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Original Article

Genotyping of JC Virus in Clinical Samples in North Eastern Iran

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Abstract

Background and Aims: There is no data about JC virus genotypes in Iran. Due to the association of some genotypes of JC virus with PML (Progressive Multifocal Leukoencephalopathy) and also for the fact that some genotypes are more likely to be associated with certain diseases, such as cancer, the importance of this research is highlighted. The aim of this study was to determine the genotype of JC virus in northeastern Iran to find any different genotype in different tissues.

Materials and Methods: Twenty two colorectal tissue samples were collected from colorectal cancer patients and 19 urine samples were collected from healthy individuals. After genomic extraction, PCR was applied to amplify the desired gene region for genotyping. The reported sequences were compared with sequences recorded in NCBI database. The 100% match was observed in most of the samples.

Results: In general, 12 urine samples and 17 tissue samples were in type 1 and 3 urine specimens belonged to type 2, and 5 tissue samples and 4 urine specimens were also found in type 3.

Conclusions: According to the statistical analysis, there was no correlation between urinary tract genotypes and tissue samples. Genotype 2 was observed only in urine specimens but not tissue samples. The most common genotype in this study was Type-1 and then Type-3. No other genotype was observed in this study.

Keywords: JC virus, Genotype, Iran.

Introduction

The JC virus was discovered in 1971 and is a member of polyomaviridae family. The ability to cause human disease from 3 viruses belonging to this family has been observed: JC-BK-SV40 (1) . The genome of the JC virus with 5.13 KB size encodes the vp1-vp2-vp3-AgT-Agt-agnopeotein (2, 3).The JC virus tends to the tonsils, kidneys, and CNS and oligodendrocytes (4). The JC virus causes a rare disease in the central nervous system of Human, the PML, which is the result of infection of oligodendrocytes and severe myelin injury .The incidence of PML is seen in people with immunodeficiency, such as those infected with HIV and cancers (5, 6). Studies have also shown that in the long-term suppression of the immune system, it is possible to re-activate the JC virus (7) The link between the different genotypes of the virus has been proven with the incidence of PML. Another important point in genotyping research has been mentioned that the isolated virus genotype is different from the brain with renal samples (8). In addition to PML, recent studies have shown that gastrointestinal cancers, and in particular colorectal cancer, are

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the most prevalent cancers in the Iranian population associated with the JC virus, which is the observation of viral protein in tumor cells that confirms this issue (9, 10). The results of the studies also indicate a significant relationship between prostate (11), bladder (12), and lung cancers (13) with the JC virus. According to the Ault and Stoner reports, IG 610bp region of the JCV genome, which contains areas that determine the differences in the sequence, is used for phylogenetic and genotyping studies (2). Of these, a portion of the VP-1 with the length of 129bp is used to determine the main type (14, 15).

Methods

Population studied. Colon specimens were collected from archive of FFPE in Mashhad University of Medical Sciences (16). Urine specimens were selected randomly from healthy individuals who had U/A Exam for checkup purpose. Given the fact that exposure to JC virus occurs in childhood (17), urine specimens were used from individuals over the age of 25 years.

Preparing samples and genome extraction. At first, paraffin-fixed tissues were cut by microtome with diameter of 8 micron with minimum paraffin entering to the microtube. For urine specimens: 15 ml liters were collected in a sterile flask, and after centrifugation at 15000 rpm, the supernatant was removed and the precipitate was transferred to the microtube. After adding 20µl of proteinase K to each microtube and shaking every 5 min, the specimens were spinned for 5 min at high speed, then the supernatant was used for the DNA extraction. We used Yektatajhiz Azma kits for DNA extraction from specimens and urine samples according to the kits instructions. After performing the above steps, the concentration of extracted DNA was measured using a nanodrop and samples with concentrations higher than 70ng /ml and a 260/280 ratio between 1.8 and 2 were placed in the next steps at -20°C freezer. In order to eliminate supercoils from genome of JCV, samples were mixed with the topoisomerase 1 enzyme M0301S (Biolabs, Canada). The enzyme (4µl) was added to 2µl of the sample and then incubated for 37 min at 45°C (18) (19). This enzyme loses activity at 60°C, while its operating temperature is at 37°C.

Primers were ordered from the pishgam Tehran Company and, based on the Agostini article (Table 1). JLP15-JLP16 primers were used as external primers (20, 21) and JLP-1-JLP4 as internal primers (22, 23) in the NESTED-PCR process. These primers are part of the VP-1 amplifier that is sequenced for genotyping. The 129 bp band is considered to be a positive JC (23, 24).

PCR. The PCR program for external primers is as follows: For initial degradation, it is placed at 95°C for 5 min. Then, 50 cycles of three steps were performed for 1 min at 95 ° for 1.5 min for 63 ° and one min for 72 °C. For final extension, it was placed at 72°C for 10 min. The PCR program for internal primers was also primed at 95°C for 15 min followed by 48 cycles of 30 seconds at 94°C for 60 sec at 63°C for 60 sec at 72°C and a final cycle at 72°C for 10 min.

Genotyping. Samples were sent to South Korea's Macrogen Corporation for sequencing

Table. 1. Information about primers includes TM and their size and sequence

Oligo Name	Sequence 5' - 3'	Oligo ID	BC	Purif.	EC	MW (Da)	Tm (°C)	OD 260nm	nmol	Normalisation# µl required for 100µM solution
JLP-15	ACA GTG TGG CCA GAA TTC ACT ACC	180731B001G12 55/93	24	Desalted	233,0	7.322	65	8,4	36,1	361
JLP-16	TAA AGC CTC CCC CCC AAC AGA AA	180731B001F12 56/93	23	Desalted	221,0	6.916	65	5,5	24,8	248
JLP-1	CTC ATG TGG GAG GCT GTK ACC T	180731X006A07 57/93	22	Desalted	203,0	6.770	65	5,9	29,0	290
JLP-4	ATG AAA GCT GGT GCC CTG CAC T	180731B001E12 58/93	22	Desalted	206,0	6.735	64	7,2	34,8	348

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by Danazist ASIA and classified according to the reference genotype registered in the NCBI. Compliance with 100% of recorded genotypes was observed.

Statistical analysis. In this study, all data were recorded in SPSS 16 software. Two descriptive and analytic methods were used. The Chi-square was used to analyze the results. T-test was used to examine the results in the age groups. The tumor group was considered as the case group and the urine group as the control group. Also, in examining the relationship between the case and control genotypes due to the lack of the components of the chi-square test, it is not possible to use this test in this area.

Ethical considerations. the study was conducted in accordance with Helsinki declaration on Human research and its further Additions. Individual samples were reviewed by their privacy and only using age and gender information. This study was reviewed and approved by the Medical Sciences University of Mashhad for ethical considerations. The ethical code of this research is IR.MUMS.fm. REC.1396.582.

Results

Of the colon specimens sent for sequencing, 17 specimens were identified as type 1 and 5 were recognized as type 3(Fig. 2). In the case of urine specimens, 12 samples were in accordance with the reference type 1 and 3 were Type 2 and 4 samples were identified as type 3 (Table 2).

Table 2. The results of genotyping of case and control sample.

Gender		Genotype 1	Genotype2	Genotype3	Number
Male	**Case**	85.7%	0	14.3%	**14**
	Control	87.5	0	12.5%	**8**
Female	**Case**	62.5%	0	37.5%	**8**
	Control	45.5%	27.3%	27.3%	**11**

Given the available resources, VP1 is used for sequencing and 129bp band implies viral positive result (Fig. 1) (25) (23).

Statistical analysis results. This study was performed using T-test and considering that the mean age of the case group (tumor samples) was 56.45 ± 18 and the mean of control group (urine specimens) was 61.5 ± 7. The P value was 0.239, which indicates no statistically significant differences, and in fact the homogeneity of the age indicator in each of the two groups is indicated.

In the examination of gender, 63.6% of the case group and 42.1% of the control group were male, which was 36.4% and 57.9% for females, respectively. There was no significant relationship between these two groups, which means that both groups were homogeneous in terms of gender. Genotypic frequency in case group: 17 samples observed in genotype 1 and 5 were genotype 3. Genotype 2 was not observed in this group. The genotypic frequency in the control samples was as follows: 12 tested specimens were genotype 1, and 3 samples were genotype 2, and finally 4 samples were also genotype 3. Because there was no genotype 2 in the case group and its frequency was equal to zero, there were no assumptions about chi-square test, but 15.8% of the control group had genotype 2 in clinical terms (Fig. 4). In terms of genotype 1, the case group was 77.3% and the control group was 63.2%, which indicates the similarity between these groups in genotype 1. Genotype 3 also had 22.7% of the case group and 21.1% of the control group; in this case both the case and control groups were similar.

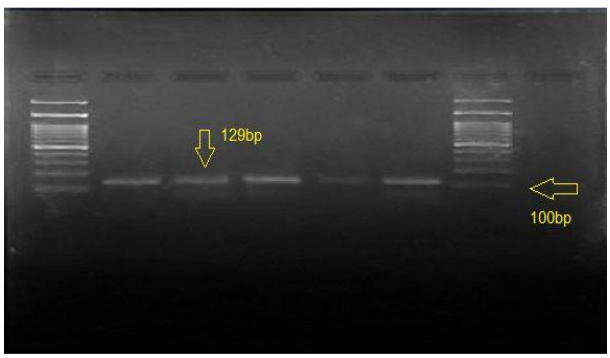

Fig. 1. VP-1 gene PCR product.

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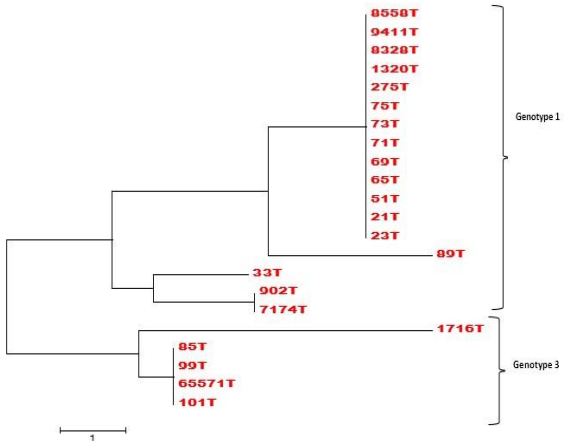

Fig. 2. Neighbor- joining phylogenetic tree of tumor samples (Case). Mega -7 software is used. The desired fragment VP-1 is from the JC virus with a length of 129 bp.

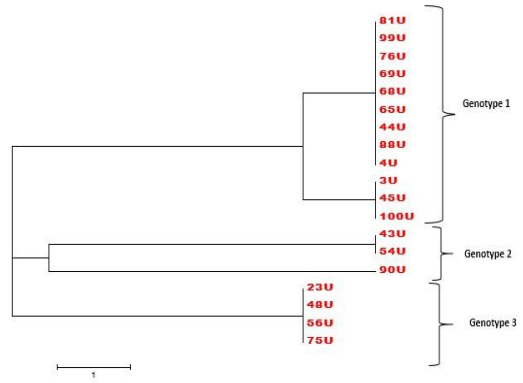

Fig. 3. Neighbor- joining phylogenetic tree of Urine samples (Control). Mega -7 software is used. The desired fragment VP-1 is from the JC virus with a length of 129 bp.

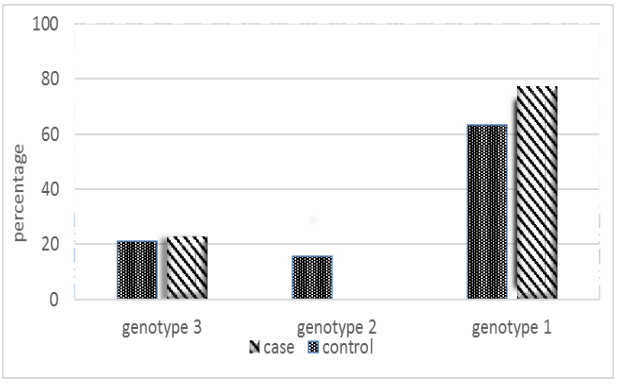

Fig. 4. The percentage of genotypes in the case and control groups.

The study of the relationship between gender and genotype distribution in this research, which resulted in a higher percentage of males in Genotype 1, on average 86.4%, for genotype 3 the majority of cases with an average of 31.6% were females. The genotype 2 belonging to this study also relates to female sex (Table 3).

Table 3. The percentage of genotypic differentiation is based on gender.

	Tissue samples case	Urine samples control	**Acc. no.**
Type 1	17	12	**U21842.1**
Type 2	0	3	**U21843.1**
Type 3	5	4	**U21844.1**

Discussion

The genome of the polyomaviruses shows a large number of sequence variations not only between JCV and BKV, but also in a variety of JCV sub-types (26). According to the previous studies, genotypes 1 and 4 are predominant in Europe and 2 and 7 in Asia. Genotypes 3 and 6 in Africa and genotype 8 have been reported exclusively in New Guinea (27). It is noteworthy that alterations in the amino acid sequence of a structural protein can lead to increased viral entry and, as a result, contribute to PML, or potentially cause disease (28). It is also important to note that the genotype distribution of the JC virus is diverse in relation to different breeds in different geographic regions (29). It is also important that the JCV genome, in addition to the sequence variation that has evolved in long time, brings new sequences by rearranging parts of its genome control area after an initial infection. While the mechanisms underlying the rearrangement of the genome or where the event one occurs is not yet understood that (30) this hypothesis stated that the existence of rearrangement results in immunosuppression, long-term person (31). Interestingly, the 2B genotype found in Italian patients with chronic inflammatory disease could represent a

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genotype selection of the JC virus in response to immunosuppressive drugs (32) and because PML is thought to be the source of cascading events that start with reactivation of JCV after immune suppression (6, 33). Since the relationship between JC genotype 2 and PML has been proven (24) and as previously mentioned, some studies (34), including the present research, referred to the increase in genotype 1 in colon cancerous tissues and reported type 4 with low risk of disease (7). For this reason, we decided to study a link between genotype and cancer and also PML, as well as the frequency of common genotypes in tumor tissue. In this study, we noted that urine specimens were collected from individuals who had no illness (diseases that affect JC prevalence and virus reactivating , such as diabetes and cancer, and various types of immunodeficiency) (35, 36).

In present research, the dominant genotype was observed in both urine specimens and tissue samples, was genotype 1. Unlike Northeast Asia where the dominant genotype is 2A and 2C, the genotype with high prevalence in Iran was genotype 1 (11). After genotype 1, genotype 3 has the highest prevalence in both urine specimens and our tissue samples.

Finding genotype 3 in Isfahan was also reported by Atyabi et al. In 2016 (37). This study also confirms the presence of genotype 3 in northeastern Iran. It should be noted that this study is one of the rare studies in the genotype of the virus circulation in Iran. In this study, in the tumor samples from 22 samples, none of them had genotype 2, which was clearly associated with the PML complication (38). Among the tumor samples, the most genotype was genotype 1 (Mad-1), which was also matched by a research done at the University of California (34). Although in this study, contrary to the research carried out in Isfahan, the highest frequency of genotype 3 was reported, genotype 1 had a higher percentage.

Conclusions

The frequency of genotype 3 and 1 was similar in both urinary and tumor groups. In this study, either in tumor samples or in urine specimens, genotype 4 was not observed. It was also hypothesized that because of the absence of genotype 2 in tumor samples, this genotype may have a protective effect on colorectal cancer. Since there is a need for a broader study in this field, it cannot be determined with certainty.

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