Table 5. TCR sequences of various BKV isolates directly obtained from urine

Isolates cloned previously (Rubinstein *et al.*, 1987; Sugimoto *et al.*, 1989) are underlined.

TCR sequence*	Subtype	Isolate
Seq-1	IV	THK-3, -7, TW-3, RYU-5, KOM-2, -7, -12
Seq-2	IV	RYU-3
Seq-3	IV	THK-8
Seq-4	III	KOM-3, RYU-7
Seq-5	Ib	THK-2, KOM-5, WW
Seq-6	Ib	KOM-1
Seq-7	Ic	THK-5, -6, -9, -11, TW-1, 2, -4 to -7, -12 to -14, RYU-1, -4, -8, KOM-6, -10, -11, -13 to –16, -21, -26 to -30
Seq-8	Ic	KOM-8, MT-1
Seq-9	Ic	TW-8
Seq-10	Ic	RYU-2

*Sequences are shown in Fig. 2.

Molecular epidemiological studies of BKV conducted thus far (see above) suggest that there is no significant correlation between BKV subtypes and geographical regions. This is in striking contrast to the established correlation between JCV subtypes and geographical regions (Sugimoto *et al.*, 1997; Agostini *et al.*, 2001; Yogo *et al.*, 2004). However, it should be noted that the subtypes of BKV are antigenically distinguished, while those of JCV are only discernable in terms of nucleotide sequences. If it can be assumed that BKV and JCV have essentially the same evolutionary rate, it may be speculated that it took longer for the BKV subtypes than JCV subtypes to be generated. Thus, it is conceivable that BKV originated before the formation of modern humans (i.e. the four BKV subtypes would have already existed in ancestral populations of modern humans). In contrast, JCV subtypes would have been generated, after the emergence of modern humans, in association with the division of human populations (Yogo *et al.*, 2004).

Nevertheless, we consider that subgroups within each subtype may have a correlation with human populations.

Table 6. Differences in TCR sequences among BKV subtypes

Sequence (subtype)	Nucleotides at indicated positions*												
	52	53	65	89	92	143	146	181	182	199	227	233	254
Seq-1 (IV)	T	T	C	G	A	C	G	A	G	C	A	C	T
Seq-4 (III)	T	T	C	C	–†	C	A	T	T	C	A	C	T
Seq-5 (Ib)	A	A	T	G	A	C	A	G	G	T	A	C	–
Seq-7 (Ic)	T	A	C	G	A	A	A	G	G	T	G	G	–

*See Fig. 2.

†Indicates deletion of a single nucleotide.

In this study, we analysed many isolates from the Japanese population, and found that a majority of Japanese isolates occurred in Ic, suggesting a correlation between Japanese isolates and subgroup Ic. If a larger number of isolates derived from Europeans, Africans and Asians (other than Japanese) are sequenced and a phylogenetic tree is constructed using the resultant sequence data, a correlation between BKV subgroups and human populations will be evident.

Relationships between TCR structures and BKV subtypes

We compared TCR structures, and found that there were several nucleotide substitutions and a few single-nucleotide deletions (or insertions) among BKV subtypes. We sometimes detected BKV TCRs with rather extensive rearrangements, but the BKVs with these rearranged TCRs rarely predominated in a population. Thus, we concluded that the basic TCR structure (the so-called archetype configuration) were conserved among subtypes of BKV.

The TCR detected in strain WW has been considered to represent the archetypal TCR of BKV (Knowles, 2001). Nevertheless, the present findings suggested that like other parts of the genome, the BKV TCR underwent evolutionary changes, involving nucleotide substitutions and single-nucleotide deletions/insertions. Thus, each subtype of BKV has a unique set of nucleotide substitutions and deletions/insertions. We therefore suggest that 'archetype' be used as a conceptual word that denotes the prototypical structure that can generate various rearranged TCRs typically observed in BKV strains passaged in cell culture (Yoshiike & Takemoto, 1986; Hara *et al.*, 1986; Rubinstein *et al.*, 1987). In this sense, TCR structures (e.g. Seq-1, -4, -5 and -7) shared by most isolates belonging to the same subtypes are all archetypal.

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Genotypes of JC virus in Southeast Asia and the western Pacific: implications for human migrations from Asia to the Pacific

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Abstract JC virus (JCV) genotyping is a novel method of tracing human migrations. In the present study, we used this method to gain insights into human expansions to the western Pacific. Using the whole genome approach, four genotypes of JCV (8A, 8B, 2E, and 7A) were previously detected in the island populations of the western Pacific. Using the same approach, we detected five genotypes (B1-a, B3-a, B3-b, SC-f, and SC-x) in Filipino populations (B3-b and SC-f corresponded to 2E and 7A, respectively). From these findings, it was concluded that seven genotypes of JCV were spread in the island populations in Southeast Asia and the western Pacific (these genotypes were tentatively designated as the ocean genotypes). Three of them (B1-a, B3-a, and SC-f/7A) occurred in the Philippines but did not occur in the western Pacific; two (B3-b/2E and SC-x) occurred in the Philippines as well as in the western Pacific excluding Guam; and two (8A and 8B) occurred only in the western Pacific. These regional distribution patterns for the oceanic genotypes suggested various human migrations to the Pacific, some restricted to islands near the Asian Continent and some extended far to Remote Oceania. Furthermore, our findings were consistent with the view that the Austronesians originated in an area of Southeast Asia, including the Philippines.

Key words: JC virus, phylogenetic analysis, western Pacific, human migrations, Philippines

Introduction

The JC virus (JCV) is a small DNA virus, belonging to the *Polyomaviridae* (Cole and Conzen, 2001). JCV causes, for immunocompromised patients, a fatal demyleinating disease in the central nervous system, known as progressive multifocal leukoencephalopathy (Padgett et al., 1971). This virus, however, is ubiquitous in the human population, infecting children asymptomatically, then persisting in renal tissue (Padgett and Walker, 1973; Chesters et al., 1983; Kitamura et al., 1990, 1994, 1997; Tominaga et al., 1992). The main mode of transmission of JCV is from parents to children through long-term cohabitation (Kunitake et al., 1995; Kato et al., 1997; Suzuki et al., 2002).

JCV isolates worldwide belong to a single serotype (Major, 2001), but they can be classified into more than ten groups (designated as genotypes) according to nucleotide variations in their genomes (Agostini et al., 2001). Each of these genotypes occupies a unique domain in the world (Sugimoto et al., 1997; Guo et al., 1998): 1) The European genotype EU is spread throughout the Europe and Mediterranean areas. 2) A genotype (B1-c) related to the Asian genotypes occurs in some regions of Europe (e.g. the Netherlands, Greek). 3) The African genotype Af2 is spread not only throughout Africa but also in West and South Asia. 4) A minor African genotype, Af1, occurs in Central and West Africa. 5) Various genotypes (e.g. B1-a, -b, -d, B2, CY, MY, and SC) are spread in Asia, with their domains partially overlapping.

Multiple genotypes of JCV occur in geographic areas where different ethnic groups are thought to have intermixed (Sugimoto et al., 1997). For instance, both African (Af2) and European (EU) genotypes equally occur in an area of North Africa facing the Mediterranean Sea. European (EU), African (Af2), and Asian (B1-b) genotypes are prevalent in West Asia. African (Af2), South Asian (B2), and Southeast Asian (SC) genotypes occur in Mauritius, an island in the Indian Sea. Various JCV genotypes occur in the Americas. Some of these are indigenous to Native Americans (Agostini et al., 1997a; Fernandez-Cobo, 2002; Sugimoto et al., 2002b; Zheng et al., 2003), but some of them represent the genotypes introduced to the Americas by recent immigrants from Europe, Africa, and Asia (Stoner et al., 2000; Suzuki et al., 2002).

The above described distribution pattern of the JCV genotypes indicate that JCV migrated over the earth, accompanying recent and ancient human migrations. It was thought

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that the genotype of JCV should serve as a new marker for tracing human migrations (Agostini et al., 1997a; Sugimoto et al., 1997). Using the JCV genotyping approach, Stoner and his colleagues (Jobes et al., 2001; Yanagihara et al., 2002) examined JCV relationships in the island populations of the western Pacific. They found that Type 2E and 8A are widely distributed in western Pacific populations, but Type 8B and 7A were confined to Papua New Guinea (PNG) and Guam, respectively. On the basis of these findings, they proposed several events of human dispersals in the western Pacific, carrying distinct genotypes of JCV (Type 8A, 8B, 2E, or 7A) (Yanagihara et al., 2002).

However, in the studies noted above (Jobes et al., 2001; Yanagihara et al., 2002), JCV isolates in the western Pacific were compared with only a small number of isolates from the Asian Continent and neighboring islands. In this study, we analyzed phylogenetic relationships among many JCV isolates in three broad areas, the Asian Continent, Southeast Asian islands (i.e. the Philippines), and the western Pacific. Complete DNA sequences of many JCV isolates in the Philippines were determined in this study and used in the phylogenetic analysis that included previously reported complete JCV DNA sequences of various Asian isolates (Kato et al., 2000; Saruwatari et al., 2002a; Sugimoto et al., 2002a) (some additional Asian isolates were sequenced in this study) and western Pacific isolates (Jobes et al., 2001; Yanagihara et al., 2002). The results obtained were discussed in the context of human dispersals in the western Pacific.

Materials and Methods

Urine Samples

Urine samples from 47 unrelated healthy subjects were collected with informed consent from each of the biggest linguistic groups in the Philippines: Tagalogs of Luzon Island, and Cebuanos of Cebu Island (Miranda et al., 2003). The subjects were native Tagalog and Cebuano speakers aged 40 years or older and residents of Nueva Ecija in Luzon Island and Cebu City in Cebu Island, respectively. In addition, we used urine samples collected previously at Pamalica Island (the Philippines), Chiang Mai (Thailand), X'ian (China), Lanzhou (China), and Urumqui (China) (Sugimoto et al., 1997; Guo et al., 2001).

DNA Analysis

Entire JCV DNAs were cloned into pUC19 at the unique *Bam*HI site as described previously (Yogo et al., 1991). The resultant complete JCV DNA clones were prepared using a QIAGEN Plasmid Maxi kit (QIAGEN GmbH, Hilden, Germany). Purified plasmids were sequenced as described previously (Sugimoto et al., 2002a).

Phylogenetic Analysis

The noncoding regulatory region of the JCV genome was excluded from phylogenetic analysis, as this region is hypervariable especially in JCV isolates derived from the brains of PML patients (Yogo and Sugimoto, 2001). DNA sequences were aligned using CLUSTAL W (Thompson et al., 1994) with a gap opening penalty of 15.00 and 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) using the CLUSTAL W program. Divergences were estimated with Kimura's two-parameter method (Kimura, 1980). To assess the confidence of branching patterns of the NJ tree, bootstrap probabilities (BPs) were estimated with 1,000 bootstrap replicates (Felsenstein, 1985) using CLUSTAL W. BPs larger than 70% were considered to be significant (Hillis and Bull, 1993). A phylogenetic tree was visualized using the TREEVIEW program (Page, 1996).

Results

By analyzing partial genomic sequences of JCV, we previously detected five genotypes of JCV, including B1-a, B3-a, B3-b, SC-f, and SC-x, in the Philippines (Sugimoto et al., 1997; Miranda et al., 2003); the general-type SC and SC/Phi (Miranda et al., 2003) were renamed SC-f and SC-x, respectively. We established complete JCV DNA clones representing these JCV genotypes (Table 1). In addition, we established seven continental clones belonging to B1-a and B3-a (Table 1). The complete JCV DNA clones (30 in total) thus obtained were sequenced. We confirmed that these complete sequences were not recombinant using the method described previously (Sugimoto et al., 2002a) (data not shown).

Table 1. JCV isolates whose complete DNA sequences were determined in this study

Isolate	Genotype	Geographic origin	Accession no.[a]
LZ-11	B1-a	Lanzhou, China	AB113216
LZ-12	B1-a	Lanzhou, China	AB113217
UR-2	B1-a	Urumqi, China	AB113143
XA-11	B1-a	X'ian, China	AB113144
PH-2	B1-a	Pamalican Is., Philippines	AB113138
PH-5	B1-a	Pamalican Is., Philippines	AB113139
PH-7	B1-a	Pamalican Is., Philippines	AB113140
Luz-4	B1-a	Luzon, Philippines	AB113135
Luz-6	B1-a	Luzon, Philippines	AB113137
Luz-11	B1-a	Luzon, Philippines	AB113127
Luz-16	B1-a	Luzon, Philippines	AB113129
UR-15	B3-a	Urumqi, China	AB113142
XA-9	B3-a	X'ian, China	AB113145
TL-6	B3-a	Chiang Mai, Thailand	AB113141
Luz-10	B3-a	Luzon, Philippines	AB113126
Luz-13	B3-a	Luzon, Philippines	AB113128
Ceb-5	B3-b/2E	Cebu, Philippines	AB113124
Luz-5	B3-b/2E	Luzon, Philippines	AB113136
Ceb-1	SC-f	Cebu, Philippines	AB113118
Ceb-2	SC-f	Cebu, Philippines	AB113122
Ceb-4	SC-f	Cebu, Philippines	AB113123
Luz-1	SC-f	Luzon, Philippines	AB113125
Luz-2	SC-f	Luzon, Philippines	AB113132
Luz-3	SC-f	Luzon, Philippines	AB113134
Ceb-14	SC-x	Cebu, Philippines	AB113119
Ceb-15	SC-x	Cebu, Philippines	AB113120
Ceb-16	SC-x	Cebu, Philippines	AB113121
Luz-18	SC-x	Luzon, Philippines	AB113130
Luz-19	SC-x	Luzon, Philippines	AB113131
Luz-20	SC-x	Luzon, Philippines	AB113133

[a] GSDB, DDBJ, EMBL and NCBI accession numbers.

We constructed an NJ phylogenetic tree from 138 complete sequences, the 30 complete JCV DNA sequences determined in this study plus the 108 complete sequences reported previously. The latter included isolates representing eleven genotypes of JCV worldwide (Kato et al., 2000; Sugimoto et al. 2002a), various intra-SC isolates in Myanmar and other Asian countries (Saruwatari et al., 2002a), and various isolates in the western Pacific (Jobes et al., 2001; Yanagihara et al., 2002); the origins of JCV isolates that were used in the phylogenetic analysis are shown in Table 2

Table 2. Origins of JCV isolates whose complete DNA sequences were used in the phylogenetic analysis (Figure 1)[a]

Geographic region	JCV isolates	Ref.[b]
Würzburg, Germany	GS/K, GS/B	1
Deventer, Netherlands	N4	2
Addis Ababa, Ethiopia	ET-3	2
Shirati, Tanzania	#308	3
Riyadh, Saudi Arabia	SA-3, -5	2
Port Louis, Mauritius	MU-3, -9	2
Varanasi, India	IN-6	2
Colombo, Sri Lanka	SL-2	2
Chiang Mai, Thailand	TL-2, -5, -6, -7	4, 5
Ulaanbaatar, Mongolia	MO-1, -3, -5, -6, -11	2
Yangon, Myanmar	MN-3, -6, -7, -11	4
Chaungtha Beach, Myanmar	CH-2, -7, -17	4
Myitkyina, Myanmar	MT-1, -2, -10, -14, -15, -22	4
Tiddim, Myanmar	TD-4, -6, -15, -19	4
Peinnebeen, Myanmar	PB-3, -4, -5	4
Jakarta, Indonesia	ID-1	2
Masai, Malaysia	ML-1, -6	2
Taipei, China	C2, C3	2, 4
Beijing, China	CB-2, -3	2
Wuhan, China	CW-2, -10	2, 4
Xi'an, China	XA-9, -11	5
Lanzhou, China	LZ-11, -12	5
Urumqi, China	UR-2, -15	5
Pamalican Is., Philippines	PH-2, -5, -7	5
Luzon, Philippines	Luz-1 to -6, -10, -11, -13, -16, -18 to -20	5
Cebu, Philippines	Ceb-1, -2, -4, -5, 14 to -16	5
Various cities, Japan	AT-8, CY, HR-7, MY, Tky-1, Tky-2, Tokyo-1	2, 6, 7
Papua New Guinea	#801 to #804, PNG8	8, 9
New Britain	#233	8
Guam	#234	8
Fiji	F53	9
Kiribati	K1	9
New Caledonia	NC23	9
Solomon Is.	SI30, SI40	9
Tonga	T10	9
Vanuatu	V16	9
Wallis and Futuna	WF4	9
Australia	L2001 to L2003	9
Chuuk	CHU	9
Hawaii, USA	HWN	9
Various cities, USA	#223 to #229, #230, #311, #312	3, 7

[a] Only isolates belonging to Type-B are shown.

[b] 1, Loeber and Dörries, 1988; 2, Sugimoto et al., 2002a; 3, Agostini et al., 1997b; 4, Saruwatari et al., 2002a; 5, this study; 6, Kato et al., 2000; 7, Agostini et al., 1998; 8, Jobes et al., 2001; 9, Yanagihara et al., 2002.

and elsewhere (Sugimoto et al., 2002a). On the resultant tree (Figure 1), we confirmed the first split of the ancestral JCV into three superclusters, Type-A, -B, and -C (Sugimoto et al., 2002a). We also confirmed that all JCV isolates detected in Southeast-Asian and western-Pacific islands belong to Type-B.

We found that JCV isolates in Southeast-Asian islands and western-Pacific islands are classified as one of the seven genotypes, 8A, 8B, B1-a, B3-a, B3-b, SC-f, and SC-x. These oceanic genotypes, except for SC-f, were identified as distinct clusters with high BPs (100%). Although the BP (62%) for SC-f was not high, all members of this cluster carried the unique pentanucleotide deletion in the regulatory region of the genome (Saruwatari et al., 2002a).

A significantly high BP (86%) was obtained for the grouping of all intra-Type-B genotypes excluding Af2, confirming that Af2 was the first to split in Type-B (Sugimoto et al., 2002a). However, no apparent grouping of intra-Type-B genotypes, including the oceanic genotypes, was supported by high BPs (Figure 1). This observation indicated that the order of the other splits in Type-B remained unclear.

According to the phylogenetic tree (Figure 1) and Table 2, we describe below the regional origins of the isolates belonging to each oceanic genotype. We tentatively classified the western-Pacific islands into Near, Middle, and Remote Oceania. Near Oceania included PNG and New Britain; Middle Oceania corresponded to the eastern part of Melanesia and included the Solomon islands, New Caledonia, Vanuatu, Fiji, and Wallis and Futuna; and Remote Oceania corresponded to Polynesia and included Kiribati, Tonga, Chuuk, and Hawaii.

1) 8A included three isolates in Near Oceania. No isolate in the Philippines and the Asian Continent belonged to 8A.
2) 8B included two isolates in Near Oceania, four in Middle Oceania, and two in Remote Oceania. No isolate in the Philippines and the Asian Continent belonged to 8B.
3) B1-a included seven isolates in the Philippines, one in Malaysia, one in Taiwan, and five in mainland China.
4) B3-a included two isolates in the Philippines and two in mainland China, and one in Thailand.
5) B3-b/2E included two isolates in the Philippines, one in Guam, one in Near Oceania, two in Middle Oceania, and one in Remote Oceania. In addition, three isolates in Australia belonged to this genotype.
6) SC-f included six isolates in the Philippines, one in Indonesia, one in Malaysia, six in Myanmar, two in mainland China, and one in Taiwan. Probably, about half of the Guam isolates whose partial genomic sequences were analyzed (Jobes et al., 2001) belonged to SC-f/7A, as they carried the pentanucleotide deletion unique to SC-f (Saruwatari et al., 2002a).
7) SC-x included six Filipino and one Hawaiian isolate (HWN). HWN was previously assigned to 7A according to a phylogenetic analysis using a data set without the SC-x sequences in the Philippines (Yanagihara et al., 2002).

Table 3 summarizes the geographic distribution of the seven oceanic genotypes.

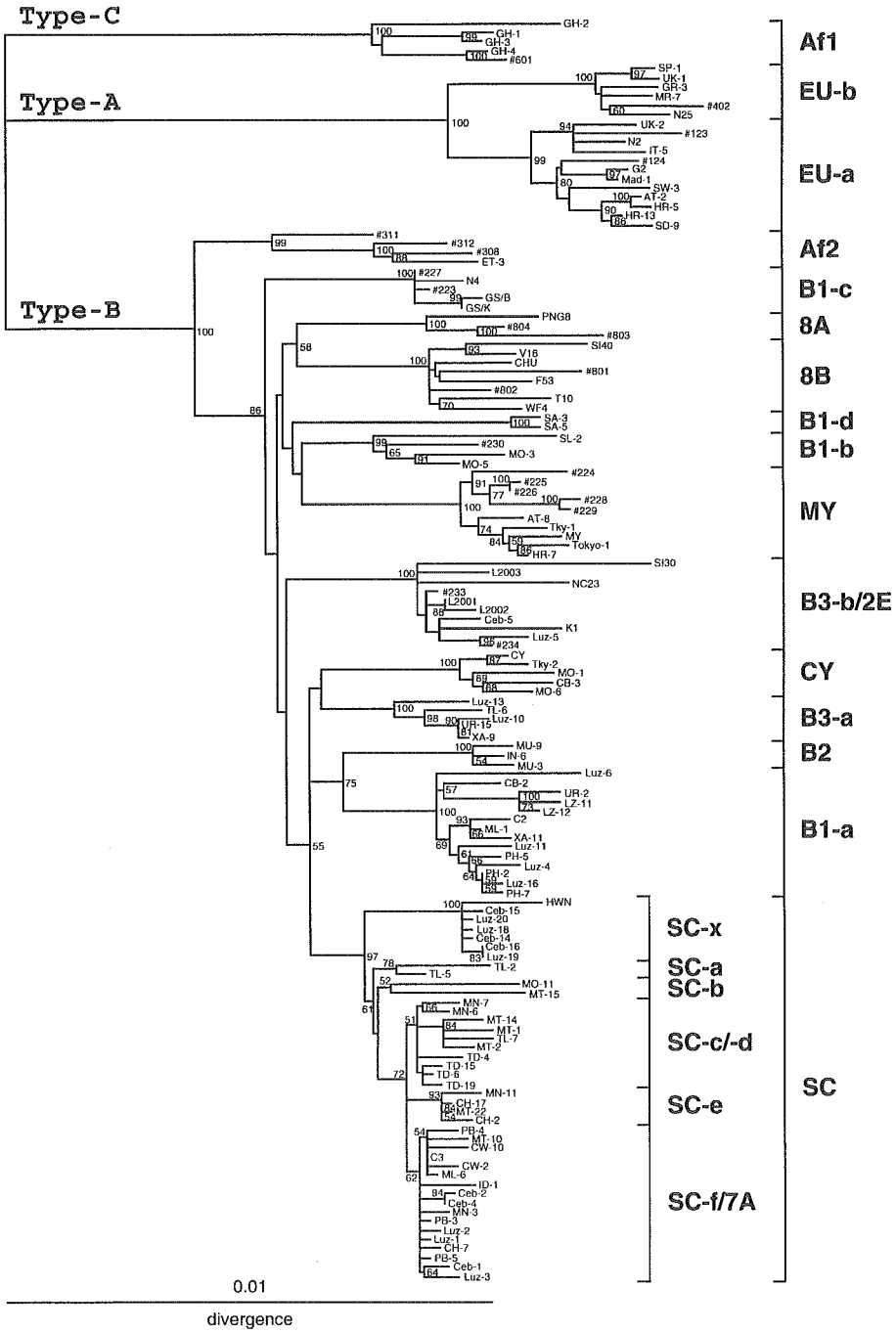

Figure 1. NJ phylogenetic tree relating 138 complete JCV DNA sequences. A phylogenetic tree was constructed from the complete sequences, excluding regulatory sequences, using the NJ method. The phylogenetic tree was visualized using the TREEVIEW program (Page, 1996). The symbols for sequences are shown in Table 2 and elsewhere (Sugimoto et al., 2002a). The numbers at nodes in the tree indicate the BPs (percent) obtained by 1,000 replicates (only those ≥50% are shown). Superclusters, subtypes of JCV, and intra-SC subgroups are indicated.

Discussion

Using the whole genome approach with which a reliable phylogeny of JCV isolates can be reconstructed (Jobes et al., 1998; Hatwell and Sharp, 2000), Stoner and his colleagues (Jobes et. al., 2001; Yanagihara et al., 2002) detected four genotypes (8A, 8B, 2E, and 7A) in the island populations of the western Pacific. In the present study, using the same approach, we detected five genotypes (B1-a, B3-a, B3-b, SC-f, and SC-x) in Filipino populations. In addition, we found that a single isolate previously detected in Hawaii (Yanagihara et al., 2002) belonged to SC-x, although this isolate was previously thought to belong to 7A (Yanagihara et al., 2002). As B3-b and SC-f corresponds to 2E and 7A, respectively, we concluded that seven genotypes of JCV (designated as the oceanic genotypes of JCV) were mainly

Table 3. Geographic distribution of the seven oceanic genotypes of JCV

Geographic region	Occurrence of genotype[a]						
	SC-f/7A	B1-a	B3-a	B3-b/2E	SC-x	8A	8B
Asian Continent	+	+	+	−	−	−	−
Philippines	+	+	+	+	+	−	−
Guam	+[c]	−	−	+	−	−	−
Near Oceania[b]	−	−	−	+	−	+	+
Middle Oceania[c]	−	−	−	+	−	−	+
Remote Oceania[d]	−	−	−	+	+	−	+

[a] Determined according to Figure 1 with a single exception of SC-f/7A in Guam.
[b] Including PNG and New Britain.
[c] Including the Solomon islands, New Caledonia, Vanuatu, Fiji, and Wallis and Futuna.
[d] Including Kiribati, Tonga, Chuuk, and Hawaii.
[e] Determined based on the presence of the distinct pentanucleotide deletion (Saruwatari et al., 2002a).

spread in the island populations in Southeast Asia and the western Pacific. We confirmed that on a phylogenetic tree all seven genotypes split from the Type-B supercluster, suggesting that they all originated from the Asian Continent.

Three oceanic genotypes of JCV (B1-a, B3-a, SC-f) were spread in the Asian Continent as well as Southeast Asian islands, but they were not detected in the western Pacific. Yanagihara et al. (2002) suggested that relatively recent movements of Asians caused the spread of SC-f/7A. Based on the present findings, we further suggested that two other genotypes of JCV (B1-a and B3-a) represent relatively recent movements from the Asian Continent to the neighboring islands.

Four oceanic genotypes of JCV (2E, 8A, 8B, and SC-x) with unique regional distributions occur in the western Pacific. 2E, 8A, and 8B occurred in Near Oceania, 2E and 8B in Middle Oceania, and 2E, 8B, and SC-x in Remote Oceania (Table 3). As these oceanic genotypes were rarely detected in the Asian Continent, they may represent ancient human migrations to the Pacific. Furthermore, our findings suggested that the island populations in Near, Middle, and Remote Oceania were formed by a different combination of a few ethnic groups, each carrying 8A, 8B, 2E, or SC-x.

SC-x was previously detected as the second most abundant genotype in the Philippines (Miranda et al., 2003). This genotype has not been detected in other Southeast Asian countries, including Malaysia, Indonesia, Thailand, Vietnam, and Myanmar (Sugimoto et al., 1997; Guo et al., 2001; Saruwatari et al., 2002a, b). In this study, however, we found that a single isolate (HWN) that was detected in a part-Hawaiian man and assigned to 7A (Yanagihara et al., 2002) belonged to SC-x. This finding suggested that SC-x might represent a novel human migration not identified so far. Further search for SC-x in other Pacific islands is required to define this migration.

The following view on the peopling of the Pacific is generally accepted. The ancestors of the so-called Melanesians settled in New Guinea around 30,000–50,000 years ago. Their expansion was limited to the surrounding islands for a long time until the Austronesians arrived 3,500–5,000 years ago. These new migrants, bearing the Lapita culture complex (Bellwood, 1978), first appeared in the Bismark Archipelago, and moved probably in canoes, via Vanuatu and New Caledonia, and to Polynesian islands, including Fiji, Tonga, and Samoa (see a review by Gibbons, 2001).

However, it has been open to debate as to how much they intermixed along the way with the indigenous people (the Melanesians). Furthermore, it remains to be clarified exactly where the sailors came from. Archaeologists, linguists, and geneticists appear to agree on some degree of mixing (Gibbons, 2001). However, disputes still remain regarding the origin of the Austronesians. Linguists suggested Taiwan, but archeologists and geneticists pointed to other areas of Southeast Asia (Gibbons, 2001).

The finding of multiple genotypes of JCV (mainly 2E and 8B) in Middle and Remote Oceania is consistent with a substantial degree of intermixture between the Austronesians and the indigenous Melanesians in Middle and Remote Oceania. Furthermore, the detection of 2E in the Philippines suggested that the Austronesians originated from an area of Southeast Asia, including the Philippines, if 2E accompanied the Austronesian movements in the Pacific, as suggested by Yanagihara et al. (2002).

On the whole, the JCV genotyping approach appears to promise to provide new insights into the peopling of the Pacific. Indeed, only one or two isolates were analyzed in each Pacific island, except for PNG and the Philippines. Therefore, in future studies, substantial numbers of samples should be analyzed in various Pacific and Asian islands.

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JC virus genotype profile in the Mamanwa, a Philippine Negrito tribe, and implications for its population history

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Abstract JC virus (JCV) is ubiquitous in the human population, usually being transmitted from parents to children during cohabitation. JCV genotyping is a useful means of elucidating the origins of various ethnic groups in the world. We used this method to gain insights into the origin of the Mamanwa, a Philippine Negrito tribe in Northeast Mindanao. We found that the Mamanwa carried two major JCV genotypes, B3-b/2E and SC-f/7A. This was in contrast with the JCV genotype profile of modern Filipinos who carry up to five genotypes, with B3-b/2E showing only a low frequency. B3-b/2E is spread throughout Oceania but rare on the Asian continent. In contrast, SC-f/7A is spread throughout Southeast Asia (including neighboring Oceanic islands) but rare in Remote Oceania. The present findings thus suggest that the Mamanwa tribe was formed by early colonization by people carrying B3-b/2E followed by an admixture of more recent immigrants carrying SC-f/7A. As the indigenous tribe (the Chamorro) in the Mariana Islands has essentially the same JCV genotype profile as the Mamanwa, other indigenous tribes in Southeast Asian and Oceanic islands may have a population history analogous to that suggested for the Mamanwa.

Key words: Mamanwa, Philippine Negrito, population history, JC virus genotype

Introduction

The Mamanwa (or Mamanua) people of Northeast Mindanao in the Philippines belong to the hunter-gatherer Negritos of Southeast Asia and the western Pacific, and are presumed to be among the oldest indigenous peoples in the region (Omoto, 1984). Direct ancestors of the present-day Mamanwa have been postulated to be either the Proto-Malay population of late Pleistocene Sundaland (Omoto, 1984), or the Negritos from Borneo, Sumatra, and Malaya who came via the still remaining land bridges some 30,000–25,000 years ago (Lagassé, 2001; Burton, 2003). The Indonesians and Malays followed in successive waves, and their descendants now account for the majority of modern Filipinos. As each new wave of migration entered the Philippines, the earlier peoples were either driven into the hinterlands, or assimilated.

More recent history, for instance, describes that for the whole duration of Spanish rule in the 1500s, indigenous populations in the Philippines, including the Mamanwa, endeavored to avoid colonization by resettling in more inaccessible and mountainous regions and practiced slash-and-burn farming along with hunting and foraging (Burton, 2003). In fact, pockets of the Philippine Negritos remain to this day in remote areas mostly along the Sierra Madre mountain range that extends along the entire eastern side of Luzon island in the northern Philippines (Headland, 2002). The Mamanwa is the only Negrito group in the southern Philippines, geographically separated from the other Negrito groups in Luzon and the central Philippines. Presently, there are about 1500 Mamanwas confined to the provinces of Agusan and Surigao in Northeast Mindanao.

To obtain information about the origins of the Mamanwa, this study attempted to elucidate JC virus (JCV) genotypes in a Mamanwa population in the province of Surigao del Norte in Northeast Mindanao, the Philippines. JCV is ubiquitous in the human population (Padgett and Walker, 1973), usually being transmitted from parents to children during cohabitation (Kunitake et al., 1995; Kato et al., 1997; Suzuki et al., 2002; Zheng et al., 2004). All JCV strains in the world constitute a single serotype (Major, 2001), but can be classified into more than ten major genotypes, with each occupying a unique geographical domain (Yogo et al., 2004). Distribution patterns of JCV genotypes have been found to be compatible with human migrations (Yogo et al., 2004). It was recently suggested that like modern humans, JCV originated in Africa (Pavesi, 2003). JCV genotyping analysis has helped gain new insights into the origins of ethnic groups worldwide (Yogo et al., 2004).

We recently investigated the JCV genotype profiles of modern urban Philippine populations, the Tagalogs of Luzon island, Cebuanos of Cebu island, and residents of Pamalican island in Palawan (Sugimoto et al., 1997; Miranda et al.,

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2003; Takasaka et al., 2004). Stoner and his colleagues reported JCV genotype profiles in Oceanic populations (Ryschkewitsch et al., 2000; Jobes et al., 2001; Yanagihara et al., 2002). Takasaka et al. (2004) elucidated the relationships among JCV genotypes in the Philippines and the western Pacific. Here, we report the JCV genotype profile in a Philippine Negrito population, the Mamanwa. We discuss the present findings in the context of human expansion in the Pacific as well as in relation to current knowledge about the genetics of the Mamanwa and other populations.

Materials and Methods

Urine samples

Urine samples were collected with informed consent from 49 unrelated Mamanwas, aged 40 years or older, residing in Mat-i, Sison and Surigao city of Surigao del Norte province in Northeast Mindanao, the Philippines. About 40 ml of urine samples were collected in 50 ml plastic tubes that contained 0.5 ml of 0.5 M EDTA, pH 8.0, and sent to Department of Urology, Faculty of Medicine, The University of Tokyo, where DNA was extracted as described previously (Kitamura et al., 1990).

DNA analysis

The 610 bp IG region was amplified by polymerase chain reaction (PCR) using primers P3 and P4 (Miranda et al., 2003). The IG region of the viral genome encompasses the 3′-terminal regions of both the T-antigen and VP1 genes, and was established as a region of the JCV genome that contains abundant type-determining sites (Ault and Stoner, 1992). The reaction was carried out for 50 cycles with PWO DNA polymerase (Roche Diagnostics, Tokyo, Japan) or ProofStart DNA polymerase (QIAGEN GmbH, Hilden, Germany). The amplified fragments were cloned into the vector pBluescript II SK (+) (Stratagene, La Jolla, USA) (Miranda et al., 2003), and purified recombinant plasmids were sequenced with an autosequencer (ABI PRISM 373S DNA Analyzer, Applied Biosystems, Foster City, USA).

Phylogenetic analysis

A neighbor-joining (NJ) phylogenetic tree (Saitou and Nei, 1987) was constructed using the CLUSTAL W program (Thompson et al., 1994). Divergences were estimated with the two-parameter method (Kimura, 1980). The phylogenetic tree was visualized using TREEVIEW (Page, 1996). To assess the confidence of branching patterns of the NJ trees, 1000 bootstrap replicates were performed (Felsenstein, 1985).

Statistic analysis

To test for genotypic differentiation between the populations, a log-likelihood (G)-based exact test was performed using Genepop version 3.1c at http://www.biomed.curtin.edu.au/genepop (Raymond and Rousset, 1995). The principle of this test is the same as the probability test (or Fisher exact test). The null hypothesis (H_0) tested was: 'the genotypic distribution is identical across populations'. The Markov chain parameters used were: dememorization: 1000; batches: 100; iterations per batch: 1000.

Results

Using a PCR that amplifies the IG region, we detected JCV DNA from 11 (22%) of the 49 urine samples collected from Mamanwas of Surigao province. This detection rate is lower than rates reported previously for the same age group (40 years or older) in many other populations (Kitamura et al., 1994; Agostini et al., 1996; Chang et al., 1999; Saruwatari et al., 2002a, b; Miranda et al., 2003). Nevertheless, lower detection rates for JCV DNA were also reported for some ethnic groups in Central Africa (Chima et al., 1998) and Northeast Siberia and the Arctic regions (Sugimoto et al., 2002).

We cloned IG regions amplified from the Mamanwa urine samples, and sequenced representative clones for each urine sample. We obtained single sequences from most urine samples, but detected two independent sequences from one urine sample (no. 29) (Table 1). From the 12 IG sequences (including both sequences from no. 29), together with reference sequences in Asia and the western Pacific (Table 2), a phylogenetic tree was constructed using the NJ method (Saitou and Nei, 1987). According to the resultant phylogenetic tree (Figure 1), half of the Mamanwa sequences were found in the B3-b/2E cluster and half in the SC-f/7A cluster. As two systems have been developed to designate JCV genotypes, we describe here a genotype named B3-b (Miranda et al., 2003) or Type 2E (Jobes et al., 2001) as B3-b/2E and a genotype named SC-f (Saruwatari et al., 2002b) or Type 7A (Agostini et al., 2001) as SC-f/7A. Although the bootstrap probabilities for these clusters were 50% or lower, representative isolates (shown by asterisks in Figure 1) formed distinct clusters with higher bootstrap probabilities in a phylogenetic analysis based on complete sequences (Takasaka et al., 2004). In addition, the genotypes of JCV based on the phylogenetic analysis were consistent with genotype-specific variations of the noncoding control region (Guo et al., 1996; Chang et al., 1999; Ryschkewitsch et al., 2000; and data not shown).

Table 3 shows the JCV genotype frequencies in three populations in the Philippines (Mamanwa, Tagalog, and Cebuano). The data for the Tagalog and Cebuano were from

Table 1. JCV isolates detected from Mamanwa urine samples

Urine samples	Isolates	Genotypes[a]	Accession no.[b]
No. 5	MMW-1	B3-b/2E	AB126814
No. 9	MMW-2	B3-b/2E	AB126815
No. 15	MMW-3	SC-f/7A	AB126816
No. 18	MMW-4	B3-b/2E	AB126817
No. 21	MMW-5	SC-f/7A	AB126818
No. 29	MMW-6	SC-f/7A	AB126819
No. 29	MMW-7	B3-b/2E	AB126820
No. 34	MMW-8	SC-f/7A	AB126821
No. 36	MMW-9	B3-b/2E	AB126822
No. 40	MMW-10	SC-f/7A	AB126823
No. 42	MMW-11	SC-f/7A	AB126824
No. 48	MMW-12	B3-b/2E	AB126825

[a] Determined according to the phylogenetic tree (Figure 1).
[b] GSDB, DDBJ, EMBL, and NCBI accession numbers for IG sequences.

Table 2. Geographic origins of JCV isolates, excluding those from the Mamanwa, whose IG sequences were used for the phylogenetic analysis (Figure 1)[a]

Isolates	Geographic origin
CB-1 to -7, -9	Beijing, China
CW-2, -3, -6, -10, -11	Wuhan, China
CD-5, -7	Chengdu, China
GZ-1, -4, -6, -9, -10, -11, -12	Guangzhou, China
HB-1 to -4, -6	Harbin, China
SJ-1, -2, -4, -5	Shenyang/Jinzhou, China
C1, C2, C3, C9	Taipei, Taiwan
D-3, -33; S-16	Nantou County, Taiwan
C-06; M-05, -06, -08, -10, -12, -14	Ishikawa Prefecture, Japan
M-11, -13	Tokyo, Japan
ON-10	Okinawa, Japan
MO-1, -3 to -7, -11	Ulaanbaatar, Mongolia
ID-1, -8, -11	Jakarta, Indonesia
ML-1, -4, -9	Masai, Malaysia
TL-1 to -5, -8	Chiang Mai, Thailand
MN-1, -8, -11, -14	Yangon, Myanmar
PH-1, -5, -6	Pamalican Is., Philippines
Ceb-1, -2, -5, -7 to -11	Cebu, Philippines
Luz-1, -3 to -7, -9 to -11, -14, -16 to -18, -20	Luzon, Philippines
IN-6	Varanasi, India
SL-1, -2, -4	Colombo, Sri Lanka
MU-3, -9	Port Louis, Mauritius
UZ-13	Tashkent, Uzbekistan
#233	New Britain
#234	Guam
#801 to #804	Papua New Guinea
G1	Illertissen, Germany

[a] Kunitake et al. (1995), Kitamura et al. (1997), Sugimoto et al. (1997), Chang et al. (1999), Jobes et al. (2001), Saruwatari et al. (2002a), and Miranda et al. (2003).

our previous report (Miranda et al., 2003). SC-f/7A and B3-b/2E each accounted for 50% of all genotypes detected in the Mamanwa. This was in contrast with the profiles for the other Philippine populations which carried up to five genotypes, with B3-b/2E showing only a low frequency. The test for genotype differentiation showed that the JCV genotypic distribution in the Mamanwa was significantly different from that in the Tagalog and Cebuano populations (Table 4).

Discussion

Results of the present study show that the JCV genotype profile of the Mamanwa is significantly different from the profiles of two other Philippine populations, the Tagalogs of Luzon island and the Cebuanos of Cebu island, in which up to five genotypes (B1-a, B3-a, B3-b/2E, SC-f/7A, and SC-x) were detected and B3-b/2E had only a low frequency (Miranda et al., 2003). The JCV genotype patterns in the Tagalogs and the Cebuanos were somewhat consistent with the traditional view about the formation of the modern Philippine population (see Introduction), although it also suggested some previously undescribed migrations to the Philippines, recent as well as ancient (Miranda et al., 2003). In contrast, the JCV genotype profile of the Mamanwa suggests that this tribe was formed by two major events of colonization, one involving carriers of B3-b/2E and the other, carriers of SC-f/7A.

The question then arises as to which colonization occurred first. Before discussing this issue, we will summarize recent findings about the JCV genotypes in Oceania. Seven oceanic JCV genotypes (B1-a, B3-a, B3-b/2E, SC-f/7A, SC-x, 8A, and 8B) have been described (Ryschkewitsch et al., 2000; Jobes et al., 2001; Yanagihara et al., 2002; Miranda et al., 2003; Takasaka et al., 2004). All of these genotypes belong to the Type-B supercluster from which most Asian genotypes were generated (Takasaka et al., 2004). The Oceanic genotypes were classified into three categories (herein designated groups 1–3) on the basis of their geographic distribution patterns (Takasaka et al., 2004). Group 1 includes three genotypes (SC-f/7A, B1-a, and B3-a) distributed on the Asian continent as well as islands near the continent; group 2 includes two genotypes (B3-b/2E and SC-x) distributed in islands near the Asian continent as well as in the western Pacific; and group 3 includes two genotypes (8A and 8B) distributed only in the western Pacific. It was suggested that groups 2 and 3, which are rarely detected on the Asian continent, represent ancient human migration, while group 1, which is detected both in the Pacific and on the Asian continent, represents recent human migration (Takasaka et al., 2004). We detected B3-b/2E and SC-f/7A equally in the Mamanwa. According to the classification above, B3-b/2E and SC-f/7A are classified as group 2 and group 1 genotypes, respectively. Thus, the occurrence of B3-b/2E in the Mamanwa may suggest ancient colonization, while the occurrence of SC-f/7A may represent recent colonization.

Ryschkewitsch et al. (2000) recently reported the JCV genotype profile of the indigenous tribe (the Chamorro) in Guam. Of the 20 isolates examined, nine (45%) were classified as B3-b/2E, and eight (40%) as SC-f/7A (the others were European types probably imported recently). This genotype profile suggests that Chamorros have a population history similar to that assumed for the Mamanwa, i.e. an earlier colonization by people carrying B3-b/2E followed by later migrants carrying SC-f/7A. It is tempting to postulate that other indigenous tribes in Southeast Asian and Oceanic islands near the Asian continent may have analogous population histories. If our hypothesis is correct, then the genetic differences previously observed between the Mamanwa and other Philippine and Asian populations (Matsumoto et al., 1979; Omoto, 1980, 1984; Horai et al., 1981; Omoto et al., 1981; Davila et al., 2002) may be explained by the difference in the ratios of the two ancestral populations carrying B3-b/2E or SC-f/7A.

Interestingly, a close correlation between the Mamanwa and the Chamorro was previously suggested by the genetic observation that a carbonic anhydrase-1 variant called CA1-3N frequently occurs in both the Mamanwa and the Chamorro (Omoto, 1980; Omoto et al., 1981). Furthermore, this variant shows a broad distribution in the western Pacific (Omoto, 1980; Omoto et al. 1981). The languages spoken by islanders in a wide area of the western Pacific, including the Mamanwas and Chamorros, belong to the Austronesian family (Ruhlen, 1987). All in all, it is likely that ancient Austronesians carried the CA1-3N variant as well as the B2-b/2E genotype of JCV and that as they spread in Oceania, the

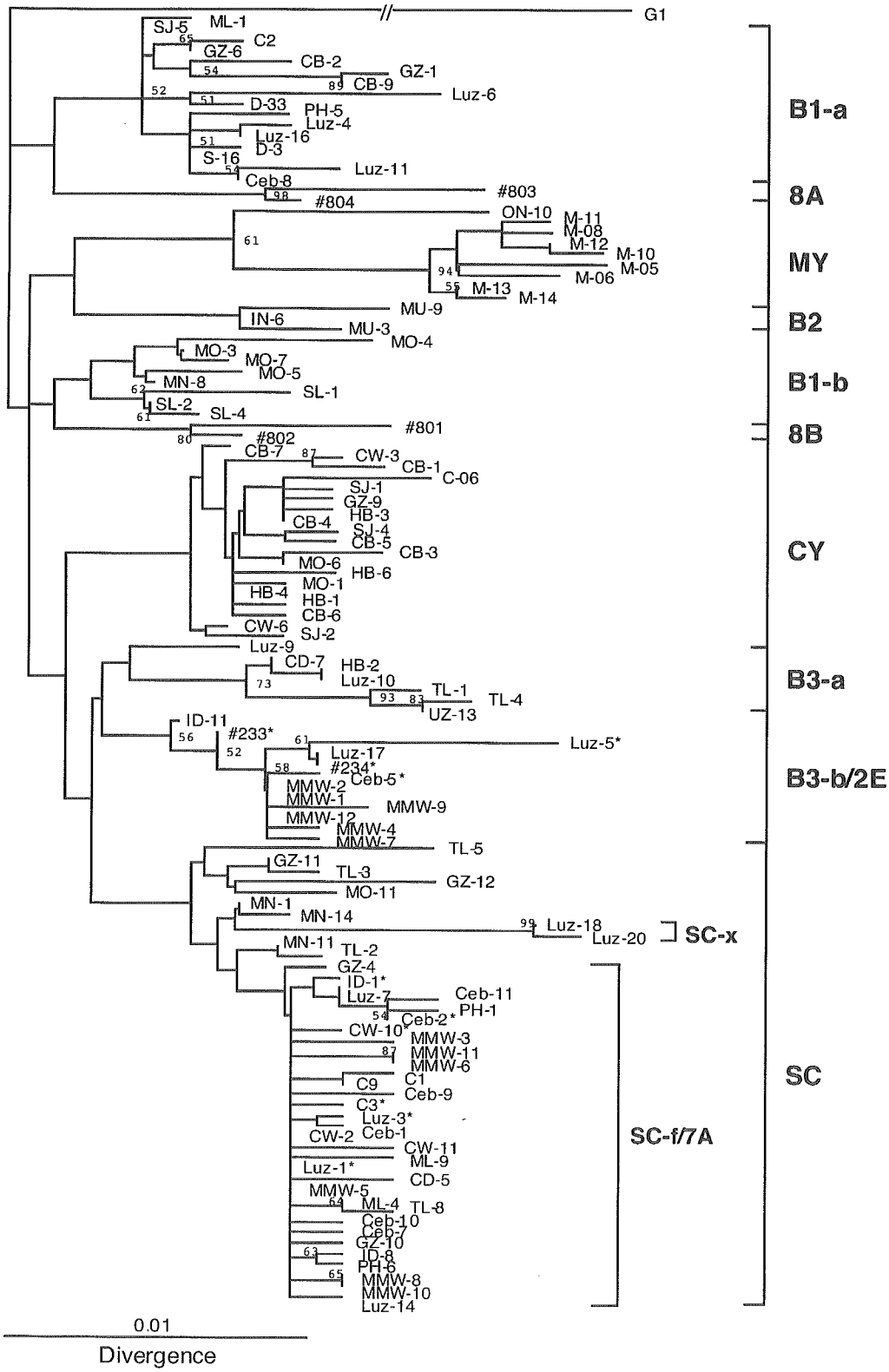

Figure 1. Phylogenetic tree used to classify the JCV isolates detected into distinctive genotypes. IG sequences in Mamanwas together with those detected previously in Asia and Oceania were used to construct an NJ phylogenetic tree using CLUSTAL W (Thompson et al., 1994). The phylogenetic tree was visualized using TREEVIEW (Page, 1996). The tree was rooted using a European isolate (G1) as the outgroup. Genotypes are indicated at the right of the tree. Asterisks identify isolates (within the B3-b/2E and SC-f/7A genotypes) previously analyzed using the whole genome approach (Takasaka et al., 2004). Origins of isolates are shown in Tables 1 and 2. The numbers at nodes give bootstrap confidence level (%) obtained for 1000 replicates (only values ≥50% are shown for major nodes).

Table 3. JCV genotype profiles of various Philippine ethnic populations

Population (island)	No. of isolates analyzed	Frequency of JCV genotype (%)				
		SC-f/ 7A[a]	SC-x[b]	B1-a	B3-a	B3-b/2E
Mamanwas (Mindanao)	12	6 (50)	0 (0)	0 (0)	0 (0)	6 (50)
Tagalogs (Luzon)[c]	22	8 (36)	5 (23)	4 (18)	3 (14)	2 (9)
Cebuanos (Cebu)[c]	19	11 (58)	6 (32)	1 (5)	0 (0)	1 (5)

[a] Previously designated as general-type SC (Miranda et al., 2003).
[b] Previously designated as SC/Phi (Miranda et al., 2003).
[c] Cited from Miranda et al., 2003.

Table 4. Pairwise test for genotypic differentiation

Populations	*P*	S.E.
Mamanwa vs. Tagalog	0.00531	0.00067 (significant)
Mamanwa vs. Cebuano	0.00574	0.00058 (significant)
Tagalog vs. Cebuano	0.27312	0.00404 (not significant)

The data in Table 3 were examined using the log-likelihood (G)-based exact test (see Materials and Methods).

genetic as well as JCV markers expanded in the region. An analogous view was proposed by Yanagihara et al. (2002), who suggested that Austronesians carrying B3-b/2E migrated into Oceania 3500 years ago or more; the earliest migrants (ancestors of the modern Papuans and Melanesians) were thought to have brought genotypes 8A and 8B about 40,000 years ago (Yanagihara et al., 2002).

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New sequence polymorphisms in the outer loops of the *JC polyomavirus* major capsid protein (VP1) possibly associated with progressive multifocal leukoencephalopathy

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JC polyomavirus (JCPyV) causes progressive multifocal leukoencephalopathy (PML) in patients with decreased immune competence. To elucidate genetic changes in JCPyV associated with the pathogenesis of PML, multiple complete JCPyV DNA clones originating from the brains of three PML cases were established and sequenced. Although unique rearranged control regions occurred in all clones, a low level of nucleotide variation was also found in the coding region. In each case, a parental coding sequence was identified, from which variant coding sequences with nucleotide substitutions would have been generated. A comparison between the parental and variant coding sequences demonstrated that all 12 detected nucleotide substitutions gave rise to amino acid changes. Interestingly, seven of these changes were located in the surface loops of the major capsid protein (VP1). Finally, 16 reported VP1 sequences of PML-type JCPyV (i.e. derived from the brain or cerebrospinal fluid of PML patients) were compared with their genotypic prototypes, generated as consensus sequences of representative archetypal isolates belonging to the same genotypes; 13 VP1 proteins had amino acid changes in the surface loops. In contrast, VP1 proteins from isolates from the urine of immunocompetent and immunosuppressed patients rarely underwent mutations in the VP1 loops. The present findings suggest that PML-type JCPyV frequently undergoes amino acid substitutions in the VP1 loops. 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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INTRODUCTION

JC polyomavirus (JCPyV) is the causative agent of a demyelinating disease of the central nervous system, progressive multifocal leukoencephalopathy (PML) (Walker, 1985), and is widespread in humans. Primary infection occurs asymptomatically during childhood (Padgett & Walker, 1973). JCPyV is then disseminated throughout the body,

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are AB183534–AB183544 and AB190446–AB190453 (see Table 1).

probably through viraemia (Ikegaya *et al.*, 2004). It is well established that JCPyV persists in renal tissue (Chesters *et al.*, 1983; Tominaga *et al.*, 1992; Kitamura *et al.*, 1997; Aoki *et al.*, 1999). Nevertheless, JCPyV also persists in other sites, including lymphoid tissues and peripheral blood lymphocytes (Gallia *et al.*, 1997; Kato *et al.*, 2004).

The genome of JCPyV has a non-coding control region (CR) between the origin of replication and the start site of the agnogene (Frisque *et al.*, 1984). JCPyV CRs in the brain of PML patients (PML-type CRs) are so variable that identical PML-type CRs have never been detected in different PML patients (Yogo & Sugimoto, 2001). In contrast, JCPyV CRs detected in the urine, kidney and tonsils of immunocompetent individuals have the same basic structure, designated the archetype (Yogo & Sugimoto, 2001; Kato *et al.*, 2004). Yogo & Sugimoto (2001) formulated a correlation between archetype and PML-type JCPyV as the archetype concept, consisting of the following five principles: (i) JCPyV with the archetype CR circulates in the human population; (ii) the archetype CR is highly conserved, in marked contrast to the hypervariable CRs (PML-type CRs) of JCPyV in the brain of PML patients; (iii) each PML-type CR is produced from the archetype by deletion and duplication or by deletion alone; (iv) the shift of the CR from archetype to PML type occurs during persistence in the host; and (v) PML-type JCPyV never returns to the human population.

The archetype concept adequately explains changes in the JCPyV CR from a molecular epidemiological standpoint. However, this concept does not address a medically important issue, i.e. whether these changes are involved in pathogenesis of PML. A few studies have challenged this issue by using *in vitro* expression assays (Sock *et al.*, 1996; Ault, 1997). According to the results of these studies, there is little doubt that JCPyV with archetype CR can propagate in the human brain. Indeed, O'Neill *et al.* (2003) recently demonstrated successful propagation of an archetypal JCPyV strain in human fetal brain cells. Thus, the question remains open as to why JCPyV DNAs in the brains of PML patients regularly undergo sequence rearrangement in their CRs.

JCPyV DNA replicates in the nucleus by using a cellular DNA polymerase with proofreading activity. Therefore, the fidelity of JCPyV DNA replication is thought to be as high as that of cellular DNA replication. Nevertheless, by sequencing five to eight complete JCPyV DNA clones established from each family member, we found that nucleotide substitutions sometimes occur in the coding region of JCPyV (Zheng *et al.*, 2004a). The coding region of JCPyV is about 4800 bp in size and encompasses several genes that encode at least six viral proteins (agnoprotein, capsid proteins VP1–3 and large and small T antigens) (Frisque *et al.*, 1984). Nucleotide changes in the coding region have been used successfully as a marker for distinguishing between JCPyV variants transmitted to offspring and those not transmitted (Zheng *et al.*, 2004a).

Furthermore, we recently analysed 29 complete JCPyV DNA sequences detected in autopsied brain tissue in a paediatric case of PML (Zheng *et al.*, 2004b). From the results obtained, it was concluded that, in the studied case, nucleotide substitution (and resultant amino acid change) was not involved in either the genesis of rearranged JCPyV or the expansion of demyelinated lesions in the brain. However, from the autopsied brain in the same PML case noted immediately above, a single nucleotide substitution occurred in three rare clones with a rearranged CR sequence. As the studied PML case was atypical in terms of the patient's age, it could be that, in adult PML patients, the stability of the coding sequences might decrease (i.e. more nucleotide substitutions might occur in the coding region).

Thus, changes in the coding region, if combined with CR rearrangements, might offer useful information about the history of JCPyV DNAs from persistence to reactivation. In this study, an overall analysis of JCPyV DNA sequences from the brain tissue of three adult PML patients was performed. It was found that all nine detected nucleotide substitutions gave rise to amino acid changes; these changes frequently occurred in the surface loops of the major capsid protein (VP1). To confirm this finding, 16 reported VP1 sequences from PML-type isolates were compared with their closest typological prototypes.

METHODS

Patients. As a detailed case report was described previously (case 1) (Hall *et al.*, 1991) or will be described elsewhere (cases 2 and 3), cases are only described briefly here.

Case 1. A 33-year-old homosexual male was admitted to North Shore University Hospital, New York, USA, with AIDS. He developed various opportunistic infections before and after hospitalization. On 2 May 1988, he exhibited a change in mental status, becoming extremely confused and progressively disoriented as to time and place. He continued to do poorly and developed cortical blindness. The patient became unresponsive and died on 4 June 1988. Pathological findings, including the detection of JCPyV DNA by *in situ* hybridization, have been documented previously (Hall *et al.*, 1991).

Case 2. A 59-year-old female with progressive impairment of memory, motor aphasia and right-sided weakness was admitted to Juntendo University Hospital, Tokyo, Japan, on 7 December 2000. Three years earlier, she had been diagnosed with mixed connective tissue disease based on the presence of antinuclear antibodies, anti-ribonucleoprotein positivity and clinical symptoms. She had been treated with predonisolone and azathioprine. Cerebral magnetic resonance imaging (MRI) performed upon administration showed progressive, confluent, non-enhancing lesions in the left frontal subcortical white matter. The cerebrospinal fluid (CSF) was positive for JCPyV DNA by nested PCR. The patient became unresponsive and died on 1 March 2001.

Case 3. A 20-year-old male had suffered from chronic candidiasis of the skin and mucosa since early childhood. As he developed a

motor disturbance of the right upper limb and difficulty in speech at the end of August 2001, he was admitted to Saitama Children's Medical Center, Iwatsuki, Japan. Based on a diagnosis of multiple sclerosis, he received γ-globulin and steroid-pulse therapy. However, his symptoms got worse and he presented quadriplegia and pseudobulbar paralysis. T2-weighted brain MRI showed a high-intensity area in a white-matter area of the right parietal lobe. JCPyV DNA was detected by nested PCR in CSF collected on 23 October 2001. He was unresponsive to either plasma-exchange therapy or intravenous interleukin 2 and Ara-C injection. He became unconscious in January 2002 and died from septicaemia on 29 October 2003.

Molecular methods. Autopsied brain tissue was digested with 100 μg proteinase K ml^{-1} at 56 °C for 1 h in the presence of 0·5 % SDS. The digest was extracted once with phenol and once with chloroform/isoamyl alcohol (24 : 1). DNA was recovered by ethanol precipitation and dissolved in water. Entire JCPyV DNAs were cloned into pUC19 at the unique *Bam*HI site as described previously (Yogo *et al.*, 1991a). The resultant recombinant plasmids containing complete JCPyV DNA sequences were prepared by using a Qiagen Plasmid Midi kit. Purified plasmids were sequenced as described previously (Sugimoto *et al.*, 2002a).

Phylogenetic analysis. The determined and reference sequences were aligned by using the program CLUSTAL W (Thompson *et al.*, 1994). Aligned sequences were subjected to phylogenetic analysis by using the neighbour-joining (NJ) method (Saitou & Nei, 1987). Phylogenetic trees were constructed by using CLUSTAL W and divergences were estimated by the two-parameter method (Kimura, 1980). Phylogenetic trees were visualized by using TREEVIEW (Page, 1996). To assess the confidence of branching patterns of the NJ trees, 1000 bootstrap replications were performed (Felsenstein, 1985). Translation of nucleotide sequences into amino acid sequences and alignment of multiple amino acid sequences were performed with GENETYX-MAC ver. 11.10 (GENETYX).

RESULTS

CR sequence variations

From DNA extracted from autopsied brain tissue, complete JCPyV DNAs were cloned by using a plasmid vector. Twelve, 14 and 14 complete JCPyV DNA clones were established in cases 1, 2 and 3, respectively. The CR and coding sequences of all these clones were determined. Two (1A and 1B), three (2A, 2B and 2C) and three (3A, 3B and 3C) rearranged CR sequences were identified in cases 1, 2 and 3, respectively (Table 1). The structures of the detected CRs are shown diagrammatically in Fig. 1, with reference to the archetype at the top. Deletions in rearranged CR sequences are shown as gaps and duplications are depicted by parallel lines. CRs 1A and 1B were identical to CRs detected previously in the brain of the same patient (Yogo *et al.*, 1994).

Coding sequence variations

Three (1-1 to 1-3), three (2-1 to 2-3) and five (3-1 to 3-5) complete coding sequences of JCPyV were detected in cases 1, 2 and 3, respectively (Table 1). Nucleotide differences were examined among detected sequences in each

Table 1. JCPyV DNA sequences determined in this study

Sequence	Case	Region of the JCPyV genome*	GenBank accession no.
1A	1	Regulatory	AB183534
1B	1	Regulatory	AB183535
2A	2	Regulatory	AB183536
2B	2	Regulatory	AB183537
2C	2	Regulatory	AB183538
3A	3	Regulatory	AB190446
3B	3	Regulatory	AB190447
3C	3	Regulatory	AB190448
1-1	1	Coding	AB183539
1-2	1	Coding	AB183540
1-3	1	Coding	AB183541
2-1	2	Coding	AB183542
2-2	2	Coding	AB183543
2-3	2	Coding	AB183544
3-1	3	Coding	AB190449
3-2	3	Coding	AB190450
3-3	3	Coding	AB190451
3-4	3	Coding	AB190452
3-5	3	Coding	AB190453

*Regulatory region: a region from the midpoint of the origin of replication to the position immediately before the start site of the agnogene. Schematic representations of the regulatory sequences are given in Fig. 1. Coding region: a region of about 4800 bp in size encompassing several genes that encode at least six viral proteins (agnoprotein, capsid proteins VP1–3 and large T and small t antigens) (Frisque *et al.*, 1984).

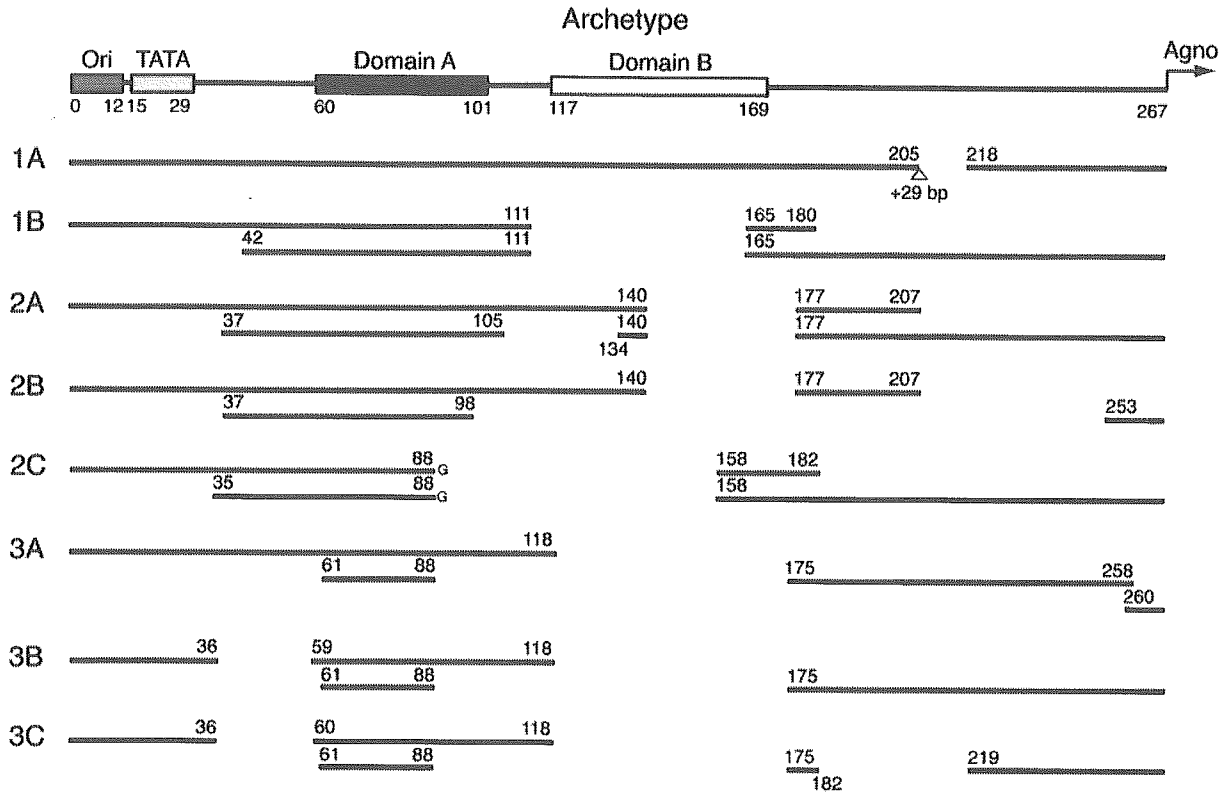

Fig. 1. Diagrammatic representation of the detected JCPyV CR sequences. The structure of the archetypal CR is shown schematically at the top. The origin of replication (Ori), TATA sequence and agnogene (Agno) are indicated (Frisque *et al.*, 1984). Domain A indicates a sequence duplicated in many PML-derived JCPyV isolates and domain B indicates a sequence deleted in many PML-derived JCPyV isolates (Iida *et al.*, 1993). The JCPyV CRs detected in this study (1A, 1B, 2A, 2B, 2C, 3A, 3B and 3C) are shown, with deletions relative to the archetype described as gaps. Reading from left to right, when a repeat is encountered, the linear representation is displaced to the line below and to a position corresponding to the sequence of the archetype. Numbers below each box and lines are nucleotide numbers indicating end locations [nucleotide numbers are those of the archetype (Yogo *et al.*, 1990)].

case (Table 2). Sequences in cases 1 and 2 showed single nucleotide polymorphisms (SNPs) at two positions, whereas those in case 3 showed SNPs at five positions. Sequences in cases 1 and 2 were distinguished by one nucleotide difference, with the exception that 1-1 and 1-2 were distinguished by two nucleotide substitutions, whereas those in case 3 were distinguished by one to four nucleotide substitutions. Numbers of clones with individual CR and coding sequences are shown in Table 3.

Relationships among the coding sequences detected in each case

Classification of JCPyV DNAs detected in the three cases into genotypes was attempted by using NJ phylogenetic analysis (Saitou & Nei, 1987) based on complete coding sequences of JCPyV. According to the resultant phylogenetic tree (not shown), the JCPyV DNAs detected in cases 1, 2 and 3 belonged to genotypes EU-a2, CY-a and MY-b, respectively. These genotypes were recently recognized as independent genotypes based on phylogenetic analysis using complete JCPyV DNA sequences (Zheng *et al.*, 2003, 2004c; Ikegaya *et al.*, 2005).

To elucidate the ancestral states for polymorphic sites in the coding sequences (Table 2), the complete coding sequences detected in each case and a number of reference sequences belonging to the same genotype of JCPyV as that to which the detected sequences belonged were aligned. The latter included 10 EU-a2, 13 CY-a and 18 MY-b sequences, shown in Table 4. A consensus nucleotide identified at each polymorphic site was considered to be the ancestral state. Thus, sequences 1-1, 2-1 and 3-1, detected in cases 1, 2 and 3, respectively, were found to contain the ancestral states at all polymorphic sites (Table 2) and thus designated parental coding sequences.

NJ phylogenetic trees were constructed from the complete coding sequences detected in cases 1, 2 and 3, together with many reported sequences grouped as EU-a1 and -a2, CY-a and -b, and MY-b, respectively (Table 4). On the resultant tree (Fig. 2a), the sequences detected in case 1 (1-1 to 1-3)

Table 2. Nucleotide variations among the coding sequences detected in each case

Nucleotides (amino acids) at positions of the Mad-1 genome (Frisque *et al.*, 1984) are shown. See text for explanation of ancestral sequences.

Coding sequence	nt 2274*	nt 3329†
Case 1		
1-1	C (S)	C (K)
1-2	T (F)	G (N)
1-3	T (F)	C (K)
Ancestral	C (S)	C (K)
	nt 1650*	**nt 4778‡**
Case 2		
2-1	C (S)	C (S)
2-2	T (L)	C (S)
2-3	C (S)	G (T)
Ancestral	C (S)	C (S)

	nt 599§	nt 840§	nt 1647*	nt 1836*	nt 2274*
Case 3					
3-1	C (S)	G (W)	A (K)	C (S)	C (S)
3-2	C (S)	G (W)	A (K)	C (S)	A (Y)
3-3	C (S)	C (C)	T (M)	C (S)	C (S)
3-4	T (L)	G (W)	A (K)	G (C)	C (S)
3-5‖	T (L)	G (W)	A (K)	G (C)	C (S)
Ancestral	C (S)	G (W)	A (K)	C (S)	C (S)

*Located in the VP1 gene.
†Located in the large T gene.
‡Located in the small t/large T genes.
§Located in the VP2 gene.
‖Other change, duplication of a 6 bp segment (nt 2744–2749) within the large T gene.

clustered together with a high bootstrap probability (96 %), with 1-1 located at the node. Similarly, the sequences detected in case 2 (2-1 to 2-3) clustered together with a high bootstrap probability (98 %), with 2-1 located at the node (Fig. 2b). The sequences detected in case 3 (3-1 to 3-5), together with eight other MY-b sequences derived from unrelated individuals, formed a cluster on the phylogenetic tree (Fig. 2c), with sequence 3-1 at the node. The observation that the case 3 sequences and many other MY-b sequences clustered together (Fig. 2c) could be explained by assuming that the parental sequence (i.e. 3-1) in case 3 happened to be the ancestral sequence for the other sequences belonging to the cluster.

Amino acid variations among VP1 sequences detected in cases 1, 2 and 3

All nucleotide substitutions detected in JCPyV DNA clones in cases 1, 2 and 3 gave rise to amino acid changes (Table 2). Interestingly, seven of these substitutions caused amino acid changes in the major capsid protein, VP1. Although the crystal structure of the JCPyV VP1 has not yet been elucidated, it can be assumed that it is similar to that of the crystallized *Simian virus 40* (SV-40) VP1 protein (Liddington *et al.*, 1991), as the amino acid sequences between JCPyV and SV-40 VP1 proteins are highly similar (Shishido-Hara & Nagashima, 2001). Thus, by analogy with the SV-40 VP1 structure (Liddington *et al.*, 1991), Chang *et al.* (1996) identified various elements in the JCPyV VP1. The amino acid changes detected in the VP1 sequences were mapped and, to our surprise, all seven amino acid changes were located within the possible surface loops, designated BC, DE and HI (Chang *et al.*, 1996) (Table 5). In the BC loop, substitutions of lysine-60 with methionine in coding sequence 3-3 and serine-61 with leucine in coding sequence 2-2 were detected; in the DE loop, a substitution of serine-123 with cysteine was detected in coding sequences 3-4 and 3-5; and in the HI loop, substitutions of serine-269 with either phenylalanine in sequences 1-2 and 1-3 or tyrosine in sequence 3-2 were detected. Most of the detected amino acid substitutions, excluding the substitution of serine-123 with cysteine in the coding sequences 3-4 and 3-5, caused changes in the amino acid properties based on a Venn diagram grouping of amino acids (Betts & Russell, 2003).

Thus, VP1 loop mutations were detected in two of the three coding sequences in case 1, in one of the three coding sequences in case 2 and in four of the five coding sequences in case 3. In terms of clone frequencies, VP1 loop mutations were identified in three of the 12 clones in case 1, two of the 14 clones in case 2 and 12 of the 14 clones in case 3 (Table 3).

Amino acid changes in VP1 sequences identified previously in the brain or CSF of PML patients

To our knowledge, complete VP1 sequences (designated PML-type VP1 sequences for convenience) have been reported for 16 JCPyV isolates derived from the brain or CSF of different PML patients [NY-1B, which was isolated from one of the patients (case 1) investigated in this study, was excluded] (Table 6). The presence of any amino acid changes in the surface loops of these VP1 sequences was examined. Naturally, detection of such amino acid changes requires parental VP1 sequences from which PML-type VP1 sequences might have been generated. The genotypic prototype was used as a substitute for the real parental sequence for each PML-type VP1 sequence. The genotypic prototype was identified as the consensus sequence of VP1 sequences detected in representative archetypal isolates (i.e. isolates derived from the urine of healthy individuals and non-PML patients) belonging to each genotype. The JCPyV genotype designation used was that of Yogo *et al.* (2004) with modifications (Saruwatari *et al.*, 2002; Ikegaya *et al.*, 2005; Takasaka *et al.*, 2005). Furthermore, B1-b was divided into B1-b1 and -b2 according to a recent phylogenetic study on Asian isolates (Cui *et al.*, 2004). The archetypal isolates used were four Af1, six Af2-a, 10 EU-a1, four B1-c,