



Lymphoproliferative disorders in Costa Rica and simian virus 40

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Background and Objectives. Simian virus 40 (SV40) is an oncogenic DNA virus implicated in some human malignancies, including lymphomas. In the present masked case-control study, we investigated the prevalence of SV40 sequences and the expression of the viral oncoprotein, large tumor antigen (T-ag), in lymphomas and control specimens from patients negative for the human immunodeficiency virus in Costa Rica.

Design and Methods. Coded specimens were analyzed by polymerase chain reaction for SV40 and Epstein-Barr virus (EBV). SV40 sequences were confirmed by Southern blot and DNA sequence analysis. Immunohistochemistry was used to detect the expression of SV40 T-ag in coded samples and to immunophenotype the lymphomas.

Results. When samples were decoded, SV40 DNA sequences were detected significantly more often in lymphomas than in control samples (30/125, 24% vs. 0/91, 0%; $p=0.001$). SV40 DNA was detected in 26% and 10% of non-Hodgkin's and Hodgkin's lymphomas, respectively. EBV DNA was detected in 10% of lymphomas and 33% of control specimens. None of the lymphomas was positive for both SV40 and EBV. Expression of SV40 T-ag was detected in 64% of B-cell lymphomas that contained T-ag DNA sequences and in none of the samples negative for viral DNA. Not all cells in a positive tumor expressed T-ag and the reactions were relatively low intensity. A germinal center B-cell-like profile was frequently associated with SV40-positive lymphomas. Of note, 20% of patients with SV40-related lymphomas were born in the 1970s and 1980s.

Interpretation and Conclusions. These results indicate that SV40 is significantly associated with some B-cell neoplasms in Costa Rica today.

Key words: SV40, T-antigen, non-Hodgkin's lymphomas, lymphomas, Epstein-Barr virus, immunohistochemistry, polymerase chain reaction, human cancer, Costa Rica.

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Lymphomas represent a diverse group of malignancies arising from the lymphopoietic system. Two major groups of lymphoproliferative disorders are non-Hodgkin's lymphoma (NHL) and Hodgkin's lymphoma. The yearly incidence rates of NHL range from over 15/100,000 in the United States to 1.2/100,000 in China, with intermediate rates in Latin America, Africa and Japan.¹⁻⁵ Although genetic and acquired immunodeficiencies are known risk factors, a viral etiology is considered likely to play a role in some lymphomas.^{3,6-9} Lymphomas are recognized as a common neoplasm arising in the hamster model of polyomavirus simian virus 40 (SV40) infection¹⁰⁻¹³ and the transforming capacity of the virus has been demonstrated in human lymphocytes *in vitro*.¹⁴ Independent studies have shown that SV40 DNA sequences are associated with some NHL and Hodgkin's lymphoma

among human immunodeficiency virus (HIV)-infected and -uninfected patients,¹⁵⁻²³ with the frequency of positive tumors ranging from 11% to 50%, and with pediatric lymphoid malignancies (77%).²⁴ A recent report described the specific promoter methylation of several tumor suppressor genes in SV40-positive NHL that were not methylated in Epstein-Barr virus (EBV)-positive NHL,²² and a meta-analysis of the molecular biology data of case-control studies of SV40 in human malignancies found a significant excess risk of SV40 associated with NHL.²⁵ The Institute of Medicine of the National Academies recognized that SV40 is an emergent human pathogen having oncogenic properties and concluded that the present biological evidence (moderate strength) indicates that SV40 infections could lead to cancer in humans under natural conditions.²⁶

The history of the discovery of SV40 and its introduction into the human population is related to the development and distribution of early forms of the poliovaccine.²⁶⁻²⁹ Early inactivated and live attenuated forms of poliovaccines were inadvertently contaminated with SV40 as those vaccine formulations were prepared in primary cultures of kidney cells derived from rhesus monkeys, which are often naturally infected with SV40. Infectious SV40 survived the killed vaccine inactivation treatments, and estimates indicate that millions of people in North and South America, Europe and Asia were exposed to live SV40 from 1955 through 1963 when administered potentially contaminated killed and live poliovaccines.^{26,27,29} Although the incidence of SV40 infections in humans linked to those vaccines is not known, data suggest that geographical differences exist in the frequency of SV40-positive human malignancies, perhaps reflecting the extent of past usage of contaminated poliovaccines and the present rate of SV40 infections in different populations.^{27,28}

Previous analyses of SV40 and human lymphomas have been limited geographically to a few countries and have focused on detection of viral DNA sequences.^{15-22,24,30} Studies in different human populations with SV40 infections are necessary to test the specificity of the relationship of SV40 with lymphoproliferative disorders. In addition, it is predicted that virus-caused tumors will express a viral transforming protein detectable in at least some cells,⁸ so human lymphomas need to be examined for expression of the SV40 oncoprotein, large tumor antigen (T-ag). Here, in a masked case-control study, we investigated the prevalence of SV40 detection, the expression of T-ag, and the immunophenotypic features of lymphoproliferative disorders showing SV40 markers in Costa Rica, a country in Latin America whose population was exposed to potentially contaminated poliovaccines.^{31,32}

Design and Methods

Poliovirus vaccinations in Costa Rica

Costa Rica suffered a severe outbreak of type 1 poliomyelitis in 1954. A limited vaccination program with the killed (Salk) vaccine was carried out in 1956–1958. A nationwide campaign was initiated in the following year, March 1959, to vaccinate all children under 11 years of age in Costa Rica with live attenuated poliovirus vaccine. The vaccine was supplied by the U.S. (Lederle Laboratories). By April 1960, 71% of the estimated total population of 382,905 children under 11 years old had been vaccinated. Vaccination was conducted in all provinces, with the geographic distribution of those vaccinated ranging from as many as 80% of children in the San Jose and Limon provinces to as few as 54% in the Guanacaste province. In the

metropolitan area of San Jose, 98% of children were vaccinated.^{31,32}

Patients and biopsy samples

Two hundred and six HIV-uninfected, chemotherapy-naïve patients diagnosed with systemic lymphomas from January 1999 through December 2003 at the Hospital San Juan de Dios and Hospital Mexico in San Jose, Costa Rica were studied. Central nervous system lymphomas were not included. Control tissues included reactive lymph nodes and tonsils from 132 HIV-negative patients without lymphoma or any type of cancer and gastric and hepatic carcinoma from 50 HIV-negative patients. Specimens from cases and controls were collected, fixed and embedded in paraffin for clinical diagnosis, coded at each hospital in San Jose, Costa Rica, and shipped to Baylor College of Medicine (Houston, TX, USA) for a masked molecular analysis. Institutional Review Board approval was obtained at each institution for this study.

Extraction of total DNA from tissue samples

Two 20-micron sections of tissue from paraffin-embedded biopsy specimens were processed to obtain total cellular DNA using a proteinase K digestion and phenol-chloroform extraction method.³³ All sample processing was performed in a laminar flow hood within a BL3 facility free from viruses and plasmids at the Department of Molecular Virology and Microbiology, Baylor College of Medicine.

Polymerase chain reaction, amplification, Southern blot and DNA sequence analysis

All polymerase chain reaction (PCR) assays were set up in the *PCR Clean Rooms* core facility of the Department of Molecular Virology and Microbiology at Baylor College of Medicine to avoid contamination of reaction mixtures. As a further precaution, positive displacement pipettors and barrier tip pipettes were used. Oligonucleotide primers used for PCR and DNA sequence analysis have been described previously.³⁴⁻³⁸ All of the DNA samples were tested for suitability for amplification using primers specific for a fragment of the human β -hemoglobin gene (P0C3/KM38).³⁹ Only specimens from which cellular β -globin gene sequences were amplified were then examined by PCR amplification using primers for SV40 (PYVfor/PYVrev) and EBV (LMP-2).³³ Primers were obtained from Integrated DNA Technologies, Inc. (Coralville, IA, USA). Positive control plasmids were added to the control PCR reactions outside of the core facility after tubes containing negative controls and test DNA had been closed. The positive control for SV40 PCR reactions was plasmid DNA containing a cloned SV40 genome.³³ The positive control for EBV reactions was DNA extracted from the EBV-positive Burkitt lymphoma cell line Namalwa.

Negative controls for PCR assays were reactions without added DNA template. Each reaction contained 1.75 µg of DNA in a 50-µL reaction volume (approximately 2.6×10^5 cell equivalents, assuming that 1 µg DNA represents about 150,000 cells). PCR amplifications were performed using a Perkin-Elmer GeneAmp PCR system 2400 thermocycler and high-stringency annealing temperatures specific for each primer set as described elsewhere.³⁴⁻³⁸ PCR amplification products were analyzed by agarose gel electrophoresis following standard protocols.³³

Specific SV40 ³²P-γ-ATP-labeled oligoprobes were used to confirm amplified sequences of the large T-ag gene.^{34,38,40,41} Electrophoresed PCR products were transferred to a nylon membrane and the DNA was cross-linked to filters by UV irradiation for 2 min. The prehybridization process was performed at 46°C for 30 min to 1 h in 30 mL 6×saline sodium phosphate-EDTA (SSPE)/0.5% sodium dodecyl sulfate (SDS)/Denhardt's reagent prewarmed to 46°C. The hybridization process was carried out overnight at 46°C in 20 mL 6×SSPE/10% dextran sulfate and labeled oligoprobes prewarmed to 46°C. The membrane was washed twice with 50 mL 2× saline sodium citrate (SSC) at room temperature for 5 min each, then twice with 50 mL 2×SSC/1% SDS at 46°C for 5 min each and twice with 50 ml 0.1×SSC at room temperature for 5 min each. Autoradiography was carried out at -80°C overnight. In addition, SV40 PCR products were sequenced to confirm the identity of the SV40-specific DNA from the positive human samples.

Immunohistochemistry

The paraffin sections (5 µm) of lymphomas and control specimens were first stained with hematoxylin and eosin and then reviewed histologically for morphologic features both at the hospitals in San Jose, Costa Rica and at Baylor College of Medicine. Lymphomas were categorized according to the World Health Organization Classification for Neoplastic Diseases of the Lymphoid Tissues.³ The antigen retrieval procedure consisted of steaming slides in citrate buffer (0.01 M, pH 6.0) or Tris-HCl buffer (0.1 M, pH 9.0) for 20 min. All slides were immersed in 3% H₂O₂ for 10 min to block the endogenous peroxidase. Primary mouse monoclonal antibodies and dilutions used for immunophenotyping were CD3 (Lab Vision Corp., Fremont, CA, USA; 1:60), CD5 (DakoCytomation, Carpinteria, CA, USA; 1:25), CD15 (BD Biosciences, San Jose, CA, USA; 1:50), CD20 (1:600), CD23 (1:50), CD30 (1:20), CD45 (1:100), CD246 (1:25) (DakoCytomation) and bcl-2 (BioGenex, San Ramon, CA, USA; 1:150). In addition, staining was performed to identify expression of markers of germinal center B-cell-like (GC) and non-GC diffuse large B-cell lymphoma (DLBCL). Sections were stained with mouse

monoclonal antibodies to CD10 (Novacastra Laboratories, Newcastle, UK; 1:50), bcl-6 (DakoCytomation, 1:100), and MUM1/IRF4 (DakoCytomation, 1:100). All the described antibodies were detected using the EnVision+ Labeled Polymer Kit (DakoCytomation) as described by the manufacturer, followed by application of the liquid Diaminobenzidine (DAB) Substrate Pack (DakoCytomation). The latter set of antibodies recognized molecules whose mRNA expression was highly associated with GC or non-GC DLBCL in cDNA microarray studies;⁴² recent studies provided evidence that immunostaining was a valid method that resulted in similar classification of this group of lymphomas.⁴³ The expression of SV40 T-ag was examined as described previously.^{44,45} Deparaffinized sections were rehydrated and rinsed using Tris-buffered saline (TBS) (Signet Laboratories, Inc., Dedham, MA, USA) three times. Sections were then incubated at 4°C overnight with monoclonal antibodies against the N-terminus (PAb416, Oncogene Research Products, San Diego, CA, USA) and C-terminus (PAb101, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) of SV40 T-ag. Both antibodies were used at a dilution of 1:1000 as recommended by the manufacturers. After rinsing, sections were incubated with a mouse probe-polymer reagent (Biocare Medical, Walnut Creek, CA, USA) for 20 min for detection. Sections were washed three times with TBS, followed by DAB, as above, then counterstained with Mayer's modified hematoxylin. The staining was examined and scored for cell type, localization of staining and percentage of lymphoma cells and as negative or positive for lymphocyte markers and SV40 T-ag reactivity. SV40-induced hamster tumors were used as positive controls.⁴⁴

Statistical analysis

Group proportions among cases and control groups were tested with the Z test of proportions using the STATA 6.0 software package (College Station, TX, USA). *p* values of <0.05 were considered statistically significant.

Results

Detection of SV40 and EBV DNA sequences in masked lymphoma and control specimens

As degradation of nucleic acids and proteins is a common problem with formalin-fixed and paraffin-embedded specimens, only those samples from which cellular β-globin gene sequences were amplified successfully were deemed suitable for further analysis in this study. Two-hundred and sixteen (56%) of the 388 biopsy specimens were positive for amplifiable β-globin gene sequences and were examined blindly for SV40 and EBV DNA sequences. Thirty samples yielded SV40

Table 1. Immunophenotypes and viral sequences in lymphoproliferative disorders in Costa Rica.

Morphology	Totals	No. of samples (%) SV40 DNA positive	EBV DNA positive	Expression of lymphocyte markers
Non-Hodgkin's	106	28 (26%)	10 (9%)	
Precursor B-cell lymphoblastic lymphoma*	4	0	1	CD3 ⁺ , CD20 ⁺
Small B-lymphocytic lymphoma	1	0	0	CD3 ⁺ , CD5 ⁺ , CD23 ⁺
Extranodal marginal zone B-cell lymphoma	3	0	0	CD3 ⁺ , CD20 ⁺ , CD45 ⁺
Follicular lymphoma	22	7 (32%)	1	CD3 ⁺ , CD20 ⁺ , bcl-2 ⁺
Mantle cell lymphoma	1	0	0	CD3 ⁺ , CD5 ⁺ , CD20 ⁺
Diffuse large B-cell	68	21 (31%)	6	CD3 ⁺ , CD20 ⁺ , CD45 ⁺ , CD246 ⁺
Burkitt lymphoma	1	0	0	CD3 ⁺ , CD20 ⁺ , CD45 ⁺
Malignant lymphoma	3	0	0	CD3 ⁺ , CD20 ⁺ , CD45 ⁺ , CD246 ⁺
Mycosis fungoides	1	0	1	CD3 ⁺ , CD20 ⁺
Mature T-cell lymphoma, NOS	1	0	0	CD3 ⁺ , CD20 ⁺ , CD45 ⁺
NK/T-cell lymphoma	1	0	1	CD3 ⁻ , CD20 ⁺ , CD45 ⁺
Hodgkin's	19	2 (10%)	2 (10%)	
Classic	2	0	1	CD15 ⁺ , CD20 ⁺ , CD30 ⁺
Mixed cellularity	4	0	1	CD15 ⁺ , CD20 ⁺ , CD30 ⁺
Nodular sclerosis	13	2	0	CD15 ⁺ , CD20 ⁺ , CD30 ⁺
Grand Totals	125	30 (24%)	12 (10%)	

*Terminal deoxynucleotidyl transferase positive.

PCR products whereas 42 samples were EBV-positive. The amplified products obtained from the biopsy samples using the universal polyomavirus primers were analyzed by Southern blot hybridization using probes for SV40. The products were confirmed as SV40-specific sequences in all cases. Next, sequence analysis was carried out on amplified products obtained from samples using the large T-ag primers. By sequence analysis the DNA sequences were identical to those of the SV40 large T-ag gene. Although the primers have the ability to amplify BK virus and JC virus sequences, the polyomavirus-positive NHL specimens did not contain either JC virus or BK virus DNA as evidenced by the nucleotide sequence and the diagnostic absence of a 9 basepair insert in the viral genomic product.

After completing the molecular analyses, samples were decoded. The 216 patients with specimens that could be analyzed included 125 lymphoma patients and 91 controls. The demographic characteristics of the two groups (lymphoma, control) were mean ages of 51 and 45 years and male/female ratios of 55/70 and 51/40, respectively. The data revealed that SV40 large T-ag gene sequences were amplified from 30 of 125 (24%) lymphoma samples (Table 1) and from none of the 51 reactive lymph nodes and tonsil samples or the 40 gastric and hepatic carcinoma control samples (Table 2). The detection of SV40 T-ag DNA sequences in lymphomas differed significantly when compared to that in non-malignant lymphoid samples (30/125, 24% vs. 0/51, 0%; $p=0.001$) or carcinoma samples (30/125, 24% vs. 0/40, 0%; $p=0.001$). EBV DNA was detected in 12 (10%) of 125 lymphoma samples (Table 1), in 24 (47%)

Table 2. Viral DNA sequences in control tissues from Costa Rica.

Type of control sample	No. of samples	SV40 DNA-positive No. (%)	EBV DNA-positive No. (%)
Lymphoid (reactive lymph nodes, tonsils)	51	0 (0)	24 (47)
Carcinomas (gastric, hepatic)	40	0 (0)	6 (15)
Total control tissues	91	0 (0)	30 (33)

of 51 samples in the non-malignant lymphoid control group, and in 6 (15%) of 40 carcinoma specimens (Table 2). It is known that EBV can colonize the tonsils, and a recent study in Taiwan detected the EBV LMP-1 gene by PCR in 29/57 (50.9%) non-neoplastic tonsils.⁴⁶ None of the lymphoma samples was positive for both EBV and SV40 sequences.

Expression of SV40 T-ag in viral DNA-positive lymphomas

The morphologic and immunophenotypic features of the 125 lymphoma cases are summarized in Table 1. SV40 DNA was detected in 26% (28/106) of NHL and 10% (2/19) of Hodgkin's lymphomas. SV40 gene sequences were found significantly more often in DLBCL and follicular lymphomas than in other types of NHL (Table 1), although few of the other types were represented among these tumor samples. No difference was observed in the mean age of patients with SV40-

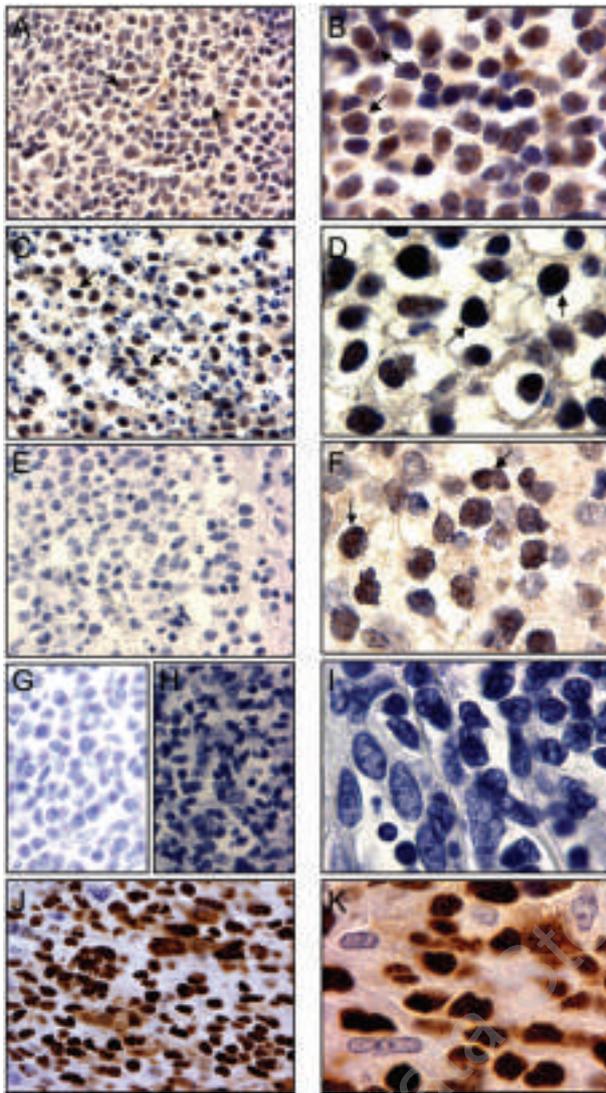


Figure 1. SV40 T-ag expression in non-Hodgkin's lymphoma from Costa Rica. (A and B) Expression of SV40 T-ag in malignant B-cells in an SV40 DNA-positive diffuse large B-cell lymphoma from a 67-year old female, stained with PAb101. (C and D) Expression of SV40 T-ag in an SV40 DNA-positive diffuse large B-cell lymphoma from a 45-year old male, stained with PAb101. (E) Same tumor as in panels C and D, stained with negative control mouse serum. (F) Expression of SV40 T-ag in an SV40 DNA-positive diffuse large B-cell lymphoma from a 70-year old male, stained with PAb101. (G) SV40 DNA-negative lymph node stained with PAb101. (H and I) An SV40 DNA-negative diffuse large B-cell lymphoma from a 29-year old male reacted with PAb101. (J and K) Expression of SV40 T-ag in an SV40-induced hamster tumor, stained with PAb101. Arrows point to T-ag positive cells in panels A-D and F. Original magnification for panels A, C, E, G, H, J, 40x, panels B, D, F, I and K, 100x.

positive or SV40-negative lymphomas (48 vs. 51 years, $p=0.6$). However, it is noteworthy that six patients with SV40-related lymphomas (20% of the positive cases) were born in the 1970s and 1980s, a period recognized as free from SV40-contaminated vaccines in the Western hemisphere.²⁶ EBV was detected in 9% (10/106) and 10% (2/19) of NHL and Hodgkin's lymphomas, respectively (Table 1). The presence of SV40

DNA does not necessarily indicate synthesis of the viral transforming protein.

We next examined 76 coded specimens that included lymphoma biopsies from lymph nodes, reactive lymph nodes, tonsils, and carcinoma samples for SV40 T-ag positivity by immunohistochemistry without knowledge of the PCR results. Eighteen samples gave a positive reaction with antibodies against SV40 T-ag. After the immunohistochemistry assays were completed, their results were correlated with the PCR results. Expression of SV40 T-ag was detected in 64% (18/28) of NHL that contained SV40 DNA sequences and in none of the lymphoma and control samples that had tested negative for viral DNA sequences (Table 3). SV40 was found only in neoplasias with a B-cell phenotype (CD20 positive).

Typical immunohistochemical staining patterns are shown in Figure 1. Three different NHL expressing SV40 T-ag are shown (Figure 1, panels A, B, C, D, F). In contrast, no T-ag reactivity was detected in SV40 DNA-negative lymph nodes (Figure 1, panel G), in SV40 DNA-negative NHL (Figure 1, panels H,I), or when SV40 DNA-positive tumors were stained with negative control antibody (Figure 1, panel E). When expression of SV40 T-ag in human lymphomas was compared to that in SV40-induced hamster tumors (Figure 1, panels J,K), human lymphoproliferative disorders had a relatively lower proportion of malignant cells expressing the viral oncoprotein and displayed a lower-intensity reaction. Expression of T-ag varied from tumor to tumor among the human samples, with the number of T-ag positive cells ranging from about 10% to 50%. However, in both the human and hamster malignancies, T-ag expression was detected in malignant cells. Antibody PAb101 gave stronger reactions than PAb416. As the samples were tested under code, the specificity of T-ag expression to lymphomas that were SV40 DNA positive indicates that the staining reactions were not spurious.

Immunophenotypic analysis of virus-positive lymphomas

Although SV40 has been detected in some human B-cell lymphomas, the phenotypic features of SV40-transformed B cells are not known. Recent gene expression profiling of DLBCL using cDNA microarray and tissue staining by immunohistochemistry have shown that this single diagnostic category includes more than one molecularly distinct disease.^{42,43} DLBCL among HIV-negative individuals consist of at least three subgroups with different gene expression profiles, known as germinal center B-cell-like (GCB), activated B-cell-like (ABC), and type 3. The last group (type 3) is a heterogeneous cluster that behaves in a manner similar to the ABC group. As SV40 was found in the DLBCL group of B-cell neoplasias (Table 1), we determined the sub-

Table 3. Expression of SV40 T-ag detected by immunohistochemistry among lymphomas and control samples.

Specimen ^a	No. tested	No. positive (%)	
		CD20	SV40 T-ag
Non-Hodgkin's^b			
SV40 DNA-positive	28	28 (100)	18 (64)
SV40 DNA-negative	16	16 (100)	0 (0)
Hodgkin's	2	N/A	0 (0)
Benign lymph nodes	10	10 (100)	0 (0)
Tonsils	10	10 (100)	0 (0)
Carcinomas	10	N/A	0 (0)

^aSpecimens were coded and tested for SV40 T-ag expression without knowledge of which tissues contained SV40 DNA sequences by PCR. ^bSV40 DNA-positive and -negative NHL cases included follicular and DLBCL.

group (GCB and non-GCB) associated with viral DNA. Markers for the GCB subgroup were the following: CD10 positive; CD10 and bcl-6 positive; or CD10 negative, bcl-6 positive, and MUM1 negative. Markers for the non-GCB subgroup were: CD10 and bcl-6 negative; or CD10 negative, bcl-6 positive, and MUM1 positive. Of the 68 DLBCL analyzed, 53 (78%) were considered to be GCB and 15 (22%) were considered to be non-GCB. A GCB profile was more frequently expressed than a non-GCB pattern in SV40-related lymphomas (16/21, 76% vs. 5/21, 24%; $p=0.001$).

Discussion

The importance of this investigation is that it used two different technical approaches – PCR for viral DNA and immunohistochemistry for expression of viral protein – to confirm an association of polyomavirus SV40 with human B-cell neoplasms. The findings described here were obtained from a geographical region (Central America) with a history of documented usage of SV40-contaminated poliovaccine that had not previously been studied for polyomavirus-associated cancer. Overall, 26% of NHL and 10% of Hodgkin's lymphomas from Costa Rica contained SV40 DNA, in general agreement with several independent studies in the United States,^{16–20,22,23} Italy,¹⁵ and Japan,²¹ but lower than the rate observed for pediatric lymphoid cancers of German origin.²⁴ However, another report from Japan⁴⁷ found a very low frequency of SV40-positive NHL and other studies from Europe^{30,48,49} failed to detect SV40 sequences in systemic lymphomas. These differences among studies may occur because of unrecognized differences among specimens available for analysis, technical variations in sample processing or assay, or because of the inclusion of many T-cell neoplasias and

EBV-positive lymphomas in surveys. The latter circumstances may reduce the probability of detecting SV40-positive tumors because SV40 has rarely been found in T-cell cancers and SV40 and EBV are seldom found together in a given tumor.^{18,19,22} However, we consider that even more important than technical issues in affecting differences between studies is that the prevalence of SV40 infections may vary among different geographic regions.²⁸ This may reflect the history of usage of contaminated poliovaccines within a given area. Unfortunately, records are often unavailable or inadequate to identify the frequency or duration of use of contaminated vaccines within a certain region, the amount of contaminating SV40 present in specific vaccine lots used, or the population groups most exposed to those contaminated vaccines. However, as lymphomas can arise from different causal factors, variation in the frequency of detection of SV40 in human lymphoproliferative disorders among population groups does not rule out a possible functional role for the virus in those malignancies in which it is present.

Immunohistochemical assays detected the expression of SV40 T-ag in 64% of B-cell lymphomas positive for SV40 DNA and in none of the control non-tumor lymphoid samples, control carcinomas, or SV40 DNA-negative lymphomas. Other researchers have also found T-ag expression in NHL.^{15,23} In our assays the T-ag staining reactions in NHL were weaker in intensity than those typically observed with SV40-induced hamster tumors and fewer lymphoma cells within a positive tumor appeared to contain T-ag. We have observed similar patterns and characteristics of T-ag expression in NHL from HIV-positive individuals.⁴⁵ We do not know whether this difference in reactivity reflects lower steady-state levels of T-ag in lymphoma cells or higher lability to the tissue fixation procedures used, as compared to T-ag in hamster tumor cells. The immunohistochemical staining reactions appeared to be specific as they correlated with the presence of SV40 DNA detectable by PCR and control lymphoid tissue, carcinomas and lymphomas lacking SV40 DNA all failed to stain for SV40 T-ag. The design of this study addresses concerns that possible laboratory plasmid contamination may yield false-positive PCR results with human cancer samples.⁵⁰ Lymphoma and control specimens were masked and analyzed in parallel and immunohistochemistry was performed without knowledge of tissue identity or PCR results. Plasmid contamination, if it were to occur, would be randomly distributed among lymphomas and control samples and would not result in T-ag expression in fixed tissues. Although NHL comprise a biologically diverse group of hematologic malignancies, SV40 was detected in this study only in B-cell neoplasias (Tables 1 and 3). B-cell lymphomas, which represent over 85% of NHL worldwide,³ are the type of lymphomas found to contain SV40 DNA sequences in

recent studies.¹⁵⁻²³ The present study showed that a GCB-like profile was frequently associated with SV40-positive lymphomas from HIV-negative individuals. A similar observation was made for SV40-positive lymphomas from HIV-positive patients.⁴⁵ GCB cells are destined to undergo apoptosis unless they are rescued by positive stimuli;⁵¹ SV40 T-antigens are known to have the capacity to stimulate infected cells to enter S phase and proliferate.^{8,52,53} Modulation of apoptosis is an important aspect of tumorigenesis by oncogenic viruses, including SV40.^{54,55} Perhaps SV40 can infect B cells in the germinal center and affect their survival.

The prevalence of SV40 in humans is not known, but observations indicate that SV40 infections are occurring in human populations at present.^{26,28,56} The infected people include individuals who received potentially SV40-contaminated vaccines, as well as persons born after 1963 who could not have been exposed to those vaccines. Previous studies have detected SV40 in brain and bone cancers and lymphoid malignancies in children and in NHL in young adult patients not exposed to contaminated poliovaccines.^{18,22,24,34,36} This study in Costa Rica found that 20% of the patients with molecular evidence of SV40-related lymphomas were born in recent decades. In addition, the detection of SV40 in non-neoplastic specimens^{40,57-63} suggests that SV40 is causing infections in the human population today. Serological studies have not yet provided certain SV40 seroprevalence data, but seropositivity rates appear to be low. Retrospective cross-sectional studies of stored serum samples using ELISA tests based on virus-like particles have encountered cross-reactive JC virus and BK virus antibodies at low serum dilutions that have hampered estimates of SV40 infections,⁶⁴⁻⁶⁹ leading to some conclusions that there was no evidence of human infections by SV40 or of an association of SV40 with disease.^{64,67-69} Polyomavirus serological surveys are complicated. ELISA tests can detect non-neutralizing antibodies and some cross-reactivity is not unexpected as the

three viruses (BKV, JCV, SV40) are partially related.⁷⁰ Human immune responses to SV40 infections have not been characterized; specific antibody responses may be low-titered and, as indicated recently, may wane over time.⁵⁶ It is worth noting that children who received a known contaminated oral poliovaccine excreted infectious SV40 in their stools for up to 5 weeks after vaccination but failed to show neutralizing antibody responses to the viral infection⁷¹ and a person with a proven SV40-positive brain tumor did not possess detectable SV40 antibodies.⁷² Therefore, we believe that modern molecular biology assays known to be highly sensitive and specific are most informative for the analysis of the prevalence of SV40 infections in humans.

In summary, this study found that polyomavirus SV40 is significantly associated with B-cell neoplasias in Costa Rica. Detectable expression of the T-ag oncoprotein in lymphoma cells is compatible with a possible role for SV40 in the development of some of these hematologic malignancies. Because of its known oncogenic properties, studies using sensitive molecular assays are warranted to determine the current distribution and prevalence of SV40 infections in humans.

AM, VM: identification of subjects and specimens, histologic evaluation of specimens in Costa Rica, data analysis; AM, RAV: processing of specimens in the USA; DLT: histologic evaluation of specimens in the USA, immunohistochemistry, data analysis; PZ: PCR, data analysis; DEK: immunohistochemistry, data analysis; RAV, JSB: conception and design of study, data analysis and interpretation, manuscript preparation and revision, approval of the final version of the manuscript.

The authors report no potential conflict of interest. There are no potentially redundant publications.

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