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Molecular epidemiology of JC polyomavirus in HIV-infected patients and healthy individuals from Iran

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Abstract

JC polyomavirus (JCPyV) is the causative agent for progressive multifocal leukoencephalopathy (PML) in immunocompromised patients. More than 40% of healthy population excretes JCPyV particles in their urine. As JCPyV is ubiquitous in human, the definition of genotype distribution can help trace population migration. In this study, to define the frequency of JCPyV in southwest of Iran, urine samples of 161 volunteers including 80 healthy individuals and 81 HIV-infected patients were collected. PCR assays and sequence analysis were performed using JCPyV-specific primers designed against VP1 coding region. JCPyV DNA was detected in 65 out of 81 urine samples (80.2%) of HIV-infected, and in 43 out of 80 urine samples (53.8%) of healthy individuals ($P = 0.001$). The shedding of JCPyV among HIV-infected patients revealed an age-related pattern while such relationship was not observed in healthy individuals group. The most common genotype found in this region was genotype 3A (80.8%), followed by genotype 2D (11.5%), 4 (3.8%), and 7 (3.8%). The frequency of JCPyV in the urine of HIV-infected patients was found significantly higher than in the healthy individuals ($P = 0.001$).

Keywords JC polyomavirus · Polyomavirus · Genotype · VP1 gene · Polymerase chain reaction

Introduction

JC polyomavirus is a member of the *Polyomaviridae* family [1]. They are small, non-enveloped DNA viruses with a 5.1 kilo base pairs (kbp) supercoiled, double-stranded DNA genome [2]. The genome has two main regions known as early and late genes which are separated by a non-bonding control region (NCCR) containing the origin of replication (*Ori*) [3]. The early gene encodes large T antigen and small t antigen, which contribute to the viral replication [4]. Large T antigen appears to be involved in tumor genesis because of its ability in cell transformation in vivo and in vitro [5]. All *Polyomaviruses*

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have also three structural proteins termed VP1, VP2, and VP3 which are encoded by late genes as well as Agno protein [6].

JCPyV is endemic so most human populations are infected with the virus during childhood [7]. The frequency of JCPyV in adult population is approximately 70%, although the virus is silently shed in the urine of ~ 40% of adults [4, 5].

There are two different classifications of JCPyV genotyping. The early genotyping was designed by Yogo et al. based on restriction fragment length polymorphism (RFLP) and later confirmed by V-T intergenic region dividing JCPyV in genotypes based on an alphabetical order (A, B, C, and D) [8, 9]. The second system of JCPyV classification was primarily based on the analyses of the sequence variation of the V-T intergenic region. Later, an upstream major capsid protein (VP1) gene fragment was used to classify different genotypes [10]. Using this method presented by Agostini et al., more than 7 genotypes numbered 1 to 8 and several subtypes were identified [6, 10, 11]. All JCPyV isolated worldwide belong to a unique serotype but there is a number of JCPyV genotypes that are used for tracing human migration in the world [6, 12, 13]. Type 1 (type A subtype EU) is prevalent in Europe and the USA, while type 2 (type B subtype B1 & MY) and type 7 (type B subtype SC) are Asian types. Type 3 (type B subtype Af2) and 6 (type C subtype Af1) are found in Africa and type 4 is associated with type 1 which has been found in the USA and Europe [14–16]. JCPyV genotype 5 is a recombinant type between type 2b and type 6 [16]. Type 8 is found in the Western Pacific and Papua New Guinea populations [17]. Type 2 is classified into 4 subtypes. The main subtypes in the northeastern part of Asia are 2a and 2c while the dominant subtype is 2b in west of Asia. Subtypes 2d and 2e are prevalent in India and Guam, respectively [15].

Latent infection is induced by the virus in the kidney, lymphocyte, spleen, and bone marrow [18]. JCPyV reactivation under immunosuppressive condition causes deadly demyelinating disease of the central nervous system known as progressive multifocal leukoencephalopathy (PML) as well as various neurological diseases including granule cell neuronopathy, meningitis, and JCPyV encephalopathy [19, 20]. PML was a rare disease before AIDS pandemic. An extreme rise in the PML incidence occurred in the world after the HIV outbreak in the early 1980s [21–23]. Recently, the number of the HIV-infected people with PML disorder has decreased because of the extensive use of combined antiretroviral therapies. Currently, PML has been observed in the patients with autoimmune disorders who take immunomodulatory medication such as natalizumab, rituximab, and efalizumab [20]. Yet, HIV infection still remains as the most frequent immunodeficiency setting concerning PML [24]. Previous studies indicated a difference in JCPyV excreted in the urine of HIV-infected patients compared with healthy individuals, albeit the difference was not significant. Similarly, the distribution of genotypes between these two groups was not different [13, 25]. Among JCPyV genotypes, type 2b has proved to be related with the high-risk PML disorder in immunodeficient patients [7]. Hence, this project was carried out to detect JCPyV prevalence and the genotype distribution in HIV-infected patients in comparison with healthy individuals as a molecular-based epidemiological survey in Ahvaz city, Iran.

Materials and methods

Patients and collection of clinical samples

Eighty-one samples were obtained from HIV-infected patients when they referred to the High Risk Behavior Guidance Center in Ahvaz for checking their CD4 count and taking medicines. Inclusion criteria involved (a) HIV-positive ELISA and Western blot serology, (b) CD4 cell count $< 600 \times 10^6$ cell/l, (c) receiving HAART regimen for at least 3 months, (d) no sign of active malignancy or other infectious diseases, and (e) no pregnant female. Any sign of other active infectious diseases or malignancy caused exclusion from the group. Also, 80 samples were collected from healthy individuals who had referred to diagnostic laboratories for checkup, without any malignancy and immunosuppression conditions. Fresh urine samples were sent immediately to the laboratory of virology department and stored at − 70°C.

DNA extraction and PCR amplification

Fifteen milliliters of the urine was centrifuged at 3500 rpm for 15 min. The cell pellet remained suspended in phosphate-buffered saline (PBS) by vortexing, recentrifuged, with the supernatant discarded. Viral DNA was then extracted using QIAamp Viral DNA isolation micro kit (Qiagen Inc., Germany) according to the manufacturer's protocol, and then stored at − 20 °C until the next phase. Two primer sets were designed using the Oligo version 7 software for amplifying 649 and 768 bp fragments in order to yield the entire VP1 coding region for JCPyV genotyping. The first and the second primer pairs are presented in Table 1.

Finally, PCR amplification was run with a total of 50 μL consisting of 25 μL PCR Master Mix (Amplicon), 2 μL (1 μM) each of FW and RV primers, 5 μL extracted DNA, and 16 μL injection grade water. The PCR reaction mixture was subjected to thermocycler (Peqlab, Germany) with the following thermal program: initial activation step at 94 °C/5 min, 35 cycles of 95 °C/45 s, 50 °C/45 s, 72 °C/45 s, and a final extension at 72 °C for 10 min. All samples along with negative control (previously known as negative sample) and positive control (previously known as positive sample) were amplified. The PCR products were detected by

Table 1 The sequences of JCPyV primers for PCR and cycle sequencing

Primer	Sequence (5′-3′)	Position	Amplicon size
F3J	ACAGCATTCAAGAAGTTACCCA	1280–1302	649
R3J	CCCCTGTAATTCTAAAGCCTCC	1928–1906	
F4J	ACTCTAATGGTCAAGCAACTC	1824–1844	768
R4J	GCAACTGTAAAGTAAAGCTGG	2591–2571	

electrophoresis on 1.5% agarose gel, where the size of the amplified products was anticipated by the migration pattern of a 100-bp DNA ladder.

Sequencing and phylogenetic analysis

Positive results of PCR amplification were sequenced in both the forward and reverse direction with the same primer sets used for PCR amplification. The DNA sequencing was conducted on an ABI 3730XL DNA Analyzer (Bioneer, South Korea) via BigDye™ cyclic Sequencing. SnapGene sequence alignment editor version 3.2.1 was used to align sequences with consensus sequences obtained. The MEGA7 software was utilized for phylogenetic analysis. The multiple sequences alignment of retrieved sequences from GenBank and the sequences of this research were obtained by CLUSTALW. Distances were evaluated via Tamura Nei-model method. The bootstrap maximum likelihood method with 1000 replicates was performed to determine the evolutionary distances.

GenBank accession numbers

The sequences reported in this research were deposited in the GenBank database under the accession numbers: MH454250, MH602988, MH643768, MH643769, MH643770, MH643771, MH643772, MH667311, MH667312, MH667313, MH667314, MH688160, MH688161, MH688162, MH688163, MH688164, MH688165, MH688166, MK028301, MK028302, MK028303, MH725628, MH725629, MH725630, MH725631, and MH725632.

Statistical analyses

In this study, as an essential part of biometric analysis, descriptive statistics was run to describe mean, standard deviation, and frequency. Also, in the inferential statistics section, non-parametric chi-square and fisher exact tests were conducted to compare the frequency of qualitative variables based on the group variable (HIV-infected patients and healthy individuals). The analyses were performed using SPSS 22 software and the significance level was less than 0.05.

Results

JCPyV DNA excretion

This study was conducted on urine samples of HIV-infected patients (81 samples) and healthy individuals (80 samples) to acquire the prevalence and genotyping of JCPyV in these two groups in southwest of Iran. HIV-infected patients included 23 females and 58 males ranging from 8 to 68 years of age with an average age of 39.8. On the other hand, the healthy group consisted of 50 females and 30 males with the age range of 2 to 71 years. The average age of the subjects in this group was 41.1 years. The results of PCR assay indicated that in HIV-infected group, 65/81 patients (80.2%) had shed JCPyV in their urine. Among healthy individuals, viral DNA was detected in 43 out of 80 (53.8%) urine samples (Table 2).

As summarized in Table 2, the excretion of JCPyV among HIV-infected group was more common (80.2%) with a significant difference compared with shedding in healthy group (53.8%) ($P = 0.001$).

While the excretion of JC polyomavirus in male HIV-infected patients was more than in female patients, and greater in healthy females than in their male counterparts, no significant difference was observed in the shedding of JCPyV between males and females ($P = 0.317$).

To find the correlation between age and incidence of viral DNA, the two groups were organized into 4 age groups: 0–19, 20–39, 40–59, and over 60 years (Table 2). The investigation revealed that in HIV-infected patients, the frequency of viral shedding increased with age and was higher in the oldest age group, though no significant difference was observed ($P = 0.015$). However, in the healthy individuals, the prevalence of JCPyV lost the age-related pattern so, in the youngest group (0–19 years, 58.3%) was more than in the two older groups (groups 20–39 and 40–59 years). Note that the relationship between age and shedding of JCPyV had no significant difference ($P = 0.320$). In both groups, the viral shedding of JCPyV in the eldest group (> 60 year) was the highest among all age population groups (100.0% in HIV-infected patients and 73.3% in healthy individuals). Overall, in all samples classified according to age population groups, the presence of JCPyV in the urine increased given the age group though no significant difference was traced ($P = 0.229$).

Table 2 Excretion of JCPyV among HIV-infected patients and healthy individuals

	JCPyV positive number/age groups (%)					JCPyV positive number/total number of individuals (%)			
Group	0–19	20–39	40–59	>60	*p* value	female	male	*p* value	total*
HIV-infected patients	0/1 (0.0)	33/46 (73.7)	27/29 (93.1)	5/5 (100)	0.015	20/23 (86.9)	45/58 (77)	0.317	65/81 (80.2)
Healthy individuals	7/12 (58.3)	12/27 (44.4)	13/26 (50.0)	11/15 (73.3)	0.320	26/50 (52)	17/30 (56.6)	″	43/80 (53.8)
Total	7/13 (53.8)	45/73 (61.6)	40/55 (72.7)	16/20 (80.8)	0.229	46/73 (63)	62/88 (70.4)	″	108/161 (67.1)

*Significant difference was observed in the excreting of JCPyV among HIV-infected patients compare with healthy individuals ($P = 0.001$)

Identification of JCPyV genotyping

In order to detect the distribution of different types of JCPyV in urine samples of both HIV-infected patients and healthy group, 26 out of 108 positive samples were picked out randomly and subjected to multiple alignments after obtaining sequences. Analysis of nucleotide blast and phylogenetic tree constructed using maximum likelihood indicated that the most common genotype was type 3, subtype A which is the most prevalent type in Africa. The other identified genotypes were 2, 4, and 7 (Table 3). Type 3A was the most predominant type in the two groups of HIV-infected patients and healthy individuals. Notably, the other types of JCV (2, 4, and 7) were also identified among the HIV-infected patients. Neither type 1 nor type 6 was identified in this study (Fig. 1).

As seen in Table 4, this subtype is different with genotype 3A in some typing sites like position 1654 that changed from *T* (genotype 3) to *A* (genotype 1) or in position 2311 that nucleotide substitution T → A was observed. This nucleotide substitutions were seen in other typing sites which were represented above.

Discussion

JCPyV is a member of the *Polyomaviridae* family infecting human in the late childhood without any symptom and then persists in the kidneys, bone marrow, and lymphoid tissues [26, 27]. According to the seroepidemiological investigations, approximately 80% of human population is JCPyV seropositive while only 40% of human population excretes JCPyV in their urine [5, 6].

Table 3 Distribution of JCPyV genotypes from HIV-infected patients and healthy individuals

Genotypes	HIV-infected patients	Healthy individuals	Total
2D	3/19(15.7%)	0	3/26(11.5)
3A	14/19(73.7%)	7/7(100%)	21/26(80.8%)
4	1/19(5.3%)	0	1/26(3.8%)
7	1/19(5.3%)	0	1/26(3.8%)

Type 3A was the most common type in both groups followed by genotypes 2D, 4, and 7 only in HIV-infected group. JCPyV genotypes 1 and 6 were not detected in either of the two groups

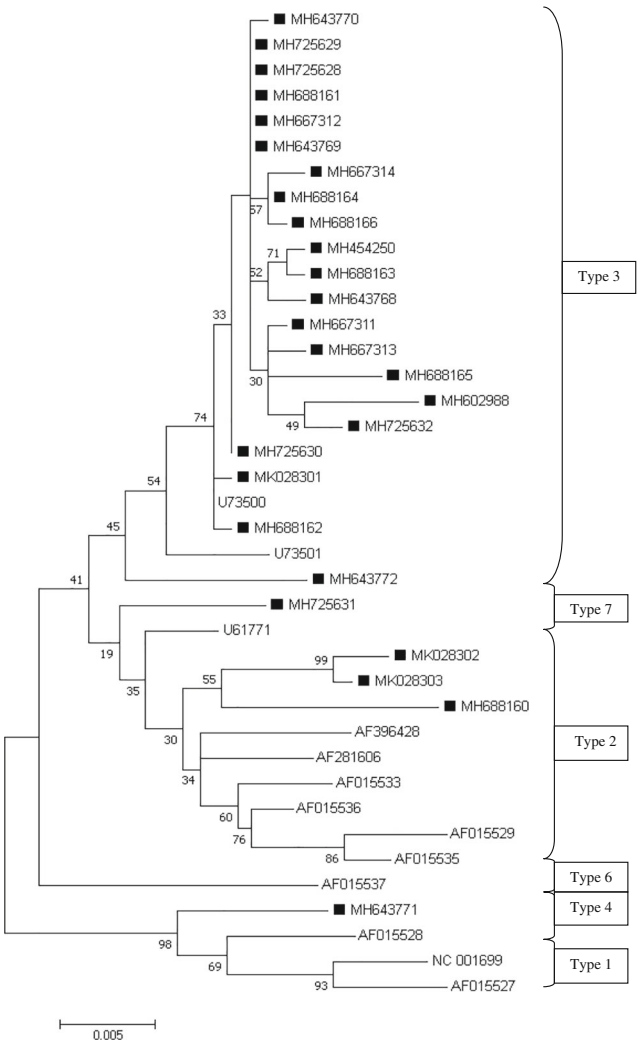

Fig. 1 Maximum likelihood phylogenetic tree of JCPyV VP1 region. Phylogenic analysis based on the VP1 coding sequences. Phylogenetic analysis of the JCPyV VP1 gene (1065 bp) was completed by PCR. The Clustal W method was used to align the sequences. The phylogenetic tree was built according to maximum likelihood under the Tamura Nei-model using MEGA 7. The scale bars demonstrate the frequency of nucleotide substitutions. The precision of the tree was assessed by 1000 bootstrap replicates. The isolations obtained in this study are shown in black squares (■). GenBank accession numbers of all sequences are presented in the tree

Table 4 Genotype variants in the VP1 gene fragment

Nucleotide positions																	
Strain	Type	1654	1753	1771	1786	1804	1843	1850	1869	1870	2311	2432	2435	2455	2482	2502	Accession no.
Mad-1	1A	**A**	**A**	**C**	**G**	**T**	**G**	A	G	G	**G**	A	A	**A**	**C**	**A**	NC001699
123	1B	A	A	C	G	T	*T*	*G*	G	G	*T*	A	A	A	C	*G*	AF015527
224	2A	*T*	A	*A*	*T*	T	*T*	A	G	*A*	*T*	A	A	*G*	C	*G*	AF015529
227	2B	*T*	A	*A*	*T*	T	*T*	*G*	G	*A*	*T*	A	A	*G*	C	*G*	AF015533
229	2C	*T*	A	*A*	*T*	T	*T*	*G*	G	*A*	*T*	A	A	*G*	C	*G*	AF015535
230	2D	*T*	A	*A*	*T*	T	*T*	*G*	G	*A*	*T*	A	A	*G*	C	*G*	AF015536
234	2E	*T*	A	*A*	G	T	*T*	A	G	*A*	*T*	A	A	*G*	C	*G*	AF281606
308	3A	*T*	*T*	*A*	*T*	*C*	*T*	A	*C*	*A*	*T*	A	A	*G*	*T*	*G*	U73500
SA296-02	3B	*T*	*T*	*A*	G	T	*T*	A	*C*	*A*	*T*	A	A	*G*	*T*	*G*	U73501
402	4	A	A	*C*	G	T	G	A	*C*	*A*	*T*	A	A	A	C	*G*	AF015528
601	6	*T*	A	*A*	G	T	*T*	A	G	G	*T*	A	A	*G*	C	*G*	AF015537
Tai-3	7	*T*	A	*A*	G	T	*T*	A	G	*A*	*T*	A	A	*G*	C	*G*	U61771
805-B	8	T	A	G	G	T	T	A	G	*A*	T	A	A	G	C	G	AF396428
11H this study	3A	**A**	**A**	**C**	**G**	**T**	**G**	A	*C*	*A*	**G**	**A**	A	A	**C**	**A**	MH643772

The comparison of isolated Ahvaz11h in the VP1 with other genotypes and subtypes of JCPyV. The different nucleotides with prototype Mad-1 are shown in italics. The similar nucleotides with strain Mad-1 which were identified in this study are shown in bold. The sequences used for comparison were provided by the National Center for Biotechnology (NCBI)

In Iran, there is little information about the prevalence and genotyping of JC polyomavirus and the relationship between the risk of PML and JCPyV infection. Therefore, this study was carried out to reveal the prevalence and genotyping of JCPyV in HIV-infected patients in comparison with healthy individuals in Ahvaz city in southwest of Iran.

Concerning all urine specimens, our findings revealed a total prevalence of JCPyV shedding of 67.1%. Among HIV-infected patients, the prevalence of JCPyV excretion (80.2%) was more than healthy individuals (53.8%) which indicated a significant difference between case and control groups ($P = 0.001$). While some previous studies reported that JCPyV excretion in urine would be not magnified by HIV infection [28, 29], Lednicky et al. showed a difference (not statistically meaningful) in urinary viral excretion between HIV-infected patients and HIV-uninfected volunteers [25]. Likewise, a study in an Irish population conducted by Schaffer et al. and a study carried out by Zanotta et al. in Northern Italy showed a significant difference in JCPyV shedding in urine between immunocompetent individuals and HIV-positive patients [3, 30]. These observations suggest that the impaired immune function system in HIV-infected patients may increase the replication of JCPyV in renal tissue, while receiving HAART treatment does not affect JCPyV excretion.

Regarding the preceding studies, the JC polyomavirus isolation rises with age [4, 6, 22, 31]. The findings of this study indicated the age-related pattern of JCPyV excretion in HIV-infected patients ($P = 0.015$), but such a typical pattern was not observed in healthy group ($P = 0.320$).

JC polyomavirus is ubiquitous in human and with all JCPyV isolates belonging to a unique serotype [32]. Different genotypes of JCPyV are identified worldwide and are linked with specific geographic regions and human migration [7]. In the present research, the most dominant genotype in the two groups was genotype 3A (80.8%), followed by genotypes 2D (11.5%), 4 (3.8%), and 7 (3.8%), respectively. Neither genotype 1 nor 6 was identified in this study. Genotype 3A (Af2) is an African genotypes which is also found in the western and southern parts of Asia such as Turkey, Saudi Arabia, and India as reported by Sugimoto et al. [32, 33]. This research confirms previous findings concerning this issue.

Genotypes 3A obtained in this investigation were very similar to isolate QLD-01 from Australia [19] except for positions 2311 and 2502. Also, some of the isolates obtained in this study were similar to isolate 308 from Tanzania (Table 5). The cycle sequencing results and comparison of sequences retrieved from GenBank, isolate "Ahvaz 11H" showed different nucleotides at some positions (Table 4). These differences revealed a potential for a new subtype. This sequence showed 99% similarity with genotype 3A, but at some positions nucleotides were like prototype Mad-1 and differed from other genotypes 3. Furthermore, the G →A substitution at positions 2432 and 2435 led to amino acid changes: Glutamine to lysine at positions 324 and 325 though changes in these sites are not type specific.

In conclusion, the results of the present study indicated that the JCPyV excretion was significantly higher among HIV-infected patients than in healthy individuals, while the prevalence of viral DNA between males and females was not

Table 5 Comparison of nucleotides at positions 2311 and 2502 in genotypes 3A in this study with strains Mad-1 (NC001699), 308 (U73500), and QLD-01 (HG764413)

Positions		
Strains	2311	2502
Mad-1(1A) (NC001699)	G	A
308 (3A) (U73500)	*T*	*G*
QLD-01(3A) (HG764413)	G	A
MH454250	G	A
MH602988	G	*G*
MH643768	G	A
MH643768	G	A
MH643770	G	A
MH643772	G	A
MH667311	G	A
MH667312	G	A
MH667313	G	A
MH667314	G	A
MH688161	G	A
MH688162	G	*G*
MH688163	G	A
MH688162	*T*	A
MH688165	G	A
MH688166	G	A
MH725628	G	A
MH725629	G	A
MK028301	*T*	A
MH725630	G	*G*
MH725632	G	*G*

Differences/similarities between the isolations obtained in this study with/to isolates Mad-1, 308, and QLD-01. Different nucleotides with isolate QLD-01 are shown in italics. All genotypes of this study presented in Table 5 belong to genotype 3A

different as among the age-related groups. Moreover, our findings revealed that the predominant genotype in southwest of Iran was genotype 3A, followed by genotypes 2D, 4, and 7 whereas neither genotype 1 nor 6 was found.

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Authors' contributions Study concept and design: Manoochehr Makvandi. Acquisition of data: Roya Pirmoradi. Anlysis and interpretation of data: Manoochehr Makvandi and Roya Pirmoradi. Drafting of manuscript: Roya Pirmoradi and Somayeh Biparva Haghighi. Critical revision of the manuscript for important intellectual concept: Manochehr Makvandi. Statistical analysis: Maryam Dastoorpoor and Roya Pirmoradi. Administrative, technical, and material support: Roya Pirmoradi, Mohammad Karimi Babaahmadi, Nastaren Khodadad, and Maryam Tabasi. Supervision: Manoocher Makvandi and Hayat Mombeini.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethnic consent The project was approved with approval number NO: B-97042 and ethical code of IR.AJUMS.REC.1397.446 by ethnic committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

Ethical issues Prior to the sampling procedures, informed consent was obtained from all participants or parents (in case of minor participants).

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