

Neonatal Herpes Encephalitis Caused by a Virologically Confirmed Acyclovir-Resistant Herpes Simplex Virus 1 Strain

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A neonate with herpes simplex virus 1 encephalitis was treated with intravenous acyclovir. During the course of therapy, the infection became intractable to the treatment and a mutation in the viral thymidine kinase gene (nucleotide G375T, amino acid Q125H) developed. This mutation was demonstrated *in vitro* to confer acyclovir resistance.

CASE REPORT

A 13-day-old boy was admitted to National Defense Medical College Hospital due to lethargy and failure to thrive. He was born at 39 weeks 5 days of gestation and 2,558 g birth weight to a healthy 35-year-old mother (gravida 2, para 2). Group B streptococcus (GBS) was detected from the mother's vagina in the third trimester, but the baby's bacterial culture tests performed at birth, including throat, skin, and blood analyses, were negative for GBS. The mother did not have a history of genital herpes. Her herpes simplex virus 1 (HSV-1) and HSV-2 serostatus was not examined, and her history of acyclovir (ACV) use was not clear. Furthermore, the genital swab culture examination for HSV was not performed. On admission, physical examination of the boy revealed skin blisters on the forehead and upper lip. A swab from the blister showed positive and negative reactions for the specific antigens of HSV-1 and HSV-2, respectively, in a direct immunofluorescent antibody assay (Denka Seiken Co. Ltd., Tokyo, Japan) performed by a qualified clinical laboratory (SRL Inc., Tokyo, Japan). A serum sample collected on admission showed positive and negative reactions in the enzyme-linked immunosorbent assay for detection of anti-HSV IgM and IgG antibody (SRL Inc.), respectively. A lumbar puncture revealed pleocytosis (547 cells/ μ l) and an elevated protein level (168 mg/dl) in the cerebrospinal fluid (CSF). The CSF was also positive for HSV-1 DNA, which was determined by a previously reported method (1) in PCR testing (SRL Inc.). The boy was diagnosed as having neonatal herpes encephalitis (NHE), and intravenous high-dose ACV (60 mg/kg/day) treatment was initiated. His general status improved with resolution of the skin lesions within a few days of the beginning of treatment. However, the viral load in the CSF determined by TaqMan-based quantitative real-time PCR (SRL Inc.), which dropped temporarily, increased again after 4 weeks from the initiation of ACV treatment (Fig. 1A) without obvious deterioration in clinical symptoms. Because the standard dose of ACV was given and drugs which have antagonistic effects for ACV were not used, we assumed that an ACV-resistant HSV-1 strain had developed. The ACV concentration in the CSF was not measured. Foscarnet, an antiviral drug recommended for treatment of ACV-resistant HSV infections (2), was not immediately available. Therefore, vidarabine

(15 mg/kg/day) was added to the therapeutic regimen from the fifth week of the treatment course. Subsequently, HSV-DNA in the CSF decreased to a level that was finally undetectable; hence, the antiviral drug treatment was terminated. Because virus isolation from the CSF of the patient was unsuccessful, as is common in herpes encephalitis cases (3), we could not perform a plaque reduction assay to test the *in vitro* inhibition concentration of ACV. Neuroimaging showed residual necrotic changes of the bilateral temporal lobes. Unfortunately, neurodevelopmental sequelae remained in this patient.

To reveal the mechanism of the clinical ACV resistance, sequencing analysis of the viral thymidine kinase (*vTK*) gene was conducted using the CSF samples collected at two different time points. As denoted by the arrows in Fig. 1A, sample 1 and sample 2 were collected before the initiation of and at the 5th week of ACV treatment, respectively. Full-length *vTK* genes were successfully amplified from both samples by a previously reported nested PCR method (4). By direct sequencing, one nucleotide substitution, G375T, leading to a Q125H amino acid substitution was detected. CSF sample 2 contained a mixture of *vTK* genes with and without this mutation (Fig. 1B). To examine whether or not this mutation induced HSV-1 ACV resistance, further analysis was conducted.

The analysis was performed according to a method developed by our group (4). The concept for the novel assay system is to assess the sensitivity of the HSV-1 to ACV and other *vTK*-associated drugs by measuring the replication capacity of the *vTK*-deficient and highly ACV-resistant HSV-1 TAR strain (5) in 293T cells expressed with the recombinant *vTK* protein of the HSV-1 strain of interest. In this study, *vTK* expression plasmid vectors were constructed using pTARGET (Promega, Madison, WI). A *vTK* expression plasmid without the G375T mutation, which was inserted with the *vTK* PCR product from sample 1, was constructed

Received 27 August 2012 Returned for modification 18 September 2012

Accepted 17 October 2012

Published ahead of print 24 October 2012

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doi:10.1128/JCM.02247-12

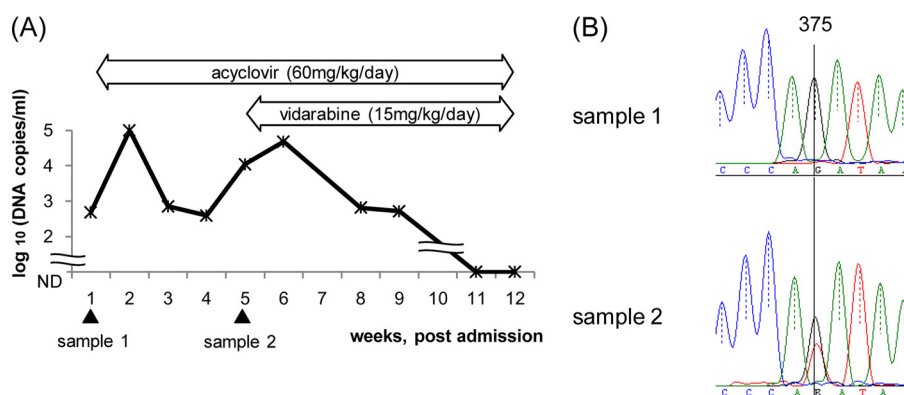


FIG 1 (A) Sequential changes in the HSV-1 DNA level in the CSF determined by quantitative real-time PCR. Arrows below the chart represent the time points of the CSF sample collection for sequencing analysis. ND, not detected. (B) Chromatogram of the *vTK* genes amplified from sample 1 and sample 2. A nucleotide substitution (G375T) was found in sample 2 (lower panel).

and designated *vTK*-375G. Subsequently, a G375T-mutant *vTK* expression plasmid was constructed by site-directed mutagenesis using the following primers, reagents, and PCR conditions and designated *vTK*-375T. Primers 5'-ATATAACAATGGGCATGCC TTATGCC and 5'-GGGCGCTTGTCATTACCAC were designed for the inverse PCR (the underlined T is the targeted nucleotide), and *vTK*-375G was used as the template. The reaction was performed using a PrimeSTAR GXL DNA polymerase kit (TaKaRa Bio, Otsu, Japan), and the amplification conditions included an initial denaturation step of 2 min at 94°C, followed by 10 cycles of 10 s at 98°C, 15 s at 55°C, and 7 min at 68°C. Digestion and self-ligation were performed with a KOD mutagenesis kit (Toyobo, Osaka, Japan). G375T substitution without other nucleotide changes was confirmed by sequencing analysis. The expression plasmid for the ACV-sensitive HSV-1 *vTK* TAS strain (5) was constructed as a positive control and designated *vTK*-TAS. Empty pTARGET served as a negative control. In the final stage of the assay, the titers of the replicated TAR were determined by the standard plaque assays. Then, $\Delta\log_{10}$ PFU values were calculated as follows: $\Delta\log_{10}$ PFU = \log_{10} (PFU per milliliter of the replicated TAR at each ACV concentration) - \log_{10} (PFU per milliliter of the replicated TAR at the ACV concentration of 0 μ g/ml). This value represents the inhibitory effect of ACV on TAR replication, which is brought about by the transfection. Thus, the higher the value is, the lower the activity of the expressed *vTK*. In this way, the *vTK*-related resistance of HSV-1 can be judged from the $\Delta\log_{10}$ PFU values. The sensitivities to ganciclovir (GCV; Sigma-Aldrich Chemical Company, St. Louis, MO), penciclovir (PCV; Wako), and brivudine (BVDU; Sigma-Aldrich) were also tested in the same way.

TAR replication in 293T cells transfected with a negative control was not affected by any concentrations of any antiviral compounds (Fig. 2). When ACV was used, $\Delta\log_{10}$ PFU values elicited by *vTK*-375G transfection were at almost the same level as those elicited by *vTK*-TAS transfection, indicating that HSV-1 in sample 1 and TAS had nearly equal levels of sensitivity to ACV. However, $\Delta\log_{10}$ PFU values elicited by *vTK*-375T transfection were significantly higher than those elicited by *vTK*-375G transfection (Welch's *t* test; $P = 0.004$, <0.001 , and $= 0.045$, at ACV concentrations of 0.4, 4, and 40 μ g/ml, respectively), indicating that the HSV-1 with the G375T mutation in the *vTK* gene had acquired ACV resistance (Fig. 2A). When GCV, PCV, and BVDU were

used, transfection of *vTK*-375G, *vTK*-375T, and *vTK*-TAS resulted in almost the same level of $\Delta\log_{10}$ PFU values (Fig. 2B to D). The HSV-1 G375T mutant was therefore considered to be sensitive to these drugs.

To our knowledge, this is the first report of a patient with ACV-resistant neonatal HSV-1 disease. Neonatal HSV infection is estimated to occur in 1 in every 3,500 to 5,000 deliveries (6). Approximately 30% of the patients are diagnosed as having NHE (7). Although the introduction of ACV has significantly improved the prognosis, NHE is still a severe disease with a mortality rate of 6%, and 70% of the survivors suffer from moderate-to-severe neurological abnormalities (7, 8). ACV-resistant HSV mainly threatens immunocompromised patients, and the prevalence among them is reported to range from 3.5% to 10%. In immunocompetent individuals, the prevalence of ACV-resistant HSV is far lower, ranging from 0.1% to 0.7% (2). Neonatal ACV-resistant HSV infections are quite rare, and all the cases previously described have been caused by ACV-resistant HSV-2 (9–11).

The present study also showed for the first time that a Q125H amino acid substitution in the *vTK* polypeptide induces ACV resistance. Using a method previously described (4), it was confirmed that the Q125H mutation was not a part of natural polymorphism. Q125 of HSV-1 TK has been shown to be located above the nucleotide binding pocket in the three-dimensional (3D) structure of the *vTK* protein (12). Several studies have shown that substitution of Q125 to other amino acids changes *vTK* activity; Q125E and Q125L are associated with resistance to ACV, and Q125N leads to hypersensitivity to ACV (13, 14). Interestingly, the Q125H mutation did not induce cross-resistance to GCV, PCV, and BVDU, suggesting that these drugs may be effective with respect to this specific mutant.

This study showed also for the first time a central nervous system infection caused by a virologically confirmed ACV-resistant HSV-1 strain. There is one report of a possibly ACV-resistant HSV-1 encephalitis adult patient (15). In that report, virus isolation from the CSF failed, but an amino acid substitution of R41H found in the *vTK* polypeptide was suspected to be responsible for the ACV resistance, although it has not been virologically confirmed whether the mutation confers ACV resistance. The method

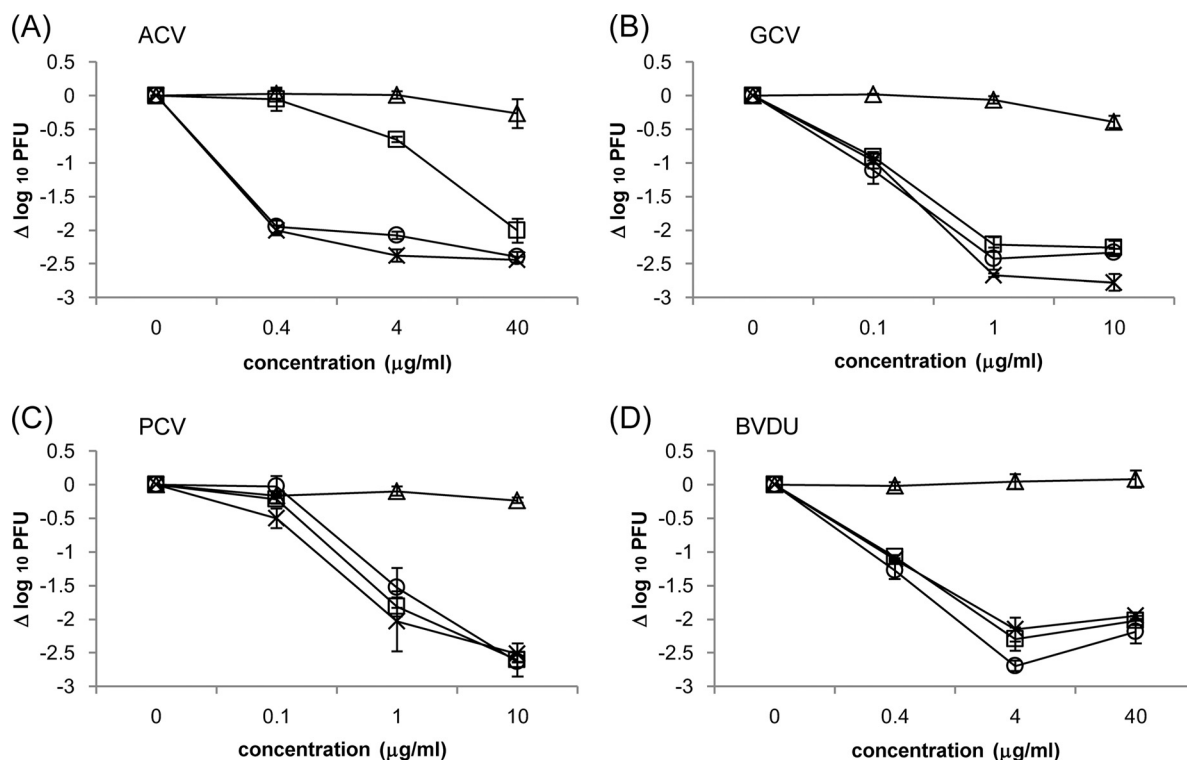


FIG 2 The inhibitory effects of antiviral compounds on replication of TAR in 293T cells transfected with each of the plasmids *vTK-375G* (○), *vTK-375T* (□), *vTK-TAS* (×), and empty pTARGET (Δ). Each experiment was performed in triplicate, and the error bars indicate standard deviations.

used in the present study may be suitable for such a condition. However, it should be kept in mind that the method can be applied only for *vTK*-related ACV resistance and not for DNA polymerase-related resistance.

ACV-resistant HSV is usually seen in patients with a history of ACV treatment (16). In the present case, administration of ACV may possibly have induced the appearance of the ACV-resistant HSV-1 strain. Although a recent study showed a benefit of oral ACV suppressive therapy for survivors of NHE (17), emergence of ACV-resistant HSV during the suppressive therapy was also reported (18). Thus, sensitivity of the causative HSV to antiviral drugs should be carefully monitored. This patient did not receive the suppressive therapy because he suffered from NHE before the beneficial effect of the therapy was reported.

It is recommended to repeat the lumbar puncture after 21 days from the initiation of ACV administration in the treatment of NHE (19). On the other hand, persistence of CSF HSV DNA is reported to be associated with poor neurodevelopmental outcomes of NHE patients (20). CSF HSV DNA quantification was conducted weekly in this patient to monitor the HSV-1 genome level in a real-time manner. We considered that the practice was beneficial, although further discussion is needed. In fact, the frequent monitoring enabled us to treat NHE with an appropriate choice of antiviral drugs.

In conclusion, ACV-resistant HSV-1 was virologically confirmed for the first time in a NHE patient. A nucleotide mutation, G375T in the HSV-1 TK gene, leading to a Q125H amino acid substitution, conferred ACV resistance.

Nucleotide sequence accession numbers. The *vTK* DNA sequence data have been deposited in the DNA Data Bank of Japan

(DDBJ) under accession no. AB713519 (CSF sample 1) and AB713520 (CSF sample 2).

ACKNOWLEDGMENT

We have no conflicts of interest.

This study was financially supported by grants-in-aid from the Japan Society for the Promotion of Science (no. 21591402 and no. 24591591).

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