BMC Cancer

RESEARCH Open Access

A new evaluation of the rearranged non-coding control region of JC virus in patients with colorectal cancer

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Abstract

Background Several studies have reported the presence of JC virus (JCV) in human tumors, The association of JCV and CRC remains controversial. This study aimed to evaluate the rearranged NCCR region of the detected JCV DNA in CRC patients' tissue samples.

Methods In this case-control study, tumor tissues ($n=60$), adjacent normal tissues ($n=60$), and urine samples ($n=60$) of the CRC patients were collected. The nested PCR was employed to detect the VP1 and NCCR regions of the JCV genome. The positive JCV PCR products were sequenced and a phylogenetic tree was constructed to determine the JCV genotypes. After extracting RNA and preparing cDNA, the expression of JCV LTAg was examined in 60 tumor tissues and 60 adjacent normal tissues. The analysis of JCV LTAg expression was performed using GraphPad Prism software version 8.

Results The analysis reveals that JCV DNA was detected in 35/60 (58.3%) tumor tissues, while 36/60 (60.0%) of adjacent normal tissues ($p=0.85$). JCV DNA was detected in 42/60 (70.0%) urine samples when compared to 35/60 (58.3%) tumor tissues of CRC patients and was not found significant ($P=0.25$). The phylogenetic tree analysis showed the dominant JCV genotype 3, followed by genotype 2D was distributed in tumor tissue, normal tissue, and urine samples of the CRC patients. Analysis of randomly selected NCCR sequences from JCV regions in tumor tissue samples revealed the presence of rearranged NCCR blocks of different lengths.: 431 bp, 292 bp, 449 bp, and 356 bp. These rearranged NCCR blocks differ from the rearranged NCCR blocks described in PML-type Mad-1, Mad-4, Mad-7, and Mad-8 prototypes. The expression of JCV LTAg was significantly different in tumor tissue compared to normal tissue, with a p-value of less than 0.002.

Conclusion A significant proportion of 35%> of the tumor tissue and urine samples of the CRC patients was found to be positive for JCV DNA ($P=0.25$). The parallel analysis of tumor and urine samples for JCV DNA further supports the potential for non-invasive screening tools. This study provides new insights into Rearranged NCCR variant isolates from patients with CRC. The significant difference in JCV LTAg expression between tumor and normal tissue indicates a latent JCV status potentially leading to cancer development.

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Highlights

- A significant proportion of 35/60 (58.33%) tumor tissue and 42/60 (70%) urine samples of the CRC patients were found to be positive for JCV DNA ($P=0.25$). The parallel analysis of tumor and urine samples for JCV DNA further supports the potential for non-invasive screening tools.
- screening JCV DNA in peripheral blood mononuclear cells (PBMCs) and stool of CRC patients may further support the potential for non-invasive screening tools.
- The study offers new insights into rearranged NCCR variants isolated from colorectal cancer (CRC) patients' tissue.
- The screening and analysis of the rrNCCR region of JCV DNA in CRC patients' stool may indicate the pathogenesis of the JCV virus in CRC development.
- The presence of JCV LTAg in tumor tissue is significantly higher than in normal tissue ($p=<0.002$), suggesting JCV's role in cancer development during the latency phase.

Keywords Colorectal neoplasms, Nested polymerase chain reaction, JC virus, Genotypes

Background

Colorectal cancer (CRC) is a prevalent form of cancer worldwide, ranking second in frequency and fourth in mortality [1]. it is a significant contributor to cancer-related deaths globally with an estimated 1.93 million new cases and nearly one million deaths projected for 2020 [2]. " The rate of colorectal cancer was estimated at *13.91* in men and *9.85* in women per 100,000 people" in Khuzestan City, Iran [3].

Lifestyle changes, diet, and decreased physical activity have resulted in an increase in the incidence rate of CRC in Iran. Between 2003 and 2008, colorectal cancer incidence rates went up in women from 5.47 to 11.12 and in men from 5.56 to 12.7 per 100,000 people [4]. Over 50% of cases and deaths can be attributed to preventable risk factors such as smoking, an unhealthy diet, excessive alcohol consumption, physical inactivity, and obesity [5].

JCV is typically acquired early in life via the fecal-oral route, leading to a lifelong infection that remains latent in the kidneys, central nervous system (CNS), upper and lower gastrointestinal tract, and CD34+lymphocytes of healthy individuals [6–9]. JCV is frequently present in the upper and lower digestive systems of individuals with normal immune function [8]. In cells where JCV cannot cause cell lysis, such as the colon mucosa, re-activated JCV might be linked to adenomas and CRC development [10].

Moreover, viruses have been suggested to play a role in the development of human cancers, with approximately 20% of all cancers believed to be associated with infectious agents [11]. The John Cunningham virus (JCV) is suspected of potentially contributing to various stages of cancer development [12]. JCV belongs to the polyomavirus family, with a genome consisting of a 5.13-kb, double-stranded, icosahedral, non-enveloped, supercoiled circular DNA [13, 14].

The genome of JCV contains coding regions for proteins in both the early and late stages of the viral life cycle. These regions are flanked by a non-coding control region (NCCR), which includes the promoter and enhancer elements required to regulate the expression of early and late genes [15].

The JCV genome's gene expression encodes the capsid's structural proteins, namely VP1, VP2, and VP3, along with a small regulatory protein called Agno protein. The Agno protein is essential in regulating the life cycle of a virus [16, 17]. The early region encodes several regulatory proteins, including T antigen (TAg) and small T antigen (tAg), as well as T′ proteins. During the latency phase, the expression of JCV LTAg showed multifunctional protein that can bind and inhibit the tumor suppressor proteins p53 and pRb, causing cell cycle misregulation and allowing replication of cells with damaged chromosomes. It was discovered that JCV LTAg can interact with β-catenin, which can dysregulate the Wnt signaling pathway and eventually lead to the development of colorectal cancer [18–21].

The NCCR is a regulatory area that includes essential sequences for both replication (ORI) and transcription [15]. JCV can exist in at least three different states in individual hosts: Silent infection does not express any protein. The latent infection will express a small amount of T/t protein but does not produce a large quantity of virions. Active infection will produce a large quantity of virions and lyse host cells, usually occurring in the brain [22]. A possible association between JCv and cancer will occur in latent infection form [19].

There are two types of JCV variants based on the structure of the NCCR: Archetype and Rearranged [23]. JCV is typically detected in urine, and its NCCR does not undergo rearrangement. The majority of JCV NCCR sequences found in urine, known as the 'archetype' (at-NCCR), have an initial regulatory region that includes the ORI, followed by six consecutive segments designated as "*a*" through "*f*" [15]. It is believed that NCCR rearrangements were formed due to deletions and/or duplications in the archetypal sequences, resulting in modifications to

the promoter activity [24]. These rearrangements were thought to contribute to the virus's pathogenesis by altering its cellular tropism [25]. The rearranged NCCR has been associated with PML [26], bladder cancer [27], and colorectal cancer [28].

According to reports, JCV was detected in patients with colorectal cancer in Tunisia at 58.1% [18], China at 40.9% [29], and Tunisia at 46% [30]. Human polyomaviruses are classified as possible carcinogens by the International Agency for Research in Cancer [31], although the role of JCV in human cancers remains unproven.

This study examined the presence of the JC virus and the rearrangement of sections in the NCCR region in urine, colorectal cancer tissue, and adjacent normal tissue samples. Moreover, the expression of JCV LTAg was achieved for colorectal cancer tissue and adjacent normal tissue samples.

Methods and materials

In this case-control study, from 60 CRC sufferers, the subsequent samples were amassed from tumor tissues (n=60), adjacent non-cancerous tissue (located 15–20 cm apart from the tumor) acquired by a health practitioner after the removal of the cancerous organ. (n=60), and urine (n=60). All the patients were hospitalized at Apadana health facility affiliated with Ahvaz Jundishapur University of Medical Sciences from April 2021 to October 2021. The inclusion criteria were newly diagnosed patients with confirmed histopathological findings, while the exclusion criteria were participants who had received preoperative adjuvant therapy such as chemotherapy and radiotherapy, as well as patients with metastatic cancer.

To prevent cross-contamination, we followed a strict aseptic procedure during specimen collection and handling of tissue samples. This standardized protocol involved using of separate disposable items such as biopsy needles and gloves for each specimen. Additionally, tissue specimens were individually placed in sterile cryo tubes (2 ml DNase/RNase free).

Tumor tissues and adjacent normal tissues were immediately placed in cryo tubes containing RNA stabilization solution to preserve RNA in tissues (RNA Later solution, Ambion, Austin, TX), then transferred to the laboratory in cold condition and at once stored at -80 °C for further tests. The urine of each patient was centrifuged and the sediment was collected in a micro-tube and stored at -80 °C until testing.

Tumor tissue, adjacent normal tissue, and urine DNA extraction were performed using the Sina-Pure DNA Kit (Cat No.: EX: 6011) according to the manufacturer's instructions. The quantity and quality of the extracted DNA were analyzed using the NanoDrop 2000 (Thermo Scientific, USA).

Polymerase chain reaction (PCR) of β-globin, JCV VP1 in tissue and urine and nested PCR for JCV NCCR

To confirm the quality of the extracted DNA, all samples were initially subjected to β-globin gene PCR using primers PCO3/PCO4 (Table 1). The PCR test was carried out with the following thermal program, initial denaturation at 95 °C for 5 min, followed by 38 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min with a final extension at 72 °C for 5 min. The positive β-globin PCR samples were subjected to JCV PCR, using a specific primer (Table 1).

JC virus genotype was determined by amplifying a partial region of the VP1 gene using specific primers [32].

The PCR program for amplification of the VP1 region of JCV was set up using JLP15/16 primers (Table 1). The PCR reaction in a final volume of 25 µl contained 1X PCR buffer, 1.5 mM MgCl2, 100 µM dNTPs, 25 pmol of each primer (JLP15/JLP16), 1 unit of Taq DNA polymerase, and 500 ng of DNA sample. The thermal conditions for the VP1 region of JCV were as follows: pre-heat at 95 °C for 5 min, then 35 cycles of 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 1 min for amplification of a 215 bp product.

Table 1 Primer sequences used in the study

Target	Primers	5′ to 3′ Sequence	Product size, bp	Accession Number	location	Reference
B-globin	PCO3	ACACAACTGTGTTCACTAGC	110	NM_000518.5	14–123	[59]
	PCO4	CAACTTCATCCACGTTCACC				
JCV-VP1	JLP15	ACAGTGTGGCCAGAATTCCACTAC	210 bp	NC_001699.1	1710–1924	
	JLP16	TAAAGCCTCCCCCCCAACAGAAA				[33]
JCV-NCCR	Outer primer-F1	AGGCCTAATAAATCCATAAGCTCCA	511 bp	NC_001699.1	4958 – 338	[58]
	Outer primer-R1	GTTCCACTCCAGGTTTTACTAACTT				
	inner primer-F2	TTTTAGCTTTTTGCAGCAAAAAATTA	409 bp	NC_001699.1	5013 – 291	
	inner primer-R2	CCTGGCGAAGAACCATGGCCAG				
LTAg-JCV	LTAg-JCV-F	CTAAACACAGCTTGACTGAGGAATG	172 bp	NC_001699.1	4212–4383	[60]
	LTAg-JCV-R	CATTTAATGAGAAGTGGGATGAAGAC				

The PCR products were then loaded onto a 1.5% agarose gel, and visualized under a UV transilluminator (Vilber Lourmat, France).

Based on the available, data, it has been demonstrated that the ORI sequence was not assessed and eliminated. As a result, the remaining sequence consists of 256 nucleotides which include sections/boxes *a* (25nt), *b* (23nt), *c* (55nt), *d* (66nt), *e* (18nt), and f (69nt). These sections were present in Archetype II. The evaluation of this study may reveal duplicate sections, triplicate sections, or deletions of sections, resulting in a sequence length greater than 256 nt, known as rearranged NCCR (*rr*NCCR), or deletions of sections may occur [15, 26]. The nested PCR test detected NCCR in two steps using specific primers. The amplification for the first round PCR was 511 bp, and for the second round PCR was 409 bp (Table 1). The first round of PCR was performed in a 25 µl mixture, which included 5 µl of extracted DNA, 12 µl PCR master mix (2x), 1 µl (10 nM) of each primer sequence, and 6 µl distilled water. Cycling conditions were as follows: denaturation at 94 °C for 5 min, followed by an amplification cycle at 94 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s for 35 cycles, and a final extension at 72 °C for 10 min. The second round was carried out with 2.5 µl of the first-round product, 1 µl (10 nM) of each primer sequence, 12 µl PCR master mix (2x), and 8.5 µl distilled water. Cycling conditions were as follows: denaturation at 94 °C for 5 min, followed by an amplification cycle at 94 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s for 35 cycles, and a final extension at 72 °C for 10 min. The PCR product was subjected to electrophoresis on a 2% agarose gel, stained with DNA-safe stain, and observed under ultraviolet light. The expected PCR product for the second round was 409 bp.

The analysis of sequencing and phylogenetic tree

The positive PCR products from adjacent normal tissues, tumor tissues, and urine of five patients were sent to Bioneer Company in Korea for sequencing of both the forward and reverse directions of the partial VP1 and NCCR regions of the JCV genome isolates.

The examination of isolates using partial VP1 sequencing was conducted by comparing them to VP1 consensus nucleotide sequences from the reference JCV genotypes 3 and 2D using SnapGene software (3.2.1) and the NCBI JCV database (https://blast.ncbi.nlm.nih.gov).

A phylogenetic tree was constructed for the partial VP1 region to determine JC polyomavirus genotypes using the Maximum Likelihood method under the Kimura 2-parameter distance model with 1000 bootstrap replicates [33]. The MEGA software version 6 was used to implement these methods.

The analysis of sequencing NCCR sections, both archetype II and rearranged NCCR sections, was determined using Snap Gene software (version 3.2.1).

LTAg expression

Total RNA was extracted from the tumor and adjacent normal tissues using the Total RNA Minipreps kit (Bio Basic, Inc.) kit, following instructions. RNA integrity was checked with gel electrophoresis for 28 S and 18 S rRNA, and yield was measured with NanoDrop 2000 (Thermo Scientific, USA). cDNAs made from 2 µg of RNA using the cDNA Synthesis Kit (cDNA Kit TaKaRa, Japan). Real-time PCR was done using the StepOnePlus system (Applied Biosystems, Foster City, USA) with specific primers for LTAg and Master Mix SYBR Green low ROX® (Amplicon, Denmark). Reactions for the LTAg target were carried out in 13 µL volume with 6.5 µL 2X master mix, 0.25 µL rox, 1 µL of cDNA, 4.25 µL water, and 0.5mM each of forward and reverse primers (Table 1).

The LTAg thermal cycling conditions were performed for 15 min at 95 °C, 40 cycles of 15 s at 94 °C, and 10 s at 57 °C. The expression level of LTAg was normalized using the housekeeping gene GAPDH. All tests were conducted twice, and the relative quantification of LTAg was calculated using the 2-ΔΔCt formula. The analysis of LTAg expression in the adjacent normal tissue and tumor tissue was determined by fold change using GraphPad Prism version 8.

Statistical analysis

Data analysis was conducted using SPSS software version 21. Chi-square and logistic regression tests were utilized for the analysis. GraphPad Prism software version 8 was employed for analyzing experimental data A P-value of less than 0.05 was considered statistically significant.

Results

The study analyzed 180 samples, including 60 tumor tissues (34 male, 26 female), 60 adjacent normal tissues, and 60 urine samples. The results revealed that 43 out of 60 (71.7%) were over the age of 60 years, while 17 out of 60 (28.3%) were under the age of 60 years. The integrity of the DNA was evaluated using PCO3/PCO4 primers, and all samples successfully passed the β-globin amplification test, indicating adequate DNA quality across all samples. Following this, positive samples underwent further analysis. The survey revealed that JCV DNA tested positive in 35 out of 60 (58.3%) tumor tissues and negative in 25 out of 60 (41.6%) tumor tissues. Additionally, JCV DNA tested positive in 36 out of 60 (60%) adjacent normal tissues and negative in 24 out of 60 (40%) adjacent normal tissues ($p=0.85$). JCV DNA was detected in 42 out of 60 (70.0%) urine samples of CRC patients. All 35 out of 60 CRC patients tested positive for JCV in both tumors and urine samples. The demographic characteristics of patients and the PCR results are displayed in Table 2.

The analyses of JCV-VP1 from four individual samples, including urine, adjacent normal tissue, and tumor tissue,

Table 2 Clinical pathological characteristics of JCV-positive and JCV-negative tumor tissues and adjacent normal tissues

Variables	Total	JCV positive in tumor tissues	JCV negative in tumor tissues	JCV positive in adjacent normal tissues	JCV negative in adjacent normal tissues	JCV positive in urine	JCV negative in urine	*P*-value (tumor tissues)	*P*-value (adjacent normal tissues)
Number of cases	**60**	**35**	**25**	**36**	**24**	**42**	**18**		
Gender								0.66	0.39
Male	34	19	15	22	12	24	10		
Female	26	16	10	14	12	18	8		
Age group (years)								0.07	0.64
≤ 60	17	13	4	11	6	14	3		
> 60	43	22	21	25	18	28	15		
Tumor location								0.55	0.89
Right colon	11	8	3	7	4	7	4		
Left colon	4	2	2	2	2	1	3		
Rectum	45	25	20	27	18	34	11		
Stage								0.64	0.18
I	14	8	6	5	9	7	7		
II	17	8	9	12	5	13	4		
III	22	14	8	15	7	19	3		
IV	7	5	2	4	3	3	4		
Family History								**0.96**	**0.64**
Yes	17	10	7	11	6	13	4		
No	43	25	18	25	18	29	14		
Tobacco								**0.96**	**0.10**
Yes	17	10	7	13	4	12	5		
No	43	25	18	23	20	30	13		

were recorded in GenBank with the accession numbers OR400616-618 and OR400622-630. The maximum likelihood method was employed to analyze the phylogenetic tree for JCV-VP1 sequencing. It was observed that the dominant samples from three patients (urine, adjacent normal tissues, and tumor tissues) belonged to JCV-VP1 genotype 3. Additionally, patient samples (urine, adjacent normal tissues, tumor tissues) contained one sample of JCV-VP1 genotype 2D. The JCV genotype was detected in the tumor tissue, as well as in the adjacent normal tissue and urine samples, indicating a match between them (Fig. 1).

The analysis of four JCV-NCCR samples was deposited in GenBank with the following accession numbers: urine (PP104437-39, PP104441), adjacent normal tissues (PP104436, PP104443-45), and tumor tissues (PP104446, PP104448-50). The NCCR amplicon sizes for four tumor tissues including PP104446 (431 bp), PP104448 (292 bp), PP104449 (449 bp), and PP104450 (356 bp), were found to be greater than archetype II blocks. However, the rearranged blocks differ from those described in PML-type Mad-1, Mad-4, Mad-7, and Mad-8 prototypes [15, 34–36].

The NCCR sequences of urine (PP104437-39, PP104441) and adjacent normal tissue samples (PP104436, PP104443-45) were identified as archetype II (Fig. 2, A). The NCCR sequences of the four tumor tissues were found to be rearranged (*rr*)NCCR. Among them, the JCV-NCCR (PP104446) *a-b-c-d-(a)-(b)-(c)-(d)-e-f* blocks showed duplication of blocks *a*, *b*, *c*, and *d* (Fig. 2, B). The JCV-NCCR (PP104448), *a-b-c-d-e-f-(c)* blocks displayed duplication of a partial "*c*" block with a deletion of 25 nucleotides (Fig. 2, C). The JCV-NCCR (PP104449), *a-b-c-(e)-d-(a)-(b)-(d)-(d)-e-f* blocks exhibited duplication of blocks *a*, *b*, *e*, and two partial "*d*" (Fig. 2, D). The JCV-NCCR (PP104450), *a-b-c-(a)-(c)-(d)-d-e-f* blocks demonstrated two partial duplications "*c*" and a partial duplication "*d* " (Fig. 2, E). Out of the four cancer patients, three were stage 3 and one was stage 1.

The fold change analysis of the expression of JCV LTAg in tumor tissue when compared with the expression of JCV LTAg in normal tissue found to be very significant (p=<0.002) (Table 3).

Figure 3 Shows the fold change expression level of LTAg in tumor and adjacent normal tissue determined by GraphPad Prism software version 8.

Discussion

JCV can induce chromosomal instability in colon cells. This specific mechanism triggers significant chromosome structure, leading to loss of heterozygosity and aneuploidy. These genetic alterations can promote the transformation of colorectal cells during different stages of tumorigenesis [37, 38]. The American Cancer

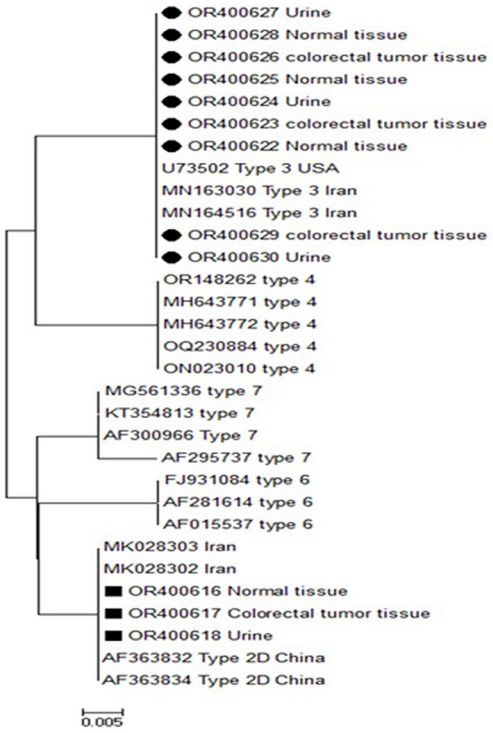

Fig. 1 The Maximum Likelihood method was used to construct a phylogenetic tree for sequences of the VP1 region of the JC polyomavirus genome isolated from urine, adjacent normal tissue, and tumor tissue from four patients. This was then compared with different JC polyomavirus genotypes retrieved from GenBank. The analysis of the phylogenetic tree revealed that the sequences of VP1 of isolates (OR400622-630) with a black circle were clustered with genotype 3 (U73502, USA), (MN164516, MN163030, Iran) while, isolates OR400616 – OR400618 with a black square was clustered with (AF363832, AF363834) type 2D isolated from China

Society changed guidelines in 2018 to start screening at 45 years old. In the current study, six CRC patients were found with CRC onset below 40 years, with the youngest patient at 33 years having JCV positive in the tumor, normal tissue, and urine samples. Health policymakers need to know and be informed about this finding. Factors like diet, lifestyle changes, and obesity may impact CRC risk in younger people [39–41].

The analysis of this survey reveals that JCV DNA was detected in 35 out of 60 (58.3%) tumor tissues, while in adjacent normal tissues, it was found in 36 out of 60 (60.0%) cases ($p=0.85$). Our results is in agreement with Ricciardiello et al. report, JCV is prevalent in the gastrointestinal tract of individuals with healthy immune systems [8].

JCV DNA was also detected in 42 / 60 (70.0%) urine samples from CRC patients when compared with. All 35 out of 60 CRC patients tested positive for JCV DNA in both their tumor tissues and urine samples. The frequency of JCV DNA among the male and female patients was not found to be significant. Interesting high frequency 42/60 (70%) JCV DNA was detected in urine samples of CRC patients and when compared with the prevalence of 10/164 (6.09%) JCV in the general population [42], it was found to be very significant ($p=0.00000$).

The conclusion of this analysis indicates the detection of JCV in urine samples of CRC patients could be used as a noninvasive and surrogate test for CRC patients associated with JCV infection.

The occurrence of JCV DNA between the 13/17 CRC patients group age>60 when compared with 22/21 CRC patients group age<60 ($p=0.07$). The rate of JCV DNA among the tumor location, right colon 8/11, left colon 2/2, and rectum 25/20 of patients was not found to be significant ($p=0.55$). The incidence of JCV DNA amongst the tomur stage, was 8/14 I, 8/17 II,14/22 II, and 5/17 IV ($p=0.6.4$). The existence of JCV DNA among family history and non-family history was 10/17 and 25/43 respectively ($p=0.96$). The presence of JCV DNA between smokers and non-smokers was 10/17 and 25/43 respectively ($p=0.96$).

Further analysis of the phylogenetic tree revealed that the sequences of VP1 isolates (OR400622-630) were clustered with genotype 3 (U73502, USA) and (MN164516, MN163030, Iran), while isolates OR400616 – OR400618 were clustered with (AF363832, AF363834) type 2D isolated from China. The prevalence of JC virus genotypes 3 and 2D has been dominant in certain regions of Iran. The present study has identified JCV rrNCCR with duplications of blocks from *a* to *f*, which were associated with an increase in the size of the NCCR among the four isolates in CRC patients. In our analysis, we found the NCCR amplicon sizes were varied for four tumor tissues including PP104446 (431 bp), PP104448 (292 bp), PP104449 (449 bp), and PP104450 (356 bp), were found to be greater than archetype II blocks. This data suggests that NCCR rearrangements may be a characteristic of individuals with CRC. The enlarged size and duplications at the NCCR blocks may result in viral replication, and gene transcription, and potentially contribute to tumorigenesis development [43].

The JCV-NCCR (PP104446) *a-b-c-d-(a)-(b)-(c)-(d)-e-f* blocks exhibited duplication of blocks *a*, *b*, *c*, and *d*. The JCV-NCCR (PP104448) displayed duplication of a partial "*c*" block. The JCV-NCCR (PP104449) showed duplication of blocks *a*, *b*, *e*, and two partial "*d* " blocks. The JCV-NCCR (PP104450) demonstrated two partial duplications of " *c*" and partial duplication of "*d* ". The arrangement of NCCR blocks in the four isolates differs from that of rr (NCCR) blocks that occurred in Mad1, Mad4, Mad7, and Mad8 blocks [15, 34–36]. In our study, the NCCR sequences of urine samples (PP104437-39, PP104441) and adjacent normal tissues (PP104436, PP104443-45) were found to be archetype II. To the best of our knowledge, this is the first documented evidence

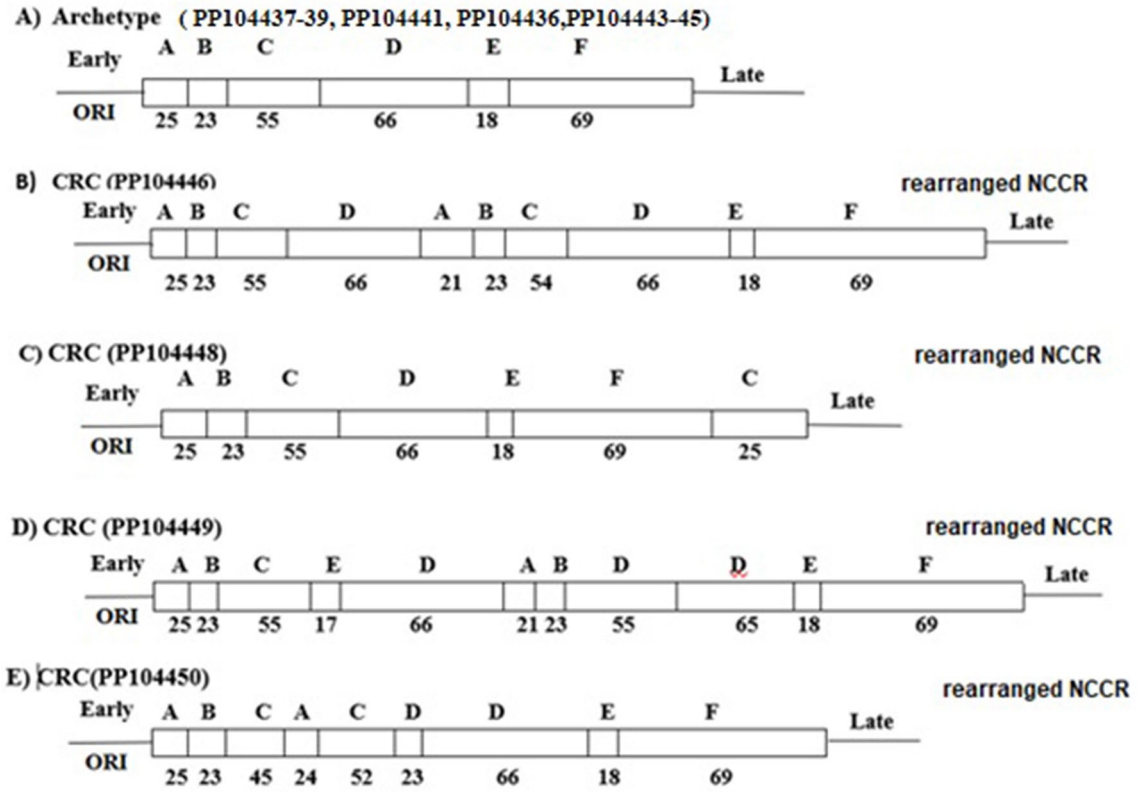

Fig. 2 depicts the Noncoding Control Region (NCCR) of JC Polyomavirus (JCV) in urine and adjacent normal tissue appearing as an archetype II (**A**). The NCCR in four patients with colorectal carcinoma (CRC) showed interesting variations. The NCCR of JCV (PP104446) exhibited a sequence arrangement of *a-b-c-d-(a)-(b)-(c)-(d)-e-f* blocks, with the occurrence of duplicated blocks *a*, *b*, *c*, and *d* (**B**). Similarly, the NCCR of JCV (PP104448) manifested the duplication of a partial "*c*" block (**C**). Furthermore, the NCCR of JCV (PP104449) displayed the duplication of blocks *a*, *b*, and *e* in addition to two partial "*d* " blocks alongside the "*d* " block (**D**). Moreover, the NCCR of JCV (PP104450) demonstrated the presence of two additional partial duplications of "*c*" and partial "*d* " blocks (**E**)

Table 3 Displays the expression of LTAg in tumor cells compared with the expression of JCV LTAg in adjacent normal tissue cells ((p = < 0.002)

	Cell type	*N*	Mean	SEM	Median	IQR	Test statistic	*P*-value
LTAg	Adjacent normal tissue cells	60	1.000	0.477	0.023	0.110	2388	0.002
	Tumor cells	60	12.018	3.623	0.177	7.860		

demonstrating the occurrence of rr (NCCR) in patients with CRS in Iran and other regions.

Uleri et al. (2019) conducted a study in Italy to evaluate the JC virus NCCR in 41 fresh samples from patients with CRC adenocarcinoma, 16 polyps with adenomatous, and 9 non-tumor controls (NTC). They discovered two types of NCCR-specific nested PCR amplicons: one with a size of the PML-type (353 bp) and a smaller one (260 bp). Additionally, they found a rearranged NCCR of the size and organization typical of the PML-type, with the classical 98 bp duplication sequenced among 22 CRC cases, 9 polyps, and 8 controls [28].

L Ricciardiello et al. (2001) documented the existence of two distinct categories of JCV-NCCR amplicons. One category, found in patients with CRC, is equivalent in size to the PML-type (369 bp). Sequencing analysis revealed that the higher band corresponds to the expected PML-type Mad-1 prototype, while the smaller band (270 bp) was identified as a Mad-1 variant with two types of rearrangements. The CRC tumor mucosa exhibited the D98a rearrangement, characterized by deleting the second 98 bp domain. [44]. This rearrangement of NCCR has been observed in the kidneys and brains of infected patients [45]. Additionally, this rearrangement has been detected in bone marrow and brain tissue from a PML patient [46]. A construct featuring this rearranged NCCR has been demonstrated in transformed Rat-2 fibroblasts compared to the Mad-1 prototype [47].

Coelho TR et al. (2010), in Portugal, postulated that the persistent presence of JCV in the gastrointestinal tract can contribute to the emergence and amplification of a tumor-prone environment [48].

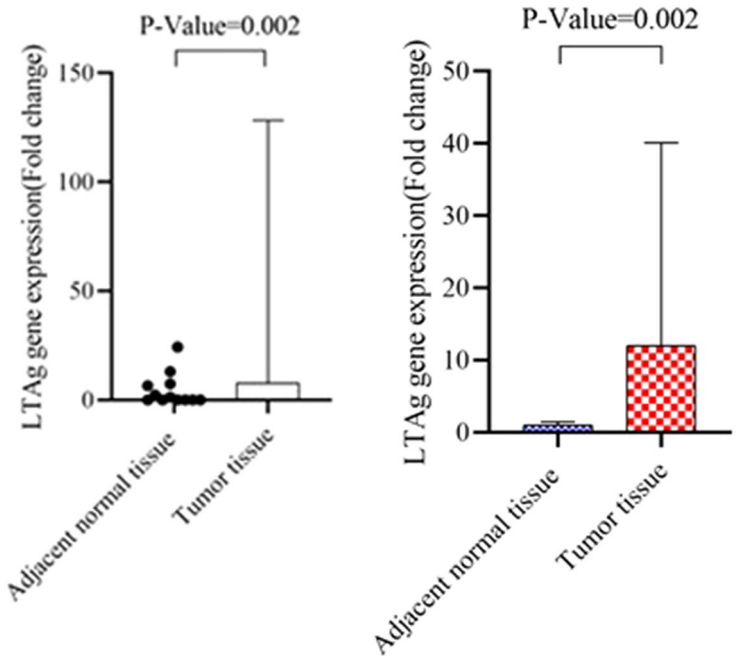

Fig. 3 Expression level of LTAg in tumor and adjacent normal tissue

Mou X et al. (2012) in China reported that the JCV viral DNA load was 40.9% (56/137) in CRC tissues, ranging from 49.1 to 10.3×10ˆ 4 copies/μg DNA. in non-cancerous colorectal tissues 34 (24.5%) tested positive for JCV, ranging from 192.9 to 4.4×10ˆ 3 copies/μg DNA (P=0.003). Additionally, 25 (18.2%) peripheral blood (PB) samples from CRC patients tested positive for JCV, ranging from 81.3 to 4.9×10ˆ 3 copies/μg DNA (P<0.001) (30).

Shoraka et al. (2020) in Iran have reviewed over 24 articles in qualitative analysis and 19 articles in quantitative analysis using immunohistochemistry and nested-PCR methods. They discovered a significant increase in JCV levels in colorectal cancer (CRC) patients compared to healthy controls [49].

Kimla L J et al. (2023) in the United Kingdom have surveyed more than 66 studies and found a significant 11-fold increase in the risk of CRC development in JCV DNA-positive samples with JCV LTAg expression compared to normal tissues [22].

The results of this study showed that the JCV-NCCR (PP104446) (431 bp) *a-b-c-d-(a)-(b)-(c)-(d)-e-f* blocks have duplications of blocks *a*, *b*, *c*, and *d*. Additionally, the JCV-NCCR (PP104448) (292 bp) displays a duplication of a partial "*c*" block. The JCV-NCCR (PP104449) (449 bp) demonstrated duplications of sections *a*, *b*, *e*, and two incomplete "*d* " sections. The JCV-NCCR (PP104450) (356 bp) showed two partial replications of "*c*" and a partial replication of "*d* ". As a result, our findings revealed original rearranged NCCR segments.

The presence of JCV LTAg protein in colorectal cancer tissues suggests an increased cancer risk [22]. In the current study, the significant difference in JCV LTAg expression between tumor and normal tissue (P=<0.002) indicates a latent JCV status leading to colorectal cancer development. Goel A. et al., in the USA, have reported that JCV LTAg protein expression was detected in 43/100 (43%) tumor tissue and 0/25 matched normal distal samples of CRC patients using PCR and immunohistochemistry tests, results found to be significant (p=0.0113) [50].

Lin et al. In Taiwan have stated JCV LTAg protein expression was detected in 14/22 (63.6%) and 0/22 in matched adjacent normal tissue samples using immunohistochemistry (IHC) and nested PCR tests, the outcomes were found to be significant (0.0037) [51].

Hori et al., in Japan, have described JCV LTAg protein expression as not detected among 23 CRC patients and 20 non-CRC control (healthy colonoscopy) samples using immunohistochemistry (IHC) and nested PCR tests.

Environmental factors affect JCV transmission in the human population. JCV DNA was found in rivers in Italy, and sewage in Egypt and Uruguay [52–54]. JCV DNA is also found in blood, urine, and feces from adult patient samples in Australia [55]. So far the detection of the JC virus has not been studied in rivers or sewages in Iran, but it is necessary to investigate it.

A significant proportion of 35/60 (58.33%) tumor tissue and 42/60 (70%) urine samples of the CRC patients were found to be positive for JCV DNA (P=0.25). The parallel

analysis of tumor and urine samples for JCV DNA further supports the potential for non-invasive screening tools.

The presence of JCV LTAg in tumor tissue is significantly higher than in normal tissue (p=<0.002), indicating JCV's involvement in cancer development during the latency phase.

ThThe limitation of this study includes, the lack of studies on JCV-NCCR rearranged variants in CRC patients, which could have been utilized and compared with our study, A large sample size is needed to properly analyze the role of JCV-NCCR in CRC development for future study.

The limitation of this study includes, the lack of studies on JCV-NCCR rearranged variants in CRC patients, which could have been utilized and compared with our study, A large sample size is needed to properly analyze the role of JCV-NCCR in CRC development for future study. The JCV DNA quantitative PCR in tumor tissue and normal tissue samples required to be evaluated. The screening of JCVDNA stool of CRC patients may be facilitated further supporting the potential for non-invasive screening tools.

The association between colorectal cancer and a variety of viral agents has been established. Examples include herpes simplex virus (HSV), human papillomavirus (HPV), Epstein-Barr virus (EBV), cytomegalovirus (CMV), and JC polyomavirus (JCV). It is important to note that these viruses have been identified as potential risk factors in the development of tumors in the gastrointestinal tract, including esophageal, gastric, and colorectal cancers [56].

Conclusion

The investigation of this work revealed that JCV genotypes 3 and 2D were predominant in urine, tumor tissues, and adjacent normal tissues of CRC. The examination of the JCV-NCCR of CRC patients' tissue identified rearranged NCCR variants, with the rearranged blocks differing from the previously described PML-associated rearranged NCCR. These findings suggest that the enlarged size and duplications at the NCCR blocks may indicate the activation of the NCCR, thereby facilitating viral replication and gene transcription however requires further investigations. "high frequency 42/60 (70%) JCV DNA was detected in urine samples of CRC patients and when compared with a significant proportion of 35/60 (58.33%) tumor tissue (p=0.25). This concluded detection of JCV in urine samples of CRC patients could be used as a non-invasive and surrogate test for CRC patients associated with JCV infection. The screening of JCV DNA in peripheral blood mononuclear cells (PBMCs) and stool of CRC patients may facilitate further support of the potential for non-invasive screening tools. The study offers new insights into rearranged NCCR variants isolated from colorectal cancer (CRC) patients' tissue. The screening and analysis of the rrNCCR region of JCV DNA in CRC patients' stool may indicate the pathogenesis of the JCV virus in CRC development. Consequently, this study offers novel insights into the rearranged NCCR variant isolates from patients with CRC tissue.

The occurrence of JCV LTAg in tumor tissue demonstrates a notably higher level compared to normal tissue (p=<0.002), revealing the participation of JCV in the progression of cancer during the latency phase. More research on JCV LTAg expression and colorectal cancer development is necessary.

Abbreviations

CRC	Colorectal Cancer
NCCR	Non-Coding Control Region
PML	Progressive Multifocal Leukoencephalopathy
JCV	John Cunningham Polyomavirus
HSV	Herpes Simplex Virus
HPV	Human Papilloma Virus
EBV	Epstein-Barr Virus
CMV	Cytomegalovirus

Acknowledgements

This work was conducted as part of a Ph.D. thesis by Azadeh Haghi Navand, a Ph.D. student in virology. The registration number CRC-0113 and financial support were provided by the Cancer, Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

Author contributions

Manoochehr Makvandi and Azadeh Haghi Navand: Writing, reviewing, and editing the original draft, software, data curation, conceptualization, formal analysis, methodology, validation, and visualization. Shahram Jalilian: formal analysis, software, review, and editing. Kambiz Ahmadi Angali: formal analysis. Mohammad Karimi Baba Ahmadi: methodology. Abdol Hassan Talaiezadeh: critical revision of the manuscript for important intellectual content.

Funding

The present study is supported by the Cancer, Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran: (NO. CRC-0113).

Data availability

Sequence data that support the findings of this study have been deposited in the https://www.ncbi.nlm.nih.gov/nucleotide/ . Accession Numbers are provided within the manuscript and supplementary information files.

Declarations

Ethics approval and consent to participate

This human subjects study adhered to the ethical standards of the 1964 Declaration of Helsinki and received approval from the Ethics Committee of Jundishapur University of Medical Sciences in Ahvaz, Iran (IR.AJUMS.MEDICINE.REC.1401.023). Written informed consent was obtained from all participants.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Received: 6 April 2024 / Accepted: 24 July 2024
Published online: 12 August 2024

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