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Short communication

Resistance of herpes simplex virus type 1 to acyclovir: Thymidine kinase gene mutagenesis study

Emilie Frobert [a,b,*], Tadamasa Ooka [b], Jean-Claude Cortay [b], Bruno Lina [a,b], Danielle Thouvenot [a,b], Florence Morfin [a,b]

[a] *Laboratoire de Virologie Est, Hospices Civils de Lyon, 8 Avenue Rockefeller, 69373 Lyon Cedex 08, France*
[b] *Laboratoire de Virologie et Pathogénèse Virale, CNRS UMR 5537, Faculté de Médecine Laennec, Université Claude Bernard Lyon 1, rue Guillaume Paradin, 69372 Lyon Cedex 08, France*

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Abstract

By site-directed mutagenesis, we investigate the role of six mutations of herpes simplex virus type 1 thymidine kinase (TK) gene in the acquisition of resistance to acyclovir (ACV). TK activity was not impaired by substitutions located at codons 17, 161 and 374 and these mutations were thus related to TK gene polymorphism. Mutations His105Pro, Leu364Pro and Asp162Ala lead to the loss of TK activity that could result in ACV-resistance.

Keywords: Herpes simplex virus; ACV resistance; Thymidine kinase; Site-directed mutagenesis

Herpes simplex virus (HSV) infections may result in severe diseases among immunocompromised patients. Since its commercialisation in the 1980s, acyclovir (ACV) is the reference treatment for these infections. ACV is a guanosine analogue that needs to be first phosphorylated by the virus-encoded thymidine kinase (TK). Emergence of ACV-resistant strains has occurred, mainly in immunocompromised patients with a mean prevalence of 5% (Danve-Szatanek et al., 2004) that can reach 27% in allogenic bone marrow transplant patients (Morfin et al., 2004). ACV resistance is mostly due to the presence of mutations in the TK gene. Among mutations that can be associated with resistance, half are nucleotide substitutions, most of them being located in active or conserved sites of the enzyme. Half are nucleotide insertions or deletions often occuring in homopolymer repeats of guanines or cytosines that are considered as mutational hot spots (Darby et al., 1986; Gaudreau et al., 1998; Morfin et al., 2000; Bestman-Smith et al., 2001). Genetic studies of drug-sensitive strains also revealed mutations which are not related to resistance, mainly located outside the active sites (Kudo et al., 1998; Morfin et al., 2000; Bodaghi et al., 2000; Chibo et al., 2004). Because of the TK gene polymorphism, site-directed mutagenesis of detected mutations have to be performed to ascertain their role in ACV resistance. Using site-directed mutagenesis, we had previously studied the implication of HSV-1 TK gene mutations located at codons 51, 77, 83 and 175 on TK enzymatic activity, using both a reticulocyte lysate system and a bacterial system to produce recombinant proteins (Frobert et al., 2005). To complete this first study, we now report the results obtained on six other HSV-1 TK gene mutations that are located at codons 17, 105, 161, 162, 364 and 374. All these mutations have previously been detected in clinical isolates (Morfin et al., 2000; Chibo et al., 2004). Most of them were described in resistant strains which presented several mutations simultaneously. Moreover, no sensitive strain had been previously isolated from the same patient and sequence comparison could not be performed. These mutations have thus been investigated to ascertain their role in the acquisition of ACV resistance.

TK gene of ACV-sensitive reference HSV-1 strain KOS was previously cloned into the pGEM-T Easy vector (Promega) (Frobert et al., 2005). This pGEM-TK KOS was used as a matrix to create, by site-directed mutagenesis and PCR, TK gene mutants pGEM-TKm17, pGEM-TKm105, pGEM-

* Corresponding author at: Laboratoire de Virologie Est, Hospices Civils de Lyon, 8 Avenue Rockefeller, 69373 Lyon Cedex 08, France.
Tel.: +33 4 78 77 70 29; fax: +33 4 78 01 48 87.
E-mail address: e.frobert@wanadoo.fr (E. Frobert).

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Table 1
Modified internal primers used for site-directed mutagenesis

Mutated codon	Forward primer	Reverse primer
17	5′-AGGCTG**T**GCGTTCTCGCGGCCAT	5′-GAACGCAC**A**GCCTGGTCGAACGC
105	5′-CACAAC**C**CCGCCTCGACCAGGGT	5′-AGGCG**G**GGTTGTGTGGTGTAGAT
161	5′-CGGTC**T**AAGATGAGGGTGAGGG	5′-ATCTT**A**GACCGCCATCCCATCG
162	5′-GGCGG**G**CGAAGATGAGGGTGAG	5′-CTTCG**C**CCGCCATCCCATCGCC
364	5′-CGACC**C**GGCGCGCATGTTTGCCC	5′-GCGCC**G**GGTCGCAGATCGTCGGT

In bold: mutated nucleotide.

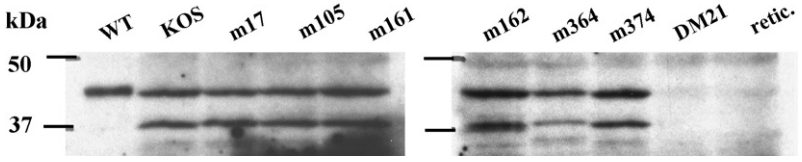

Fig. 1. Western blot analysis of thymidine kinase (TK) proteins. WT: TK from cultured wild-type KOS HSV-1, positive control; KOS: ACV-sensitive reference HSV-1 strain; DM21: ACV-resistant reference HSV-1 strain presenting a 816 bp deletion between codon 98 and codon 370 in its TK gene (Efstathiou et al., 1989); m17, m105, m161, m162, m364 and m374 are recombinant proteins of the respective mutants; Retic.: control of residual activity of reticulocytes (reaction without any plasmid DNA).

TKm161, pGEM-TKm162, pGEM-TKm364 and pGEM-TKm374. To create TK gene mutants pGEM-TKm105, pGEM-TKm161, pGEM-TKm162, the external forward primer TKf (5′-GATCTTGGTGGCGTGAAACTCC-3′), the external reverse primer TKr (5′-GGTTCCTTCCGGTATTGTCTCC-3′) and internal modified primers (Table 1) were used, as previously described (Frobert et al., 2005). To create the mutation 17, the external forward primer TKf was replaced by the forward primer 17TK-5′-CGGCGAACGTGGCGAGAAAGGAA designed on the pGEM-T easy plasmid, upstream of the TK gene. To create the mutation 364, the external reverse primer TKr was replaced by the reverse primer 364TK-5′-CGAACGACCGAGCGCAGCGAGTC designed on the pGEM-T easy plasmid, downstream from the TK gene. As HSV-TK is a 376-amino-acid protein, to create the mutation 374 located close to the C-terminus of the protein, only one PCR was performed using the forward primer TKf and the modified reverse primer 374TK-5′-GTTTCAGTTAGCC**G**CCCCCATC. TK proteins were produced using 1 μg of plasmid DNA using the TNT®-coupled reticulocyte lysate system (Promega, France) in a final volume of 50 μl (Frobert et al., 2005). Identification of TK proteins was performed by Western blotting analysis on 5 μl (∼30 ng according to the Manufacturer's instructions) of the reticulocyte system product used for TK enzymatic activity assays. The incubation with polyclonal rabbit antibodies directed against HSV-1 proteins diluted 1: 2500 (kindly provided by William C. Summers, Yale University) went on one night at 4 °C. TK enzymatic activity was measured on 45 μl of the reticulocyte system product (∼270 ng according to the Manufacturer's instructions). The substrate medium contained 150 mM phosphate buffer pH 7.5, 20 mM ATP, 20 mM $MgCl_2$, 40 mM KCl, 1 mM dithiothreitol, 10 mM NaF, 20 μM cold thymidine and 2.5 μCi/50 μl of [^{3}H]thymidine. Adsorption of the reaction product onto a DEAE-cellulose paper after 0, 15, 30 and 60 min incubation, allowed to separate the phosphorylated thymidine from the non-phosphorylated substrate. The monophosphorylated thymidine was counted by scintillation (UltimaGold MV, Packard). Means and standard deviations of enzymatic activities were calculated from three independent assays performed for each mutant.

Before enzymatic activity assays, the recombinant TK proteins, expected at the molecular weight of ∼41 KDa, were checked by Western blot analysis (Fig. 1). Production of proteins with the reticulocyte lysate system showed reproducibility as the enzymatic activity was at similar levels in three independent assays (Fig. 2). The results revealed that mutants Ala17Val, Phe161Leu and Glu374Ala presented a phosphorylating activity of respectively 4.84 ± 1.01, 3.30 ± 0.30 and 4.17 ± 0.37 pmol of thymidine/h/20 μl of reactional mixture, which was similar to the activity of the reference strain KOS (4.13 ± 0.25 pmol of thymidine/h/20 μl of reactional mixture). These mutants were defined as TK-positive mutants. Mutations His105Pro, Asp162Ala and Leu364Pro abolished the phosphorylating activity of the respective mutated proteins, defining them as TK-negative mutants.

The mutations studied herein were first described in clinical strains isolated from immunocompromised patients chronically infected with HSV. These isolates have been previously tested by phenotypic antiviral assays which revealed resistance to ACV

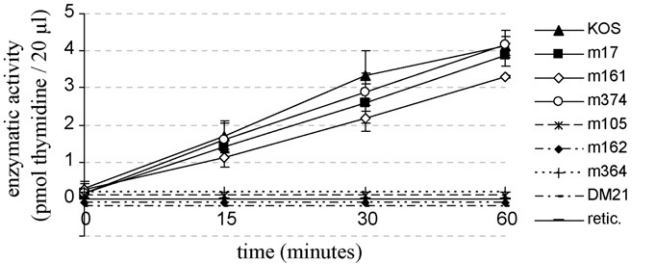

Fig. 2. Enzymatic activity of TK recombinant proteins. KOS: ACV-sensitive reference HSV-1 strain; DM21: ACV-resistant reference HSV-1 strain; m17, m105, m161, m162, m364 and m374 are recombinant proteins of the respective mutants; Retic.: control of residual activity of reticulocytes (reaction without any plasmid DNA).

Table 2
Clinical context, phenotypic and genetic results of HSV-1 isolates

Clinical context	Source of virus	Phenotypic study (codons mutated)	TK gene mutations	Reference
Immunocompromised patient	Eye	CI_{90} = 444 μmol/l	Asp162Ala	Chibo et al. (2004)
Bone marrow transplant patient	BAL	CI_{50} = 100 μmol/l	His105pro, Ala17Val, Glu374Ala	Morfin et al. (2000)
Immunocompromised patient	Not available	Not available	Phe161Leu, other mutations known to confer resistance	Chibo et al. (2004)
Bone marrow transplant patient	Vesicle	CI_{50} = 42 μmol/l	Leu364Pro, Ala175Val	Morfin et al. (2000)

CI_{90}: ACV concentration causing 90% inhibition of viral replication. Cutoff value for ACV resistance: 44.4 μmol/l (Chibo et al., 2004); CI_{50}: ACV concentration causing 50% inhibition of viral replication. Cutoff value for ACV resistance: 6.5 μmol/l (Morfin et al., 2000); BAL: bronchoalveolar lavage.

(Table 2). Mutated TKs were produced by site-directed mutagenesis and tested with an enzymatic assay using thymidine as substrate. This technique detects TK-deficient mutants which phosphorylate neither thymidine nor ACV. TK-altered strains, which phosphorylate thymidine but not ACV, are not detected by this assay. These strains do exist in clinical situation but remain very uncommon (Gilbert et al., 2002). In addition, Saijo et al. (2002), testing TK mutations, reported similar results using either thymidine or ACV as substrate in enzymatic assays. Mutation Asp162Ala is located in a conserved region of the TK gene and this mutation induced a loss of TK activity. Even if not located in a conserved part of the TK gene, His105Pro substitution induced a loss of TK activity. Chatis and Crumpacker (1991) had previously reported the mutation Gln105Pro in an ACV-resistant HSV-2 strain. Mutations Ala17Val and Glu374Ala have been described in a phenotypically resistant strain also presenting the His105Pro mutation (Morfin et al., 2000). As the Ala17Val and Glu374Ala mutations were located outside of active or conserved sites of the TK gene, they were presumed to be associated with TK gene polymorphism. Producing proteins presenting only one of these two mutations confirmed that both did not modify TK activity and that they were indeed not related to ACV resistance. Mutation Phe161Leu, located outside the conserved sites, was first described by Chibo et al. (2004) in a resistant strain harboring a second mutation known to induce ACV resistance. It was thus associated with TK gene polymorphism and this is confirmed by the results here presented. Mutation Leu364Pro, located outside of any active or conserved sites, was initially related to TK gene polymorphism because it has been detected in an ACV-resistant isolate that also presented the well defined mutation Ala175Val (Morfin et al., 2000). Nevertheless, our study showed that mutation Leu364Pro was itself sufficient to induce a loss of TK activity and may be responsible for ACV resistance. This clinical strain thus harbored two mutations involved in ACV resistance. No additional isolate was available from this patient to determine whether these two mutations were simultaneously or successively selected. This observation proves that, although substitutions located outside the TK-conserved sites are likely to be associated with TK gene polymorphism, their role, in each case, needs to be confirmed by site-directed mutagenesis. Using this technique, the effect of several HSV TK mutations on enzyme activity could also be considered in combination. Michael et al. (1995) has indeed described synergistic effects of residues in position 251, 321 and 348 of HSV-1 TK in selective substrate recognition. Pilger et al. (1999) also showed that mutation His58Leu had a compensatory effect as this mutation restored TK enzyme activity of the double mutant Met128Phe/Tyr172Phe.

Our results complete the database allowing the discrimination between TK gene mutations associated with ACV resistance and those related to gene polymorphism. This database has to be expanded as new mutations will be reported in the literature for ACV-resistant isolates. At the present time, detection of HSV resistance is performed by phenotypic antiviral assays that require isolation of the virus, which is time-consuming, and sensitivity or resistance evaluation results cannot be given to the physician prior to 7–10 days. Switching to an alternative treatment based on *in vitro* sensitivity can thus be delayed. Establishing a database of TK gene mutations related to ACV resistance will be essential for the interpretation of TK gene sequencing results and for the implementation of rapid molecular biology tests to detect ACV-resistant HSV directly in clinical specimens.

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