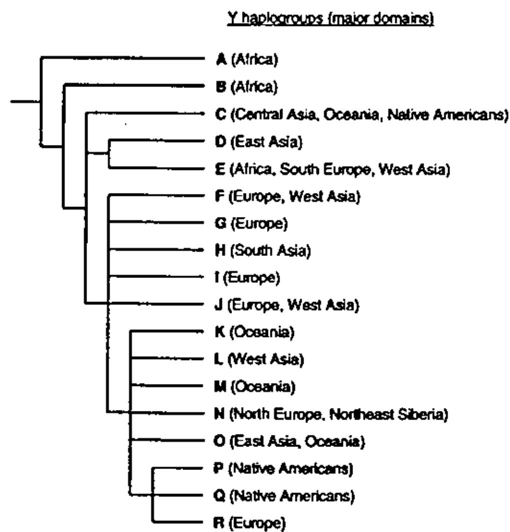

Figure 7. Schematic representation of the phylogenetic tree relating various Y-haplogroups. The tree was depicted based on the most parsimonious tree of Y-haplogroups [62]. YCC haplogroups and their major domains are given at the right of the tree [29]. The lengths of branches are not proportional to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

1. Kitamura T, Sugimoto C, Ebihara H, *et al*. Peopling of Japan as revealed by genotyping of urinary JC virus DNA. *Anthropol Sci* 1998; **106**: 311–325.
2. Guo Z, Zheng SP, Sugimoto C, *et al*. JC virus genotypes in northwestern China: implications for its population history. *Anthropol Sci* 2001; **109**: 203–212.

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3. Saruwatari L, Zheng HY, Takasaka T, *et al.* Peopling of Myanmar as demonstrated by genotyping of urinary JC virus DNA. *Anthropol Sci* 2002; **110**: 235–249.
4. Yogo Y, Zheng HY, Hasegawa M, *et al.* Phylogenetic analysis of JC virus DNAs detected in Ainus: an attempt to elucidate the origin and diversity of the Ainu. *Anthropol Sci (Japanese Series)* 2003; **111**: 19–34.
5. Takasaka T, Miranda JJ, Sugimoto C, *et al.* Genotypes of JC virus in Southeast Asia and the western Pacific: implications for human migrations from Asia to the Pacific. *Anthropol Sci* 2004; in press.
6. Fernandez-Cobo M, Agostini HT, Britez G, *et al.* Strains of JC virus in Amerind-speakers of North America (Salish) and South America (Guarani), Na-Dene-speakers of New Mexico (Navajo), and modern Japanese suggest links through an ancestral Asian population. *Am J Phys Anthropol* 2002; **118**: 154–168.
7. Miranda JJ, Sugimoto C, Paraguison R, *et al.* Genetic diversity of JC virus in the modern Filipino population: implications for the peopling of the Philippines. *Am J Phys Anthropol* 2003; **120**: 125–132.
8. Cole CN, Conzen SD. Polyomaviridae: the viruses and their replication. In *Fields Virology*, 4th edn, Knipe DM, Howley PM, Griffin DE, *et al.* (eds). Lippincott: Philadelphia, 2001; 2141–2173.
9. Padgett BL, Walker DL. Prevalence of antibodies in human sera against JC virus, an isolate from a case of progressive multifocal leukoencephalopathy. *J Infect Dis* 1973; **127**: 467–470.
10. Walker DL, Padgett BL. The epidemiology of human polyomaviruses. *Prog Clin Biol Res* 1983; **105**: 99–106.
11. Chesters PM, Heritage J, McCance DJ. Persistence of DNA sequences of BK virus and JC virus in normal human tissues and in diseased tissues. *J Infect Dis* 1983; **147**: 676–684.
12. Tominaga T, Yogo Y, Kitamura T, *et al.* Persistence of archetypal JC virus DNA in normal renal tissue derived from tumor-bearing patients. *Virology* 1992; **186**: 736–741.
13. Kitamura T, Sugimoto C, Kato A, *et al.* Persistent JC virus (JCV) infection is demonstrated by continuous shedding of the same JCV strains. *J Clin Microbiol* 1997; **35**: 1255–1257.
14. Kitamura T, Aso Y, Kuniyoshi N, *et al.* High incidence of urinary JC virus excretion in nonimmunosuppressed older patients. *J Infect Dis* 1990; **161**: 1128–1133.
15. Kitamura T, Kunitake T, Guo J, *et al.* Transmission of the human polyomavirus JC virus occurs both within the family and outside the family. *J Clin Microbiol* 1994; **32**: 2359–2363.
16. Agostini HT, Ryschkewitsch CF, Stoner GL. Genotype profile of human polyomavirus JC excreted in urine of immunocompetent individuals. *J Clin Microbiol* 1996; **34**: 159–164.
17. Kunitake T, Kitamura T, Guo J, *et al.* Parent-to-child transmission is relatively common in the spread of the human polyomavirus JC virus. *J Clin Microbiol* 1995; **33**: 1448–1451.
18. Suzuki M, Zheng HY, Takasaka T, *et al.* Asian genotypes of JC virus in Japanese-Americans suggest familial transmission. *J Virol* 2002; **76**: 10074–10078.
19. Zheng HY, Kitamura T, Takasaka T, *et al.* Unambiguous identification of JC virus strains transmitted from parents to children. *Arch Virol* 2004; **149**: 261–273.
20. Kato A, Kitamura T, Sugimoto C, *et al.* Lack of evidence for the transmission of JC polyomavirus between human populations. *Arch Virol* 1997; **142**: 875–882.
21. Sugimoto C, Kitamura T, Guo J, *et al.* Typing of urinary JC virus DNA offers a novel means of tracing human migrations. *Proc Natl Acad Sci USA* 1997; **94**: 9191–9196.
22. Guo J, Sugimoto C, Kitamura T, *et al.* Four geographically distinct genotypes of JC virus are prevalent in China and Mongolia: implications for the racial composition of modern China. *J Gen Virol* 1998; **79**: 2499–2505.
23. Agostini HT, Jobes DV, Stoner GL. Molecular evolution and epidemiology of JC virus. In *Human Polyomaviruses: Molecular and Clinical Perspectives*, Khalili K, Stoner GL (eds). John Wiley & Sons: New York, 2001; 491–526.
24. Hatwell JN, Sharp PM. Evolution of human polyomavirus JC. *J Gen Virol* 2000; **81**: 1191–1200.
25. Sugimoto C, Hasegawa M, Kato A, *et al.* Evolution of human Polyomavirus JC: implications for the population history of humans. *J Mol Evol* 2002; **54**: 285–297.
26. Cann RL, Stoneking M, Wilson AC. Mitochondrial DNA and human evolution. *Nature* 1987; **325**: 31–36.
27. Underhill PA, Passarino G, Lin AA, *et al.* The phylogeography of Y chromosome binary haplotypes and the origins of modern human populations. *Ann Hum Genet* 2001; **65**: 43–62.
28. Hammer MF, Karafet TM, Redd AJ, *et al.* Hierarchical patterns of global human Y-chromosome diversity. *Mol Biol Evol* 2001; **18**: 1189–1203.
29. Jobling MA, Tyler-Smith C. The human Y chromosome: an evolutionary marker comes of age. *Nat Rev Genet* 2003; **4**: 598–612.
30. Padgett BL, Walker DL, ZuRhein GM, *et al.* Cultivation of papova-like virus from human brain with progressive multifocal leucoencephalopathy. *Lancet* 1971; **1**(7712): 1257–1260.
31. Walker DL. Progressive multifocal leukoencephalopathy. In *Demyelinating Diseases. Handbook of Clinical*

Copyright © 2004 John Wiley & Sons, Ltd.

Neurology. Vol. 3, Vinken PJ, Bruyn GW, Klawans HL, *et al.* (eds). Elsevier: Amsterdam, 1985; 503–524.
32. Arthur RR, Shah KV. Occurrence and significance of papovaviruses BK and JC in the urine. *Prog Med Virol* 1989; 36: 42–61.
33. Chang D, Sugimoto C, Wang M, *et al.* JC virus genotypes in a Taiwan aboriginal tribe (Bunun): implications for its population history. *Arch Virol* 1999; **144**: 1081–1090.
34. Saruwatari L, Sugimoto C, Kitamura T, *et al.* Asian domains of four major genotypes of JC virus, Af2, B1-b, CY and SC. *Arch Virol* 2002; **147**: 1–10.
35. Chima SC, Ryschkewitsch CF, Stoner GL. Molecular epidemiology of human polyomavirus JC in the Biaka Pygmies and Bantu of Central Africa. *Mem Inst Oswaldo Cruz* 1998; 93: 615–623.
36. Sugimoto C, Hasegawa M, Zheng HY, *et al.* JC virus strains indigenous to northeastern Siberians and Canadian Inuits are unique but evolutionally related to those distributed throughout Europe and Mediterranean areas. *J Mol Evol* 2002; 55: 322–335.
37. Zheng HY, Sugimoto C, Hasegawa M, *et al.* Phylogenetic relationships among JC virus strains in Japanese/Koreans and Native Americans speaking Amerind or Na-Dene. *J Mol Evol* 2003; 56: 18–27.
38. Yogo Y, Kitamura T, Sugimoto C, *et al.* Isolation of a possible archetypal JC virus DNA sequence from nonimmunocompromised individuals. *J Virol* 1990; 64: 3139–3143.
39. Yogo Y, Sugimoto C. The archetype concept and regulatory region rearrangement. In *Human Polyomaviruses: Molecular and Clinical Perspectives*, Khalili K, Stoner GL (eds). John Wiley & Sons: New York, 2001; 127–148.
40. Nathanson N. Epidemiology. In *Fields Virology*, 4th edn, Knipe DM, Howley PM, Griffin DE, *et al.* (eds). Lippincott: Philadelphia, 2001; 371–392.
41. Daniel R, Shah K, Madden D, *et al.* Serological investigation of the possibility of congenital transmission of papovavirus JC. *Infect Immun* 1981; 33: 319–321.
42. Taguchi F, Kajioka J, Miyamura T. Prevalence rate and age of acquisition of antibodies against JC virus and BK virus in human sera. *Microbiol Immunol* 1982; 26: 1057–1064.
43. Ault GS, Stoner GL. Two major types of JC virus defined in progressive multifocal leukoencephalopathy brain by early and late coding region DNA sequences. *J Gen Virol* 1992; 73: 2669–2678.
44. Walker DL, Frisque RJ. The biology and molecular biology of JC virus. In *The Papovaviridae. The Polyomaviruses*, Salzman NP (ed.). Plenum Press: New York, 1986; 327–377.
45. Yogo Y, Iida T, Taguchi F, *et al.* Typing of human polyomavirus JC virus on the basis of restriction fragment length polymorphisms. *J Clin Microbiol* 1991; 29: 2130–2138.
46. Guo J, Kitamura T, Ebihara H, *et al.* Geographical distribution of the human polyomavirus JC virus type A and B and isolation of a new type from Ghana. *J Gen Virol* 1996; 77: 919–927.
47. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987; 4: 406–425.
48. Jobes DV, Friedlaender JS, Mgone CS, *et al.* New JC virus (JCV) genotypes from Papua New Guinea and Micronesia (type 8 and type 2E) and evolutionary analysis of 32 complete JCV genomes. *Arch Virol* 2001; 146: 2097–2113.
49. Yanagihara R, Nerurkar VR, Scheirich I, *et al.* JC virus genotypes in the western Pacific suggest Asian mainland relationships and virus association with early population movements. *Hum Biol* 2002; 74: 473–488.
50. Ryschkewitsch CF, Friedlaender JS, Mgone CS, *et al.* Human polyomavirus JC variants in Papua New Guinea and Guam reflect ancient population settlement and viral evolution. *Microbes Infect* 2000; 2: 987–996.
51. Agostini HT, Yanagihara R, Davis V, *et al.* Asian genotypes of JC virus in Native Americans and in a Pacific Island population: markers of viral evolution and human migration. *Proc Natl Acad Sci USA* 1997; 94: 14542–14546.
52. Agostini HT, Ryschkewitsch CF, Brubaker GR, *et al.* Five complete genomes of JC virus type 3 from Africans and African Americans. *Arch Virol* 1997; 142: 637–655.
53. Agostini HT, Deckhut A, Jobes DV, *et al.* Genotypes of JC virus in East, Central and Southwest Europe. *J Gen Virol* 2001; 82: 1221–1231.
54. Dubois V, Moret H, Lafon ME, *et al.* JC virus genotypes in France: molecular epidemiology and potential significance for progressive multifocal leukoencephalopathy. *J Infect Dis* 2001; 183: 213–217.
55. Pagani E, Delbue S, Mancuso R, *et al.* Molecular analysis of JC virus genotypes circulating among the Italian healthy population. *J Neurovirol* 2003; 9: 559–566.
56. Jeong BH, Lee KH, Choi EK, *et al.* Genotyping of the JC virus in urine samples of healthy Korean individuals. *J Med Virol* 2004; 72: 281–289.
57. Jobes DV, Chima SC, Ryschkewitsch CF, *et al.* Phylogenetic analysis of 22 complete genomes of the human polyomavirus JC virus. *J Gen Virol* 1998; 79: 2491–2498.
58. Li WH. Unbiased estimation of the rates of synonymous and nonsynonymous substitution. *J Mol Evol* 1993; 36: 96–99.
59. Dodo Y, Ishida H. Population history of Japan as viewed from cranial nonmetric variation. *Anthropol Sci* 1990; 98: 269–287.

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60. Hanihara K. Dual structure model for the population history of the Japanese. *Japan Rev* 1991; **2**: 1–33.
61. Stoner GL, Jobes DV, Fernandez-Cobo M, *et al.* JC virus as a marker of human migration to the Americas. *Microbes Infect* 2000; **2**: 1905–1911.
62. YCC (The Y Chromosome Consortium). A nomenclature system for the tree of human Y-chromosomal binary haplogroups. *Genome Res* 2002; **12**: 339–348.
63. Underhill PA, Shen P, Lin AA, *et al.* Y chromosome sequence variation and the history of human populations. *Nat Genet* 2000; **26**: 358–361.
64. Bosch E, Calafell F, Comas D, *et al.* High-resolution analysis of human Y-chromosome variation shows a sharp discontinuity and limited gene flow between northwestern Africa and the Iberian Peninsula. *Am J Hum Genet* 2001; **68**: 1019–1029.
65. Cruciani F, Santolamazza P, Shen P, *et al.* A back migration from Asia to sub-Saharan Africa is supported by high-resolution analysis of human Y-chromosome haplotypes. *Am J Hum Genet* 2002; **70**: 1197–1214.
66. Semino O, Santachiara-Benerecetti AS, Falaschi F, *et al.* Ethiopians and Khoisan share the deepest clades of the human Y-chromosome phylogeny. *Am J Hum Genet* 2002; **70**: 265–268.
67. Pavesi A. African origin of polyomavirus JC and implications for prehistoric human migrations. *J Mol Evol* 2003; **56**: 564–572.
68. Semino O, Passarino G, Oefner PJ, *et al.* The genetic legacy of Paleolithic *Homo sapiens sapiens* in extant Europeans: a Y chromosome perspective. *Science* 2000; **290**: 1155–1159.
69. Karafet TM, Osipova LP, Gubina MA, *et al.* High levels of Y-chromosome differentiation among native Siberian populations and the genetic signature of a boreal hunter-gatherer way of life. *Hum Biol* 2002; **74**: 761–789.
70. Karafet TM, Zegura SL, Posukh O, *et al.* Ancestral Asian source(s) of new world Y-chromosome founder haplotypes. *Am J Hum Genet* 1999; **64**: 817–831.
71. Su B, Xiao J, Underhill P, *et al.* Y-Chromosome evidence for a northward migration of modern humans into Eastern Asia during the last Ice Age. *Am J Hum Genet* 1999; **65**: 1718–1724.
72. Wells RS, Yuldasheva N, Ruzibakiev R, *et al.* The Eurasian heartland: a continental perspective on Y-chromosome diversity. *Proc Natl Acad Sci USA* 2001; **98**: 10244–10249.
73. Karafet T, Xu L, Du R, *et al.* Paternal population history of East Asia: sources, patterns, and microevolutionary processes. *Am J Hum Genet* 2001; **69**: 615–628.
74. Zerjal T, Wells RS, Yuldasheva N, *et al.* A genetic landscape reshaped by recent events: Y-chromosomal insights into central Asia. *Am J Hum Genet* 2002; **71**: 466–482.
75. Kayser M, Brauer S, Weiss G, *et al.* Reduced Y-chromosome, but not mitochondrial DNA, diversity in human populations from West New Guinea. *Am J Hum Genet* 2003; **72**: 281–302.
76. Greenberg JH, Turner II CG, Zegura SL. The settlement of the Americas: a comparison of the linguitic, dental and genetic evidence. *Curr Anthropol* 1986; **27**: 477–497.
77. Tarazona-Santos E, Carvalho-Silva DR, Pettener D, *et al.* Genetic differentiation in South Amerindians is related to environmental and cultural diversity: evidence from the Y chromosome. *Am J Hum Genet* 2001; **68**: 1485–1496.
78. Lell JT, Sukernik RI, Starikovskaya YB, *et al.* The dual origin and Siberian affinities of Native American Y chromosomes. *Am J Hum Genet* 2002; **70**: 192–206.
79. Bosch E, Calafell F, Rosser ZH, *et al.* High level of male-biased Scandinavian admixture in Greenlandic Inuit shown by Y-chromosomal analysis. *Hum Genet* 2003; **112**: 353–363.
80. Bortolini MC, Salzano FM, Thomas MG, *et al.* Y-chromosome evidence for differing ancient demographic histories in the Americas. *Am J Hum Genet* 2003; **73**: 524–539.
81. Zegura SL, Karafet TM, Zhivotovsky LA, *et al.* High resolution SNPs and microsatellite haplotypes point to a single, recent entry of native American Y chromosomes into the Americas. *Mol Biol Evol* 2004; **21**: 164–175.

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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).

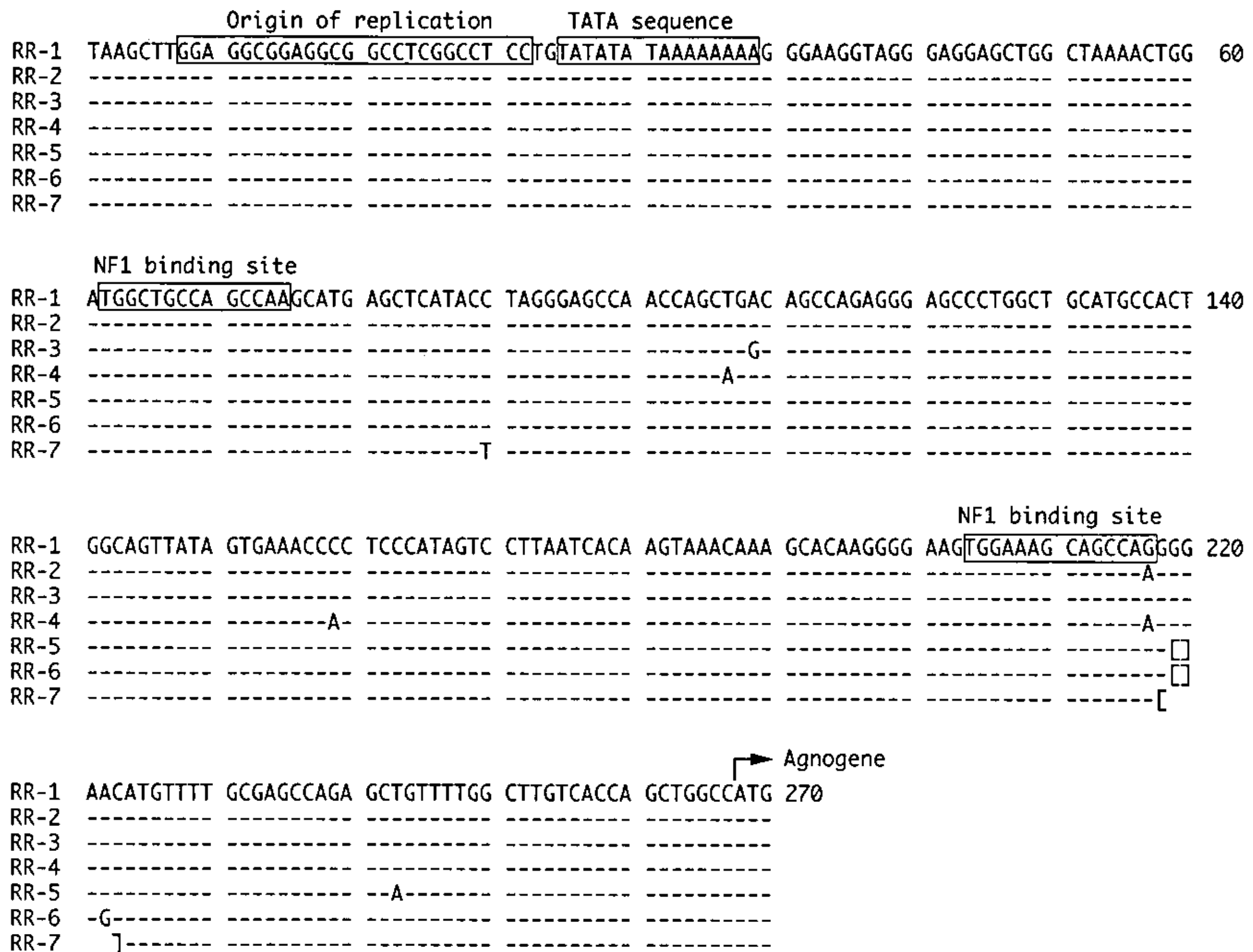

Figure 1 JCV RR sequences detected in tonsil tissue. The nucleotide sequence of RR-1, spanning from the origin of replication to the start site of the agnogene, is shown at the top. The RR-1 sequence is identical with the CY archetype (Yogo *et al*, 1990). The origin of replication, TATA sequence, NF-1 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 tonsillar 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 srl, 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.

References

Agostini HT, Deckhut A, Jobes DV, Girones R, Schlunck G, Prost MG, Frias C, Perez-Trallero E, Ryschkewitsch CF, Stoner GL (2001). Genotypes of JC virus in East, Central and Southwest Europe. *J Gen Virol* **82:** 1221–1231.

Agostini HT, Ryschkewitsch CF, Brubaker GR, Shao J, Stoner GL (1997a). Five complete genomes of JC virus type 3 from Africans and African Americans. *Arch Virol* **142:** 637–655.

Agostini HT, Ryschkewitsch CF, Singer EJ, Stoner GL (1997b). JC virus regulatory region rearrangements and genotypes in progressive multifocal leukoencephalopathy: two independent aspects of virus variation. *J Gen Virol* **78:** 659–664.

Agostini HT, Ryschkewitsch CF, Stoner GL (1996). Genotype profile of human polyomavirus JC excreted in urine of immunocompetent individuals. *J Clin Microbiol* **34:** 159–164.

Aoki N, Kitamura T, Tominaga T, Fukumori N, Sakamoto Y, Kato K, Mori M (1999). Immunohistochemical detection of JC virus in nontumorous renal tissue of a patient with renal cancer but without progressive multifocal leukoencephalopathy. *J Clin Microbiol* **37:** 1165–1167.

Ault GS (1997). Activity of JC virus archetype and PML-type regulatory regions in glial cells. *J Gen Virol* **78:** 163–169.

Ault GS, Stoner GL (1993). Human polyomavirus JC promoter/enhancer rearrangement patterns from progressive multifocal leukoencephalopathy brain are unique derivatives of a single archetypal structure. *J Gen Virol* **74:** 1499–1507.

Chesters PM, Heritage J, McCance DJ (1983). Persistence of DNA sequences of BK virus and JC virus in normal human tissues and in diseased tissues. *J Infect Dis* **147:** 676–684.

Dolei A, Pietropaolo V, Gomes E, Di Taranto C, Ziccheddu M, Spanu MA, Lavorino C, Manca M, Degener AM (2000). Polyomavirus persistence in lymphocytes: prevalence in lymphocytes from blood donors and healthy personnel of a blood transfusion centre. *J Gen Virol* **81:** 1967–1973.

Dörries K, Sbiera S, Drews K, Arendt G, Eggers C, Dörries R (2003). Association of human polyomavirus JC with peripheral blood of immunoimpaired and healthy individuals. *J NeuroVirol* **9(Suppl 1):** 81–87.

Eash S, Tavares R, Stopa EG, Robbins SH, Brossay L, Atwood WJ (2004). Differential distribution of the JC virus receptor-type sialic acid in normal human tissues. *Am J Pathol* **164:** 419–428.

Flœgstad T, Sundsfjord A, Arthur RR, Pedersen M, Traavik T, Subramani S (1991). Amplification and sequencing of the control regions of BK and JC virus from human urine by polymerase chain reaction. *Virology* **180:** 553–560.

Frisque RJ, Bream GL, Cannella MT (1984). Human polyomavirus JC virus genome. *J Virol* **51:** 458–469.

Gallia GL, Houff SA, Major EO, Khalili K (1997). Review: JC virus infection of lymphocytes-revisited. *J Infect Dis* **176:** 1603–1609.

Guo J, Kitamura T, Ebihara H, Sugimoto C, Kunitake T, Takehisa J, Na YQ, Al-Ahdal MN, Hallin A, Kawabe K, Taguchi F, Yogo Y (1996). Geographical distribution of the human polyomavirus JC virus types A and B and isolation of a new type from Ghana. *J Gen Virol* **77:** 919–927.

Hernandez Losa J, Fernandez-Soria V, Parada C, Sanchez-Prieto R, Ramon Y Cajal S, Fedele CG, Tenorio A (2003). JC virus and human colon carcinoma: an intriguing and inconclusive association. *Gastroenterology* **124:** 268–269.

Iida T, Kitamura T, Guo J, Taguchi F, Aso Y, Nagashima K, Yogo Y (1993). Origin of JC polyomavirus variants associated with progressive multifocal leukoencephalopathy. *Proc Natl Acad Sci U S A* **90:** 5062–5065.

Ikegaya H, Iwase H, Yogo Y (2004). Detection of identical JC virus DNA sequences in both human kidneys. *Arch Virol*, **49:** 1215–1220.

Jeong BH, Lee KH, Choi EK, Kim K, Kim YS (2004). Genotyping of the JC virus in urine samples of healthy Korean individuals. *J Med Virol* **72:** 281–289.

Kato K, Guo J, Taguchi F, Daimaru O, Tajima M, Haibara H, Matsuda J, Sumiya M, Yogo Y (1994). Phylogenetic comparison between archetypal and disease-associated JC virus isolates in Japan. *Jpn J Med Sci Biol* **47:** 167–178.

Kitamura T, Sugimoto C, Kato A, Ebihara H, Suzuki M, Taguchi F, Kawabe K, Yogo Y (1997). Persistent JC virus

(JCV) infection is demonstrated by continuous shedding of the same JCV strains. *J Clin Microbiol* **35:** 1255–1257.
Kwok S, Higuchi R (1989). Avoiding false positives with PCR. *Nature* **339:** 237–238.
Laghi L, Randolph AE, Chauhan DP, Marra G, Major EO, Neel JV, Boland CR (1999). JC virus DNA is present in the mucosa of the human colon and in colorectal cancers. *Proc Natl Acad Sci U S A* **96:** 7484–7489.
Markowitz RB, Eaton BA, Kubik MF, Latorra D, McGregor JA, Dynan WS (1991). BK virus and JC virus shed during pregnancy have predominantly archetypal regulatory regions. *J Virol* **65:** 4515–4519.
Martin JD, King DM, Slauch JM, Frisque RJ (1985). Differences in regulatory sequences of naturally occurring JC virus variants. *J Virol* **53:** 306–311.
Monaco MCG, Atwood WJ, Gravell M, Tornatore CS, Major EO (1996). JC virus infection of hematopoietic progenitor cells, primary B lymphocytes, and tonsillar stromal cells: implications for viral latency. *J Virol* **70:** 7004–7012.
Monaco MCG, Jensen PN, Hou J, Durham LC, Major EO (1998). Detection of JC virus DNA in human tonsil tissue: evidence for site of initial viral infection. *J Virol* **72:** 9918–9923.
Padgett BL, Walker DL (1973). Prevalence of antibodies in human sera against JC virus, an isolate from a case of progressive multifocal leukoencephalopathy. *J Infect Dis* **127:** 467–470.
Pagani E, Delbue S, Mancuso R, Borghi E, Tarantini L, Ferrante P (2003). Molecular analysis of JC virus genotypes circulating among the Italian healthy population. *J NeuroVirol* **9:** 559–566.
Sock E, Renner K, Feist D, Leger H, Wegner M (1996). Functional comparison of PML-type and archetype strains of JC virus. *J Virol* **70:** 1512–1520.
Sugimoto C, Ito D, Tanaka K, Matsuda H, Saito H, Sakai H, Fujihara K, Itoyama Y, Yamada T, Kira J, Matsumoto R, Mori M, Nagashima K, Yogo Y (1998). Amplification of JC virus regulatory DNA sequences from cerebrospinal fluid: diagnostic value for progressive multifocal leukoencephalopathy. *Arch Virol* **143:** 249–262.
Tominaga T, Yogo Y, Kitamura T, Aso Y (1992). Persistence of archetypal JC virus DNA in normal renal tissue derived from tumor-bearing patients. *Virology* **186:** 736–741.
Walker DL (1985). Progressive multifocal leukoencephalopathy. In: *Handbook of clinical neurology; vol 47 (3). Demyelinating diseases.* Vinken PJ, Bruyn GW, Klawans HL, Koetsier JC (eds). Amsterdam: Elsevier, pp 503–524.
Yogo Y, Iida T, Taguchi F, Kitimura T, Aso Y (1991). Typing of human polyomavirus JC virus on the basis of restriction fragment length polymorphisms. *J Clin Microbiol* **29:** 2130–2138.
Yogo Y, Kitamura T, Sugimoto C, Ueki T, Aso Y, Hara A, Taguchi F (1990). Isolation of a possible archetypal JC virus DNA sequence from nonimmunocompromised individuals. *J Virol* **64:** 3139–3143.
Yogo Y, Sugimoto C (2001). The archetype concept and regulatory region rearrangement, In: *Human polyomaviruses: molecular and clinical perspectives*, Khalili K, Stoner GL (eds). New York: John Wiley & Sons pp 127–148.

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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.

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.

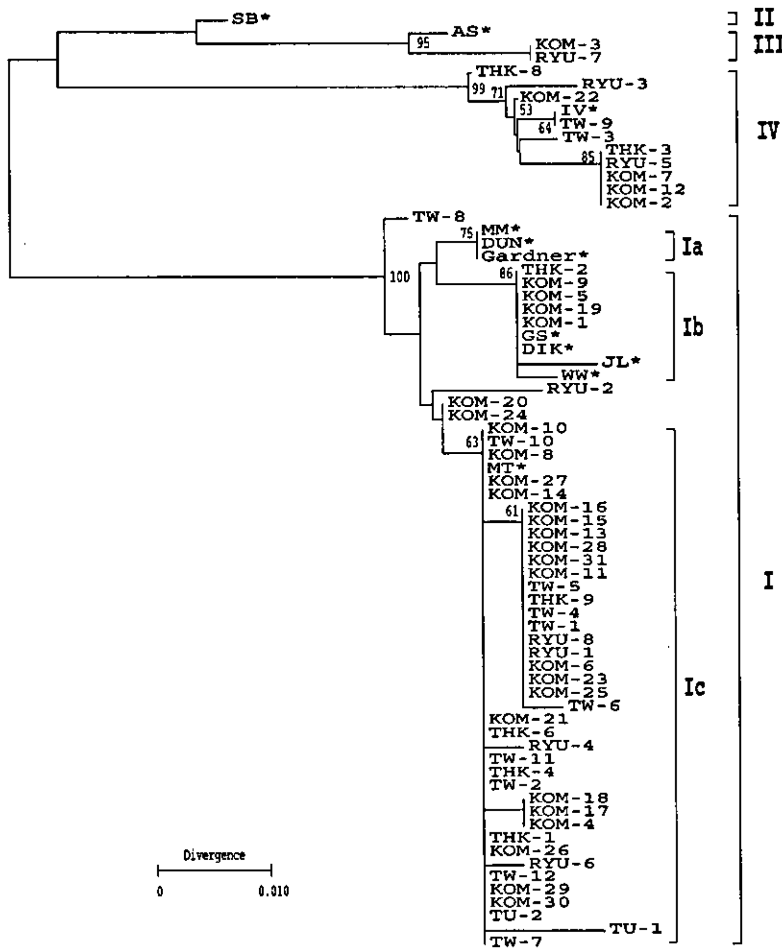

Fig. 1. Phylogenetic tree used to classify the BKV isolates into subtypes. The 287 bp typing sequences detected in the present and previous studies were used to construct an NJ phylogenetic tree using CLUSTAL W (Thompson *et al.*, 1994). The phylogenetic tree was visualized using DendroMaker for Macintosh 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.

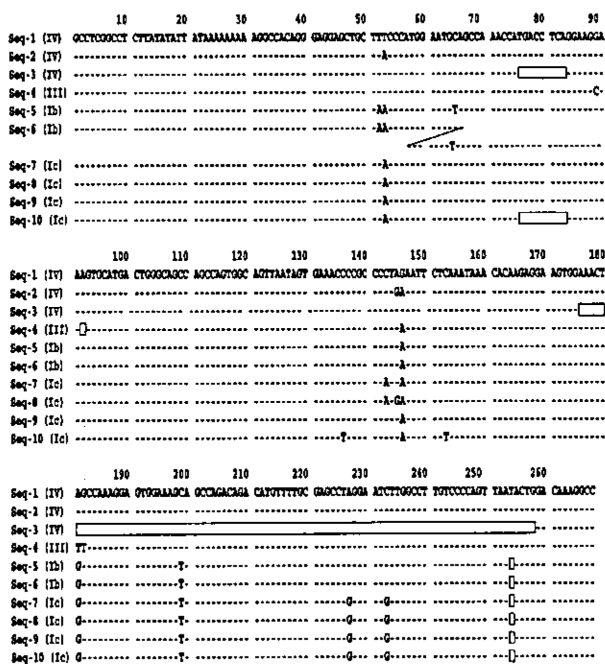

Fig. 2. Ten TCR sequences detected from the urine of RT and BMT patients. Sequences between the midpoint of the origin of replication and the start site of the agnogene are shown (for convenience, the nucleotide numbering starts at the midpoint of the origin of replication). Seq-1 (the TCR commonly found 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.

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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REFERENCES

Agostini, H. T., Brubaker, G. R., Shao, J., Levin, A., Ryschkewitsch, C. F., Blattner, W. A. & Stoner, G. L. (1995). BK virus and a new type of JC virus excreted by HIV-1 positive patients in rural Tanzania. *Arch Virol* **140**, 1919–1934.

Agostini, H. T., Jobes, D. V. & Stoner, G. L. (2001). Molecular evolution and epidemiology of JC virus. In *Human Polyomaviruses: Molecular and Clinical Perspectives*, pp. 491–526. Edited by K. Khalili & G. L. Stoner. New York: Wiley.

Akiyama, H., Kurosu, T., Sakashita, C. & 9 other authors (2001). Adenovirus is a key pathogen in hemorrhagic cystitis associated with bone marrow transplantation. *Clin Infect Dis* **32**, 1325–1330.

Baksh, F. K., Finkelstein, S. D., Swalsky, P. A., Stoner, G. L., Ryschkewitsch, C. F. & Randhawa, P. (2001). Molecular genotyping of BK and JC viruses in human polyomavirus-associated interstitial nephritis after renal transplantation. *Am J Kidney Dis* **38**, 354–365.

Chauhan, S., Lecatsas, G. & Harley, E. H. (1984). Genome analysis of BK (WW) viral DNA cloned directly from human urine. *Intervirology* **22**, 170–176.

Chesters, P. M., Heritage, J. & McCance, D. J. (1983). Persistence of DNA sequences of BK virus and JC virus in normal human tissues and in diseased tissues. *J Infect Dis* **147**, 676–684.

Coleman, D. V., Wolfendale, M. R., Daniel, R. A., Dhanjal, N. K., Gardner, S. D., Gibson, P. E. & Field, A. M. (1980). A prospective study of human polyomavirus infection in pregnancy. *J Infect Dis* **142**, 1–8.

Di Taranto, C., Pietropaolo, V., Orsi, G. B., Jin, L., Sinibaldi, L. & Degener, A. M. (1997). Detection of BK polyomavirus genotypes in healthy and HIV-positive children. *Eur J Epidemiol* **13**, 653–657.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

Gardner, S. D., Field, A. M., Coleman, D. V. & Hulme, B. (1971). New human papovavirus (B.K.) isolated from urine after renal transplantation. *Lancet* **1**, 1253–1257.

Hara, K., Oya, Y., Kinoshita, H., Taguchi, F. & Yogo, Y. (1986). Sequence reiteration required for the efficient growth of BK virus. *J Gen Virol* **67**, 2555–2559.

Heritage, J., Chesters, P. M. & McCance, D. J. (1981). The persistence of papovavirus BK DNA sequences in normal human renal tissue. *J Med Virol* **8**, 143–150.

Imanishi, T. (1998). DendroMaker for Macintosh ver. 4.1. http://www.cib.nig.ac.jp/dda/timanish/dendromaker/home.html.

Jin, L. (1993). Rapid genomic typing of BK virus directly from clinical specimens. *Mol Cell Probes* **7**, 331–334.

Jin, L., Gibson, P. E., Knowles, W. A. & Clewley, J. P. (1993a). BK virus antigenic variants: sequence analysis within the capsid VP1 epitope. *J Med Virol* **39**, 50–56.

Jin, L., Gibson, P. E., Booth, J. C. & Clewley, J. P. (1993b). Genomic typing of BK virus in clinical specimens by direct sequencing of polymerase chain reaction products. *J Med Virol* **41**, 11–17.

Jin, L., Pietropaolo, V., Booth, J. C., Ward, K. H. & Brown, D. W. (1995). Prevalence and distribution of BK virus subtypes in healthy people and immunocompromised patients detected by PCR-restriction enzyme analysis. *Clin Diag Virol* **3**, 285–295.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

Kitamura, T., Aso, Y., Kuniyoshi, N., Hara, K. & Yogo, Y. (1990). High incidence of urinary JC virus excretion in nonimmunosuppressed older patients. *J Infect Dis* **161**, 1128–1133.

Knowles, W. A. (2001). The epidemiology of BK virus and the occurrence of antigenic and genomic subtypes. In *Human Polyomaviruses: Molecular and Clinical Perspectives*, pp. 527–559. Edited by K. Khalili & G. L. Stoner. New York: Wiley.

Knowles, W. A., Gibson, P. E. & Gardner, S. D. (1989). Serological typing scheme for BK-like isolates of human polyomavirus. *J Med Virol* **28**, 118–123.

Moens, U. & Rekvig, O. P. (2001). Molecular biology of BK virus and clinical and basic aspects of BK virus renal infection. In *Human Polyomaviruses: Molecular and Clinical Perspectives*, pp. 359–408. Edited by K. Khalili & G. L. Stoner. New York: Wiley.

Negrini, M., Sabbioni, S., Arthur, R. R., Castagnoli, A. & Barbanti-Brodano, G. (1991). Prevalence of the archetypal regulatory region and sequence polymorphisms in nonpassaged BK virus variants. *J Virol* **65**, 5092–5095.

Rubinstein, R., Pare, N. & Harley, E. H. (1987). Structure and function of the transcriptional control region of nonpassaged BK virus. *J Virol* **61**, 1747–1750.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Seif, I., Khoury, G. & Dhar, R. (1979). The genome of human papovavirus BKV. *Cell* **18**, 963–977.

Sugimoto, C., Hara, K., Taguchi, F. & Yogo, Y. (1989). Growth efficiency of naturally occurring BK virus variants in vivo and in vitro. *J Virol* **63**, 3195–3199.

Sugimoto, C., Hara, K., Taguchi, F. & Yogo, Y. (1990). Regulatory DNA sequence conserved in the course of BK virus evolution. *J Mol Evol* **31**, 485–492.

Sugimoto, C., Kitamura, T., Guo, J. & 16 other authors (1997). Typing of urinary JC virus DNA offers a novel means of tracing human migrations. *Proc Natl Acad Sci U S A* **94**, 9191–9196.

Tavis, J. E., Walker, D. L., Gardner, S. D. & Frisque, R. J. (1989). Nucleotide sequence of the human polyomavirus AS virus, an antigenic variant of BK virus. *J Virol* **63**, 901–911.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Yang, R. C. & Wu, R. (1979). BK virus DNA: complete nucleotide sequence of a human tumor virus. *Science* **206**, 456–462.

Yogo, Y., Sugimoto, C., Zheng, H.-Y., Ikegaya, H., Takasaka, T. & Kitamura, T. (2004). JC virus genotyping offers a new paradigm in the study of human populations. *Rev Med Virol* **14**, 179–191.

Yoshiike, K. & Takemoto, K. K. (1986). Studies with BK virus and monkey lymphotropic papovavirus. In *The Papovaviridae, vol. 1, The Polyomaviruses*, pp. 295–326. Edited by N. P. Salzman. New York: Plenum.

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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 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.

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.

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.