Molecular Identification of Simian Virus 40 Infection in Healthy Italian Subjects by Birth Cohort

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Simian virus SV40, an oncogenic virus in rodents, was accidentally transmitted to humans through the Poliovirus vaccine during the years 1955 to 1963. If the vaccination program were the major source of human infection, then differences in SV40 infection rates by cohort of birth should be observed. The aim of this study was to address this issue. In 134 healthy Italian Caucasian subjects, 15 DNA samples were positive for SV40 by nested polymerase chain reaction and DNA sequencing. The prevalence of genomic infection did not differ across cohorts of birth from 1924 to 1983, however DNA sequencing revealed viral strain differences in individuals born before 1947 and after 1958. While horizontal transmission following the introduction of the polio vaccine could explain the presence of SV40 DNA in younger people, our results also suggest the possibility that other sources of the virus may also be involved in human SV40 infection.

INTRODUCTION

Simian virus SV40, an oncogenic virus in rodents, has been detected in human tumors and tissues at variable frequencies. The virus was inadvertently transmitted to humans through the Poliovirus vaccine between 1955 and 1963 in the United States (1). Epidemiological studies do not indicate an increased incidence of cancer related to such contamination (2,3), and the role of SV40 sequences in human tumorigenesis remains controversial. Some of the controversy is due to methodological issues related to detection of the virus (4). High levels of SV40 viral infection have been reported in some tumors, and from some laboratories (1,5), whereas others have not found consistent evidence for widespread SV40 infection and propose that laboratory contamination, hypersensitivity of the polymerase chain reaction (PCR) or cross reactivity of the virus with other common viruses are responsible for the positive results (6,7). At present, there is, therefore, no clear consensus as to the actual degree of SV40 positivity in either human tumors or even in human blood and other nondiseased tissues. Studies that have investigated the presence of SV40 by DNA sequence or serological methods have found infection rates varying from 0 to more than 20% (8–13). One of the unresolved questions involves the discovery of SV40 DNA in tumors of persons born after or long before the polio vaccination program of the early 1960s. Such findings have raised the possibility that SV40 infection has been transmitted horizontally among the human population, and therefore can be found in people never directly exposed to the vaccine (14,15). To address the issue of SV40 infection as a direct result of vaccination as opposed to transmission by other routes, we have examined blood DNA from individuals ranging in age from 26 to 77, born between 1924 and 1983. The presence of SV40 DNA, determined by PCR and DNA sequencing, was analyzed according to cohort of birth. No such study of the presence of SV40 genomes in subjects without cancer by cohort of birth has been performed so far.

MATERIALS AND METHODS

We studied 134 solid organ donors of Caucasian Italian origin (56.7% males), whose anonymous DNA, isolated from peripheral blood lymphocytes, was stored in the repository of the North Italian Transplant Reference Center located in the Policlinico Hospital of Milano, Italy, since 1972. No identifying linkers exist for these samples. Informed consent for organ donation and evaluation of organ safety, as required by Italian law, was obtained by family members at the time of organ donation. The program of solid organ donation was approved by the Hospital Ethical Committee. A random sample of these DNAs, stratified by cohort of birth, was extracted from the whole set of DNA samples currently stored (n = 10707). None of the subjects were clinically affected by cancer at the time of donation. DNA was isolated from blood samples using the method of Gustincich et al. (16). DNA concentration was measured by spectrophotometry, and ranged from 100 ng/µL to 450 ng/µL.

PCRs were performed to determine the presence of SV40 sequences in our samples. Negative and positive controls were included in all sets of reactions. The pBRSV (ATCC 45019, from G. Khoury), plasmid, containing the entire genome of reference strain SV40–776, was used as the positive control in PCR amplification. DNA (1 µg) was amplified in a total volume of 50 µL.

The regulatory region of the SV40 viral genome was analyzed by 2 nested PCRs, using the primer set RA3 (GCGTGACAGCCG-

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Table 1. Presence of sv40 genomic regions in healthy subjects according to cohort of birth[a]

Birth cohort	Nr	Males (%)	SV40 Positive		Percent (95% CI)
			PCR	Sequencing[b]	
1924-1933	14	6 (42.9)	3	3/3 (2F, 1M)	21.4 (0-42.9)
1934-1943	34	17 (50.0)	5	5/5 (3F, 2M)	14.7 (2.8-26.6)
1944-1953	25	10 (40.0)	1	1/1 (1F)	4.0 (0-11.7)
1954-1963	19	15 (78.9)	3	3/3 (1F, 2M)	15.8 (0-32.2)
1964-1973	17	11 (64.7)	1	1/1 (1M)	5.9 (0-17.1)
1974-1983	25	17 (68.0)	3	2/3 (1F, 1M)	8.0 (0-18.6)
Total	134	76 (56.7)	15	15/16 (8F, 7M)	11.2 (5.9-16.5)

[a]Chi square = 4.33, NS = not significant.
[b]M, male; F, female.

GCGCAGCACCA)-RA4 (GTCCATTAGCTGCAAAGATTCCTC) (10 μM) for the 1st PCR, and RA1 (AATGTGTGTCAGTTAGGGT-GTG)-RA2 (TCCAAAAAAGCCTCCTCACTACTT) (10 μM) for the 2nd (17). The 1st reaction was carried out for 30 cycles at a denaturing temperature of 94°C for 1 min, an annealing temperature of 52°C for 1 min, and a primer extension temperature of 72°C for 1 min. The product of PCR analysis was then analyzed on agarose gel (2%) visualized by ethidium bromide staining (SV40 positive sample showed a 483 bp band). The 2nd reaction was carried out (with 1 μL of the 1st PCR product) for 30 cycles at a denaturing temperature of 94°C for 1 min, an annealing temperature of 55°C for 1 min, and a primer extension temperature of 72°C for 1 min. Absence or presence of the specific PCR products (314 bp) was analyzed on agarose gel at 2% visualized by ethidium bromide staining.

The tag COOH region of SV40 was analyzed by a single PCR with the primer set TA1 (GACCTGTGGCTGAGTTTGCTCA)-TA2 (GCTTTATTTGTAACCATTATAAG); reaction was carried out for 30 cycles at a denaturing temperature of 94°C for 1 min, an annealing temperature of 58°C for 1 min, and a primer extension temperature of 72°C for 1 min. The presence of the viral genome (441 bp) was analyzed on agarose gel (2%) visualized by ethidium bromide staining.

Special precautions were taken to avoid laboratory contamination with SV40 sequences. Access to the laboratory was limited, and no work using molecular reagents containing plasmids was done in the same laboratory, either concurrently or before the present study. PCR reactions were set up in dedicated ultraviolet-irradiated hoods, and all post-PCR procedures were done in a dedicated space. Dedicated pipettes with sterile filter tips were used for PCR setup and post-PCR analyses. Samples that were positive at PCR were repeated to confirm the results.

DNA from samples that were positive for SV40 by PCR were sequenced twice in both directions using RA1 and RA2 primers according to the manufacturer's protocol (ABI PRISM© Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems, Foster City, CA, USA).

Data are presented as percentage of positive samples, the 95% Confidence Intervals (CI) of the percentage of positive samples is also reported. Differences among proportions were calculated by Chi-square test for trend.

RESULTS

Sixteen DNA samples of 134 investigated were positive for SV40 using PCR, and 15 of these (11.2%, 95% CI: 5.9% to 16.5%) were confirmed by sequencing. One sample was positive by PCR was not confirmed by sequencing. One of the positive samples proved to be archetypal, containing only one 72-bp repeat, whereas the other fourteen were nonarchetypal, with two 72-bp repeats. The frequency of positive samples by birth date cohort are shown in Table 1, ranging from 4.0% to 21.4% without statistically significant differences across cohorts. The positive samples belonged to 7 out of 76 male subjects (9.2%, 95% CI: 2.7% to 29.5%), and to 8 of 58 female subjects (13.8%, 95% CI: 4.9% to 22.7%). Examination of the sequence data revealed 3 variants of SV40 DNA sequence. Six of the samples had the same sequence as strain SV40-776 (SV40ITVAR1); Variant 2 (SV40-ITVAR2) included 4 samples with an insertion of A at position 56, a deletion at position 159, and a C-to-T base substitution at position 178. Another variant (SV40-ITVAR3) included 4 samples with a G-to-A base substitution at position 65, and the same C-to-T base substitution at 178. Other mutations were found in some of the samples, which are therefore labeled as substrains (VAR3A, etc.) as indicated in Table 2. None of the samples showed polymorphism at position 5209, and in none of the samples could the TAG region be amplified. None of the sequence variants matched any of the previously published sequence variations in the regulatory region (18–21), nor any of those listed in GENBANK. Therefore, definitive strain identification is not possible. Strikingly, the distribution of these sequence variants was not uniform across birth cohorts, with all of the 6 ITVAR1 samples belonging to persons born after 1958, whereas the samples with SV40 ITVAR2 and ITVAR3 variants were all from subjects born before 1947 (Table 2), where a more diverse mixture of variants is observed. None of the samples showed sequence homology to either JCV or BKV sequences (including the single sample that was negative by DNA sequencing).

DISCUSSION

The present study did not show any significant difference in SV40 infection rates according to year of birth. Poliovirus vacci-

Table 2. SV40 sequence variants

Year of birth	Donation date	Sex	Sequence variant
1928	2002	F	ITVAR3 (65G-A, 178C-T)
1932	2002	M	ITVAR2 (56INS-A, 159DEL, 178C-T, 105G-C)
1933	2002	F	ITVAR2A (56INS-A, 159DEL, 178C-T)
1936	2002	M	ITVAR3A (65G-A, 178C-T + 141C-A)
1938	2002	F	ITVAR2
1939	2002	M	ITVAR3
1939	2002	F	ITVAR3B (65G-A, 178C-T + 105G-C)
1942	2003	F	ARCHETYPAL, ONE 72 BP REPEAT
1947	2003	F	ITVAR2
1958	2003	F	ITVAR1A (141C-A)
1963	2003	F	ITVAR1B (DEL28)
1963	2003	M	ITVAR1
1972	2003	M	ITVAR1
1974	2003	F	Negative
1977	2003	F	ITVAR1
1979	2003	M	ITVAR1

nation was a requirement for admission to both nursery and primary school in Italy starting from 1959, and it became mandatory within the 1st year of life in 1966. Therefore, if the only source of the infection were polio vaccination, SV40 positive samples should be found to a larger degree in the cohort of subjects born between 1953 and 1963. In our study, 19 subjects were born in this time frame, and 3 of them were positive for SV40, with a prevalence of infection (14.3%, 95% C.I.: 0% to 34.7%) similar to that observed in subjects born in previous or subsequent years, indicating no difference in the frequency of infection according to cohort of birth. One study (7) of SV40 infection in normal blood also found no differences in rate between persons born before (4.3%) and after (8.2%) 1962. However, these authors suggest all of the SV40 serological positivity was due to cross reactivity to antibodies to the common human JC and BK viruses (7,22).

Although the past few years have witnessed a large number of publications on the presence of SV40 sequences in human tumors (23–27), surprisingly little information exists on the proportion of SV40 infection in the blood of healthy individuals. This report of SV40 DNA in 134 persons represents the largest such study in a control population done to date. The published frequency of SV40 DNA in normal tissues or in people without cancer is quite variable. In Italians, 1 group has found frequencies of 13% (28), 23% (29), and 29% (11) in blood, and 45% (29) in sperm, whereas another Italian group has failed to find any positive control samples among 84 examined (30,31). We found a frequency of SV40 positives of 11%, similar to the average of that found in other studies. The frequency of infection was similar in females and in males. We were unable to find positive subjects for the Tag COOH region of SV40. A possible explanation could be that the Tag COOH region was analyzed by a less sensitive approach than that used for the regulatory region.

It is supposed that SV40 viral infection entered the human population through poliovirus vaccination in Italy during the years 1957 to 1963. The vaccination started on a voluntary basis in 1957, and it became mandatory for school admission starting from 1959. The vaccine used at that time was killed Salk vaccine type produced in Italy, Belgium, and United States, whereas the live attenuated Sabin vaccine was introduced in Italy starting from 1964. Although cytopathic tests were routinely performed on the vaccine stocks, it cannot be excluded that some degree of contamination did exist. The explanation for the existence of SV40 DNA in persons too old or too young to have been directly inoculated with the vaccine is horizontal transmission of the virus from the cohort (born between 1953 and 1961) that was inoculated (14,15). Our results are consistent with this interpretation in that we did find SV40 DNA in people who were not inoculated. However, it is puzzling that the rate of infection is not higher in those who could have been directly inoculated than in people whose exposure was due to transmission. While preliminary, our results on sequence variation among the 15 positive samples are consistent with findings of natural sequence variation in the regulatory region of SV40 (32). The nonrandom distribution of the specific variants among birth cohorts calls into question the uniform spread of the virus from a single inoculated cohort. An alternative possibility is that different sources of human SV40 infection might have existed (33), and that while 1 strain present in an adenovirus type 1 vaccine stock spread by horizontal transmission from the inoculated cohort, other strains, found in older people, might have come from these alternative sources. In addition to these hypotheses, a possible explanation could be that ITVAR1 is more transmissible among humans and has gradually become the most prevalent strain in the population studied. Further research in larger cohorts, and from different global regions, is needed to resolve the issue of how and when SV40 infection entered and spread among the human species.

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