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Resistance testing of clinical herpes simplex virus type 2 isolates collected over 4 decades

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ABSTRACT

There is only little information about the role of mutations of the thymidine kinase (TK) and DNA polymerase (pol) genes of herpes simplex virus type 2 (HSV-2) for the development of antiviral resistance. In this study, the polymorphism of TK and DNA pol genes was examined in 82 clinical isolates collected routinely between 1973 and 2013. If novel, presently unclear or resistance-related mutations were found, the resistance phenotype against acyclovir (ACV) and foscarnet (FOS) was analyzed. The four novel amino acid changes G150D, A157T, R248W, L342W and the hitherto phenotypically unclear substitution T131M within the TK gene were identified as natural polymorphisms. Within the DNA pol gene, 17 novel substitutions and the to-date unclear change R628C were characterized as part of natural gene polymorphism. Two novel DNA pol mutations were linked to resistance (M910T) and weak susceptibility to ACV (684 insertion ED), respectively. In one isolate, the genomic cause of ACV resistance could not be identified. Phylogenetic analysis including sequences of this study and of the GenBank revealed a hierarchy of mutation clusters in TK displaying G39E as first common mutation step, followed by N78D and L140F. In conclusion, the present findings allow a deeper insight in the variability of HSV-2 TK and DNA pol genes. The most common substitution G39E can be excluded as unique cause of HSV-2 resistance. This study supports once more the importance of phenotypic adjustment of genotypic results to enhance the clinical utility of genotypic findings.

Introduction

Herpes simplex virus type 2 (HSV-2) is the most frequent virus found in genital herpes and, therefore, responsible for one of the most prevalent sexually transmitted diseases worldwide. The standard treatment of symptomatic primary and recurrent HSV-2 infections is the administration of acyclovir (ACV), an acyclic analog of guanosine. The inactive form is phosphorylated to monophosphate by the viral thymidine kinase (TK), encoded by the *UL23* gene and then converted by cellular kinases to the ACV di- and triphosphate. Acyclovir triphosphate acts as a competitive inhibitor of the viral DNA polymerase (pol) encoded by the *UL30* gene and if incorporated into the nascent DNA chain induces a stop of the viral genome replication by chain termination (Gilbert et al., 2002). A previous study has shown that a daily antiviral therapy with ACV may reduce the rates of clinical and subclinical HSV-2 reactivations (Corey et al., 2004). In addition, it minimizes the risk of virus transmission to uninfected partners by 50%. In case of ACV resistance, tri-sodium-phosphono-formate (foscarnet, FOS), a direct inhibitor of the HSV-2 DNA pol has been recommended for alternative treatment (Sauerbrei et al., 2011). Due to different mode of action, previous reports could show that infections caused by ACV-resistant HSV-2 strains can be treated successfully with FOS (Alvarez-McLeod et al., 1999; Jones and Paul, 1995; Khurana et al., 2005). Possibly, the novel helicase–primase inhibitor pritelivir may improve significantly the treatment of ACV-resistant HSV infections in future (Wald et al., 2014). The TK and DNA pol genes of HSV-2 exhibit a high natural polymorphism, which, however, is lower than the diversity of the corresponding HSV-1 genes (Bohn et al., 2011; Sauerbrei et al., 2011, 2010). As a natural phenomenon, spontaneous mutations in the absence of drug selection may result in the accumulation of approximately six to eight TK-deficient variants per 10,000 plaque-forming viruses in the whole population (DasGupta and Summers, 1978; Duffy et al., 2002; Hall et al., 1984).

Under drug-induced selective pressure, clinical HSV-2 strains show a high frequency of spontaneous resistance-associated mutations toward ACV especially in genital herpes. Hence, resistant

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Table 1
Periods of time during which 82 HSV-2 strains were collected.

Periods of time	Number of strains
1973–1978	5
1979–1984	8
1985–1990	4
1991–1996	11
1997–2002	26
2003–2008	8
2009–2013	20
Total	82

isolates have been described in up to 5% of the cases (Danve-Szatanek et al., 2004; Fife et al., 1994; Reyes et al., 2003). The majority of mutations related to resistance are localized in the TK gene (Duffy et al., 2002). Furthermore, mutations within the viral DNA pol gene may affect spontaneously the viral mutation rates (Hall et al., 1984). Additionally, the immunocompetence of the patient has a major influence on the development of ACV resistance. In immunocompromised individuals, the viral replication is not disrupted because of the weakened immune response. Thus, natural spontaneous mutations occur more often and resistant or less pathogenic viruses that would be eliminated by normal immune response survive under antiviral treatment (Piret and Boivin, 2011).

The laboratory identification of resistance to antiviral drugs needs rapid, reliable and standardized methods. Conventionally, analysis of HSV resistance against antiviral drugs is carried out by the time-consuming determination of the viral phenotype allowing a clear interpretation of experimental findings. Genotypic analysis by sequencing of TK and DNA pol genes has been established stepwise as the method of choice. The advantage is that HSV mutants can be characterized within short time intervals and without the need to grow the virus in cell culture. Thus, the rapid detection of drug resistance can improve significantly the treatment of infections with HSV-2. However, studies are needed to verify the role of substitutions in the TK and DNA pol genes for the development of antiviral resistance.

The objective of the present study was to examine the genotype of the TK and DNA pol of 82 unselected clinical HSV-2 strains, which were isolated over a time period of 40 years. In case of novel, unclear or resistance-related mutations, the phenotype to ACV and FOS was determined. It was expected that the results would allow deeper insights in the variability of these genes. Additionally, phylogenetic analysis of TK and DNA pol genes was performed to reveal changes in the rates and patterns of mutations.

Materials and methods

Patients and viral strains

In this study, 82 clinical HSV-2 isolates from 75 different patients were included to examine the TK and the DNA pol genes for non-synonymous mutations. In five cases, two strains each were sampled from one patient, and in one case, three strains were obtained. The viruses were isolated between 1973 and 2013 (Table 1) from patients with the following clinical diagnoses: recurrent herpes genitalis ($n = 14$), herpes integumentalis ($n = 12$), herpes glutealis ($n = 10$), herpes progenitalis ($n = 8$), herpes zoster ($n = 3$), herpes facialis ($n = 1$), eczema herpeticatum ($n = 1$), herpes simplex meningitis ($n = 1$), and herpes simplex encephalitis ($n = 1$). In 24 patients, the clinical diagnosis was unknown. Patients' specimens were sent to the German Consulting Laboratory for HSV and VZV for virological diagnostics. The age of 57 patients ranged between 17 and 80 years (mean: 44.7 ± 16.4 years; median: 41.5 ± 16.2 years). One neonate was 12 days, another baby eight months old, and the age of 16 patients was unknown. Samples were collected from 39 female and 17 male patients. There was no information on the gender of 19 patients. Six patients were immunocompromised (Table 2: 06-95, 625-98, 464-00, 2688-08, 267-09, 13-13/14-13) because of transplantation or iatrogenic immunosuppression. For 69 patients, information about the immunocompetence was not available. A clinical resistance to ACV was diagnosed in one patient from whom two samples (Table 2: 13-13 and 14-13) had been collected. Resistance was assumed because of the absence of clinical improvement of HSV disease under administration of the drug for at least 10 days (Balfour et al., 1994). For 74 patients, there were no data about antiviral treatment. Herpes simplex virus type 2 was identified in all cases by diagnostic polymerase chain reaction (PCR) using primers specific for the TK gene (Sauerbrei et al., 2000). All viral strains were isolated and titrated in human embryonic lung fibroblasts or African green monkey kidney Vero 76 cells (ATCC, CRL 1587). The method of viral growth has been described previously (Sauerbrei et al., 2010). Viral stocks were obtained when the titer reached between 10^6 and 10^8 tissue culture infective doses 50% per ml and then stored at −80 °C until use.

Genotypic analysis

For genotypic analysis of resistance, the viral DNA of cell culture isolates was obtained from 200 μl of viral stocks using QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany). The TK gene of all isolates was amplified as single fragment with a size of 1300 base pairs (bp) whereas the DNA pol gene had to be separated into four fragments as previously reported (Bohn et al., 2011). Configuration of primers for amplification and sequencing of TK and DNA pol genes was based on the reference strain HSV-2 HG 52 (GenBank ID: Z86099.2) as described previously (Bohn et al., 2011). In addition, the novel primers Pol-1d (5′-ATC CCA CCC CGA GCT GTT GG-3′) and Pol-r4b (5′-CAC ATG AGC TTG TAG GCC G-3′) were used to improve amplification and sequencing of the first DNA pol fragment. All amplified fragments contained a high concentration of HSV-2 DNA (50–200 ng). Standard stock PCR mixture contained Q-Solution, 5 × PCR buffer, 10 mM dNTP mix, 10 μM forward as well as reverse primers and was diluted in a final volume of 50 μl. Before amplification, 250 ng template DNA and 1.25 U of the HiFidelity HotStar Taq polymerase (Qiagen) were added. Amplification conditions included an initial denaturation step for 5 min at 95 °C followed by 45 cycles of 15 s at 94 °C, 60 s at 60 °C, 3 min at 68 °C and a final extension step of 10 min at 72 °C. All amplified samples were purified using QIAquick PCR Purification Kit (Qiagen) and DNA fragments were sequenced by Eurofins MWG Operons (Ebersberg, Germany). Finally, the alignment of sequence data and the comparison with the published sequence of reference strain HG 52 (GenBank ID: Z86099.2) was performed using the software MEGA 5.2. All specified nucleotide (nt) sequences corresponded to nt positions in this reference strain.

Phenotypic analysis

Twenty-two viral isolates, which contained unknown or resistance-related non-synonymous mutations in the TK and/or DNA pol genes, were tested for sensitivity to ACV (GlaxoSmithKline, Uxbridge, UK). Additionally, two isolates (Table 2: 06-95 and 625-98) from immunocompromised patients and with the single TK substitution G39E were also tested for resistance phenotype to ACV. Finally, three strains with hitherto unclear phenotype (169-99 with TK substitution T131M; 436-10 and 437-10 with R628C in DNA pol gene) as well as two isolates (13-13, 14-13) collected sequentially from one patient with clinical resistance to ACV were also tested against ACV. Twenty-one isolates with novel or unclear substitutions in the DNA pol were analyzed for resistance phenotype to FOS (AstraZeneca, Wilmslow, UK). All phenotypic assays were carried