

Analysis of the origin of inherited chromosomally integrated human herpesvirus 6 in the Japanese population

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Abstract

Integration of the complete human herpesvirus 6 (HHV-6) genome into the telomere of a chromosome has been reported in some individuals (inherited chromosomally integrated HHV-6; iciHHV-6). Since the proportion of iciHHV-6-positive individuals with integration in chromosome 22 is high in Japan, we hypothesized a founder effect. In this study, we sought to elucidate the reason for the high proportion of viral integrations into chromosome 22. We analyzed six cases of iciHHV-6A and two cases of iciHHV-6B, including one iciHHV-6A case with a matched sample from a father and one iciHHV-6B case with a matched sample from a mother. In iciHHV-6A, the same copy numbers of viral telomeric repeat sequences (TRS) and the same five microsatellite markers were detected in both the index case and paternal sample. Moreover, the same five microsatellite markers were demonstrated in four cases and the same copy numbers of viral TRS were demonstrated in two pairs of two cases. The present microsatellite analysis suggested that the viral genomes detected in some iciHHV-6A patients were derived from a common ancestral integration.

INTRODUCTION

Human herpesvirus 6 (HHV-6) is categorized into two distinct species, HHV-6A and HHV-6B, which are closely related herpesviruses with an overall nucleotide sequence identity of 90% [1, 2]. Although HHV-6B is a ubiquitous virus in developed countries, prevalence of HHV-6A infection is very low in those countries [3]. Primary HHV-6B infection generally occurs in infancy and causes a common febrile exanthematous disease, exanthem subitum [4, 5]. In contrast, the clinical features of primary HHV-6A infection remain unclear. Horizontal transmission of HHV-6 is considered to be the main route of viral infection from parents to children because HHV-6 is frequently excreted in saliva in seropositive individuals [6, 7].

In addition to horizontal viral transmission, HHV-6 is transmitted from parent to child as an inherited chromosomally integrated HHV-6 (iciHHV-6) [8]. The complete HHV-6 genome is integrated into the telomere of a

chromosome in some individuals [9, 10]. The prevalence of iciHHV-6 in the USA [11] and UK [12] is approximately 0.8% compared to only 0.2% in Japan [13]. As iciHHV-6 patients have one viral copy per cell, the result of real-time polymerase chain reaction (PCR) analysis demonstrates extremely high copy numbers of viral DNA; generally, over 1 million copies ml⁻¹ in whole blood and over 3000 copies ml⁻¹ in serum [14]. Furthermore, an individual with a high viral load may be misdiagnosed with an active HHV-6 infection and unnecessarily prescribed antiviral drugs [15]. Although iciHHV-6 has been associated with several clinical manifestations such as encephalitis [16, 17], cognitive dysfunction and fatigue [18], and angina pectoris [19], the precise role of iciHHV-6 in these clinical manifestations is still under debate. Additionally, viral reactivation from integrated HHV-6 genomes has also been suggested in immunocompromised patients [20] and pregnant women [21, 22], as well as under *in vitro* experimental conditions [23].

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Abbreviations: FISH, fluorescence *in situ* hybridization; HHV-6, human herpesvirus 6; iciHHV-6, inherited chromosomally integrated HHV-6; TRS, telomeric repeat sequences; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; PHA, phytohemagglutinin.

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The HHV-6 genome can be integrated into a host chromosome by homology-mediated integration between human telomere repeat sequences and viral telomere repeat-like sequences and the integrated viral genome is transmitted by Mendelian inheritance [24, 25]. Furthermore, a recent study has demonstrated that TRS, in particular the perfect TRS, is crucial for integration and maintenance of a viral genome in HHV-6A integration [26]. Several sites of viral integration have been reported to date based on fluorescence *in situ* hybridization (FISH) analysis [27, 28]. Although the HHV-6 genome is believed to be randomly integrated into the telomeric region of host chromosomes, we discovered several individuals and families with *ici*HHV-6 integration into chromosome 22. Additionally, although HHV-6A infection rarely occurs in developed countries, it has been demonstrated that frequency of *ci*HHV-6A was higher than that of *ci*HHV-6B [29]. These findings suggest that HHV-6A may have higher potency for integration into a host genome or chromosome 22 may be more susceptible to viral genome integration than other chromosomes. Another possible hypothesis for explaining the unique findings is the founder effect. Therefore, we sought to elucidate the mechanism of viral integration into chromosome 22, particularly the founder effect.

RESULTS

Virological and microsatellite analyses of the two matched parent and index cases

FISH analysis confirmed that the virus genome was integrated into chromosome 22 in all of the analyzed subjects (one representative image is shown in Fig. 1). As an initial experiment, in order to evaluate the reliability of the analysis, microsatellite and TRS analyses were carried out in the matched parent and index cases. The data for virological and microsatellite analyses of two families (families 1 and 7) are shown in Fig. 2. In family 1, 16 HHV-6A TRS were detected in both the index case and paternal genomes. Additionally, the PCR products were exactly the same sizes for all five microsatellite markers on one of the alleles. The haplotypes for the integration of HHV-6A in chromosome 22 were estimated as follows: 242 bp for rs71772361; 102 bp for rs386395698; 273 bp for rs71810967; 215 bp for rs746349407; and 141 bp for rs762747991. Similarly, in family 7, 32 HHV-6B TRS were demonstrated in both the index case and maternal genomes. Moreover, the PCR products were exactly the same sizes for all of the microsatellite markers. Haplotypes of chromosome 22, in which HHV-6B might be integrated, were estimated to be 232 or 238 bp for rs71772361, 106 bp for rs386395698, 271 bp for rs71810967, 213 or 217 bp for rs746349407 and 141 bp for rs762747991. As expected, TRS and microsatellites were matched completely between each parent and index case suggesting the analysis is highly reliable.

Virological and microsatellite analyses of all cases of *ici*HHV-6A

Next, the TRS and microsatellite analysis were carried out in other unrelated families (index cases) to examine a founder effect. As assumed by the analysis of family 1, PCR product

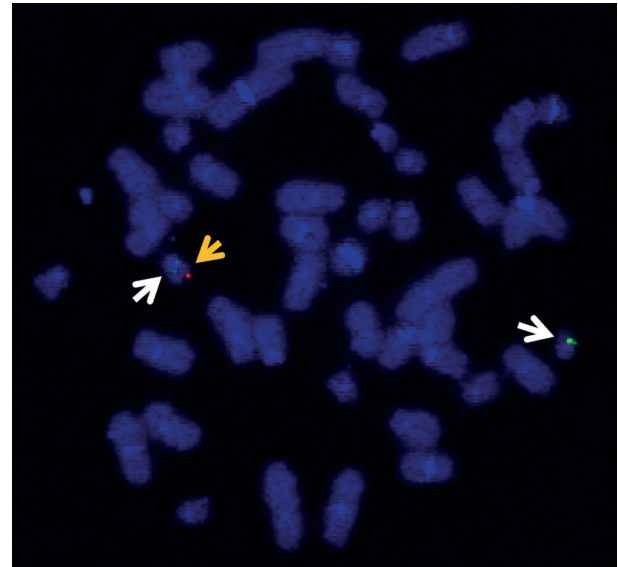


Fig. 1. FISH analyses of metaphase chromosomes derived from the *ici*HHV-6A study subjects. The red signal from HHV-6 is detectable (yellow arrowhead) at the end of chromosome 22q (family 5 index case). The green reference signals for chromosome 22q11.21 are indicated by white arrowheads.

sizes of the microsatellite markers at rs71772361, rs386395698, rs71810967, rs746349407 and rs762747991, representing the integration of the HHV-6A genome, were 242, 102, 273, 215 and 141 bp, respectively. As shown in Table 1, the five haplotypes of one allele in families 2, 3 and 4 were exactly the same as family 1; however, these haplotypes were different in families 5 and 6. Additionally, 16 HHV-6A TRS were found to be identical in families 1 and 2, but not in families 3 and 4 that had 14 HHV-6A TRS. Families 5 and 6 contained 15 and 12 HHV-6A TRS, respectively.

Virological and microsatellite analyses of *ici*HHV-6B cases (Table 2)

In addition to *ici*HHV-6A, we compared the microsatellite markers of *ici*HHV-6B in families 7 and 8. PCR product sizes for microsatellite markers at rs71772361, rs386395698, rs71810967, rs746349407 and rs762747991 in family 8 were 232, 106, 255 or 277 bp, 215 or 211 bp, and 141 or 139 bp, respectively. Thus, distinct haplotypes of chromosome 22 were detected for family 8 relative to family 7. We detected 32 HHV-6B TRS in family 7 compared to 29 HHV-6B TRS in family 8.

DISCUSSION

A microsatellite is a tract of repetitive DNA ranging in length from two to five base pairs that is typically repeated 5–50 times [30]. Microsatellites have been identified in thousands of locations throughout the human genome, and are associated with a high mutation rate as well as high diversity in the population. Therefore, microsatellite marker

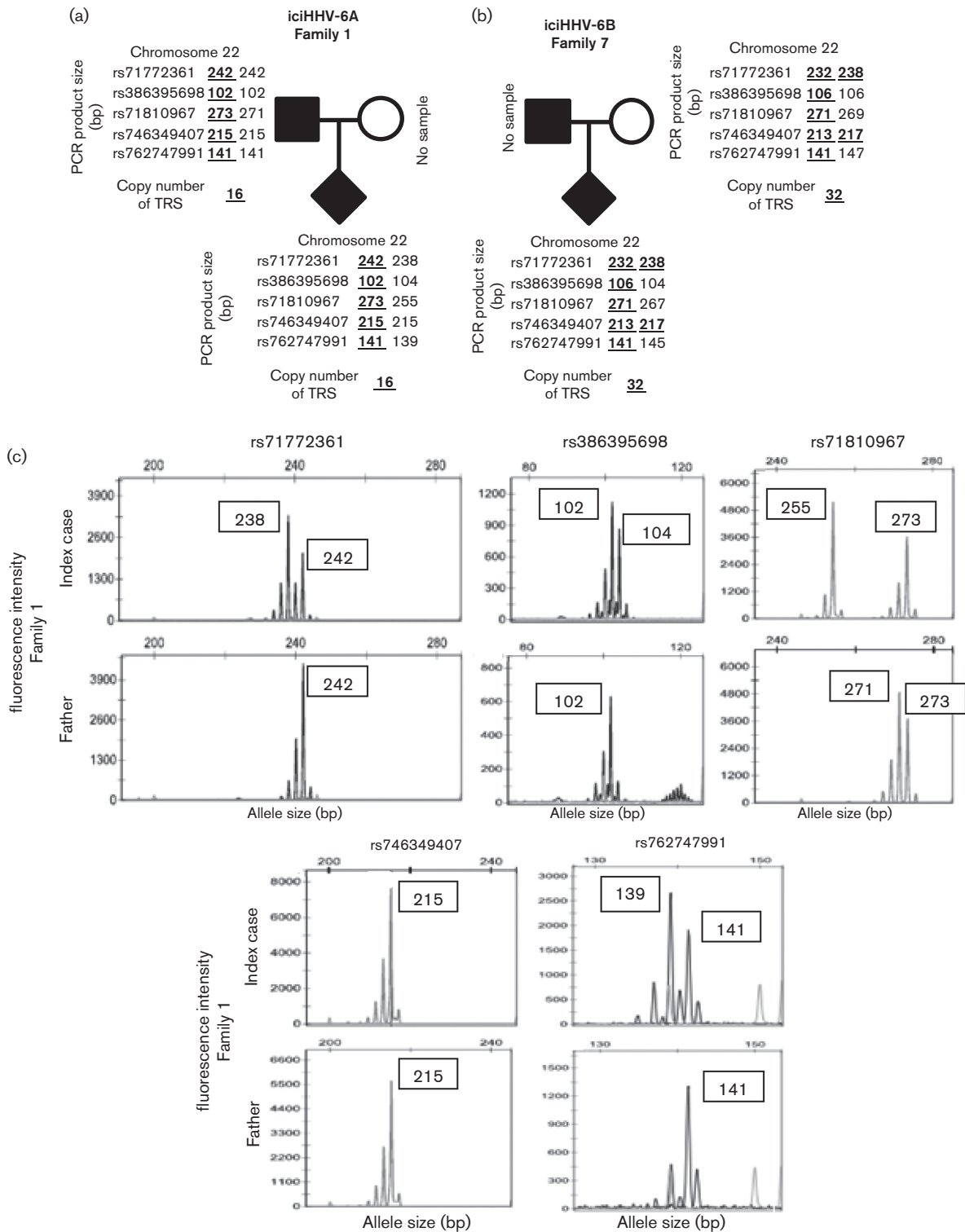


Fig. 2. Haplotypes of microsatellite markers and copy number of TRS for (a) the index case of iciHHV-6A and matched father and (b) the index case of iciHHV-6B and matched mother. (c) Genotyping microsatellite data. The dye labeled PCR amplicons of each microsatellite were separated by size using electrophoresis and were then identified by fluorescence detection. The data were genotyped detecting the peaks. The data of the index case and father from family 1 are shown here as a representative.

Table 1. PCR product size (bp) of microsatellite markers and copy number of TRS in iciHHV-6A patients and families

Family	1		2		3		4		5		6			
Case	Index	Father	Index	Index	Index	Index	Index	Index	Index	Index	Index			
rs71772361	242	238	242	242	242	242	242	236	242	236	242	242	242	232
rs386395698	102	104	102	102	102	102	102	102	102	102	110	110	102	102
rs71810967	273	255	273	271	273	271	273	255	273	271	273	269	273	269
rs746349407	215	215	215	215	215	213	215	209	215	217	215	213	219	227
rs762747991	141	139	141	141	141	143	141	141	141	139	141	143	143	139
Copy no. of TRS	16		16		16		14		14		15		12	

The bold underlined numbers indicate matched haplotypes among different families.

iciHHV-6, inherited chromosomally integrated human herpesvirus 6; no., number; TRS, telomeric repeat sequences.

analysis has been used to discover founder effects in several important genetic diseases [31–33]. In this study, the haplotypes of the microsatellites that are located close to the HHV-6 genome integration site in chromosome 22q were exactly the same between the index cases and matched parental samples for both HHV-6A and HHV-6B. Previous reports using FISH analysis [20, 23, 25, 34], revealed that if two generations had the same HHV-6 genome integration sites then the integrated viral genome was likely inherited from a parent. Our present data further support the hypothesis for hereditary transmission of iciHHV-6 from parent to child. Three of the five iciHHV-6A index cases (families 2, 3 and 4) from unrelated families had the same type of microsatellite markers as family 1. These results suggested that the HHV-6A genome was integrated into chromosome 22 of their ancestors and passed on to offspring in Japan. Although lack of basic data for the present microsatellites in the Japanese population is considered to be limitation of this study, as shown in Tables 1 and 2, the diversity of these microsatellites in iciHHV-6A patients was different from those of iciHHV-6B patients. Therefore, it is considered that the microsatellites measured in this study have sufficient diversity for evaluation of the founder effect.

Recently, Tweedy *et al.* demonstrated a founder HHV-6A integration in the telomere of chromosome 17p by phylogenetic analysis and Sanger sequencing of integration sites [35]. To the best of our knowledge, this is the first data that suggests the founder effect on iciHHV-6 in chromosome 22q, which is relatively common for the integration site in the Japanese population. Despite the fact that HHV-6A is not commonly detected in children in Japan [36], the USA and Europe [37–39], the HHV-6A genome was integrated into six of the eight iciHHV-6 cases in this study. Thus, this curious phenomenon further supports the notion of the founder effect of iciHHV-6A.

Two families of iciHHV-6B were analyzed in this study. The haplotypes of the microsatellites were the same in the index case and matched maternal sample (family 7). However, distinct haplotypes were identified for families 7 and 8. Thus, inherited transmission of iciHHV-6B genomes between generations was demonstrated, but it is unclear

whether founder effects also play a role in iciHHV-6B. Future studies on a large number of cases are needed to elucidate the founder effect on iciHHV-6B in the Japanese population.

In order to determine whether integrated HHV-6 genomes were the same among the families, we examined the numbers of TRS located in the direct repeats of the HHV-6 genome. Previous studies determined that the number of TRS in the HHV-6 genome was highly variable among laboratory strains and clinical specimens [40]. In our previous study, the number of TRS was stable after at least 17 passages of cultured cells, and was useful for the differentiation of HHV-6 strains [41]. In contrast to our expectation, the number of HHV-6A TRS was different in some families with the same haplotype of microsatellites. For example, families 1 and 2 had 16 TRS and families 3 and 4 had 14 TRS. Although the number of TRS are stable after at least 17 passages of cultured cells [41], it is unclear whether the number of TRS is stable after more than 17 passages and is stable *in vivo*. It is possible that mutations may occur in a unique region of integrated HHV-6A causing the number of TRS to change over many generations after the initial integration of the viral genome in a Japanese ancestor. The

Table 2. PCR product size (bp) of microsatellite markers and copy numbers of TRS in iciHHV-6B patients and families

Family	7		8			
Case	Index	Mother	Index	Index		
rs71772361	232	238	232	238	232	232
rs386395698	106	104	106	106	106	106
rs71810967	271	217	271	269	255	277
rs746349407	213	217	213	217	215	211
rs762747991	141	145	141	147	141	139
Copy no. of TRS	32		32		29	

The bold underlined numbers indicate matched haplotypes among different families.

iciHHV-6, inherited chromosomally integrated human herpesvirus 6; no., number; TRS, telomeric repeat sequences.

Table 3. Clinical characteristics of iciHHV-6 patients and families

Family	Cases	Trigger for the diagnosis of iciHHV-6
1	Index case and father	Examination for the causative agent of hemophagocytic lymphohistiocytosis
2	Index case	Virus monitoring in ALL patients after bone-marrow transplantation
3	Index case	Examination for the causative agent of uveitis
4	Index case	Examination for the causative agent of encephalitis
5	Index case	Examination for the causative agent of multiform exudative erythema
6	Index case	Virus monitoring in AML patients after bone-marrow transplantation
7	Index case and mother	Examination for the causative agent of meningitis
8	Index case	Virus monitoring in patients with aplastic anemia after bone-marrow transplantation

iciHHV-6, inherited chromosomally integrated human herpesvirus 6; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia.

copy numbers of TRS were different between iciHHV-6A patients (from 12 to 16) and iciHHV-6B patients (from 29 to 32). These results were consistent with previous studies that demonstrated 15 to 25 TRS in HHV-6A strains and 22 to 90 TRS in HHV-6B strains [40, 41]. Although the underlying mechanism responsible for the variance in the copy number of TRS is unknown, these differences may depend on the HHV-6 species.

Most iciHHV-6 patients are diagnosed based on real-time PCR analysis. However, in transplant recipients, PCR monitoring may lead to the misdiagnosis of active HHV-6 infection and unnecessary administration of toxic antiviral drugs [15, 42]. FISH analysis is required for identification of the viral integration site, but it is a time-consuming method that requires informed consent from the patient. We understand that the small number of cases analyzed was a limitation of the current study. Therefore, we believe a longitudinal study enrolling a large number of cases is

needed to confirm our hypothesis. Additionally, an international collaborative study is also important to elucidate the origin of the first integration of either the HHV-6A or HHV-6B genome into the human genome.

Based on our findings, the detection of iciHHV-6 in some patients is likely due to viral integration in their ancestors. Although previous reports have demonstrated the integration of HHV-6 into telomeres of naïve cells upon their infection *in vitro* [23], reports of this phenomenon has not yet been established *in vivo*. It is important to highlight the fact that iciHHV-6 represents a risk factor for the development of angina [19]. Therefore, we believe that it is critically important to elucidate the mechanism of viral integration into hosts to clarify the pathogenic relationship between reactivation and various diseases.

METHODS

Human subjects

Eight Japanese cases with iciHHV-6 integrated into chromosome 22, including two cases with matched parental samples, were analyzed in this study. All of the cases were from different regions in Japan, and there were no known blood relationships among them. iciHHV-6 was confirmed by real-time PCR analysis, which detected high copy numbers of viral DNA in peripheral blood mononuclear cells (PBMCs), hair follicle and nail samples [43], and by FISH of PBMCs [27]. Restriction digestion of PCR products was used to discriminate between integration of HHV-6A and HHV-6B viruses [44]. The clinical features of each case are summarized in Table 3.

FISH

FISH was conducted to identify the integration sites of HHV-6 on the human chromosome as previously described [27]. Briefly, phytohemagglutinin (PHA)-stimulated lymphocytes or Epstein Barr virus-transformed lymphoblasts were treated with colcemid to induce cell-cycle arrest. Metaphase preparations were obtained using 0.075 M KCl followed by methanol/acetate fixation. FISH probes were made from PCR products and a plasmid containing HHV-6 sequences that were labeled by nick-

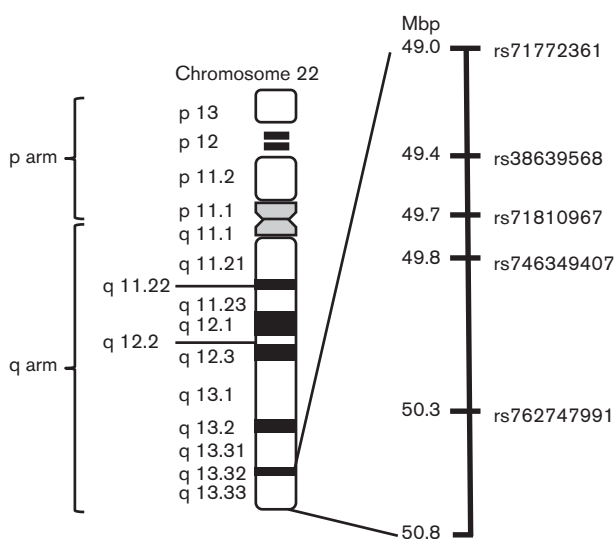


Fig. 3. Schematic of chromosome 22 including microsatellite markers at 22q13.33.

Table 4. Position and primer sequences for the PCR of each microsatellite

Name	Microsatellite		Primer sequence	
	Start position	End position	Forward	Reverse
rs71772361	49 006 333	49 006 366	5'-FAM-ACAACCTCCAGCAGACGTGGTG-3'	5'-CACACTCACTCACACAAAATCATG-3'
rs386395698	49 424 694	49 424 743	5'-FAM-TACCTCCTCCTCCTTGAGAC-3'	5'-GATCAGGGAGGAAGCAACAG-3'
rs71810967	49 706 585	49 706 632	5'-VIC-GCACAGATGGGATCCTAGAGTG-3'	5'-GATCCATTGACCAAAGATGACTTC-3'
rs746349407	49 832 718	49 832 765	5'-VIC-CAGACAGAGCTAGGAAATAAGTATG-3'	5'-AGCTACTAAGTGAAGGCATAGATAG-3'
rs762747991	50 296 194	50 296 230	5'-FAM-ATAGTGGTGAACCTTGGGAGCAAG-3'	5'-CGGTCTAGTTCAAGTTCCTTGAAC-3'

translation with biotin-16-dUTP or digoxigenin-11-dUTP. After hybridization, the probes were visualized using either Alexa Fluor 488-conjugated streptavidin or rhodamine-conjugated anti-digoxigenin in cells that were counter stained with 4',6-diamino-2-phenylindole (DAPI). As reference standards, we used Bac clone RP11-18608 (22q11.21) or TelVysion 22q SpectrumOrange (5J04-022, Abbott Molecular, Illinois, USA).

DNA extraction and PCR assay for the viral TRS of HHV-6

TRS analysis was conducted as previously described [41]. DNA was extracted from the stored PBMCs using a QIAamp DNA blood minikit (QIAGEN, Chatsworth, CA, USA) according to the manufacturer's instructions. Extracted DNA was eluted in 100 µl buffer and stored at -20 °C until ready to be assayed using PCR. TRS of HHV-6A and HHV-6B were amplified using primers that were specific to sequences containing the perfect telomere repeat known as TRS-2 in the direct repeat region (DR)-L of each virus as previously described [40, 41]. LA Taq (TaKaRa Bio, Otsu, Japan) and the following conditions were used for the PCRs: denaturation at 94 °C for 1 min, followed by 30 cycles at 94 °C for 30 s, 63 °C for 30 s and 72 °C for 1 min. The sizes of the amplified products were analyzed by electrophoresis on a 1 % agarose gel with ethidium bromide staining.

Sequence analysis of the TRS of HHV-6

PCR products were purified using a PCR purification kit (QIAGEN), and directly sequenced using a BigDye Terminator cycle sequencing kit and a Prism 3100 Avant analyzer (Applied Biosystems, Foster City, CA, USA). The purified PCR products were sequenced using H6 TRS F (5'-CTCGGACCCATGCTATCCT-3') for HHV-6B cases and using HHVA (5'-CTACCACGGACGCGTACACACG-3') for HHV-6A cases according to the manufacturer's instructions. The size of the TRS was enumerated by counting the copy number of TAACCC repeats.

PCR assay of microsatellites and microsatellite analysis using fragment analysis

Genotyping was performed by selecting five microsatellites using Tandem Repeats Finder on chromosome 22q13.33 (UCSC Genome Browser on Human Dec. 2013 (GRCh38/hg38) Assembly) [45] (Fig. 3). There were at least 15 perfect

dinucleotide repeat sequences that were highly polymorphic in our population. These microsatellite loci were amplified by PCR using primer pairs consisting of a forward primer that was labeled at the 5'-end with either FAM or VIC and unlabeled reverse primers (Applied Biosystems). Since these microsatellites have not been submitted to GenBank, the rs number which includes each microsatellite region from the Single Nucleotide Polymorphism Database was used as the microsatellite name in this study, and the positions at chromosome 22 and primer sequences of each microsatellite are shown in Table 4. AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA) and the following conditions were used for the PCRs: denaturation at 95 °C for 10 min, followed by 30 cycles at 94 °C for 1 min, 60 °C for rs71772361, rs71810967, and rs746349407, 65 °C at rs386395698, and 62 °C at rs762747991 for 1 min, and 72 °C for 1 min. The PCR products were separated by capillary electrophoresis, and were detected by the intensity of emitted fluorescence on an ABI Prism 3100-Avant Genetic analyzer (Applied Biosystems, Foster City, CA, USA). Raw data were analyzed with the GeneMapper Software v.3.7 (Applied Biosystems, Foster City, CA, USA). The sizes of these PCR products were determined based on the peak detection of fluorescence intensity, and this size was used to identify the haplotypes of each allele.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

This study was approved by the Ethical Review Board Human Genome Studies at Fujita Health University (Accession number 90, approved on 24 March 2010). Written informed consents were obtained from all of the participants or their guardians.

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