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Detection of Human Polyomavirus Proteins, T-Antigen and Agnoprotein, in Human Tumor Tissue Arrays

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Abstract

Expression of the human polyomavirus JCV genome in several experimental animals induces a variety of neural origin tumors. The viral proteins, T-antigen and Agnoprotein, contribute to the oncogenesis of JCV by associating with several tumor suppressor proteins and dysregulating signaling pathways, which results in uncontrolled cell proliferation. In addition, T-antigen and Agnoprotein have been associated with DNA damage and interfering with DNA repair mechanisms. In this study, we have utilized commercially available tissue arrays of human tumors of various origins and demonstrated the expression of both T-antigen and Agnoprotein in some, but not all, tumors of neural and non-neural origin. Most notably, more than 40% of human glioblastomas and greater than 30% of colon adenocarcinomas express viral proteins. The detection of viral transforming proteins, T-antigen and Agnoprotein in the absence of viral capsid proteins suggests a role for JCV in the development and/or progression of human tumors. These results invite further large-scale investigation on the role of polyomaviruses, particularly JCV in the pathogenesis of human cancer.

Keywords

JCV; tumors; polyomavirus; cancer; T-antigen

INTRODUCTION

The human polyomavirus JCV is an opportunistic pathogen infecting greater than 90% of the human population worldwide [Taguchi et al., 1982; Walker and Padgett, 1983; Berger and Concha, 1995]. Initial infection with JCV, which usually occurs in early childhood, is subclinical and yields no symptomatology. Repeated detection of JCV in kidney epithelial cells and its occasional presence in urine of individuals with mild immunosuppressive conditions and in the urine of pregnant women led to the assumption that the kidney may serve as a site for virus latency [Coleman et al., 1977; Kitamura et al., 1997; Bendiksen et al., 2000; Behzad-Behbahani et al., 2004; Rossi et al., 2007]. Subsequent studies on the detection of JCV in B-lymphocytes [Tornatore et al., 1992] and more recently in astrocytes and oligodendrocytes of normal brain [Delbue et al., 2008; Perez-Liz et al., 2008] suggested alternative sites in which JCV may remain in a non-productive state. Reactivation of the virus, which usually occurs in the context of immunosuppressive conditions, leads to the expression of viral genome proteins and replication of viral DNA in oligodendrocytes, thus causing cytolytic destruction of these cells and the development of the fatal demyelinating

disease, progressive multifocal leukoencephalopathy (PML) [Del Valle and Piña-Oviedo, 2006].

The absence of viral replication in any other cells and tissues indicates that the productive lytic cycle of JCV is cell type-specific and occurs almost exclusively in oligodendrocytes and astrocytes. However, expression of the viral early protein, T-antigen, has been detected in several tumors of non-glial origin [Del Valle et al., 2004, 2005]. Similar to its well-studied counterpart from SV40, T-antigen of JCV is a pleiotropic protein with the ability to interact with several tumor suppressor proteins including p53, pRb, and NF2 [Tan et al., 1986; Dyson et al., 1990; Krynska et al., 1997, 1999; Shollar et al., 2004], suggesting a role for JCV in the dysregulation of cell proliferation by its early protein, T-antigen. In connection with this finding, several studies in experimental animals have revealed that inoculation of JCV into primates and small animals including hamsters and rats resulted in the development of a broad range of tumors including neuroectodermal and glial origin tumors [Zu Rhein and Varakis, 1979; London et al., 1983; Ohsumi et al., 1985]. Further, several lines of transgenic mice expressing JCV T-antigen developed a variety of tumors including primitive neuroectodermal tumors, glial tumors, and pituitary tumors, once again verifying the oncogenic potential of JCV T-antigen in the absence of viral replication in the experimental animals [Del Valle et al., 2001a].

In human brain tumors, there have been several reports indicating the detection of the JCV genome and more importantly the presence of its T-antigen in neoplastic cells [Del Valle et al., 2002a,b]. Of interest is the observation by five independent laboratories on the detection of JCV in colon cancer tissues [Laghi et al., 1999; Enam et al., 2002; Hori et al., 2005; Lin et al., 2008; Noshio et al., 2009]. While these observations may not establish an etiological role for JCV in the development of human cancer, its accepted role in the initiation of tumors in animal models and the transforming ability of T-antigen may ascribe a role for this protein in the disruption of pathways that involve tumor cell progression. In addition to its capacity to interact with several tumor suppressors, JCV T-antigen has the ability to dysregulate several signaling pathways, such as WNT/ β -catenin, which are responsible for the control of cell proliferation [Gan et al., 2001; Enam et al., 2002; Bhattacharyya et al., 2007]. Another important pathway affected by JCV is the IGF-IR/IRS-1 pathway. Several reports have shown that T-antigen is capable to translocate IRS-1 to the nucleus, where it interferes with DNA repair fidelity, resulting in the accumulation of mutations [Lassak et al., 2002; Trojanek et al., 2006].

The late accessory protein of JCV, Agnoprotein has also received significant attention in tumorigenesis of JCV as several studies showed its expression in brain and non-brain tumors [Del Valle et al., 2002b; Enam et al., 2002]. Of particular interest is the observation indicating the ability of JCV Agnoprotein to dysregulate the cell cycle and interfere with the pathways involved in DNA repair and chromosomal stability [Darbinyan et al., 2002, 2007].

To gain some insights regarding the possible involvement of JCV in a broader range of human tumors, in this study, we evaluated the expression of JC viral proteins in several commercially available tissue arrays by immunohistochemistry. We evaluated the expression of the early protein, T-antigen and the late auxiliary product, Agnoprotein. Results further confirm the detection of T-antigen and Agnoprotein in various human tumors.

MATERIALS AND METHODS

Tissue Arrays

Three different sets of cancer tissue microarrays were purchased from AccuMax (ISU/ABXIS Co., Seoul, Korea): slide no. A201, containing 27 different types of human cancer samples (3 slides); slide no. A203 (I), containing 50 cases of human adenocarcinomas of the colon and 2 normal colon controls (3 slides); and slide no. A221 (II), containing 4 grades of human brain tumors of glial origin, ranging from low-grade astrocytomas to GBM (30 cases in total), and 2 normal brain samples (3 slides). The tissue in all slides had been previously fixed in formalin and embedded in paraffin.

Immunohistochemical Analysis

Immunohistochemistry was performed using the avidin–biotin–peroxidase methodology, according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA). Our modified protocol includes paraffin melting at 58°C in a regular oven for 20 min, deparaffination in xylene, rehydration through descending grades of alcohol up to water, and non-enzymatic antigen retrieval in 0.01 mol/L sodium citrate buffer, pH 6.0, heated to 95°C for 40 min in a vacuum oven. After a cooling period of 30 min, the slides were rinsed in PBS and treated with 3% H₂O₂ in methanol for 25 min to quench endogenous peroxidase. Sections were then blocked with 5% normal horse serum (for mouse monoclonal antibodies), or normal goat serum (for rabbit polyclonal antibodies) in 0.1% PBS/BSA for 2 hr at room temperature. Primary antibodies were incubated overnight at room temperature in a humidifier chamber. Primary antibodies utilized in the present study included a mouse monoclonal against the viral early product T-antigen, which recognizes the T-antigen from JCV and SV40 (Clone pAb416, 1:100 dilution, Calbiochem, San Diego, CA), and rabbit polyclonal antibodies for Agnoprotein generated in our laboratory using peptides derived from JCV Agnoprotein (Del Valle et al., 2002b) (1:2000 dilution) and VP-1 (1:1000 dilution, kindly provided by Dr. Walter Atwood, Brown University, Providence RI) that recognizes JCV as well as SV40 capsid proteins. In the second day, slides were thoroughly rinsed with PBS, and biotinylated secondary anti-mouse or anti-rabbit antibodies were incubated for 1 hr at room temperature (1:200 dilution). Then sections were incubated with avidin–biotin–peroxidase complexes (Vectastain ABC Elite kit; Vector Laboratories) for 1 hr at room temperature, rinsed with PBS, and developed with diaminobenzidine (DAB tablets, Sigma, St. Louis, MO) for 3 min. Finally, the sections were counterstained with Hematoxylin and mounted with Permount (Fisher Scientific, Fair Lawn, NJ).

RESULTS AND DISCUSSION

Results from immunohistochemical experiments performed in a glial brain tumor tissue array demonstrated the expression of the JCV oncoprotein T-antigen in 1 out of 2 cases of diffuse fibrillary astrocytomas, 4 out of 7 cases of anaplastic astrocytomas, and 6 out of 13 cases of Glioblastoma multiforme. Interestingly none of the eight cases of pilocytic astrocytomas in the array showed T-antigen immunoreactivity. These percentages of positive cases are in accordance with the results reported in our previous study [Del Valle et al., 2001b]. The late accessory product, Agnoprotein was found in the same number of cases of glial tumors, with the exception of one Glioblastoma (Table I). The capsid protein VP-1 was consistently negative, ruling out productive viral infection in all the samples.

Results from a colon cancer array showed that 17 of 50 samples were immunoreactive for T-antigen. Again, 15 out of those 17 positive samples also expressed Agnoprotein, while the 2 samples of normal colon were negative for all viral proteins. In the tissue array containing different phenotypes of human cancers, T-antigen was found expressed in one case of GBM,

one out of two cases of esophageal carcinoma, four out of four cases of thyroid malignancies (papillary and follicular), one out of two cases of breast cancer, four out of six cases of lung carcinomas, one case of nephroblastoma (Wilms' tumor), and one out of four cases of ovarian cancer. Table I shows the total number of cases and the expression of viral proteins in the three different tissue arrays.

In all positive cases, expression of T-antigen is confined to the nuclear compartment of neoplastic cells and Agnoprotein is located to the cytoplasm. Within the same slides, tissue cylinders of normal colon and normal brain parenchyma show no expression of T-antigen, and work as an internal control. Representative cases in which T-antigen was detected are presented in Figure 1 and expression of Agnoprotein is shown in Figure 2.

Despite the well-characterized transforming abilities of polyomaviruses in vitro, and the established oncogenic potential of JCV in animal models, either after direct inoculation of JCV or expression of the transgene encompassing the JCV early genome in transgenic animals [Zu Rhein and Varakis, 1979; London et al., 1983; Ohsumi et al., 1985; Small et al., 1986; Franks et al., 1996], the association of JCV with human tumors has been debated during the last decade. While several studies have successfully detected viral genomic sequences by PCR and viral proteins by immunohistochemistry in several types of brain tumors and other organs including the gastrointestinal, urinary, and respiratory tracts, other studies have failed to establish the frequent presence of JCV in human tumors [Rollison et al., 2005]. These discrepancies may be due to technical difficulties and especially to the quality of the available tissue. For example, it is well known that long periods of fixation in formalin contribute to DNA degradation [Ben-Ezra et al., 1991; Ferrer et al., 2007], making PCR amplification challenging. Other methodologic issues including the efficiency of DNA extraction, sensitivity of PCR and Southern blot, and the copy number of positive controls that are used in the assay may also contribute to the observed discrepancy in the detection of JCV DNA in the clinical samples [Gordon et al., 2005]. Of note, in a number of studies where JCV was detected in the tumor cells, the more sophisticated technique of laser capture microdissection was utilized to avoid any difficulties associated with copy number, DNA contamination, etc. The relatively newly developed technology of tissue microarrays [Rimm et al., 2001] presents a great source for standardized tissue, which is available to different groups and presents a large number of samples in the same slide, reducing costs, experimental time, and reagents.

The results of our study shown in this report, although limited in its scope, are critical as for the first time they demonstrate the expression of the polyomavirus proteins in commercially available tissue arrays, and thus can serve as a benchmark for other laboratories to obtain duplicate samples derived from the same tissue bank for studying JCV expression in human tumors. Consistent with recent observations, expression of the viral proteins was absent in the normal brain samples included in the array [Delbue et al., 2008; Perez-Liz et al., 2008]. Of note, none of the tumor samples showed the presence of viral capsid proteins, ruling out productive infection of JCV in these tumor cells. It is also important to note that the antibodies used for the detection of JCV T-antigen and Agnoprotein also react with their counterparts from SV40 and BKV. Thus, while our results may not exclude the possible presence of SV40 and/or BKV proteins in these samples, based on several previous studies, using gene amplification [Del Valle et al., 2001a,b, 2002a,b, 2004, 2005; Enam et al., 2002] have shown limited, if any, association of BKV and SV40 with those adult tumors derived from brain and colon. Thus, it is most likely that the observed T-antigen and Agnoprotein in these samples are produced by JCV. These observations, along with mounting evidence on the presence of JCV induced tumors in animals and humans, suggest a bi-potential role for JCV in the development of CNS diseases. On one hand, by completing its lytic cycle in glial cells, JCV can induce demyelinating disorders, such as PML, in immunodeficient patients

[Berger and Concha, 1995; Piña-Oviedo et al., 2007]. On the other hand, in the absence of viral DNA replication and lytic infection, expression of its genome including T-antigen and possibly Agnoprotein can promote cell proliferation and tumor formation.

Activation of the JCV promoter in a non-glial context is also of particular interest as it implies that under certain physiological conditions, the JCV genome can be expressed in non-neural cells. With this notion, one may speculate that precancerous cells containing a broad and dysregulated repertoire of transcription factors, may gain the capacity to activate transcription of the JCV promoter in these cells. Under these circumstances, it is likely that expression of viral proteins accelerates the development and progression of a broad range of non-neural cancers. The results from our studies may open the door for new experiments in different human cancers that were not suspected for their association with human oncogenic viruses including JCV.

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Fig. 1. Detection of T-antigen in human cancer tissue arrays. Immunohistochemistry for the JCV early protein T-antigen performed in different human tissue arrays shows robust expression in the nuclei of neoplastic cells from astrocytic tumors, including low-grade diffuse astrocytomas (**Panel A**), anaplastic gliomas (**Panel B**), and GBMs (**Panel C**). In contrast, no expression of T-antigen was found in any cell type from normal brain cylinders present in the same array (**Panel D**). T-Antigen expression is robust in neoplastic epithelial cells from colon adenocarcinomas (**Panel E**), while is undetectable in the normal colon (**Panel F**). T-Antigen expression was also found in the nuclei of malignant epithelial cells from lung carcinomas (**Panel G**), breast lobular carcinomas (**Panel I**), and serous adenocarcinomas

from the ovary (**Panel J**), while stromal cells in the same sections remain negative. Nephroblastomas, primitive tumors of the kidney, also show expression of T-antigen (**Panel H**). Finally nuclear expression of T-antigen was noted in epithelial cells from serous and follicular carcinomas of the thyroid gland (**Panels K and L**, respectively). All low power views (upper panels) original magnification 40×. All high power views (lower panels) original magnification 400×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 2.

Detection of Agnoprotein in human cancer tissue arrays. Immunohistochemical studies performed in tissue arrays of different human tumor phenotypes demonstrates the expression of the viral accessory product, Agnoprotein in the cytoplasm of neoplastic astrocytes in different degrees of glial tumors ranging from low-grade astrocytomas (**Panel A**), to anaplastic tumors (**Panel B**) and glioblastoma multiforme (**Panel C**). In a different array, Agnoprotein is detected in the cytoplasm of neoplastic epithelial cells from colon adenocarcinomas (**Panel D**), esophageal carcinomas (**Panel E**), and cases of breast lobular carcinomas (**Panel F**). All panels original magnification 400×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I**Immunohistochemical Detection of JC Viral Proteins in Human Cancer Arrays**

Tumor	Total cases	T-antigen	Agno protein	VP-1
AccuMax A221(II) brain cancer tissues				
Pilocytic astrocytomas	8	0	0	0
Diffuse fibrillary astrocytomas	2	1	1	0
Anaplastic astrocytomas	7	4	4	0
Glioblastoma multiforme	13	6	5	0
Normal brain	2	0	0	0
AccuMax A203(I) colon cancer tissues				
Adenocarcinomas	50	17	15	0
Normal colon	2	0	0	0
AccuMax A201 various cancer tissues				
Glioblastoma multiforme	2	1	1	0
Esophageal carcinoma	2	1	1	0
Larynx squamous cell carcinoma	2	0	0	0
Thyroid papillary carcinoma	2	2	2	0
Thyroid follicular carcinoma	2	2	2	0
Breast ductal carcinoma	1	0	0	0
Breast lobular carcinoma	1	1	1	0
Gastric adenocarcinoma	2	0	0	0
Gastric signet ring cell carcinoma	2	0	0	0
Pancreatic adenocarcinoma	2	0	0	0
Lung squamous cell carcinoma	2	2	1	0
Lung adenocarcinoma	2	0	0	0
Poorly differentiated lung tumors	2	2	2	0
Hepatic colangiocarcinoma	1	0	0	0
Hepatic adenocarcinoma	1	0	0	0
Hepatocarcinoma	2	0	0	0
Kidney Wilm's tumor	1	1	1	0
Renal cell carcinoma	1	0	0	0
Ovary serous adenocarcinoma	2	1	1	0
Ovary mucinous adenocarcinoma	2	0	0	0
Endometrial squamous cell carcinoma	2	0	0	0
Endometrial adenocarcinoma	2	0	0	0
Colon adenocarcinoma	2	0	0	0
Rectal maltoma	1	0	0	0
Cecal adenocarcinoma	1	0	0	0
Dermatofibrosarcoma protuberans	1	0	0	0
Skin squamous cell carcinoma	1	0	0	0