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**Virulence of herpes simplex virus 1 harbouring a UAG stop codon between the first
and second initiation codon in the thymidine kinase gene**

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Running title: Novel mechanism of HSV-1 attenuation

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ABSTRACT: Herpes simplex virus 1 (HSV-1)-TK(8UAG) expresses a truncated thymidine kinase (TK) translated from the second initiation codon due to a stop UAG codon at the 8th position (counted from the first initiation codon). Here, we showed that the sensitivity of HSV-1-TK(8UAG) to acyclovir (ACV) is similar to that of control HSV-1 wild-type (WT), which expresses an intact TK protein. However, HSV-1-TK(44UAG), which expresses a truncated TK due to a UAG codon at position 44, showed lower sensitivity to ACV. A mouse infection model was used to compare the virulence of HSV-1-TK(8UAG) and HSV-1-TK(44UAG) with that of HSV-1 wild-type (WT). The 50% lethal dose (LD₅₀) value of HSV-1-TK(44UAG) was 7.8-fold higher than that of HSV-1-TK(8UAG), whereas the LD₅₀ value of HSV-1-TK(8UAG) was the same as that of the parental HSV-1 WT. There were no statistically significant differences between HSV-1-TK(44UAG), HSV-1-TK(8UAG), and HSV-1 WT with respect to replication capacity and viral TK mRNA expression in mouse brain. Thus, the virulence of HSV-1 expressing a truncated viral TK translated from the second initiation codon might depend on the position of the UAG stop codon.

INTRODUCTION

Herpes simplex virus 1 (HSV-1; family *Herpesviridae*) is a ubiquitous human virus. HSV-1 infection recurs frequently and causes severe complications in immunocompromised patients (1-5). 9-(2-Hydroxyethoxy)-methyl guanine (acyclovir, ACV) is a standard drug for treatment of patients with HSV-1 infection. ACV is converted to ACV-monophosphate via phosphorylation of the viral thymidine kinase (vTK); it is then phosphorylated further by cellular kinases to yield the active form, ACV-triphosphate (ACV-TP). ACV-TP inhibits activity of the viral DNA polymerase (DNApol) and acts as a chain terminator at the site of ACV-TP incorporation into the viral genome (6-8). Ninety-five percent of ACV-resistant HSV-1 cases arise due to mutations in the vTK gene (9-16), the remaining 5% being due to mutations in the DNApol gene (17, 18).

vTK-deficient HSV-1 shows reduced virulence and replication capacity *in vivo* (17, 19, 20, 21), and viral TK-associated ACV-resistant (ACVr) HSV-1 is less virulent than parental HSV-1 in animal models (22-25). Moreover, mouse models of intracerebral inoculation revealed a positive correlation between ACV sensitivity and the virulence among vTK-associated ACVr HSV-1s (25).

Previously we conducted an *in vitro* study using a recombinant HSV-1-TK(44UAG), which possesses a UAG stop codon at position 44 between the 1st initiation AUG codon (1st position) and the 2nd initiation AUG codon (46th position) of the vTK gene, and identified ACV resistance. By contrast, another recombinant, HSV-1-TK(8UAG), whose vTK gene has a UAG stop codon at the 8th position between the 1st and 46th positions,

showed similar ACV sensitivity to that of the parental recombinant HSV-1 wild-type (WT), which does not have a UAG codon between the 1st and 2nd initiation codons. The position of the UAG stop codon between the 1st and 2nd AUG initiation codons affects expression of the vTK polypeptide in a temperature-dependent manner, and is responsible for the vTK-associated antiviral drug sensitivity profiles (26).

Because HSV-1 expressing C-terminally truncated vTK is circulating in nature (27), the virulence of such HSV-1 viruses is of interest. In this study, we used a mouse model to examine the virulence of recombinant HSV-1-TK(8UAG), HSV-1-TK(44UAG), and the parental recombinant HSV-1 WT, in addition to that of HSV-1 TAS (isolated from a patient with congenital immunodeficiency) (27) and HSV-1 I4-2 (a clinical isolate that possesses a UAG stop codon at the 8th position, counted from the 1st initiation codon) (27,28). Furthermore, we investigated the viral replication kinetics and expression of the vTK mRNA in the brain of mice infected with each of these HSV-1 viruses.

MATERIALS AND METHODS

Cells: African green monkey kidney (Vero) and human embryonic lung fibroblast (HELFL) cells were used for virus titration and susceptibility testing. Eagle's minimal essential medium (MEM; Wako Pure Chemical Industries, Ltd., Osaka, Japan), supplemented with 10% foetal bovine serum (Biological Industries, Beit Haemek, Israel), 7.5% sodium bicarbonate, and 200mM L-glutamine (MEM-10FBS), was used for culture of HELFL cells. Dulbecco's modified Eagle's essential medium containing penicillin-G (100 U/ml),

streptomycin (100 µg/ml), and 5% calf serum (DMEM-5CS; Thermo Fisher Scientific, Waltham, MA) was used for Vero cell culture.

Viruses: The characteristics and the origins of HSV-1 viruses are summarized (Table 1). HSV-1 TAS and HSV-1 I4-2, both of which were isolated from patients, were used (5, 27, 28). HSV-1 I4-2 possesses a UAG stop codon at the 8th position, counted from the 1st initiation codon of the vTK gene (27, 28). The nucleotide sequences of the vTK gene of HSV-1 TAS and HSV-1 I4-2 were determined and deposited in the DNA Data Bank of Japan (AB047358 and AB078742, respectively) (27, 28). Recombinant HSV-1 viruses HSV-1 WT, HSV-1-TK(8UAG), and HSV-1-TK(44UAG) were generated in a previous study by two-step recombination (26). HSV-1 WT was pYEbac102-derived HSV-1 strain F virus without stop codon between the 1st and 2nd initiation codon on the vTK gene (26, 29). HSV-1-TK(8UAG) and HSV-1-TK(44UAG) harbour a UAG stop codon at the 8th and 44th position, counted from the 1st initiation codon of the vTK gene, respectively.

Mice: Three week-old female BALB/c mice (SLC Japan, Kurume, Japan) were used.

Compounds: ACV (Tokyo Chemical Industry, Tokyo, Japan) was used for susceptibility testing.

Body temperature measurement: Prior to inoculation with virus, the body temperature of 20 mice was measured using an infrared thermometer (Tecnimed srl, Vedano O, Italy). Temperature was measured through the surface of the skin.

Susceptibility to ACV: The 50% inhibitory concentration (IC₅₀) values of ACV against HSV-1 TAS, HSV-1 I4-2, HSV-1 WT, HSV-1-TK(8UAG), and HSV-1-TK(44UAG) were

determined in a plaque reduction assay (PRA), as described previously (26). Briefly, approximately 50 plaque-forming units (pfu) of virus was absorbed onto a HELF cell monolayer at 37°C for 1 h. The inoculum was then removed. Cells were fed with MEM-2FBS containing the designated concentrations of ACV and gamma-globulin (v/v 100:2) and incubated at 37°C/5% CO₂ for 3 days. The cells were fixed and stained with crystal violet solution. The plaques were counted visually. The concentrations of ACV used in this sensitivity assay were 0, 0.01, 0.04, 0.1, 0.4, and 1 µg/ml.

Determination of virulence by intracerebral inoculation of mice with each virus:

Female mice (3 weeks old; five mice per group) were inoculated intracerebrally with 50 µl of virus solution containing each HSV-1 virus at the designed infectious dose (1×10^0 , 1×10^1 , 1×10^2 , and 1×10^3 pfu/50 µl DMEM-2CS per head). Five mice were inoculated with 50 µl DMEM-2CS per head as mock-inoculated control. Body weight and survival were recorded daily for 10 days post-infection (p.i.). Loss of >20% body weight from baseline was defined as significant. The 50% lethal dose (LD₅₀) was determined at 10 days p.i. using the Reed and Muench method (30). The experiment was repeated twice, independently.

Determination of replication capacity and vTK mRNA expression in the brain: 15

mice from each group were inoculated with 50 µl of each virus solution (dose = 10 pfu in DMEM-2CS per head). Six mice were inoculated with 50 µl DMEM-2CS per head as mock-inoculated control. At Days 1, 3, and 5 p.i., five mice from each group were sacrificed by exposing to CO₂, and brain tissues were collected and stored at -80°C until they were used for DNA and RNA extraction and infectious virus measurement. At Days 1, and 5 p.i., 3 mice in control group were sacrificed and collected the brain tissues.

Extraction of DNA and RNA from brain: The whole brain tissue from each mouse was soaked in 400 μ l DMEM-2CS and homogenized mechanically. DNA and RNA were extracted using NucleoSpin RNA/Protein (Takara Bio, Inc., Shiga, Japan) and RNA/DNA Buffer Set (Takara Bio, Inc.) kits, respectively.

Evaluation of replication capacity of HSV-1 in the brain: Replication capacity of each HSV-1 in the brain was determined in a plaque assay on Vero cells (26). Briefly, 100 μ l brain homogenate was 1×10^1 to 1×10^2 times diluted with DMEM-2CS and inoculated onto Vero cell monolayers. After adsorption for 1 h, the inoculate was removed, and the cells were fed with DMEM-2CS containing gamma-globulin (v/v 100:2) and incubated at 37°C/5% CO₂ for 3 days. The cells were fixed and stained for approximately 20 min at room temperature with staining solution. Cells were washed with water, and the plaque number was counted visually under a microscope.

The amount of HSV-1 genome in the brain of inoculated mice was measured by quantitative real-time polymerase chain reaction (qPCR) using the TaqMan Universal Master Mix (Thermo Fisher Scientific). DNA was extracted from brain tissue as described above. Primers targeting the HSV-1 envelope glycoprotein B (gB) gene were as follows: HSV-1 gB forward primer 5'-CGCATCAAGACCACCTCCTC-3' and HSV-1 gB reverse primer 5'-GCAGCTCGCACCACGCGA-3'. PCR-based reaction conditions were as follows: 50°C for 2 min (annealing and elongation), 95°C for 10 min (denaturation), and 45 cycles of 95°C for 30 sec (denaturation) and 60°C for 1 min (elongation). The amount of viral genome was expressed in terms of copy number per 1 ng of extracted DNA.

Expression of HSV-1 TK mRNA: HSV-1 TK mRNA expressed in mouse brain was measured by quantitative reverse transcription PCR (qRT-PCR) using the TaqMan RNA-to-Ct 1-Step Kit (Thermo Fisher Scientific). The primer set used for qRT-PCR was designed as reported previously (31). The primers were HSV-1-TK mRNA forward primer 5'-GTACCCGAGCCGATGACTTACT-3' and HSV-1-TK mRNA reverse primer 5'-CCCGGCCGATATCTCA-3', and the TaqMan probe was 5'-CTTCCGAGACAATCGCGAACATCTACACC-3'. The PCR conditions were as follows: 94°C for 10 min (denaturation), followed by 40 cycles of 95°C for 15 sec (denaturation) and 60°C for 1 min (extension).

Statistical analysis: Differences in the LD₅₀, viral load, and vTK mRNA expression by each HSV-1 strain in mice were evaluated using the Student's *t*-test. A P-value < 0.05 was considered statistically significant. The statistical calculation were performed with Excel statistical program file (ystat2008.xls).

Ethical statement: All animal experiments were conducted under the approval of the Animal Care and Use Committee of the National Institute of Infectious Diseases (NIID; No. 117026) and followed the guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan.

RESULTS

Body temperature of mice: Previous studies show that the temperature at which HSV-1 TK mutants undergo replication affects the level of vTK expression (26). Therefore, we

measured the body temperature of mice prior to inoculation with virus. The average temperature was 35.8°C.

Susceptibility of HSV-1s to ACV: The IC₅₀ of ACV was determined in a PRA based on HELF cells infected with HSV-1 WT, HSV-1-TK(8UAG), HSV-1-TK(44UAG), HSV-1 TAS, and HSV-1 I4-2 at doses of 0.31, 0.24, 2.54, 0.02, and 0.15 µg/ml, respectively (Table 1). The sensitivity profile was consistent with the ACV sensitivity of these viruses determined in Vero cells (26).

Virulence of HSV-1 strains in mice: To evaluate virulence *in vivo*, we inoculated groups of 3-week-old BALB/c mice intracerebrally with each of the five HSV-1 strains. The LD₅₀ values for HSV-1 WT, HSV-1-TK(8UAG), HSV-1-TK(44UAG), HSV-1 TAS, and HSV-1 I4-2 measured in a single experiment at 10 days p.i. were 75, 68, 500, 1.7, and 1.3 pfu, respectively (Table 1). When the same experiment was repeated, the LD₅₀ values were 68, 83, 682, 1.7, and 1.0 pfu, respectively. The LD₅₀ values for HSV-1-TK(8UAG) were the same as those for HSV-1 WT, and the LD₅₀ values for HSV-1 I4-2 were the same as those for HSV-1 TAS.

The replication capacity of HSV-1, as evaluated by PRA and qRT-PCR: Because the virulence of HSV-1-TK(44UAG) was less than that of HSV-1-TK(8UAG) and HSV-1 WT, we evaluated the replication capacity of HSV-1 strains *in vivo*. No infectious HSV-1 WT, HSV-1-TK(8UAG), or HSV-1-TK(44UAG) viruses in homogenates prepared from the brains of mice at 1 day p.i. were detected upon virus isolation using a cell culture system; however, these viruses were isolated from brain homogenates at 3 days p.i. (Fig.

1A). Recombinant HSV-1 WT, HSV-1-TK(8UAG), and HSV-1-TK(44UAG) showed equivalent replication capacity in the brain at each time point tested. In the case of HSV-1 TAS and HSV-1 I4-2, infectious viruses were isolated from brain tissue homogenates prepared at 1 day p.i. The titres of infectious recombinant HSV-1 WT, HSV-1-TK(8UAG), and HSV-1-TK(44UAG) at 5 days p.i. were approximately 10 times lower than those of clinically isolated HSV-1 TAS and HSV-1 I4-2 (Fig. 1A).

Viral DNA copy numbers in the brain of mice intracerebrally infected with each HSV-1 were also evaluated by qPCR (Fig. 1B). The trend at each time point was similar to that observed after measurement of the infectious viral load (Fig. 1A, 1B); however, the number of viral DNA copies in the brain of mice in all groups increased further from 3 days p.i. to 5 days p.i. (Fig. 1B), whereas the infectious virus titres determined at 5 days p.i. were the same as those determined at 3 days p.i. (Fig. 1A).

Expression of HSV-1 TK mRNA in mouse brain homogenates: HSV-1 TK mRNA expression in mouse brain homogenates was examined by qRT-PCR (Fig. 2). At each time point examined, the level of HSV-1 TK mRNA expression in the brain of mice infected with HSV-1-TK(44UAG) was similar to that in mice infected with HSV-1 WT and HSV-1-TK(8UAG). At 5 days p.i., expression of HSV-1 TK mRNA in mouse brain infected with either HSV-1 TAS or HSV-1 I4-2 was >10 times higher than that in brain homogenates prepared from mice infected with HSV-1 WT, HSV-1-TK(8UAG), or HSV-1-TK(44UAG), with no statistical difference between the HSV-1 strains tested.

DISCUSSION

Data from mouse infection models reveal that ACVr HSV-1 harbouring ACV resistance-associated mutations in vTK show reduced neurovirulence and cause less severe disease and mortality than their parental strains (19, 22, 24, 25, 32, 33). Here we also showed that ACVr was associated with less virulence in mice using recombinant HSV-1 harbouring a UAG stop codon on the vTK gene. Although both HSV-1-TK(8UAG) and HSV-1-TK(44UAG) express the truncated vTK translated from the 2nd initiation codon, HSV-1-TK(44UAG) was less virulent than HSV-1-TK(8UAG), whose LD₅₀ value was similar to that of the parental HSV-1 WT. The virulence of HSV-1 I4-2 in mice was as high as that of HSV-1 TAS. Although a careful confirmation of whole genome sequence of BAC clones is needed to exclude the possibility of unexpected mutation during BAC mutagenesis that affect attenuation phenotype, the results in this study suggest that the attenuated virulence of HSV-1-TK(44UAG) is highly possible due to the difference in the position of UAG stop codon in the vTK gene. Furthermore, the position of the stop codon at the 8th position did not attenuate HSV-1-TK(8UAG) or HSV-1 I4-2. The nucleotide sequence of the vTK gene of HSV-1-TK(44UAG) amplified from the brain tissue of the virus-infected mice at day 5 post infection was determined and showed no unexpected mutations in the vTK (data not shown), indicating that HSV-1-TK(44UAG) carrying revertant mutation on vTK did not appear during these experiments.

Expression of HSV-1 TK is essential for viral replication in mouse neurons and for virulence (34). A previous study showed that the *in vitro* expression level of HSV-1-TK(44UAG)-truncated TK was much lower than that of

HSV-1-TK(8UAG)-truncated vTK and HSV-1 WT-full length vTK (26); however, expression of vTK in mouse brain was not studied. Therefore, vTK expression level was quantitatively examined with measurement of vTK mRNA expression level, a very sensitive assay system, because expression of the vTK polypeptide in the brain could not be evaluated by western blotting (data not shown). The amount of vTK mRNA expressed by HSV-1-TK(44AUG) was similar to that expressed by the other HSV-1 recombinants (Fig. 2). Moreover, the replication capacity of HSV-1-TK(44UAG) in the brain of mice was almost the same as that of HSV-1-TK(8UAG) and HSV-1 WT (Fig. 1). These results indicate that the virulence and pathogenicity of HSV-1, whose TK gene possesses a stop codon between the 1st and 2nd initiation codons, are not simply related to the total viral load and total vTK mRNA expression in the brain. Attenuation of HSV-1-TK(44UAG) might be due to lower expression of vTK in the brain. When HSV-1-TK(44UAG) replicated in the mouse brain, active N-terminally truncated vTK was expressed, allowing the virus to replicate to the same level as HSV-1 WT. However, there is a limitation in this study because the correlation between the vTK mRNA and vTK polypeptide expression *in vivo* was not clear. There is a possibility that expression of N-terminally truncated vTK in brain tissue infected with HSV-1-TK(44UAG) might be less than that in brain tissue infected with HSV-1 WT or HSV-1-TK(8UAG), although expression of vTK mRNA was the same in HSV-1-TK(44UAG)-, HSV-1-TK(8UAG)-, and HSV-1 WT-infected brain tissues. Studies focusing on expression of the vTK protein and its dynamics in the brain of infected mice at the molecular level are needed if we are to elucidate the mechanism underlying virulence attenuation.

The mechanism of attenuation of HSV-1-TK(44UAG) might be associated with other factors rather than the replication capacity of HSV-1-TK(44UAG), HSV-1-TK(8UAG), and HSV-1 WT. Multiple factors contribute to the virulence of HSV-1, including viral factors and host factors. HSV-1 replicating in the hippocampus area of the mouse brain causes more severe disease than virus replicating in the cerebellum, indicating that the area of replication in the brain affects virulence (35). It is unclear whether the replication site of HSV-1-TK(44UAG) in the mouse brain differs from that of HSV-1-TK(8UAG) and HSV-1 WT. In addition, the vTK protein is a component of the viral tegument, which modulates the host innate immune response by inhibiting the production of beta interferon (36, 37). However, the morphology of the HSV-1-TK(44UAG) vTK protein has not been investigated *in vivo* or *in vitro*, nor compared with that of HSV-1-TK(8UAG) and HSV-1 WT.

In conclusion, it was demonstrated that HSV-1-TK(44UAG) was less virulent than HSV-1-TK(8UAG), while the virulence of HSV-1-TK(8UAG) was the same as that of HSV-1 WT. The difference in virulence might be due to the difference in the position of the UAG stop codon. The mechanism responsible for this difference in virulence remains unclear.

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Conflict of interest

None declared.

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Figure legends

Fig. 1. Viral load of HSV-1-TK(44UAG), HSV-1-TK(8UAG), HSV-1 WT, HSV-1 TAS, and HSV-1 I4-2 in the brain of mice intracerebrally inoculated with 10 pfu of each HSV-1 strain. The infectious viral load of HSV-1 in the brain at 1, 3, and 5 days p.i. was measured in a plaque assay on Vero cells (A). The viral genome load of each HSV-1 was measured by quantitative qPCR (B). Values are expressed as $\log_{10}$ pfu/head (A) and $\log_{10}$ copies/ngDNA (B) (with the standard deviation). The kinetics of HSV-1 WT genome production (grey) overlap with those of HSV-1-TK(44UAG); therefore, the kinetics of HSV-1 WT cannot be seen clearly.

Fig. 2. Expression of HSV-1 TK mRNA in mouse brain homogenate, as measured by quantitative RT-PCR at 1, 3, and 5 days p.i. (mice were inoculated intracerebrally with 10 pfu of each HSV-1 strain). Values are expressed as $\log_{10}$ copies/ngRNA (with the standard deviation).

Table 1. The characteristics and origins of HSV-1 strains used in the study.

Viruses		Presence of UAG stop codon between the first and the second initiation codons (Position)	IC ₅₀ (μg/ml) ^{*1}	LD ₅₀ (pfu) ^{*2}
HSV-1-TK(44UAG)	Recombinant viruses	Positive (44 th position)	2.54	500
HSV-1-TK(8UAG)		Positive (8 th position)	0.24	68
HSV-1 WT		Negative	0.31	75
HSV-1 TAS	Clinically isolated	Negative ^{*3}	0.02	1.7
HSV-1 I4-2		Positive (8 th position) ^{*4}	0.15	1.3

*1: IC₅₀: 50% inhibitory concentration

*2: LD₅₀: 50% lethal dose; pfu: plaque forming unit

*3: The nucleotide sequence is deposited in GenBank with the accession number of AB047358.

*4: The nucleotide sequence is deposited in GenBank with the accession number of AB078742.

A)

B)

Accepted