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Short Communication

Significance of amino acid substitutions in the thymidine kinase gene of herpes simplex virus type 1 for resistance

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ABSTRACT

The analysis of the viral thymidine kinase (TK) genotype is of rising significance for testing resistance of herpes simplex virus (HSV) to antivirals especially acyclovir. However, numerous of the described amino acid (aa) substitutions are diagnostically less conclusive because of the pronounced natural polymorphism of this gene. In this study, several aa substitutions in the TK sequence of HSV-1 with unclear significance for resistance were analyzed by expression of recombinant TK proteins and determination of enzymatic activity on the basis of an enzyme linked immunosorbent assay using bromodeoxyuridine (BrdU) as TK substrate. The recombinant TK wild-type protein resulted in high TK activity and TK mutant with stop of translation showed negative results. The recombinant TK proteins containing the aa substitutions R41H or V348I had high phosphorylation activities suggesting most likely natural gene polymorphisms. By contrast, the aa changes Y53H, L139V, R163H, L298A and L315S were accompanied by negative or weakly positive TK activities indicating resistance association. In conclusion, the combination of methods described here represents a useful tool to evaluate the significance of aa substitutions for resistance of clinical HSV-1 strains.

Non-synonymous mutations in the thymidine kinase (TK) gene (Morfin and Thouvenout, 2003) are considered the genetic basis of herpes simplex virus (HSV) resistance to acyclovir (ACV) and one half is due to single nucleotide polymorphisms (SNP). They can either lead to a stop of amino acid (aa) translation (Sauerbrei et al., 2010, 2011) also associated with the lack of TK activity or can result in aa substitutions (Chibo et al., 2004) which can be connected with an alteration or a loss of enzyme activity or a change of substrate specificity. Recently, novel resistance-associated aa substitutions have been reported in several studies and have enriched the knowledge about the genetic alteration of TK (Burrel et al., 2010; Sauerbrei et al., 2010, 2011). In clinical HSV strains, genotypic data can be interpreted easily when frame-shift mutations and/or premature stop codons were detected. By contrast, single aa substitutions can hardly be associated with resistance especially when they have not been defined experimentally, they are located outside active sites or conserved regions and/or there is no information available about the corresponding susceptible phenotype. The reason for this is the pronounced natural polymorphism of the TK gene (Bohn et al., 2011). Accordingly, several aa substitutions of the TK gene with unclear significance for ACV resistance have been described in the literature. Thus, the objective of the present study was to examine the significance of seven aa substitutions in the TK gene for ACV resistance by the use of cloning experiments, site-directed mutagenesis, expression of recombinant TK proteins and determination of enzymatic activity by the use of a non-radioactive TK test with bromodeoxyuridine (BrdU) as substrate. The substitutions R41H and V348I have been described mainly as natural polymorphisms of the HSV-1 TK gene (Burrel et al., 2010; Bohn et al., 2011), but they have also been reported as possible cause of ACV resistance (Schulte et al., 2010; Sauerbrei et al., 2011). Y53H, L139V, R163H, L298R and L315S have been identified recently as possible novel substitutions associated with resistance sometimes combined with other resistance mutations (Sauerbrei et al., 2010, 2011). Since L139V, L298R and L315S are located outside active or conserved gene regions, the significance of these substitutions has to be clarified.

The ACV-sensitive HSV-1 isolate 2110/08 was used as wild-type (WT-2110/08) strain (Bohn et al., 2011). Genotypic analysis of the TK gene revealed the aa substitutions D14Y, N23S, K36E, L42P and A265T that were components of the natural TK polymorphism. The HSV-1 isolate 3579/09 served as ACV-resistant mutant (TK-R-3579/09; Sauerbrei et al., 2011). The TK gene exhibited the

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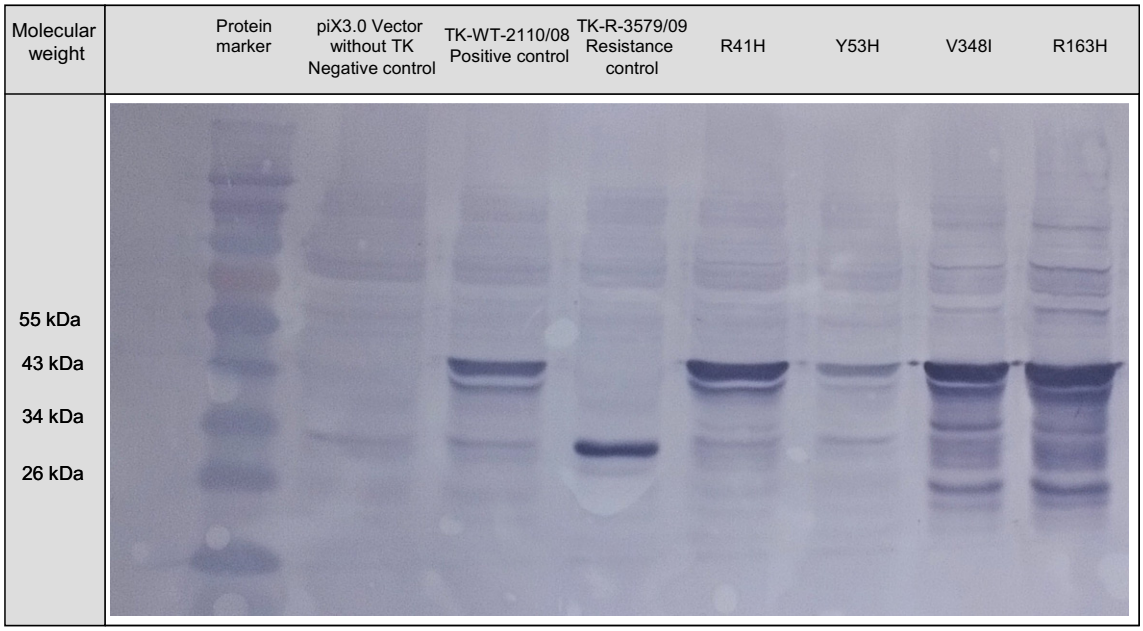

Fig. 1. Detection of recombinant HSV-1 TK using Western blot analysis. Wild-type TK protein of HSV-1 has a size of about 41 kDa and slightly higher molecular weight of the second band can be explained by an isoform of protein containing the additional His- and *Strep*-tag components of the corresponding DNA. Negative control consisting of the pIX3.0 vector without TK did not show any signal and confirmed the specificity of the methods used. Recombinant TK protein TK-R-3579/09 used as resistance control comprised amino acid change Q250stop and led to a smaller visible TK protein with molecular weight of approximately 27 kDa.

aa substitutions N23S, K36E, R89Q attributed to gene polymorphism and the SNP C > T at nucleotide position 748 resulting in a premature stop of translation (Q250stop). Genotypic analysis was based on the TK-positive HSV-1 reference strain 17 (GenBank Accession No. X14112). After DNA extraction by the use of QIAmp® DNA Blood Mini Kit (Qiagen, Hilden, Germany), the TK genes of the HSV-1 wild-type and resistant mutant were constructed and cloned using the EasyXpress® Linear Template Kit Plus and the pIX 3.0 vector (Qiagen) following instructions for use (IFU). All primer sequences and conditions of polymerase chain reaction (PCR) are available on request. To construct thymidine kinase mutants, site-directed mutagenesis was performed using the Geneart® Site-Directed Mutagenesis System Kit (Invitrogen, Darmstadt, Germany) according to IFU. The modified forward and reverse TK-specific primers for the methylation and mutagenesis PCR and information on PCR conditions are also available on request. The PCR products were gel-purified and used as template (0.35–1.12 μg) for the reaction of *in vitro* recombination. Recombinant TK protein was *in vitro* transcribed/translated from plasmids using the EasyXpress Protein Synthesis Kit (Qiagen) according to IFU. The plasmid DNA of vector pIX 3.0 without TK gene was used as negative control while the plasmid DNA of WT2110/08 served as positive control. Bradford protein assay was used to control the protein synthesis and to estimate protein concentrations. Unpurified recombinant protein samples were analyzed by standard sodium dodecyl sulfate polyacrylamide gel electrophoresis. Blotted Protran® nitrocellulose membranes with a 0.45 μm pore size (Whatman, Dassel, Germany) were incubated with primary anti-HSV-1 TK goat antibody diluted 1:500 (Santa-Cruz Biotechnology, Heidelberg, Germany). Alkaline phosphatase-conjugated rabbit anti-goat antibody (Acris, Herford, Germany) diluted 1:2000 was applied as secondary antibody. Proteins were visualized using the nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3′-indolylphosphate detection system (Roche, Grenzach-Wyhlen, Germany). Each of the seven recombinant TK proteins was produced in two independent experiments. The enzymatic TK activity was determined using the DiviTum® test system (Biovica International AB, Uppsala, Sweden) according to IFU. One per cent of BrdU was used as substrate for the TK and the enzyme immunoassay reaction of alkaline phosphatase with the chromogenic substrate was evaluated after incubation for 180 min. Two assays were performed to verify the TK activity of each recombinant protein and the TK activity was given in the arbitrary units DU per 2.8 ng protein since this concentration resulted in values within the measuring range of the microtiter plate reader used. Arithmetic means and standard deviations (SD) were computed from two replicates per two independent preparations used. For each assay run, the described positive (TK-WT-2110/08), negative (pIX3.0 vector without TK) and resistant controls (TK-R-3579/09) were used. Cut-off values for TK activity expressed in DU per 2.8 ng protein were calculated

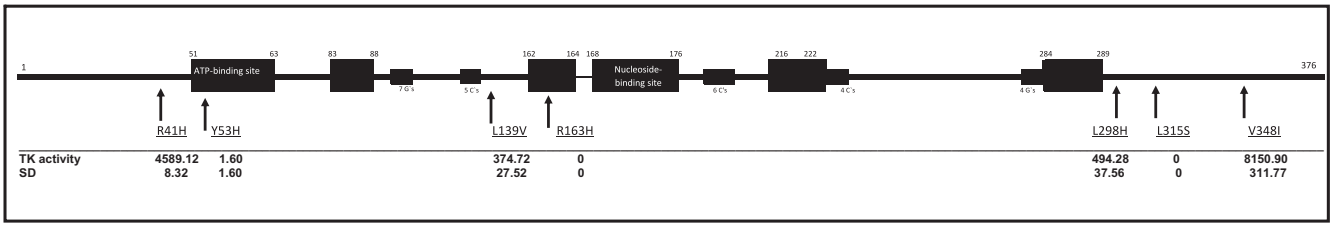

Fig. 2. Activities of recombinant TK containing different amino acid changes in association with the positions of substitutions studied. Activities are given in DU per 2.8 ng protein plus standard deviation (SD). Vector pIX3.0 without TK revealed TK activity value of 0.96 (SD 1.66) DU. On the basis of this negative control, a cut-off value of 13.10 DU was calculated. Positive control of wild-type protein TK-WT-2110/08 resulted in highly positive value of 5196.72 (SD 1264.30) DU and resistant control TK-R-3579/09 showed negative result at 0 (SD 0) DU comparable with negative control.

as five times the mean values plus SD of the negative control. The TK activities were defined as weakly positive when the results in DU per 2.8 ng protein ranged between >cut-off and ⩽10% of the positive control. Activities were considered positive when the results were >10% of the positive control.

All recombinant TK proteins exhibiting the aa substitutions R41H, Y53H, L139 V, R163H, L298H, L315S or V348I were detected by Western blotting. In each, double bands at a molecular weight of about 41 and 43 kDa could be identified. In Fig. 1, this is shown exemplarily for the TK proteins containing the R41H, Y53H, V348I and R163H changes. The TK activities detected according to the positions of the mutations studied are summarized in Fig. 2. The recombinant TK proteins containing the aa substitutions R41H or V348I had high phosphorylation activities regarded as positive. By contrast, the aa changes Y53H, R163H and L315S were accompanied by negative TK activity values. In addition, the recombinant TK proteins with the aa changes L139V or L298A revealed low TK activity <10% of the positive control.

The findings of this study reveal that the substitutions R41H and V348I are most likely part of TK polymorphism. There are several reports on this substitution presented in HSV-1 strains with ACV-resistant phenotype (Bestman-Smith et al., 2001; Chibo et al., 2004). Recently, R41H has been described as the only substitution most likely associated with ACV resistance in one HSV-1 strain, which caused herpes simplex encephalitis but whose phenotype could not be tested (Schulte et al., 2010). The aa change V348I was detected in one ACV-resistant HSV-1 isolate specified only by further well-characterized polymorphism-associated non-synonymous mutations in both the TK and DNA polymerase genes (Sauerbrei et al., 2011). A possible reason for the resistance phenotype might be a mixture of resistant and sensitive viral strains (Nugier et al., 1992) that may be prevented an unambiguous evaluation of the genotypic status. Without doubt, the substitutions Y53H, R163H and L315S could be attributed to an ACV-resistant phenotype of HSV-1 since the viruses represent TK-negative mutants. Y53H is located within the ATP-binding site of TK gene and has been reported firstly by Sauerbrei et al. (2010) in combination with ACV-resistant viral phenotype. This viral isolate also comprised the substitution R163H that could also be clarified as resistance-related in clinical HSV-1 strains. The aa change L315S has also been reported recently as novel resistance-associated mutation (Sauerbrei et al., 2010). Even though nucleotide substitutions related to resistance are usually in the conserved sites of the TK (Gaudreau et al., 1998; Morfin et al., 2000), several mutations have also been shown to confer resistance to ACV despite their location outside conserved gene regions (Chatis and Crumpacker, 1991; Duan et al., 2009). This is also relevant for L139V and L298R reported as novel substitutions (Sauerbrei et al., 2011). L139V was detected in combination with the resistance-related substitution T287M (Sarisky et al., 2001) in one HSV-1 strain with ACV-resistant phenotype (Sauerbrei et al., 2011). L298R was found in one non-viable HSV-1 strain obtained from a patient with clinical resistance to ACV (Sauerbrei et al., 2011). Both recombinant TK proteins revealed activities less than 10% of wild-type TK. Therefore, the corresponding viruses represent most likely TK-deficient HSV-1 mutants. The assay proposed here has the advantages of using commercially available cloning, site-directed mutagenesis, *in vitro* expression of recombinant protein and determination of TK activity based on a non-radioactive test system. Limitations are the use of unpurified *in vitro* translated material because of their small quantities and the use of BrdU and not ACV as substrate for the TK. Therefore, mutants with low TK activity level in the assay are linked to ACV resistance, but high TK level cannot be unequivocally associated with genetic polymorphisms.

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