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Expression of herpes simplex virus type 1 recombinant thymidine kinase and its application to a rapid antiviral sensitivity assay

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ABSTRACT

Antiviral-resistant herpesvirus infection has become a great concern for immunocompromised patients. Herpes simplex virus type 1 (HSV-1) infections are treated with viral thymidine kinase (vTK)-associated drugs such as acyclovir (ACV), and most ACV-resistance (ACV^r) is due to mutations in the vTK. The standard drug sensitivity test is usually carried out by the plaque reduction assay-based method, which requires over 10 days. To shorten the time required, a novel system was developed by the concept, in which 293T cells transiently expressing recombinant vTK derived from the test sample by transfection of the cells with an expression vector were infected with vTK-deficient and ACV^r HSV-1 (TAR), and then cultured in a maintenance medium with or without designated concentrations of ACV, ganciclovir (GCV) and brivudine (BVdU). The replication of TAR was strongly inhibited by ACV, GCV and BVdU in 293T cells expressing recombinant vTK of the ACV-sensitive HSV-1, whereas replication was not or slightly inhibited in cells expressing the recombinant vTK of highly resistant or intermediately resistant HSV-1, respectively. An inverse correlation was demonstrated in the 50% effective concentrations (EC_{50}s) and inhibitory effects of these compounds on the replication of TAR among ACV^s and ACV^r HSV-1 clones. These results indicate that the EC_{50}s of the vTK-associated drugs including ACV can be assumed by measuring the inhibitory effect of drugs in 293T cells expressing recombinant vTK of the target virus. The newly developed antiviral sensitivity assay system for HSV-1 makes it possible to estimate EC_{50} for vTK-associated drugs, when whole vTK gene is available for use by gene amplification directly from lesion's samples or from virus isolates.

1. Introduction

Antiviral-resistant herpesvirus infections represent a major concern for immunocompromised patients, such as patients with acquired immune deficiency syndrome (AIDS) and organ transplant recipients (Danve-Szatanek et al., 2004; Morfin et al., 2004; Stranska et al., 2005). Chronic and intractable herpes simplex virus type 1 (HSV-1) infections in such patients require long-term acyclovir (ACV) administration, resulting in the risk of ACV-resistance (ACV^r) in some patients (Boivin et al., 1993; Chen et al., 2000; Erlich et al., 1989; Morfin et al., 2000b; Saijo et al., 1999, 1998; Wade et al., 1982).

Resistance to ACV came about through mutations in viral thymidine kinase (vTK) and/or viral DNA polymerase (vDNApol) genes, with approximately 95% of incidences of ACV^r due to mutations in the vTK gene. vTK-associated ACV^r HSV expresses vTK deficient in viral TK activity (TK^-), mainly due to a frameshift mutation and nucleotide substitution resulting in the early appearance of a stop codon and amino acid substitution, and with altered TK activity (TK_A) due to reduced thymidine- and ACV-phosphorylation activity. The TK_A phenotype has amino acid substitutions in the vTK (Collins and Ellis, 1993; Hill et al., 1991; Nugier et al., 1992; Pottage and Kessler, 1995). Although the incidence is small, mutations in the vDNApol gene are also known to result in ACV-resistance (Collins et al., 1989; Parker et al., 1987; Sacks et al., 1989; Saijo et al., 2002b).

As ACV^r HSV infections should be treated with alternative drugs, such as foscarnet and cidofovir, a rapid and accurate susceptibility

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assay system is required to ascertain the proper treatment. An antiviral sensitivity assay in combination with a plaque reduction assay (PRA) in mammalian cells, such as green monkey kidney cell line (Vero cells) and human embryonic lung fibroblast (HEL) cells is the standard method for determining sensitivity (Collins et al., 1982; Field, 2001; Field and Darby, 1980; McLaren et al., 1983; Safrin et al., 1994). However, this method takes a relatively long period to draw results as number of steps including virus isolation, titration of the isolate with plaque assay, and the sensitivity assay with PRA are required.

To reduce the time required, a rapid sensitivity assay system was developed. This novel antiviral sensitivity assay made it possible to estimate the 50% effective concentration (EC_{50}) by measuring the inhibitory effect of antiviral drugs on the replication of vTK-deficient HSV-1 in 293T cells expressing recombinant vTK of the target HSV-1 cultured in a medium with or without designated concentrations of antiviral drugs.

2. Materials and methods

2.1. Cells

The Vero cell line was used for virus passage and susceptibility testing to ACV and ganciclovir (GCV). HEL cells were also used for the susceptibility test for brivudine (BVdU). 293T cells (American Type Culture Collection, Manassas, VA) were also used. All cells were cultured in Eagle's minimal essential medium (MEM) containing 5% fetal bovine serum (FBS) and antibiotics, penicillin-G and streptomycin (MEM-5FBS).

2.2. Viruses

The TK-positive (TK^+) and ACV-sensitive (ACV^s) HSV-1 TAS and TK^- TAR were used (Saijo et al., 1999). TAR was resistant to ACV, GCV, and BVdU due to of a frameshift mutation in the TK gene. Eight ACV^s HSV-1 strains including TAS were also used (Saijo et al., 1999). Fourteen ACV^r HSV-1 strains generated from the parent TAS strain in a previous study were used (Table 1) (Saijo et al., 2002a).

2.3. Antiviral compounds

ACV, GCV (Wako, Osaka, Japan), and BVdU (Sigma–Aldrich Chemical Company, St. Louis, Mo) were used.

2.4. Western blotting

Western blotting was performed, as reported previously (Saijo et al., 2002a, 1999), to determine the size of the TK polypeptides and to analyze the relative levels of vTK expression.

2.5. Nucleotide sequencing

The nucleotide sequences of the TK genes of the ACV^r HSV-1 strains in this study were determined by direct sequencing methods, as described previously (Saijo et al., 1999).

2.6. Plaque reduction assay

All the HSV-1 strains used in the study were tested for sensitivity to ACV, GCV, and BVdU using PRA (Saijo et al., 1992). Sensitivity to ACV and GCV was determined in Vero cells, while that to BVdU was determined in HEL cells.

2.7. Construction of vectors for the expression of the recombinant vTK of HSV-1

The expression vector was constructed as reported previously (Saijo et al., 2008). The TK gene of HSV-1 was amplified from each HSV-1 strain (Table 1) using the primer set full(CAG)Hind (5′ AGAAGCTTGTAGAAGCGGGTATGGCTTCGTACCCCGGCCATCAGCACGC-3′, the *Hind*III restriction site is underlined) and TK1R(BamHI)

Table 1
Characteristics of the HSV-1 strains used in the study. EC_{50} values, mutations or variations in the TK gene, size of vTK polypeptides, and accession numbers.

HSV-1	EC_{50} (μg/ml) for ACV	Mutations or variations in the TK gene in comparison with that of TAS (amino acid substitutions)	Number of amino acid residues	Accession number
ACV^r HSV-1 clones (Saijo et al., 2002a)				
CL1	>100	G added within 7-Gs (430–436)	227	AB047359
TAR	>100	C deleted within 4-Cs (1061–1064)	407	AB047365
CL11	>100	C deleted within 6-Cs (548–553)	407	AB047366
CL13	>100	Deletion of nucleotide residues (340–835)	262	AB047367
CL14	>100	C310T	103	AB047368
CL15	>100	C325T	108	AB047369
CL17	70	G163A (Asp 55 Asn)	376	AB047371
CL18	4.8	C194A (Thr 65 Asn)	376	AB047372
CL19	16	C250T (Pro 84 Ser)	376	AB047373
CL20	>100	C518G(Pro 173 Arg)	376	AB047374
CL21	10	T598G(Gly 200 Cys)	376	AB047375
CL22	80	C734T(Thr 245 Met)	376	AB047376
CL23	40	C860T(Thr 287 Met)	376	AB047377
CL24	72	G1006A(Cys 336 Tyr)	376	AB047378
ACV^s HSV-1 isolates (Saijo et al., 1999)				
TAS	0.6	No mutations	376	AB047358
96-435	–	C228A, C477G	376	Not registered
96-586	–	No mutations	376	Not registered
Il-6	–	C348T	376	Not registered
I4-32	–	C578G, G631T, A1144G	376	Not registered
I5-48	–	C348T	376	Not registered
I3-19	–	A580C, G1148A	376	Not registered
I5-15	–	C802A, G1144A	376	Not registered

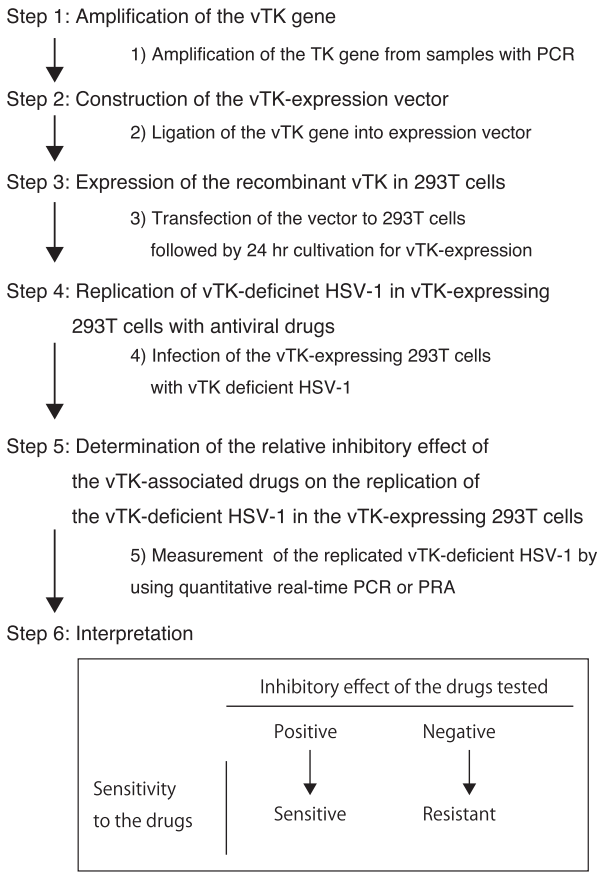

Fig. 1. Schematic representation of the strategy for the novel sensitivity assay system. Step 1: Amplification of the vTK gene by PCR. Step 2: A vTK-expression vector was constructed using pcDNA3.1(+) or pTargeT. Step 3: Expression of recombinant vTK in 293T cells by transfection of 293T cells with the expression vector. Step 4: Infection of the 293T cells expressing the recombinant vTK with HSV-1 TAR allowing the replication of TAR with or without antiviral drugs. Step 5: Determination of TAR load by PRA or qPCR.

(5′-TGGATCCGTGTTTCAGTTAGCCTCCCCCATC-3′, the *Bam*HI restriction site is underlined). These DNA products were digested with *Hind*III and *Bam*HI, and then the DNAs were inserted into the cloning site of the *Hind*III and *Bam*HI restriction sites of pcDNA3.1(+) (Invitrogen, Carlsbad, CA). Another mammalian expression vector, pTargeT (Promega, Madison, Wisconsin), was also used for a construction of vTK-expression vector, because the expression vector can be constructed with a TA-cloning method and without an endonuclease restriction step in the process.

2.8. Expression of recombinant vTK in 293T cells

The recombinant vTK was expressed in 293T cells by transfection of the cells with the expression vector (Saijo et al., 2008).

2.9. Determination of the inhibitory effect of the compounds on the replication of TAR in the vTK-expressing 293T cells

Schematic representation of the assay is shown in Fig. 1. 293T cells were seeded on the bottom of Collagen I 96-well Microplates (Becton, Dickinson and Company, Franklin Lakes, NJ) and cultured to approximately 50% confluence. The cells were then transfected with each of the expression vectors using Fugene HD transfection reagent (Roche Diagnostics, Basel, Switzerland) and cultured in MEM-5FBS for 24 h. These cells were subsequently infected with TAR at multiplicity of infection (m.o.i.) of approximately 1 PFU per cell for 1 h and the virus solutions were gently removed. The cells were then washed gently with phosphate-buffered saline solution (PBS) and refed with MEM containing 2% fetal bovine serum (FBS) and antibiotics, penicillin-G and streptomycin (MEM-2FBS) containing each drug at the designated concentrations. The wells for each concentration were prepared in triplicate. The 293T cells transfected with the expression vectors in additional wells were collected before infection to determine the relative levels of vTK expression by Western blotting. ß-actin was detected by Western blotting using an anti-ß-actin monoclonal antibody (Sigma–Aldrich Chemical Company, St. Louis, Mo) as an internal control. The 293T cells infected with TAR were cultured for 12 h in MEM-2FBS with ACV, GCV, or BVdU at a concentration of 0, 0.4, 1.0, 4.0, 10, or 40 μg/ml. The medium as well as the cells were collected, frozen and thawed. The virus titers of each well were then determined by plaque assay in Vero cells. In addition, the viral genome was extracted from the samples using a Viral Nucleic Acid Extraction Kit (Roche Diagnostics, Basel, Switzerland). The purified genomes were tested for quantitation of the HSV-1 genome by SYBR-green-based real-time quantitative PCR (qPCR) using a LightCycler® 2.0 Real-Time PCR System and LightCycler® FastStart DNA Master SYBR Green I (Aldea et al., 2002).

2.10. Normalization of the inhibitory effect of the compounds between independent experiments

In each experiment, the inhibitory effect of tested compounds on the replication of TAR in 293T cells was normalized as follows. The inhibitory effect on TAR replication was always measured not only in 293T cells expressing the vTK of the target viruses but also in those expressing TAS vTK as a positive control and in those transfected with an empty vector, pcDNA3.1(+), as a negative control. The normalization was performed using the equation below, in which ID indicates "infectious dose (PFU/ml)", and the value was defined as the relative inhibitory value (RIV). The RIV in the cells transfected with the empty vector (RIV_{NC}) and that for TAS (RIV_{PC}) are always normalized as zero and -3, respectively, in each assay. As shown in the results section, the TAR replication was always inhibited by 10^3–10^4 times in the 293T cells expressing TAS vTK (positive control) at an ACV concentration of 40 μg/ml, while it was not at all in those without expressing vTK (negative control) at the same ACV concentration (Fig. 2A). Therefore, RIV for the positive and negative controls were normalized as described above.

$$\mathrm{RIV} = -3X\frac{\mathrm{Log}_{10}\ \mathrm{ID\ of\ negative\ control} - \mathrm{Log}_{10}\ \mathrm{ID\ of\ the\ virus\ tested}}{\mathrm{Log}_{10}\ \mathrm{ID\ of\ negative\ control} - \mathrm{Log}_{10}\ \mathrm{ID\ of\ positive\ control}} \quad (1)$$

2.11. Determination of the RIV cut-off value

Two-graph receiver operating characteristic analysis (TG–ROC) was used to set the cut off value to distinguish ACV-resistance from ACV-sensitivity (Greiner et al., 1995).

2.12. Development of nested PCR for an amplification of full TK gene from the clinical samples

Nested PCR for full genome amplification of vTK gene was developed. The clinical samples were treated with a QIAamp DNA Mini Kit (QIAGEN) for viral DNA extraction and purification according to the manufacture's instruction. Five μl of the purified sample was subjected to the first round PCR using the primer set, S6f (5′-ACAGCGTGCCGCAGATCTTG-3′, 0.2 μM) and S1r (5′-TATCGACAGAGTGCCAGCCC-3′, 0.2 μM) using GC-Rich PCR System (Roche Diagnostics) at a Resolution Buffer concentration of 1 M to amplify the entire vTK gene and its boundary sequences. The

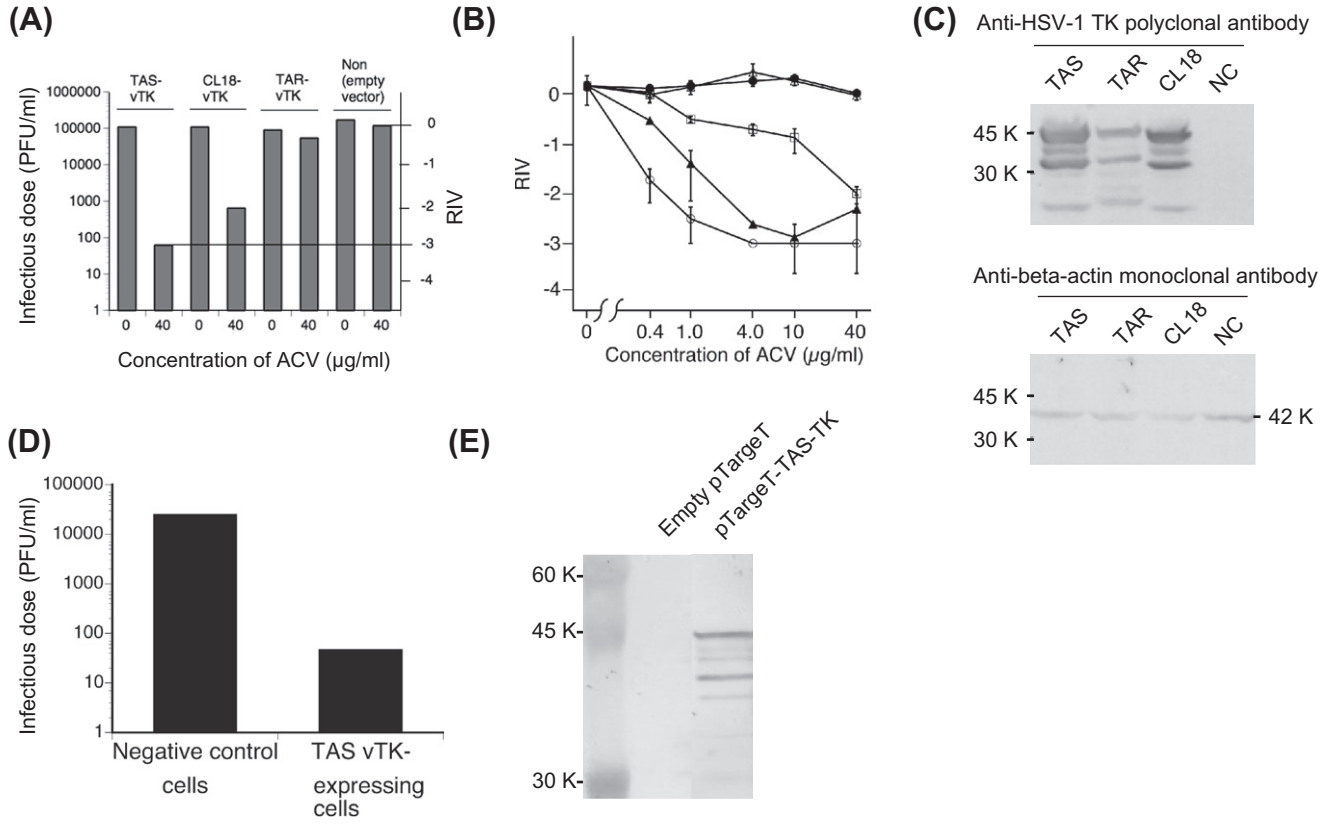

Fig. 2. Expression of recombinant vTK of HSV-1 in 293T cells and replication of HSV-1 TAR in these cells with or without ACV. (A) One step growths of TAR in 293T cells with expression of TAS-vTK, CL18-vTK, TAR-vTK, or without expression of vTK (negative control), at a concentration of 0 and 40 μg/ml of ACV are shown as a form of raw data (PFU/ml). (B) The inhibitory effect of ACV on the replication of TAR in 293T cells expressing TAS-vTK (○), TAR-vTK (△), CL18-vTK (□) by transfection with pcDNA3.1(+)-based expression vector, and in 293T cells transfected with an empty pcDNA3.1(+) vector (●) is shown as a form of RIVs. As the control for the efficacy of ACV, the inhibitory effect of ACV on ACV^s TAS in 293T cells transfected with an empty vector (▲) was included. RIVs were expressed as means ± standard errors (error bars) calculated from 3 wells at each ACV concentration. (C) vTK expression was confirmed by Western blotting with anti-HSV-1 TK rabbit polyclonal antibody and the corresponding bands appeared in the near 45 KDa molecular weight bar (upper panel). The expression level of ß-actin was also confirmed by Western blotting as an internal control (lower panel). 293T cells transfected with pcDNA3.1 empty vector were used as negative control (NC). (D) One step growths of TAR in 293T cell with expression of TAS-vTK by transfection with pTarget-based expression vector and those transfected with an empty pTargeT as a negative control. (E) vTK expression with pTargeT-based expression vector was confirmed by Western blotting with anti-HSV-1 TK rabbit polyclonal antibody and the corresponding bands appeared in the near 45 KDa molecular weight bar.

amplification condition included denaturing at 94 °C for 5 min, 30 cycles of denaturing at 94 °C for 40 s annealing at 56 °C for 30 s, and extension at 72 °C for 2 min, and then an extension step at 72 °C for 5 min. One microliter of the PCR product in the first round PCR was subjected to the second round PCR. The second round PCR was performed in the same way as the 1st round PCR except for the primer set, a forward primer (TKnestedF, 5′-GCGCCTTGTAGAAGCGCGTATG-3′, 0.2 μM) and a reverse primer (TKnestedF, 5′-GGTATTGTCTCCTTCCGTGTTTC-3′, 0.2 μM). After electrophoretic separation of the PCR products, it was stained and visualized with GelGreen Nucleic Acid Gel Stain (Biotium Inc., Hayward, CA) in 1% agarose.

2.13. Clinical samples for the assessment of applicability of the developed drug sensitivity assay

Dried FTA papers (Whatman, Tokyo, Japan) applied with each of the 5 HSV-1 virus solutions were sent for an antiviral sensitivity analyses by Dr. Maria Jose Martinez, Faculty of Medicine, University of Chile, Santiago, Republic of Chile. The 5 HSV-1 isolates (Chile No. 1–5) were recovered from 5 patients with ACV-therapy-resistant keratitis. The virus solution-applied and dried FTA is not infectious, therefore, the paper can be sent by airmail.

2.14. Clinical samples for the assessment of efficacy of the nested PCR in amplification of full genome of vTK gene from the clinical samples

Twenty throat swab specimens were collected from hematopoietic stem cell transplantation recipients in Toranomon Hospital, Tokyo, Japan. The samples were used for the evaluation of the developed nested PCR to amplify full genome of vTK gene. The samples were subjected to the nested PCR and virus isolation using HEL and Vero cells.

2.15. Statistic analyses

The relationship between EC_{50}s and RIVs among the ACV^s and ACV^r HSV-1 clones were assessed by Spearman's correlation coefficient by rank.

3. Results

Inhibitory effect of ACV on the replication of TAR in 293T cells expressing the vTKs of ACV^s, ACV^r, intermediately ACV^r HSV-1, TAS, TAR and CL18.

The inhibitory effect of ACV on TAR replication in 293T cells expressing each of the recombinant vTKs of the 3 representative strains, ACV^s TAS (EC_{50} = 0.6 μg/ml), highly ACV^r TAR (EC_{50} >

100 μg/ml), and intermediately ACV^r CL18 (EC_{50} = 4.8 μg/ml) were determined in a preliminary study (Table 1). The vTK of each HSV-1 was found to be expressed, and the expression level of CL18-vTK was the same as that of TAS-vTK, whereas the expression level of the internal control, ß-actin, was also confirmed to be the same between TAS and CL18 (Fig. 2C, upper and bottom panel). The CL18-vTK was confirmed to be the same size as the intact vTK. The TAR-TK was larger than the intact vTK and the intensity of the band was slightly weaker than those of TAS-vTK and CL18-vTK in Western blotting. The replication of TAR in 293T cells expressing TAS-vTK was inhibited by ACV in a dose-dependent manner, but not in 293T cells expressing TAR-vTK (Fig. 2B). ACV intermediately inhibited the replication of TAR in the cells expressing CL18-vTK. According to these results, we determined that the RIV should be calculated at a concentration of 40 μg/ml of ACV for the differentiation of ACV-resistance from ACV-sensitivity. Although the data are not shown here, similar results were demonstrated for GCV and BVdU.

3.1. Relations between EC_{50} and RIV

There was a positive correlation between the RIVs of ACV and the EC_{50}s among the ACV^r HSV-1 clones (Fig. 3A), with the EC_{50} increasing with increases in RIV. In addition, similar results were demonstrated in the sensitivity assay for GCV and BVdU (Fig. 3B and C, respectively). The correlation coefficients between the RIVs and EC_{50}s in the assays for ACV, GCV, and BVdU were 0.96, 0.76, and 0.81, respectively.

3.2. Cut-off value for the differentiation of resistance from sensitivity

The RIV cut-off value for the differentiation of ACV-resistance from ACV-sensitivity was determined using TG–ROC analysis with 8 ACV^s HSV-1 isolates including TAS and 14 ACV^r HSV-1 clones (Table 1). The RIV of ACV for ACV^s isolates varied between −4 and −2, whereas the RIV of ACV for ACV^r clones ranged between −2 and 1. Therefore, the tested viruses showing an RIV of <-2 and $\geqslant -2$ could be judged to be sensitive and resistant, respectively (Fig. 4).

3.3. Application of the novel sensitivity assay of the clinical samples

Full genome of the HSV-1 TK was successfully amplified from the 5 HSV-1 isolates, which were sent as a form of dried materials in the FTA papers (Whatman) by PCR using the primer set of full(CAG)Hind and TK1R(BamHI). Then the expression vectors for the recombinant vTK of these isolates were constructed, and subjected to the novel sensitivity assay. The RIVs of ACV, GCV, and BVDU for one isolates (Chile No. 5) out of 5 isolates were between 0 and −1, while those for the other isolates (Chile No. 1–4) were less than −2, suggesting that Chile No. 5 was resistant to the drugs tested.

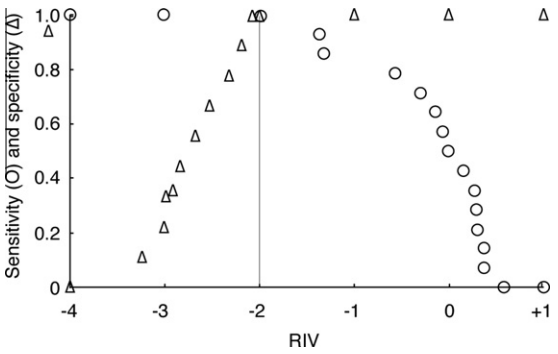

Fig. 4. The RIV cut-off value for the determination of resistance in the novel sensitivity assay system evaluated using TG–ROC analyses. Blank circles and triangles indicate the sensitivity and specificity for ACV-resistance calculated using 8 ACV^s and 14 ACV^r clones. The intersection point of the two graphs indicates the cut-off point between -3 and -2, at which sensitivity = specificity can be achieved. In the case of a cut-off value of −2.0, all the 8 ACV^s HSV-1 and all the 14 ACV^r HSV-1 clones were confirmed to be ACV^s and ACV^r by the novel assay system, respectively.

3.4. Correlation between RIVs calculated by plaque assay and those calculated by qPCR

To shorten the time required for the assay, qPCR was substituted for plaque assay for the determination of relative amount of virus replicated. There was a strong linear correlation between RIV obtained by plaque assay and that obtained by qPCR (Fig. 5). The correlation coefficient was 0.93, indicating that a comparable level of TAR replication can be determined by qPCR.

3.5. Inhibitory effect of ACV on the replication of TAR in 293T cells expressing the vTKs of HSV-1 TAS by transfection using pTargeT expression vector

To shorten the time required for the assay, the mammalian expression vector, pTargeT (Promega) was tested for the availability in the assay instead of the original vector, pcDNA3.1(+). The recombinant vTK of TAS was efficiently expressed (Fig. 1E). Furthermore, the replication of TAR in the TAS vTK-expressing 293T cells by transfection with the pTargeT-based expression vector was inhibited at the same level as in the TAS vTK-expressing 293T cells by transfection with pcDNA3.1(+)-based expression vector (Fig. 1A and D).

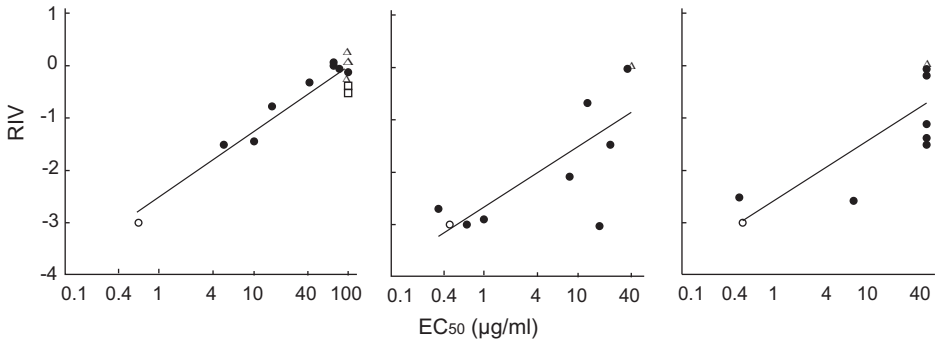

Fig. 3. Relationship between the EC_{50}s and RIV. Relationship between the EC_{50}s of ACV (A), GCV (B), and BVdU (C) for TAS (○), ACV^r HSV-1 clones with frameshift mutations in the TK polypeptide (△), those with mutations leading to the early appearance of a stop codon in the TK gene (□), and those with amino acid substitutions in the TK polypeptide (●).

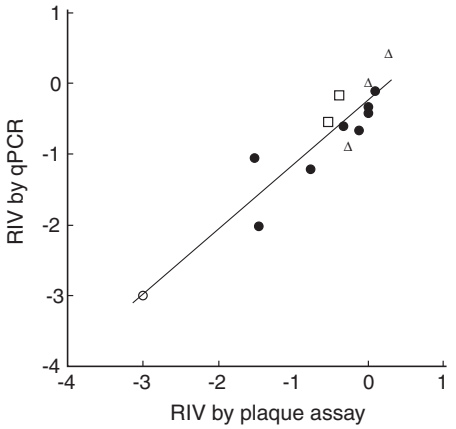

Fig. 5. Relationship between the RIVs of ACV determined with plaque assay and those determined by qPCR. Relationship between the RIVs of ACV determined with plaque assay and those determined by qPCR is shown for ACV^s HSV-1 (○), ACV^r HSV-1 with frameshift mutations in the TK gene (△), ACV^r HSV-1 with nucleotide substitutions resulting in the early appearance of a stop codon (□), and ACV^r HSV-1 with amino acid substitutions in the vTK polypeptide (●).

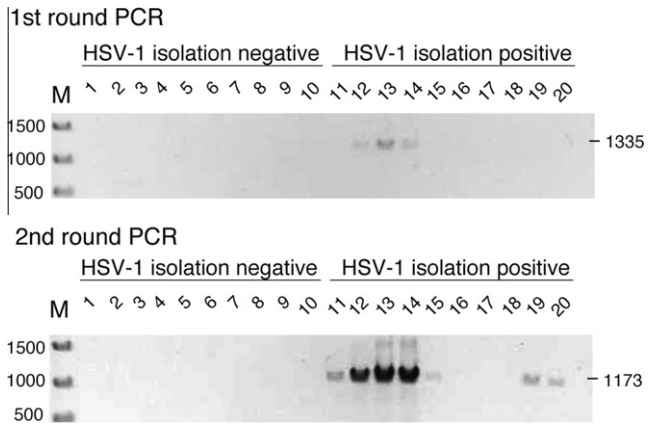

Fig. 6. Direct amplification of the full genome of vTK gene by the nested PCR. Three and 7 out of the 10 HSV-1 isolation positive samples showed a positive reaction in the 1st round and 2nd PCR, respectively. None of the HSV-1 isolation negative samples showed a positive reaction in the assay.

3.6. Nested PCR for an amplification of full genome of vTK gene from clinical samples

Because the sensitivity assay could be performed when full genome of the vTK gene was amplified, it is expected that the time required for the assay can be shortened if full genome of the vTK gene was amplified directly from clinical samples. Nested PCR methods for the amplification of the full genome of the vTK gene was developed and evaluated. HSV-1 was isolated from 10 samples, while it was not from another 10 samples. Full genome of the vTK gene was amplified in 7 of the 10, while none of the 10 virus isolation negative samples showed a positive reaction in the nested PCR (Fig. 6).

4. Discussion

More than 30 years have passed since the introduction of ACV in clinical use for the treatment of HSV. ACV^r HSV infections are of great concern, particularly in immunocompromised patients such as those with hematopoietic stem cell transplantation or HIV infections.

In the present study, a rapid sensitivity assay system for testing HSV-1 sensitivity to vTK-associated drugs was developed. To our knowledge, this assay system is based on a novel concept. We confirmed that the sensitivity of HSV-1 could be assessed by determination of the relative inhibitory effect of the compounds on the replication of vTK$^-$ HSV-1 (TAR) in 293T cells transiently expressed with the recombinant vTK of the target HSV-1.

The advantages of this novel assay are summarized as follows. **First, the time required is short**. PRA is usually used for sensitivity assays, and it usually requires approximately 10 days when applied to a sensitivity test, because the virus amplification step including the storage of the virus solution, titration of the amplified virus with plaque assay, and the sensitivity assay with PRA require approximately 3–4 days, 3–4 days, and 3–4 days, respectively. On the other hand, the time required for the novel sensitivity assay are approximately 0.5 day for full genome amplification of vTK gene directly from clinical samples, approximately 2 days for the construction of the expression vector, 1 day for the expression of recombinant vTK in 293T cells by transfection with the expression vector, 0.5 day for cultivation of TAR in the cells with or without drugs, and 0.5 day for quantitation of TAR by quantitative PCR, being the minimum total time for the assay less than 5 days. **Secondly, EC_{50} values can be measured**. It is evident that nucleotide sequencing is a rapid and efficacious tool for the differentiation of ACV-resistance from ACV-sensitivity if frameshift mutations or mutations that result in the early appearance of a stop codon are demonstrated. However, phenotypical analyses are required in cases in which only differences in amino acids are demonstrated in the vTK polypeptide. In contrast, EC_{50} values could be estimated by this novel assay. **Thirdly, the sensitivity of the isolates could be tested, not only to ACV, but also to other vTK-associated drugs**. The information regarding EC_{50} values of the isolates, not only to ACV, but also to GCV and BVdU, suggesting that this novel assay might be applied to all the vTK-associated anti-alpha herpesviruses including ACV and pencilovir that is effective in treatment of HSV-1 infections (Lazarus et al., 1999). These advantages offer usefulness for the treatment of drug-resistant HSV infections in terms of dosage, route of administration, and drug selection.

A rapid system was reported for the differentiation of ACV^r HSV-1, HSV-2, and VZV by measuring the vTK activity of *in vitro*-synthesized vTK polypeptides (Suzutani et al., 2000). The method requires only a short time of less than 2 days. The assay enabled the differentiation of resistant from sensitive viruses, but intermediately resistant viruses were all classified as resistant viruses, indicating the level of resistance cannot be evaluated by the assay. Two heterologous systems based on recombinant vTK expression have also been reported. One system is based on the expression of the vTK gene by the protozoan parasite *Leishmania*, normally devoid of TK activity (Bestman-Smith et al., 2001). The sensitivity of the target virus to ACV could be evaluated by measuring the inhibitory effect of GCV on the growth of the recombinant *Leishmania*. This protocol requires a relatively longer time of more than 5 days. The other system is based on vTK gene expression in bacteria: the TK-deficient strain *Escherichia coli* B SY211, in which is TK activity is artificially abolished (Sahli et al., 2000). This bacteria-based method requires only 3 days for the evaluation of sensitivity to ACV by measuring the inhibitory effect of 5-fluorodeoxyuridine (FUdR), a nucleotide analog, on the colony forming level of the TK-deficient *E. coli* transformed with the vTK-expression vector compared with that in bacteria in the absence of FUdR (Summers and Raksin, 1993). Both assay systems are able to differentiate resistant from sensitive viruses. However, it is not possible to evaluate the sensitivity level, as shown by EC_{50} values.

The novel sensitivity assay system was applied to the assessment of HSV-1 isolates, Chile No. 1–5, recovered from 5 patients

with ACV-therapy-resistant keratitis in the Republic of Chile. No sensitivity assay of HSV to antiviral drugs is generally commercially available, and testing is performed in only a limited number of laboratories. As shown in this study, the sensitivity of HSV-1 isolates can be tested without transfer of the infectious virus, relying instead on the far safer transfer of the virus genome. This is considered to be an additional advantage over the conventional PRA for drug sensitivity assay.

Numerous vTK mutations of ACV^r HSV-1 were reported, therefore the direct sequencing with the genotypic information enables rapid estimation of ACV-resistance (Gaudreau et al., 1998; Graham et al., 1986; Hwang and Chen, 1995; Morfin et al., 2000a; Nugier et al., 1992; Pramod et al., 2000; Saijo et al., 2002a). If the sensitivity assay were relied only on the genotypic assay for the vTK gene, the ACV-resistance would not be assessed in the case that the resistance to ACV is due to a novel amino acid mutation in the TK polypeptide. Genotypic characteristics of GCV- or BVdU-resistant HSV-1 in vTK gene are limited, while those of ACV^r HSV-1 are accumulated. The advantage of the novel assay is that the sensitivity of the test virus can be measured not only to ACV but also to the other vTK-associated drugs. The novel antiviral sensitivity assay system grasps the resistant viruses, which cannot be determined for sensitivity by genotypic assay. As a result, it is considered that the combination of direct sequencing method with the novel assay system would be recommended as promising workflow to evaluate the sensitivity in a short time.

The direct amplification method of the full genome of HSV-1 TK from clinical samples using nested PCR was developed. Seven out of the 10 HSV-1 isolation positive-throat swab specimens showed a positive reaction in the nested PCR. In combination with the full genome amplification from the clinical samples, it becomes possible to shorten the time required in the novel drug sensitivity assay system.

Unfortunately, it must be noted that there is one disadvantage to this novel assay. The resistance of HSV-1 to ACV due to the mutations in the DNA polymerase gene will be missed, although ACV-resistance due to this mechanism accounts for approximately 5% of all ACV^r HSV-1 isolates.

In conclusion, a novel and rapid antiviral sensitivity assay system was developed for HSV-1. The assay system makes it possible, not only to differentiate ACV-resistance from ACV-sensitivity, but also to measure the level of resistance to ACV. Furthermore, the sensitivity to other vTK-associated drugs could be determined by this novel assay. The time required from the receipt of samples to completion of the assay was also much shorter than that for the standard PRA. The system might be a useful tool in the diagnosis and treatment of HSV-1 infections.

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