Is the simian virus SV40 associated with idiopathic focal segmental glomerulosclerosis in humans?

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ABSTRACT: *Background:* **Glomerulosclerosis was reported in mice transgenic for the simian polyomavirus SV40 early region that contains the transforming sequences encoding the SV40 large T-antigen (TAG). This was discovered when an SV40 epidemic occurred following the use of contaminated polio vaccines during 1955-1963, and led to investigations that showed an association between SV40 infection and tumors in humans. We investigated the possible association of SV40 infection and idiopathic focal segmental glomerulosclerosis (FSGS).**
Methods: **The study was performed in 17 Bouin-fixed, paraffin-embedded renal biopsies from FSGS patients and 10 matched biopsies from patients with IgA glomerulonephritis; all patients had undergone polio vaccination in the early 1960s. Extracted DNA was polymerase chain reaction (PCR) amplified using SV.for3/SV.rev primers and GabE1/GabE2 primers; both sets of primers map in the region of SV40 TAG sequences, and amplify a fragment of respectively 105-bp and 135-bp. The biopsies considered were those in which the DNA was sufficiently intact to allow amplification of a fragment of 102-bp of the ApoE gene.**
Results: **Three FSGS and none of the IgA biopsies were positive for the SV.for3/SV.rev fragment. Conversely, amplification with GabE1/GabE2 primers did not lead to any specific product in either the IgA or FSGS biopsies. Restriction fragment length polymorphism and sequencing analyses revealed that the positive results obtained with the SV.for3/SV.rev primers were due to amplicons generated by multiple dimerization of forward and reverse primers.**
Conclusions: **With the limited number of patients investigated, this study excludes the hypothesis that SV40 is associated with idiopathic FSGS.**

Key words: *Focal segmental glomerulosclerosis, PCR assay, Renal biopsies, SV40 infection, SV40 large T-antigen*

INTRODUCTION

Simian virus 40 (SV40) is a papovavirus that naturally infects rhesus macaques; it also induces a variety of tumors in experimentally infected newborn hamsters, and in SV40 transgenic mice (1-4). Concerns regarding SV40 infection in humans were first raised in the 1960s following the inadvertent contamination of initial stocks of polio and influenza vaccines with this virus during 1955-1963. Although epidemiological studies have not indicated an increased incidence of any disease in the recipients of the contaminated vaccines, it was demonstrated that a productive infection with SV40 was established in these individuals (5). Recent reports have challenged the view that SV40 is not associated with human disease. SV40 DNA footprints have been detected in certain human tumor tissues (6-9). Most of these reports specifically link SV40 to the tumor tissue; therefore, supporting a role for the virus in human cancer.

This study investigated whether SV40 is also associated with focal segmental glomerulosclerosis (FSGS) in humans. This hypothesis arose from observations of SV40 transgenic mice. Some of the lines of transgenic mice established with DNA containing the early region of SV40 that codes for the large and small T-anti-

gens (TAG and tag) develop FSGS in up to 90% of individuals (10) with no association with the rarely occurring extrarenal pathology (thymomas and hepatic tumors) possibly responsible for secondary forms of glomerulosclerosis. Therefore, it was advanced that glomerular lesions could develop because of alterations in glomerular cell behavior induced by the local expression of the transforming gene encoding the TAG (10).

Patients and methods

We reviewed all the native kidney biopsies at the Verona center performed in 1975-1984 on patients who were polio-vaccinated in the late 1950s to early 1960s, according to Italian law; 17 caucasian cases were identified (out of 32) of idiopathic FSGS with enough paraffin-embedded tissue to perform further analysis. A group of 10 sex-, age-, and duration of storage-matched biopsies from patients with the common IgA mesangioproliferative glomerulonephritis was also identified and used as the control group. All FSGS patients had nephrotic range proteinuria. No patients had putatively SV40-related pathologies (thymoma, lymphoma, etc) or clinical conditions (i.e. reflux nephropathy, heroin assumption, kidney dysplasia and obesity) possibly responsible for secondary forms of FSGS. Finally, although no patients were checked for HIV infection, it is worth noting that the first AIDS case in Italy was described in 1984. All FSGS patients disclosed typical histopathology; particularly, no collapsing variant was found. All biopsies had been fixed overnight with Bouin's acid fixative, and then paraffin-embedded.

DNA extraction

Five 10 µm thick paraffin sections were first de-paraffinized by two incubations in 1 mL xylene (10 min at room temperature), followed by centrifugation at 20.000 rpm / 5 min after the first incubation, and 1.300 rpm / 3 min after the second. These steps were followed by three washes with 1 mL of 100%, 70% and then 50% ethanol. The pellet was vacuum dried for 60 min, and then incubated with 150-250 µl digestion buffer (20 mM TrisHCl pH 8, 0.5 mM EDTA-Na_2, 0.5% NP40, 0.5% Tween 20, 0.05% Na-azide, 0.125 mg / mL proteinase K [Sigma, St Louis, MO, USA]) for 18 hr at 56°C. Following heat inactivation of proteinase K (15 min at 95°C), genomic DNA was purified and concentrated by precipitation with absolute ethanol at -80°C overnight, and pelleted by centrifugation at 20.800 rpm for 1 hr at 4°C. The pellet was vacuum dried, and then resuspended in 50 µl TE buffer (10 mM TrisHCl pH 9, and 0.5 mM EDTA).

Mesangial cells obtained from SV40 early region transgenic mouse (11) (ATCC, Manassas, VA, USA) were washed twice in PBS buffer, Bouin-fixed, pelleted, and then incubated in digestion buffer, as described above.

Two negative controls (buffer only) were included in each vacuum drying step, to check for carry-over. The negative controls were treated exactly as the DNA samples.

Polymerase chain reaction

To assess the quality of the DNA specimens extracted from the archives material, two different sets of primers for two different human genome regions were employed; the first one, for HLA-DQ (amplification fragment of 242-bp), and the second, for apolipoprotein E (102-bp, a size very similar to that of the SV40 amplicons). PCR cycling parameters used for the HLA-DQ region were: an initial denaturation at 95°C / 2 min followed by 45 cycles of 94°C / 25 sec, 60°C / 30sec, 72°C / 30 sec, and a final extension step of 72°C / 10 min. PCR cycling parameters for ApoE were: an initial denaturation at 95°C / 5 min followed by 42 cycles of 94°C / 30 sec, 60°C / 20 sec, 72°C / 20 sec, and a final extension step of 72°C / 10 min.

Samples were screened for the presence of SV40 using the SV.for3 / SV.rev primers originally described by Bergsagel et al (6), which allow amplification of a very specific and highly conserved region of the SV40 genome. The PCR product is a 105-bp fragment close to the N-terminus of TAG.

For this PCR assay we followed Lednicky and Butel's recommendation (12) to use low stringency PCR conditions (i.e. lower annealing temperature) for archives samples, and a second round of PCR amplification after the completion of the first PCR run.

For the first PCR run, the cycling parameters used were: an initial denaturation at 94°C / 3 min followed by 55 cycles of 94°C / 1 min, 55°C / 1 min, 72°C / 1 min, and a final extension step of 72°C / 10 min. For the second PCR round, we employed the same cycling parameters for 20 or 30 cycles, as above.

All amplifications were performed with a positive control (DNA extracted from mesangial cells of SV40 early region transgenic mouse), and at least three negative controls to guard against possible misinterpretation due to contaminating DNA.

A further, not previously described, set of primers was designed to identify the SV40 genome. This set, indicated as GabE1 / GabE2, gives an amplification product of 135-bp and was chosen by analyzing the SV40 genome sequence in the region coding for TAG. According to information by Blast Search (NCBI) this primer set is highly specific for SV40 DNA, and annealing with the DNA of other polyomaviruses like BKV and JCV is unlikely. Two subsequent PCR rounds

with the primers GabE1/GabE2 were performed. The cycling parameters used in the first round were: an initial denaturation at 94°C/3 min followed by 50 cycles of 94°C/1 min, 60°C/1 min, 72°C/1 min, and a final extension step of 72°C/10 min. For the second round, the cycling parameters were: an initial denaturation at 94°C/2 min followed by 20 cycles of 94°C/30 sec, 60°C/30 sec, 72°C/30 sec, and a final extension step of 72°C/5 min.

Electrophoretic analysis of PCR products

For the qualitative analysis of PCR assay, 10 µl of each PCR reaction mix were electrophorezed at 190 V for 40 min on a 10% polyacrylamide gel in TBE buffer, and then stained with ethidium bromide; the image was collected with a Gel Doc 1000 instrument (BIO-RAD, Hercules CA, USA). To detect digestion products, the samples were electrophorezed on a 20% polyacrylamide gel.

DNA sequencing

The 105-bp amplicons generated by PCR amplification with the SV.for3/SV.rev primer set and the 102-bp amplicons generated with the primer set ApoE-112 of samples and controls, were separated on a 3% agarose gel in tris-acetate-EDTA (TAE) buffer; after ethidium bromide staining, individual bands of correct size were cut-off and treated for DNA extraction following the instructions given in the "Agarose Gel DNA Extraction Kit" (Boehringer, Mannheim, Germany). These extracts were directly sequenced with an ABI PRISM 310 automated DNA sequencer (PE Applied Biosystems, Norwalk, CT, USA). Raw data were analyzed by alignment with SV40 and ApoE-112 complete genome sequence (NCBI-J02400).

RESULTS

The recoverable DNA from Bouin's acid fixed and paraffin-embedded tissues, appears highly degraded on gel, and is generally considered refractory to any attempt at PCR amplification. However, some success has been reported with this type of material (13, 14) and a method was recently described to overcome the problem of Bouin-fixed tissues (15). However, as very little tissue was available to us, and to avoid any unnecessary loss of DNA, contrary to Longy et al (15) we decided to develop a method not employing DNA purification through columns and/or ultrafiltration through minicolumns but instead through ethanol precipitation steps.

To assess the quality of the DNA specimens extracted from our archives material, two sets of primers were em-

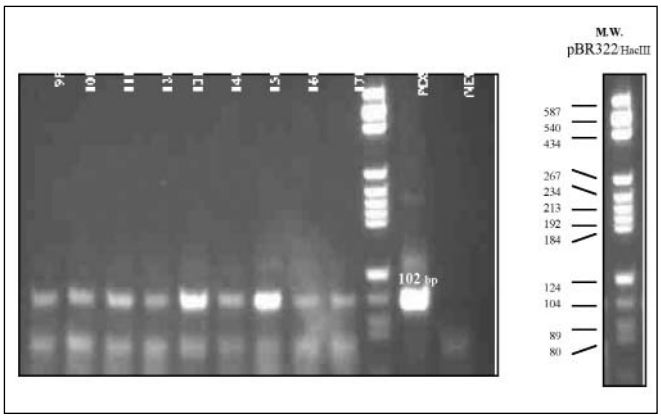

Fig. 1 - Evaluation of quality of DNA samples - 10% PAGE analysis of samples 9P-17P amplified with ApoE-112 primers. DNA was prepared from Bouin-fixed and paraffin-embedded archives biopsies of kidneys from patients with FSGS; positive control (POS): human DNA; negative control (NEG): primer buffer.

ployed for two different human genome regions, HLA-DQ and ApoE genes. Amplification of the HLA-DQ gene (PCR product of 242-bp) gave negative results in all samples, most likely due to excessive DNA fragmentation. Amplification of the apolipoprotein E gene by the primer set ApoE-112 (102-bp, a size very similar to that of the SV40 sequence we were looking for) gave positive results in all cases but one, in both the FSGS and IgA groups. By DNA sequencing we demonstrated the alignment of these amplicons with the ApoE-112 genome sequence. The search for SV40 footprints was conducted only on the ApoE-112 positive samples (25 biopsies) (Fig. 1). As a positive control DNA, we employed DNA extracted from SV40 early region transgenic mouse mesangial cells. PCR amplification of control DNA by both SV.for3/SV.rev (6) and GabE1/GabE2 primers produced a single PCR product of the expected size, respectively 105-bp and 135-bp. A series of positive control dilutions were used to investigate the sensitivity of the two sets of primers. Based on the established amplification profiles, specificity was identical for the two sets whereas sensitivity was higher for the GabE1/GabE2 set (Fig. 2). Sequencing confirmed the SV40 nature of the PCR product.

Samples from control and FSGS biopsies were initially screened for the presence of SV40 using SV.for3/SV.rev primers. Two successive sessions were carried out on different tissue slices to confirm positive results. As shown in Table I and Figure 3, all control biopsies in both sessions were negative for the SV40 genome. On the contrary, 6 of 16 FSGS biopsies (16 out of 17 samples gave a valuable signal for ApoE-112) in the first session and 7 of 14 biopsies (14 out of 17 samples gave a valuable signal for ApoE-112 or had sufficient RNA to be analyzed) in the second session were positive on PCR assay; three samples were positive in both sessions.

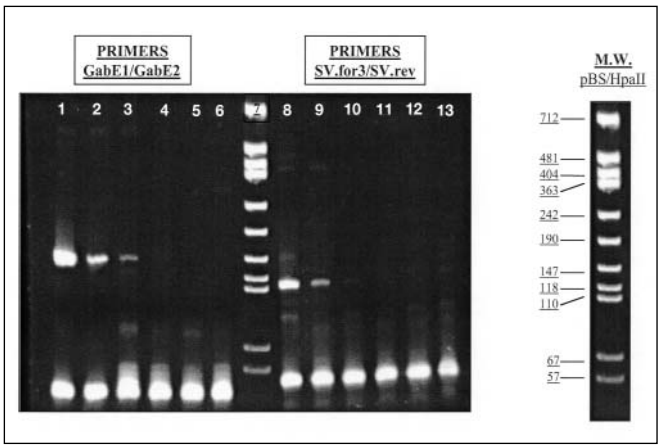

Fig. 2 - Sensibility and specificity of SV40 PCR assays - 10% PAGE analysis of PCR products obtained by amplification of serial dilutions of SV40 positive control with SV.for3/SV.rev and GabE1/GabE2 primers. Lane 1-5 and 8-12: positive control dilutions: 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}; Lane 6 and 13 negative control.

Sequencing was performed to confirm the PCR results. Sequencing of the forward strand was conducted on four positive samples, and revealed that the 105-bp PCR product contained only forward and reverse primer sequences. In particular, sequencing of both strands allowed the definition of the artifactual origin of the amplicons, due to primer dimerization during the PCR reaction.

The second set of primers called GabE1/GabE2 was designed in the same TAG-region, but amplifies a larger SV40 portion (135-bp). With these primers none of the samples in either the control or the FSGS series were positive for SV40 (Fig. 4). These findings were confirmed in two sessions.

DISCUSSION

The major result of this study is that it excludes the hypothesis that SV40 is associated with idiopathic

TABLE I - RESULTS OF SV40 DETECTION ASSAY PERFORMED WITH SV.FOR3/SV.REV PRIMERS

SAMPLE	primers ApoE-112 (102-bp)	primers SV.for3/SV.rev (105-bp)	SAMPLE	primers ApoE-112 (102-bp)	primers SV.for3/SV.rev (105-bp)
	FIRST SESSION			SECOND SESSION	
1P	Pos	Neg	21P	Pos	Neg
2P	Pos	Neg	22P	Pos	Neg
3P	Pos	Neg	23P	Pos	Pos
4P	Pos	Neg	24P	Pos	Pos
5P	Pos	Neg	25P	Pos	Neg
6P	Neg	n.d.	26P	Pos	Neg
7P	Pos	Neg	27P	Neg	n.d.
8P	Pos	Neg	28P	Pos	Pos
9P	Pos	Pos	29P	Pos	Neg
10P	Pos	Pos	30P	Pos	Pos
11P	Pos	Neg	31P	Pos	Pos
12P	Pos	Neg	32P	Pos	Neg
13P	Pos	Pos	33P	Pos	Neg
14P	Pos	Pos	34P	i.m.	i.m.
15P	Pos	Pos	35P	Pos	Pos
16P	Pos	Pos	36P	Pos	Pos
17P	Pos	Neg	37P	Neg	n.d.
1N	Pos	Neg	11N	Pos	Neg
2N	Pos	Neg	12N	Pos	Neg
3N	Pos	Neg	13N	Pos	Neg
4N	Neg	n.d.	14N	Pos	Neg
5N	Pos	Neg			
6N	Pos	Neg			
7N	Pos	Neg			
8N	Pos	Neg			
9N	Pos	Neg			
10N	Pos	Neg			

The FSGS samples assayed in the second session were identified by using the original code plus 20; for example, sample 1P = 21P.
The IgAN samples assayed in the second session were identified by the original code plus 10, for example, sample 1N = 11N.
n.d. = not determined; i.m. = insufficient material.

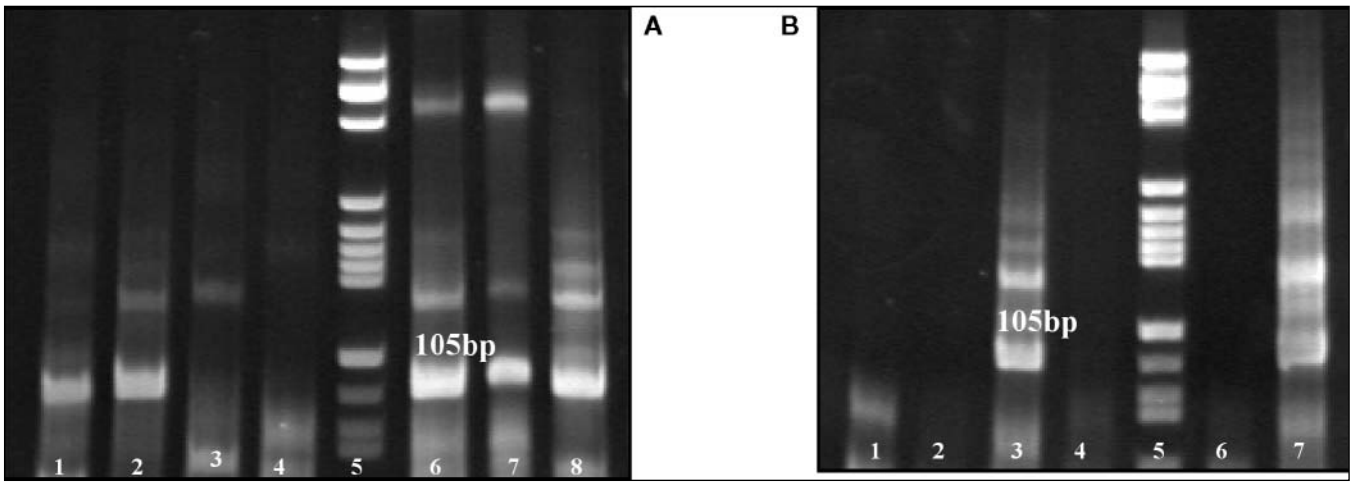

Fig. 3 - SV40 PCR assay with SV.for 3/SV.rev primers - (A) First session Lane 1-4 and 8: samples 9P, 10P, 11P, 12P, 14P; lane 5: M.W. (pBR322/HaeIII); lane 6-7: SV40 positive control (tested in duplicate). (B) Second session Lane1-2: negative control; lane 3-4 and 6-7: samples 30P, 31P, 33P, 35P; lane 5: M.W. (Pbr322/HaeIII). 10P and 30P, 11P and 31P were taken from the same biopsy.

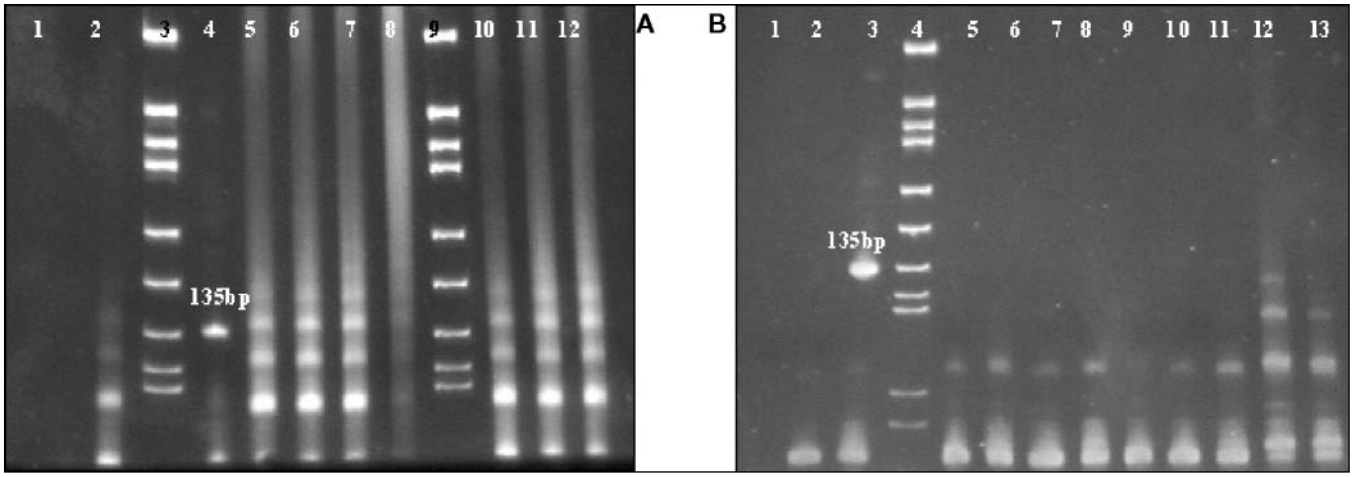

Fig. 4 - SV40 PCR assay with GabE1/GabE2 primers - (A) Patients with FSGS: lane 1-2: negative control; lane 3-9: M.W. (pBS/HpaII); lane 4: SV40 positive control; lane 5-8/10-12: samples 30P, 31P, 32P, 33P, 35P, 36P, 37P. (B) patients with IgA nephropathy: lane 1-2: negative control; lane 3: SV40 positive control; lane 4: M.W. (pBS/HpaII); lane 5-13: samples 1N, 2N, 3N, 4N, 9N, 11N, 12N, 13N, 14N.

FSGS. While the etiology of idiopathic FSGS remains obscure, its similarities with HIV-associated nephropathy suggest a possible viral origin. A variable percentage of patients show systemic symptoms that could be manifestations of an occult infectious process. Furthermore, transgenic mice produced from HIV-1 (16), and SV40 genomes (4) have been shown to develop FSGS. In idiopathic collapsing glomerulopathy, another specific form of glomerulosclerosis, parvovirus B19 DNA was detected in renal biopsies (17). Three considerations led us to search for a possible association between SV40 infection and idiopathic FSGS in patients exposed to contaminated polio vaccines in the late 1950s to early 1960s:

1. The observation of glomerulosclerosis in transgenic SV40 mice, and that the kidney is a major site of SV40 infection in infected macaques (18);
2. The notion that a latent epidemic of SV40 that occurred in humans could possibly be implicated in human pathology;
3. Renal infections by a strictly related polyomavirus, the BK strain, although rare, have been reported in kidney transplant recipients (19). It is supposed that in these cases the virus reservoir is the engrafting kidney. Therefore, in the human kidney a similar latent infection by polyomaviruses can occur as in macaques (18).

To investigate virus infections in tissues, PCR based methods are generally employed because of their higher sensitivity and specificity compared to

immunological methods. In reference to SV40, TAG-expression can be studied by immunohistochemistry with specific antibodies. However, even in tumor pathology very few cells are infected by SV40, and only < 1% of cells express TAG (9). False positive results have also been reported with immunological detection techniques (20).

For this reason, and due to the small quantity of tissue available to us, we decided to use PCR methods to investigate the working hypothesis of this study. We had available only Bouin's fixed biopsies. It is generally considered that this kind of acid fixative limits the use of tissues for molecular analysis. However, we succeeded in extracting suitable DNA for PCR amplification from Bouin-fixed archives material that was more than two decades old by numerous ethanol precipitation steps after xilene de-paraffinization; this method allowed the recovery of intact DNA fragments sufficiently large to recognize a 102-bp amplicon of the ApoE-112 gene.

We assumed that some SV40 could be integrated, and speculated that the entire TAG gene would be present. Therefore, we chose to search for a sequence of the TAG early gene in the renal biopsies. TAG is a protein associated with various DNA viruses, including SV40 and other papovaviruses, among which is the BK and JC strain of human polyomavirus. The TAG amino-terminus region contains the RB-pocket binding domain, which binds and inactivates the retinoblastoma gene product (21). TAG association with tumor suppressor genes is thought to be important in SV40-mediated transformation i.e., oncogenesis and possibly glomerulosclerosis (4). Therefore, if TAG played any role in the development of glomerulosclerosis, it was unlikely that this region would be mutated or deleted. For this reason, the first set of primers we used for PCR was SV.for3/SV.rev, which amplifies a specific and highly conserved SV40 sequence of 105-bp which encodes the N-terminal region of TAG.

Two experimental sessions confirmed PCR products with molecular weight similar to the 105-bp of the positive control in three of 16 FSGS specimens, and none in the 10 IgA biopsies and constituted a preliminary result supporting the association of SV40 with FSGS. It is well known that a number of mechanisms can cause false positive results in these kinds of study and confirmation of results by different methods is mandatory. We employed three strategies: (a) RFLP analysis of the SV.for3/SV.rev amplicons using restriction enzymes MboII and Sau3AI; (b) search for a further SV40 genome fingerprint using the GabE1/GabE2 set of primers and; (c) DNA sequencing of some of the 105-bp amplification products.

Since RFLP analysis with different restriction enzymes gave negative results we considered the hypothesis that possible artifacts in the SV.for3/SV.rev PCR could lead to the formation of amplicons of the expected molecular weight, and performed a second amplification with GabE1/GabE2 primers. According to information by Blast Search, this set of primers is highly specific for SV40 DNA. The use of this second set of primers gave negative results in every FSGS and IgA sample.

Subsequent DNA sequencing demonstrated that the putative SV40 fingerprints obtained by SV.for3/SV.rev PCR in some FSGS specimens were indeed DNA hetero-duplex artifacts, which had formed because of dimerization of the primers. Although this is well known, to our knowledge it very rarely leads to the formation of a sequence whose length overlaps with that of the expected PCR product. It was most likely favored by our need to study relatively small sequences due to the degradation of the DNA extracted from old Bouin-fixed biopsies, and that the length of each primer was essentially a submultiple of the expected 105-bp fragment. We cannot explain why this occurred only in the FSGS samples, and not in the IgA specimens, nor why it was confirmed only in the FSGS specimens in different experimental sessions; one could speculate on the existence of some very specific biological background predisposition in the FSGS biopsies.

In conclusion, the study does not support the hypothesis of the association of SV40 with idiopathic FSGS and IgA nephropathy. Certainly the number of patients investigated was small, although it was casually derived from a larger group of 32. In addition, we cannot rule out either that an FSGS subpopulation, particularly in different geographical areas could harbor SV40, or infections by other papovaviruses because we did not use specific primers for these viruses. However, our data support the conclusion that the SV40 virus does not have a causative role in the pathogenesis of human idiopathic FSGS and IgA nephropathy, at least in the more common clinical and histopathological presentations. In the collapsing form of FSGS, for which a viral pathogenesis has found some confirmation (17), a role for SV40 cannot be excluded; however this morphopathological picture is very different from that observed experimentally in transgenic mice (4).

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