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JC Virus T-Antigen Expression in Anal Carcinoma

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Abstract

Background & Aims—Anal carcinoma is thought driven by HPV infection through interrupting function of cell regulatory proteins such as p53 and pRb. JCV expresses a T-antigen (T-Ag) that causes malignant transformation through development of aneuploidy and interaction with some of the same regulatory proteins as HPV. JCV T-Ag is present in brain, gastric and colon malignancies, but has not been evaluated in anal cancers. We examined a cohort of anal cancers for JCV T-Ag and correlated this with clinicopathologic data.

Methods—Archived anal carcinomas were analyzed for JCV T-Ag expression. DNA from tumor and normal tissue was sequenced for JCV with viral copies determined by qPCR and Southern blotting. HPV and MSI status was correlated with JCV T-Ag expression.

Results—Of 21 cases of anal cancer (mean age 49 years, 38% female), 12 (57%) were in HIV-positive individuals. All 21 cancers expressed JCV T-Ag, including 9 HPV-negative specimens. More JCV copies were present in cancer vs. surrounding normal tissue (mean 32.54 copies/μg DNA vs. 2.98 copies/μg DNA, P=0.0267). 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=0.7804). In subset analysis, we found no association between JCV T-Ag expression and HPV or MSI status.

Conclusions—Anal carcinomas uniformly express JCV T-Ag and contain more viral copies compared to surrounding normal tissue. JCV and its T-Ag oncogenic protein, presumably through interruption of cell regulatory proteins, may play a role in anal cancer pathogenesis.

Keywords

JC virus; anal carcinoma; T-antigen; human papilloma virus; HIV

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INTRODUCTION

JC Virus (JCV), a member of the polyomaviridae family, is a 5.13kb 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 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 AIDS 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 (T-Ag) 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 HIV positive community presumably because of the inherent immunocompromised state that allows reactivation of latent 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 connection of human papilloma virus (HPV) with invasive anal squamous carcinoma through its potential association with cell cycle regulatory proteins such as p53 and pRb [19-21]. High JC viral 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 insulin-like growth factor-1 receptor (IGF-1R) [26]. To our knowledge, there has been no study to date demonstrating an association of JCV and T-Ag 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 (MMR) 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 (LOH) 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-Ag expression within anal cancer specimens compared to matched surrounding normal tissue and to determine any relevant clinicopathological correlation. We also sought to evaluate the association of JCV T-Ag expression with HPV infection and microsatellite instability. We observed that anal squamous cancer tissue had a higher JCV load compared to 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-Ag protein expression, HPV infection, or microsatellite instability in anal cancer tissue.

METHODS

Anal Cancer specimens

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

DNA extraction

The pathologic blocks were cut and marked by one pathologist (KM). 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-micron slides was performed in order 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, CA) in accordance to the manufacturer's specifications.

Microsatellite instability analysis

We used 5 NCI recommended microsatellite markers (BAT25, BAT26, D5S346, D2S123, D17S250) [29]. P^{32} labeled PCR products were separated on 8% polyacrylamide gel containing 7.5M urea and then exposed to x-ray film. Product DNA bands from anal cancer tissue 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 to normal controls, MSI-L if only 1 locus demonstrated instability [29]. 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 17mer 5′ extension sequence (GTTTCCCAGTCACGATC) to the original GP5+/GP6+ primers. HPV DNA was amplified with use of these modified primers via 2 rounds of PCR. Prior to second round of PCR amplification, the initial PCR product was labeled with amino-allyl dUTP. The labeled product was then coupled with Cy-3 NHS 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 (TMA) that our lab had previously constructed to type 37 different HPV variants (manuscript in preparation) 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 & 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 & 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 [15,33]. Sequencing of the PCR products was performed using an ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA). The data were aligned to GeneBank reference sequences for JCV.

Following 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 which consisted of 12.5 μl of Power SYBR green mix (Applied Biosystems, Foster City, CA), 250 nmole of forward and reverse primers, and 2 microliters 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 two copies of actin per cell. At least two independent experiments were performed for each sample.

Immunohistochemistry for JCV T-Ag

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 times. 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 10mM 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 non-specific 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-Ag, which cross-reacts with JCV T-Ag (clone PAb416, 1:40 dilution, Calbiochem, CA) followed by incubation in Dako EnVision™ labeled polymer (Dako Cytomation Inc., Carpinteria, CA). Staining was developed by incubation of the tissue sections with diaminobenzadine (DAB) chromagen for 5-10 minutes and then further counterstaining with hematoxylin. Presence of brown nuclear staining was indicative of T-Ag expression. Both anal squamous tissue as well as corresponding adjacent normal squamous tissue was analyzed for T-Ag expression. All determinations of T-Ag expression were performed by blinded independent pathologists. All immunohistochemistry against JCV T-Ag were performed in one lab (AG and CRB).

Statistical analysis

Analysis of differences between anal cancer and normal tissue groups were done using students t test or one-way ANOVA when comparing means and fisher exact test for categorical variables. $P<0.05$ was considered significant.

RESULTS

Patient demographics

The 21 anal cancer patients in our cohort consisted of 13 males (62%) and 8 females (38%). 12 (57%) of the patients were HIV-positive (11 male, 1 female). Their mean age at time of diagnosis of anal cancer was 49 ± 11 years (range 39-77). 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-Ag expression is correlated with anal cancer

All 21 anal cancer specimens demonstrated JCV DNA, and all 21 specimens stained positive for JCV T-Ag protein expression via immunohistochemistry (Figure 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 to 2.98 copies/μg DNA in corresponding normal anal tissue (p=0.0262, CI 3.94 to 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 respectively (28.74 vs. 36.34, p=0.7804, CI: −67.8059 - 52.5259). The full breakdown of T-Ag expression and mean JCV viral load data is illustrated in Table 2.

Relationship of JCV T-Ag expression to anal cancer stage

In our cohort of 21 patients with anal squamous cancer, 8 patients were stage 1 and had 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 were stage 3 and interestingly the 3 patients with stage 4 disease had the lowest mean viral load (7.37 copies). Utilizing one-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-Ag expression to MSI status

MSI data was available for 16 patients (11 HIV-positive, 5 HIV-negative). Only 1 patient (6%) demonstrated MSI-H. One other patient (6%) was MSI-L and the remaining 14 patients (88%) were MSS (Table 4). The one patient that demonstrated MSI-H was HIV-positive. We found no association between HIV status and MSI-H (1/8, 11% MSI-H HIV positive vs. 0/7, 0% MSI-H HIV negative, p=1). No significant association existed between T-Ag 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-Ag expression.

Relationship of JCV T-Ag 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 (8/12, 67% vs. 4/9, 44%, p= 0.3964). We also found no association between HPV status and JCV T-Ag expression (Table 5).

DISCUSSION

The oncogenic potential of JCV was initially discovered when Walker et al injected the virus into Syrian hamster brains and induced aneuploid tumors [34]. Since then, numerous studies have linked JCV and in particular, its T-Ag 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 to surrounding normal tissue, which suggests a biological and mechanistic role for this virus in the pathogenesis of this disease. Furthermore, JCV T-Ag expression is highly predominant in anal cancer tissue.

JCV T-Ag is a multifunctional oncogenic protein that has the ability to transform mammalian cells. It does this by binding and inactivating p53 and pRb, two key tumor suppressor proteins that regulate cell cycle progression [35,36]. 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-Ag has also been shown to inhibit homologous recombination DNA repair, part of the system that maintains genomic stability [37]. Previous work has also demonstrated that the activation of IGF-1R, along with the interaction between JCV T-Ag and IRS-1 could potentially induce carcinogenesis via triggering cell proliferation, anti-apoptotic signaling and inhibition of homologous DNA recombination repair [38].

Our data demonstrating higher JCV viral copies in anal cancer tissue compared to surrounding normal epithelium is 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-Ag 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, while 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-Ag protein expression via IHC in anal cancer is important as 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 [39]. The finding of JCV T-Ag 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-Ag 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-Ag expression is attenuated in anal dysplastic compared to anal cancer cells, and with the addition of a control group of benign anorectal disease, to rule out the possibility that JCV T-Ag is an innocent bystander or associated with inflammation or chronic infection. JCV is believed to facilitate carcinogenesis at its early stages, the so-called “hit and run” hypothesis, where late stage tumors do not exhibit high viral numbers after genetic damage has been done [40]. 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-Ag protein expression and MSI status in anal cancer tissue, which is also consistent with prior data demonstrating no relation between MSI and JCV T-Ag expression in colon adenomas [15]. A relationship between JCV T-Ag protein expression and promoter methylation of 4 tumor suppressor genes, including *hMLH1*, has been observed [33]. Hypermethylation of *hMLH1* is the main cause of MSI in sporadic colorectal cancers, and in MSI cancers that expressed JCV T-Ag, a higher methylation index, a measure of the degree of methylation, was present [33]. Taken together, the data suggests that the association of hypermethylation, MSI, and JCV T-Ag 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-Ag thus suggesting a potential for JCV T-Ag to drive carcinogenesis through an HPV-independent process. JCV T-Ag is well known to induce chromosomal instability (aneuploidy) as it’s presumed prime mode of genomic instability, and could account for this mechanism as the dominant one observed in anal cancer [41]. These mechanisms of action may act synergistically or cumulatively to potentiate the transformation of tissue by HPV, but would need be studied in an experimental model to show any conclusive interaction. In bronchial cancers, HPV was detected in 10 of 78 lesions, while JCV sequences was amplified in only 1 of 78 tumors, suggesting a lack of synergistic effect within lung cancer [42]. In colorectal cancers, HPV

was detected in 22 of 66 samples while no JCV viral sequences were detected, again suggesting no synergistic effect for this tumor [43].

Building on the previously described interactions between JCV T-Ag and various proto-oncogenic and tumor suppressor proteins in various cancers, it seems plausible that T-Ag 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 to surrounding normal anal epithelium. In addition, JCV T-Ag protein expression is also highly predominant in anal cancer tissue thus further suggesting a potential role for JCV T-Ag in the development of anal cancer.

CONDENSED ABSTRACT

The pathogenesis of anal carcinoma is linked to HPV, but this is less clear in HPV-negative anal cancers. JCV is present in 100% of anal cancers and contains a higher viral load in cancer compared to normal tissue. JCV may play a role in the development of anal cancer.

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Abbreviations

MSI	microsatellite instability
MSI-H	microsatellite instability-high
MSI-L	microsatellite instability-Low
MSS	microsatellite stable
HPV	human papilloma virus
JCV	JC virus
T-Ag	T-antigen
IRS-1	insulin receptor substrate 1
PML	progressive multifocal leukoencephalopathy
IGF-1R	insulin-like growth factor-1 receptor
qPCR	quantitative polymerase chain reaction

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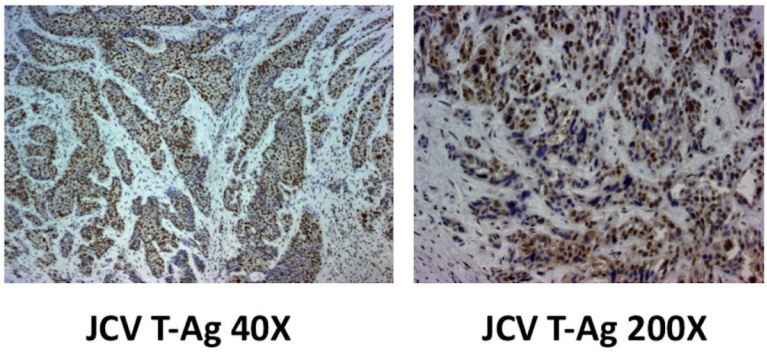

Figure 1.
JCV T-Ag expression in anal cancer. Immunohistochemistry was performed as described in the methods. *Left panel*, 40X; *Right panel* 200X, and showing nuclear expression. All specimens assessed expressed JCV T-Ag.

Table 1

Anal Cancer patient demographics

	HIV + (n=12)	HIV Neg (n=9)	Total (n=21)
Mean Age ± S.D.	43 ±(5	56 ±12	49 ±11
Gender (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%)

Table 2

Correlation of JCV copies and T-Ag expression in anal cancers

		Anal cancer tissue	Normal tissue	*p* value (CI)
Mean JCV copy (μg/DNA)	All matched pts (n=11)	32.54	2.98	0.0267 (3.94 - 56.06)
	Matched HIV pts (n=5)	28.75	1.08	0.0786 (−4.04 - 60.04)
	Matched HIV neg pts (n=6)	36.34	4.89	0.1784 (−16.73 - 78.73)
T-Ag expression (n=21,%)		100%	0%	N/A

Table 3

Anal Cancer Stage and JCV copies/ug DNA

Stage	N (%)	Mean JCV copy (μg/DNA)	*p* value
1	8 (38%)	59.91 *	0.651
2	10 (48%)	60.92 **	
3	0 (0%)	N/A	
4	3 (14%)	7.37 ***	

* JCV copy data available for 7 pts with stage 1 anal cancer

** JCV copy data available for 9 pts with stage 2 anal cancer

*** JCV copy data available for 2 pts with stage 4 anal cancer

Table 4

MSI status and JCV T-Ag expression

	MSI-H	MSI-L/MSS	*p* value
Total (n=16)	1 (6%)	15 (94%)	N/A
HIV + (n=9)	1 (11%)	8 (89%)	1.0
HIV neg (n=7)	0 (0%)	7 (100%)	
T-Ag (n=21)	1 (5%)	20 (95%)	N/A

Table 5

HPV status and JCV T-Ag expression

	HPV +	HPV neg	*p* value
Total (n=21)	12 (57%)	9 (43%)	N/A
HIV + (n=12)	8 (67%)	4 (33%)	0.3964
HIV neg (n=9)	4 (44%)	5 (56%)	
T-Ag (n=21)	12 (57%)	9 (43%)	N/A