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# Subclinical generation of acyclovir-resistant herpes simplex virus with mutation of homopolymeric guanosine strings during acyclovir therapy

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ABSTRACT

*Background:* Suppressive therapy in patients with genital herpes has been used in Japan since 2006. Susceptibility and resistance of herpes simplex virus (HSV)-2 to acyclovir were examined in genital isolates from patients receiving suppressive therapy and compared with those from those naïve to acyclovir and receiving episodic treatment with acyclovir.
*Objective:* The aim of this study was to analyze the effect of acyclovir use on the susceptibility to acyclovir and analysis of the thymidine kinase gene by acyclovir treatment.
*Methods:* Genital HSV isolates were obtained from three patients groups. Susceptibility to acyclovir, the frequency of acyclovir-resistant clones and mutations in the thymidine kinase gene of acyclovir-resistant clones were determined.
*Results:* Susceptibility to ACV was significantly higher in isolates from patients receiving suppressive therapy than those naïve to acyclovir and receiving episodic treatment, but the frequencies of resistant clones were similar among the three groups. Mutation in guanosine homopolymeric strings (G-string mutation) was significantly more frequent in clones during episodic treatment and suppressive therapy than clones from patients naïve to ACV. The frequency of G-string mutation was significantly less frequent in isolates from patients naïve to ACV than those experienced ACV therapy.
*Conclusion:* The frequency of acyclovir-resistant mutants was not increased by episodic and suppressive therapy, but exposure to acyclovir significantly generated G-string mutations, possibly induced by acyclovir. Acyclovir therapy had no substantial effects on the susceptibility of HSV-2 or frequency of resistant virus but did generate subclinical G-string mutants in patients' HSV-2.


## 1. Introduction

Acyclovir (ACV) has been safely used for episodic treatment and suppressive therapy of genital herpes, and ACV-resistant mutants have not been observed in immunocompetent and immunocompromised hosts without prolonged ACV treatment for lesions [1–3]. We have shown the susceptibility of genital herpes to ACV in immunocompetent women in Japan between 1977 and 1996 by drug concentration for 50% plaque reduction ($IC_{50}$), and demonstrated that the number of ACV-resistant virus was not increased by quantitation of the ACV-resistant virus in $10^4$ plaque forming units (PFU) of clinical isolates. The mean frequencies of ACV-resistant viruses per $10^4$ PFU for all strains of HSV-1 and HSV-2 were $0.31 \pm 0.41$ and $9.74 \pm 14.83$, respectively, and were not affected by ACV treatment [1].

Japan has used suppressive therapy as the standard treatment for recurrent genital herpes since 2006. Suppressive therapy has been reported to be safe and to not induce resistance in patients

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with genital herpes [4–7]. We previously developed a sensitive assay to detect resistant viruses by direct quantitation of $10^4$ plaques that is at least 1000 times more sensitive than the $IC_{90}$ assay that detects more than 10% of resistant viruses [1]. The appearance of ACV resistance of genital specimens from patients receiving suppressive therapy was directly characterized by direct quantification of ACV-resistant virus in the virus population as the frequency of resistant virus among patients naïve to ACV, receiving episodic treatment, and receiving suppressive therapy. ACV-resistant viruses directly isolated from genital swabs were characterized by their thymidine kinase gene. A mutation in the guanosine homopolymeric string (at least 5 homopolymeric guanosines) of the TK gene was defined as a G-string mutation, and we compared the frequency of G-string mutants among isolates from the three patient groups. Resistance-associated mutations within the TK gene consisted of 64% non-synonymous frameshift mutations within the homopolymer region of guanosines and cytosines in 11 ACV-resistant isolates generated from clinical isolates [8]. The G-string mutation is closely related to the ACV-induced mutation [9,10], and the significance of G-string mutation in ACV treatment was evaluated from ACV-resistant clones of the isolates of the patients with genital herpes in this study.

The susceptibility of genital isolates to ACV was slightly but significantly less in patients underwent suppressive therapy than those naïve to ACV and underwent episodic treatment, but the frequency of resistant virus was not significantly different among the three patients groups. Surprisingly, G-string mutants were observed significantly more frequently in patients exposed to ACV, indicating generation of G-string mutants by ACV. However, G-string mutants were isolated from recurrent lesions before ACV administration, suggesting that the generated G-string mutants became newly latent in the ganglia incrementally to the preexisting latent virus population. Generation and accumulation of G-string mutation might be important in transmission source of ACV-resistant mutant during ACV therapy, especially, in immunocompromised patients.

## 2. Materials and methods

### 2.1. Clinical isolates and laboratory strain

Vero cells were grown and maintained in Eagle's minimum essential medium supplemented with 5% or 2% bovine serum, respectively.

HSV were isolated from 79 swab samples obtained at the Department of Dermatology of the Jikei University School of Medicine, Miyamoto Central Clinic in Kawasaki City, and the Obstetrics and Gynecology Department of the University of Tokyo. A total of 230 ACV-resistant clones were isolated from 50 patients. The inhibitory concentration of ACV ($IC_{50}$) of 89 isolates was determined, and the UL23 TK gene of 74 clones was sequenced. The frequency of ACV-resistant clones in $10^4$ PFU was observed in a total of 50 clones. The profiles of patients used for analysis of G-string mutation in the isolates are summarized in Table 1.

### 2.2. Determination of susceptibility of clinical isolates to ACV

The susceptibility of viruses to ACV was determined by examining the concentration for 50% inhibition of plaque formation ($IC_{50}$) [1,11,12] [13–16]. Briefly, confluent Vero cell monolayers in 60 mm plastic dishes were infected in duplicate or triplicate with 100 PFU of the virus for 1 h and incubated in a nutrient methylcellulose medium containing ACV at various concentrations. After appearance of the cytopathic effect (3 d), the cells were fixed with 5% neutral buffered formalin and stained with methylene blue, and the number of plaques was counted by

**Table 1**
Patients with ACV-resistant clones in the isolates and G-string mutants.

| Category | Gender | Age (years) | Period since first episode of HSV (years) | Period on ACV therapy (years) | Period on suppressive therapy (years) | Frequency of recurrence per year | No. of clones examined | No. of G-string clones |
|---|---|---|---|---|---|---|---|---|
| Naïve | Female | 24 | 0.1 | 0 | 0 | 0 | 3 | 0 |
| Naïve | Female | 35 | 0.1 | 0 | 0 | 0 | 1 | 0 |
| Naïve | Female | 25 | 0.1 | 0 | 0 | 0 | 1 | 0 |
| Naïve | Female | 40 | 0.1 | 0 | 0 | 0 | 1 | 0 |
| Naïve | Female | 47 | 0 | 0 | 0 | 0 | 4 | 0 |
| Naïve | Female | 34 | 0 | 0 | 0 | 1 | 3 | 0 |
| Naïve | Female | 33 | 0 | 0 | 0 | 0 | 3 | 0 |
| Naïve | Male | 31 | 0 | 0 | 0 | 0 | 5 | 0 |
| Naïve* | Female | 30 | 0.9 | 0 | 0 | 2 | 1 | 0 |
| Naïve* | Female | 39 | 1.2 | 0 | 0 | 2 | 1 | 1 |
| Naïve* | Female | 26 | 0.3 | 0 | 0 | 3 | 2 | 0 |
| Episodic | Female | 19 | 0.1 | 0.01 | 0 | 0 | 4 | 1 |
| Episodic | Male | 46 | 16 | 0 | 0 | 18 | 6 | 3 |
| Episodic | Female | 25 | 2 | 0 | 0 | 1 | 3 | 1 |
| Episodic | Female | 30 | 5 | 2 | 0 | 3 | 5 | 3 |
| Suppressive | Female | 55 | 13 | 2 | 2 | 2 | 6 | 1 |
| Suppressive | Male | 39 | 6 | 2 | 2 | 3 | 11 | 3 |
| Suppressive | Female | 44 | 4 | 1 | 1 | 1 | 1 | 0 |
| Suppressive | Male | 44 | 10 | 9 | 9 | 1 | 3 | 1 |
| Suppressive | Female | 50 | 10 | 6 | 3 | 1 | 2 | 2 |
| Suppressive | Male | 66 | 13 | 7 | 7 | 5 | 5 | 1 |
| Suppressive | Male | 44 | 13 | 9 | 9 | 1 | 3 | 2 |