Original Article

John Cunningham Virus T-Antigen Expression in Anal Carcinoma

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BACKGROUND: Anal carcinoma is thought to be driven by human papillomavirus (HPV) infection through interrupting function of cell regulatory proteins such as p53 and pRb. John Cunningham virus (JCV) expresses a T-antigen that causes malignant transformation through development of aneuploidy and interaction with some of the same regulatory proteins as HPV. JCV T-antigen is present in brain, gastric, and colon malignancies, but has not been evaluated in anal cancers. The authors examined a cohort of anal cancers for JCV T-antigen and correlated this with clinicopathologic data. **METHODS:** Archived anal carcinomas were analyzed for JCV T-antigen expression. DNA from tumor and normal tissue was sequenced for JCV with viral copies determined by quantitative polymerase chain reaction and Southern blotting. HPV and microsatellite instability (MSI) status was correlated with JCV T-antigen expression. **RESULTS:** Of 21 cases of anal cancer (mean age 49 years, 38% female), 12 (57%) were in human immunodeficiency virus (HIV)-positive individuals. All 21 cancers expressed JCV T-antigen, including 9 HPV-negative specimens. More JCV copies were present in cancer versus surrounding normal tissue (mean 32.54 copies/μg DNA vs 2.98 copies/μg DNA, $P = .0267$). There was no correlation between disease stage and viral copies, nor between viral copies and HIV-positive or -negative status (28.7 vs 36.34 copies/μg DNA, respectively, $P = .7804$). In subset analysis, no association was found between JCV T-antigen expression and HPV or MSI status. **CONCLUSIONS:** Anal carcinomas uniformly express JCV T-antigen and contain more viral copies compared with surrounding normal tissue. JCV and its T-antigen oncogenic protein, presumably through interruption of cell regulatory proteins, may play a role in anal cancer pathogenesis. ***Cancer* 2011;117:2379–85.** © *2010 American Cancer Society.*

KEYWORDS: John Cunningham virus, anal carcinoma, T-antigen, human papilloma virus, human immunodeficiency virus.

John Cunningham virus (JCV), a member of the *Polyomaviridae* family, is a 5.13 kb closed, supercoiled, double-stranded DNA virus. JCV is thought to ubiquitously infect humans, with as much as 60% to 80% of adults in the United States and Europe having JCV-specific antibodies.[1,2] Initial infection is thought to occur in the tonsils[3] or more likely the gastrointestinal (GI) tract.[4] The virus then remains latent in the GI tract[5] and tubular epithelial cells of the kidneys.[6] Classically, JCV association with human pathology has been limited to immunosuppressed patients, such as in acquired immunodeficiency syndrome and organ transplantation. In the immunosuppressed setting, reactivation of JCV occurs and can induce the fatal demyelinating disease progressive multifocal leukoencephalopathy (PML) as well as polyomavirus-associated nephropathy.[7,8] More recently, there has been mounting evidence for a potential role of JCV in human cancers in the absence of immunosuppression or PML. JCV DNA sequences and its oncogenic T-antigen expression have been demonstrated in a variety of human cancers, including brain,[9] gastric,[10,11] esophageal[12] and colon cancer.[13-16]

Most anal cancers are squamous in cell origin.[17] Anal cancer is more prevalent in the human immunodeficiency virus (HIV)-positive community, presumably because of the inherent immunocompromised state that allows reactivation of latent human papillomavirus (HPV). Anal cancer develops through stepwise transformation of normal squamous cells to dysplastic and then eventually malignant cells.[18] The vast majority of studies have focused on the well-established

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DOI: 10.1002/cncr.25793, **Received:** August 18, 2010; **Revised:** October 7, 2010; **Accepted:** October 11, 2010, **Published online** December 14, 2010 in Wiley Online Library (wileyonlinelibrary.com)

connection of HPV with invasive anal squamous carcinoma through its potential association with cell cycle regulatory proteins such as p53 and pRb.[19-21] High JCV load has also been implicated as a potential risk factor for other squamous-based carcinomas, including tongue[22] and lung.[23] The precise mechanisms behind JCV-mediated cellular oncogenesis are not completely understood but it is believed that T-antigen plays a vital role in malignant transformation via interaction with a variety of regulatory and growth signaling pathway proteins, including p53,[24] pRb,[25] and insulinlike growth factor 1 receptor (IGF-1R).[26] To our knowledge, there has been no study to date demonstrating an association of JCV and T-antigen expression with anal squamous cancer. It would be of interest to know if there might be a role for JCV and in particular its oncogenic T-antigen in the development of anal squamous carcinoma.

Microsatellite instability (MSI) is a hallmark of DNA mismatch repair dysfunction and is detected by instability at mono- or dinucleotide microsatellite DNA sequences. In sporadic colorectal cancer, MSI is seen in approximately 15% of cases. The prevalence of MSI in sporadic anal carcinoma is less clear, with most studies describing loss of heterozygosity at chromosomes 5p, 11q, and 18q in relation to integration of HPV DNA and subsequent expression of E6 and E7 genes.[27,28] To our knowledge, there are no reports describing the prevalence of MSI in anal cancer.

The objective of our study was to determine the prevalence of JCV and T-antigen expression within anal cancer specimens compared with matched surrounding normal tissue and to determine any relevant clinicopathological correlation. We also sought to evaluate the association of JCV T-antigen expression with HPV infection and MSI. We observed that anal squamous cancer tissue had a higher JCV load compared with corresponding surrounding normal squamous tissue. In addition, in our patient cohort, JCV T-antigen protein expression was present in all anal squamous cancers, including HPV-negative cancers, and absent in normal squamous tissue. We did not find any association with JCV T-antigen protein expression, HPV infection, or microsatellite instability in anal cancer tissue.

MATERIALS AND METHODS

Anal Cancer Specimens

Our patient cohort consisted of 21 patients with anal squamous cancer diagnosed at the University of California, San Diego from 2000 to 2007. Paraffin-embedded tissue slides were obtained for each of the patients with approval of the University of California, San Diego Institutional Review Board. Our patient cohort consisted of 13 men and 8 women. In addition, 12 of the patients were HIV positive. Pathological staging and histology were all performed by board-certified pathologists at University of California, San Diego and Baylor University Medical Center, Dallas. Clinicopathological data were obtained via retrospective analysis of our patient cohort.

DNA Extraction

The pathologic blocks were cut and marked by 1 pathologist (K.M.). Under high-power microscopy, the area of tumor tissue was marked by the pathologist as separate from normal and dysplastic tissue. This area was then sharply microdissected using a scalpel under high-power microscopy. Microdissection under microscopy of paraffin-embedded 5 μm slides was performed to isolate anal cancer tissue as well as corresponding normal tissue from the same patient. Genomic DNA was extracted from both anal cancer tissue and normal tissue isolated from the same paraffin-embedded slide using the QIAamp DNA minikit (Qiagen, Valencia, Calif) in accordance with the manufacturer's specifications.

Microsatellite Instability Analysis

We used 5 National Cancer Institute-recommended microsatellite markers (BAT25, BAT26, D5S346, D2S 123, D17S250).[29] P^{32}-labeled polymerase chain reaction (PCR) products were separated on 8% polyacrylamide gel containing 7.5 M urea and then exposed to x-ray film. Product DNA bands from anal cancer tissue was compared against matched normal control tissue from the same patient to determine MSI status. Classification of microsatellite instability was performed in accordance with previously established protocols; tumors were classified as MSI-H if 2 or more loci showed instability compared with normal controls and MSI-L if only 1 locus demonstrated instability.[29] Microsatellite stable (MSS) tumors were classified when no instability occurred at any locus.

Determination of HPV Status

HPV DNA was amplified via PCR using modified primers from the standard GP5+/GP6+ protocol.[30] The modified primers contained a 17 mer 5′ extension sequence (GTTTCCCAGTCACGATC) to the original GP5+/GP6+ primers. HPV DNA was amplified with the use of these modified primers via 2 rounds of PCR.

Before the second round of PCR amplification, the initial PCR product was labeled with aminoallyl deoxyuridine triphosphate. The labeled product was then coupled with Cy-3 N-hydroxysuccinimidyl ester for array hybridization. The described method allowed for an unbiased amplification of the HPV DNA and increased sensitivity.[31,32]

We used a tissue microarray that our laboratory had previously constructed to type 37 different HPV variants[33] for the determination of HPV status in our patient cohort. The HPV variants included 14 presumed high-risk variants: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. Also included were 23 presumed low risk variants: 6, 11, 26, 34, 40, 42, 43, 44, 53, 54, 55, 57, 61, 70, 71, 72, 73, 81, 82/MM4, 82/IS39, 83, 84, and CP6108. High-risk variants are those HPV serotypes that are known to cause malignant transformation in normal tissue. Low-risk variants are serotypes that have low or unknown malignant potential, but are seen in patients who are HPV positive.[21]

Detection of JCV Copies

DNA was extracted from anal cancer and normal tissue specimens by careful microdissection of the paraffin-embedded tissues. Thereafter, we first performed PCR amplification followed by DNA sequencing to identify and validate the presence of JCV sequences in the anal cancer tissues as described previously.[15,34] Sequencing of the PCR products was performed using an ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, Calif). The data were aligned to GeneBank reference sequences for JCV.

After confirmation of JCV sequences in anal cancer tissues, we subsequently performed quantitative analysis for JCV copy number determinations using JCV-specific primers and PCR reactions that consisted of 12.5 μL of Power SYBR green mix (Applied Biosystems), 250 nmol of forward and reverse primers, and 2 μL of each sample DNA. Amplification of β-actin DNA was used as an endogenous control. Standard curves were generated for both JCV and β-actin. Each sample was run in duplicate to ensure quantitative accuracy. The data were expressed in JCV DNA copies per cells assuming 2 copies of actin per cell. At least 2 independent experiments were performed for each sample.

Immunohistochemistry for JCV T-Antigen

Five-micron paraffin-embedded slide sections consisting of anal squamous cancer and normal squamous epithelium were placed in an oven and heated to 60°C for 40 minutes to melt the paraffin. The tissue sections were then deparaffinized in xylene for 30 minutes. This step was repeated 3×. The sections were then rehydrated through a graded series of alcohols as previously described.[15] Antigen retrieval was performed via immersion of tissue slide sections in 10 mM citrate buffer (pH 6.0) and autoclaved at 100°C for 15 minutes. Sections were then allowed to cool for 20 minutes at room temperature. To prevent nonspecific antibody binding, we blocked the tissue sections with 10% goat serum at room temperature for 1 hour. Slides were incubated overnight with primary mouse monoclonal antibody against SV40 T-antigen, which cross-reacts with JCV T-antigen (clone PAb416, 1:40 dilution, Calbiochem, San Diego, Calif) followed by incubation in Dako EnVision-labeled polymer (Dako Cytomation, Carpinteria, Calif). Staining was developed by incubation of the tissue sections with diaminobenzidine chromogen for 5 to 10 minutes and then further counterstaining with hematoxylin. Presence of brown nuclear staining was indicative of T-antigen expression. Both anal squamous tissue and corresponding adjacent normal squamous tissue were analyzed for T-antigen expression. All determinations of T-antigen expression were performed by blinded independent pathologists. All immunohistochemistry against JCV T-antigen was performed in 1 laboratory (A.G. and C.R.B.).

Statistical Analysis

Analysis of differences between anal cancer and normal tissue groups were done using Student *t* test or 1-way analysis of variance (ANOVA) when comparing means and Fisher exact test for categorical variables. $P < .05$ was considered significant.

RESULTS

Patient Demographics

The 21 anal cancer patients in our cohort consisted of 13 men (62%) and 8 women (38%). Twelve (57%) of the patients were HIV positive (11 men, 1 women). Their mean age at time of diagnosis of anal cancer was 49 ± 11 years (range, 39-77 years). HPV status was available for all patients. Of these, 9 (43%) patients were negative for HPV. Table 1 illustrates the patient cohort demographic data.

JCV Viral Load and T-Antigen Expression Are Correlated With Anal Cancer

All 21 anal cancer specimens demonstrated JCV DNA, and all 21 specimens stained positive for JCV T-antigen

Table 1. Anal Cancer Patient Demographics

Characteristic	HIV^+, n = 12	HIV^-, n = 9	Total, n = 21
Mean age, y ± SD	43 ± 5	56 ± 12	49 ± 11
Sex (M/F)	11/1 (92%/8%)	2/7 (18%/82%)	13/8 (62%/38%)
HPV status (+/–)	8/4 (67%/33%)	4/5 (44%/56%)	12/9 (57%/43%)

HIV indicates human immunodeficiency virus; SD, standard deviation; M, male; F, female; HPV, human papillomavirus.

protein expression via immunohistochemistry (Fig. 1), with expression exclusively nuclear. We obtained matching data of JCV viral copies in both normal and anal cancer tissue for 11 patients. The mean JCV viral load in anal cancer tissue was 32.54 copies/μg DNA, compared with 2.98 copies/μg DNA in corresponding normal anal tissue ($P = .0262$; 95% confidence interval [CI], 3.94-56.06). Interestingly, we found no correlation between HIV status and mean JCV copies on comparison of mean JCV viral copies in HIV-positive and HIV-negative patients (28.74 vs 36.34, respectively; $P = .7804$; 95% CI, −67.8059 to 52.5259). The full breakdown of T-antigen expression and mean JCV viral load data are illustrated in Table 2.

Relationship of JCV T-Antigen Expression to Anal Cancer Stage

In our cohort of 21 patients with anal squamous cancer, 8 patients were stage 1 and had a mean JCV viral load of 59.91 copies. Ten patients were stage 2, and their mean viral load was slightly increased at 60.92. None of our patients was stage 3, and interestingly the 3 patients with stage 4 disease had the lowest mean viral load (7.37 copies). Using 1-way ANOVA for analysis of anal cancer stage data, we found no significant correlation between stage and mean JCV viral load (Table 3).

Relationship of JCV T-Antigen Expression to MSI Status

MSI data were available for 16 patients (11 HIV positive, 5 HIV negative). Only 1 patient (6%) demonstrated MSI-H. One (6%) other patient was MSI-L, and the remaining 14 (88%) patients were MSS (Table 4). The 1 patient who demonstrated MSI-H was HIV positive. We found no association between HIV status and MSI-H (1 of 8, 11% MSI-H HIV positive vs 0 of 7, 0% MSI-H HIV negative, $P = 1$). No significant association existed between T-antigen expression and MSI status. Thus, in our patient cohort of anal cancer, MSI-H is rare and does not appear to be associated with HIV status or JCV T-antigen expression.

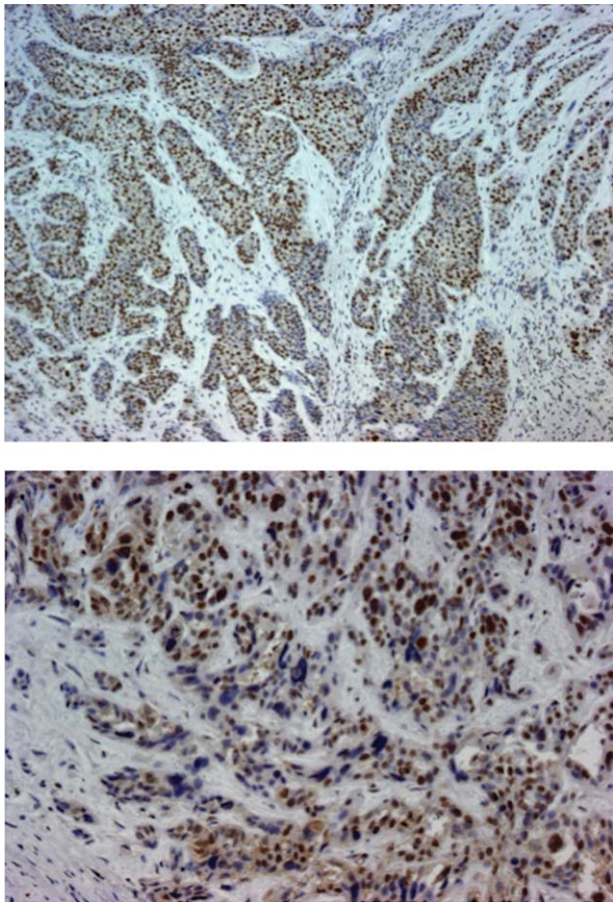

Figure 1. John Cunningham virus (JCV) T-antigen expression in anal cancer is shown. Immunohistochemistry was performed as described in Materials and Methods. Nuclear expression is shown; original magnification, ×40 (*top*) and ×200 (*bottom*). All specimens assessed expressed JCV T-antigen.

Relationship of JCV T-Antigen Expression to HPV Status

HPV status was available for all 21 patients in our cohort. Interestingly, only 12 (57%) of our anal cancer patients were HPV positive (11 high-risk HPV, 1 low-risk HPV). Of these, 8 (67%) were HIV positive. In our cohort, there was no significant difference between HIV and HPV status (8 of 12, 67% vs 4 of 9, 44%, $P = .3964$). We also found no association between HPV status and JCV T-antigen expression (Table 5).

Table 2. Correlation of JCV Copies and T-Antigen Expression in Anal Cancers

	Anal Cancer Tissue	Normal Tissue	*P* (CI)
Mean JCV copy, μg/DNA			
All matched pts, n = 11	32.54	2.98	.0267 (3.94 to 56.06)
Matched HIV$^+$ pts, n = 5	28.75	1.08	.0786 (−4.04 to 60.04)
Matched HIV$^-$ pts, n = 6	36.34	4.89	.1784 (−16.73 to 78.73)
T-antigen expression, n = 21, %	100%	0%	N/A

JCV indicates John Cunningham virus; CI, confidence interval; HIV, human immunodeficiency virus; pts, patients; N/A, not applicable.

Table 3. Anal Cancer Stage and JCV Copies per Microgram DNA

Stage	No. (%)	Mean JCV Copies/μg DNA	*P*
1	8 (38%)	59.91[a]	.651
2	10 (48%)	60.92[b]	
3	0 (0%)	N/A	
4	3 (14%)	7.37[c]	

JCV indicates John Cunningham virus; N/A, not applicable.
[a] JCV copy data available for 7 patients with stage 1 anal cancer.
[b] JCV copy data available for 9 patients with stage 2 anal cancer.
[c] JCV copy data available for 2 patients with stage 4 anal cancer.

Table 4. MSI Status and JCV T-Antigen Expression

	MSI-H	MSI-L/MSS	*P*
Total, n = 16	1 (6%)	15 (94%)	N/A
HIV$^+$, n = 9	1 (11%)	8 (89%)	1.0
HIV$^-$, n = 7	0 (0%)	7 (100%)	
T-antigen, n = 21	1 (5%)	20 (95%)	N/A

JCV indicates John Cunningham virus; MSI, microsatellite instability; H, high; L, low; MSS, microsatellite stable; N/A, not applicable; HIV, human immunodeficiency virus.

Table 5. HPV Status and JCV T-Antigen Expression

	HPV$^+$	HPV$^-$	*P*
Total, n = 21	12 (57%)	9 (43%)	N/A
HIV$^+$, n = 12	8 (67%)	4 (33%)	.3964
HIV$^-$, n = 9	4 (44%)	5 (56%)	
T-antigen, n = 21	12 (57%)	9 (43%)	N/A

JCV indicates John Cunningham virus; HPV, human papillomavirus; N/A, not applicable.

DISCUSSION

The oncogenic potential of JCV was initially discovered when Walker et al injected the virus into Syrian hamster brains and induced aneuploid tumors.[35] Since then, numerous studies have linked JCV and in particular, its T-antigen protein expression to a variety of cancers. We were interested in extending this correlation to anal cancer. In this study, we have demonstrated that in our cohort of patients, JCV viral copies are elevated in anal cancer tissue compared with surrounding normal tissue, which suggests a biological and mechanistic role for this virus in the pathogenesis of this disease. Furthermore, JCV T-antigen expression is highly predominant in anal cancer tissue.

JCV T-antigen is a multifunctional oncogenic protein that has the ability to transform mammalian cells. It does this by binding and inactivating p53 and pRb, 2 key tumor suppressor proteins that regulate cell cycle progression.[36,37] Through its inactivation of p53 and pRb, JCV sets itself up in an optimal cellular environment for its replication and assembly during its lytic phase of infection and concurrently facilitates transformation of normal cells. Furthermore, through its interaction with insulin receptor substrate 1 (IRS-1), JCV T-antigen has also been shown to inhibit homologous recombination DNA repair, part of the system that maintains genomic stability.[38] Previous work has also demonstrated that the activation of IGF-1R, along with the interaction between JCV T-antigen and IRS-1, could potentially induce carcinogenesis via triggering cell proliferation, antiapoptotic signaling, and inhibition of homologous DNA recombination repair.[39]

Our data demonstrating higher JCV viral copies in anal cancer tissue compared with surrounding normal epithelium are consistent with numerous previous studies demonstrating increased JCV sequences in gastric, esophageal, and colon cancers.[10-16] To our knowledge, the present study is the first demonstration correlating JCV viral copies and T-antigen protein expression in anal cancer tissue against surrounding normal epithelium. Of note, we did not find any significant correlation between level of JCV viral copies and stage progression in anal cancer. This suggests that JCV, although potentially having a role in carcinogenesis, may not play a big role in prognosis. However, our small sample size could be the other reason that we found no stage correlation with level of JCV copies. To show T-antigen protein expression via immunohistochemistry in anal cancer is important, because the finding of JCV viral copies alone is not sufficient to demonstrate biological activity of this oncogenic protein, as JCV DNA sequences have been shown to be frequently present in cells of normal individuals.[40] The finding of JCV T-antigen expression exclusively in the nuclei of anal squamous cells suggests

there might be a role for JCV in the pathogenesis of anal cancer. A prior report has illustrated that JCV T-antigen expression in adenomas was lower than in colorectal carcinoma (16% vs 40%-50%, respectively).[15] Given this, it would also be of interest to know if the level of JCV copies as well as predominance of T-antigen expression is attenuated in anal dysplastic compared with anal cancer cells, with the addition of a control group of benign anorectal disease, to rule out the possibility that JCV T-antigen is an innocent bystander or associated with inflammation or chronic infection. JCV is believed to facilitate carcinogenesis at its early stages, according to the so-called "hit and run" hypothesis, where late stage tumors do not exhibit high viral numbers after genetic damage has been done.[41] Our observation appears to be similar in our anal cancer cohort.

Our finding that anal cancer tissue in our cohort demonstrated extremely low levels of MSI is to our knowledge a new finding. We found no association between JCV T-antigen protein expression and MSI status in anal cancer tissue, which is also consistent with prior data demonstrating no relation between MSI and JCV T-antigen expression in colon adenomas.[15] A relationship between JCV T-antigen protein expression and promoter methylation of 4 tumor suppressor genes, including *hMLH1*, has been observed.[34] Hypermethylation of *hMLH1* is the main cause of MSI in sporadic colorectal cancers, and in MSI cancers that expressed JCV T-antigen, a higher methylation index, a measure of the degree of methylation, was present.[34] Taken together, the data suggest that the association of hypermethylation, MSI, and JCV T-antigen expression may occur later in the adenoma-to-carcinoma sequence for colorectal cancers. Our results suggest that the underlying genomic instability pattern behind anal squamous cell cancer carcinogenesis is less likely dependent on defects in the DNA mismatch repair system. Of interest, there were 9 (43%) anal cancer patients in our cohort who were HPV negative but had expression of JCV T-antigen, thus suggesting a potential for JCV T-antigen to drive carcinogenesis through an HPV-independent process. JCV T-antigen is well known to induce chromosomal instability (aneuploidy) as its presumed prime mode of genomic instability, and could account for this mechanism as the dominant 1 observed in anal cancer.[42] These mechanisms of action may act synergistically or cumulatively to potentiate the transformation of tissue by HPV, but would need to be studied in an experimental model to show any conclusive interaction. In bronchial cancers, HPV was detected in 10 of 78 lesions, whereas JCV sequences was amplified in only 1 of 78 tumors, suggesting a lack of synergistic effect within lung cancer.[43] In colorectal cancers, HPV was detected in 22 of 66 samples, whereas no JCV viral sequences were detected, again suggesting no synergistic effect for this tumor.[44]

Building on the previously described interactions between JCV T-antigen and various proto-oncogenic and tumor suppressor proteins in various cancers, it seems plausible that T-antigen might likewise interact with similar proteins that could then lead to carcinogenesis in anal cancer. However, this has yet to be determined. In conclusion, our results demonstrate that JCV viral copies are increased in anal squamous cancer compared with surrounding normal anal epithelium. In addition, JCV T-antigen protein expression is also highly predominant in anal cancer tissue, thus further suggesting a potential role for JCV T-antigen in the development of anal cancer.

CONFLICT OF INTEREST DISCLOSURES

Supported by a University of California, San Diego institutional grant from the American Cancer Society (CCT05SR), the US Public Health Service (DK067287 and CA98572), the University of California, San Diego Digestive Diseases Research Development Center (DK080506), the San Diego State University/University of California, San Diego Comprehensive Cancer Center Partnership (CA132379 and CA132384), and a KL2 grant from the University of California, San Diego Clinical and Translational Science Award. There are no conflicts of interest for any authors on this article.

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