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Characterization and phylogenetic analysis of Varicella-zoster virus strains isolated from Korean patients[§]

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Varicella-zoster virus (VZV) is a causative agent of chickenpox in primary infection and shingles after its reactivation from latency. Complete or almost-complete genomic DNA sequences for various VZV strains have been reported. Recently, clinical VZV strains were isolated from Korean patients whose genome was sequenced using high-throughput sequencing technology. In this study, we analyzed single nucleotide polymorphism (SNP) of VZV strains to genetically characterize Korean clinical isolates. Phylogenetic analyses revealed that three Korean strains, YC01, YC02, and YC03, were linked to clade 2. Comprehensive SNP analysis identified 86 sites specific for the 5 VZV clades. VZV strains isolated from Korea did not form a phylogenetic cluster. Rather, YC02 and YC03 clustered strongly with Chinese strain 84-7 within clade 2, more specifically cluster 2a. Signature sequences for the cluster 2a were identified and found to play an important role in the separation of cluster 2a strains from other clade 2 strains, as shown in substitution studies. Further genetic analysis with additional strains isolated from Japan, China, and other Asian countries would provide a novel insight into the significance of two distinct subclades within clade 2.

Keywords: Varicella-zoster virus, Korean strains, Clade 2

Introduction

Varicella-zoster virus (VZV) is a member of alpha-herpesviridae and contains a double-stranded DNA genome with an approximate length of 124 kb. Primary infection of VZV results in varicella or chickenpox and establishes a latent infection in nerve cells of dorsal root ganglia (DRG). Varicella is typically characterized by generalized rash and skin vesicles. Although most cases of varicella are mild, rare central nervous system (CNS) complications may become severe or fatal (Gershon and Gershon, 2013). VZV reactivation from latency can cause zoster or shingles especially in the elderly and immunocompromised people. Herpes zoster is typically unilateral and dermatomal, and causes a wide spectrum of illnesses, ranging from pain with no rash to mild rash, to severe rash with dissemination (Gershon and Gershon, 2013). VZV infection can be prevented with live attenuated vaccines (Creed *et al*., 2009; Flatt and Breuer, 2012; Gershon, 2013). Currently, live attenuated VZV vaccines, which have been developed based on Japanese Oka strain (Takahashi *et al*., 1974; Yamanishi, 2008) and Korean MAV/06 strain (Sohn *et al*., 1994; Kim *et al*., 2011b), are available worldwide. VZV vaccines are licensed for routine use in countries including Australia, Brazil, Canada, China, Germany, Greece, Israel, Italy, Japan, Qatar, South Korea, Spain, Taiwan, United States of America, and Uruguay (Gershon and Gershon, 2013).

Genetic classification and characterization of VZV DNA have been evaluated by several methods and applied to compare sequences of wild-type VZV to sequences of VZV strains or distinguishing vaccine-type strains. In the absence of DNA sequence information, restriction fragment length polymorphism (RFLP) patterns of selected open reading frames (ORFs), such as ORF38 with *Pst*I, ORF54 with *Bgl*I, and ORF62 with *Sma*I, had been commonly used (LaRussa *et al*., 1992; Loparev *et al*., 2000; Sauerbrei *et al*., 2003). Recently, DNA sequence information was employed for genotyping using partial DNA sequences of certain ORFs. Common genotyping methods include the use of concatenated sequences of 5 glycoproteins (gB, gE, gH, gI, gL) and IE62 (Faga *et al*., 2001; Wagenaar *et al*., 2003), nucleotide sequences of ORF22 amplified by PCR (Loparev *et al*., 2004), and scattered single nucleotide polymorphism (SNP) sequences from ORFs 1, 21, 50, 54, and 62 (Quinlivan *et al*., 2002; Barrett-Muir *et al*., 2003). With the availability of full-genome sequences, phylogenetic analysis of 18 VZV strains revealed four major clades (Peters *et al*., 2012). Following the analysis, a common and universal nomenclature of VZV genotyping has been proposed (Breuer *et al*., 2010). This new genotyping scheme separates five clades (1 to 5) and two provisional genotypes (VI, VII). After sequencing additional 21 VZV genomes, Zell *et al* has proposed two novel genotypes, genotype VIII and IX (Zell *et al*., 2012).

Of the five major clades, clade 2 is characterized by VZV strains of Asian origin. Oka and Oka-derived vaccine strains are of Japanese-origin (Takahashi *et al*., 1974), and while Suduvax is of Korean-origin (Kim *et al*., 2011b). LAX1 is also suspected to be of Asian origin, since the strain was

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collected in Los Angeles, a city with a large Asian population (Park *et al.*, 2008; Chow *et al.*, 2013). Phylogeographic studies suggest an out-of-Africa model of VZV evolution. VZV appears to have migrated with modern humans from Africa to Europe and Asia (Grose, 2012). Full genomic sequence analysis of VZV can lead to a better understanding of the evolutionary development of VZV lineage (McGeoch, 2009). Although clade 2 contains several strains, whose full genome has been sequenced, most strains, except for pOka and LAX1, are vaccine or overlapping strains derived from pOka. More genetic information from clade 2 clinical strains is needed to understand the evolution and characteristics of the clade 2.

This study reports the sequencing and characterization of three clinical strains of VZV isolated from Korean patients. Three clinical strains, YC01, YC02, and YC03, all belong to the clade 2. Furthermore, YC02 and YC03, but not YC01, grouped together with the Chinese strain 84-7 to form a phylogenetically meaningful subcluster within clade 2.

Table 1. List and genotyping of VZV strains analyzed in this study

Strain	Accession number	gB,E,H,I,L + IE62	ORF22	Scattered SNP	ORI	Full (Clade)
Dumas	NC001348	A	E	C	A	1
SD	DQ479953	A	E	C	A	1
Kel	DQ479954	A	E	C	A	1
36	DQ479958	A	E	C	A	1
49	DQ479959	A	E	C	A	1
32p5	DQ479961	A	E	C	A	1
NH29_3	DQ674250	A	E	C	A	1
SVETA	EU154348	A	E	C	A	1
MSP	AY548170	A	E	C	A	1
BC	AY548171	A	E	C	A	1
243/2000	JN704690	A	E	C	A	1
1256/2004	JN704691	A	E	C	A	1
551/2005	JN704692	A	E	C	A	1
667/2005	JN704693	A	E	C	A	1
1883/2007	JN704694	A	E	C	A	1
432/2008	JN704695	A	E	C	A	1
925/2008	JN704696	A	E	C	A	1
11	DQ479955	D	E	B	D	3
22	DQ479956	D	E	B	D	3
03-500	DQ479957	D	E	B	D	3
HJO	AJ871403	D	E	B	D	3
2308/2003	JN704699	D	E	B	D	3
3/2005	JN704700	D	E	B	D	3
52/2007	JN704701	D	E	B	D	3
405/2007	JN704702	D	E	B	D	3
1219/2007	JN704703	D	E	B	D	3
Ellen	JQ972913	D	E	B	D	3
413/2000	JN704704	C	M1	A1	D	5
875/2004	JN704705	C	M1	A1	D	5
134/2005	JN704706	C	M1	A1	D	5
446/2007	JN704707	C	M1	A2	D	5
1805/2007	JN704708	C	M1	A1	D	5
CA123	DQ457052	C	M1	A1	A	5
M2DR	DQ452050	C	M2	J1	A	4
8	DQ479960	C	M2	J1	C	4
1483/2005	JN704709	C	M2	A1	C	Genotype8
457/2008	JN704710	D	M1	A1	D	Genotype9
LAX1	JQ972914	B	J	J1	B	2
pOka	AB097933	B	J	J1	B	2
YC01	KJ767491	B	J	J1	B	2
YC02	KJ767492	B	J	J1	B	2
YC03	KJ808816	B	J	J1	B	2
84-7	KC847290	B	J	J1	B	2
092	KY037796	B	J	J1	B	2

Materials and Methods

Acquisition of VZV genome sequences

When this study first began, complete genomic DNA sequences were available from 47 VZV strains. Since the major scope of this study was to perform a molecular phylogenetic analysis of wild-type strains, sequences from mutant or redundant strains were arbitrarily excluded for routine analysis. Therefore sequences of the following eight strains were excluded from this study: five vaccine strains which are mutants of clinical strains, two strains with different passage history, and one strain from a different lot. In addition, the genomic DNA sequences, determined as described previously (Jeon *et al*., 2016), from three Korean patients (KJ767491, KJ767492, KJ808816), and two VZV sequences (KC847290, KY037796) known to belong to clade 2 were added later for analysis. Complete genomic sequences of 44 VZV strains were used in this study (Table 1).

Genotyping

Several methods for genotyping of VZV, were used and applied to determine the genotype of YC01, YC02, YC03, as well as the other VZV strains analyzed in this study. The first method utilizes concatenated sequences of 5 glycoproteins (gB, gE, gH, gI, gL) and IE62 (Faga *et al*., 2001; Wagenaar *et al*., 2003). The second method is based on the nucleotide sequences of ORF22 amplified by PCR using forward primer p22R1f (5′-GGGTTTTGTATGAGCGTTGG-3′) and reverse primer p22R1r (5′-CCCCCGAGGTTCGTAATATC-3′) (Loparev *et al*., 2004). The third method uses scattered single nucleotide polymorphic (SNP) sequences from ORFs 1, 21, 50, 54, and 62 (Quinlivan *et al*., 2002; Barrett-Muir *et al*., 2003). The fourth method uses sequences around replication origins for genotyping (Peters *et al*., 2012). Corresponding sequences were retrieved from the genomic sequences of each VZV strain and aligned using ClustalX program (version 2.0, http://www.clustal.org/clustal2). The resulting *.phy files were used to calculate genetic distances. Phylogenetic trees were constructed using Phylip package (version 3.6a, http://evolution.gs.washington.edu/phylip/getme.html).

Phylogenetic analysis

DNA sequences were obtained from NCBI GenBank database and aligned with the sequences of YC01, YC02, and YC03 using ClustalW program (version 2.1, http://www.clustal.org/clustal2) and examined with Bioedit (version 7.0.5.3, http://www.mbio.ncsu.edu/bioedit/bioedit.html) and CLC Sequence Viewer (version 6.4, http://www.clcbio.com/products/clc-sequence-viewer/) programs. The aligned files were used to determine the genetic distances among the strains using Dnadist program with Kimura-2 parameter in Phylip package. Neighbor joining and maximum likelihood trees were constructed using Neighbor and Dnaml program in Phylip package. Constructed trees were then confirmed and viewed with Treeview program (version 1.6.6, http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Inferring the most recent common ancestor (MRCA) sequence

MRCA of clade 2 strains was inferred using maximum likelihood (ML) method. Dnamlk program in PHYLIP package (ver. 3.6a3), with the options of transition/transversion ratio = 2.0 and no rate variation among sites, was used for ML-based reconstruction of MRCA. Other parameters were set as defaults. The root sequences thus represented the inferred MRCA sequences.

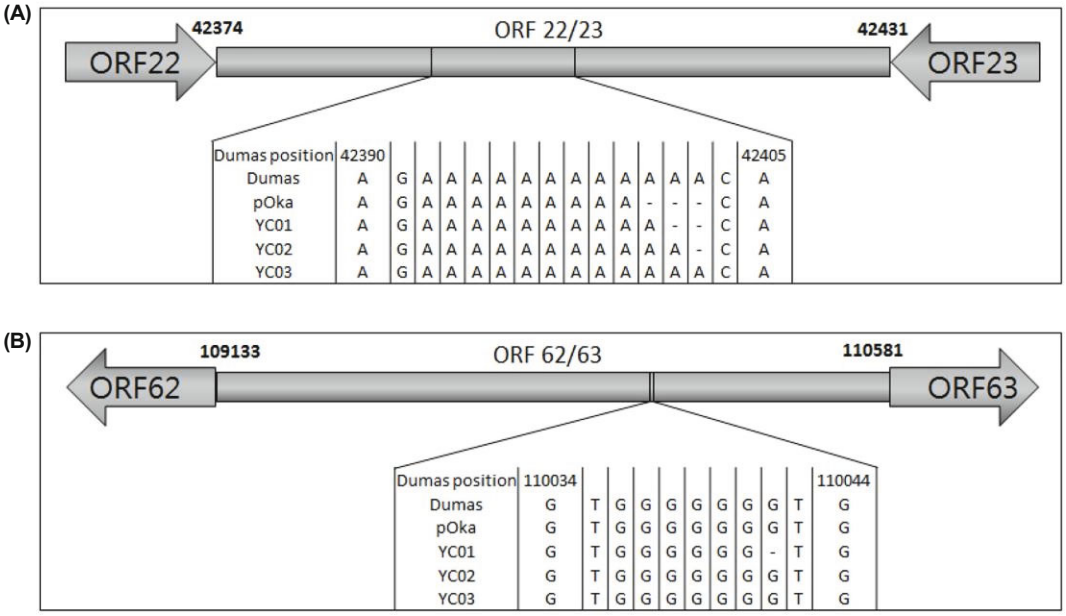

Fig. 1. Two examples of in/dels found among 3 Korean strains (YC01, YC02, YC03). (A) In/del at position 42402 and 42403 in ORF22/23 of YC01 and YC02 was detected after a run of 10 consecutive A's. ORF22/23 is located between rightward ORF22 and leftward ORF23. (B) Similarly, in/del at position 110042 in ORF62/63 of YC01 was found after a run of 6 consecutive G's. ORF 62/63 is located between leftward ORF62 and rightward ORF63.

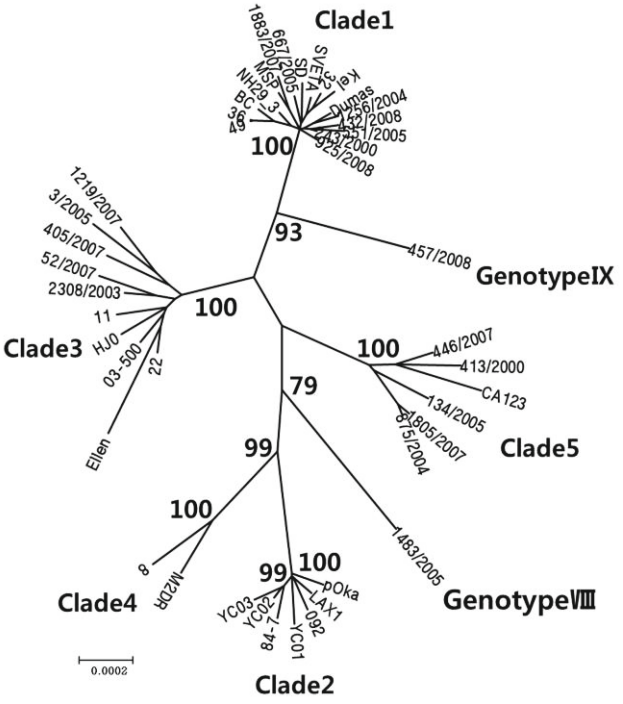

Fig. 2. Phylogenetic trees of 44 VZV strains. The full genome sequences of 41 VZV strains obtained from NCBI GenBank combined with 3 Korean strains (YC01, YC02, and YC03) were used to generate the phylogenetic trees using neighbor-joining (NJ) or maximum likelihood (ML) method. The tree shown was generated by ML method and bootstrap values were included. Similar tree was generated by NJ method.

Results

Characterization of Korean VZV strains

The complete genomic DNA sequences of YC01, YC02, and YC03 were compared and characterized in this study. The genome size of the three strains was as follows: 125,144 bp for YC01, 125,150 bp for YC02, and 125,162 bp for YC03. The difference in genome size among the strains was due to 17 insertion/deletion (in/del) in non-coding regions. Although nucleotide sequences varied, the lengths of ORFs among YC01, YC02, and YC03 were found to be identical (see below). Interestingly, all in/dels occurred after a run of the same 6 to 10 nucleotides. For example, in/del at position between 42402 and 42403 located in ORF22/23 occurred after a run of 10 tandem A's and in/del at position 110042 located in ORF62/63 occurred after a run of 6 consecutive G's (Fig. 1). Other in/dels are described in Supplementary data Fig. S1. In addition, 11 of the 17 in/dels were located in regions where the polarity of VZV ORF changes. For example, the direction or polarity of the ORF changed in the non-coding region ORF22/23. The direction of ORF22 was rightward while the direction of ORF23 was leftward. Similar change was also observed in ORF62/63 (Fig. 1). The lengths of the highly variable reiteration (R) regions and replication origin (Ori) were invariable among YC01, YC02, and YC03.

Genotyping and phylogenetic analysis of Korean strains

Several methods have been used to genotype VZV based on the available information from limited sequences. Each VZV genotyping method resulted in different clustering of VZV strains. VZV strains that belong to the same genotype according to one method are separated into two or more genotypes when another genotyping method is used, and vice versa (Table 1). For example, genotype E strains in ORF22 method were separated into two different genotypes when other methods were used. On the other hand, YC01, YC02, and YC03 consistently and unequivocally classified with strains pOka, LAX1, 84-7, and 092, forming a distinct genotype in all genotyping methods (Table 1). The phylogenetic tree constructed using complete sequences of 44 VZV strains demonstrates that YC01, YC02, and YC03 belong to clade 2 with pOka, LAX1, 84-7, and 092 (Fig. 2). Other strains belong to the clades as described in Table 1.

SNPs specific for each clade

Previous studies suggest a minimum complement of 27 single nucleotide polymorphisms (SNPs) defining five clades and two genotypes (Breuer *et al.*, 2010). In this study, we attempted to identify SNPs specific to each clade. SNPs were defined as positions on the VZV genome where the base sequence of at least one strain was different from another. Redundant sequences at terminal repeat sequences (TR_L and TR_S) were excluded when considering SNPs. The clade-specific SNPs were defined as SNPs commonly found among the strains in each clade, but not found in the strains of other clades. Accordingly, 86 clade-specific SNPs were identified; 3 for clade 1, 43 for clade 2, 7 for clade 3, 15 for clade 4, and 18 for clade 5 (Fig. 3 and Supplementary data Table S1). The number of genotype VIII- and IX-specific SNPs was 49 and 31, respectively (data not shown). High numbers of SNPs may have originated from a single strain for genotype VIII or genotype IX. The distribution of the clade-specific SNPs along VZV genome appeared to be random as shown in Fig. 3.

The number of clade-specific SNPs was identified for clade

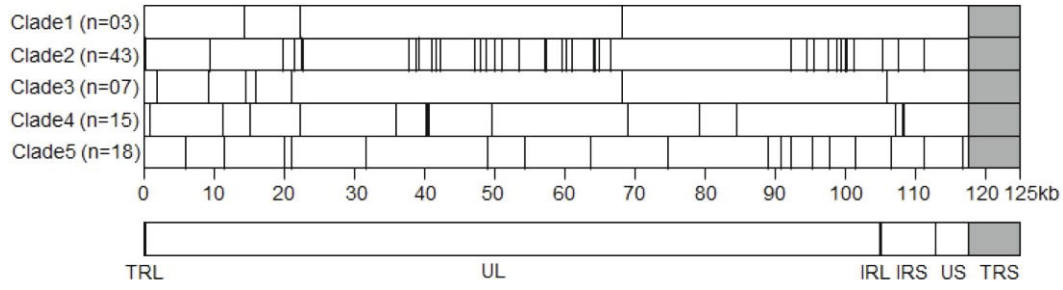

Fig. 3. Distribution of the clade-specific markers on the VZV genome. SNP site specific for each clade was marked. Reiterated (R) region, replication origin, and TRL, TRS (shaded) were excluded from analysis.

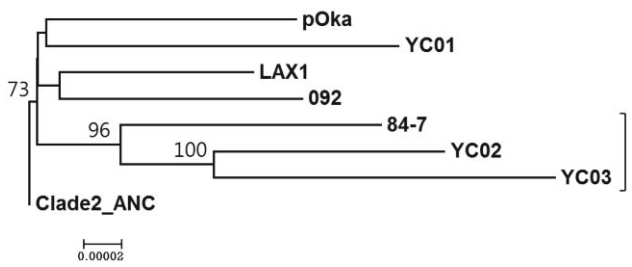

Fig. 4. Phylogenetic analysis of clade 2 strains using most recent common ancestor (MRCA) sequence as an outgroup. The cluster 2a strains (YC02, YC03, and 84-7) formed a strong cluster with bootstrap value of 96. MRCA sequence of the clade 2 was shown as Clade2_ANC. Cluster 2a is shown with a bracket.

2 was 43, including 2 insertions and 41 substitutions. Insertions were found in non-coding regions (NCR) ORF31/32 and ORF32/33. NCR also contained 3 substitutions. Thirty-eight substitutions were located in open reading frames (ORFs). Forty-one substitutions included 31 transitions and 10 transversions. Interestingly, transversions were found only in ORFs, not in NCR. The clade 2-specific SNP sites appear to be clustered in two regions, one between ORF22 and 37, and another between ORF52 and 63 (Fig. 3 and Supplementary data Table S1). In fact, 37 of the 43 (86%) clade 2-specific SNP sites were located in these 2 regions, together spanning approximately 48 kb and 38% of the genome.

Cluster 2a within clade 2

The phylogenetic tree shown in Fig. 2 did not support the idea that YC01, YC02, and YC03 might form a cluster distinguishable from the other clade 2 strains. Rather, YC02 and YC03 appeared to group with 84-7, while YC01 appeared to group with pOka, LAX1, and 092 (Fig. 2). Possibility of subgrouping with the clade 2 was examined by obtaining the genetic distances among the clade 2 strains. The genetic distance ($1.94\pm0.35\times10^{-4}$) among YC02, YC03, and 84-7 was significantly smaller than the genetic distance ($2.83\pm0.42\times10^{-4}$) between these three strains and the other four strains ($P = 0.0036 < 0.01$). On the other hand, the genetic distance ($2.68\pm0.37\times10^{-4}$) among the four strains (YC01, pOka, LAX1, and 092) was not statistically smaller than the genetic distance between the four strains and YC02, YC03, and 84-7 ($P = 0.237$). Thus, only YC02, YC03, and 84-7 appear to form a statistically and phylogenetically meaningful cluster within clade 2, and was named to be cluster 2a. To confirm the presence of the cluster 2a and separation from the other clade 2 strains, the most recent common ancestor (MRCA) se-

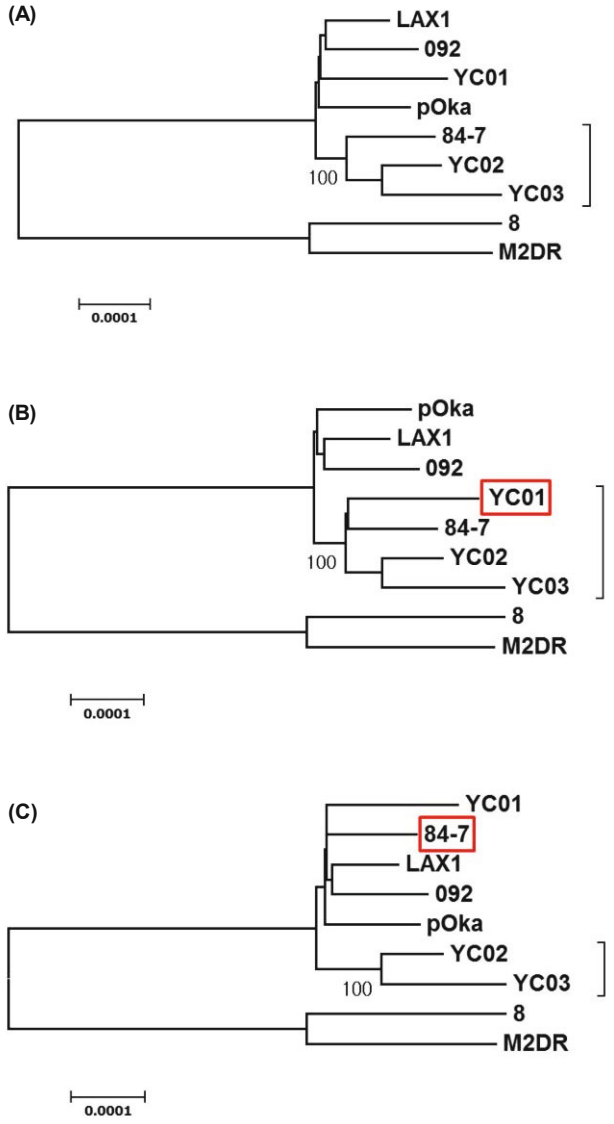

Fig. 5. Substitution analysis. Cluster 2a-specific signature sequences in clade 2 strains at 6 SNP sites shown in Table 2 were replaced with other sequences. The following substituted sequences were used to reconstruct phylogenetic trees along with sequences from other clades. A part of the phylogenetic tree including Clade 4 strains as outgroup is shown in this figure. (A) Unsubstituted original tree showing the separation of cluster 2a strains from the other clade 2 strains. (B) Corresponding sequences of YC01 were replaced with Cluster 2a-specific signature sequences. (C) Cluster 2a signature sequences of 84-7 were replaced with non-signature sequences. Clade 4 strains (8, M2DR) were used as outgroup since the clade 4 is the nearest neighbor of the clade 2. Cluster 2a is shown with a bracket. Note that the bootstrap value for the cluster 2a is 100.

Table 2. SNPs separating cluster 2a from the other clade 2 strains

ORF	Dumas position	Dumas sequence	Cluster 2a			Other clade 2			
			YC02	YC03	84-7	pOka	LAX1	YC01	092
22	40130	C	T	T	T	C	C	C	C
22	40370	A	G	G	G	A	A	A	A
26	44995	T	C	C	C	T	T	T	T
41	76131	G	T	T	T	G	G	G	G
51	88867	C	T	T	T	C	C	C	C
59	100653	C	T	T	T	C	C	C	C

quence of the clade 2 was constructed. The phylogenetic tree of clade 2 strains was generated using MRCA, shown as Clade 2_ANC in Fig. 4, as the outgroup. Cluster 2a strains formed a distinct cluster (bootstrap value: 96), separated from the other clade 2 strains (Fig. 4). The phylogram analysis also suggested that cluster 2a strains arose later than the other clade 2 strains. The average distance from MRCA to cluster 2a strains was calculated to be $2.30\pm0.39\times10^{-4}$, which was significantly greater than the distance from MRCA to the other clade 2 strains ($1.50\pm0.30\times10^{-4}$).

Six sites of SNPs separating cluster 2a from the other clade 2 strains were identified (Table 2). The sequences of these six sites were specific to cluster 2a, since they were not detected in other clade 2 strains or in the other clades. The significance of cluster 2a-specific signature sequences in clustering and separation from the others was investigated using substitution studies. SNP sequences of one clade 2 strain, such as YC01, were replaced by cluster 2a-specific sequences listed in Table 2, and phylogenetic trees were constructed. YC01 with cluster 2a-specific sequences clustered with cluster 2a strains, 84-7, YC02, and YC03, separated from the other clade 2 strains YC01, 092, and LAX1 (Fig. 5B). On the other hand, when cluster 2a signature sequences were removed, a cluster 2a strain, such as 84-7, separated from other cluster 2a strains and grouped with other clade 2 strains (Fig. 5C). Similar results were obtained from the substitution studies of other strains (Supplementary data Fig. S2). Our observation of the presence of a cluster composed of YC02, YC03, and 84-7 within clade 2 was verified by extensive phylogenetic analysis that included additional VZV strains that were reported to belong to clade 2 (Depledge *et al.*, 2014; Norberg *et al.*, 2015). The addition of 24 strains did not affect the clustering of the three strains (Supplementary data Fig. S3). Thus, cluster 2a-specific signature sequences at the 6 SNP sites play an important role in the separation of cluster 2a strains from other clade 2 strains.

Discussion

In this study, genomic DNA sequences of three clinical strains of VZV from Korean patients with different clinical history were characterized. YC01 was isolated from a zoster patient, YC02 from a varicella patient, and YC03 from a varicella vaccinee. Although YC03 was isolated from a patient with a history of vaccination, VZV sequences recovered did not match the sequences from a vaccine strain. Given that YC03 lacked vaccine-type sequences at 24 vaccine-specific sites suggested by Jeon *et al.* (2016), it is highly likely that the vaccinee was superinfected with the wild-type strain. YC01 was isolated in 2013 from a 40-year-old male patient with zoster and may represent a VZV strain that circulated around in 1980. YC02 and YC03, on the other hand, may represent VZV strains that circulated around in 2010.

Despite different clinical history, YC01, YC02, and YC03 shared high sequence similarity and all belonged to clade 2, which is considered to be of Asian origin (McGeoch, 2009; Schmidt-Chanasit and Sauerbrei, 2011). Before the inclusion of YC01, YC02, and YC03, clade 2 contained pOka and its related strains of Japanese origin, Suduvax, of Korean origin, and LAX1. Although LAX1 was isolated from Los Angeles, USA, it has been suggested to have originated in Asia (Chow *et al.*, 2013). Later we added two strains whose complete genomic sequences are registered in GenBank database and known to belong to clade 2. The strain 092, which is suspected to be of Asian origin as in the case of LAX1, was isolated from a patient of unknown ethnic origin in California, USA. The strain 84-7 was isolated from China (Li *et al.*, 2010).

Considering a world-wide distribution of VZV clades, clade 2 strains were detected mainly in Asian countries based on genotyping of VZV strains using RFLP or SNPs of limited regions of VZV genome (Schmidt-Chanasit and Saubrei, 2011). In China and Korea, 100% of the clinical strains of VZV belonged to clade 2 (Liu *et al.*, 2009; Kim *et al.*, 2011a; Jiang *et al.*, 2013). Most (77/79 = 98%) of the VZV strains from Japan belonged to clade 2 (Loparev *et al.*, 2004). On the other hand, VZV strains isolated from Southwestern Asia, including India, Bangladesh, and Nepal, belonged to clades 4 or 5, with none belonging to clade 2 (Loparev *et al.*, 2004). Clade 2 strains were also found in Singapore, where approximately 3/4 of the population is of Chinese-origin (Wagenaar *et al.*, 2003), and in Australia, where there is a prominent population of Asian immigrants (Loparev *et al.*, 2007; Toi and Dwyer, 2010). Therefore, clade 2 appears to be specific for the natives of Far-Eastern Asian countries such as China, Japan, and Korea. These three countries, although ethnically different, are geographically, historically, and culturally closely related.

Most genotyping methods depend on panels of a certain number of single nucleotide polymorphisms (SNPs), such as 16 SNPs (Peters *et al.*, 2006) or 27 SNPs (Breuer *et al.*, 2010). A combination of SNPs may be useful to differentiate clades, but does not provide information on which SNPs are specific to a certain clade. In this study, we identified the position and number of SNP sites specific to each clade. Due to the relatively small number (n = 5) of strains constituting clade 2, the number for clade 2 (n = 43) was much greater than the numbers for other clades (n = 3–18). On the other hand, although clade 4 has only two members, the number of sequences specific for clade 4 was found to be 16. The clade 2-specific sequences were also found in other vaccine strains such as vOka, Varilrix, Varivax, and Suduvax (data not shown). Recently, additional strains were reported to belong to clade 2 (Depledge *et al.*, 2014; Norberg *et al.*, 2015), and these strains were found to contain all the clade 2-specific sequences except for Zos/Cli/Ves/UK/1801/2012 (accession number: KP771925) which had a deletion at position 60279. These additional strains were isolated from rashes in patients with histories of vaccination (Depledge *et al.*, 2014; Norberg *et al.*, 2015). Interestingly, these strains, as well as the reference vaccine strains, clustered strongly together with pOka in a phylogram (Supplementary data Fig. S3). In addition, Norberg *et al.* (2015) proposed a new clade, clade 6, that is composed of Var160 (KC112914) and Var/Cli/Ves/FRA/98/2013 (KP771914). Since these clade 6 strains were not included in the study to identify clade-specific sequence, their full genomic sequences (KC112914, KP771914) were examined to see if they possessed any of the five clade-specific sequences. Since pre-existing clade-specific sequences

were not found in clade 6 strains, the classification of clade-specific sequence in this study, as well as Norberg *et al.*'s proposition of clade 6, is appropriate.

Our phylogenetic tree analysis of the VZV strains based on the genomic DNA sequence suggested that two Korean strains YC02 and YC03 form a phylogenetically significant cluster with 84-7. The clustering does not, however, reflect the geographical difference since another Korean strain YC01 was not included in the cluster. The separation of this cluster from other clade 2 strains was also evident when MRCA sequence of clade 2 was inferred and included for the phylogenetic analysis. The clustering is likely to depend on 6 SNPs specifically found in cluster 2a strains. These nucleotides can be regarded as a signature sequence, defined as conserved inserts or deletions shared by specific taxa (Gupta, 1998), for cluster 2a. Based on the presence or absence of a signature sequence, the taxa (or clusters) can be divided into two unambiguous groups. Signature sequences play an important phylogenetic marker, with evolutionary significance in some cases (Gupta, 1998).

Further studies with more isolates from China, currently circulating strains from Japan, and other Asian countries, would provide a better understanding of genetic characteristics and evolutionary significance of the division of clade 2 into two subgroups.

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