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Differences in the Likelihood of Acyclovir Resistance-Associated Mutations in the Thymidine Kinase Genes of Herpes Simplex Virus 1 and Varicella-Zoster Virus

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ABSTRACT Acyclovir (ACV) resistance-associated mutations in two recombinant herpes simplex virus 1 (HSV-1) clones were compared. Recombinant HSV-1 lacking its thymidine kinase (TK) and expressing varicella-zoster virus (VZV) TK ectopically had no mutations in the VZV TK gene. In contrast, recombinant HSV-1 expressing HSV-1 TK ectopically harbored mutations in the HSV-1 TK gene. These results suggest that the relatively low frequency of ACV-resistant VZV is a consequence of the characteristics of the TK gene.

KEYWORDS DNA polymerase, acyclovir, acyclovir resistant, herpes simplex virus 1, thymidine kinase, varicella-zoster virus

Herpes simplex virus 1 (HSV-1) and varicella-zoster virus (VZV) establish latency in ganglion cells and reactivate under certain conditions to cause recurrent vesicular lesions (1). Typically, reactivation of HSV-1 results in herpes labialis, periorbital herpes, herpes keratitis, or recurrent genital herpes, whereas reactivation of VZV causes zoster (1). These recurrent diseases occur more frequently, and are more severe, in immunocompromised patients than in immunocompetent patients (2). Acyclovir (ACV), a guanosine analog, is a drug used to treat patients with HSV-1 and VZV diseases. The mechanism of action of ACV is as follows: ACV is phosphorylated by the viral thymidine kinase (TK) to yield ACV monophosphate and further phosphorylated by host cellular kinases to yield ACV triphosphate, which then competes with dGTP for the viral DNA polymerase (DNApol) (3–5). Occasionally, ACV-resistant (ACVr) HSV-1 and VZV are induced in immunocompromised patients (2, 6, 7). It is suggested that the occurrence of viral TK-associating ACVr mutations in VZV is much less frequent than in HSV-1. ACVr VZV is reported rarely in immunocompromised patients (8–23). In contrast, ACVr HSV-1 occurs in 3.5% to 10% of immunocompromised patients (2, 24–28). It is thought that the emergence of ACVr VZV is less likely than that of HSV-1, because VZV is approximately 100 times less sensitive to ACV than HSV-1 (29). The ACV sensitivity of a chimeric HSV-1 lacking the original HSV-1 TK gene, but harboring the VZV TK gene, is the same as that of VZV (29). However, the precise mechanism underlying the difference in the likelihood of emergence of ACVr viruses is unclear.

One possible mechanism may be based on the particular characteristics of VZV TK and HSV-1 TK; i.e., viral TK determines the sensitivity to ACV (29). To clarify the hypothesis, we generated two recombinant HSV-1 viruses: HSV-1_VZV-TK expressing the TK gene of VZV strain vOka (GenBank accession AB097932.1) under the control of the cytomegalovirus (CMV) promoter and from which the HSV-1 TK gene was deleted,

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TABLE 1 Inhibitory effect of ACV on parental and recombinant viruses, as determined in a plaque reduction assay in Vero and MRC-5 cells

Virus	IC_{50} (μg/ml) in[a]:	
	Vero cells	MRC-5 cells
HSV-1 F	0.3 ± 0.5	0.1 ± 0.04
HSV-1_VZV-TK	2.1 ± 0.5	3.4 ± 1.4
HSV-1_HSV1-TK	0.3 ± 0.03	0.1 ± 0.03
VZV vOka	ND[b]	1.0 ± 0.5

[a]Values include standard deviations from three independent tests.
[b]ND, not determined.

and HSV-1_HSV1-TK, in which the VZV TK gene of HSV-1_VZV-TK was replaced with that for HSV-1 TK. We then compared the ACV resistance-associated mutations in recombinant HSV-1 clones.

HSV-1_VZV-TK was generated using a two-step Red recombination system, as described previously (30–32). A DNA fragment containing nucleotides (nt) 1 to 44 of the VZV TK gene, the I-SceI restriction site and the kanamycin resistance gene from pEP-KanS (32), the CMV promoter, and the VZV TK gene and its poly(A) region were amplified using primers 5′-GCGGTACCATGTCAACGGATAAAACCGATGTAAAAATGGGCG TTTTGCGTATGATGACGACGATAAGTAGGG-3′ and 5′-GCGGTACCGCCAGTGTTACAACCA ATTAACC-3′. The DNA fragment was then inserted between the UL50 and UL51 genes using primers 5′-ATCTCATCTTTCCTGTGTGTAGTTGTTTCTGTTGGAGGCCTGTGGGTCTAAC ATTGATTATTGACTAGTTATTAA-3′ and 5′-TTCATCCAACCCGTGTGTTCTGTGTTTGTGGGAT GGAGGGGCGGGTGTGAATCTTTTTACTGGTACATACGTAAA-3′, as described previously (30–32). Next, the HSV-1 TK gene was replaced with the kanamycin resistance gene using primers 5′-TTATTGCCGTCATAGCGCGGGTTCCTTCCGGTATTGTCTCCTTCCGTGT TAGGATGACGACGATAAGTAGGG-3′ and 5′-TCCGCCTGGAGCAGAAAATGCCCACGCTAC TGCGGGTTTATATAGACGGTAACACGGAAGGAGACAATACCAACCAATTAACCAATTCTGAT TAG-3′ (30–32). HSV-1_HSV1-TK was constructed by replacing the VZV TK gene of HSV-1_VZV-TK with the TK gene of HSV-1 strain F (GenBank accession GU734771.1). The DNA fragment containing nt 1 to 44 of the HSV-1 TK gene, the I-SceI restriction site and the Zeocin resistance gene from pUC-Zeo (33), the CMV promoter, and the HSV-1 TK gene and its poly(A) region were amplified using primers 5′-GCGGTACCATGGCTTCGT ACCCCTGCCATCAACACGCGTCTGCGTTCGACCACGGGGATCTAGGGATAACAG-3′ and 5′-GCGGTACCATTACGCCAAGCTTGCATGC-3′ and then inserted between the UL50 and UL51 genes using primers 5′-ATCTCATCTTTCCTGTGTGTAGTTGTTTCTGTTGGAGGCCTGT GGGTCTAACATTGATTATTGACTAGTTATTAA-3′ and 5′-TTCATCCAACCCGTGTGTTCTGTG TTTGTGGGATGGAGGGGCGGGTGTGATTTATTCTGTCTTTTTATTGCCGTC-3′. The half maximal inhibitory concentration (IC_{50}) of ACV to HSV-1_VZV-TK was higher than those to HSV-1 F and HSV-1_HSV1-TK in both Vero cells and MRC-5 cells; also, the sensitivity of HSV-1_VZV-TK in MRC-5 cells to ACV was similar to that of VZV vOka (Table 1). In contrast, the sensitivities of HSV-1_HSV1-TK to ACV were similar to those of HSV-1 F in both cells (Table 1). These results suggest that the ACV sensitivity of HSV-1 and VZV depends on the characteristics of viral TK.

ACVr HSV-1_VZV-TK and ACVr HSV-1_HSV1-TK clones were generated by serial passage of HSV-1_VZV-TK and HSV-1_HSV1-TK, respectively, in the presence of increasing concentrations of ACV, as described previously (34, 35). Thirty-two ACVr HSV-1_VZV-TK clones were obtained, and the sequences of the viral TK and DNApol genes were determined. Compared with the original HSV-1_VZV-TK clones, none of the ACVr HSV-1_VZV-TK clones harbored mutations in the TK gene. However, 31 ACVr clones harbored mutations in the DNApol gene; all were single or double nucleotide mutations that resulted in the substitution of one or two amino acids, respectively (Fig. 1A). Twenty-five ACVr clones harbored amino acid substitutions in the conserved regions of DNApol, whereas the other six harbored amino acid substitutions in nonconserved regions (Fig. 1). Twelve ACVr clones harbored substitutions at amino acid 719 of DNApol (Fig. 1B). L356R, F733S, E798G, C830G, and I952T in DNApol were identified as

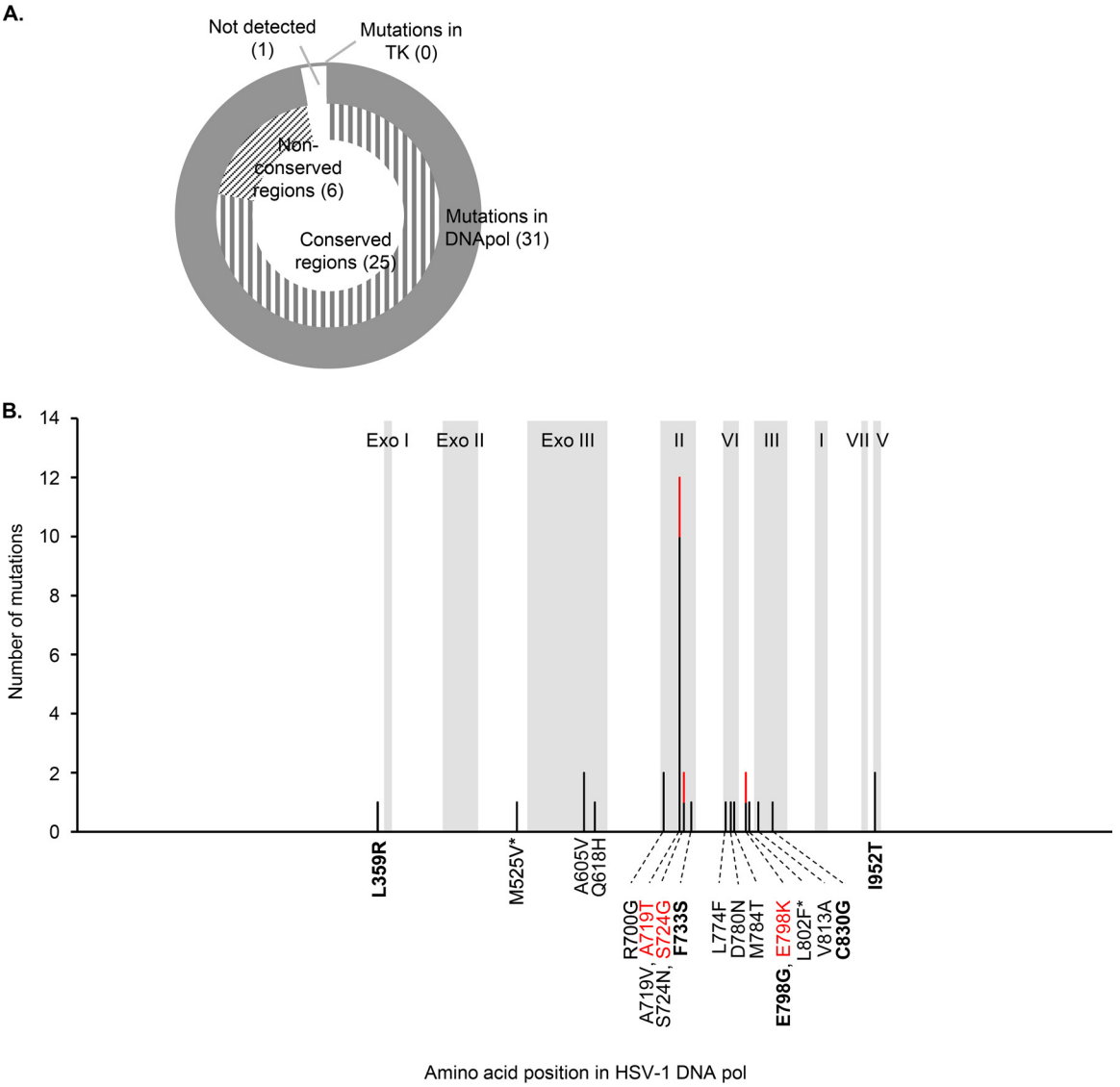

FIG 1 ACV resistance-associated mutations detected in ACVr HSV-1_VZV-TK clones. (A) The pie chart shows the regions in which ACV resistance-associated mutations were detected. The numbers in parentheses denote the number of ACVr HSV-1_VZV-TK mutations. (B) Mutations detected, and their frequencies, in HSV-1 DNApol. The *y* axis indicates the number of clones harboring the mutations detected at a given amino acid position. Amino acid changes are shown on the *x* axis, and the color of the letters corresponds to the color of the bars. The gray boxes indicate the conserved regions in DNApol of HSV-1 strain F (GenBank accession GU734771.1): Exo I to III (amino acids 363 to 373, 437 to 469, and 531 to 627), II (amino acids 694 to 736), VI (amino acids 772 to 791), III (amino acids 805 to 845), I (amino acids 881 to 896), VII (amino acids 938 to 946), and V (amino acids 953 to 963). Novel mutations are shown in boldface font. Two amino acid substitutions were detected in one ACVr HSV-1_VZV-TK clone; these are indicated by asterisks.

novel amino acid substitutions that conferred ACV resistance (Fig. 1B). Of 31 nucleotide substitutions, 12 (approximately 40%) were an A/T to G/C switch in the HSV-1 DNApol gene. Although VZV TK has affinity not only for thymidine but also for deoxycytidine (36), there was no significant preference with respect to nucleotide substitutions in the HSV-1 DNApol gene. One ACVr clone harbored no mutations in either the TK or DNApol genes compared to those of the original HSV-1_VZV-TK (Fig. 1A), suggesting that not only viral TK and DNApol but also other factors affect the functional mechanism of action of ACV in this recombinant virus. Further studies are needed to clarify the mechanism underlying the generation of clones that are resistant to ACV.

Forty-seven ACVr HSV-1_HSV1-TK clones were obtained as described above, and all harbored mutations in the HSV-1 TK gene but not in the DNApol gene. These results suggest that ACV resistance-associated mutations are less likely to occur in VZV TK than

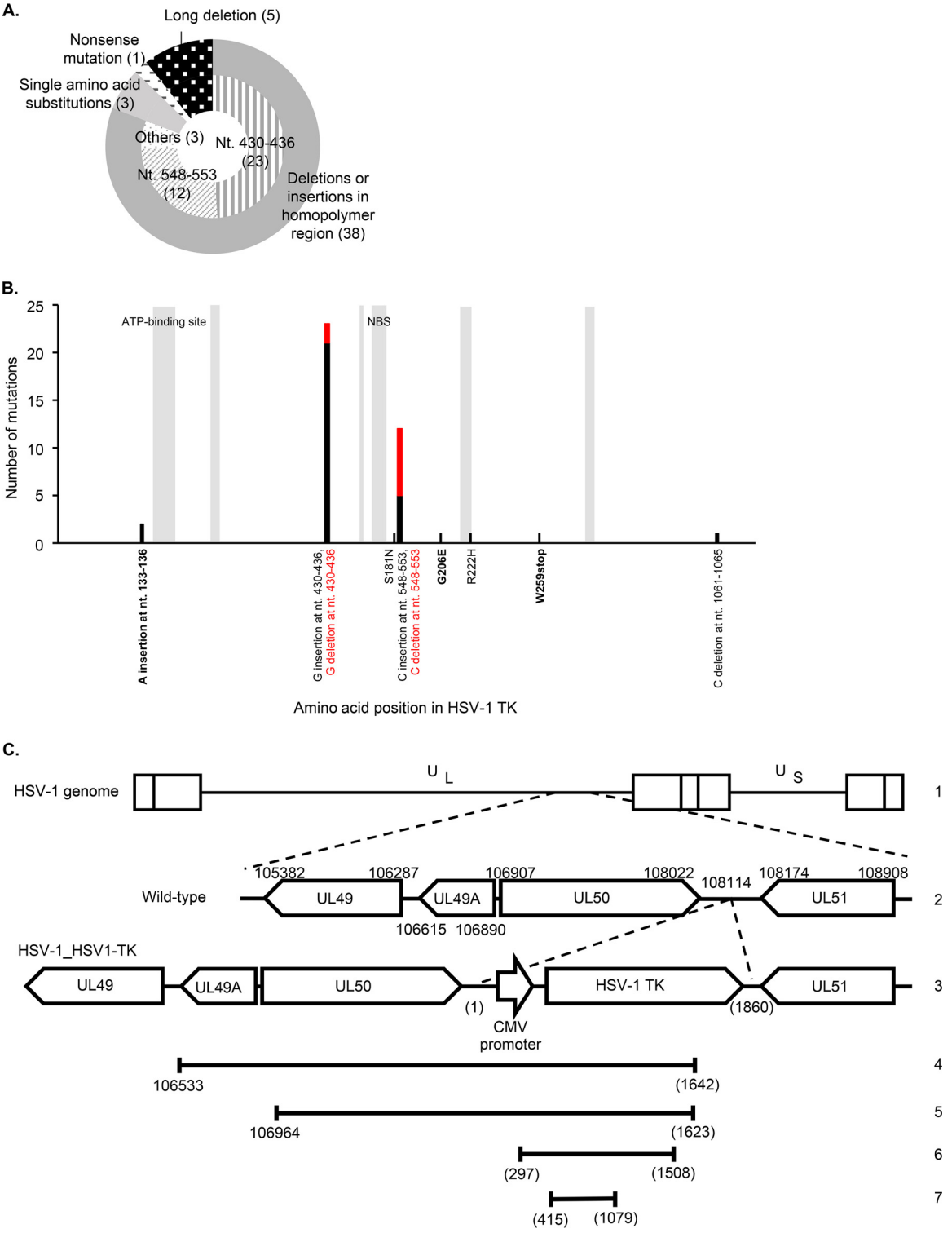

FIG 2 ACV resistance-associated mutations detected in ACVr HSV-1_HSV1-TK clones. (A) The pie chart shows the patterns of mutations in the HSV-1 TK gene. Nt, nucleotide. The numbers in parentheses denote the number of ACVr HSV-1_HSV1-TK clones. (B) Frequency of each mutation detected in HSV-1 TK. The *y* axis indicates the number of mutations detected at a given amino acid position. The mutations detected are shown on the *x* axis, and the color of the letters corresponds to the color of the bars. The gray boxes indicate the conserved regions within HSV-1 TK. NBS, nucleoside binding site. Novel mutations are shown in boldface font. (C) Schematic representation of the long sequence deletions detected in five ACVr HSV-1_HSV1-TK clones. Line 1, the whole-genome structure of wild-type HSV-1 F; line 2, the related domains of HSV-1 F; line 3, the related domains of HSV-1_HSV1-TK; lines 4 to 7, nucleic acid deletions detected in ACVr HSV-1_HSV1-TK clones. The same deletion (line 6) was detected in two clones. Other deletions (lines 4, 5, and 7) were each detected in a single clone. Numbers indicate the location of nucleic acids in the HSV-1 F genome (GenBank GU734771.1). The numbers in parentheses indicate the location of nucleic acids within the region inserted.

in HSV-1 TK. This is supported, at least partially, by the fact that the HSV-1 TK gene contains homopolymer regions in which nucleotide insertions or deletions are common (37–40). Thirty-eight clones harbored insertion or deletion mutations in the homopolymer stretch regions, and 23 of the 38 clones harbored a single insertion of guanine at nt 430 to 436 in the 7-G homopolymer stretch within the HSV-1 TK gene (Fig. 2A). Four clones harbored single point nucleotide substitutions at different locations, resulting in a single amino acid substitution or a nonsense mutation (Fig. 2A). A single insertion of adenine at nt 133 to 136 and W259stop and G206E substitutions in HSV-1 TK were novel mutations (Fig. 2B). Five clones lost a long sequence of nucleotide residues, including part of the inserted HSV-1 TK gene (Fig. 2C). One clone lost a long nucleotide sequence stretching from the UL49 gene to the HSV-1 TK gene (Fig. 2C, line 4). Another clone lost a long sequence stretching from the UL50 gene to the HSV-1 TK gene (Fig. 2C, line 5). The other three clones lost a long sequence within the transfer region (Fig. 2C, lines 6 and 7); two of these deletions showed the same pattern (Fig. 2C, line 6). Such long deletions may be due to homologous recombination, although no homologous regions were found in and around the deleted regions. It is possible that the phenomenon was specific to recombinant HSV-1_HSV1-TK, as it has not been reported in any other ACVr HSV-1 isolates examined in *in vitro* or clinical studies.

In conclusion, the data presented herein suggest that the mechanism underlying differences in the likelihood of emergence of ACVr HSV-1 and ACVr VZV is due to the characteristics of HSV-1 TK and VZV TK. In addition, as HSV-1_VZV-TK induced ACV resistance-associated mutations only in the HSV-1 DNApol gene, this system could be used to enrich the database of HSV-1 DNApol gene-associated ACVr mutations.

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