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Detection of the archetypal regulatory region of JC virus from the tonsil tissue of patients with tonsillitis and tonsilar hypertrophy

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The regulatory regions of JC virus (JCV) DNAs in the brain of patients with progressive multifocal leukoencephalopathy (PML) (designated as PML-type regulatory regions) are hypervariable, whereas those in the urine and renal tissue of individuals without PML have the same basic structure, designated as the archetype. It is thought that JCV strains with the archetypal regulatory region circulate in the human population. Nevertheless, Monaco *et al* (J Virol 70: 7004–7012, 1996) reported that PML-type regulatory regions occur in human tonsil tissue. The purpose of this study is to confirm their findings. Using nested polymerase chain reaction (PCR), the authors detected the regulatory region of JCV DNA in the tonsil tissue from 14 (44%) of 32 donors with tonsillitis and tonsilar hypertrophy. Sequencing of the detected regulatory regions indicated that they were identical with the archetypal regulatory regions detected previously or, in a few cases, slightly deviated from the archetype. This finding suggests not only that tonsil tissue is the potential site of initial JCV infection but also that archetypal JCV strains circulate in the human population.
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Introduction

Human polyomavirus JC (JCV) is the causative agent of the demyelinating disease in the central nervous system known as progressive multifocal leukoencephalopathy (PML) (Walker, 1985). This virus, however, is ubiquitous in humans. The primary infection with JCV asymptomatically occurs during childhood (Padgett and Walker, 1973). JCV is then disseminated throughout the body, probably through viremia (Ikegaya *et al*, 2004). It is well established that JCV persists in renal tissue (Chesters *et al*, 1983; Tominaga *et al*, 1993; Kitamura *et al*, 1997; Aoki *et al*, 1999). It is likely that JCV also persists in other sites, including lymphoid tissues and peripheral blood lymphocytes (PBLs) (Gallia *et al*, 1997). Nevertheless, there is debate about the occurrence of JCV in the brain (Gallia *et al*, 1997) and colon (Laghi *et al*, 1999; Hernandez Losa *et al*, 2003).

The genome of JCV has a noncoding regulatory region (abbreviated as RR) between the origin of replication and the start site of the agnogene (Frisque *et al*, 1984). JCV RRs (PML-type RRs) in the brain of PML patients are hypervariable (Martin *et al*, 1985). In contrast, JCV RRs detected in the urine and renal tissue of immunocompetent individuals have the same basic structure designated as the archetype (Yogo *et al*, 1990). The wide geographical distribution of JCV strains with the archetypal RR suggested that they circulate in the human population (Yogo *et al*, 1991; Flœgstad *et al*, 1991; Markowitz *et al*, 1991; Agostini

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et al, 1996, 1997a, 2001; Guo *et al*, 1996; Pagani *et al*, 2003; Jeong *et al*, 2004). From a structural comparison between PML-type and archetypal RRs, it was concluded that various PML-type RRs were generated from the archetype by deletion and duplication or by deletion alone (Yogo *et al*, 1990; Ault and Stoner, 1993; Agostini *et al*, 1997b). Furthermore, phylogenetic comparison between JCV strains with archetypal RRs and those with PML-type RRs indicated that the shift of the JCV RR from archetype to PML-type occurs during persistence in the hosts (Iida *et al*, 1993; Kato *et al*, 1994). These findings were recently formulated as the archetype concept (Yogo and Sugimoto, 2001).

Indeed, the archetype concept explains well the changes in the JCV RR from a molecular-epidemiological standpoint. However, this concept does not address a medically important issue, i.e., whether these changes are involved in the pathogenesis of PML. A few studies challenged this issue using *in vitro* expression assays (Sock *et al*, 1996; Ault, 1997). According to the results of these studies, there is little doubt that JCV with the archetype RR can propagate in human brain. Thus, the question remains open as to why JCV DNAs in the brains of PML patients regularly underwent sequence rearrangement in their RRs.

Although a significant number of studies have been conducted in relation to the archetype concept, Monaco *et al* (1998) reported that they detected PML-type RRs (mainly of strain Mad-1) in the tonsil tissue of immunocompetent individuals. (They also detected the archetypal RR, but they considered that it was derived from PBLs.) Based on their findings, they suggested that archetypal strains represent variants selected for the adaptation to specific cell types (Monaco *et al*, 1998). As this suggestion sharply contrasts with the archetype concept, we decided to reexamine whether JCV DNA can be detected in tonsil tissue of immunocompetent patients and, if so, to clarify the structures of the detected JCV RRs.

Results

A nested polymerase chain reaction (n-PCR) was previously developed for the detection of the JCV RR from the cerebrospinal fluid of PML patients (Sugimoto *et al*, 1998). Using this n-PCR, the JCV RR could be detected at least at 25 genome equivalents from JCV isolates belonging to 6 major genotypes of JCV (CY, MY, SC, B1-c, EU-a, and Af2) (Sugimoto *et al*, 1998). We repeated the n-PCR 10 times using various amounts of standard JCV (MY) DNA as the template. The JCV RR was amplified in 8 of the 10 trials at 25 genome equivalents of the JCV DNA, whereas the JCV RR was amplified in 1 of the 10 trials at 2.5 genome equivalents. These results indicated that if the n-PCR is repeated 10 times, the JCV RR can be detected even at 2 to 3 genome equivalents.

Table 1 Detection of the JCV RR in tonsil from various donors

Donors	*Gender/age in years*	*Tonsil*	*Detection rates*	*Detected RRs*
1	M/24	L	2/10	RR-1
		R	1/10	RR-1
2	M/47	L	1/10	RR-7
5	M/21	L	4/10	RR-1
		R	8/10	RR-1
7	M/38	R	1/10	RR-1
10	M/49	R	3/10	RR-1, RR-4
12	F/32	L	3/10	RR-1, RR-4
13	M/34	L	7/10	RR-1
14	F/23	R	1/10	RR-5
19	M/50	L	1/10	RR-1
23	F/61	L	1/10	RR-1
		R	0/10	—
25	F/42	L	0/10	—
		R	2/10	RR-2
30	M/23	L	1/10	RR-3
		R	1/10	RR-3
31	F/46	L	1/10	RR-3
		R	0/10	—
32	F/25	L	1/10	RR-6
		R	0/10	—

Note. Only donors from whom the JCV RR was detected are shown. Sequences of RRs are presented in Figure 1. Detection rates are expressed as number of positives/number of n-PCR trials. L, left tonsil; R, right tonsil.

Thus, we repeated the n-PCR 10 times for each tonsil tissue derived from 28 individuals. Aliquots of the PCR mixtures were electrophoresed on 1% agarose gels stained with ethidium bromide, and photographed under a ultraviolet (UV) light (data not shown). We cloned and sequenced the amplified fragments to confirm that they contained JCV RRs. The results of the detection are shown in Table 1 in detail, and can be summarized as follows. The JCV RR was detected in 14 of the 32 donors (44%). JCV-positive donors were all adults. The JCV RR was detected in 8 (38%) of the 21 males and in 6 (55%) of the 11 females. The JCV RR was detected in 7 (41%) of the 17 donors for whom both tonsils were examined, and in 7 (47%) of the 15 donors for whom a single tonsil was examined. The detection rate for the JCV RR (number of positives/number of n-PCR trials) was 0/10 in 65% of tonsils, 1/10 in 20% of tonsils, and 2/10 to 8/10 in 14% of tonsils.

We detected seven different RR sequences (RR-1 to -7) (Figure 1). RR-1, -2, -3, -4, and -6 were identical with the regulatory sequences of urine-derived isolates CY, N1, UA, MY, and MO-2, respectively (Yogo *et al*, 1990, 1991; Guo *et al*, 1996). RR-5 and -7 were not found previously. In reference to RR-1 (identical with the CY archetype) (Yogo *et al*, 1990), RR-2 through -4 had one or a few nucleotide mismatches, whereas RR-5 through -7 had single nucleotide mismatches and short deletions involving two- or five-nucleotide stretches. The deletions in RR-5 through -7 were identical with those in some urine-derived JCV isolates described previously (Guo *et al*, 1996).

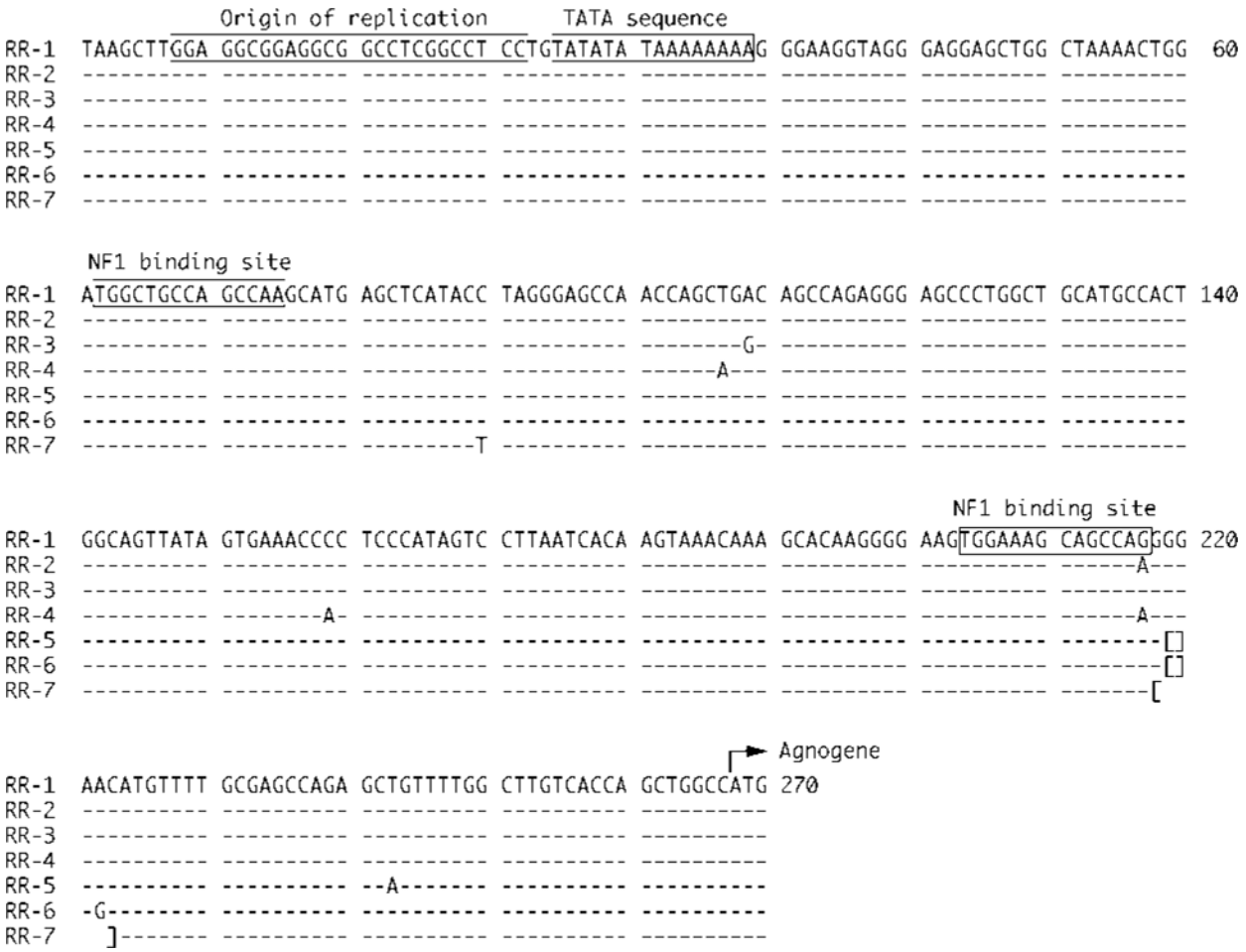

Figure 1 JCV RR sequences detected in tonsil tissue. The nucleotide sequence of RR-1, spanning from the origin of replication to the start site of the agnogene, is shown at the top. The RR-1 sequence is identical with the CY archetype (Yogo *et al*, 1990). The origin of replication, TATA sequence, NF-1 binding motifs, and the start site of agnogene are indicated. Below this sequence, the other sequences are shown. Dashes denote nucleotides identical to those in RR-1. Brackets denote deletions relative to RR-1.

Frequencies of various RRs are shown in Table 2. Thus, it can be concluded that JCV RRs in tonsil tissue are the archetype or slightly deviated from it.

We examined whether the JCV RR was detectable in PBL samples derived from 15 immunocompetent patients, as there has been some debate about the presence of the JCV DNA in PBLs of immunocompetent individuals (Dolei *et al*, 2000; Dörries *et al*, 2003). The n-PCR described above was repeated 10 times for each DNA sample extracted from PBLs of 15 patients without obvious immunodeficiency. (It should be noted that the high sensitivity of our n-PCR [see above] did not require further analysis of PCR products using the Southern blot hybridization to enhance detection sensitivity.) No sample gave positive amplification at all. Our result suggests not only that JCV DNA rarely occurs in the PBLs of immunocompetent individuals, but also that false positives due to contamination are rare with our n-PCR.

Table 2 Frequency of various JCV RRs

Regulatory region	*No. of donors*	*No. of tonsils*
RR-1 (CY)	7	10
RR-2 (N1)	1	1
RR-3 (UA)	2	3
RR-4 (MY)	2	2
RR-5	1	1
RR-6 (MO-2)	1	1
RR-7	1	1
Total	15	19

Note. Frequency of JCV RRs was estimated from Table 1. Sequences of RRs are presented in Figure 1. Urine-derived isolates with the same RRs are indicated within parentheses (Yogo *et al*, 1990, 1991; Guo *et al*, 1996).

Discussion

Before discussing the implications of our findings, we examine the possibility that we detected false positives due to contamination. First, we made every

effort to avoid false positives in conducting the n-PCR (see Materials and methods). Second, as a control experiment, we attempted to detect the JCV RR in PBL samples derived from 15 immunocompetent patients. No PBL sample gave a positive amplification, and we are thus confident that false positives were rare using our n-PCR.

The donors from whom JCV RRs were detected in the tonsil were all adults. As primary JCV infection usually occurs during childhood (Padgett and Walker, 1973), the detection of the JCV RR in tonsil tissue does not necessarily suggest that primary infection with JCV was ongoing in the tonsil tissue examined. It seems more reasonable to assume that the tonsil tissue is persistently infected with JCV. Nevertheless, the detection of the JCV RR in tonsil tissue suggests that a fraction of the cells constituting the tonsil tissue can support JCV replication. Indeed, it was reported that JCV can productively grow in tonsilar cells (Monaco *et al*, 1996). Furthermore, Eash *et al* (2004) recently reported that the receptor-type sialic acid is highly expressed on B lymphocytes in normal human tonsil. Altogether, it appears that tonsil tissue is the potential site of initial JCV infection.

Monaco *et al* (1998) reported the occurrence of JCV DNA in human tonsil tissue (although the conditions of the tonsil donors were not specified, it is reasonable to assume that they were patients with tonsillitis or tonsi11ar hypertrophy). Most of the JCV RRs detected in dissected and nondissected tonsil tissue were identical with the rearranged RRs of three PML-type strains (Mad-1, Mad-4, and Mad-8), with Mad-1 mainly detected. (Although the archetypal RR, together with Mad-1 and Mad-4 RRs, were detected in tonsillar lymphocytes, it was thought that this archetypal RR was derived from PBLs.) In the present study, however, we found that JCV DNA with the archetype RR occur in tonsil tissue. Although we detected the archetype RR from nondissected tonsil tissue, the possibility that we detected it from PBLs possibly included in the tonsil specimens can be excluded, as JCV DNA was undetectable in PBLs derived from immunocompetent patients. However, it remains to be elucidated which component of tonsil tissue carry JCV DNA.

Inasmuch as JCV DNAs with the archetype RR had been detected only in the urine and kidney tissue, Monaco *et al* (1998) raised the possibility that "the archetype is a variant strain that cells in different organs can select in order to survive after primary infection." In the present study, however, we demonstrated that JCV with the archetype RR, rather than PML-type RRs, persist in the tonsil tissue of patients with tonsillitis or tonsillar hypertrophy. Thus, the present study excluded the possibility noted above that JCVs with the archetype RR are tissue-adapted variants, and provided further support for the archetype concept proposed recently (Yogo and Sugimoto, 2001).

Materials and methods

Tonsils and PBLs

Tonsils surgically excised because of tonsillitis or tonsillar hypertrophy were used. The tonsil donors included 2 children aged 4 and 7 years and 26 adults aged 21 to 47 years (average, 28 years). The donors (or the parents if the donors were minors) gave their informed consent regarding their inclusion in this study. Both tonsils were obtained from 17 donors, and single tonsils were obtained from 15 donors. PBLs were obtained from 15 general patients without immunosuppression aged from 42 to 87 years (average, 68 years). This study was approved by the Human Subjects Committee, Faculty of Medicine, The University of Tokyo.

Extraction of DNA

Tonsil tissue was digested with 100 μg/ml of proteinase K at 56°C for 1 h in the presence of 0.5% sodium dodecyl sulfate (SDS). The digest was extracted once with phenol and once with chloroform/isoamyl alcohol (24/1), and DNA was recovered by ethanol precipitation and dissolved in water. PBL DNA was extracted from buffy coat preparations using the GENOMIX kit (Talent sr1, Trieste, Italy). The entire procedures for the DNA extraction from tonsil tissue and PBLs were carried out in laboratories never exposed to a high level of JCV DNA.

n-PCR

n-PCR was conducted as described previously with some modifications (Sugimoto *et al*, 1998). A1 and A3 were used as outer primers, and B1 and B3 were used as inner primers. A1, B1, and B3 were described previously (Sugimoto *et al*, 1998). A3, a version of A2 (Sugimoto *et al*, 1998), was 5′ CATTACTTACCTATGTAGCTTTTGGTTCAGGC 3′, nucleotides (nt) 502 to 471 in the JCV (Mad-1) genome (Frisque *et al*, 1994). The total reaction volume of 50 μl in the first round contained 1 to 2 μg of sample DNA, 125 units of HotStar Taq DNA polymerase (QIAGEN GmbH, Hilden, Germany), 200 μM of each dNTP, 1.5 mM $MgCl_2$, 0.5 μM primers (A1 and A3), and a PCR Buffer supplied by the manufacturer. A 2.5 μl volume of the first amplification product was transferred into the second PCR mixture (50 μl) that contained 125 units of HotStar Taq DNA polymerase, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 0.5 μM primers (B1 and B3), and the PCR Buffer. After activation at 95°C for 15 min, the first round amplification was performed for 40 cycles, and after activation under the same condition, the second round amplification was performed for 30 cycles. The cycle profile was 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. Both activation and amplification were carried out in a Thermal Sequencer (Asahi Techno Glass Corporation, Tokyo, Japan).

In preparing template DNA as well as in performing PCR amplifications, we took all precautions to avoid

contamination (Kwok and Higuchi, 1989). We used three isolated rooms, one for the first-round PCR, one for the second round PCR, and one for the analysis of PCR products.

Cloning and sequencing

The amplified fragments were digested with a combination of *Hin*dIII and *Pst* I, which excises a fragment containing the JCV RR (Sugimoto *et al*, 1998). The digested DNA was ligated to *Hin*dIII- and *Pst*I-digested, alkaline phosphatase–treated pBluescript II SK (+) (Stratagene, La Jolla, USA), and was used to transform *Escherichia coli* HB101 competent cells (Takara Shuzo, Kyoto, Japan). Recombinant clones containing the JCV RR were selected by digestion with a combination of *Hin*dIII, *Pst*I, and *Sst*I (*Sac*I) followed by agarose gel electrophoresis (*Sst*I cleaves the archetype RR at a single site [Sugimoto *et al*, 1998]). For each PCR product, three recombinant clones carrying the JCV RR were purified using a Quiaprep 8 Turbo Miniprep kit (QIAGEN), and purified recombinant clones were sequenced using the T3 and T7 primers and an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, USA). The three sequences were usually identical. However, one sequence sometimes differed from the others by a single nucleotide mismatch (probably due to errors during PCR); in such cases we adopted the latter sequence as a consensus.

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