

coding sequences were identified, distinguished in each case by the presence or absence of one or two nucleotide substitutions and a short duplication (only in case 3). It is unlikely that variations were introduced during molecular cloning because of the high fidelity of DNA synthesis in bacterial cells. To clarify the relationships among the detected coding sequences, two analyses were performed (see above). From these results, it is concluded that 1-1, 2-1 and 3-1 were the parental sequences in cases 1, 2 and 3, respectively, from which the other coding sequences (e.g. variant coding sequences) were generated. A comparison between the parental and variant coding sequences in each PML case enabled us to detect VP1 loop mutations. Furthermore, 16 reported VP1 sequences from PML-type isolates were compared with their genotypic prototypes, generated as consensus sequences of representative archetypal isolates belonging to the same genotypes. It was found that 13 VP1 proteins underwent amino acid changes in the surface loops. From these findings, it can be concluded that PML-type JCPyV frequently undergoes amino acid substitutions in the VP1 loops.

The frequency of JCPyV DNA clones with VP1 loop mutations varied significantly among the three cases. Thus, 25, 14 and 86% of the analysed clones in cases 1, 2 and 3 showed the VP1 loop mutations. In cases 1 and 2, the patients died within 6 months of the onset of symptoms, whereas the patient in case 3 survived for about 2 years. The observations noted above suggest that the ratio of VP1 loop mutations increases with time as virus propagation continues in the central nervous system.

Although amino acid changes in the VP1 loop occurred frequently in JCPyV isolates derived from brain tissue and CSF of PML patients, they occurred rarely in JCPyV isolates derived from the urine of both healthy individuals and immunosuppressed patients. These findings suggest that the VP1 loop mutations are somehow associated with the development of PML.

By analogy with the crystallized VP1 structure of a related polyomavirus, i.e. SV-40 (Liddington *et al.*, 1991), the VP1 loops of JCPyV are considered to be involved in functions such as interactions with cell receptors and antigenic responses. Therefore, there are two possible explanations for the frequent occurrence of VP1 loop mutants in PML-type JCPyV isolates. First, VP1 loop mutants have a higher affinity for receptors on the cell surface, thereby being able to grow more efficiently. Second, VP1 loop mutants are escape mutants that are not neutralized by the antibodies against JCPyV with non-mutated VP1 loops.

Gee *et al.* (2004) reported that arginine-56 and arginine-75 on the BC loop and arginine-273 on the HI loop are the potential sialic acid-binding sites for JCPyV infection. None of these amino acids was found to be altered in the PML-type JCPyV shown in this study (Tables 5 and 6). This would suggest that these PML-type JCPyV underwent no change in their ability to bind to cellular receptors, if the

indirect effect of the VP1 loop mutations detected in this study could be excluded.

In summary, it was found that VP1 loop mutations occur frequently in JCPyV isolates from the brain and CSF of PML patients and that these mutations occur rarely in isolates from the urine of patients, regardless of their immunological state. These polymorphisms should serve as a new marker for the identification of JCPyV isolates associated with PML. The biological significance of these mutations, however, remains unclear.

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Characterization of the VP1 loop mutations widespread among *JC polyomavirus* isolates associated with progressive multifocal leukoencephalopathy

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Abstract

Recently, we found that *JC polyomavirus* (JCPyV) associated with progressive multifocal leukoencephalopathy (PML) frequently undergoes amino acid substitutions (designated VP1 loop mutations) in the outer loops of the major capsid protein, VP1. To further characterize the mutations, we analyzed the VP1 region of the JCPyV genome in brain-tissue or cerebrospinal fluid samples from 20 PML patients. VP1 loop mutations occurred far more frequently than silent mutations. Polymorphic residues were essentially restricted to three positions (55, 60, and 66) within the BC loop, one (123) within the DE loop, and three (265, 267, and 269) within the HI loop. The mutations at most polymorphic residues showed a trend toward a change to specific amino acids. Finally, we presented evidence that the VP1 loop mutations were associated with the progression of PML. These findings should form the basis for elucidating the biological significance of the VP1 loop mutations.

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Keywords: *JC polyomavirus*; Progressive multifocal leukoencephalopathy; Capsid protein; VP1; Outer loop; Amino acid substitution

JC polyomavirus (JCPyV) is ubiquitous in human populations, infecting children asymptotically [1]. This virus, however, causes a demyelinating disease in the central nervous system, progressive multifocal leukoencephalopathy (PML), among individuals with reduced immune competence [2]. PML was once a rare disease, but is now a common opportunistic infection in patients with acquired immunodeficiency syndrome (AIDS) [3].

The JCPyV genome, 5100 bp in length, encodes six viral proteins [two early regulatory proteins (large and small T), a late regulatory protein (agnoprotein), and three capsid proteins (VP1-3)] [4]. A non-coding control region (CR) spanning from the origin of replication to the start site of the agnoprotein gene is well known for its variability. CRs of JCPyV isolates derived from the urine, kidney, and tonsil of immunocompetent individuals have the same basic structure designated the archetype. In contrast, those of JCPyV isolates from the brain and cerebrospinal fluid (CSF) of PML patients have divergent structures generated from the archetype

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by sequence rearrangements (deletion and amplification, or deletion alone) [5]. It is considered that JCPyVs with archetypal CRs represent those circulating in the human population [5].

While it remains to be elucidated why JCPyV DNAs in the brains of PML patients regularly undergo sequence rearrangements in their CRs, new genetic changes, named VP1 loop mutations, were recently detected in PML-type JCPyV isolates [6]. These mutations were first detected in some JCPyV DNA clones obtained from brain biopsic tissues of three PML patients, and then in various PML-type JCPyV isolates whose VP1 sequences were available in the DNA database. In contrast, archetypal JCPyV isolates derived from the urine of immunosuppressed as well as immunocompetent patients did not carry the VP1 loop mutations [6].

The VP1 loop mutations are characterized by the variations of affected amino acid residues [6]. According to previously reported data [6], eight polymorphic amino acid residues were detected in the VP1 loops, four within the BC loop, one within the DE loop, and three within the HI loop. However, the frequencies of mutations at individual residues varied greatly, with a single mutation at four residues, two or three mutations at three residues, and eight mutations at one residue. Therefore, the question arose as to whether all of these mutations are biologically important. Furthermore, it remained to be clarified if there is a trend toward a change to specific amino acids.

In this study, we analyzed VP1 sequences of many PML-type JCPyV isolates derived from not only brain tissue but also CSF samples previously used for the diagnosis of PML. Furthermore, to test the previously proposed hypothesis that the ratio of VP1 loop mutations increases during the period when viral propagation continues in the brain, we analyzed VP1 loop sequences in various parts of the brain autopsied from two long-surviving PML patients, one with a full progression of PML, the other with a stabilization of PML.

Materials and methods

Brain-tissue and CSF DNA. Brain-tissue samples (biopsies and autopsies) and CSF samples of patients with suspected PML had been sent to our laboratory from various hospitals in Japan. DNA was extracted from these samples and assayed for the presence of JCPyV DNA using the polymerase chain reaction (PCR). Of these samples, those from which JCPyV DNA was detected were used in this study. They included three brain biopsic, seven brain autopsic, and 11 CSF samples. The underlying diseases of the patients are shown in Table 1. DNA extracted from the samples had been stored at -20°C prior to use.

Patients 1 and 2. DNA, previously extracted from various parts of the brain obtained at autopsy from two PML patients (1 and 2), was examined in this study for the presence of VP1 loop mutations. Case reports for these patients were described elsewhere [7–9]. In brief, patient 1 was admitted to hospital in January 1994 with a slowly progressing dementia. The underlying generalized disease in this pa-

Table 1

Underlying diseases of the PML patients whose brain-tissue or CSF samples were analyzed in this study

Underlying disease	No. of patients	
	Brain tissue	CSF
Acquired immunodeficiency syndrome	1	5
Autoimmune diseases	1	2
Chronic renal failure	1	1
Adult T-cell leukemia	1	1
Chronic myeloid leukemia	1	0
Wiskott–Aldrich syndrome	1	0
Primary aldosteronism	0	1
Polycythemia vera	0	1
Unknown	3	0
Total	9	11

tient was diagnosed as Wiskott–Aldrich syndrome. In July 1994, a diagnosis of PML was made on the basis of brain biopsy findings, including the detection of JCV DNA by PCR and in situ hybridization. Multiple diffuse spotty lesions were found initially in the frontal lobe, but then in other parts of the cerebrum, the cerebellar white matter, and the brain stem. The patient survived in a vegetative state for 4 years after the diagnosis was made. He eventually died from central cardiorespiratory failure in October 1998. Necropsy findings showed severe brain atrophy and diffuse demyelination of the white matter of the cerebrum, cerebellum, and spinal cord.

Patient 2 was a 33-year-old male infected with human immunodeficiency virus (HIV) (his CD4 cell count was $1/\mu\text{l}$ and viral load was 2.9×10^5 copies/ml). He was hospitalized because of cytomegalovirus-associated meningitis in February 1998. Highly active antiretroviral therapy (HAART) was commenced in March 1998. About 2 months later, he developed dementia and right homonymous hemianopia. Brain MRI revealed T2 hyperintense lesions in the right occipital and frontal subcortical areas. A nested PCR for JCV DNA demonstrated the presence of a rearranged CR in the CSF, confirming the diagnosis of PML based on neurological symptoms and brain MRI. He developed right hemiplegia in June 1998, but survived for about 2 years without further changes to his neurological symptoms. He died from an overall worsening of his condition in February 2000. Autopsy findings showed severe cerebral softening, involving the left temporal lobe, parietal lobe, and bilateral occipital lobes. Demyelinated lesions were found in these regions, with rare oligodendroglia containing enlarged nuclei.

Standard and semi-nested PCR. Standard and semi-nested PCR were used to amplify a VP1 region of the JCPyV DNA from brain tissue and CSF, respectively (see Fig. 1). The target of amplification was a 737-bp region of the VP1 gene encompassing five VP1 loops, BC, DE, EF, GH, and HI loops. The primers used in the standard and semi-nested PCR are shown in Table 2. Standard PCR and the first round of the semi-nested PCR were performed using primers 1 and 4a, while the second round of the semi-nested PCR was performed using primers 1 and 2a or 1.5 and 4a. The standard PCR and the first round of the semi-nested PCR generated a 797-bp fragment (note that this fragment contained primer sequences and therefore was slightly longer than the target region). The second round of the semi-nested PCR generated two fragments, 1–2a (481 bp) and 1.5–4a (490 bp). The total reaction volume of $50 \mu\text{l}$ in the standard PCR and in each round of the semi-nested PCR contained 125 units of HotStar Taq DNA polymerase (Qiagen GmbH, Hilden, Germany), $200 \mu\text{M}$ of each dNTP, 1.5 mM MgCl_2 , $0.5 \mu\text{M}$ primers, and a PCR buffer supplied by the manufacturer. A $2.5\text{-}\mu\text{l}$ volume of sample DNA or the first amplification product was added to the PCR mixture ($50 \mu\text{l}$). After activation at 95°C for 15 min, the standard PCR amplification was performed for 50 cycles, and the first and second rounds of the

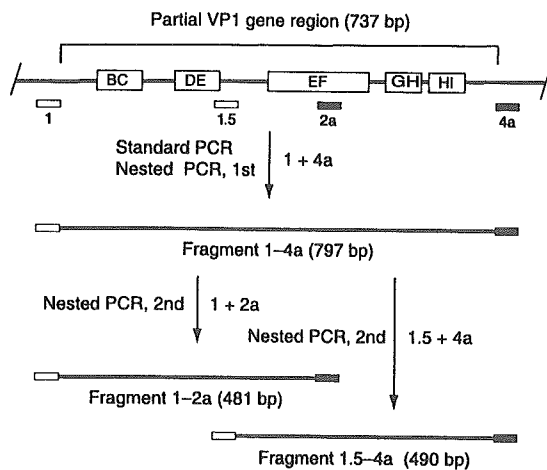


Fig. 1. Standard and semi-nested PCR used in the present study. A 737-bp VP1 gene region encoding outer loops (BC, DE, EF, GH, and HI) together with neighboring regions are schematically shown at the top. Below the VP1 region are shown the sites of primers 1, 1.5, 2a, and 4a. The 737-bp region was amplified by the standard PCR or by the first round of the semi-nested PCR using primers 1 and 4a. From the fragment generated (fragment 1–4a, 797 bp), two fragments, 1–2a (481 bp) and 1.5–4a (490 bp), were amplified by the second-round semi-nested PCR using primers 1 and 2a and 1.5 and 4a, respectively.

semi-nested PCR were performed for 40 and 30 cycles, respectively. Both activation and amplification were carried out in a Thermal Sequencer (Asahi Techno Glass, Tokyo, Japan). Standard and semi-nested PCR were performed in duplicate. In preparing template DNA as well as in performing PCR amplifications, we took all precautions to avoid contamination. We used three isolated rooms, one for the first round of the semi-nested PCRs, one for the standard PCR and the second round of the semi-nested PCR, and one for the analysis of PCR products.

Cloning and sequencing of amplified fragments. The amplified fragments were digested with a combination of *Hind*III and *Pst*I, which excised fragment 1–4a, 1–2a, and 1.5–4a (see Fig. 1) containing partial VP1 gene sequences. The digested DNA was ligated to *Hind*III- and *Pst*I-digested, alkaline phosphatase-treated pBluescript II SK (+) (Stratagene, La Jolla, USA), and was used to transform competent *Escherichia coli* HB101 cells (Takara Shuzo, Kyoto, Japan). Recombinant clones containing partial VP1 gene sequences were selected by digestion with a combination of *Hind*III and *Pst*I followed by agarose gel electrophoresis. For each PCR product, two recombinant clones carrying partial VP1 gene sequences were purified using a Quiaprep 8 Turbo Miniprep kit (Qiagen), and sequenced using the T3 and T7 primers and an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, USA).

Results

Detection of nucleotide substitutions originally occurring in tissue or CSF specimens

Using standard PCR, we amplified a 737-bp region of the VP1 gene that encompassed five VP1 loops, including BC, DE, EF, GH, and HI loops (see Fig. 1), from bioptic or autoptic brain-tissue specimens derived from PML patients. With semi-nested PCR (Fig. 1), which has a higher sensitivity, we amplified the same VP1 gene region from CSF samples from PML patients. Each PCR was performed in duplicate. The amplified fragments were cloned, and two representative recombinant clones for each amplification were sequenced. The nucleotide sequences obtained were compared to the consensus sequence of the VP regions as obtained by aligning various archetypal isolates belonging to the same genotype of JCPyV (i.e., either of the two genotypes, CY and MY-b, prevalent in Japan [10–12]). Although various nucleotide substitutions were detected in reference to the archetypal isolates, we regarded, for each DNA sample, nucleotide substitutions detected in all or most sequences from both PCR products as having originally occurred in the tissue or CSF specimens (those detected in rare sequences probably were generated during PCR). (VP1 region sequences corrected for PCR errors are shown in Table 3.)

Synonymous versus non-synonymous substitutions in the VP1 loop region

Nucleotide substitutions detected in the VP1 region of JCPyV isolates from the brain or CSF of individual PML patients are shown in Table 4. We detected two kinds of nucleotide substitutions, i.e., synonymous and non-synonymous (the former do not cause amino acid substitutions, but the latter do). The synonymous substitutions were detected in the brain or CSF of only five of the 20 patients analyzed and in only five of the 22 nucleotide substitutions detected. The non-synonymous substitutions, on the other hand, were detected in the brain or CSF of PML patients very frequently (in 17 of the 20 patients and in 17 of the 22 nucleotide substi-

Table 2
Primers used for the standard and semi-nested PCR

Primer	Positions	Sequence (5' → 3')	Restriction site
1	nt 1569–1592	<u>GCAAGCTT</u> GACTCAATTACAGAGGTAGAAT	<i>Hind</i> III
2a	nt 2037–2014	GCTGCAGGTACGCCTTGTGCTCTGTGTTTC	<i>Pst</i> I
1.5	nt 1877–1898	<u>GGAAGCTT</u> GTGCAGGGCACCAGCTTTCAT	<i>Hind</i> III
4a	nt 2353–2330	GCTGCAGAAATTGGGTAGGGGTTTTTAAC	<i>Pst</i> I

Nucleotide (nt) numbers are those of Mad-1 [4]. Sequences added to create restriction sites are underlined. Restriction sites added for cloning are indicated.

Table 3
VP1 region sequences (737 bp) detected in the brain tissue or CSF of PML patients

Patient	Sequence	Genotype	GenBank/EMBL/DDBJ Accession No.
1	JVL-1a	CY	AB214912
	JVL-1b	CY	AB214913
	JVL-1c	CY	AB214914
	JVL-1d	CY	AB214915
2	JVL-2	MY-b	AB214916
3	JVL-3	CY	AB214917
4	JVL-4	CY	AB214918
5	JVL-5	MY-b	AB214919
7	JVL-7	MY-b	AB214920
8	JVL-8	CY	AB214921
9	JVL-9	CY	AB214922
10	JVL-10	MY-b	AB214923
11	JVL-11	CY	AB214924
12	JVL-12	MY-b	AB214925
13	JVL-13	MY-b	AB214926
16	JVL-16	CY	AB214927
17	JVL-17	MY-b	AB214928
18	JVL-18	CY	AB214929
19	JVL-19	CY	AB214930
20	JVL-20	CY	AB214931

Only sequences with nucleotide substitutions are shown.

tutions). All of the non-synonymous substitutions occurred in VP1 loops and were considered to represent the VP1 loop mutations [6].

We statistically analyzed the trend towards a more frequent occurrence of non-synonymous mutations in

the VP1 region of PML-type isolates. As nucleotide substitutions were rare in the VP1 region of archetypal isolates from the urine of individuals without PML [6], we used, as the control group, the genetic data previously obtained when we analyzed complete JCPyV DNA sequences detected in the urine of healthy individuals [12]. In this control group [12], non-synonymous mutations accounted for three of the 11 substitutions detected, while synonymous mutations accounted for eight. In the VP1 region of PML-type isolates (the test group), non-synonymous substitutions accounted for 17 of the 22 substitutions detected, while synonymous substitutions accounted for five (this study). Using the Fisher exact test, we examined the difference between the test and control groups and confirmed that the difference was highly significant ($P = 0.0023$).

The frequencies of VP1 loop mutation in the brain or CSF of PML patients

VP1 loop mutations were detected in the brain in seven of the nine PML patients examined in this study, while they were previously detected in the brain in 11 of 14 PML patients [6]. Similarly, VP1 loop mutations were detected in CSF samples in eight of the 11 PML patients in this study, while they were previously detected in CSF samples in five of five PML patients [6]. Combining data obtained in both studies, VP1 loop mutations were detected in the brain in 18 (78%) of 23 PML

Table 4
Nucleotide substitutions in the VP1 region of JCPyV isolates from the brain or CSF of PML patients

Patient	Source	Position	Nucleotide substitution	Amino acid substitution	Affected regions of VP1
1	Brain	nt 2218	C → T	None	GH
1	Brain	nt 1646	A → G	K → E	BC
1	Brain	nt 1664	G → A	D → N	BC
1	Brain	nt 1664	G → C	D → H	BC
2	Brain	nt 2268	C → T	S → F	HI
3	Brain	nt 1647	A → T	K → M	BC
4	Brain	nt 1664	G → C	D → H	BC
5	Brain	nt 2274	C → T	S → F	HI
7	Brain	nt 2268	C → T	S → F	HI
8	Brain	nt 2305	T → C	None	β-I
9	Brain	nt 2274	C → T	S → F	HI
10	CSF	nt 1858	C → T	None	DE
10	CSF	nt 2261	A → G	N → D	HI
11	CSF	nt 1631	C → T	L → F	BC
12	CSF	nt 2261	A → G	N → D	HI
13	CSF	nt 1836	C → G	S → C	DE
16	CSF	nt 2274	C → T	S → F	HI
17	CSF	nt 2248	T → C	None	β-H
17	CSF	nt 1836	C → G	S → C	DE
18	CSF	nt 1631	C → T	L → F	BC
19	CSF	nt 1798	T → A	None	β-D
20	CSF	nt 2261	A → G	N → D	HI

Nucleotide substitutions were not detected in the brain of patient 6 or in the CSF of patients 14 and 15. Multiple regions of the brain were analyzed in patients 1 and 2 (see text), while a single region of the brain or single CSF sample was analyzed in the other patients. Nucleotide (nt) numbers are those of Mad-1 [4]. BC, DE, and HI, outer loops; β-D, β-H, and β-I, β-sheets [14].

patients, and in the CSF in 13 (81%) of 16 PML patients. The difference in the rate of detection between the brain and CSF was not significant ($P = 0.47$, the Fisher exact test). Herein, the findings for brain and CSF will be described in combination.

Amino acid residues susceptible to VP1 loop mutations

Table 5 shows the frequencies of VP1 loop mutations at various VP1 amino acid residues detected in this and a previous study [6]. Amino acid substitutions were previously detected at four, one, and three residues within the BC, DE, and HI loops, respectively [6]. In this study, amino acid substitutions were detected at most of these residues, with the single exception of residue 61 (an amino acid substitution at this residue was detected only once in the previous study [6]). There was no additional residue at which an amino acid substitution was detected only in this study. Combining the data from this and the previous study [6], the frequency of amino acid substitutions of most polymorphic residues, excluding residue 61 (see above), ranged from 3 to 10. Residue 269 was highly polymorphic with 10 amino acid substitutions. Thus, we concluded that polymorphic residues are essentially restricted to three positions (55, 60, and

66) within the BC loop, one (123) within the DE loop, and three (265, 267, and 269) within the HI loop.

Amino acid specificity of VP1 loop mutation

A single nucleotide substitution within a codon potentially causes various amino acid substitutions, depending on not only the position where the nucleotide substitution occurs but also the nucleotide substituted. For example, a single nucleotide substitution in the codon (CUU) for amino acid residue 55 (Leu) potentially generates six amino acid substitutions (L55P, L55H, L55R, L55F, L55I, and L55V). Nevertheless, we detected only one of them, L55P, caused by the change in codon from CUU to UUU (Table 6). Similar analyses were carried out for the seven other polymorphic amino acid residues (Table 6). In summary, of four to eight potential amino acids, one to three amino acid substitutions occurred at these polymorphic residues.

Furthermore, there was a bias toward a particular amino acid substitution at individual polymorphic residues (i.e., residues 60, 66, 265, 267, and 269) where multiple substitutions were detected (Table 6). Interestingly, in a comparison among amino acid substitutions regard-

Table 5
Amino acid substitutions at various positions within the outer loops of VP1

Study	No. of isolates with amino acid substitutions in indicated VP1 loops (amino acid residues)								Total no. of isolates with VP1 loop mutations
	BC (55)	BC (60)	BC (61)	BC (66)	DE (123)	HI (265)	HI (267)	HI (269)	
[6]	3	2	1	1	1	1	2	7	18
This study	2	2	0	3	2	3	2	3	17
Total	5	4	1	4	3	4	4	10	35

Table 6
Codons (amino acids) potentially generated by single nucleotide substitutions and those actually detected in the brain or CSF of PML patients

VP1 loop (amino acid residue)	Codon (amino acid) in archetypal isolates	No. of substituted codons (amino acids) potentially generated by a single nucleotide substitution	Substituted codons (amino acids) actually detected in PML-type isolates	No. of isolates with substituted codons (amino acids)
BC (55)	CUU (L)	6	UUU (F)	5
BC (60)	AAG (K)	7	AUG (M)	2
			AAC (N)	1
			GAG (E)	1
BC (61)	UCA (S)	4	UUA (L)	1
BC (66)	GAU (D)	8	CAU (H)	3
			AAU (N)	1
DE (123)	UCU (S)	7	UGU (C)	3
HI (265)	AAC (N)	8	GAC (D)	3
			ACC (T)	1
HI (267)	UCU (S)	8	UUU (F)	3
			UUA (L)	1
HI (269)	UCC (S)	6	UUC (F)	7
			UAC (Y)	2
			UGC (C)	1

The codon (amino acid) [UUA (L)] detected at residue 267 within the HI loop was produced by dual substitutions.

Table 7
Frequencies of individual amino acids substituted (estimated based on Table 6)

Amino acid substituted	Frequency
F	15
C	4
H	3
D	3
M	2
N	2
L	2
Y	2
E	1
T	1
Total	35

less of polymorphic residues, we noticed that phenylalanine was most frequently substituted (Table 7).

VP1 loop mutations in long-surviving PML patients

Using the method described above, we analyzed VP1 sequences of JCPyV in various parts of the brain autopsied from two long-surviving PML patients, one (patient 1) with a full progression of PML, the other (patient 2) with a stabilization of PML (see Materials and methods for their case reports). In patient 1, we detected three amino acid substitutions (K60E, D66H, and D66N) in various parts of the brain (Table 8). One (D66H) was widespread throughout the brain, and two (K60E and D66N) were localized to specific areas (pons and parietal lobe, respectively). The prototypal VP1 was rarely detected in the brain of patient 1. In contrast, the prototypal VP1 was widespread in the brain of patient 2, and a VP1 carrying the substitution S267F, together with the prototype VP1, was detected only in the temporal lobe and cerebellum (Table 8).

Discussion

We characterized VP1 loop mutations frequently occurring in PM-type JCPyV isolates. First of all, we found that the number of polymorphic residues is essentially restricted to three (55, 60, and 66), one (123), and three (265, 267, and 269) in the BC, DE, and HI loops, respectively. For all of these polymorphic residues, amino acid substitutions occurred in three or more indepen-

dent JCPyV isolates from the brain or CSF of PML patients, and therefore, it is likely that amino acid substitutions of these residues have a biological significance that remains to be clarified. The amino acid substitution at residue 61 (S61L) was detected in a minor JCPyV DNA clone obtained from the brain of a PML patient (case 2) [6]. As we detected no further amino acid substitution at residue 61, we tentatively exclude this residue from the biologically significant VP1 loop mutations.

We found that substitutions of most polymorphic residues showed a trend toward a change to specific amino acids. In addition, in a comparison among amino acid substitutions regardless of polymorphic residues, we noticed that phenylalanine was most frequently substituted. These findings, together with the observation that silent mutations were rather rare in the VP1 gene region encoding VP1 loops, suggest that VP1 loop mutations were selected at a high rate because they were advantageous, although their biological significance remains to be understood.

Outer loops of JCPyV VP1 are thought to contain not only sites reacting with cellular receptors [13] but also epitopes recognized by the anti-JCPyV antibody [14]. Therefore, amino acid changes in the outer loops potentially modify the reaction between JCPyV and cellular receptors or between JCPyV and anti-JCPyV antibodies, leading to the expansion of demyelinated lesions in the brain. It was recently reported that Arg-56 and Arg-75 on the BC loop and Arg-273 on the HI loop are the potential sialic acid-binding sites for JCPyV infection [13]. None of these amino acids were among the potential residues where VP1 loop mutations occur (see above). This would suggest that PML-type JCVs with VP1 loop mutations underwent no change in the ability to bind to cellular receptors, if the indirect effect of the mutations could be excluded.

One piece of evidence supports that VP1 loop mutations modify the interaction between JCPyV and anti-JCPyV antibody. An amino acid substitution (D66H) in the BC loop was detected in the isolate Mad-11 from the brain of a PML patient [6]. The same mutation was found in two additional isolates from the brains of PML patients (this study). Interestingly, Mad-11 is the only antigenic variant of JCPyV documented so far [15]. As Mad-11 carries no other amino acid substitution in its VP1 protein (unpublished data), it is likely that D66H is responsible for the antigenic alteration found in Mad-11.

Table 8
VP1 sequences detected in various parts of the brain

Patient	Frontal lobe	Parietal lobe	Temporal lobe	Occipital lobe	Pons	Cerebellum
1	D66H	D66N	D66H	D66H	D66H + K60E	D66H
2	Proto	Proto	Proto + S267F	Proto	Proto	Proto + S267F

D66H, D66N, K60E, and S267F indicate VP1 sequences with these mutations, while Proto indicates the prototype VP1 without VP1 loop mutations. Patient 1, a long-term survivor with a full progression of PML; patient 2, a long-term survivor with a stabilization of PML (see text).

We previously reported that the frequencies of JCV DNA clones with the VP1 loop mutations were significantly increased in a long-surviving PML patient as compared with two short-surviving PML patients [6]. In the present study, we analyzed VP1 loop sequences in various parts of the brain autopsied from two long-surviving PML patients, one (patient 1) with a full progression of PML, the other (patient 2) with a stabilization of PML. In patient 1, three VP1 loop mutations were detected, one spread throughout the brain, and two localized to a few areas of the brain, while a single VP1 loop mutation was detected only in the temporal lobe and cerebellum. These findings, together with those in the previous study [6], suggest that the ratio of VP1 loop mutations increases during the time when viral propagation continues in the brain.

In summary, we obtained valuable information regarding the VP1 loop mutations possibly associated with the development of PML. The present findings should form the basis for elucidating the biological significance of the VP1 loop mutations.

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Two distinct genotypes (MY-x and MX) of JC virus previously identified in Hokkaido Ainu

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Abstract Genotyping the urinary JC virus (JCV) DNA is a useful means to gain new insights into the origin of ethnic groups. We recently detected thirteen JCV isolates from the Ainu, an indigenous population living on Japan's northernmost island (Hokkaido). Based on phylogenetic analysis, these isolates were classified into five genotypes: two (MX and MY-x) were first identified in the Ainu, two (EU-a/Arc and EU-c) are prevalent in northeastern Siberians and an Arctic tribe, and one (MY-b) is widespread among Hondo Japanese, i.e. contemporary Japanese excluding the Ainu. Although these findings have several potential implications for the development of the modern Ainu, further studies are required to reach a definite conclusion. In this report, an isolate in a forensic subject whose ethnic origin was Ainu belonged to the MX genotype and two isolates recently identified in South Koreans and grouped as Native American isolates belonged to the MY-x genotype. The present findings suggest that the MX genotype of JCV is unique to the Ainu, whereas MY-x is spread among some Northeast Asian populations.

Key words: Ainu, JC virus, genotype, MX, MY-x

Introduction

JC virus (JCV) is a member of the *Polyomaviridae* family. Its genome is a single molecule of covalently closed, circular double-stranded DNA about 5100 bp in length (Cole and Conzen, 2001). Although JCV causes progressive multifocal leukoencephalopathy (PML) in immunocompromised patients (Walker, 1985), it is ubiquitous in the human population; it infects children asymptotically, then persists in renal tissue throughout life (Padgett and Walker, 1973; Chesters et al., 1983; Tominaga et al., 1992; Kitamura et al., 1997). In most adults, renal JCV is not latent, but instead replicates and its progeny are excreted into urine (Kitamura et al., 1990, 1994; Agostini et al., 1996). JCV strains around the world can be classified into more than ten major genotypes, with each genotype occupying a unique geographical domain (Sugimoto et al., 1997; Yogo et al., 2004). Analysis of JCV genotypes has thus provided new insights into the origins of various ethnic groups throughout the world (e.g. Kitamura et al., 1998; Guo et al., 2001; Saruwatari et al., 2002; Miranda et al., 2004; Takasaka et al., 2004; Yogo et al., 2004).

Using phylogenetic analysis, Yogo et al. (2003) recently

analyzed thirteen JCV isolates from the Ainu, an indigenous population living on Hokkaido, the northernmost island of Japan, and classified them into five genotypes: two (MX and MY-x) were first identified in the Ainu, two (EU-a/Arc and EU-c) were prevalent in northeastern Siberians and an Arctic tribe, and one (MY-b) was widespread among Hondo Japanese (i.e. contemporary Japanese, excluding the Ainu). Although two new genotypes were recognized in Yogo et al.'s (2003) study, the data size was too small for them to draw definite conclusions about the ethnic distribution of these genotypes. In the present study, we performed a detailed phylogenetic analysis of three unique JCV isolates, one identified by ourselves in an Ainu subject and two detected by Cui et al. (2004) in Koreans living in Seoul, South Korea. The latter two isolates were described as Type 2A2, which is characteristic of Native Americans (Cui et al., 2004).

Materials and Methods

Subject

A 70-year-old male Ainu who was born in Makubetsu, southern Hokkaido, and died of pneumonia was the subject of this study. A medico-legal autopsy was performed at his family's request. His ethnic origin was found to be Ainu according to interviews with the family.

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Table 1. JCV isolates whose complete DNA sequences were used for the phylogenetic analysis

Genotype	Isolate	Geographic origin (ethnic origin)	Accession no. ¹	Reference
Af1	#601	USA	AF015537	Agostini et al. (1998c)
Af1	GH-1	Ghana	AB038252	Kato et al. (2000)
Af1	GH-2	Ghana	AB038253	Kato et al. (2000)
Af1	GH-3	Ghana	AB048545	Sugimoto et al. (2002a)
Af1	GH-4	Ghana	AB048546	Sugimoto et al. (2002a)
EU-a	G2	Germany	AB038251	Kato et al. (2000)
EU-a	N2	Netherlands	AB048574	Sugimoto et al. (2002a)
EU-a	SW-3	Sweden	AB048575	Sugimoto et al. (2002a)
EU-a	UK-2	UK	AB048576	Sugimoto et al. (2002a)
EU-a	IT-5	Italy	AB048568	Sugimoto et al. (2002a)
EU-a	#123	USA	AF015527	Agostini et al. (1998a)
EU-a	#124	USA	AF015526	Agostini et al. (1998a)
EU-a	Mad-1	USA	J02226	Frisque et al. (1984)
EU-a/JK ²	AT-2	Japan	AB048569	Sugimoto et al. (2002a)
EU-a/JK	AT-4	Japan	AB048570	Sugimoto et al. (2002a)
EU-a/JK	HR-5	Japan	AB048572	Sugimoto et al. (2002a)
EU-a/JK	HR-13	Japan	AB048571	Sugimoto et al. (2002a)
EU-a/JK	SD-9	Japan	AB048573	Sugimoto et al. (2002a)
EU-a/JK	SK-6	South Korea	AB183152	Yogo et al. (2004)
EU-a/Arc	ES-1	Canada (Inuit)	AB074578	Sugimoto et al. (2002b)
EU-a/Arc	ES-3	Canada (Inuit)	AB074579	Sugimoto et al. (2002b)
EU-a/Arc	KO-2	Siberia (Koryak)	AB074585	Sugimoto et al. (2002b)
EU-a/Arc	KO-3	Siberia (Koryak)	AB074586	Sugimoto et al. (2002b)
EU-a/Arc	KO-5	Siberia (Koryak)	AB074587	Sugimoto et al. (2002b)
EU-a/Arc	AN-12	Japan (Ainu)	AB092586	Yogo et al. (2003)
EU-b	GR-3	Greece	AB048563	Sugimoto et al. (2002a)
EU-b	MR-7	Morocco	AB048564	Sugimoto et al. (2002a)
EU-b	N25	Netherlands	AB048565	Sugimoto et al. (2002a)
EU-b	SP-1	Spain	AB048566	Sugimoto et al. (2002a)
EU-b	UK-1	UK	AB048567	Sugimoto et al. (2002a)
EU-b	#402	USA	AF015528	Agostini et al. (1998a)
EU-c	AM-5	Siberia (Nanai)	AB074576	Sugimoto et al. (2002b)
EU-c	AM-7	Siberia (Nanai)	AB074577	Sugimoto et al. (2002b)
EU-c	AM-18	Siberia (Nanai)	AB074575	Sugimoto et al. (2002b)
EU-c	SI-1	Siberia	AB074589	Sugimoto et al. (2002b)
EU-c	SI-7	Siberia	AB074590	Sugimoto et al. (2002b)
EU-c	AN-3	Japan (Ainu)	AB092579	Yogo et al. (2003)
EU-c	AN-5	Japan (Ainu)	AB092581	Yogo et al. (2003)
EU-c	AN-11	Japan (Ainu)	AB092585	Yogo et al. (2003)
Af2	#308	Tanzania	U73500	Agostini et al. (1997)
Af2	#311	USA (African American)	U73501	Agostini et al. (1997)
Af2	#312	USA (African American)	U73502	Agostini et al. (1997)
Af2	ET-3	Ethiopia	AB048547	Sugimoto et al. (2002a)
B1-a	ML-1	Malaysia	AB048548	Sugimoto et al. (2002a)
B1-a	C2	Taiwan	AB048549	Sugimoto et al. (2002a)
B1-a	CB-2	China	AB048550	Sugimoto et al. (2002a)
B1-b	MO-3	Mongolia	AB048551	Sugimoto et al. (2002a)
B1-b	MO-5	Mongolia	AB048552	Sugimoto et al. (2002a)
B1-b	SL-2	Sri Lanka	AB048553	Sugimoto et al. (2002a)
B1-b	#230	USA (African American)	AF015536	Agostini et al. (1998b)
B1-c	N4	Netherlands	AB048554	Sugimoto et al. (2002a)
B1-c	GS/K	Germany	AF004349	Loeber and Dörries (1988)
B1-c	GS/B	Germany	AF004350	Loeber and Dörries (1988)
B1-c	#223	USA (African American)	AF015532	Agostini et al. (1998b)
B1-c	#227	USA (European American)	AF015533	Agostini et al. (1998b)
B1-d	SA-3	Saudi Arabia	AB048555	Sugimoto et al. (2002a)
B1-d	SA-5	Saudi Arabia	AB048556	Sugimoto et al. (2002a)
B2	IN-6	India	AB048557	Sugimoto et al. (2002a)
B2	MU-3	Mauritius	AB048558	Sugimoto et al. (2002a)
B2	MU-9	Mauritius	AB048559	Sugimoto et al. (2002a)
CY	CY	Japan	AB038249	Kato et al. (2000)
CY	Tky-2a	Japan	AB038255	Kato et al. (2000)
CY	CB-3	Japan	AB048560	Sugimoto et al. (2002a)
CY	MO-1	Mongolia	AB048561	Sugimoto et al. (2002a)
CY	MO-6	Mongolia	AB048562	Sugimoto et al. (2002a)

Table 1. (continued)

Genotype	Isolate	Geographic origin (ethnic origin)	Accession no. ¹	Reference
MX	AN-9	Japan (Ainu)	AB092584	Yogo et al. (2003)
MX	AN-13	Japan (Ainu)	AB092587	Yogo et al. (2003)
MX	ANF	Japan (Ainu)	AB185020	This study
MY-a	Aic-1	Japan	AB081005	Zheng et al. (2003)
MY-a	AT-8	Japan	AB048577	Sugimoto et al. (2002a)
MY-a	JP-7	Japan	AB081016	Zheng et al. (2003)
MY-a	YI	Japan	AB081030	Zheng et al. (2003)
MY-b	Tokyo-1	Japan	AF030085	Agostini et al. (1998b)
MY-b	Tky-1	Japan	AB038254	Kato et al. (2000)
MY-b	HR-7	Japan	AB048578	Sugimoto et al. (2002a)
MY-b	MY	Japan	AB038250	Kato et al. (2000)
MY-b	SK-1	South Korea	AB081028	Zheng et al. (2003)
MY-b	SK-4	South Korea	AB081029	Zheng et al. (2003)
MY-b	AN-4	Japan (Ainu)	AB092580	Yogo et al. (2003)
MY-b	AN-6	Japan (Ainu)	AB092582	Yogo et al. (2003)
MY-b	AN-8	Japan (Ainu)	AB092583	Yogo et al. (2003)
MY-c	#224	USA (Hispanic)	AF015529	Agostini et al. (1998b)
MY-c	ME-5	Mexico (Tarahumalan)	AB081021	Zheng et al. (2003)
MY-c	ME-14	Mexico (Tarahumalan)	AB081018	Zheng et al. (2003)
MY-c	ME-16	Mexico (Tarahumalan)	AB081019	Zheng et al. (2003)
MY-c	GU-8	Guatemala (Mayan)	AB081015	Zheng et al. (2003)
MY-d	#225	USA (Navaho)	AF015530	Agostini et al. (1998b)
MY-d	#226	USA (Navaho)	AF015531	Agostini et al. (1998b)
MY-e	GU-4	Guatemala (Mayan)	AB081014	Zheng et al. (2003)
MY-e	GU-15	Guatemala (Mayan)	AB081011	Zheng et al. (2003)
MY-e	GU-21	Guatemala (Mayan)	AB081012	Zheng et al. (2003)
MY-e	GU-25	Guatemala (Mayan)	AB081013	Zheng et al. (2003)
MY-e	ME-8	Mexico (Tarahumalan)	AB081022	Zheng et al. (2003)
MY-f	PE-1	Peru (Andean)	AB081023	Zheng et al. (2003)
MY-f	PE-11	Peru (Andean)	AB081024	Zheng et al. (2003)
MY-f	PE-12	Peru (Andean)	AB081025	Zheng et al. (2003)
MY-f	PE-16	Peru (Andean)	AB081026	Zheng et al. (2003)
MY-f	PE-21	Peru (Andean)	AB081027	Zheng et al. (2003)
MY-g	#228	USA (Navaho)	AF015534	Agostini et al. (1998b)
MY-g	#229	USA (European American)	AF015535	Agostini et al. (1998b)
MY-g	CN-1	Canada (Beaver/Dene Tha')	AB081007	Zheng et al. (2003)
MY-g	CN-13	Canada (Beaver/Dene Tha')	AB081006	Zheng et al. (2003)
MY-g	CN-15	Canada (Beaver/Dene Tha')	AB081008	Zheng et al. (2003)
MY-g	CN-25	Canada (Beaver/Dene Tha')	AB081009	Zheng et al. (2003)
MY-g	CN-28	Canada (Beaver/Dene Tha')	AB081010	Zheng et al. (2003)
MY-x	AN-1	Japan (Ainu)	AB092578	Yogo et al. (2003)
MY-x	255A	South Korea	AY121910	Cui et al. (2004)
MY-x	256A	South Korea	AY121911	Cui et al. (2004)
MY	ME-4	Mexico (Tarahumalan)	AB081020	Zheng et al. (2003)
MY	ME-12	Mexico (Tarahumalan)	AB081017	Zheng et al. (2003)
SC	CW-2	China	AB048579	Sugimoto et al. (2002a)
SC	ID-1	Indonesia	AB048580	Sugimoto et al. (2002a)
SC	ML-6	Malaysia	AB048581	Sugimoto et al. (2002a)
SC	MO-11	Mongolia	AB048582	Sugimoto et al. (2002a)
2E	#233	New Britain (Tolai)	AF281605	Jobes et al. (2001)
2E	#234	Guam (Chamorro)	AF281606	Jobes et al. (2001)
8A	#801	PNG (Highlander)	AF281623	Jobes et al. (2001)
8A	#802	PNG (Highlander)	AF281624	Jobes et al. (2001)
8B	#803	PNG (Highlander)	AF281625	Jobes et al. (2001)
8B	#804	PNG (Highlander)	AF281626	Jobes et al. (2001)

¹ GSDB, DDBJ, EMBL, and NCBI accession numbers.² EU-a/Arc has been renamed EU-a/JK (Yogo et al., 2004).

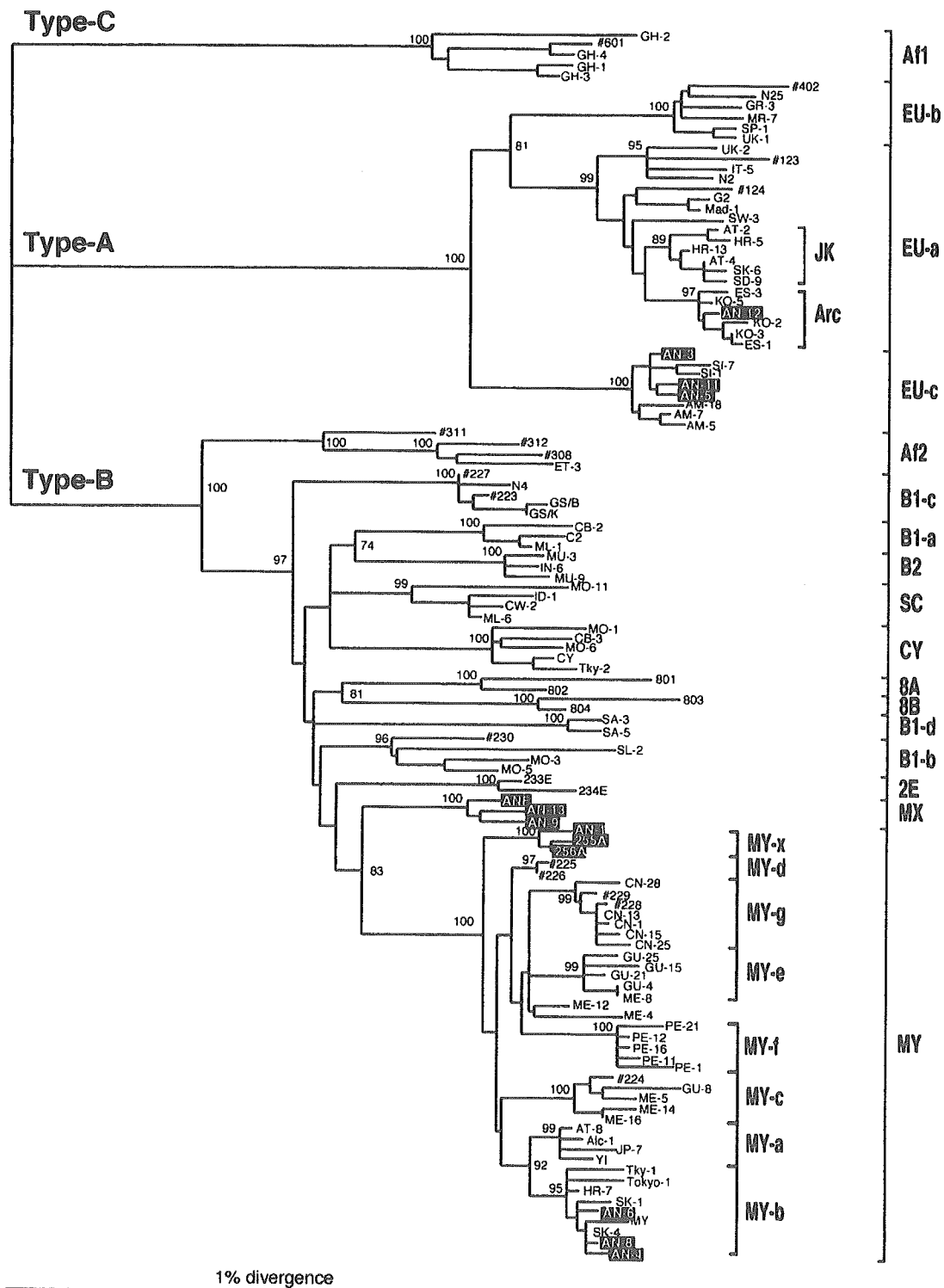


Figure 1. Neighbor-joining (NJ) phylogenetic tree relating 118 complete JCV DNA sequences. A phylogenetic tree was constructed from complete sequences, excluding regulatory sequences, using the NJ method. The phylogenetic tree was visualized using the TREEVIEW program (Page, 1996). The tree was rooted using Af1 as the outer group, as this genotype probably represents the ancestral type (Pavesi, 2003). The symbols for sequences are shown in Table 1. The number at each node in the tree indicates the bootstrap probability (BP, %) obtained from 1000 replicates (only values >70% are shown for major clusters). Superclusters (Types A, B, and C), genotypes (Af1, Af2, EU-a, EU-b, EU-c, B1-a, B1-b, B1-c, B1-d, B2, CY, MX, MY, 2E, 8A, and 8B), intra-EU-a subgroups (EU-a/Arc and EU-a/JK), and intra-MY subgroups (MY-a to MY-g and MY-x) are indicated. Isolates from the Ainu (Yogo et al., 2003; this study) and from Koreans (Cui et al., 2004) are shown in white on black backgrounds.

DNA extraction and analysis

About 5 ml of urine was collected from the subject's bladder and was used as the source for our extraction of viral DNA, as described by Kitamura et al. (1990). A 610-bp IG region was amplified by means of polymerase chain reaction (PCR) using the P1 and P2 primers (Kunitake et al., 1995). The entire JCV DNA was cloned into pUC19 at the unique *Bam*HI site, as described by Yogo et al. (1991), and the resultant clone, which carried the entire JCV DNA, was sequenced as described by Sugimoto et al. (2002a).

Reported complete DNA sequences of JCV

The complete JCV DNA sequences reported previously and used in this study are shown in Table 1, along with their GSDB, DDBJ, EMBL, and NCBI accession numbers.

Phylogenetic Analysis

The non-coding regulatory region of the JCV genome was excluded from our phylogenetic analysis, as this region is hypervariable, especially in JCV isolates derived from the brains of patients with PML (Yogo and Sugimoto, 2001). DNA sequences were aligned using the CLUSTAL W program (Thompson et al., 1994) with a gap-opening penalty of 15.00 and a gap-extension penalty of 6.66. To evaluate the phylogenetic relationships among DNA sequences, we used the neighbor-joining (NJ) method (Saitou and Nei, 1987) in CLUSTAL W. Divergences were estimated using Kimura's two-parameter method (Kimura, 1980). To assess the confidence limit of the branching patterns of the NJ tree, bootstrap probabilities (BPs) were estimated using 1000 bootstrap replicates (Felsenstein, 1985) in CLUSTAL W. BPs greater than 70% were considered to be significant (Hillis and Bull, 1993). A phylogenetic tree was then visualized using the TREEVIEW program (Page, 1996).

Results

As the urine sample from the Ainu subject gave positive amplification in the PCR process, we attempted to establish complete clones of the JCV DNA from the urine. We obtained a complete JCV DNA clone, which we designated ANF. We then sequenced this clone completely. Cui et al. (2004) recently reported various JCV isolates in Asia, including China, South Korea, and India. Among these isolates, two from Seoul (255A and 256A) attracted our attention because despite their Asian origin, they were described as a genotype (Type 2A2) formerly considered to be characteristic of Native Americans (Cui et al., 2004). We constructed an NJ phylogenetic tree from the complete DNA sequences of ANF, 255A, and 256A, together with 118 complete sequences that had been collected from around the world, including ten detected in the Ainu (Yogo et al., 2003). Based on the resulting tree (Figure 1), we conclude that ANF and two isolates (AN-9 and AN-13) that had previously been detected in the Ainu (Yogo et al., 2003) formed a distinct clade (designated MX), with a high bootstrap probability (100%). In contrast, the Korean isolates (255A and 256A) together with an isolate (AN-1) from an Ainu formed another distinct clade (MY-x) with a 100% BP (Figure 1). MY-x and the other MY subgroups (MY-a to MY-g) consti-

tuted a superclade, designated MY. As previously shown (Yogo et al., 2003), the MX and MY grouping has been demonstrated with a significantly high BP (83%; Figure 1).

Discussion

Yogo et al. (2003) detected MY-x and MX in only one or a few subjects. Therefore, the ethnic distribution of these genotypes required further study. In the present study, we demonstrated the presence of the MX genotype in a forensic subject whose origin was Ainu. MX was previously detected at two distant sites (Asahikawa and Shiraoi) on Hokkaido (Yogo et al., 2003). In the present study, we detected MX in a subject from another site on Hokkaido (i.e. Makubetsu), which suggests that the MX genotype of JCV is widespread among the Ainu, albeit at a lower frequency.

We found that two isolates from Seoul belonged to MY-x, a subgroup within genotype MY (Yogo et al., 2003). These isolates were previously described as belonging to the Native American subgroup of MY (named Type 2A2; Cui et al., 2004), but the result of our analysis suggests that this classification should be revised. Although MY-x was distributed in two distinct human populations (i.e. the Ainu and Koreans), further division of the MY-x isolates was not demonstrated. This suggests that peoples carrying the MY-x genotype migrated to the Korean Peninsula and Hokkaido relatively recently (i.e. not earlier than 10000 years ago).

Acknowledgments

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JC virus genotyping suggests a close contact or affinity between Greenland Inuit and other circumarctic populations

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Abstract We used the JC virus genotyping method to investigate the origins of Greenland Inuits. Using polymerase chain reaction, we detected six JC virus isolates in urine samples collected from Inuits in northwestern and southeastern Greenland. Phylogenetic analysis of the complete and partial DNA sequences of these isolates demonstrated that all isolates belonged to the EU-a1/Arc cluster previously identified in native northeastern Siberians (e.g. Chukchis and Koryaks) and Canadian Inuits. This finding suggests a close contact or affinity between Greenland Inuits and other circumarctic populations.

Key words: JC virus, Inuit, circumarctic populations, population affinity

Introduction

The population history of Greenland Inuits has been inferred based on studies using human mitochondrial DNA (mtDNA) and the Y chromosome as markers. The study using mtDNA (Saillard et al., 2000) indicated that Greenland Inuits essentially descend from an ancestral people in Alaska. In contrast, the Y chromosome study (Bosch et al., 2003) suggested a high-level of male-mediated admixture with Scandinavians, although no European mtDNA type was detected in the same Inuit sample (Saillard et al., 2000). In this study, we used a complementary approach based on the JC virus (JCV) to resolve this issue, as JCV genotyping has recently been established as a novel means of elucidating the origins of various ethnic groups (Yogo et al., 2004).

Materials and Methods

Urine samples were collected with informed consent from Greenland Inuits aged 40 years or older at Siorapaluk and Kuummiut/Tasiilaq, located in northwestern and southeastern Greenland, respectively. Based on historical records, these sites have not been colonized by Europeans (Bosch et al., 2003). DNA was extracted from urine, then the 610-bp IG region of the JCV genome was amplified by polymerase chain reaction (PCR) (Kunitake et al., 1995) and the entire viral genome cloned (Sugimoto et al., 2002). The partial and complete JCV DNAs were sequenced and used to construct neighbor-joining (NJ) phylogenetic trees (Sugimoto et al., 2002).

Results

We PCR-amplified the IG region in four of the ten and two of the three urine samples collected at Siorapaluk and Kuummiut/Tasiilaq, respectively. The amplified fragments were sequenced, and the determined sequences, together with reference IG sequences, were used to construct an NJ tree. On the resultant tree [the tree is not shown to save space, but an analogous tree can be seen elsewhere (Sugimoto et al., 2002)], all IG sequences from Greenland Inuits and various sequences from northeastern Siberians (e.g. Koryaks and Chukchis) and Canadian Inuits formed a sub-cluster within the EU-a cluster.

We then performed a phylogenetic analysis based on complete JCV DNA sequences. Two complete JCV DNA sequences (one each from Siorapaluk and Kuummiut/Tasiilaq), together with 52 complete DNA sequences of Type A JCVs (see Table 1), were used to construct an NJ phylogenetic tree (Figure 1). The two Greenland isolates, together with three from Koryaks, two from Canadian Inuits, and one from Ainus (Sugimoto et al., 2002; Yogo et al., 2003), formed a single cluster (designated Arc) within EU-a1, with a high bootstrap probability (99%). Arc isolates from different ethnic groups intermingled among themselves, suggesting the lack of significant evolutionary distinction between Arc isolates derived from different ethnic groups.

Discussion

The present findings suggest a close contact or affinity between Greenland Inuits and other circumarctic populations, such as Canadian Inuits, Koryaks, and Chukchis.

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Table 1. Type A JCV isolates whose complete DNA sequences were analyzed in this study

Geographic origin (ethnic origin)	Isolate	Genotype (subgroup) ¹	Accession no. ²
Germany	G2	EU-a1	AB038251
Germany	G4	EU-a2	AB074580
Germany	G5	EU-a2	AB074581
United Kingdom	UK-1	EU-b	AB048567
United Kingdom	UK-2	EU-a2	AB048576
Netherlands	N2	EU-a2	AB048574
Netherlands	N5	EU-a1 (Sam)	AB074588
Netherlands	N25	EU-b	AB048566
Spain	SP-1	EU-b	AB048566
Spain	SP-7	EU-a2	AB074591
Italy	IT-2	EU-a1	AB074582
Italy	IT-3	EU-a2	AB074583
Italy	IT-5	EU-a2	AB048568
Italy	IT-8	EU-a2	AB074584
Sweden	SW-3	EU-a1	AB048575
Greece	GR-3	EU-b	AB048563
Finland	FL-3	EU-a1	AB127347
Finland	FL-4	EU-a1	AB127348
Finland	FL-7	EU-a2	AB127349
Finland	FL-8	EU-b	AB127351
Finland	FL-11	EU-a2	AB127350
Finland	FL-13	EU-a2	AB127353
Finland (Saami)	Sam-3	EU-a1 (Sam)	AB127342
Finland (Saami)	Sam-8	EU-a1	AB127345
Finland (Saami)	Sam-9	EU-a1 (Sam)	AB127344
Finland (Saami)	Sam-12	EU-a1 (Sam)	AB127343
Finland (Saami)	Sam-15	EU-a1 (Sam)	AB127346
Morocco	MR-7	EU-b	AB048564
United Sates	Mad-1	EU-a1	J02226
United Sates	#123	EU-a2	AF015527
United Sates	#124	EU-a1	AF015526
United Sates	#402	EU-b	AF015528
Japan	AT-2	EU-a1 (JK)	AB048569
Japan	AT-4	EU-a1 (JK)	AB048570
Japan	HR-5	EU-a1 (JK)	AB048572
Japan	HR-13	EU-a1 (JK)	AB048571
Japan	SD-9	EU-a1 (JK)	AB048573
Japan (Ainu)	AN-3	EU-c	AB092579
Japan (Ainu)	AN-5	EU-c	AB092581
Japan (Ainu)	AN-11	EU-c	AB092585
Japan (Ainu)	AN-12	EU-a1 (Arc)	AB092586
South Korea	SK-6	EU-a1 (JK)	AB183152
Siberia (Nanai)	AM-5	EU-c	AB074576
Siberia (Nanai)	AM-7	EU-c	AB074577
Siberia (Nanai)	AM-18	EU-c	AB074575
Siberia (Evenki)	SI-1	EU-c	AB074589
Siberia (Yakut)	SI-7	EU-c	AB074590
Siberia (Koryak)	KO-2	EU-a1 (Arc)	AB074585
Siberia (Koryak)	KO-3	EU-a1 (Arc)	AB074586
Siberia (Koryak)	KO-5	EU-a1 (Arc)	AB074587
Canada (Inuit)	ES-1	EU-a1 (Arc)	AB074578
Canada (Inuit)	ES-3	EU-a1 (Arc)	AB074579
Greenland (Inuit)	GL-2	EU-a1 (Arc)	AB195639
Greenland (Inuit)	GL-5	EU-a1 (Arc)	AB195640

¹ Based on Figure 1.² GSDB, DDBJ, EMBL, and NCBI nucleotide sequence database.

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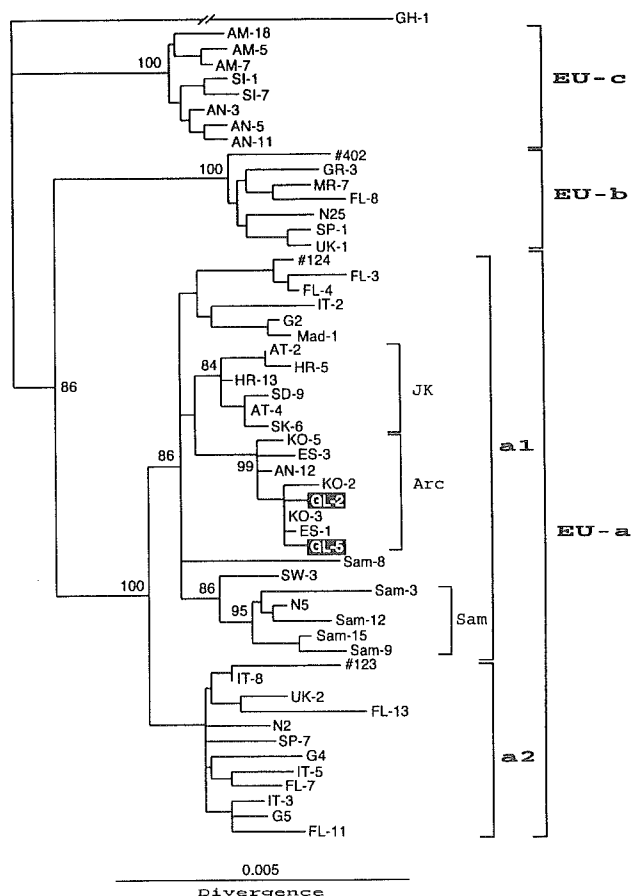


Figure 1. NJ phylogenetic tree relating 54 complete Type A sequences. A phylogenetic tree was constructed from complete sequences, excluding regulatory sequences, using the NJ method. An isolate [GH-1 (GSDB, DDBJ, EMBL, and NCBI accession number, AB038252)] belonging to Type C was used as the outgroup. The phylogenetic tree was visualized using the TREEVIEW program. The symbols for sequences are shown in Table 1. The numbers at nodes in the tree indicate bootstrap probabilities (percent) obtained with 1,000 replicates (only those $\geq 50\%$ are shown for major nodes).

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