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Presence and Incidence of DNA Sequences of Human Polyomaviruses BKV and JCV in Colorectal Tumor Tissues

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Abstract. *The human polyomaviruses JCV and BKV are widespread within population, as shown by serological studies. However, exposure to these viruses does not seem to have pathological consequences in immunocompetent individuals, while in immunocompromised or immunosuppressed patients, polyomaviruses can be activated, giving rise to serious pathologies. Viral DNA sequences were also found in cells from a number of human tumors of mesothelial origin, suggesting that activation of BKV and JCV, taking place in genetically predisposed and/or in immunodepressed individuals, might be involved in the mechanisms of tumor transformation. In this study, samples obtained from 18 patients with colon rectal carcinoma were probed for the presence of JCV and BKV by three different techniques: Southern blot, PCR and in situ hybridization. Our results demonstrate that viral DNA sequences were present in 16 out of the 18 cases considered (88.9%). In the large majority of cases, viruses were detected both in the tumor mass and in the surrounding healthy tissues. Lymphocytes in the investigated areas were also found to be infected by polyomaviruses. These data indicate, for the first time, a possible involvement of polyomaviruses in the pathogenesis of tumors of endothelial origin, like the human colon rectal carcinoma.*

The family of polyomaviruses encompasses the murine polyomavirus, the simian polyomavirus SV40 and also two strains of human polyomaviruses, JCV and BKV. These viruses have been intensively studied because of their tumorigenicity in appropriate animal models and their strong ability to mediate tumor transformation in cultured cells (1, 2). The viral large T antigen seems to be the molecule mainly involved in neoplastic transformation both *in vivo* and *in vitro* (2, 3). In our laboratory, we have studied the structure/function relationship in the genome of murine polyomavirus (4) and, recently, we have used this virus as a tool for testing the antiviral potential of natural substances (5, 6).

The two strains of human polyomaviruses, JCV and BKV, are widespread within the human population. Serological studies demonstrate that infection by JCV and BKV is extremely frequent since more than 80% of the population is seropositive (7, 8), the only exception being small and isolated communities in South America and Papua New Guinea. Viral infection, that may occur very early during childhood, usually proceeds in a persistent fashion, with a lifelong persistence of the viruses in the central nervous system, in the kidney and in the CD 34^+ lymphocytes (8, 9). In the large majority of cases virus infection is silent, without any pathological manifestation. However, in individuals with severe immunological failures, like the immunodeficiency associated with lymphoproliferative diseases and AIDS, or the immunosuppressive state induced in patients undergoing transplantation, latent polyomaviruses can be re-activated (8, 10), leading to unrestricted virus growth with high virus load in affected tissues and cytolytic destruction of viral target cells. This virus re-activation may results in serious pathologies. Progressive multifocal leukoencephalitis has been found to be associated with JCV (11), while BKV seems to be responsible for relatively serious diseases of the urogenital tract (7, 10). In addition, literature data exist demonstrating the presence of polyomavirus DNA sequences in cells from tumors of the central nervous system and of the urogenital tract (12-15) and, on this basis, it has been suggested that activation of BKV and JCV might play a role in the pathogenesis of these tumors.

To our knowledge, very little if any data have been reported on the correlation between tumors of endothelial

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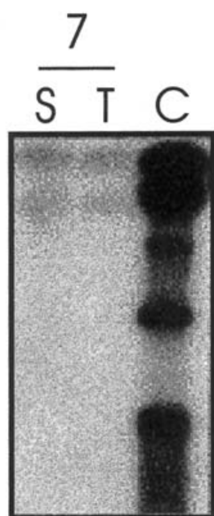

Figure 1. *Southern blot analysis of DNA obtained from a sample of tumor tissue and from the correspondent sample taken from the surrounding healthy colon mucosa (case n° 7 in Table I). DNA was extracted from the biopsy specimens, digested with endonucleases and then subjected to agarose gel electrophoresis. The probes employed for the Southern blot assay are reported in Methods. S = healthy tissue; T = tumor tissue; C = positive control (early region of BKV).*

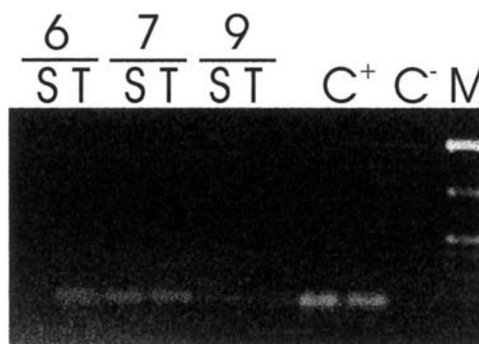

Figure 2. *PCR analysis of DNA obtained from three samples of tumor tissue and from the corresponding samples of healthy colon mucosa (cases N° 6, 7 and 9 in Table I). Following endonuclease digestion, DNA extracted from the biopsy specimens was subjected to 35 amplification cycles, using the two probes Multi V1: 5'-TAG GTG CCA ACC TAT GGA ACA GA-3' and Multi V2: 5'-GAA AGT CTT TAG GGT CTT CTA CC-3'. Amplification procedures, agarose gel electrophoresis and gel staining were performed according to standard procedures. S = healthy tissue; T = tumor tissue; M = molecular weight markers; C^+ = positive controls (JCV e BKV); C^- = negative control.*

origin and infection by human polyomaviruses. In this work, focused on colon rectal tumors, we present evidence that viral DNA sequences are detectable in a relevant proportion of cases, and that they are usually present both in the tumor mass and in the surrounding tissues. These data suggest a possible involvement of polyomaviruses in the pathogenesis of tumors of endothelial origin.

Materials and Methods

Sample collection. A group of 18 patients suffering from colon rectal carcinomas was utilized in this study. Two specimens were taken from each individual patient: one from the tumor mass and one from the healthy colon tissue in the surrounding area. The tissues samples were fixed in formalin, embedded in paraffin and then subjected to routine histological analysis by optical microscopy. All the 18 cases considered were diagnosed as adenocarcinomas; 11 showed a low level of differentiation, 5 were moderately-differentiated and 2 well-differentiated. All samples taken from tissues adjacent to the tumor mass did not show any sign of invasion or neoplastic transformation.

Southern blotting. Extraction of DNA, digestion with endonucleases, agarose gel electrophoresis and Southern blot analysis were performed according to standard methods (16, 17). Two different probes were employed for DNA hybridisation: a) the full-length JCV genoma (cloned in pBR322); b) a DNA fragment of BKV, enclosing the early region of the virus (with the coding sequences for the large T antigen) cloned in pBR322.

PCR analysis. Prior to amplification, DNA was digested with *Nar*I restriction enzyme. Amplification of an internal region of the large T antigen (highly conserved in all polyomaviruses) was performed employing the primers Multi V1: 5'-TAG GTG CCA ACC TAT GGA ACA GA-3' and Multi V2: 5'-GAA AGT CTT TAG GGT CTT CTA CC-3'. DNA was subjected to 35 amplification cycles: annealing and polymerization temperatures were 55° and 75°C, respectively. Amplification products were visualized after electrophoresis on 2% agarose gel and ethidium bromide staining.

In situ hybridization. DNA probes were biotinylated by nick translation of the whole viral genome and hybridized to histological sections previously treated with xylol, then rehydrated with washes in ethanol/water at decreasing alcohol concentration. The sections were deproteinized with DNase-free Proteinase K and endogenous peroxidases were blocked by addition of 0.9% H_2O_2 in methanol for 15 min at room temperature. DNA was denatured for 10 min at 95°C, probes were added and hybridization continued for 60 min at 37°C. Washing was performed for 20 min at 55°C. All the operations of visualization, staining and counterstaining were carried out according to the instructions of the commercial kit (Gene Point, DAKO).

Results

This study was performed on a set of biopsy specimens collected from colon rectal adenocarcinomas and, in parallel, from the healthy colon tissues surrounding the tumor mass.

In a first set of experiments, 14 samples (7 obtained from tumor tissues and 7 obtained from healthy colon tissues) were assayed by the Southern blot technique. Positive

Table I. *Comprehensive presentation of the data obtained by ISH and PCR assays.*

		ISH				PCR
		JCV		BKV		
		Epithelial	Lymphocyte	Epithelial	Lymphocyte	
1	Tumor	-	+	+	+	+
	Healthy	-	-	+	+	nd
2	Tumor	+	+	-	+	+
	Healthy	+	+	-	+	nd
3	Tumor	+	+	-		+
	Healthy	+	+	-	-	+
4	Tumor	+	+	+	+	+
	Healthy	+	+	+	+	+
5	Tumor	-	-	-	-	-
	Healthy	+	-	+	-	+
6	Tumor	-	-	+	-	+
	Healthy	-	-	-	-	-
7	Tumor	+	+	+	+	+
	Healthy	+	+	+	+	+
8	Tumor	+	-	+	+	+
	Healthy	+	-	+	-	+
9	Tumor	-	+	+	+	+
	Healthy	+	+	+	+	+
10	Tumor	+	+	+	+	+
	Healthy	+	+	+	+	+
11	Tumor	+	+	++	+	+
	Healthy	+	+	++	+	+
12	Tumor	-	-	-	-	-
	Healthy	-	-	-	-	-
13	Tumor	-	+	-	+	+
	Healthy	-	+	-	+	+
14	Tumor	-	-	-	-	-
	Healthy	-	-	-	-	-
15	Tumor	+	-	-	-	+
	Healthy	+	-	-	-	+
16	Tumor	-	+	+	+	+
	Healthy	-	+	+	+	+
17	Tumor	-	+	+	+	+
	Healthy	-	+	+	+	+
18	Tumor	+	+	+	+	+
	Healthy	+	+	+	+	+

results were obtained for 3 samples only, while the remaining 11 samples were found to be negative. Figure 1 illustrates the results obtained with patient n° 7, which resulted to be positive both for the tumor and for the healthy colon.

Since the low level of positive cases detected with the Southern blot assay might possibly be related to the low sensitivity of this technique, further experiments were performed to assess for the presence of JCV and/or BKV in colon carcinoma-bearing patients. Therefore, 34 specimens (18 from tumor tissues and 16 from healthy colon tissues) were examined by PCR using the primers reported in Materials and Methods. The complete results of this assay are reported in Table I, and 3 representative cases are illustrated in Figure 2. As apparent from Table I, viral DNA sequences were detected in 28 out of the 34 specimens tested (15 tumor tissues and 13 healthy ones). It should be noted that in 12 patients viral DNA sequences were observed both in the tumor and in the surrounding healthy tissue, in 3 patients tumor tissues were positive and the correspondent healthy colon tissues were negative or could not be tested and in one patient the healthy tissue was virus-positive while the tumor was virus-free. The remaining 2 cases (cases n° 12 and 14 in Table I) were negative both for the tumor tissue and for the surrounding healthy one.

It must be considered that neither Southern Blot nor PCR give a definitive answer as to which viral agent (JCV or BKV) is actually present in the samples under examination, since the probes in the Southern blot technique and the primers in the PCR assay do not discriminate between the two viral genomes. In light of this consideration, the biopsy specimens obtained from the 18 tumor-bearing patients were further probed by *in situ* hybridization (ISH), making use of the whole genomes of JCV and BKV polyomaviruses. By this technique, it is possible to detect specifically the presence of JCV and BKV in tumor cells and in colon epithelial cells, as well as in the lymphocytes infiltrating the tumor mass or present in the healthy colon. Samples showing the presence of virus in more than 5% of the nuclei were considered as positive (+), while the cases in which the staining was particularly intensive were considered as very positive (++) (Figure 3).

The results obtained by ISH are reported in Table I and some representative cases are illustrated in Figures 4, 5 and 6. When the ISH assay was performed making use of the JCV genome, in 9 out of the 18 tumor samples cancer cells were found to host JCV viral sequences. In all these 9 virus-positive tumors, JCV viral sequences could also be detected in the endothelial cells of the surrounding healthy mucosa. In 2 other samples (cases n° 5 and 9 in Table I), the endothelial cells in the healthy surrounding tissue were found to be positive for the virus that, on the contrary, could not be observed in the cancer cells of the correspondent samples obtained from the tumor mass. It is worth noting that in 7 out of the 9 virus-positive tumor samples JCV was present not only in cancer cells, but also in the lymphocytes infiltrating the tumor mass. Similarly, in 8 out of the 11 virus-positive samples of healthy tissue JCV was present both in endothelial cells and in the resident lymphocytes. Very interestingly, positive staining for lymphocytes was also detected in 5 samples of tumor tissues and in 3 samples of healthy colon tissues in which tumor and endothelial cells, respectively, were virus-free.

The presence of BKV viral sequences in cancer cells was detected in 11 out of the 18 tumor specimens. In 10 out of these 11 cases, positive staining was also monitored in the endothelial cells of the adjacent area. In another case (case n° 6 in Table I), the tumor cells were positive while the virus could not be observed in the healthy colon. Conversely, in case n° 5, the endothelial cells of the healthy colon were virus-positive, while in the correspondent tumor sample cancer cells were virus-negative. As far as lymphocytes are concerned, they were positive to BKV in 10 virus-positive and 3 virus-negative tumor samples and, moreover, in all the 11 samples of virus-positive healthy tissues.

Taken together, the ISH results for JCV and BKV demonstrated that polyomavirus DNA sequences were present in 30 out of the 36 biopsy specimens under consideration. In 14 samples (6 tumors and 8 healthy tissues), both JVC and BKV were found to be present, while in 6 samples (3 tumors and 3 healthy tissues) only JCV was present and 8 samples (5 tumors and 3 healthy tissues) were positive for BKV only. It must also be noted that in 1 case (case n° 13 in Table I) the positive staining observed both in the tumor sample and in the correspondent sample of healthy mucosa was due exclusively to the presence of both JCV and BKV in the lymphocytes, while both cancer cells and endothelial cells resulted virus-free.

To gain some insight into the role played by polyomaviruses in the process of neoplastic transformation, 5 virus-positive tumor samples and 5 virus-positive healthy colon samples were further assayed for the presence of the protein p53, making use of a conventional immunohistochemical technique. In this assay, 3 out of 5 tumor samples and 2 out of 5 colon samples were found to be positive for the presence of p53.

Discussion

Literature data report on the presence of DNA sequences of JCV in cells obtained from tumors of the central nervous system (7, 13). Also, BKV sequences were found in pancreatic, urinary and brain tumors, as well as in Kaposi sarcoma (12, 14, 15). However, to our knowledge, little if any information exists about the correlation between tumors of endothelial origin and infection by polyomaviruses. Therefore, we undertook a study addressed at detecting the possible presence of viral DNA sequences in colon rectal adenocarcinomas. For this purpose, three different strategies were used: Southern blot, PCR and ISH.

The first technique did not give satisfactory results, due to its relatively low sensitivity and to the low viral load present in the specimens under investigation. In effect, a number of samples which, by Southern blot, resulted to be negative for viral DNA turned out to be positive after PCR analysis.

By PCR, viral DNA sequences were detected in the large majority (28/34) of the samples under investigation, thus demonstrating that polyomaviruses are, in effect, widespread among colon rectal adenocarcinoma-bearing patients. In fact, polyomavirus DNA sequences in neoplastic and/or healthy colon tissues were detected in 16 out of the 18 patients considered (88.9%). However, PCR analysis does not allow discrimination between JCV and BKV sequences, due the extremely high DNA homology between the two viruses.

This problem was solved by *in situ* hybridization, using as a probe the entire viral genome after biotin labelling. In addition, ISH allows the localization of the viral sequences in the various cell types present in the samples under investigation. The results obtained by ISH demonstrated the presence of both JCV and BKV together in 6 virus-

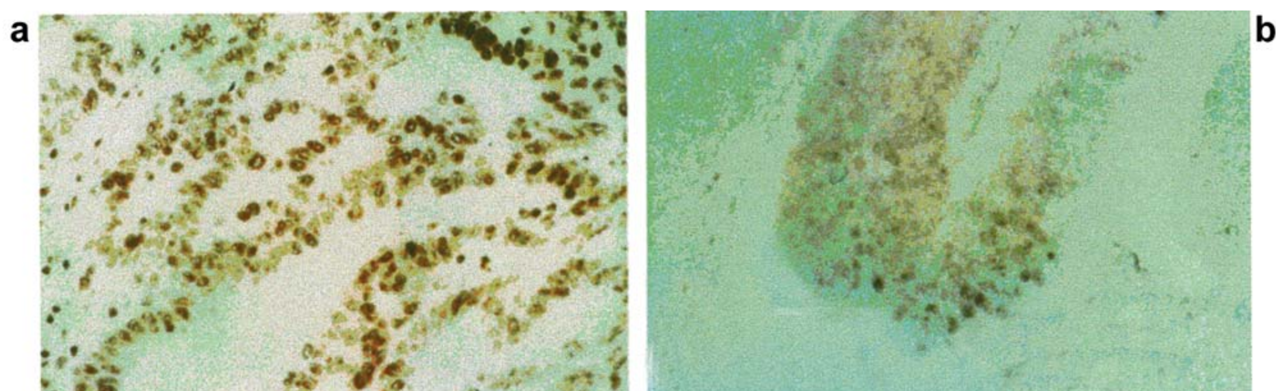

Figure 3. *Panel A: Example of a strong nuclear staining (++) in the nuclei of neoplastic cells obtained by ISH with the BKV primer (magnification 20x). Panel B: Example of a weak and focal positive nuclear staining obtained by ISH with the JCV primer (magnification 40x).*

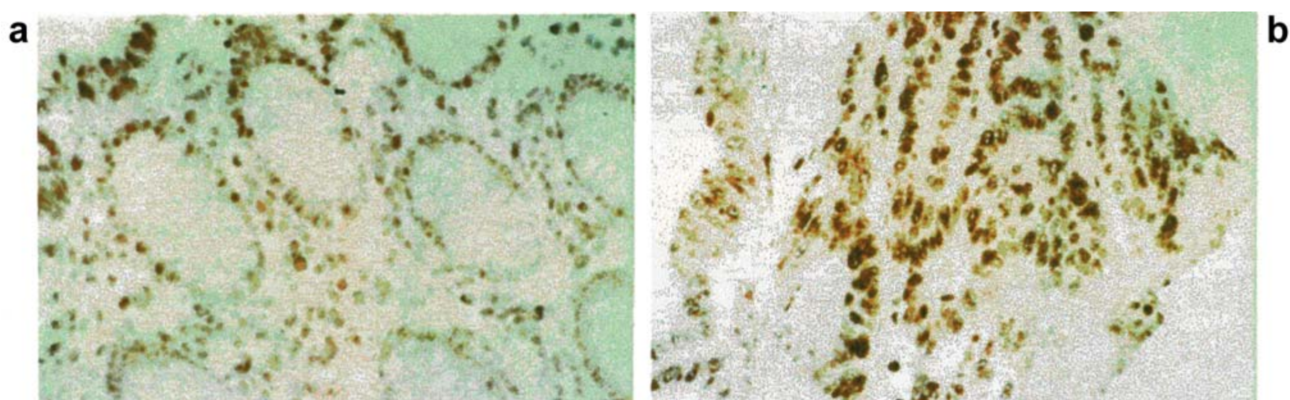

Figure 4. *Panel A. ISH for JCV in a sample of healthy mucosa: positive staining can be observed in the nuclei of endothelial cells as well as in lymphocytes present in this area (magnification 40x). Panel B. ISH for JCV in a tumor sample, showing positive staining of tumor cells (magnification 40x).*

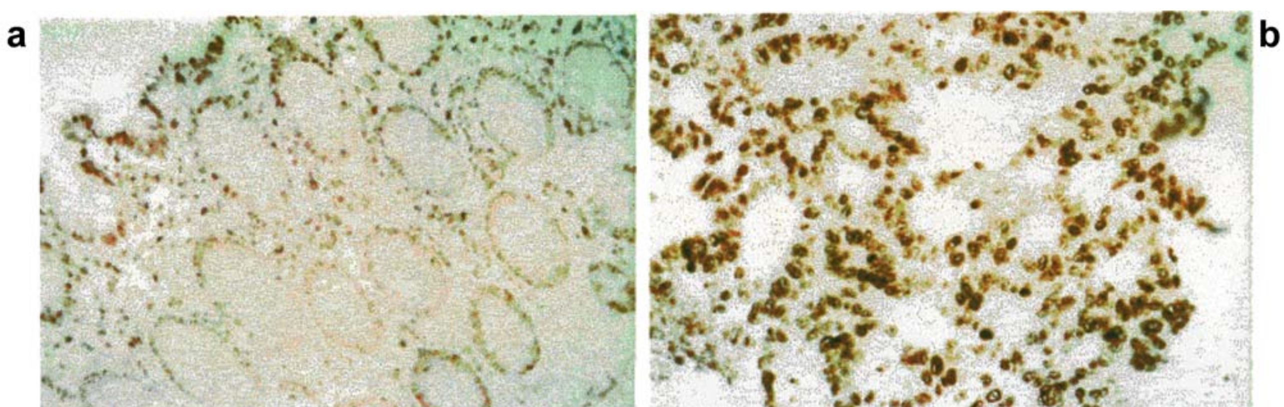

Figure 5. *Panel A. ISH for BKV in a sample of healthy mucosa: positive staining can be observed in the nuclei of endothelial cells as well as in lymphocytes present in this area (magnification 40x). Panel B. ISH for BKV in a tumor sample, showing positive staining of tumor cells (magnification 40x).*

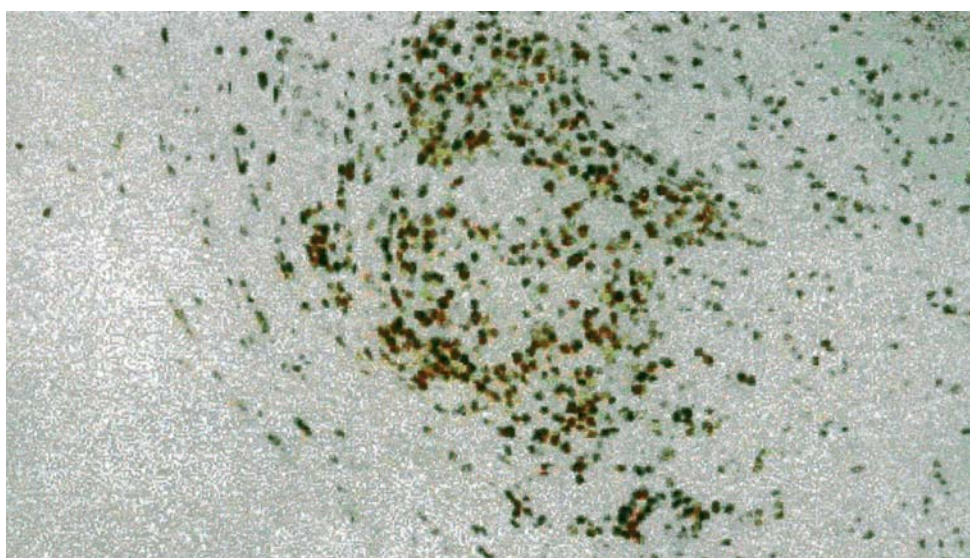

Figure 6. *ISH for BKV in a sample taken up from the healthy mucosa surrounding a colon adenocarcinoma. Positive staining was present in the nuclei of lymphocytes of a lymphatic follicle present in this area (magnification 40x).*

positive tumor samples and in 8 virus-positive healthy tissues, while only one type of virus was detected in the remaining 8 virus-positive tumors and 6 virus-positive normal tissues. ISH analysis also showed that, in the majority of samples, positive staining can be observed not only in tumor and endothelial cells, but also in the lymphocytes. Moreover, in some instances (see case n° 13), viral sequences were present only in the lymphocytes, while the other cells were virus-free. It might be argued that lymphocytes are reservoirs of latent infection and that they are responsible for the diffusion of the infection. A condition of particular immune deficiency could be at the basis of virus activation.

Our results show, that in all virus-positive tumor samples, polyomaviruses are also present in the surrounding healthy areas. However, in 3 cases (2 for JCV and 1 for BKV) viruses were found in the healthy area but were absent in tumors. An explanation of this interesting finding might be that, in the tumor cells, a dedifferentiation process, involving genomic rearrangement, could have caused the loss of the viral sequences. The surrounding healthy tissue, where these phenomena have not taken place, could therefore still host viruses. An alternative explanation is that transformation could have occurred by the "hit and run" mechanism described in papillomaviruses, but never reported in polyomaviruses. Anyway, the data clearly indicate that the presence of polyomaviruses in the colon mucosa is intrinsic and possibly related to tumor development. In agreement with our results, previous studies on BKV (18) demonstrated the presence of the virus in healthy and tumor areas, suggesting that the development of the tumor is due to multifactorial events in which the virus participates, although its presence may not be *per se* responsible for the neoplastic transformation.

We also evaluated the correlation between the presence of early viral sequences and the expression of the protein p53. This protein is involved in a number of phenomena including the control of cell proliferation. The concomitant presence of polyomaviruses and p53 was detected only in 5 out of 10 virus-positive samples. Clearly, the number of cases which were tested for the presence of p53 was too limited to draw definitive conclusions, but the above reported results are not in favor of a close relationship, in the process of neoplastic transformation, between the presence of polyomaviruses and the inactivation of p53.

In conclusion, we would like to stress that this study evidences the presence of polyomavirus DNA sequences in a type of tumor very little investigated previously. The data presented here suggest that, in some genetically predisposed and/or immunocompromised individuals, virus re-activation takes place and colon endothelial cells become virus-infected. This viral infection might be involved in the further process of neoplastic transformation.

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