

*Short Communication***No Evidence of an Association of JC Virus and Colon Neoplasia**Polly A. Newcomb,¹ Angela C. Bush,¹ Gerald L. Stoner,² Johanna W. Lampe,¹ John D. Potter,¹ and Jeannette Bigler¹¹Cancer Prevention Research Program, Fred Hutchinson Cancer Research Center, Seattle, WA and ²Neurotoxicology Section, National Institute of Neurological Disorders and Stroke, NIH, Bethesda, MD**Abstract**

JC virus (JCV) is an ubiquitous human polyomavirus that frequently resides in the kidneys of healthy individuals and is excreted in the urine of a large proportion of the adult population. Polyomaviruses are associated with disease largely in immunocompromised individuals (progressive multifocal leukoencephalopathy). Colorectal cancers can show chromosome instability and it was hypothesized that JCV may account for some of this instability. We screened urine from 45 healthy donors and 233 colorectal cancer/normal tissue pairs for the presence of JCV sequences

using a Taqman assay. This assay could detect 1 virus genome in 10 human genomes. In the urine samples, we found an infection rate of approximately 70%. The JCV isolates in these samples could be categorized into four JCV types (2B, 4, 7, and 8), none of which had a rearranged regulatory region. Among the colon tissues, one normal tissue (<0.5%) and none of the matched tumors tested positive for JCV. There is no evidence in these data to indicate that JCV is the cause of genetic instability in colorectal cancer. (Cancer Epidemiol Biomarkers Prev 2004;13(4):662–666)

Introduction

JC virus (JCV) is an ubiquitous human polyomavirus that frequently resides in the kidneys of healthy individuals and is excreted in the urine of a large proportion of the population. Infection with JCV is subclinical and leads to lifelong latency. Infection rates vary among populations and range from 20% to 80% (1). The mode of transmission of JCV among individuals has not been defined; the fact that individuals seroconvert during childhood, along with the detection of JCV in tonsillar tissue, supports a respiratory route (for review, see Ref. 2). Transmission of JCV through the gastrointestinal tract by means of contaminated water has also been proposed (3). JCV can persist in the kidney, brain, and lymphocytes (4).

Polyomaviruses are associated with disease only in immunocompromised individuals (4).

Similar to other polyomaviruses, the genome of JCV consists of a single double-stranded, circular DNA molecule, which contains sequences for the viral early proteins large- and small T antigens, and the late capsid proteins, VP1, VP2, and VP3, as well as the auxiliary agnoprotein (5). Natural variation caused by point mutations throughout the JCV genome has led to the identification of several distinct JCV genotypes (for review, see Ref. 1), which can be defined in a short segment of VP1. Pathogenic variation in the viral control region (VCR) can occur through rearrangements—deletion and duplication of promoter/enhancer elements—and is associated with progressive multifocal leukoencephalopathy (PML), a fatal neurological disease. These VCR rearrangements are thought to facilitate viral replication in glial cells. The rearranged VCR is called “PML-type” in distinction to the “archetype” found in the urinary tract. The vast majority of PML rearrangements are unique (6, 7).

The large T antigen is essential for viral replication in the nucleus. Once the DNA has been replicated and the structural proteins are produced, new viral particles are assembled and released from the cell (8). Infrequently, the viral DNA becomes integrated into the host cell genome and is subsequently inherited as if it were a cellular gene. If the arrangement of viral DNA sequences

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following integration permits continued expression of the large T antigen, the host cell can become transformed (8). Large T can induce resting cells to enter the cell cycle by inactivating the Rb family of proteins, block cell cycle arrest and apoptosis by sequestering p53, and inducing chromosomal damage (for review, see Ref. 2).

Evidence supporting a possible role for JCV in human cancer is not plentiful. However, JCV sequences and protein have been found in medulloblastomas (9, 10) and tumors of the central nervous system (11). Colorectal cancers often show chromosome instability and it has been hypothesized that JCV may account for some of this instability (12, 13). In two small studies, JCV sequences were reported in normal as well as neoplastic gastrointestinal tissue (12, 14). Cloning and sequencing of large T (12) and the VCR (15) revealed a number of point mutations, small deletions or insertions and a variable number of the 98-bp sequence that is duplicated in Mad-1. Most of the clones were unique and clones carrying different mutations were isolated from the same individual. One recent study reported JCV sequences as well as large T and agnoprotein expression in colon adenomas and carcinomas but not in normal, surrounding tissue (16).

In this study, we tested 233 colon cancer cases on which we had both tumor and normal tissue as well as urine from 45 healthy volunteers for the presence of JCV using a Taqman assay. Sequence comparison of parts of the VCR, VP1, and large T was used to determine the major JCV genotype.

Materials and Methods

Tissue Specimens. Two populations were used for this study. To evaluate the sensitivity and specificity of the assay, we used stored urine samples from 45 healthy female volunteers, aged 20–40 (17). For our investigation of JCV in tumor tissue, we used a population-based sample of invasive colorectal cancer cases enrolled as participants in the Seattle Colorectal Cancer Registry (18). Briefly, cases aged 20–74 years were identified from the Puget Sound SEER registry. Participants completed a risk-factor interview (81% of eligible individuals) and provided blood and access to diagnostic tumor tissue (75%). Activities were reviewed and approved by The Institutional Review Board at Fred Hutchinson Cancer Research Center.

For this study, we used 233 invasive colorectal cancer cases with matched colorectal tumor/normal tissue available as well as white blood cells from 196 of these cases. In the Pathology Shared Resource laboratory at FHCRC, the tissue blocks were cut into 5- μ m sections, which were transferred to Dr. Bigler's laboratory in microcentrifuge tubes. Microtome blades were changed after each study participant. DNA was extracted from paraffin-embedded tissue using the QIAGEN tissue kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. DNA was extracted from lymphocytes using the QIAGEN blood midi-kit. Urine was processed and aliquoted for storage by the FHCRC Specimen Processing Shared Resource, which does not conduct molecular analyses. Urine samples were stored at -80°C until DNA was extracted from the urine (2 ml) using the QIAGEN blood midi-kit.

Screening of Tissue for JCV. A full-length clone of JCV strain 803A (19), obtained from the American Type Culture Collection (Manassas, VA), was used as a positive control. JCV plasmid purification was performed in a different laboratory from all the screening assays. The JCV plasmid was mixed with human genomic DNA to obtain final ratios of 10:1, 1:1, and 0.1:1 virus genomes per human genome. One hundred nanograms of these three DNA mixes were included as positive controls in each batch of samples screened. The presence of JCV in the genomic DNA samples was detected using a real-time PCR assay. Test samples were added to the amplification reactions and sealed. The positive controls were added to their respective wells after sealing the other wells. Fragments from two JCV regions, large T and VP1, were amplified using the primers and probes listed in Table 1. In addition to the TaqMan PCR core reagents (Applied Biosystems, Foster City, CA), the reactions contained 4 mM MgCl_2 for VP1 (6 mM MgCl_2 for large T), 200 nM amplification primers for VP1 (300 nM for large T), 150 nM TaqMan probe, 50 ng of genomic DNA, and 0.5 units AmpliTaq Gold DNA polymerase. The cycling conditions on an Applied Biosystems 7900HT Sequence Detection System were 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 s, and 60°C for 1 min.

Strain Identification. For samples that were positive for the presence of JCV, sequencing was performed to identify the particular strain of the virus. Fragments from VP1, large T, and the regulatory region (RR) were amplified by nested PCR (see Table 1 for primer sequences). The primary PCR reactions contained $1\times$ buffer II (Applied Biosystems), 1 mM MgCl_2 for VP1 (2 mM for large T and RR), 200 μM dNTPs, 300 nM

Table 1. Primers and probes for JCV screening and sequencing

Primers and probes	
Taqman	
VP1	
FP	5' AGGAGGTGCAAATCAAAGATCTG3'
RP	5' GGGCCATCTTCATATGCTTCA3'
Probe	5' 6FAM-ATGTGCTTACTTTTAGGGTTG TACGGGACTGTA-TAMRA3'
Large T	
FP	5' GTTAGGCCATTCCTTGCAATAAA3'
RP	5' CGAAGACAAGATGAAGAGAATGAATT3'
Probe	5' 6FAM-CTCTGAACACTATCCATGTACCAAAAT CAGGCTGA-TAMRA3'
Nested PCR and sequencing	
VP1 ^a	
FP (1°)	5' CCCAAATAGGGACATGCTTCCTT3'
FP (2°) ^a	5' ACAGTGTGGCCAGAATCCACTACC3'
RP (both)	5' AATGAAAGCTGGTGCCCTGCA3'
Large T	
FP (both)	5' TGTCAACCCTTTGTTGGCT3'
RP (1°)	5' GCCTTAAGGAGCATGACTTT3'
RP (2°)	5' CCAGAAGAACCAGAAGAAAC3'
RR	
FP (1°)	5' CCTCTAAAAAGCCTCCACG3'
FP (2°)	5' CTTCTGAGTAAGCTTGGAGG3'
RP (both)	5' CCCTTGGCTGCTTCCACTT3'

^aAgostini *et al.* (1998).

Table 2. Sensitivity and specificity of JCV detection

JCV regions						
	RR	VP1	Large T	VP1 only	Large T only	VP1 and large T
Taqman	ND	32/45	21/45	11	0	21
	RR	VP1	Large T	Sequence for ≥ 2 fragments		
Sequencing	30/32	29/32	30/32	32		

Note: ND, not done.

amplification primers for VP1 (200 nM for large T and RR), 50 ng genomic DNA, and 0.5 units AmpliTaq DNA polymerase. The cycling conditions on a PTC-100 thermal cycler (MJ Research, Inc., Watertown, MA) were: 94°C for 3 min, followed by 25 cycles of 94°C for 30 s, annealing (60°C for 1 min for VP1 and large T, 64°C for 45 s for RR), and 72°C for 1 min, and 1 cycle of 72°C for 5 min.

After amplification, the PCR reactions were diluted with three volumes sterile, PCR-grade water. The conditions for the secondary PCR were the same as for the primary PCR, with the exception of the MgCl₂ concentrations (1.5 mM for VP1 and large T, 2.5 mM for RR) and 35 amplification cycles. Sequencing was performed using the BigDye Terminator v3.0 cycle sequencing kit (Applied Biosystems) and a 3100 Genetic Analyzer (Applied Biosystems).

Results

JCV Assay. Because sequence variation between different JCV types can be significant, it is critical to design a screening assay that can detect if not all, then at least a large majority of strains. Instead of using degenerate primers and PCR conditions with relatively low specificity (12), we selected regions of the VP1 and large T genes that are highly preserved among strains for the screening assay. In screening assays, it is important to include positive controls for PCR amplification, in addition to the essential negative, or reagent, controls.

These controls usually consist of cloned virus and there is the possibility of contamination of the tissue sample with the controls. For this reason, we chose a recently identified JCV strain from Papua New Guinea, 803A (19) that we did not expect to find in our study population. Unlike Mad-1, the laboratory strain commonly used as a control, JCV 803A does not have a rearranged regulatory region. Our assays for both, VP1 and large T, could dependably detect one JCV 803A genome per 10 human genomes in a total of 100 ng of DNA template.

Because JCV is normally found in urine, we first used urine samples to test our screening method. The screening assay detected VP1 sequences in 32/45 samples and large T sequences in 20/45 samples (see Table 2). All the samples that were positive for large T were also positive for VP1. However, in 11 samples, only the VP1 assay detected JCV. The VP1 PCR was at least 10 times more sensitive in detecting JCV sequences than the large T PCR. This difference in sensitivity accounted for the difference in positive scores between the VP1 and large T screens. The presence of JCV DNA in all the samples that were scored positive in the VP1 assay was confirmed by sequencing.

As noted, sequences in less well-conserved regions of the JCV genome allow classification of viruses into different types (6, 20). All the samples that tested positive for JCV in the VP1 screen were subjected to sequencing of variable regions in the VP1 and large T genes, as well as the regulatory region. This VP1 fragment provides sites that identify at least seven genotypes and additional subtypes and has been validated by the analysis of complete JCV genomes (6, 21). VP1 sequences were obtained for 29/32 samples and large T and regulatory region sequences for 30/32 samples each. All the samples yielded sequence information for at least two of the fragments. Table 3 shows sequence differences between the different isolates in this study. Other nucleotide positions that distinguish between types not identified in this study are not listed. The majority of isolates belonged to JCV types 7 and 8, three to type 4 and one to type 2B. The regulatory regions were characterized by greater sequence variation than VP1. This variation consisted of base changes and small deletions, but not rearrangements (data not shown). Furthermore, identical regulatory regions were found in different individuals.

Table 3. JCV types in urine samples from healthy volunteers

# Individuals	VP1 fragment at nt positions										JCV type
	1741	1756	1771	1786	1805	1837	1843	1850	1852	1869	
1	A	C	A	T	A	C	T	G	T	G	2B
1	A	C	C	G	A	T	T	A	T	C	4
2	A	C	C	G	A	T	G	A	T	C	4
1	A	T	A	G	T	T	T	A	T	G	7
5	A	C	A	G	A	T	T	A	C	G	7
1	A	C	A	G	T	T	G	A	T	G	7
3	A	C	A	G	A	T	T	A	C	G	7
12	A	T	A	A	A	T	T	A	T	G	8
1	A	T	A	T	A	T	T	A	T	G	8

None of the isolates was identical to JCV 803A, which was used as a positive control. These data showed that our screening assay was able to detect JCV sequences from different virus types.

JCV in Colorectal Tissue. Colorectal tumor/normal tissue pairs were then screened for the presence of JCV VP1 and large T sequences. Of the 233 tissue pairs tested, one normal specimen (<0.5%) was positive for JCV. However, none of the neoplastic tissues carried detectable levels of JCV. Because JCV can persist in lymphocytes, we also tested lymphocyte DNA for JCV. Lymphocyte DNA was available for 196 of the 233 colon cancer cases. In two of these samples (1%), we detected JCV sequences. Neither one was from the same study participant as the positive colon tumor tissue sample.

Discussion

JCV was detected using our assay in the urine of 70% of healthy volunteers. However, it was present in <1% of colorectal tumor or normal tissue and lymphocytes from colorectal cancer cases. We believe these results reflect the actual prevalence of JCV in colorectal cancer because we demonstrated JCV carrier frequencies in urine comparable to other published reports (6).

PCR is a powerful tool in the detection of DNA sequences of interest. However, problems can arise if there is the possibility of carry-over of amplified DNA into genomic DNA. This is especially true in cases such as screening for virus infection, where it is necessary to include positive controls in the assay. These positive controls usually consist of cloned virus sequences. Even when all the precautions are taken to prevent carry-over, it is important to have mechanisms in place to detect carry-over should it occur. For this reason, we chose as a positive control a JCV type that we did not expect to find in our population. By sequencing of the isolates, carry-over of the positive control can be detected.

Earlier reports showed the presence of Mad-1 sequences in the colon, the same strain that was used as a positive control (12). The authors observed heterogeneity in the VCR clones, which consisted of different numbers of repeats of a 98-bp sequence. Different copy numbers were observed between clones from the same sample as well as clones from different samples. Whereas a different copy number argues against contamination with the positive control, the point mutations and single-base deletions or insertions in the VCR as well as large T (12, 15) are more likely due to errors introduced by Taq polymerase than sequence differences within and between isolates. High variability of the JCV genome in an individual is not supported by published work on geographical differences in JCV sequences (6, 22) and the fact that isolates taken from the same individuals several years apart were the same (23). In addition, VCR rearrangements that consist exclusively of different copy numbers of a 98-bp sequence are unusual, because most PML rearrangements are unique (6, 7).

Our screening assay was sensitive by reproducibly detecting one virus genome per 10 human genome equivalents, but apparently less sensitive than the nested PCR/Southern hybridization approach used in an earlier report (12). Our assay did not include treatment with

topoisomerase, which has been reported to enhance the sensitivity of JCV detection (12). In urine samples, which contain intact virus, we determined a carrier rate of approximately 70%. This rate is at the higher end of reported infection rates (6) and indicated that our assay was a suitable and sensitive screening tool for JCV sequences and that the use of topoisomerase was not critical in this assay. Because the JCV genome is a circular molecule, supercoils can occur in the virus particle. It was proposed that by relaxing negatively supercoiled virus DNA, topoisomerase treatment could improve the detection of JCV (12). To our knowledge, there are no reports showing productive infection of colon tissue by JCV. The low level of JCV load found in colon supports this notion (12). Therefore, if this virus is present in colon tissue, it would be integrated in the genome and not be in a superhelical form. Thus, topoisomerase treatment would be unnecessary. Furthermore, our DNA was isolated from tissue blocks, which yield highly fragmented DNA. Despite the relatively low DNA quality, 10 microsatellite markers in single-copy genes have been successfully amplified from all the tissue samples.³

In contrast to a previous report (12), which scored samples as positive for JCV if at least one out of up to four amplifications was successful, we classified samples as positive only if sequence data could be obtained after a positive Taqman score. For all the samples, sequence data could be obtained for at least two of the three fragments tested. Identical sequences were obtained from different individuals and the isolates could be assigned to different JCV types based on their VP1 sequence (6). The detection of different strains and the high carrier rate show that our screening assay did not discriminate between strains.

For studies such as this one, the choice of analysis methods greatly influences the result obtained. PCR is a sensitive assay and at the same time prone to artifacts. Despite the stringent conditions for our amplifications (highest possible annealing temperature and no degenerate primers), from one of our tissue samples, we amplified a fragment of the correct size, which we were not able to sequence. In our opinion, this was a spurious result, especially because we could amplify neither the RR nor the large T fragment from this sample. Such non-specific amplification is even more of a problem when low-stringency PCR conditions are used. This emphasizes the necessity of assay designs that include sequence verification of virus-positive samples to eliminate false-positives due to amplification of non-specific or contaminating sequences.

Transformation of cells can occur after integration of the viral genome into the host cell genome in an arrangement that permits continued expression of large T antigen (8). If JCV integration was a cause of colon neoplasia, one could expect to find approximately one virus genome/human genome in neoplastic tissue. Laghi *et al.* (12) found JCV levels of 0.1 virus genomes/human genome in colon tumor tissue, which is lower than expected but could potentially be explained by tumor clonality. Even though our assay was capable of

³J. Bigler, unpublished data.

detecting JCV at these levels, all the tissue samples, except for one normal sample, were negative for JCV. Furthermore, in none of our isolates did we find evidence of rearrangements in the JCV regulatory region as has been reported (15). Thus, this study provides no evidence for a causative role of JCV in colon carcinogenesis.

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