Original Article

Significant Association Between Human Osteosarcoma and Simian Virus 40

Elisa Mazzoni, PhD[1]; Maria Serena Benassi, PhD[2]; Alfredo Corallini, PhD[3]; Giovanni Barbanti-Brodano, MD[4]; Angelo Taronna, PhD[3]; Piero Picci, MD[2]; Giovanni Guerra, PhD[5]; Antonio D'Agostino, MD[6]; Lorenzo Trevisiol, MD[6]; Pier Francesco Nocini, MD[6]; Maria Vittoria Casali, MD[7]; Giuseppe Barbanti-Brodano, MD[3]; Fernanda Martini, PhD[1]; and Mauro Tognon, PhD[1]

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 immunoadsorbent 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,[9-17] whereas other studies have not reported SV40 in tumor samples, including OS.[18-20] 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]

Corresponding author: Mauro Tognon, PhD, or Fernanda Martini, PhD, Section of Pathology, Oncology and Experimental Biology, School of Medicine, University of Ferrara, 64/B, Fossato di Mortara St, 44121 Ferrara, Italy; Fax: (011) 39-0532-455533; tgm@unife.it or mrf@unife.it

[1]Section of Pathology, Oncology and Experimental Biology, School of Medicine, University of Ferrara, Ferrara, Italy; [2]Laboratory of Experimental Oncology, Rizzoli Orthopaedic Institute, Bologna, Italy; [3]Section of Microbiology, Schools of Medicine and Sciences, University of Ferrara, Ferrara, Italy; [4]Spine Unit, Rizzoli Orthopaedic Institute, Bologna, Italy; [5]Clinical Laboratory Analysis, University Hospital, Ferrara, Italy; [6]Section of Oral and Maxillofacial Surgery, Department of Surgery, University of Verona, Verona, Italy; [7]Hospital Headquarter Department, State Hospital, Institute for the Social Security, Republic of San Marino, Italy

We would like to thank Dr. Eugene O. Major of the Laboratory of Molecular Medicine and Neuroscience of the National Institute of Neurological Disorders and Strokes at the National Institutes of Health in Bethesda, Maryland for the hyperimmune serum against the John Cunningham (JC) virus.

Dr. Elisa Mazzoni is a postdoctoral fellow of the Fondazione Veronesi in Milan, Italy.

DOI: 10.1002/cncr.29137, **Received:** July 21, 2014; **Revised:** October 14, 2014; **Accepted:** October 15, 2014, **Published online** November 6, 2014 in Wiley Online Library (wileyonlinelibrary.com)

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.[26,27] 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*/*tag* bind and abolish the functions of tumor suppressor *p53* and *pRB* gene products.[24] 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.[7] 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,[1] as well as in healthy individuals.[7,28-31] 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.[7] 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.[8]

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.[25]

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.[18,32-36] It is possible that these results have influenced the negative statistical data reported in subsequent studies.[8]

Recent investigations performed with an indirect enzyme-linked immunoadsorbent 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).[28-30,37-39]

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).[37] 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/nuccore). The amino acid similarity analysis of BKV and JCV and other polyomaviruses with SV40 has been previously reported.[28,29] 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.[28,29] The 2 SV40 peptides, known as VP1B and VP2/3C, have the following sequences:

TABLE 1. Osteosarcoma Types and SV40-Positive Samples

Bone Malignancy	No. of Samples	Male, %	Median Age ± SD (Range), Years	OS Grade (WHO)/Type	SV40-Positive Sample/OS Samples Analyzed (%)
OS	55	53	18 ± 16.77 (7-76)	Hemorrhagic OS	2/2 (100)
				Hemorrhagic OS with pulmonary metastases from sarcoma	1/1 (100)
				Parosteal low-grade OS (grade 1)	1/1 (100)
				High-grade OS in fibrous dysplasia (grade 4)	1/1 (100)
				High-grade OS with pulmonary metastases since diagnosis and fibrous dysplasia (grade 4)	1/1 (100)
				High-grade OS and pulmonary metastases from sarcomas (grade 4)	2/4 (50)
				High-grade OS and bone metastases from sarcomas (grade 4)	1/2 (50)
				High-grade OS (grade 4)	15/37 (40)
				High-grade OS with pulmonary metastases since diagnosis	0/1
				OS surface	0/1
				Multicentric OS	0/1
				OS in Paget disease	0/1
				OS in other benign damage with alterations from hyperparathyroidism	0/1
				OS (grade 3)	0/1
				Total sera	24/55 (44)

Abbreviations: OS, osteosarcoma; SD, standard deviation; SV40, simian virus 40.

TABLE 2. Prevalence of Serum IgG Antibodies Reacting With SV40 VP Mimotopes in Serum Samples From Patients With OS, BC, and UNPC and HS

Human Sera	No. of Patients/ Individuals	Median Age, Years	Male Sex, %	No. of Positive Samples (%)		
				VPB	VPC	VPB+ VPC
OS	55	18	53	24 (44)	25 (45)	24 (44)[a]
HS	114	18	58	30 (26)	21 (18)	20 (17)
BC	78	42	—	14 (18)	13 (17)	12 (15)[b]
UNPC	64	57	81	16 (25)	17 (27)	16 (25)

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.

[a] 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 ($P<.001$).

[b] The prevalence of SV40 antibodies in sera from patients with OS differed statistically from that of patients with BC ($P<.001$) and from that of patients with UNPC ($P<.05$). Statistical analysis was performed using the chi-square test.

VP1B: NH2-NPDEHQKGLSKSLAAEKQFDDS P-COOH

VP2/3C: NH2-IQNDIPRLTSQELERRTQRYLR D-COOH

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.[28,29] The polypeptides were synthesized as previously reported.[28]

The hyperimmune serum samples that reacted with SV40 and BKV were obtained from rabbits immunized with purified viral stocks, as previously reported.[28] The serum against JCV was kindly provided by Dr. Eugene O. Major of the Laboratory of Molecular Medicine and Neuroscience of the National Institute of Neurological Disorders and Strokes at the National Institutes of Health in Bethesda, Maryland. The immune serum anti-BKV was titered using the hemagglutination inhibition (HAI) test

using human erythrocytes from the 0, Rh factor-positive group.[28] Anti-SV40 serum was titered by neutralization assay.[7]

Indirect ELISA

Indirect ELISA was developed and standardized to detect specific antibodies against SV40 in human sera using synthetic peptides.[28,29]

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.[7]

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 variants[28,29] 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

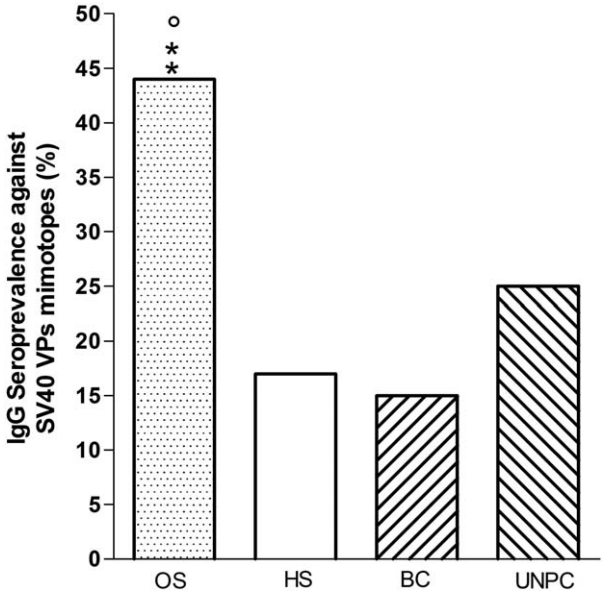


Figure 1. Prevalence of simian virus 40 (SV40)-positive serum samples from patients with osteosarcoma (OS), undifferentiated nasopharyngeal carcinoma (UNPC), and breast cancer (BC), as well as from healthy subjects (HS), is shown. To compare the SV40 prevalence among patients with OS with that detected in HS used as controls, HS with the same median age and sex as the patients were selected. Statistical analyses revealed significant differences in SV40 prevalence among patients with OS and the relative controls, cohorts of HS and patients with BC ($P<.001$), and patients with UNPC ($P<.05$). Statistical analysis was performed using the chi-square test. IgG indicates immunoglobulin G; VPs, viral capsid proteins.

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.

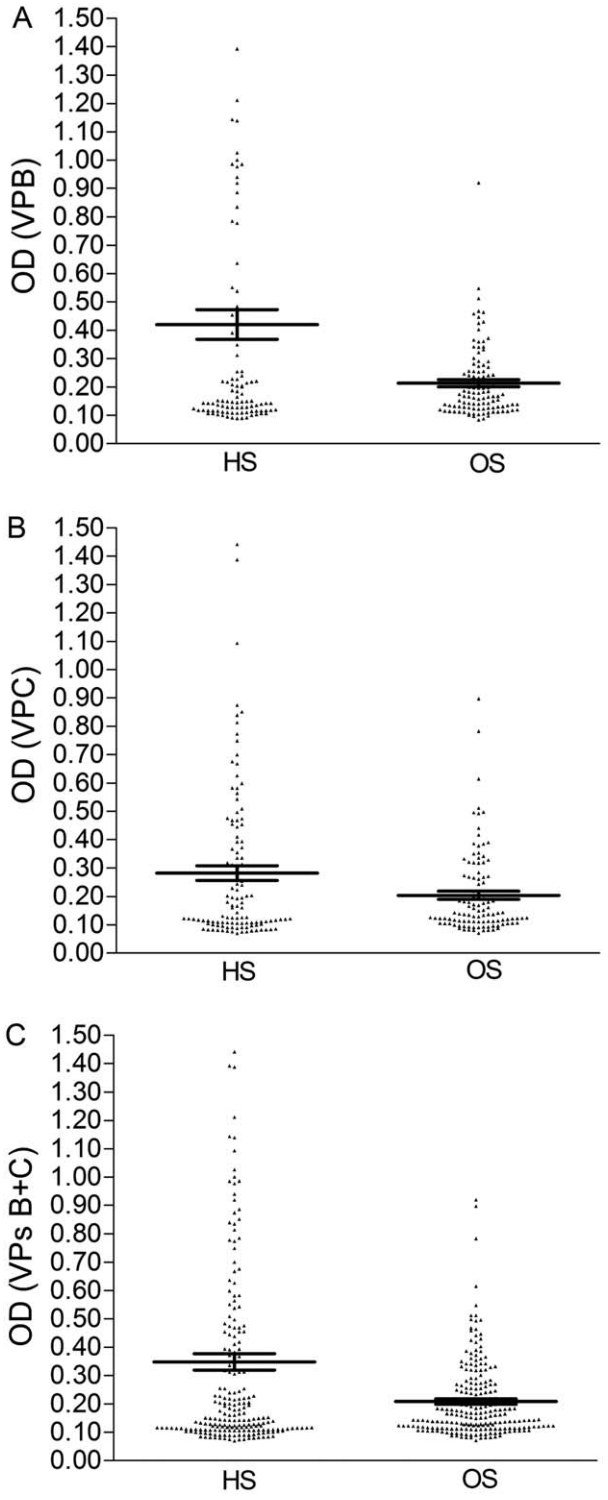
ELISA With an Automatic Processing System

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.[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.[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.[31] Moreover, SV40 footprints in the peripheral blood mononucleated cells of HS were noted with a similar prevalence using polymerase chain reaction techniques.[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.[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.[42-48] These results indicate that different SV40 transmission routes exist in humans.[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.[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.

FUNDING SUPPORT

Supported in part by grants from ASLEM, Republic of San Marino; Regione Emilia-Romagna, Bologna; ISS, Rome; Fondazione Cassa di Risparmio di Bologna, Bologna; Fondazione Cassa di Risparmio di Cento, Cento; EU FP7 Eurosarc; MIUR PRIN COFIN 2008; University of Verona and University Hospital of Verona, Verona; University Hospital Sant'Anna, Ferrara; University of Ferrara, FAR Projects, Ferrara, Italy.

CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

REFERENCES

1. Gorlick R, Janeway K, Lessnick S, Randall RL, Marina N; COG Bone Tumor Committee. Children's Oncology Group's 2013 blueprint for research: bone tumors. *Pediatr Blood Cancer*. 2013;60:1009-1015.
2. Diamandopoulos GT. Leukemia, lymphoma, and osteosarcoma induced in the Syrian golden hamster by simian virus 40. *Science*. 1972;176:173-175.
3. Knowles BB, McCarrick J, Fox N, Solter D, Damjanov I. Osteosarcomas in transgenic mice expressing an alpha-amylase-SV40 T-antigen hybrid gene. *Am J Pathol*. 1990;137:259-262.
4. Link BG, Phelan J. The social shaping of health and smoking. *Drug Alcohol Depend*. 2009;104(suppl 1):S6-S10.
5. Wilkie TM, Schmidt RA, Baetscher M, Messing A. Smooth muscle and bone neoplasms in transgenic mice expressing SV40 T antigen. *Oncogene*. 1994;9:2889-2895.
6. Malkin D, Chilton-MacNeill S, Meister LA, Sexsmith E, Diller L, Garcea RL. Tissue-specific expression of SV40 in tumors associated with the Li-Fraumeni syndrome. *Oncogene*. 2001;20:4441-4449.
7. Barbanti-Brodano G, Sabbioni S, Martini F, Negrini M, Corallini A, Tognon M. Simian virus 40 infection in humans and association with human diseases: results and hypotheses. *Virology*. 2004;318:1-9.
8. International Agency for Research on Cancer. Malaria and Some Polyomaviruses (SV40, BK, JC, and Merkel Cell Viruses). In: World Health Organization, ed. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Monograph 104, Lyon: IARC, 2013:133-214.
9. Carbone M, Rizzo P, Procopio A, et al. SV40-like sequences in human bone tumors. *Oncogene*. 1996;13:527-535.
10. Lednicky JA, Stewart AR, Jenkins JJ 3rd, Finegold MJ, Butel JS. SV40 DNA in human osteosarcomas shows sequence variation among T-antigen genes. *Int J Cancer*. 1997;72:791-800.
11. Mendoza SM, Konishi T, Miller CW. Integration of SV40 in human osteosarcoma DNA. *Oncogene*. 1998;17:2457-2462.
12. Gamberi G, Benassi MS, Pompetti F, et al. Presence and expression of the simian virus-40 genome in human giant cell tumors of bone. *Genes Chromosomes Cancer*. 2000;28:23-30.

13. Yamamoto H, Nakayama T, Murakami H, et al. High incidence of SV40-like sequences detection in tumour and peripheral blood cells of Japanese osteosarcoma patients. *Br J Cancer*. 2000;82:1677-1681.
14. Heinsohn S, Scholz RB, Weber B, et al. SV40 sequences in human osteosarcoma of German origin. *Anticancer Res*. 2000;20:4539-4545.
15. Martini F, Lazzarin L, Iaccheri L, et al. Different simian virus 40 genomic regions and sequences homologous with SV40 large T antigen in DNA of human brain and bone tumors and of leukocytes from blood donors. *Cancer*. 2002;94:1037-1048.
16. Heinsohn S, Szendroi M, Bielack S, Stadt UZ, Kabisch H. Evaluation of SV40 in osteosarcoma and healthy population: a Hungarian-German study. *Oncol Rep*. 2009;21:289-297.
17. Ziadi S, Boughamoura H, Ben Maitig M, et al. Immunodetection of SV40 T/t-antigens in human osteosarcoma in a series of Tunisian patients. *Pathol Oncol Res*. 2012;18:691-696.
18. Carter JJ, Madeleine MM, Wipf GC, et al. Lack of serologic evidence for prevalent simian virus 40 infection in humans. *J Natl Cancer Inst*. 2003;95:1522-1530.
19. Shah KV, Galloway DA, Knowles WA, Viscidi RP. Simian virus 40 (SV40) and human cancer: a review of the serological data. *Rev Med Virol*. 2004;14:231-239.
20. Engels EA. Cancer risk associated with receipt of vaccines contaminated with simian virus 40: epidemiologic research. *Expert Rev Vaccines*. 2005;4:197-206.
21. Klein G, Powers A, Croce C. Association of SV40 with human tumors. *Oncogene*. 2002;21:1141-1149.
22. Carbone M, Rdzanek MA, Rudzinski JJ, et al. SV40 detection in human tumor specimens. *Cancer Res*. 2005;65:10120-10121.
23. Fisher SG, Weber L, Carbone M. Cancer risk associated with simian virus 40 contaminated polio vaccine. *Anticancer Res*. 1999;19:2173-2180.
24. Martini F, Corallini A, Balatti V, Sabbioni S, Pancaldi C, Tognon M. Simian virus 40 in humans. *Infect Agent Cancer*. 2007;2:13.
25. Stratton K, Almario DA, McCormick MC, eds. Immunization Safety Review: SV40 Contamination of Polio Vaccine and Cancer. Washington, DC: The National Academies Press, 2003.
26. Alaribe FN, Mazzoni E, Rigolin GM, et al. Extended lifespan of normal human B lymphocytes experimentally infected by SV40 or transfected by SV40 large T antigen expression vector. *Leuk Res*. 2013;37:681-689.
27. Mazzoni E, Rigolin GM, Alaribe FN, et al. Simian virus 40 efficiently infects human T lymphocytes and extends their lifespan. *Exp Hematol*. 2012;40:466-476.
28. Corallini A, Mazzoni E, Taronna A, et al. Specific antibodies reacting with simian virus 40 capsid protein mimotopes in serum samples from healthy blood donors. *Hum Immunol*. 2012;73:502-510.
29. Taronna A, Mazzoni E, Corallini A, et al. Serological evidence of an early seroconversion to Simian virus 40 in healthy children and adolescents. *PLoS One*. 2013;8:e61182.
30. Mazzoni E, Tognon M, Martini F, et al. Simian virus 40 (SV40) antibodies in elderly subjects. *J Infect*. 2013;67:356-358.
31. Wong C, Vilchez RA, Quiroz J, Adam E, Butel JS. Ethnic differences in polyomavirus simian virus 40 seroprevalence among women in Houston, Texas. *J Infect*. 2013;66:67-74.
32. Viscidi RP, Rollison DE, Viscidi E, et al. Serological cross-reactivities between antibodies to simian virus 40, BK virus, and JC virus assessed by virus-like-particle-based enzyme immunoassays. *Clin Diagn Lab Immunol*. 2003;10:278-285.
33. Lundstig A, Eliasson L, Lehtinen M, Sasnauskas K, Koskela P, Dillner J. Prevalence and stability of human serum antibodies to simian virus 40 VP1 virus-like particles. *J Gen Virol*. 2005;86(pt 6):1703-1708.
34. Kjaerheim K, Roe OD, Waterboer T, et al. Absence of SV40 antibodies or DNA fragments in prediagnostic mesothelioma serum samples. *Int J Cancer*. 2007;120:2459-2465.
35. Kean JM, Rao S, Wang M, Garcea RL. Seroepidemiology of human polyomaviruses. *PLoS Pathog*. 2009;5:e1000363.
36. Ribeiro T, Fleury MJ, Granieri E, et al. Investigation of the prevalence of antibodies against neurotropic polyomaviruses BK, JC and SV40 in sera from patients affected by multiple sclerosis. *Neurol Sci*. 2010;31:517-521.
37. Martini F, Mazzoni E, Corallini A, et al. Breast cancer and simian virus 40 infection. *Epidemiology*. 2013;24:464-465.
38. Mazzoni E, Corallini A, Cristaudo A, et al. High prevalence of serum antibodies reacting with simian virus 40 capsid protein mimotopes in patients affected by malignant pleural mesothelioma. *Proc Natl Acad Sci U S A*. 2012;109:18066-18071.
39. Mazzoni E, Gerosa M, Lupidi F, et al. Significant prevalence of antibodies reacting with simian virus 40 mimotopes in sera from patients affected by glioblastoma multiforme. *Neuro Oncol*. 2014;16:513-519.
40. Chan KC, Hung EC, Woo JK, et al. Early detection of nasopharyngeal carcinoma by plasma Epstein-Barr virus DNA analysis in a surveillance program. *Cancer*. 2013;119:1838-1844.
41. Pancaldi C, Balatti V, Guaschino R, et al. Simian virus 40 sequences in blood specimens from healthy individuals of Casale Monferrato, an industrial town with a history of asbestos pollution. *J Infect*. 2009;58:53-60.
42. Butel JS, Arrington AS, Wong C, Lednicky JA, Finegold MJ. Molecular evidence of simian virus 40 infections in children. *J Infect Dis*. 1999;180:884-887.
43. Comar M, Zanotta N, Bovenzi M, Campello C. JCV/BKV and SV40 viral load in lymphoid tissues of young immunocompetent children from an area of north-east Italy. *J Med Virol*. 2010;82:1236-1240.
44. Li RM, Branton MH, Tanawattanacharoen S, Falk RA, Jennette JC, Kopp JB. Molecular identification of SV40 infection in human subjects and possible association with kidney disease. *J Am Soc Nephrol*. 2002;13:2320-2330.
45. Li RM, Mannon RB, Kleiner D, et al. BK virus and SV40 co-infection in polyomavirus nephropathy. *Transplantation*. 2002;74:1497-1504.
46. Patel NC, Vilchez RA, Killen DE, et al. Detection of polyomavirus SV40 in tonsils from immunocompetent children. *J Clin Virol*. 2008;43:66-72.
47. Vanchiere JA, White ZS, Butel JS. Detection of BK virus and simian virus 40 in the urine of healthy children. *J Med Virol*. 2005;75:447-454.
48. Vanchiere JA, Abudayyeh S, Copeland CM, Lu LB, Graham DY, Butel JS. Polyomavirus shedding in the stool of healthy adults. *J Clin Microbiol*. 2009;47:2388-2391.