North America. MY occurs in not only Japan and South Korea but also the Americas.

Based on the unique properties of JCV outlined above, including the mode of transmission and persistence in hosts throughout life, it appears that the geographical distribution patterns of JCV genotypes represent the division and expansion of human populations.

EVOLUTIONARY RELATIONSHIPS AMONG VARIOUS JCV GENOTYPES IN THE WORLD

Although a phylogenetic analysis based on the 610 bp IG region is useful for identifying genotypes of JCV, the information (i.e. the number of nucleotide substitutions) in this region is not sufficient to clarify evolutionary relationships among JCV genotypes. Sugimoto *et al.* [25] adopted a whole-genome approach with which a highly reliable phylogeny can be reconstructed [24,57] to analyse the phylogenetic relationships among 65 JCV isolates derived from various regions of the world. The same approach has been used to phylogenetically characterise the JCV genotypes detected in various ethnic groups (e.g. western Pacific Islanders, native Siberians and Native Americans) [3,5,6,36,37,48,49]. In the following, the phylogenetic relationships among 82 JCV isolates, including 65 worldwide [25], 6 Oceanian [48], 10 Siberian and Arctic [36] and a South Korean (SK-6) [46] isolate (see Figure 3).

(i) The ancestral JCV first evolved into three superclusters, Type-A, -B and -C.

(ii) A split in Type-A generated three genotypes, EU-a, -b and -c. Both EU-a and -b are spread throughout Europe and Mediterranean areas. Nevertheless, two subgroups belonging to EU-a also occur in Northeast Japan (EU-a/SK) and Northeast Siberia and Arctic areas of North America (EU-a/Arc). The implications of these findings will be described below.

(iii) The first split in Type-B generated Af2, a genotype spread in Africa and West and South Asia. The second split in Type-B generated B1-c, a minor genotype in Europe. Subsequent splits in Type-B generated various Asian (B1-a, -b, -d, B2, CY, MY and SC) and Oceanian (8A, 8B and 2E) genotypes.

(iv) Type-C generated a single genotype, Af1. Af1 is distributed in West/Central Africa, including the Central African Republic, Ghana and Mauritania.

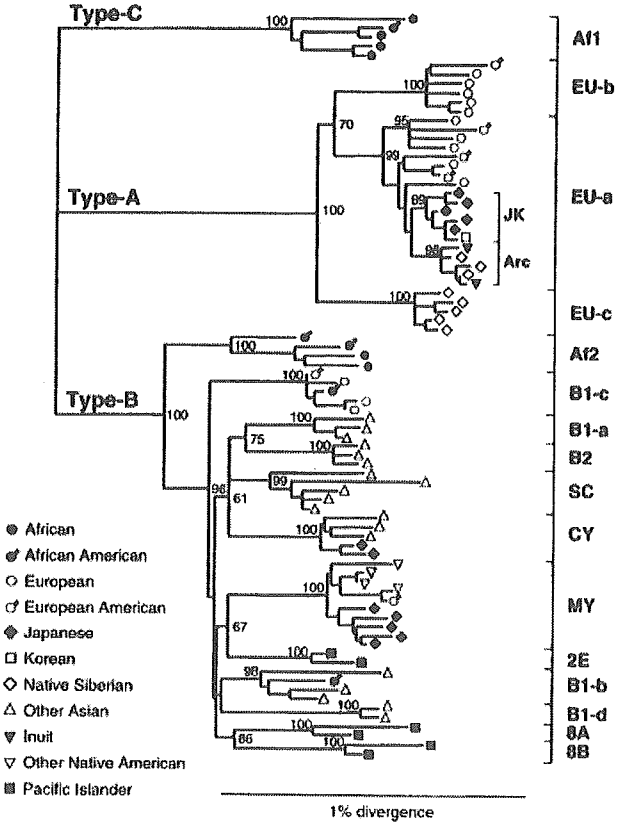

Figure 3. Neighbor-joining phylogenetic tree relating 82 JCV isolates worldwide. The complete sequences, excluding the regulatory sequences, were aligned and used to construct a tree with the neighbor-joining method [47]. The sources of the sequences are indicated [25,36,48]. The tree was visualised using the Af1 isolates as the outgroup [67]. The numbers at nodes give bootstrap confidence level (%) obtained for 1000 replicates (only values ⩾ 50% are shown for major nodes)

Assuming that JCV coevolved with humans (i.e. the first division of JCV into Type-A, -B and -C (Figure 3) occurred 100 000 years ago), Hatwell and Sharp [24] and Sugimoto *et al.* [25] estimated the rate of synonymous substitutions to be about 4×10^{-7}/synonymous site/year. This estimate is two orders of magnitude higher than the rate of synonymous substitutions in human nuclear genes [58].

Modern humans are often grouped into three major races, Caucasoids, Negroids and Mongoloids, on the basis of their morphological features and the geographical regions where they live. Judging from the geographical regions where human races or JCV genotypes are prevalent, Caucasoids correspond to humans carrying Type-A; Negroids correspond to those carrying Type-C and Type-B/

Af2; and Mongoloids correspond to those carrying Type-B excluding Af2 and B1-c.

JCV GENOTYPE PROFILE OF THE JAPANESE ARCHIPELAGO

The presence of two major JCV genotypes in Japan was first discovered in 1994, when DNA fragments amplified from JCV isolated in two distant areas of Japan were analysed using RFLP [15]. These genotypes were named CY and MY after representative isolates in each genotype. This finding was greatly extended to clarify the overall distribution of JCV genotypes in the Japanese Archipelago [1].

Thus, urine samples were collected at 34 sites along the Japanese Archipelago, from the northern edge of Honshu (Goshogawara) down to one of the southernmost islands (Miyako-jima) (sites of urine collection are shown in Figure 4). The northern island, Hokkaido (Figure 4), was not included in this study, as the general population on this island mainly consists of descendants of recent immigrants from Honshu. It was reported elsewhere the genotypes of JCV detected in an aboriginal tribe (the Ainu) in Hokkaido [4]. Amplified IG fragments derived from about 100 urine samples were sequenced, phylogenetic trees were constructed from the sequences obtained, and genotypes of isolates in urine samples were determined according to the resultant phylogenetic tree. The rest of the amplified IG fragments (about 900) were subjected to RFLP analysis, and the genotypes of isolates in urine samples were determined based on the results of the analysis.

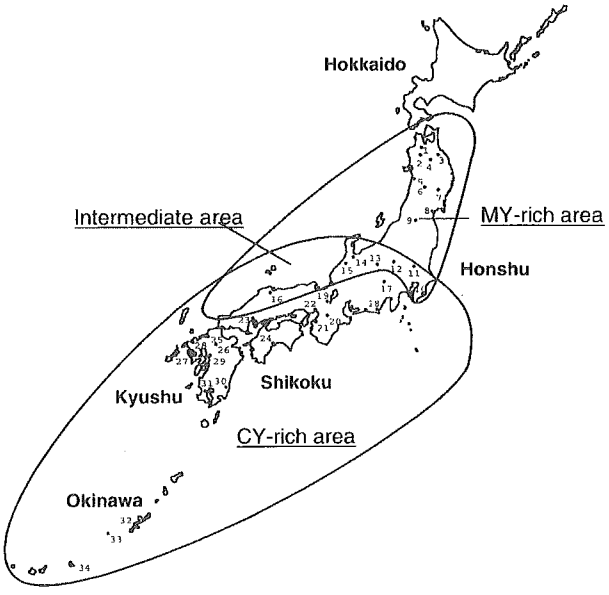

Figure 4. Map showing the domains of the two major JCV genotypes (CY and MY) in Japan. Sites of urine collection are indicated by dots with site numbers, the names of which were described elsewhere [1]. The areas in which CY and MY were found more frequently (i.e. at rates $\geqslant$ 75%) are indicated as CY-rich (or MY-rich) areas, respectively. The area designated an intermediate area is where CY and MY were found at almost the same frequencies. This map was depicted based on the data reported by Kitamura *et al.* [1]

The JCV genotype profile in modern Japanese thus established [1] is summarised below. (i) Two genotypes, CY and MY, are prevalent in modern Japanese. (ii) Both CY and MY have unique domains in the Japanese Archipelago. MY is more prevalent in Northeast Japan, while CY is more prevalent in Southwest Japan (see Figure 4). (iii) There are three minor genotypes (EU-a, B1-a and SC), each showing a unique regional distribution pattern. EU-a occurs predominantly in northeastern areas facing the Japanese Sea; B1-a occurs in the central area of Honshu; and SC is localised to South Japan.

ANCESTRAL POPULATIONS THAT CONTRIBUTED TO THE FORMATION OF MODERN JAPANESE

It is generally accepted by anthropologists that modern Japanese were formed by two distinct ethnic groups, the Jomon people who colonised Japan in the Neolithic period and later 'immigrants' from the Asian Continent during the Aeneolithic Yayoi and prehistoric Kofun periods [59,60]. Here, we infer the origin of modern Japanese based on the JCV genotype profile in Japan. As there are two major JCV genotypes (CY and MY) in Japan, it can be inferred that modern Japanese were formed mainly by two ancestral populations, carrying CY or MY. In general, this inference is in agreement with the 'immigrants' hypothesis on the formation of modern Japanese. Nevertheless, it was found that three minor JCV genotypes (EU-a, B1-a and SC) occur in the Japanese Archipelago. Two major genotypes (CY and MY) and two minor genotypes (B1-a and SC) belong to Type-B, while one minor genotype (EU-a) belongs to Type-A. As described above, people carrying Type-B JCVs excluding Af2 and B1-c correspond to Mongoloids, while those carrying Type-A JCVs correspond to Caucasoids. Thus, we may state that not only Mongoloids but also Caucasoids contributed to the formation of modern Japanese. This statement is particularly

meaningful in the area of Northeast Japan where EU-a JCVs occur at higher rates. It should be noted that no studies using other anthropological methods have detected a Caucasoid lineage in modern Japanese.

The following three sections detail the origins of people who carried three JCV genotypes (i.e. CY, MY and EU-a) to the Japanese Archipelago.

ORIGIN OF PEOPLE CARRYING CY TO JAPAN

Most Northeast Asians are characterised by the presence of JCV genotype CY [1,2,21,22]. However, its frequencies vary significantly among regions. The regional difference in the frequencies of CY may have been caused by the dispersal of ancestral people carrying CY from their homeland (probably somewhere in Northeast China) to surrounding areas and by the subsequent admixture of this group, at various rates, with indigenous groups carrying other JCV genotypes. Recently insights were obtained into the dispersals of this CY-carrying group [Zheng *et al.*, submitted]. Thus, it was found that CY diverged into two clades, designated CY-a and -b. Interestingly, the ratio of CY-a to -b significantly varied among Northeast Asian populations. CY-a was more abundant than CY-b in various Chinese and Japanese populations, but CY-b was more abundant than CY-a in South Koreans. These findings allow the following inference. A Northeast Asian population carrying proto-CY diverged into two populations, one carrying CY-a and the other carrying CY-b. The former expanded throughout North China and to the western part of the Japanese Archipelago; the latter expanded mainly along the Korean Peninsula.

ORIGIN OF PEOPLE CARRYING MY TO JAPAN

In East Asia, MY shows a very narrow domain restricted to Japan and South Korea (North Korea remains to be investigated) [1,21]. This genotype, however, is widespread among various Native American populations excluding Eskimo-Aleuts [6,37,51]. Zheng *et al.* [37] examined the phylogenetic relationships among many MY isolates worldwide using the whole genome approach, i.e. the most reliable method with which to construct phylogenetic trees [24,57]. The MY isolates analysed were derived from Japanese/Koreans and Native Americans in Canada, the United States, Guatemala, Mexico and Peru. MY isolates worldwide diverged into seven subgroups designated MY-a to -g. Interestingly, each of the seven intra-MY subgroups showed a unique regional distribution pattern. The evolution and migration of MY, based on these findings, are schematically shown in Figure 5.

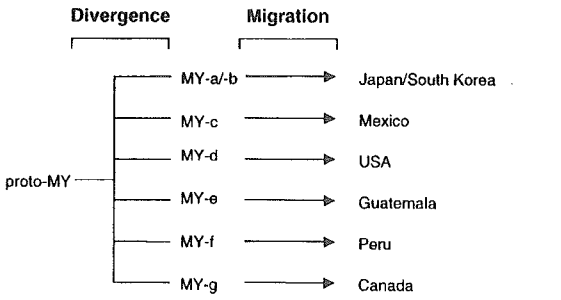

Figure 5. Schematic representation of the divergence and migration of MY JCVs. The scheme is based on a phylogenetic analysis of 81 MY isolates and the geographical distributions of various intra-MY subgroups (MY-a to -g) [37]

Wherever humans have migrated on earth, JCV has accompanied them [18,61]. If an original human population diverged into sub-populations and migrated to different areas, JCV would have accompanied these sub-populations. JCVs in sub-populations would have evolved into unique genotypes during long persistence in the sub-populations. Therefore, the pattern of the division and migration of MY (Figure 5) indicates the division and migration of human populations carrying MY. The timing of the MY division was estimated to be 10 000–30 000 years ago, from synonymous mutations per synonymous site (Ks) and the proposed rate of JCV evolution (4×10^{-7} substitutions per synonymous site per year) [37]. Thus, people carrying MY migrated to Japan/South Korea and North, Central and South America 10 000–30 000 years ago. However, the study of JCV could not reveal the geographical origin of MY.

ORIGIN OF PEOPLE CARRYING TYPE-A TO JAPAN

Type-A JCVs are mainly distributed in Europe and Mediterranean areas (Type-A JCVs occur on the American Continents, but most of them were introduced by recent immigrants from Europe [61]). However, as described above, minor Type-A JCVs were detected in Northeast Japan. Furthermore, Type-A JCVs were found among aboriginal tribes (Nanais, Chukuchis, Koryaks) in Northeast Siberia and those (Inuits) in the Arctic regions of

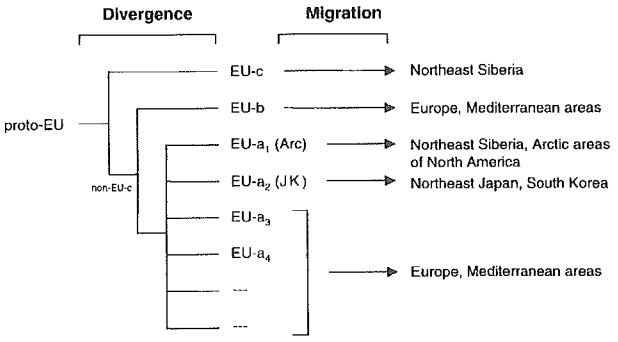

Figure 6. Schematic representation of the divergence and migration of Type-A JCVs. The scheme is based on a phylogenetic analysis and the geographical distributions of various intra-Type-A genotypes (Figure 5 in [36] was reproduced with some modification)

Canada [36]. Sugimoto *et al.* [36] made a phylogenetic comparison among Type-A isolates worldwide based on fully determined JCV DNA sequences, and established an interesting pattern of evolution of Type-A JCVs as briefly described below (see Figure 6).

Thus, Type-A JCVs worldwide diverged into three genotypes, EU-a, -b and -c. The first split in Type-A generated EU-c and non-EU-c, and the subsequent split in non-EU-c gave rise to EU-a and -b. There was a close correlation between intra-Type-A genotypes and human populations. EU-c contained only Northeast Siberian isolates, derived mainly from Nanais living in the lower Amur River region; EU-b contains only those from Europeans and Mediterranean people; and EU-a contained various isolates derived from not only Europeans and Mediterranean people but also Siberians, Arctic tribes and Northeast Japanese. Most Siberian/Arctic isolates derived from Chukchis, Koryaks, and Canadian Inuits formed one distinct cluster (EU-a/Arc) within the EU-a genotype, and all from Northeast Japanese formed the other cluster (EU-a/JK). This cluster was previously named EU-a/Jpn [61] but renamed here, as it also contained a single isolate (SK-6) [46] found in Seoul, South Korea [Takasaka *et al.*, unpublished]. A comparison of synonymous substitution rates among the intra-Type-A genotypes revealed that both the division of proto-EU into EU-c and non-EU-c and that of non-EU-c generating EU-a and -b occurred 33 000–40 000 years ago, and that the divergence of EU-a generating various subgroups occurred 10 000–20 000 years ago.

As repeatedly described in this review, JCV has always accompanied humans when they moved. Therefore, the division and migration patterns of Type-A JCVs (Figure 6) represent the division and migration of human populations carrying Type-A JCVs. The people carrying Type-A JCVs are tentatively designated here as Caucasoids. There are some remarkable features in the migration of Caucasoids to the Far East. First, the population that first diverged from the ancestral Caucasoid population moved not to Europe but to the eastern edge of the Asian Continent. Second, the major migration of Caucasoids occurred at least three times. People carrying EU-c were the first to migrate to the Far East, as described above. People carrying EU-a/Arc or EU-a/JK migrated at about the same time as people carrying other intra-EU-a subgroups moved to Europe and Mediterranean areas.

CORRELATION BETWEEN JCV GENOTYPES AND Y-CHROMOSOME HAPLOGROUPS

Recently, the genetic variation in the non-recombining portion of the Y-chromosome has been extensively examined to investigate ancient human migrations and the population structures of human groups [29]. In these studies, haplotypes and haplogroups are usually used to represent the genetic variation in the Y-chromosome. A haplotype is a set of closely linked DNA polymorphisms, such as single nucleotide polymorphisms, insertion/deletion and numbers of short tandem repeats, inherited as a unit, while a haplogroup is defined as a cluster of similar haplotypes. Geographical regions where various haplogroups are spread have been determined on a global scale [29]. We found significant agreement in geographical distribution patterns between Y-chromosome haplogroups (abbreviated as Y-haplogroups) and JCV genotypes, and describe below several such examples. The haplogroup designations, A-R, described by YCC (The Y Chromosome Consortium) [62], are used.

Four Y-haplogroups (A, B, E and R) are mainly spread in Africa [63–66]. E is spread at high frequencies in the whole of Africa and at lower frequencies in West Asia. The JCV genotype that shows a similar geographical distribution is Af2, and therefore it is likely that Af2 is the counterpart of haplogroup E. In other words, the same population expanded throughout Africa, carrying both the Y-haplogroup E and JCV-genotype Af2. A minor haplogroup (A) in Africa, presumably ancestral to all Y-haplotypes, may correspond to

Af1 which was recently suggested to be the ancestral type of JCV [67]. R, one of the major European Y-haplogroups, is localised to Cameroon. This finding was interpreted as evidence of a prehistoric migration back to sub-Saharan Africa [65]. Although the JCV genotype pattern of Cameroon is not clear, rare European-type JCVs (EU-a) were detected in neighboring Central-African populations [21,35].

Several Y-haplogroups occur in Europe, each having distinct clinal distribution patterns [63,68,69]. Haplogroups E, I, J, N and R show clines pointing to origins in the south, north, southeast, east and west, respectively. Two major (EU-a and EU-b) and some rare (Af2, B1-b and -c, MY) JCV genotypes have been detected [21,36,53–55; Ikegaya *et al.*, submitted]. Furthermore, EU-a isolates in Europe are divided into two groups, EU-a1 and -a2 (also named types 1A and 1B, respectively) [53–55; Ikegaya *et al.*, submitted]. It has been suggested that some European genotypes of JCV (EU-a1, -a2 and -b) show geographical clines [53; Ikegaya *et al.*, submitted]. As described above, we suggested that the JCV counterpart of Y-haplogroup E is Af2. Indeed, rare Af2 isolated have been detected in South Europe (i.e. France, Spain, Italy, Greece and Macedonia) where Y-haplogroup E occurs at lower frequencies [21,53–55; Takasaka *et al.*, unpublished]. Thus, JCV genotypes in Europe appear to mimic the distribution of Y-haplogroups. We believe that further study of the distribution of JCV genotypes in Europe will eventually clarify the correspondence between JCV genotypes and Y-haplogroups in the region.

Three major Y-haplogroups (C, D and O) occur in East and Central Asia [63,68,70–75]. Haplogroup O shows the widest distribution in the area, with high frequencies in China, Taiwan, South Korea and Japan. Haplogroup D is restricted to certain areas of Asia, including Japan, South Korea and Tibet. Haplogroup C occurs in Central Asia at high frequencies but in East Asia at low frequencies. In reference to the patterns of JCV genotype distribution in East and Central Asia [1–3,7,21,22,34], we may correlate haplogroups C, D and O to JCV genotypes B1-b, MY (MY-a and -b) and CY + SC, respectively. Thus, East Asian men who have the Y-haplogroup O are separated into two groups, carrying either of the two JCV genotypes, CY and SC. There is an apparent discrepancy between the Y-haplogroup and JCV-genotype profiles in Tibet. Although B1-b is a major JCV genotype in this region [34; Takasaka *et al.*, unpublished], haplogroup C (the proposed counterpart of JCV-genotype B1-b) was not detected, but instead haplogroup D (the proposed counterpart of JCV-genotype MY) was detected at a high frequency (MY was not detected at all in Tibet [34; Takasaka *et al.*, unpublished]).

One major (C) and a few minor (including R and N) haplogroups occur in Central-South and Northeast Siberia, and two major haplogroups (N and Q) equally occur at the eastern edge of Northeast Siberia [69]. The JCV genotype profiles in some Northeast Siberians are known [36]. One single JCV genotype (EU-a/Arc) occurs in two Northeast-Siberian tribes, Chukuchi and Koryaks, and one major (EU-c) and a few minor (B1-b, CY, EU-a/Arc) JCV genotypes occur in the Nanai, a tribe in the lower Amur River region. As haplogroup N frequently occurs in the Saami [68] where EU-a1 (one of the Type-A subtypes) is the major JCV genotype [Ikegaya *et al.*, submitted], it is suggested that this haplogroup corresponds to EU-a1. The Y-haplogroup counterpart of JCV-genotype EU-c has not been identified. The presence of B1-b in some Northeast Siberians [36; Sugimoto *et al.*, unpublished] is consistent with our suggestion that B1-b is the counterpart of haplogroup C. Haplogroup Q is the major haplogroup in Native Americans as described below.

Native Americans are frequently divided into three linguistic groups: Eskimo-Aleuts, Na-Denes and Amerinds [76]. One major (Q) and a few minor (C, P and R) Y-haplogroups occur in all three linguistic groups of Native-Americans [70,77–81]. A single JCV genotype (MY) occurs in Native American populations excluding Eskimo-Aleuts [6,37,51]. As MY isolates in the world diverge into two Japanese/Korean (MY-a and -b) and five Native American subgroups (MY-c to -g) [37], MY-c to -g may correspond to Y-haplogroup Q, while MY-a and -b may correspond to Y-haplogroup D (see above). However, this speculation is not consistent with the observation that haplogroups D and Q are not closely linked to each other on a phylogenetic tree relating various Y-haplogroups (see Figure 7).

PHYLOGENY OF Y-HAPLOGROUPS

In the previous section, the geographical distribution patterns of JCV genotypes and Y-haplogroups

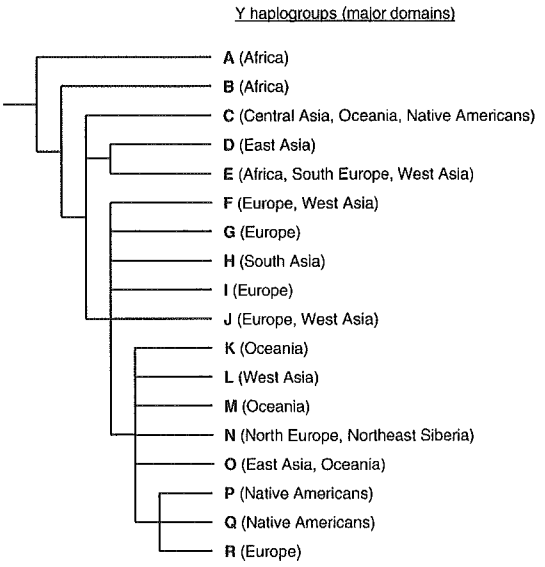

Figure 7. Schematic representation of the phylogenetic tree relating various Y-haplogroups. The tree was depicted based on the most parsimonious tree of Y-haplogroups [62]. YCC haplogroups and their major domains are given at the right of the tree [29]. The lengths of branches are not proportional to the numbers of mutation

were compared. On the whole, there appears to exist considerable correspondence in the geographical distribution patterns between JCV genotypes and Y-haplogroups. However, some problems emerged when the phylogenetic relationships among various Y-haplogroups were examined in the light of JCV genotypes. As described above, there is significant correlation between JCV genotypes and major human populations (i.e. Africans, Europeans, Asians and Oceanians) (see Figure 3). Most Africans carry either Af1 belonging to Type-C or Af2 first generated from the ancestor of Type-B JCVs; most Europeans carry JCV genotypes (i.e. EU-a and -b) belonging to Type-A; and most Asians and Oceanians carry JCV genotypes belonging to Type-B. In contrast, there is no clear-cut correlation between Y-haplogroups and major human populations (a schematic representation of the most parsimonious tree relating various Y-haplogroups is shown in Figure 7). Although two minor African haplogroups (A and B) are the first two branches of the tree, the major African haplogroup (E), being linked to an East Asian haplogroup (D), is included in a supercluster (designated here as the non-A/non-B cluster) containing all European, Asian and Oceanian haplogroups (C to R). The major European haplogroups (I, J, N and R) do not cluster together. Various Asian haplogroups are widespread in the non-A/non-B cluster, intermingling with European haplogroups. Therefore, the phylogenetic tree relating various Y-haplogroups (Figure 7) may not faithfully represent the relationships among various human groups, although it may indicate the origin of modern humans.

CONCLUDING REMARKS

The basic properties of JCV, the regional distributions of JCV genotypes and the phylogenetic relationships among various JCV genotypes have been reviewed. On the basis of such knowledge, an example in which the origins of an ethic group (modern Japanese) was successfully investigated in detail. Finally, the relationships between JCV genotypes and Y-chromosome haplogroups were examined.

It was first thought that it would be hard to find a consensus between the anthropological findings obtained using host-chromosomal and viral markers. Unexpectedly, it was found that there exists considerable correspondence in the geographical distribution between Y-haplogroups and JCV genotypes. Nevertheless, there emerged some problems in the phylogeny of Y-haplogroups when it was examined in reference to that of JCV genotypes. Further investigation, of both the JCV genotype and the Y-chromosome, will eventually solve these problems. It is believed that the findings based on one marker will complement those based on the other so that the whole picture of human migrations and the population structures of various ethnic groups will be unequivocally clarified.

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

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The regulatory regions of JC virus (JCV) DNAs in the brain of patients with progressive multifocal leukoencephalopathy (PML) (designated as PML-type regulatory regions) are hypervariable, whereas those in the urine and renal tissue of individuals without PML have the same basic structure, designated as the archetype. It is thought that JCV strains with the archetypal regulatory region circulate in the human population. Nevertheless, Monaco *et al* (J Virol 70: 7004–7012, 1996) reported that PML-type regulatory regions occur in human tonsil tissue. The purpose of this study is to confirm their findings. Using nested polymerase chain reaction (PCR), the authors detected the regulatory region of JCV DNA in the tonsil tissue from 14 (44%) of 32 donors with tonsillitis and tonsilar hypertrophy. Sequencing of the detected regulatory regions indicated that they were identical with the archetypal regulatory regions detected previously or, in a few cases, slightly deviated from the archetype. This finding suggests not only that tonsil tissue is the potential site of initial JCV infection but also that archetypal JCV strains circulate in the human population. *Journal of NeuroVirology* (2004) **10**, 244–249.

Keywords: DNA sequence rearrangement; lymphoid tissue; polyomavirus; viral persistence

Introduction

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

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

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

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

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

Results

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

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

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

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

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

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

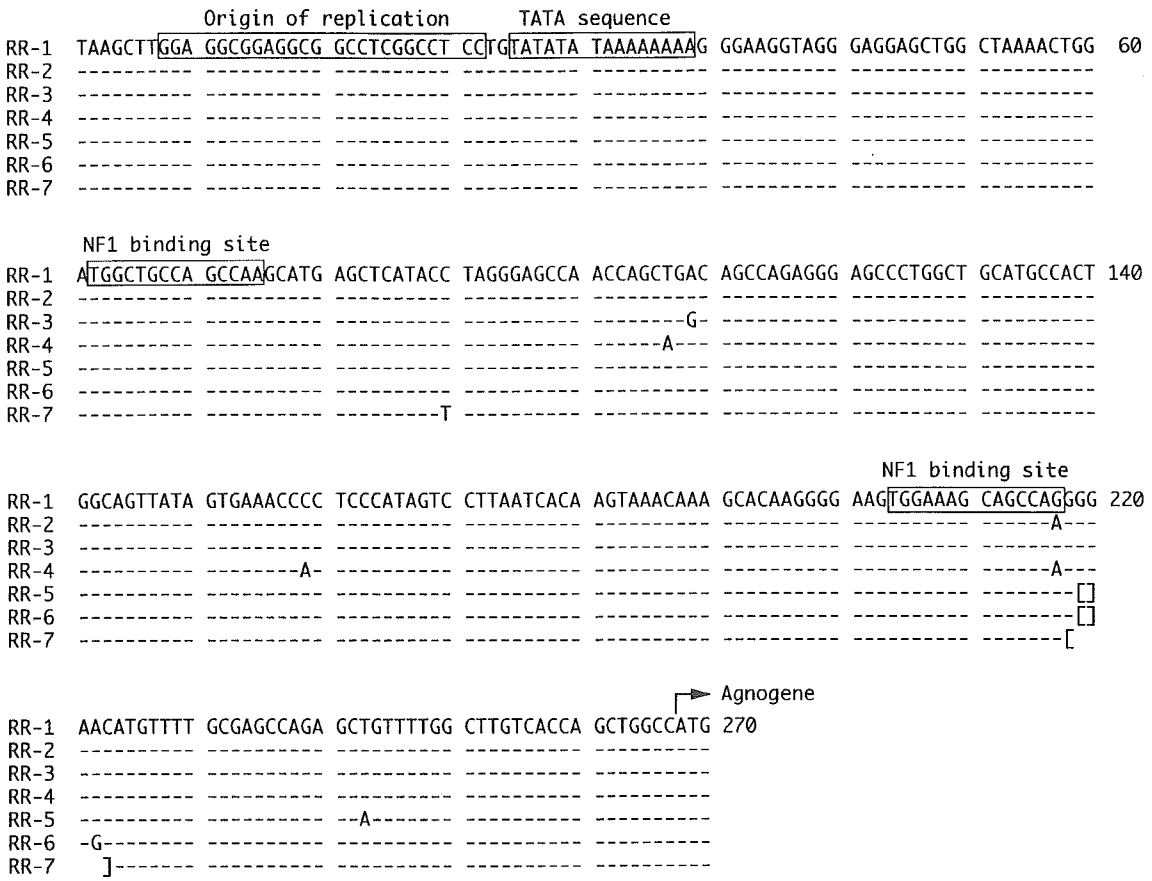

Figure 1 JCV RR sequences detected in tonsil tissue. The nucleotide sequence of RR-1, spanning from the origin of replication to the start site of the agnogene, is shown at the top. The RR-1 sequence is identical with the CY archetype (Yogo *et al*, 1990). The origin of replication, TATA sequence, NF-1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

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

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

Table 2 Frequency of various JCV RRs

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

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

Discussion

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

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

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

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

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

Materials and methods

Tonsils and PBLs

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

Extraction of DNA

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

n-PCR

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

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

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

Cloning and sequencing

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

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Subtypes of BK virus prevalent in Japan and variation in their transcriptional control region

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BK polyomavirus (BKV) is ubiquitous in the human population, infecting children without obvious symptoms, and persisting in the kidney in a latent state. In immunosuppressed patients, BKV is reactivated and excreted in urine. BKV isolates have been classified into four subtypes (I–IV) using either serological or genotyping methods. To elucidate the subtypes of BKV prevalent in Japan, the 287 bp typing region in the viral genome was PCR-amplified from urine samples of 45 renal transplant (RT) and 31 bone-marrow transplant (BMT) recipients. The amplified fragments were subjected to a phylogenetic or RFLP analysis to determine the subtypes of BKV isolates in urine samples. Subtypes I, II, III and IV were detected, respectively, in 70–80, 0, 2–3 and 10–20 % of the BKV-positive patients in both patient groups. This pattern of distribution was virtually identical to patterns previously demonstrated in England, Tanzania and the United States, suggesting that BKV subtypes are distributed similarly in various human populations. Furthermore, transcriptional control regions (TCRs) were PCR-amplified from the urine samples of 25 RT and 20 BMT recipients, and their nucleotide sequences were determined. The basic TCR structure (the so-called archetype configuration) was observed in most isolates belonging to subtypes I, III and IV (subtype II isolates were not available), albeit with several nucleotide substitutions and a few single-nucleotide deletions (or insertions). Only three TCRs carried extensive sequence rearrangements. Thus, it was concluded that the archetypal configuration of the BKV TCR has been conserved during the evolution of BKV.

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INTRODUCTION

Human polyomavirus BK virus (BKV) was first isolated in the urine of a renal transplant (RT) patient (Gardner *et al.*, 1971). Seroepidemiological surveys conducted in various countries have since demonstrated that this virus is ubiquitous in humans (Knowles, 2001). Infection most frequently occurs during childhood, with adult levels of seroprevalence (65–90 %) reached between the ages of 5 and 10 years (Knowles, 2001). It is thought that BKV persists in renal tissue (Heritage *et al.*, 1981; Chesters *et al.*, 1983). The urinary excretion (viruria) of BKV is rather rare in immunocompetent individuals, but is frequent in immunocompromised individuals, including organ transplant recipients, HIV-infected patients and pregnant women (Knowles, 2001). In immuocompromised patients, the reactivation of BKV sometimes results in renal dysfunction, such as BKV-associated nephropathy (Moens & Rekvig, 2001).

BKV is the only primate polyomavirus that has subtypes distinguishable by immunological reactivity (Knowles, 2001). Knowles *et al.* (1989) introduced a typing scheme using a set of rabbit antisera against various isolates shown to differ from the prototype BKV (Gardner strain) either

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antigenically or by restriction enzyme cleavage patterns. However, this typing method requires BKV isolates previously obtained by viral culture. Jin *et al.* (Jin *et al.*, 1993b; Jin, 1993) developed a direct and convenient method based on the polymerase chain reaction. A partial VP1 gene sequence probably containing nucleotide substitutions responsible for antigenic diversity (Jin *et al.*, 1993a) was PCR-amplified from clinical samples (usually urine specimens), and the resultant amplified fragments were subjected to either DNA sequencing or restriction enzyme analysis. Based on these analyses, Jin *et al.* (Jin *et al.*, 1993b; Jin, 1993) classified various laboratory BKV strains as well as clinical isolates into four subtypes, I–IV, which corresponded well to groups based on the serological assay.

It is of interest to examine whether a correlation exists between BKV subtypes and human populations, as JC virus (JCV), a related human polyomavirus, shows a close correlation between subtypes and human populations (Agostini *et al.*, 2001; Yogo *et al.*, 2004). The distribution of BKV subtypes has been studied in several patient groups in England (Jin *et al.*, 1993b, 1995), Italy (Di Taranto *et al.*, 1997), Tanzania (Agostini *et al.*, 1995) and the United States (Baksh *et al.*, 2001), and it was found that subtype I was predominant in all of these studies. Nevertheless, no information is available about the distribution pattern of BKV subtypes in Asia. To gain an overall picture of the distribution of BKV subtypes in the world, here, we genotyped BKV isolates detected in the urine of RT and bone-marrow transplant (BMT) recipients in Japan.

The BKV genome has a transcriptional control region (TCR) between the origin of replication and the start site of the late leader protein (agnoprotein) (Seif *et al.*, 1979). The BKV TCR readily undergoes DNA sequence rearrangement during passage of the virus in cell culture (Yoshiike & Takemoto, 1986; Hara *et al.*, 1986; Rubinstein *et al.*, 1987). Therefore, the TCRs of BKV isolates obtained by viral culture could contain alterations introduced *in vitro*. In contrast, those obtained by molecular cloning or PCR should represent naturally occurring BKV TCRs. Analysis of BKV TCRs isolated using the latter method thus revealed that naturally occurring BKV TCRs have a common structure named the archetype (Moens & Rekvig, 2001).

Nevertheless, information about BKV TCRs has been obtained mainly for subtype I, as this subtype is most prevalent (Knowles, 2001). A complete DNA sequence was reported for a strain (AS) belonging to subtype III, but this strain was isolated by viral culture (Coleman *et al.*, 1980). Indeed, AS carries a 32 bp deletion encompassing an origin-distal region of the TCR and the start site of the agnogene (Tavis *et al.*, 1989). Furthermore, Negrini *et al.* (1991) detected AS-like TCRs in 2 of 13 isolates from the urine of BMT recipients, but they did not sequence the origin-distal portion of these TCRs. Thus, the relationship between BKV subtype and TCR structure remains to be clarified. In this study, we examined TCR structures for many isolates belonging to various subtypes of BKV.

METHODS

Urine samples. Urine samples were collected with informed consent from 186 RT recipients, who underwent a renal transplantation at Tokyo Women's Medical University, University of the Ryukyus Faculty of Medicine, Oyokyo Kidney Research Institute and Iwate Medical University School of Medicine. No patient had active graft rejection episodes or developed nephropathy during the study. About 40 ml urine was collected in a 50 ml plastic tube that contained 0·5 ml 0·5 M EDTA, pH 8·0, and the samples were sent to the Department of Urology, Faculty of Medicine, University of Tokyo, where DNA was extracted as described previously (Kitamura *et al.*, 1990). Urine samples previously obtained from BMT recipients and shown to contain BKV DNA (Akiyama *et al.*, 2001) were also used.

PCR. The 287 bp typing region and the TCR were amplified from urinary DNA by PCR using ProofStart DNA polymerase (Qiagen). The 287 bp region spanned from 1650 to 1936 nt in the BKV (Dunlop) genome (GenBank accession no. V01108; NCBI no. NC_001538), and contained the whole effective sequence within the 327 bp typing region (Jin *et al.*, 1993b). Primers used to amplify the typing region were 327-1PST (5′-GCCTGCAGCAAGTGCCAAAAC-TACTAAT-3′; nt 1630–1649) and 327-2HIN (5′-GCAAGCTTGCA-TGAAGGTTAAGCATGC-3′; nt 1956–1937). Those used to amplify the TCR were RR-1PST (5′-GCCTGCAGGCCTCAGAAAAAGCCT-CCACAC-3′; nt 49–72) and RR-2HIN (5′-CGAAGCTTGTCGTG-ACAGCTGGCGCAGAAC-3′; nt 412–391). Underlined nucleotides were added to create a *Pst*I or *Hin*dIII cleavage site. Primers 327-1PST and 327-2HIN were similar to 327-1 and 327-2 reported by Jin *et al.* (1993b) but carried restriction sites for *Pst*I and *Hin*dIII, respectively. The total reaction volume of 50 µl contained 2·5 µl crude viral DNA, 1·25 U ProofStart DNA polymerase (Qiagen), 200 µM each dNTP, 0·5 µM primers and PCR buffer, supplied by the manufacturer. After activation at 95 °C for 5 min, the amplification reaction was performed for 50 cycles. The cycle profile was 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min. Both activation and amplification were carried out in a Thermal Sequencer (Asahi Techno Glass Corporation).

Molecular cloning. The amplified fragments were cleaved with a combination of *Hin*dIII and *Pst*I (Takara Bio), and ligated to pBluescript II SK (+) (Stratagene), which was previously digested with *Hin*dIII and *Pst*I and dephosphorylated with bacterial alkaline phosphatase (Takara Bio). The ligation products were used to transform competent cells (*Escherichia coli* HB101; Takara Bio). Recombinant plasmids were prepared using a plasmid mini kit (Qiagen).

Sequencing. Purified plasmids were used for a cycle sequencing reaction set up using the DYEnamic ET Terminator cycle sequencing kit (Amersham Biosciences). Primers used were the T3 and T7 promoters (Toyobo). The primers were added to a final concentration of 0·25 pmol μl^{-1} in a final reaction volume of 20 µl. The cycling conditions were 25 cycles of 30 s at 96 °C, 15 s at 50 °C and 60 s at 60 °C. The reaction was terminated at 4 °C. Cycle sequencing products were purified on Centri-Sep columns (Princeton Separations). DNA sequencing was performed using an automated sequencer (ABI Prism 373S DNA sequencer; Applied Biosystems).

Phylogenetic analysis. A neighbour-joining (NJ) phylogenetic tree (Saitou & 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 DendroMaker for Macintosh ver. 4.1 (Imanishi, 1998). The confidence of branching patterns of the NJ trees was assessed based on 1000 bootstrap replicates (Felsenstein, 1985).

RFLP analysis. Sequences of 287 bp typing fragments were available for several BKV isolates belonging to I–IV (Seif *et al.*, 1979; Yang & Wu, 1979; Tavis *et al.*, 1989; Sugimoto *et al.*, 1990;

Table 1. BKV subtype discrimination according to the RFLP analysis

A 342 bp fragment containing the 287 bp typing region was PCR-amplified from each BKV subtype as described in Methods. The sizes of subfragments detected after digestion of the amplified fragment with indicated restriction enzymes are shown.

Restriction enzyme	Fragment patterns for indicated subtype (bp)			
	I	II	III	IV
*Alu*I	193	193	342	342
	149	149	–	–
*Cfr*13I	245	245	245	342
	97	97	97	–
*Rsa*I	294	342	212	342
	48	–	130	–

Jin, 1993). Using a computer program, we examined these 287 bp sequences, together with those determined in this study (see below), for the presence or absence of restriction sites that would identify the four BKV subtypes. It was found that RFLPs generated by three restriction enzymes, *Alu*I, *Cfr*13I and *Rsa*I, can be used to identify BKV subtypes (Table 1). The RFLP analysis involving these enzymes was carried out as follows. PCR mixtures were extracted with phenol and filtered through spin-columns containing Sephadex G-25 superfine (Amersham Biosciences). Typically, 2·5 µl aliquot of a purified PCR mixture was digested at 37 °C for 1 h with 10–20 U each enzyme. The digest was resolved by electrophoresis on a 3 % NuSieve agarose gel (Takara Bio) stained with ethidium bromide.

RESULTS

BKV subtypes prevalent in Japan

Using PCR to amplify the 287 bp typing region, we screened urine samples of 186 RT recipients for the presence of BKV DNA. We detected BKV DNA in 45 urine samples in total. We determined the subtypes of these BKV DNAs by phylogenetic analysis ($n=27$) and RFLP analysis ($n=18$). (We will describe the phylogenetic analysis in the following section.) We also determined the subtypes of BKV DNA previously detected in 31 urine samples from BMT recipients (Akiyama *et al.*, 2001) by the phylogenetic method.

BKV subtype frequencies in the RT and BMT recipients analysed in this study are shown in Table 2. The RT recipients were classified into three groups according to their geographical origins, and BKV subtype frequencies are shown for each of these groups (Table 2). Subtypes I, III and IV were detected in 70–80, 2–3 and 10–20 %, respectively, of the BKV-positive patients in both patient groups. Nevertheless, subtype II was not detected in either RT or BMT recipients. Subtype distribution patterns were apparently similar among the two patient groups and geographical origins of patients (Table 2).

Phylogenetic analysis of BKV isolates based on the 287 bp sequences

We cloned 287 bp typing regions amplified from urine samples, and sequenced representative clones for each urine sample (Table 3). We obtained single sequences from all urine samples examined. From these sequences, together with reference sequences reported previously (Table 4), a phylogenetic tree was constructed using the NJ method (Saitou & Nei, 1987). According to the resultant phylogenetic tree (Fig. 1), all isolates detected in this study diverged into three clusters corresponding to subtype I, III and IV. Nevertheless, isolate SB (reference for subtype II) did not cluster with any of the present isolates. It should be noted that the grouping of isolates belonging to subtype I, III or IV was supported by higher bootstrap probabilities (95–100 %) (Fig. 1). In addition, subtype I apparently subdivided into at least three subclusters, Ia, Ib and Ic. Ia contained one isolate from Sudanese and two isolates from Americans; Ib contained one isolate from a South African, one isolate from English, two isolates from Dutch and five isolates from Japanese; and Ic contained 41 isolates from Japanese. Bootstrap probabilities ranged from 63 to 86 %.

Table 2. BKV subtype in RT and BMT recipients in Japan

Patient	Geographical region*	No. of isolates examined	No. (%) of isolates classified for indicated subtypes†			
			I	II	III	IV
RT	Tokyo	23	20 (87)	0	0	3 (13)
	Okinawa	8	5 (63)	0	1 (13)	2 (25)
	Tohoku	14	10 (71)	0	0	4 (29)
	Total	45	35 (78)	0	1 (2)	9 (20)
BMT	Tokyo	31	26 (84)	0	1 (3)	4 (13)

*Areas where patients lived are indicated.

†BKV subtypes were determined by phylogenetic or RFLP analysis of 342 bp typing fragments amplified from urine samples.

Table 3. BKV isolates analysed in this study

Isolates analysed by RFLP are not included.

Patient	Geographical region	Isolate used for analysis	
		Phylogenetic	TCR
RT	Tohoku	THK-1 to -4, -6, -8, -9	THK-2, -3, -5 to -9, -11
RT	Tokyo	TU-1, -2, TW-1 to -12	TW-1 to -8, -12 to -14
RT	Okinawa	RYU-1 to -8	RYU-1 to -5, -7, -8
BMT	Tokyo	KOM-1 to -31	KOM-1 to -3, -5 to -8, -10 to -16, -21, -26 to -30

Table 4. BKV isolates used as references in the phylogenetic analysis (Fig. 1)

Subtype	Isolate	Clinical state/geographical origin	Reference for sequences
I	Gardner	RT/Sudan	Jin *et al.* (1993a)
I	Dun	Wiskott-Aldrich/USA	Seif *et al.* (1979)
I	MM	Wiskott-Aldrich/USA	Yang & Wu (1979)
I	Dik	BMT/Netherlands	Sugimoto *et al.* (1990)
I	JL	BMT/Netherlands	Sugimoto *et al.* (1990)
I	WW	RT/South Africa	Sugimoto *et al.* (1990)
I	MT	Systemic lupus erythematosus/Japan	Sugimoto *et al.* (1990)
I	GS	RT/England	Jin *et al.* (1993a)
II	SB	Lymphocytic lymphoma/England	Jin *et al.* (1993a)
III	AS	Pregnant/England	Tavis *et al.* (1989)
IV	IV	RT/England	Jin *et al.* (1993a)

TCR sequences detected from urine-derived isolates

We cloned TCRs amplified from 45 urine samples (Table 3), and sequenced a few representative clones for each urine sample. We obtained single sequences from all urine samples examined. We also sequenced the TCRs of 2 complete BKV DNA clones (WW and MT-1) obtained directly from urine previously (Chauhan *et al.*, 1984; Sugimoto *et al.*, 1989). Alignment of the resultant 47 sequences gave rise to 10 unique sequences, designated Seq-1 to Seq-10 (Fig. 2). In Fig. 2, Seq-1 (the TCR commonly found in subtype IV isolates, see below) is shown at the top, and the other sequences are shown below in relation to Seq-1. BKV isolates carrying each TCR sequence are shown in Table 5.

Interestingly, we found a correlation between BKV subtypes and TCR structures. Thus, Seq-1, -4, -5 and -7 TCRs were commonly detected in subtypes IV, III, Ib and Ic, respectively (the TCRs of naturally occurring BKV strains belonging to subtypes II and Ia were not available). Seq-5 was detected in strain WW frequently referred to as the representative archetypal BKV strain. The subtype-specific TCR sequences (Seq-1, -4, -5 and -7) were distinguished from each other by single-nucleotide substitutions at 11 positions and by single-nucleotide deletions at 2 positions (Table 6).

The other TCR sequences (Seq-2, -3, -6, -8, -9 and -10) were probably derived from the consensus sequences in individual subtypes by nucleotide substitutions or sequence rearrangements (deletions or duplications). Thus, it was inferred that Seq-2 and -3 were generated from Seq-1; Seq-6 from Seq-5; and Seq-8 to -10 from Seq-7. Three sequences (Seq-3, -6 and -10) carried rather extensive rearrangements, involving deletions or duplications. However, these were detected in only three isolates.

DISCUSSION

Distribution of BKV subtypes in human populations

BKV is the only primate polyomavirus that can be classified into antigenically distinct subgroups. In this study, we attempted to clarify the correlation between BKV subtypes and human populations. The distribution of BKV subtypes in human populations was previously studied in England, Tanzania and the United States (Jin *et al.*, 1993b, 1995; Agostini *et al.*, 1995; Baksh *et al.*, 2001). In this study, we clarified the distribution of BKV subtypes in Japan. Although the populations studied varied in terms of the clinical state of the subjects, the results of the studies conducted so far suggest that subtype I is predominant in all human populations around the world.

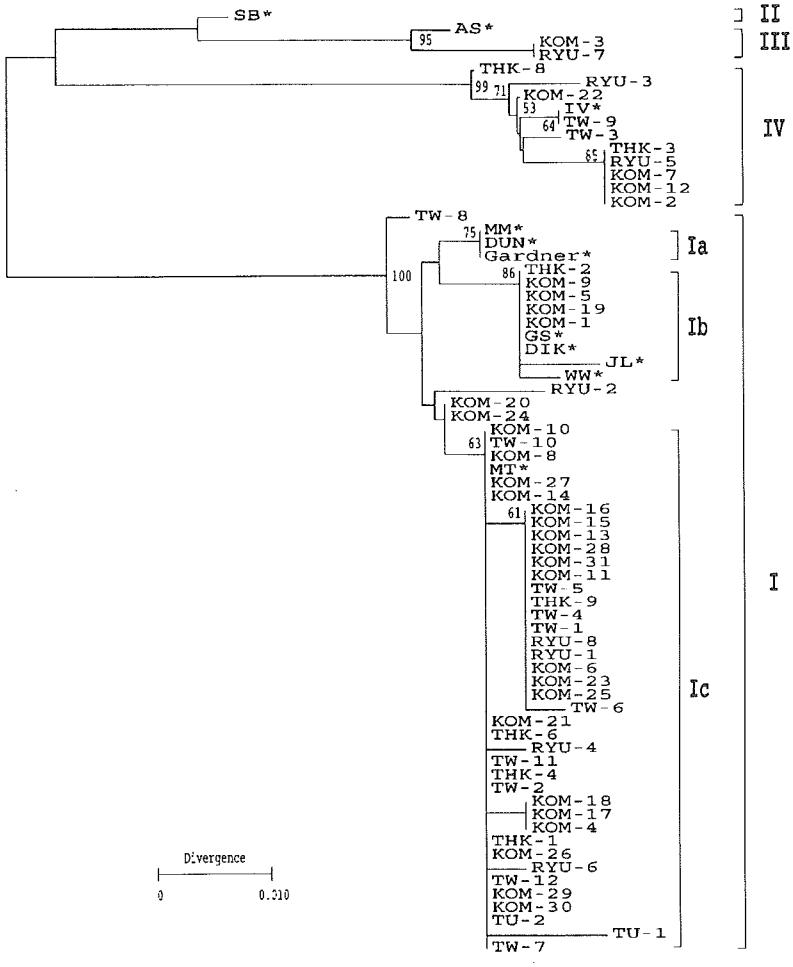
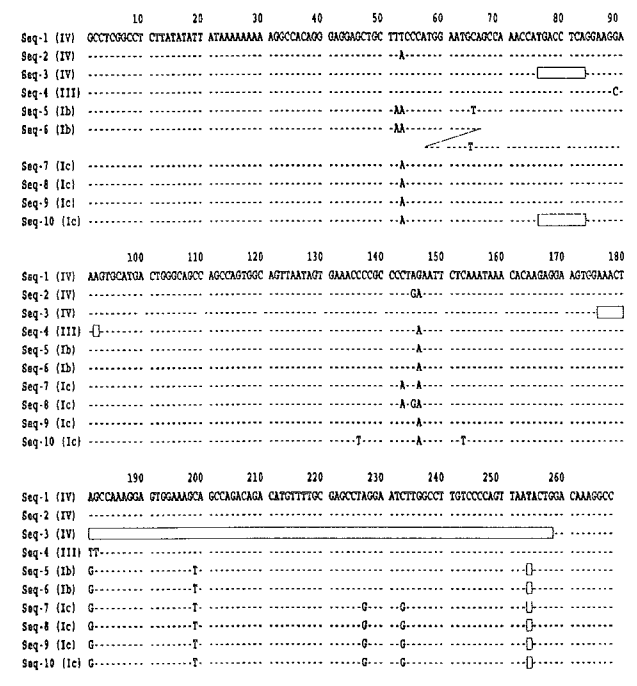


Fig. 1. Phylogenetic tree used to classify the BKV isolates into subtypes. The 287 bp typing sequences detected in the present and previous studies were used to construct an NJ phylogenetic tree using CLUSTAL W (Thompson *et al.*, 1994). The phylogenetic tree was visualized using DendroMaker for Macintosh ver. 4.1. The tree was rooted at the midpoint, assuming various BKV strains evolved at roughly the same rate. Subtypes and possible subgroups within subtype I are indicated to the right of the tree. Asterisks identify isolates reported previously and used as references. Origins of isolates are shown in Tables 3 and 4. The numbers at nodes give the bootstrap confidence level (%) obtained for 1000 replicates (only values ⩾50% are shown for major nodes).



However, relative proportions of the minor subtypes differed among populations studied. In renal transplant recipients (Jin *et al.*, 1993b, 1995; Baksh *et al.*, 2001; this study), subtype IV was detected at lower rates and subtypes II and III were not or rarely detected. Jin *et al.* (1995) reported that dual BKV infection frequently occurred in HIV-infected patients and that subtype III was more often detected than in the other subject groups. The reactivation of BKV in HIV-positive patients remains to be investigated further.

Fig. 2. Ten TCR sequences detected from the urine of RT and BMT patients. Sequences between the midpoint of the origin of replication and the start site of the agnogene are shown (for convenience, the nucleotide numbering starts at the midpoint of the origin of replication). Seq-1 (the TCR commonly found in subtype IV isolates, see text) is shown at the top. The other sequences (Seq-2 to Seq-10) are shown below in relation to Seq-1, with the same nucleotides indicated by dashes and deletions by rectangles. Parallel sequences connected with an oblique line in Seq-6 indicate a duplication. The subtype in which each sequence was detected is indicated within parentheses.