Full paper

Detection of a new JCV strain of genotype A in a subpopulation of colorectal adenocarcinomas in Tunisia

Wafa Toumi[1], Alessandro Ripalti*[2], Luigi Ricciardiello[3], Abderraouf Cherif[4], Dalila Gargouri[1,5], Ahmed Bouhafa[4], Jamel Kharrat[1,5], Slim Jarboui[6], Hichem Benrhouma[7], Mohamed Zili[1], Ridha Khelifa[1]

[1] Viral and Molecular Tumor Diagnostics Unit, Laboratory Services, Habib Thameur Hospital, Tunis, Tunisia
[2] Microbiology Unit, S.Orsola- Malpighi General Hospital, Bologna, Italy
[3] Gastroenterology Unit, S.Orsola- Malpighi General Hospital, Bologna, Italy
[4] General Surgery Services, Habib Thameur Hospital, Tunis, Tunisia
[5] Gastroenterology Services, Habib Thameur Hospital, Tunis, Tunisia
[6] General Surgery Services, Mahmoud El Matri Ariana Hospital, Tunis, Tunisia
[7] Anatomy and Histopathology Laboratory, 34 Haroun Errachid Street 1002 Mutuelleville, Tunis, Tunisia

Running title: JCV in Tunisian colon cancer

SUMMARY

The etiology of colorectal cancer (CRC) remains elusive in spite of major advances in knowledge of this disease and related risk factors. Several studies report the detection of human polyomavirus JC (JCV) in colorectal tumors and some suggest its association with CRC. Since many known human virus associations with cancer are linked to factors such as ethnic and geographical origin, it is interesting to search for the postulated association of JCV with CRC in different populations and regions. In this perspective, the present work was undertaken to assess the presence of JCV in CRC tumors in Tunisia. Fresh biopsies were obtained from both colorectal tumors and adjacent normal tissues of 47 CRC patients. Only tumors diagnosed as adenocarcinomas were included in the present study. Twenty patients with other gastroenterological disorders were taken as controls. DNA was extracted from fresh biopsies or formalin-fixed, paraffin-embedded tissue sections. A region of the viral T-Ag gene was amplified by PCR and the DNA amplicons were subjected to automated sequencing. JCV DNA was found in 22 (46%) of the adenocarcinomas but in none of the normal mucosa biopsies of either CRC or control patients. Sequence analysis indicated that the amplified DNA belonged to a new JCV variant of genotype A. The presence of JCV DNA was correlated with tumor location and grade. The data obtained suggest that JCV may be associated either with a subpopulation of colorectal tumors or with CRC in general, possibly through a hit and run mechanism.

*Key words***:** Colorectal cancer, JC virus, Transformation antigen (T-Ag), Cancer etiology.

***Corresponding author:** Alessandro Ripalti

U.O. Microbiologia, Dipartimento della Medicina Diagnostica e della Prevenzione A.O.-U. di Bologna Policlinico S.Orsola-Malpighi, via Massarenti, 9, I - 40136 Bologna

alessandro.ripalti@unibo.it

00 39 051 4290921

INTRODUCTION

Colorectal cancer is a major cause of mortality in the world (Goel et al., 2006; Parkin, 2006; Giuliani et al., 2008). Several risk factors, both genetic and environmental, including viral infections, have been linked to colorectal carcinogenesis. JCV has been repeatedly suggested as a virus candidate for an association with CRC (Boland, 2004; Niv et al., 2005; Coelho et al., 2010; Samaka et al., 2013). JCV is the etiologic agent of progressive multifocal leukoencephalopathy (PML), and JCV disease is generally associated with populations of immunocompromised patients, although it has been associated with infertility in otherwise healthy males (Comar et al., 2012). The JCV genome consists of a 5.13 Kb closed circular supercoiled double-stranded DNA. The early region of this genome encodes the large (T-Ag) and small (t-Ag) transformation antigens (Ichaso et al., 2001; Ricciardiello et al., 2001; Darbinyan et al., 2002; Murai et al., 2007). The association of JCV with human malignancies has been hypothesized primarily on the basis of the transforming abilities of its T-Ag (Pipas, 1992; Enam et al., 2002; Nosho et al., 2009). This antigen possesses a multifunctional modular structure with ATPase, helicase, polymerase and DNA binding activities, all being essential for DNA replication, cell transformation and eventually tumorigenesis (Sheng et al., 1997; Srinivasan et al., 1997). Mechanistically, T-Ag inactivates many proteins in the pRb protein family (Dyson et al., 1990) through stable association of its LXCXE domain with these proteins eventually leading to aneuploidy, cellular transformation, and tumor development (Sheng et al., 1997; Srinivasan et al., 1997). T-Ag may also exert an oncogenic activity, in part, through its ability to dysregulate the Wnt signaling pathway and to increase the levels of β-catenin and c-myc expression (Enam et al., 2002; Ricciardiello et al., 2003).

Like other members of the Polyomaviridae family, JCV can transform cells in culture and is oncogenic in laboratory animals (Del Valle et al., 2002; Khalili et al., 2003). The virus has also been shown to induce chromosomal instability in colon cancer cell lines expressing wild-type adenomatous polyposis coli (APC), p53 and β-catenin (Staib et al., 1996; Enam et al., 2002; Niv et al., 2005).

JCV DNA has been found in a variety of human malignancies, including brain, colon, gastric, and esophageal cancers as well as in adenomatous polyps of the colon (Del Valle et al., 2002; Ricciardiello et al., 2003; Del Valle et al., 2005; Murai et al., 2007; Jung et al., 2008; Ksiaa et al., 2010). Reports on the site of JCV DNA detection either in colorectal tumors, polyps or normal colonic tissues appear to be contradictory (Laghi et al., 1999; Ricciardiello, 2001, 2003, 2000; Enam et al., 2002; Casini et al., 2005). Indeed, a few reports describe the presence of JCV DNA in tumors or adenomatous polyps only (Hori et al., 2005; Theodoropoulos et al., 2005; Jung et al., 2008) and others found the viral DNA in both tumors and normal gastrointestinal tissues (Laghi et

al., 1990; Ricciardiello et al., 2001, 2000; Enam et al., 2002; Casini et al., 2005). In addition, Ricciardiello et al. (2000) reported a high prevalence of JCV DNA in gastric and colonic tissues from more than 70% of patients without gastrointestinal neoplasia. JCV DNA is also detectable in healthy subjects throughout the gastrointestinal tract that appears to be a major reservoir of the virus (Ricciardiello et al., 2003, 2000). On the other hand, two other groups found little (Hernandez Losa et al., 2003) or no (Newcomb et al., 2004) JCV DNA in CRC tumors and normal colorectal tissues. Laghi et al. (1999) reported JCV DNA in more than 80% of tumors and normal colonic tissues of CRC patients, but with at least a 10 fold higher number of viral copies in tumors. The latter observation, together with reports on the expression of T-Ag exclusively in tumor cells (Goel et al., 2006; Jung et al., 2008), indicate that specific types of virus-cell interactions must be established for JCV to exert any role in carcinogenesis.

Virus association with tumors may be influenced by geographic and ethnic factors, as clearly illustrated by the Epstein-Barr virus (Abdel-Hamid et al., 1992; Abdel-Hamid et al., 1992; Ogwang et al., 2008) and the human T-cell lymphotropic virus types one and two (De Rivera et al., 1995; Yoshida, 2010). The putative contribution of JCV to CRC oncogenesis might similarly be influenced by such factors. The geographic distribution of JCV subtypes indicates that JCV co-evolved with human populations and followed their migrations and expansion patterns (Kunitake et al., 1995; Sugimoto et al., 1997, 2002). Therefore, epidemiological data on JCV prevalence and expression in colorectal tumors from different parts of the world are needed to better assess the association of this virus with colorectal oncogenesis. To date, only one report is available on JCV infection in CRC tumors from Tunisia or its surrounding countries (Ksiaa et al. 2015).

Here we report on the detection of JCV DNA in colorectal tumors of Tunisian patients, confirming the possible correlation of the virus with onset of CRC.

MATERIALS AND METHODS

Patients

The test group consisted of 47 CRC patients, 24 men and 23 women (mean age: 64 years) from the Surgery Department of the Habib Thameur Hospital, Tunis, Tunisia. Fresh colorectal mucosa biopsies were taken from both colorectal tumors and adjacent normal tissues of these patients. Formalin-fixed, paraffin-embedded (FFPE) biopsy samples were used to prepare 5µm sections for DNA extraction. Twenty patients, 9 men and 11 women (mean age: 60 years) presenting at the hospital with various gastrointestinal disorders other than CRC were taken as controls. Samples from the control population were obtained during colonoscopy at different subsites. Written informed consent was obtained from all patients according to the regulations of the National Ethics Committee.

DNA extraction

DNA was extracted from 20 mg of fresh and FFPE-fixed biopsy sections using the Purelink DNA mini Kit of Invitrogen according to manufacturer's instructions. DNA quantification was made using the Quant-iT^{TM}ds DNA HS Assay Kit and a Qubit Fluorometer (Invitrogen). Tumor histological types were determined according to established criteria.

Detection of JCV DNA

Strict precaution measures were applied to avoid contamination by previous PCR products. These measures included the use of appropriate sample and reagent controls, separate working compartments and different micropipette sets in each working area. JCV DNA amplification was performed by simple or by nested PCR. For simple PCR, a pair of primers reported by Goel et al. (2006) was used. These primers, which were designed within the T-Ag gene will, hereafter, be referred to as JCTS (sense: ATGTATTCCACCAGGATTCCCATTCATC) and JCTAS (anti-sense: AGTTCTTGGAGACACCCCCTACAG). They also served as external primers in the first round of nested PCR. For the second round of nested PCR we used a pair of internal primers, (sense: CATCTGTTCCATAGGTTG) and (anti-sense: AGCTTTAAGGTAAACCAC), designed using OLIGO Primer Analysis Software (Molecular Biology Insights, Cascade, CO 80809, USA). Each PCR reaction consisted of a 25-µL reaction mixture containing 1x Taq polymerase buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each primer, 0.03 U/µl Hot Start Taq DNA polymerase (Invitrogen) and 20 to 50 ng DNA extract or 2 µl of first round product, for the simple PCR or first round of nested PCR, respectively. Thermocycler program for JCV DNA amplification by simple PCR consisted of initial activation of the Hot Start Taq polymerase at 94°C for 15 minutes followed by 40 cycles of denaturation at 94°C for 30s, annealing at 58°C for 30s and extension at 72°C for 30s and a final extension step at 72 °C for 10 minutes. Thermocycler program for the second stage of nested PCR was: initial activation at 94°C for 3 minutes, 30 cycles of 30s steps of denaturation at 92°C, annealing at 50°C, and extension at 70°C; a final extension step at 70°C for 10 minutes was included. PCR reactions were carried out in a PTC 200 Peltier DNA Engine MJ Research Thermal Cycler. Each PCR amplification series included a positive control DNA from neuroblastoma JCI cells transfected with the Mad-1 strain of JCV (Ricciardiello et al., 2003), a negative control DNA from Raji cells (Pulvertaft, 1964), and template blanks to assess for any contamination by previously amplified products. The positive control was also used to assess amplifiability of extracted material: an aliquot corresponding to the one planned to be analyzed for the presence of JCV DNA was added with a fixed amount of positive control amplicon for each extract. The amount of positive amplicon to be used was determined by limiting dilutions and the last dilution giving a clear positive PCR result was picked as a standard. These parallel aliquots were then

subjected to PCR amplification as described above and only samples showing effective amplification were selected for further analysis. Samples failing the amplifiability test were re-extracted until positive to the test. A portion of each amplification product was analyzed by electrophoresis on 2% agarose in the presence of ethidium bromide.

Statistical analyses

Statistical analysis was performed using the Graph-Pad Prism Version 6 (GraphPad Software, Inc., San Diego, CA, USA). The Chi-Squared or Fisher's exact test were used where appropriate. Differences were considered significant when $P < 0.05$.

DNA sequencing and analysis

Sequencing of the purified DNA amplicons was performed using the ABI Prism 3100 Avant-Genetic Analyser and Big Dye Terminators technology (Applied Biosystems ThermoFisher). Sequencing was performed on both strands of amplicons using the forward inner primer or the reverse inner primer for each sample. The obtained DNA sequences were aligned in BLAST (Zhang Z et al., 2000) and in MEGA 6 (Tamura et al., 2013) against reference sequences retrieved from GenBank. Phylogenetic trees were generated by the distance-based Maximum Likelihood method and visualized using MEGA 6 software. The robustness of the groupings in the Maximum Likelihood analysis was assessed with 1000 bootstrap.

Nucleotide sequence accession number

The nucleotide sequence obtained in this study was deposited in GenBank data library under accession number: KU721998.

RESULTS

Detection of JCV DNA in colorectal tissues

DNA was extracted from fresh biopsies and from fixed biopsy sections of 47 CRC patients. Both extracts were analysed for each patient for a total of 94 samples. In preliminary experiments, the presence of JCV DNA was explored by simple PCR. Each PCR series included a positive control consisting of DNA extracted from JCI cells transfected with the Mad-1 strain of JCV (Ricciardiello et al., 2003), and a negative control consisting of DNA from the Burkitt's lymphoma cell line Raji (Pulvertaft, 1964). Agarose gel electrophoresis of the obtained PCR products revealed 154 bp bands as expected for JCV DNA, in 12 (25%) of the 47 CRC tumors examined. Positive samples in fresh-frozen extracts corresponded to positives in FFPE extracts. We, therefore, switched to nested PCR to increase assay sensitivity. This procedure produced 98 bp bands as expected for the nested product. Ten more JCV DNA-positive tumors were thus found, to make a total of 22 (46%) viral DNA-positive biopsies (data not shown). Again, results for fresh-frozen and FFPE samples were superimposable. None of the 47 samples from normal tissues adjacent to tumors showed JCV DNA,

neither by simple nor by nested PCR. No viral DNA was found in any of the gastrointestinal biopsies of the 20 control patients (fresh-frozen samples only) tested by either assay.

Correlation with clinicopathological characteristics

We looked for possible correlations between the presence of JCV DNA and some clinicopathological parameters as described in Table 1. The presence of JCV DNA was significantly correlated with polyps (p= 0.010). It is noteworthy that the well-differentiated grade of adenocarcinoma was predominant among the studied tumors. It is interesting to note that JCV DNA was significantly prevalent in polyps and tended to increase among the well-differentiated adenocarcinomas. No significant correlation between the presence of JCV DNA and the remaining clinicopathological parameters was found (Table 1).

Sequencing data analysis.

The nested PCR products were sequenced and the DNA sequences obtained were initially aligned in BLAST. They showed a high degree of homology with JCV (>87 % nucleotide identities, 98 % homology to strain UK-2) thus confirming their JCV identity. These sequences were then aligned against each other in MEGA 6 software and appeared to be identical; they will hereafter be referred to as sequence WT.

Sequence WT was aligned against 18 reference JCV strains from all known genotypes retrieved from GenBank (Table 2; Figure 1) and a phylogenetic tree was constructed (Figure 2). Sequence WT shared a close relationship with the European strains of genotype A, e.g. Mad-1, SP-1 and MR-7, and showed a deletion in position 47 nt (T). This deletion was neither strain nor genotype-specific since it was also found in strains of genotype B such as ZJ-1, NY, MO-6 and Tky-2a. The deletion found in the WT sequence is within the T-Ag intron at position 4451.

Taken together, these data suggest that isolates detected here represent a new JC virus strain of genotype A.

DISCUSSION

The detection of JCV DNA and proteins, especially T-Ag, in colorectal tumors (Laghi et al., 1999; Ricciardiello et al., 2000; Mou et al., 2012) led many authors to suggest the implication of JCV in CRC carcinogenesis. Indeed, the presence of JCV DNA exclusively in tumors or adenomatous polyps (Hori et al., 2005; Jung et al., 2008), or in both tumoral and normal gastrointestinal tissues (Laghi et al., 1999; Ricciardiello et al., 2001, 2000; Enam et al., 2002; Casini et al., 2005; Theodoropoulos et al., 2005) is well documented. Two other studies, however, reported little (Hernandez Losa et al., 2003) or no (Newcomb et al., 2004) JCV DNA either in CRC tumors or normal colorectal tissues. In our hands, JCV DNA was found exclusively in the tumors, with a frequency comparable to that reported by other data. Such discrepancies in the reported data could

be due to either one or a combination of the following possibilities: (i) differences in sensitivity of the PCR assays used; (ii) higher loads of JCV DNA in the tumors and (iii) differences in prevalence of JCV infection in the populations studied.

Laghi et al. (1999) reported at least a 10 fold higher number of JCV DNA copies in tumors than in normal colorectal tissues. Therefore, more copies of target viral DNA would be available in tumors for PCR amplification, resulting in easier detection in tumors than in normal tissues.

More information on the prevalence of JCV in the different parts of the world is needed, however, to assess the importance of the third possibility. The American CRC population shows a prevalence of JCV-positive CRC tumors ranging from 0 to 96% (Laghi et al., 1999; Enam et al., 2002; Hernandez Losa et al., 2003; Newcomb et al., 2004; Goel et al., 2006). In Asian CRC populations, the prevalence of JCV-positive CRC tumors ranged from 26.1 to 86.4% (Bofill-Mas et al., 2001; Hori et al., 2005; Lin et al., 2008; Mou et al., 2012). Two contradictory Italian studies, however, reported the detection (Casini et al., 2005) or not (Campello et al., 2010) of JCV DNA in Italian CRC patients. Thus the prevalence of the virus in CRC tumors would reflect the prevalence of the virus in the population and its association with the ethnic and the geographic distribution, but could possibly be influenced by a lack of standardization in the technical procedure used to detect viral DNA.

In our study, JCV DNA was present only in CRC adenocarcinomas and was absent in normal mucosa surrounding colonic lesions. If JCV infection played no etiologic role, the prevalence of viral DNA would be similar, which is not the case in this study. But, if for some reason, JCV presence facilitates tumor development or progression, it should be detected at a higher prevalence in tumors. The comparison between colonic lesions and surrounding colonic mucosa showed that the detection of viral DNA was limited to tumor tissue, and statistically significant. It is also important to note that there are no significant differences between the JCV DNA prevalence in normal mucosa adjacent to the tumor versus normal mucosa of control patients.

The detection of JCV DNA in only a subpopulation of Tunisian colorectal tumors suggests that a biological selection for JCV infected cells has occurred during tumorigenesis or the cells containing JCV might be selected while the rest of the tumors would be associated with other etiologic factors. In this case, any JCV presence in the tumor would be a companion and not a causative agent. However, JCV DNA absence in normal adjacent tissues can be explained by two possibilities (i) its genome can be silenced or (ii) it can be lost entirely or in part during tumor progression “hit and rest transformation” (White et al., 2004; Avanzi et al., 2013). In the case of malignant tumors, the lack of detection of viral DNA might be a consequence of viral integration, which is always accompanied by viral DNA deletions (Coelho et al., 2013).

In the present study, JCV DNA was unrelated to patient age, sex, tobacco exposure, alcohol use, residence, family history of CRC, histologic type and tumor site but tended to increase with the differentiation of CRC tissues and the pathogenic stage of tumors, although this trend was not statistically significant. These findings suggest a correlation with promoting the onset of tumor cells and were consistent with other studies (Ksiaa et al. 2015; Hori et al., 2005; Theodoropoulos et al., 2005). We found no correlation between the presence of JCV DNA and medical characteristics. Interestingly, we note that tissues from CRC patients who received chemotherapy prior to sample collection had a higher incidence of JCV infection compared with the patients without chemotherapy, although the difference was not significant. The association between immunosuppression and increased susceptibility to infection is well recognized. As demonstrated by Selgrad et al. (2008) in a retrospective study among liver transplant recipients, immunosuppressed patients have a significantly higher presence of JCV compared with immunocompetent controls. Chemotherapy drugs can inhibit the immune response and enable the reactivation of potentially oncogenic viruses. CRC patients with a family history have a higher prevalence of JCV DNA, which is consistent with the results described by Vilkin *et al*. (2012). JCV infection is usually asymptomatic and commonly occurs in later childhood and adolescence, after which the virus remains latent in the kidney. Besides the immune condition mentioned above, genetic background might also be associated with the susceptibility of JCV infection in CRC. Furthermore, our results demonstrate statistical significance ($p< 0.05$) to the frequent presence of JCV DNA in polyps, which have been correlated with the well differentiated grade of adenocarcinoma. In addition, most of the observed polyps were tubular adenomas in an advanced pathogenic stage. Therefore, this study suggests that JCV DNA was associated with the premalignant precursors of colorectal tumors and provides additional evidence that the JCV may play an important role in the early stages of colorectal tumorigenesis.

We have identified a Tunisian isolate sharing a close relationship with the European strains of genotype A, especially Mad-1, SP-1 and MR-7, as shown in the phylogenetic analysis. It should be noted that we chose as a target for amplification a particularly short, well conserved domain of the viral genome, and one associated with the production of a functional large T oncogene, including the 3'acceptor splice site for the large T mRNA and part of the intron between exon 1 and 2 of large T coding sequence. The lower primer actually spans the best conserved motif in the consensus 3' acceptor splice site of the polyomaviridae family (Cantalupo et al., 2005) over its 3' last eight nucleotides. We were therefore surprised to find a novel polymorphism that allows the association of our amplified samples with a new viral strain. On the other hand, we were not surprised that all amplicons had an identical sequence since we did not expect much variation in our samples given

the very short region amplified, its well conserved status and the relatively limited geographic area of origin of the CRC patient population. Finally, we rule out the possibility of an artifact given our scrupulous use of blank and negative control samples, and the fact that most samples in our CRC collection remained negative. This raises the question of the origin of this strain in Tunisia. It is possible that our viral strain was imported from Europe through travel or other routes such as the importation of food products contaminated by JCV (Burnett-Hartman et al., 2008; Giuliani et al., 2008; Campello et al., 2010). However, it is interesting to note that the nucleotide deletion was identified within an intron. It is tempting to hypothesize that this mutation might indirectly facilitate, or contribute to, the transformation of JCV-infected normal cells. The deletion found in our strain was neither strain nor genotype-specific since it was also found in strains of genotype B such as ZJ-1, NY, MO-6 and Tky-2a. We used as a positive control DNA from JCI cells transfected with the Mad-1 strain. However, all our amplicons differ from the Mad-1 strain, suggesting that our findings were not the result of contamination. Further detection and analysis of other isolates from Tunisian patients with CRC would be needed to confirm the presence of Tunisian variants and strain identity. Our data cannot be taken as a clear-cut indication that JCV is a causative agent, a cofactor, or a passive passenger in human tumors. However, our work clearly supports a potential role of JCV in the etiology of CRC in a subpopulation of Tunisian patients. It might participate in different ways in the pathogenesis of CRC directly and indirectly, ranging from initiation, promotion, and morphological progression with different biological characteristics to tumor maintenance and dissemination (Bofill-Mas et al., 2003; White et al., 2004). The less frequent detection of our target JCV genomic segment in MDAC and the lack of detection in PDAC strongly suggest that JCV DNA sequence, or at least the one we chose as a target, are not required for tumor maintenance or progression. In fact, our data support a transient effect of JCV in cellular transformation, with tumor promoting sequence being possibly silenced or lost during cancer progression, as in a "hit and run" mechanism (Popescu et al., 1989; Giuliani et al., 2008; Houben et al., 2012) or in a “hit and rest” mechanism (Avanzi et al., 2013). The frequency of reactivation and viral loads during reactivation episodes could be key in promoting cancerogenesis in this context, while sociodemographic factors, population genetics, immunocompetence and iatrogenic conditions (Puri et al., 2014; Frank, 2004; Costal et al., 2010) could contribute in ways that have yet to be uncovered.

As a whole, our result strongly prompts further investigation in a quest for decisive evidence supporting the etiologic implication of JCV in CRC tumors.

In conclusion, our results indicate for the first time, that JCV DNA was frequently present in colorectal adenocarcinomas of Tunisian patients with CRC and not in the corresponding normal epithelial cells. Therefore, this study suggests that JCV is associated with at least a subpopulation of Tunisian colorectal adenocarcinomas in general possibly through a hit and run mechanism. Furthermore, our assay detected the JCV genomic sequence specifically in tumoral tissues and the phylogenetic analysis of amplified DNA indicated that our virus isolates represent a new JCV strain of European genotype. Moreover, because T-Ag is capable of participating in several of the mechanistic steps involved in carcinogenesis and recent epidemiological findings link JCV to colorectal carcinogenesis, the presence of the JCV viral genomic sequence throughout the human gastrointestinal tract might be considered a potential etiologic agent. Our data contribute to building a much-needed body of statistical data in an effort to significantly link the presence of JCV with the risk of developing CRC.

List of abbreviations

CRC: Colorectal cancer; JCV: Jamestown Canyon virus APC: Adenomatous Polyposis Coli PML: Progressive multifocal leukoencephalopathy PCR: Polymerase chain reaction DNA: Deoxyribonucleic acid T-Ag: Large T-Antigen t-Ag: Small t-Ag pRb: Retinoblastoma protein nt: nucleotideEBV: Epstein-Barr virus IHC: Immunohistochemistry ANCT: Adjacent non-cancerous tissues NGM: Normal gastrointestinal mucosa HBV: Hepatitis B virus XMRV: Xenotropic murine leukemia virus-related virus E1A: Adenovirus early region 1 AFFPE: formalin fixed, paraffin-embedded tissues

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Table 1. Correlation between CRC clinicopathological variables and the presence of JCV DNA in tumors

Clinical or pathologic feauture	JCV DNA status of tumors			P value
	Positive (%)	Negative (%)	Total (positive + negative) (%)	
Age				
<60	13 (59.09)	11 (44)	24 (51.06)	>0.05*
>60	9 (40.90)	14 (56)	23 (48.93)	
Sex				
Men	10 (45.45)	11 (44)	21 (44.68)	>0.05*
Women	12 (54.54)	14 (56)	26 (55.31)	
Tabacco exposure				
Yes	10 (45.45)	11 (44)	21 (44.68)	>0.05*
No	12 (54.54)	14 (56)	26 (55.31)	
Alcohol use				
Yes	3 (13.63)	8 (32)	11 (23.40)	>0.05*
No	19 (86.36)	17 (68)	36 (76.59)	
Residence				
City	12 (54.54)	14 (56)	26 (55.31)	>0.05*
Countryside	10 (45.45)	11 (44)	21 (44.68)	
Location				
Right colon	4 (18.18)	5 (20)	9 (19.14)	>0.05**
Left colon	1 (4.54)	4 (16)	5 (10.63)	
Transverse colon	0 (0)	2 (8)	2 (4.25)	
Sigmoid	9 (40.90)	6 (24)	15 (31.91)	
Rectum	8 (36.36)	8 (32)	16 (34.04)	
Family history of CRC				
Yes	2 (9.09)	3 (12)	5 (10.63)	>0.05*
No	20 (90.90)	22 (88)	42 (89.36)	
Polyp presence				
Polyps	19 (86.36)	13 (52)	32 (68.08)	<0.05*
No polyps	3 (13.63)	12 (48)	15 (31.91)	
Differentiation				
WDAC	17 (77.27)	15 (60)	32 (68.08)	>0.05**
MDAC	5 (22.72)	9 (36)	14 (29.78)	
PDAC	0 (0)	1 (4)	1 (2.12)	
Histologic type				
Tubular	13 (59.09)	14 (56)	27 (57.44)	>0.05*
Tubulovillous	9 (40.90)	11 (44)	20 (42.55)	
Pathogenic stage				
I, II	5 (22.72)	11 (44)	16 (34.04)	>0.05*
III, IV	17 (77.27)	14 (56)	31 (65.95)	
Chemotherapy				
Yes	15 (68.18)	13 (52)	28 (59.57)	>0.05*
No	7 (31.81)	12 (48)	19 (40.42)	

WDAC: well-differentiated adenocarcinoma

MDAC: moderately differentiated adenocarcinoma

PDAC: poorly differentiated adenocarcinoma.

*P value was determined by the Fisher Exact Test.

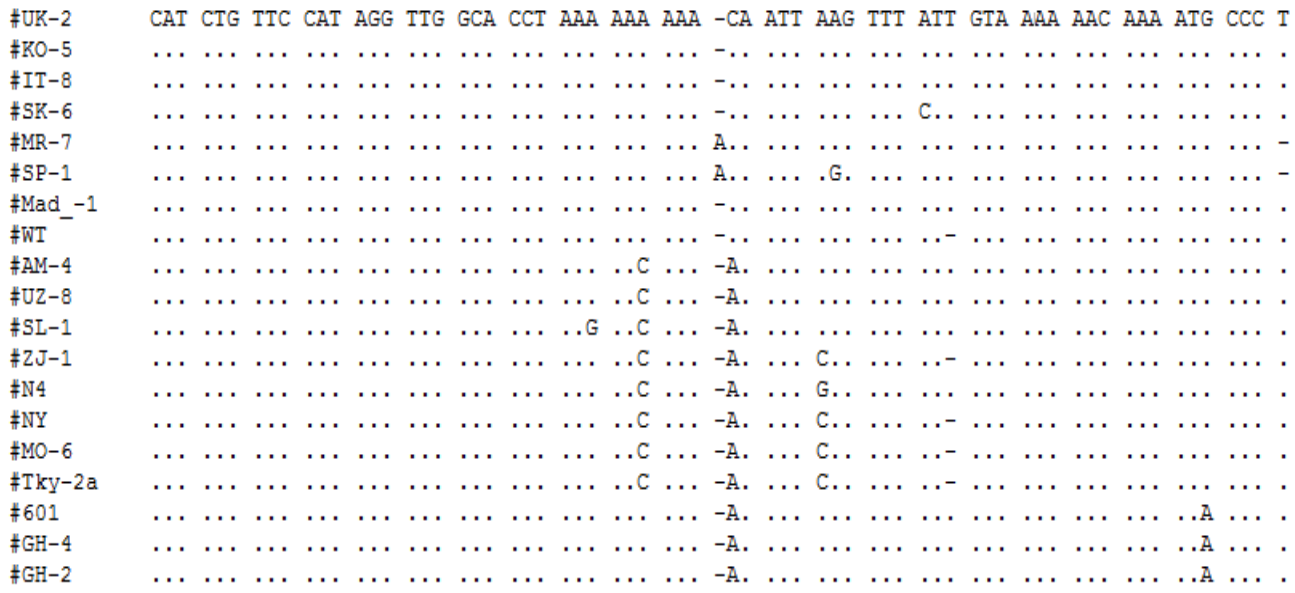
**P value was determined by the Chi-Squared test.

Table 2. GenBank of JCV strains included in the study

JCV Strain	Accession number	Origin	Reference
Genotype A			
Mad-1	J02226.1	USA	Miyamura. T et al, 1983
KO-5	AB074587.1	Russia	Yogo. Y et al, 2001
IT-8	AB074584.1	Italy	Yogo. Y et al, 2001
UK-2	AB048576.1	United Kingdom	Sugimoto. C et al, 2002
SK-6	AB183152.1	South Korea	Yogo. Y et al, 2004
MR-7	AB048564.1	Morocco	Sugimoto. C et al, 2002
SP-1	AB048566.1	Spain	Sugimoto. C et al, 2002
Genotype B			
-Subtype B1-b			
AM-4	AB262403.1	Russia: Naihin	Zheng. H et al, 2007
SL-1	AB262398.1	Sri Lanka: Colombo	Zheng. H et al, 2007
UZ-8	AB262407.1	Uzbekistan: Tashken	Zheng. H et al, 2007
-Subtype B1-c			
ZJ-1	JQ237146.1	China	Liu. X et al, 2011
N4	AB048554.1	Netherlands	Sugimoto. C et al, 2002
-Subtype CY			
NY	AB118233.1	Japan	Zheng. HY et al, 2004
MO-6	AB048562.1	Mongolia	Sugimoto. C et al, 2002
Tky-2a	AB038255.1	Japan	Kato. A et al, 2000
Genotype C			
#601	AF015537.1	USA	Agostini. HT et al, 1998
GH-4	AB048546.1	Ghana	Sugimoto. C et al, 2002
GH-2	AB038253.1	Ghana	Kato. A et al, 2000

Genotype and subtype were assigned according to Sugimoto et al., 1997, 2002.

Figure 1. Alignment of sequence WT with reference strains.



Sequence WT was aligned in MEGA 6 against 18 sequences representing the three JCV genotypes A, B and C retrieved from GenBank. See Table 4 for accession numbers.

Figure 2. Phylogenetic tree

The tree was generated by the distance-based maximum likelihood method and visualized using MEGA 6 program. The reliability of the tree was assessed by bootstrapping using 1000 replications. The numbers at the nodes indicate bootstrap confidence levels obtained from 1000 replicates. The length of the horizontal lines is proportional to the minimum number of nucleotide differences required to join nodes. The vertical lines are for spacing branches and labels.