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Single nucleotide polymorphisms of thymidine kinase and DNA polymerase genes in clinical herpes simplex virus type 1 isolates associated with different resistance phenotypes

Axel Schubert [a,1], Eva Gentner [a,b,1], Kathrin Bohn [c], Maximilian Schwarz [c], Thomas Mertens [a], Andreas Sauerbrei [c,*]

[a] Institute of Virology, German Reference Laboratory for CMV, University Hospital Ulm, Ulm, Germany
[b] Institute for Experimental Cancer Research, University Hospital Ulm, Ulm, Germany
[c] Institute of Virology and Antiviral Therapy, German Reference Laboratory for HSV and VZV, Jena University Clinic, Jena, Germany

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ABSTRACT

The role of mutations in the thymidine kinase (TK, *UL23*) and DNA polymerase (pol, *UL30*) genes of herpes simplex virus (HSV) for development of different resistance phenotypes has to be exactly determined before genotypic resistance testing can be implemented in patient's care. Furthermore, the occurrence of cross-resistance is of utmost clinical importance. In this study, clinical HSV-1 isolates obtained between 2004 and 2011 from 26 patients after stem cell transplantation were examined in parallel by phenotypic and genotypic resistance testing. Thirteen isolates, which were phenotypically cross-resistant to acyclovir (ACV), penciclovir (PCV) and brivudin (BVDU), exhibited consistently frameshift or non-synonymous mutations in the TK gene known to confer resistance. One of these mutations (insertion of C at the nucleotide positions 1061–1065) has not been described before. Seven strains, phenotypically resistant to ACV and PCV and, except one each, sensitive to BVDU and resistant to foscarnet (FOS), carried uniformly resistance-related substitutions in the DNA pol gene. Finally, 3 isolates, resistant to ACV, PCV and 2 out of these also resistant to BVDU, had known but also unclear substitutions in the TK and DNA pol genes, and 3 isolates were completely sensitive. In conclusion, clinical ACV-resistant HSV-1 isolates, carrying resistance-associated mutations in the TK gene, can be regarded as cross-resistant to other nucleoside analogs such as BVDU. In contrast, clinical FOS-resistant HSV-1 strains which are cross-resistant to ACV may be sensitive to BVDU. This has to be considered for drug changes in antiviral treatment in case of ACV resistance.

1. Introduction

Herpes simplex virus type 1 (HSV-1) is one of the most common viruses infecting humans. Primary infections occur often during infancy. In Germany and other developed countries, the seroprevalence of HSV-1 increases gradually during childhood and adolescence and reaches levels up to about 90% in adults (Sauerbrei et al., 2011; Wutzler et al., 2000). After newly acquired infections, the virus remains latent lifelong in sensory ganglia. Endogenous viral reactivations may occur resulting in recurrent manifestations such as labial herpes in nearly 30% of adults (Dréno et al., 2012; Pica and Volpi, 2012). Both, primary and recurrent HSV-1 infections may lead to substantial physical and psychological morbidity.

In contrast to the self-limited course in immunocompetent patients, HSV-1 reactivations are very common among individuals with an impaired immune system, and diseases can be a major cause of morbidity. The risk of HSV disease after allogenic hematopoietic stem cell transplantation (HSCT) without prophylaxis, mainly caused by HSV-1, is approximately 80% (Sandherr et al., 2006). This includes almost exclusively virus reactivations in the first weeks after HSCT during bone marrow aplasia or in the presence of stomatitis (Meyers et al., 1980). Herpes simplex virus type 1 disease, occurring mostly as ulcerative mucositis (van der Beck et al., 2012), is markedly invasive with the risk of dissemination but antiviral therapy using acyclovir (ACV) is highly efficacious. Thus, antiviral prophylaxis is recommended for all HSV-seropositive patients because it has been proven to lower the incidence of HSV disease (Kawamura et al., 2013; Sandherr et al., 2006).

* Corresponding author. Address: Institute of Virology and Antiviral Therapy, Jena University Clinic, Hans-Knoell-Strasse 2, D-07745 Jena, Germany. Tel.: +49 3641 9395700; fax: +49 3641 9395702.
E-mail address: Andreas.Sauerbrei@med.uni-jena.de (A. Sauerbrei).
[1] These authors contributed equally to this work.

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However, prolonged antiviral therapy with ongoing viral replication may favor the selection and emergence of drug-resistant mutants complicating the treatment of HSCT patients. While resistance to ACV has a low prevalence of <1% in immunocompetent individuals (Collins and Ellis, 1993; Danve-Szatanek et al., 2004; Englund et al., 1990), approximately 5% of immunocompromised patients develop ACV resistance (Illán et al., 2004), and the highest prevalence rates up to 30% have been reported in patients after allogenic bone morrow transplantation (Morfin and Thouvenot, 2003). In 95% of the cases, ACV resistance is mediated by mutations in the thymidine kinase (TK) gene (Morfin and Thouvenot, 2003; Bestman-Smith et al., 2001) which is necessary for activation of the drug. In the remaining 5% of the cases, mutations in the DNA polymerase (pol) gene can be detected (Larder and Darby, 1986) representing the target of the triphosphorylated ACV. Consequently, in clinical HSV isolates, resistance to ACV has been shown to be regularly linked to cross-resistance against penciclovir (PCV) whose prodrug is famciclovir (Sarisky et al., 2001; Sauerbrei et al., 2010, 2011). Furthermore, previous studies have demonstrated that ACV/PCV-resistant clinical HSV-1 isolates are most likely cross-resistant to brivudin (BVDU) (Sauerbrei et al., 2010, 2011). In these cases, where resistance is due to mutations in the TK gene, foscarnet (FOS), a direct inhibitor of the DNA pol, or cidofovir (CDV), which also targets the DNA pol, independently of viral TK, can be recommended as alternative treatment (Balfour et al., 1994; Chilukuri and Rosen, 2003). To date, ACV cross-resistance to FOS (Burrel et al., 2010; Sacks et al., 1989; Sauerbrei et al., 2010; Schmit and Boivin, 1999) or resistance to CDV (Wyles et al., 2005) has only been reported very rarely in clinical HSV isolates.

The purpose of the present study was to examine 26 clinical HSV-1 isolates from different patients undergoing peripheral blood stem cell transplantation (PBSCT) by both phenotypic and genotypic resistance testing. By correlating the resistance pheno- and genotypes, the role of specific mutations in the TK and DNA pol genes for the development of the resistance phenotypes can be estimated.

2. Materials and methods

2.1. Viral isolates

In this study, clinical HSV-1 isolates from 26 different patients with peripheral blood stem cell transplantation (PBSCT) were included. In 7 patients, acute myeloid leukemia was diagnosed (No. 2, 4, 5, 9, 11, 14, 23), in one patient each Hodgkin's lymphoma (No. 13) and chronic lymphocytic leukemia (No. 26) and, in 17 patients, the clinical diagnosis was unknown. Strains were obtained from throat wash (No. 1, 3–14, 17, 18, 20, 23, 26), bronchoalveolar lavage (No. 2), tongue swab (No. 24), tongue (No. 24) or cheek swab (No. 25; kind of sample unknown: No. 15, 16, 19, 21) of 11 male and female patients each, and the gender was unknown in 4 cases. The age of patients ranged between 6 and 66 years (mean 43.0 ± 17.3 years). Strains were isolated between 2004 and 2011, and the time interval after PBSCT was between <1 and 19 months (unknown time interval in 17 patients). There was information on the antiviral therapy provided by the clinicians in only 8 patients (No. 2, 4, 5, 9, 11, 13, 14, 26). Acyclovir (No. 2, 5, 13, 26), brivudin (BVDU; No. 5, 9, 13, 14, 26), foscarnet (No. 5), ganciclovir (No. 4), famciclovir (No. 11) and/or trifluridine (No. 4, 9, 13, 26) were administered. In all patients, there was a clinical resistance (therapy failure), which was the cause for virological testing of resistance. For the patients for whom information on antiviral treatment was provided, clinical resistance was defined as the absence of clinical improvement of HSV infection after at least 10 days of antiviral administration (Balfour et al., 1994). All HSV-1 strains were propagated in African green monkey kidney Vero76 cells (ATCC, CRL 1587) and human embryonic lung fibroblasts (HELF). The method for viral growth has been described previously (Sauerbrei et al., 2010). Viral stocks were stored at −80 °C when the titer reached between 10^6 and 10^8 tissue culture infective doses 50% ($TCID_{50}$) ml^{-1} after one to three cell culture passages. In all viral strains, HSV-1 was identified and HSV-2 could be excluded by diagnostic polymerase chain reactions (PCR) using primers specific for the UL 42 region of HSV-1 and the TK gene of HSV-2, respectively (Sauerbrei et al., 2000; Vogel et al., 1994).

2.2. Characterization of resistance phenotype

Resistance phenotype was characterized using the following antiviral compounds which are in clinical use: acyclovir (ACV; GlaxoSmithKline, Uxbridge, UK), penciclovir (PCV; GlaxoSmithKline, Uxbridge, UK), brivudin ((E)-5-(2-bromovinyl)-2′-deoxyuridine, BVDU); Berlin-Chemie AG, Berlin), tri-sodium-phosphonoformiate (Foscarnet, FOS; AstraZeneca, Wilmslow, UK), and cidofovir (CDV; Vistide®, Pharmacia & Upjohn, Luxembourg). The viral isolates were tested according to a method described previously (Sauerbrei et al., 2010). In short, human Caucasian fetal lung fibroblasts of the cell line Wi 38 (European Collection of Cell Cultures, Salisbury, UK) or Vero76 cells were seeded at a density of 2×10^4 cells ml^{-1} in 96-well flat bottom microtiter plates and grown for 2 days. The cells were infected with a multiplicity of infection of 0.01 corresponding to about 10^3 $TCID_{50}$ ml^{-1}, and the antiviral compounds were added at a final half log dilution over a range between 0.28 and 35.2 μM ACV, 0.25 and 31.6 μM PCV, 0.19 and 24.0 μM BVDU, 0.23 and 29.6 μM CDV or between 13.3 and 844.8 μM FOS. The TK-positive reference strain HSV-1 Mac Intyre (ATCC No. VR-539) was used as a sensitivity control. After incubation for 5 days, the virus-induced cytopathic effect was assessed microscopically, and a cell proliferation assay using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) was carried out. From the dose–response curves, substance concentrations at half-maximum virus inhibition (effective concentration 50%, EC_{50}) were calculated by linear regression analysis using the software SigmaStat, Version 1.01 (Jandel Corporation, San Rafael, CA, USA). Final results were calculated as mean values from two experiments each. Herpes simplex virus type 1 isolates were regarded as resistant to ACV, PCV, BVDU or CDV if the mean EC_{50} measured five times higher than the mean value of the sensitive control strain HSV-1 Mac Intyre. Taking into account the recommendation for a resistance index of 3–5 times the mean value of ACV-sensitive reference strain (Morfin and Thouvenot, 2003), the upper limit value was selected. For resistance to FOS, EC_{50} values >300 μM were considered (Safrin et al., 1991).

2.3. Characterization of resistance genotype

Viral DNA was obtained from 200 μl of viral stock by means of QIAamp® DNA blood mini kit (Qiagen, Hilden, Germany). The oligonucleotide primers for the amplification and sequencing of TK (*UL23*) and DNA pol (*UL30*) genes based on the HSV-1 reference strain 17 (GenBank Accession No. X14112) (McGeoch et al., 1985) were used as described previously (Sauerbrei et al., 2011). The TK gene of all HSV-1 cell culture isolates could be amplified as one fragment whereas the DNA pol gene was amplified in four separate fragments. The PCR mix for amplification of both genes consisted of Q-Solution, 10 mM dNTPs, 10× PCR buffer, 25 mM $MgCl_2$, 10 μM forward and reverse primers, 1.25 U of the proofreading enzyme HiFidelity HotStar polymerase (Qiagen), and 250 ng template DNA. Amplification conditions included an initial denaturation step over 15 min at 95 °C followed by 35 cycles of

60 s at 94 °C, 60 s at 55 °C and 90 s at 72 °C and a final extension step for 7 min at 72 °C. After purification using the QIAquick® PCR Purification Kit (Qiagen), the amplified fragments were sequenced by the company Eurofins MWG Operon (Ebersberg, Germany). Finally, the alignment of sequence data and the comparison with the published sequences of reference strain 17 was carried out using the software MEGA 5.1. All specified nucleotide sequences corresponded to nucleotide positions in this reference strain.

3. Results

Resistance phenotypes of all 26 HSV-1 isolates against ACV, PCV, BVDU, FOS and CDV are summarized in Table 1. Table 2 shows genotypic findings concerning TK and Table 3 the genotypes regarding DNA pol. According to phenotypic as well as genotypic results, the HSV-1 isolates were divided into 4 groups. Group 1 consisted of 3 strains (No. 1–3) sensitive to all antiviral compounds used. The TK and DNA pol exhibited amino acid substitutions which have been described as natural polymorphisms. Additionally, the novel substitution V521M could be detected in the DNA pol outside of active or conserved gene regions (Fig. 1).

Group 2 contained 13 HSV-1 isolates (No. 4–16) that were cross-resistant to the nucleoside analogs ACV, PCV and BVDU but sensitive to FOS and CDV. All these strains consistently exhibited known resistance-related frameshift mutations or amino acid changes in the TK gene. Among these, the novel insertion of C within the nucleotides (nt) 1061–1065 was observed. In 4 isolates (No. 5, 7, 9 and 15), a mixture of viral strains with drug-sensitive wild type genotypes and the resistant mutants was found. In addition to the resistance-associated mutations, the following polymorphisms with unclear significance were found in the TK and DNA pol genes: E43D (TK gene) and L49I, E421V, E662D, P829S, D1126N, M1226I (DNA pol gene). Whereas E43D (TK gene), L49I, E421V, E662D, D1126N, M1226I (DNA pol gene) are clustered outside of active or conserved gene centers of the TK gene, P829S is located within the conserved region III of DNA pol (Fig. 1). Because the resistance of the isolate No. 12 is obviously related to the insertion G nt 429–437, the substitutions E43D (TK gene) and E421V (DNA pol gene) can be most likely considered as natural polymorphisms. Taken together, in these 13 isolates, the cross-resistance to ACV, PCV and BVDU was caused by resistance-related frameshift or non-synonymous mutations in the TK gene.

In the group 3, 7 phenotypically resistant HSV-1 isolates (No. 17–23) have been summarized containing resistance-associated amino acid substitutions in the DNA pol. All strains were resistant to ACV and PCV. Furthermore, all isolates, except one (No. 20), were sensitive to BVDU and CDV and all, apart from one (No. 23) were resistant to FOS. The isolates carried uniformly known resistance-related amino acid substitutions in the DNA pol. One isolate (No. 17) contained a mixture of the resistant and sensitive genotypes. Three so far not described substitutions have been detected, namely G264V as well as G271V in the TK and I182V in the DNA pol. All clustered outside of active or conserved gene regions (Fig. 1).

Group 4 exhibited 3 HSV-1 isolates (No. 24–26) phenotypically resistant to ACV and PCV. In addition, 2 out of these 3 strains (No. 24 and 25) were resistant to BVDU, and all strains were sensitive to FOS and CDV. Both isolates resistant to ACV, PCV and BVDU carried the unknown amino acid changes H58N and R32H in the TK. The third strain (No. 26) resistant against ACV and PCV contained the

Table 1
Phenotypic resistance of HSV-1 strains against acyclovir (ACV), penciclovir (PCV), brivudin (BVDU), foscarnet (FOS) and cidofovir (CDV).

No.	Results (effective concentration 50%, EC_{50}, μM)				
	ACV*	PCV*	BVDU*	FOS*	CDV*
Sensitive strains containing polymorphism in TK and DNA pol (group 1)					
1	*s* (1.01)	*s* (1.42)	*s* (<0.18)	*s* (237.4)	*s* (3.29)
2	*s* (0.79)	*s* (3.08)	*s* (<0.18)	*s* (152.1)	*s* (4.51)
3	*s* (<0.26)	*s* (1.15)	*s* (<0.18)	*s* (224.4)	*s* (0.78)
Resistant strains containing res.-related substitutions in TK (group 2)					
4	**r (>35.2)**	**r (>31.6)**	**r (>24.0)**	*s* (249.5)	*s* (0.81)
5	**r (>35.2)**	**r (>31.6)**	**r (>24.0)**	*s* (52.7)	*s* (3.74)
6	**r (>35.2)**	**r (>31.6)**	**r (>24.0)**	*s* (220.0)	*s* (1.67)
7	**r (>35.2)**	**r (>31.6)**	**r (1.56)**	*s* (72.5)	*s* (0.96)
8	**r (>35.2)**	**r (>31.6)**	**r (>24.0)**	*s* (73.4)	*s* (1.85)
9	**r (>35.2)**	**r (>31.6)**	**r (>24.0)**	*s* (180.0)	*s* (0.96)
10	**r (>35.2)**	**r (>31.6)**	**r (16.8)**	*s* (83.9)	*s* (0.93)
11	**r (23.7)**	**r (8.18)**	**r (20.6)**	*s* (44.3)	*s* (1.04)
12	**r (9.52)**	**r (7.86)**	**r (>24.0)**	*s* (47.7)	*s* (3.63)
13	**r (>35.2)**	**r (>31.6)**	**r (>24.0)**	*s* (90.1)	*s* (3.29)
14	**r (>35.2)**	**r (>31.6)**	**r (>24.0)**	*s* (36.7)	*s* (1.59)
15	**r (>35.2)**	**r (>31.6)**	**r (>24.0)**	*s* (84.3)	*s* (1.52)
16	**r (>35.2)**	**r (>31.6)**	**r (>24.0)**	*s* (30.5)	*s* (2.11)
Resistant strains containing res.-related substitutions in DNA pol (group 3)					
17	**r (>35.2)**	**r (9.56)**	*s* (0.39)	**r (>422.4)**	*s* (7.22)
18	**r (>35.2)**	**r (14.3)**	*s* (<0.18)	**r (>422.4)**	*s* (4.11)
19	**r (6.64)**	**r (>31.6)**	*s* (<0.18)	**r (>422.4)**	*s* (3.55)
20	**r (6.03)**	**r (>31.6)**	**r (>24.0)**	**r (>422.4)**	*s* (1.52)
21	**r (9.11)**	**r (>31.6)**	*s* (<0.18)	**r (>422.4)**	*s* (8.21)
22	**r (7.21)**	**r (10.4)**	*s* (<0.18)	**r (>422.4)**	*s* (5.14)
23	**r (16.2)**	**r (>31.6)**	*s* (<0.18)	*s* (24.8)	*s* (1.92)
Resistant strains containing novel substitutions in TK and DNA pol (group 4)					
24	**r (>35.2)**	**r (>31.6)**	**r (1.38)**	*s* (135.1)	*s* (3.52)
25	**r (>35.2)**	**r (>31.6)**	**r (1.74)**	*s* (297.9)	*s* (4.88)
26	**r (>35.2)**	**r (>31.6)**	*s* (0.57)	*s* (55.0)	*s* (4.55)

* Mean EC_{50} values of sensitive HSV-1 control strain Mac Intyre and resistance indices given in parentheses: ACV, 0.62 ± 0.48 μM (3.10 ± 2.40 μM); PCV, 1.03 ± 0.04 μM (5.15 ± 0.20 μM); BVDU, ⩽0.18 μM (>0.90 μM); FOS, 178.2 ± 84.5 μM (>300 μM); CDV 3.22 ± 1.41 μM (16.10 ± 7.05 μM).

Table 2
Genotypic resistance of HSV-1 strains with amino acid substitutions and insertions/deletions of nucleotides in the thymidine kinase (TK) gene. All positions correspond to that of reference strains 17 (HSV-1, GenBank Accession No. X14112). Novel amino acid exchanges are in bold. Nt – nucleotide, pol – polymerase.

No.	Natural polymorphism	Polymorphism associated with resistance	Polymorphism with unclear significance
Sensitive strains containing polymorphism in TK and DNA pol			
1	C6G, N23S, K36E, R41H, A192V, G251C, A265T, V267L, P268T, D286E, N376H	–	–
2	N23S, K36E, R89Q, G240E, A265T	–	–
3	N23S, K36E, L42P, G240E, A265T, V267L, P268T, D286E, N376P	–	–
Resistant strains containing res.-related substitutions in TK and polymorphism in DNA pol			
4	N23S, K36E, R89Q, A265T	C336Y	–
5*	N23S, K36E, R89Q, G251C, A265T, V267L, P268T, D286E, N376H	Insertion G nt 429–437, L170P	–
6	N23S, K36E, L42P, A265T	Deletion C nt 665–670	–
7*	N23S, K36E, R89Q, A265T	R216C	–
8	N23S, K36E, R89Q, A265T	M121R	–
9*	N23S, K36E, R89Q, G240E, A265T	R220H	–
10	N23S, K36E, G251C, A265T, P268T	**Insertion C nt 1061–1065**	–
11	N23S, K36E, R89Q, A265T, V348I	R222C	–
12	C6G, N23S, K36E, R41H	Insertion G nt 429–437	**E43D**
13	N23S, K36E, R89Q, G240E, A265T	R176Q	–
14	N23S, K36E, R89Q, A265T	R222H	–
15*	N23S, K36E, R89Q, A265T	Deletion C nt 460–463	–
16	N23S, K36E, R89Q, A265T	G200D	–
Resistant strains containing res.-related substitutions in DNA pol and polymorphism in TK			
17	N23S, K36E, L42P, A265T	–	–
18	N23S, K36E, R89Q, A265T	–	–
19	C6G, N23S, K36E, L42P, A265T, V348I	–	–
20	N23S, K36E, L42P, A265T	–	**G271V**
21	C6G, N23S, K36E, R41H, A192V, A265T	–	–
22	N23S, K36E, I78F, R89Q, G240E, S332L	–	–
23	N23S, K36E, R89Q, G240E, A265T	–	**G264V**
Resistant strains containing novel substitutions in TK and DNA pol			
24	C6G, N23S, K36E, L42P, A265T, V348I	**H58N**	–
25	N23S, K36E, R89Q, A265T	**R32H**	–
26	N23S, K36E R89Q, A265T	–	**A93V**

* Mixed population consisting of wild type and mutated virus.

Table 3
Genotypic resistance of HSV-1 strains with amino acid substitutions in the DNA polymerase (pol) gene. All positions correspond to that of reference strains 17 (HSV-1, GenBank Accession No. X14112). Novel amino acid exchanges are in bold. TK – thymidine kinase.

No.	Natural polymorphism	Polymorphism associated with resistance	Polymorphism with unclear significance
Sensitive strains containing polymorphism in TK and DNA pol			
1	S33G, A330R, **V521M**, V905M, A1203T, T1208A	–	–
2	S33G, A330R, V905M, T1208A	–	–
3	S33G, A330R, V905M, T1208A	–	–
Resistant strains containing res.-related substitutions in TK and polymorphism in DNA pol			
4	S33G, A330R, V905M, P920S, P1124H, T1208A	–	–
5	S33G, A330R, A646T, P1124H, T1208A, R1229I	–	**P829S**
6	S33G, A330R, D672N, V905M	–	–
7	S33G, A330R, V905M, P920S, P1199Q, T1208A	–	**D1126N**
8	S33G, A330R, D672N, V905M	–	–
9	S33G, A330R, V905M, T1208A	–	–
10	S33G, A330R, A646T, P920S, P1124H, T1208A	–	**E662D**, **M1226I**
11	S33G, A330R, V905M, A1203T, T1208A	–	–
12	S33G, A330R, V905M, A1203T, T1208A	–	**E421V**
13	S33G, A330R, V905M, A1203T, T1208A	–	**L49I**
14	S33G, A330R, V905M, P1124H, T1208A	–	–
15	S33G, A330R, V905M, P1124H, T1208A	–	–
16	S33G, A330R, V905M, P1124H, T1208A	–	–
Resistant strains containing res.-related substitutions in DNA pol and polymorphism in TK			
17*	S33G, A330R, V905M, P1124H, T1208A	S724N	–
18	S33G, A330R, P1124H	A719V	–
19	S33G, A330R, V905M, S1123L, P1124H, T1208A	S724N	–
20	S33G, A330R, D672N, V905M	G841S	**I182V**
21	S33G, A330R, G749D, V905M, P920S, P1124H, T1208A	S724N	–
22	S33G, A330R, V905M	S724N	–
23	S33G, A330R, V905M, T1208A	N815S	–
Resistant strains containing novel substitutions in TK and DNA pol			
24	S33G, A330R, V905M, A1203T, T1208A	–	–
25	S33G, A330R, D672N, V905M	–	–
26	S33G, A330R, D672N, V905M, P1124H, T1208A	–	**T1121M**

* Mixed population consisting of wild type and mutated virus.

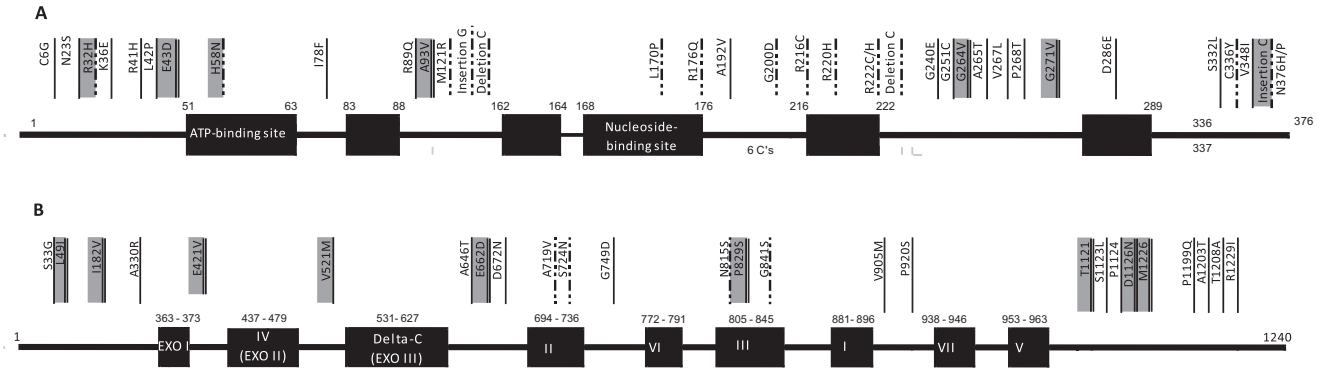

Fig. 1. Polymorphism- (– – –), resistance-associated (- ·· -) and unclear (===) amino acid substitutions and insertions/deletions of nucleotides within *UL23* thymidine kinase gene (A) and *UL30* DNA polymerase gene (B) of 26 clinical HSV-1 isolates from patients with peripheral blood stem cell transplantation in relation to the conserved and active gene regions. Novel substitutions are marked in gray color.

substitutions A93V in the TK and T1121M in the DNA pol whose significance is unknown. Both are localized outside of active or conserved gene centers (Fig. 1).

4. Discussion

Studies are needed to obtain more information about the clinical relevance of mutations in the TK and DNA pol genes of HSV-1, before the analysis of resistance genotypes may result in significant improvements of antiviral treatment, especially among patients after HSCT. Furthermore, the occurrence of cross-resistance is of utmost clinical importance. To date, the best method to verify the role of special mutations for HSV resistance is the correlation of resistance pheno- and genotype of viral isolates. Non-radioactive functional TK assays with the use of recombinant TK proteins, such as mass spectrometry (van Velzen et al., 2012, 2013), high performance liquid chromatography (Malartre et al., 2012), or enzymatic assays measuring TK activity (Burrel et al., 2012; Sauerbrei et al., 2012), which have been reported recently, can show different results which have to be interpreted with caution. The confirmation of putative resistance mutations by the generation of recombinant mutant viruses with a set of overlapping cosmids and plasmids has been described for the HSV-1 TK (Sergerie and Boivin, 2006) and DNA pol genes gene (Bestman-Smith and Boivin, 2003) only in a few studies. Furthermore, assays to examine the functionality of DNA pol in resistant HSV-1 mutants have been rarely reported (Dorsky and Plourde, 1993; Matthews et al., 1989).

This study presents the results of pheno- and genotyping in 26 HSV-1 isolates obtained from patients undergone PBSCT within a time interval of 7 years. One drawback, however, is the limited clinical information regarding antiviral treatment of patients. Only 3 out of these 26 HSV-1 isolates were sensitive to the nucleoside analogs ACV, PCV and BVDU as well as the pyrophosphate analog FOS and the acyclic nucleoside phosphonate CDV. In combination with well-known natural polymorphisms (Bohn et al., 2011; Burrel et al., 2010), the novel amino acid substitution V521M was found in the DNA pol. This substitution is located outside of active or conserved gene centers (Hwang et al., 1992; Matthews et al., 1993). It has to be considered that a resistant mutant clone could have been lost in these sensitive HSV-1 isolates during cell culture passages, because in 5 HSV-1 isolates of this study a mixture of different genotypes of the sensitive wild type and the resistant mutant was found. Therefore, isolates were used exclusively for resistance testing after one to three cell culture passages.

The highest number of HSV-1 isolates (13) was cross-resistant to ACV, PCV and BVDU but sensitive to FOS and CDV. The limited information concerning the antiviral therapy shows that mainly nucleoside analogs were administered in these patients. The resistance to the tested nucleoside analogs ACV, PCV and BVDU was caused by either frameshift or non-synonymous mutations in the TK gene. The frameshift mutations insertion of G nt 429–437 (Sauerbrei et al., 2011), deletion of C nt 460–463 (Sauerbrei et al., 2011) or 665–670 (Sauerbrei et al., 2010) as well as the amino acid substitutions M121R (Sauerbrei et al., 2011), R176Q (Bestman-Smith et al., 2001; Chibo et al., 2004), R216C (Bae et al., 2006), G200D (Sajo et al., 2002; Sauerbrei et al., 2011), R220H (Andrei et al., 2007), R222C/H (Bestman-Smith et al., 2001), and C336Y (Burrel et al., 2010; Sajo et al., 2002) have been reported in the literature to be associated with a resistant phenotype to ACV and partially to PCV and/or BVDU. The substitution L170P located in the nucleoside-binding site of the TK gene has been described by Burrel et al. (2013) and was associated with reduced ACV phosphorylation in an enzymatic assay (Burrel et al., 2012). However, the insertion of C nt 1061–1065 is novel. Studies have shown that this constellation, i.e. resistance to nucleoside analogs caused by mutations in the TK gene, can be observed in the overwhelming majority of resistant HSV-1 strains (Burrel et al., 2010, 2013; Sauerbrei et al., 2010, 2011). It can be assumed that the novel substitutions E43D in the TK as well as L49I, E421V, E662D, P829S, D1126N and M1226I in the DNA pol, which are presumably natural polymorphisms, are not relevant for the nucleoside resistance of these HSV-1 isolates. Concerning E43D and E421V, similar substitutions (E43G, E421D) have been reported as natural polymorphisms (Burrel et al., 2010; van Velzen et al., 2013). However, this assumption must be confirmed especially for P829S located in the conserved region III of DNA pol using functional TK and DNA pol assays.

The most interesting findings of this study were documented for 7 HSV-1 isolates phenotypically resistant to the cyclic nucleoside analogs ACV and PCV as well as, apart from one, to the pyrophosphate analog FOS. Except one, all isolates were sensitive to the acyclic nucleoside analog BVDU and the acyclic nucleoside phosphonate CDV. The strains carried the known resistance-related amino acid substitutions A719V, S724N, N815S and G841S clustering in the conserved regions II or III of DNA pol. The 3 unknown substitutions G264V as well as G271V in the TK and I182V in the DNA pol, all outside of active or conserved gene regions (Balasubramaniam et al., 1990; Graham et al., 1986; Hwang et al., 1992; Matthews et al., 1993) are most likely not relevant for any resistance. The substitution N815S has been described by Larder et al. (1987) and Matthews et al. (1989,

1993) to confer resistance to ACV and susceptibility to FOS by using recombinant virus and enzymatic assay, respectively. Whereas Larder et al. (1987) also reported ACV/FOS-cross-resistant HSV *in vitro* mutants with the substitutions A719V, S724N and G841S, the changes A719V and G841S were detected in clinical HSV-1 isolates resistant to ACV, PCV and BVDU but sensitive to FOS and CDV in a recently published study (Sauerbrei et al., 2011). The resistance to BVDU in the isolate with the A719V substitution, in contrast to the isolate No. 18 of this study, may be explained by the additional frameshift mutation (deletion of A nt 1065) in the TK gene. Using selective pressure of FOS, Andrei et al. (2005) produced *in vitro* mutants from the HSV-1 strain KOS exhibiting the substitutions A719V or S724N. The mutant clones were resistant to ACV and FOS but sensitive to BVDU and CDV. Here, we describe the first clinical HSV-1 isolates with this resistant phenotype (ACV/FOS resistant and BVDU/CDV sensitive). Unfortunately, there is no information available about the antiviral compounds administered in these patients. On the basis of the results obtained by Andrei et al. (2005), these patients might have been treated with FOS over an extended period. The findings of this study suggest that clinical ACV-resistant HSV-1 strains, whose resistance is caused by mutations in the DNA pol gene, are likely cross-resistant to FOS but sensitive to brivudin. This fact offers an alternative antiviral treatment of HSV-1 infections using brivudin in case of ACV/FOS resistance. However, resistance testing is necessary, because this phenotype cannot be predicted.

The data discussed are underlined by the findings of additional 3 HSV-1 isolates summarized in group 4. Two isolates, resistant to ACV, PCV and BVDU but sensitive to FOS and CDV, had the novel most likely resistance-related amino acid changes H58N and R32H in the TK. The resistance pattern is similar to that of group 2. Concerning H58N, a substitution at the same position (H58L) has been shown to impair drastically ACV phosphorylation (Pilger et al., 1999). However, the resistance association of R32H has to be confirmed by functional TK assays using recombinant proteins since code variations of the first 46 amino acids do usually not affect the phosphorylation activity of the TK (Haarr et al., 1985; Saijo et al., 2008). Additionally, the substitution R32C has been previously reported to be a natural polymorphism (Bohn et al., 2011; Frobert et al., 2008; Sauerbrei et al., 2011). The remaining strain, only resistant to ACV/PCV, but sensitive to BVDU, FOS and CDV, comprised the substitutions A93V in the TK and T1121M in the DNA pol, which are localized outside of active or conserved gene centers. The significance of these substitutions has not been clarified to date. According to the results of the strains of groups 2 and 3, the resistance is most likely caused by the DNA pol substitution T1121M, whereas the TK substitution A93V may reflect natural gene polymorphism. Recently, A93V has been reported in 3 ACV-resistant HSV-1 isolates, but the resistance association remained unclear (Karaba et al., 2012).

In conclusion, the present study demonstrates that the correlation of results of pheno- and genotypic resistance testing in clinical HSV-1 isolates obtained from immunocompromised patients provides essential data for alternative antiviral treatment of severe HSV-1 infections.

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