to SV40 VP Mimotopes in Serum Samples From Patients With OS and Controls
Serum samples from patients with OS as well as HS controls were analyzed by indirect ELISA for the presence of
IgG class antibodies against SV40 VP mimotopes, designated as VP1B and VP2/3C.28,29 ELISA indicated that samples that were seropositive for the SV40 VP1B peptide were also positive for the SV40 VP2/3C peptide. Conversely, samples that were seronegative for the SV40 VP1B peptide failed to react with the SV40 VP2/3C peptide. The exceptions were negligible, represented by a few serum samples that were found to be negative for VP1B while testing positive for VP2/3C peptide and vice versa. The difference was not found to be statistically significant (P > .05).

In the current study, sera were considered to be SV40 positive at the time of reaction with both the VP1B and VP2/3C peptides.

Specifically, in serum samples from patients with OS (55 patients), the prevalence of specific SV40 VP antibodies was 44%, whereas it was 17% in the control group of HS (114 individuals) (Tables 1 and 2) (Fig. 1). Patients with OS and the HS control group had the same median age (18 years). Results of the comparative analysis indicated that the prevalence of SV40-positive sera is higher among patients with OS than in young HS. This difference was found to be statistically significant (P < .001) (Table 2) (Fig. 1).

During the second step of the current investigation, we analyzed the prevalence of SV40 VP mimotopes in a cohort of patients with UNPC (64 patients) by indirect ELISA. This control group of patients with cancer was introduced into the study because UNPC is a human tumor associated with herpetic Epstein-Barr virus infection.40 The prevalence of SV40 antibodies determined in serum samples from patients with UNPC was 25%. This difference between OS and UNPC was found to be statistically significant (P < .05) (Table 2). It is interesting to note that the difference in the prevalence of SV40 antibodies between young HS (114 individuals: 17%) and patients with UNPC (64 patients: 25%) was not statistically significant (P > .05) (Table 2) (Fig. 1).

We then analyzed the prevalence of SV40 VP mimotopes in a cohort of patients with BC (78 patients) by indirect ELISA. This control group was introduced into the immunologic analysis because BC is another human tumor that to the best of our knowledge has not been found to be associated with SV40 infection.37 The prevalence of SV40 antibodies determined in serum samples from patients with BC was 15%. The difference between the prevalence of SV40 antibodies in patients with OS versus those with BC was found to be statistically significant (44% vs 15%; P < .001) (Table 2) (Fig. 1).

Serologic profiles of serum samples from patients with OS, UNPC, and BC, as well as HS, analyzed by indirect ELISA are shown in Figure 2.

**ELISA With an Automatic Processing System**

Forty serum samples from patients with OS, processed using the automatic system, gave the same results as previously obtained by manually performed ELISA. This result is of interest because it indicates that our serologic tests can be easily transferred to an automatic processing system in common clinical laboratory analyses without variability in the results.

**SV40 Neutralization Activity**

An inhibition test was performed to verify the neutralization activity of SV40 immune sera. Six SV40 sera samples with high OD (range, 0.300-0.910; diluted 1:20) were assayed to inhibit the SV40 CPE in permissive CV1-infected cells. These sera were negative for BKV and JCV, as determined by HAI testing. SV40 CPE was hampered to different degrees by sera with an OD ranging from 0.315 to 0.775 (grades 1-3) or abolished with a serum
sample with an OD of 0.910 (grade 4), suggesting that immune sera were positive for neutralizing antibodies. We concluded that exposure to SV40 had occurred in those immunized individuals and that the immune serum was elicited by SV40 infection and not by BKV or JCV.

DISCUSSION

In the current study, sera from young patients with OS and HS with the same median age (18 years) were investigated. The presence of IgG antibodies against SV40 antigens was examined by indirect ELISAs with synthetic peptides that mimic VP epitopes.

OS serum samples reacted with SV40 VP mimotopes with a prevalence of 44%, which is statistically significantly higher than in HS, who were the controls.

Sera from patients with BC and UNPC were also investigated to test for the prevalence of SV40 antibodies in other oncologic cohorts, which were used as controls. The rate of SV40 positivity in sera samples from patients with BC was 15%, which is statistically significantly different from what was noted among patients with OS (44%; P < .001), whereas the prevalence did not differ statistically from that detected in HS (17%). The prevalence of SV40 antibodies in patients with UNPC was 25%, which differed significantly from that of patients with OS (44%; P < .05). The prevalence detected in patients with UNPC versus HS was not found to be statistically significantly different.

These data indicate a statistically significant association between OS and SV40, whereas an association was not detected in other patients with BC and UNPC. It is interesting to note that a high prevalence of viral antibodies in serum samples from patients with OS does not prove a causal relationship between SV40 and the onset of OS.

Similar to other cancers, OS is associated with specific chromosome alterations and mutations. However, to the best of our knowledge, the agents responsible for the onset of OS are not known. SV40 is an oncogenic polyomavirus in rodents, whereas it induces transformation of

![Figure 2. Serologic profiles of serum antibody reactivity to the simian virus 40 mimotopes (A) viral capsid protein (VP) 1B, (B) VP2/3C, and (C) VPB plus VPC are shown. Immunologic data were obtained from serum samples from healthy subjects (HS) and patients with osteosarcoma (OS). Results are presented as values of optical density (OD) readings at a wavelength of 405 nanometers and of serum samples diluted at 1:20 detected in indirect enzyme-linked immunoadsorbent assay. In scatter dot plotting, each plot represents the dispersion of OD values to a mean level indicated by the line inside the scatter with the standard error (SE) of the mean for each group of subjects analyzed. The serologic profile of serum antibody reactivity to SV40 mimotopes was statistically analyzed using a Student t test for unpaired data. (A) The mean OD of sera (VPB ± SE) in HS (0.41 ± 0.05) was lower than that in patients with OS (0.21 ± 0.012) (P < .001). (B) The mean OD of sera (VPC ± SE) in HS (0.28 ± 0.025) was lower than that in patients with OS (0.20 ± 0.014) (P < .01). (C) The mean OD (VPB + VPC ± SE) of sera in HS (0.35 ± 0.03) was lower than that in patients with OS (0.21 ± 0.01) (P < .001).]
animal and human cells of different types. Indeed, SV40 demonstrates mutagenic and clastogenic activities in a variety of eukaryotic cells.\textsuperscript{7,24} It is also possible that SV40, as a passenger virus, multiplies better in some transformed cells than in normal cells, which are only semipermissive for its replication.\textsuperscript{26,27}

In healthy young individuals, the prevalence of IgG antibodies against SV40 VP mimotopes is approximately 17%. It is interesting to note that the prevalence of SV40 antibodies reported in the current study is similar to that published by earlier investigations performed with human sera in the United States, which used neutralization assays with the plaque reduction technique to test SV40 infectivity. This assay is highly specific for the neutralization activity of SV40 antibodies.\textsuperscript{31} Moreover, SV40 footprints in the peripheral blood mononucleated cells of HS were noted with a similar prevalence using polymerase chain reaction techniques.\textsuperscript{41} These data indicate that SV40 circulates in humans with a prevalence that is much lower than that of other human polyomaviruses such as BKV and JCV.\textsuperscript{32} It has been proposed that SV40 infection spreads by human-to-human contact in the natural environment. Many studies have reported the detection of SV40 sequences in human samples of different kinds, such as blood, stool, and urine.\textsuperscript{42-48} These results indicate that different SV40 transmission routes exist in humans.\textsuperscript{42-48}

The presence of specific antibodies against SV40 was investigated in serum samples in different tests using immunologic techniques, mainly virus-like particles used as antigens, but the high polypeptide similarity noted among JCV, BKV, and SV40 resulted in cross-reactivity. It was found that our indirect ELISA using SV40 mimotopes is specific and provides reliable results without cross-reactivity.

Our indirect ELISA using SV40 mimotopes as antigens could be used by other investigators in the SV40 field to verify its association with specific cancers such as OS. It is interesting to note that this indirect ELISA has been successfully transferred to an automatic processing system without any resulting variability.

The results of the current study demonstrate that SV40 infectivity inhibition using SV40-positive serum samples is highly significant. Indeed, these results confirm and extend the data from earlier investigations, which indicated a correlation between the level of antibodies in immune sera and the grade of SV40 infection inhibition.\textsuperscript{37,38} In this comparison, it should be recalled that the SV40 immune sera used were negative for both JCV and BKV, thereby indicating that there were no cross-inhibitions.

The current study on the association between OS and SV40 does not prove that SV40 is involved in the development of this malignancy. At the same time, it cannot be excluded that SV40, after infecting the human host, may exert its tumorigenic power during the multistep mechanism of oncogenesis. If SV40 is a tumor agent in the onset of OS, new antiviral therapies could be used in clinical cases.

As an alternative explanation of our immunologic data, it is possible that another human polyomavirus similar to SV40, which is still unknown, could be responsible for the results noted herein.

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**CONFLICT OF INTEREST DISCLOSURES**

The authors made no disclosures.

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