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Herpes simplex virus resistant to acyclovir: A single-centre experience from the Czech Republic

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ABSTRACT

Introduction and aim: Infections caused by herpes simplex viruses (HSV) are frequent in the human population. Because of the widespread use of long-term treatment or prophylaxis by anti-herpetic antivirals in various specific medical contexts (immunosuppression, recurrent infections), the level of antiviral resistance is increasing. According to previous studies, there is a low resistance level in immunocompetent populations but a relatively high level in populations with immunodeficiency. However, there has been no study from the Czech Republic. This study presents results of a single-centre retrospective study from the Czech Republic.

Materials and methods: Deep frozen DNA from patients with suspected clinical antiviral failure over a long time period (2009–2016) – a total of 15 isolates of HSV1 and seven of HSV2 – were examined for the presence of mutations associated with antiviral resistance. Sequence analysis was performed using an ABI PRISM 3500xL Genetic Analyzer (Applied Biosystems®).

Results: There were no mutations associated with resistance to antivirals inside the UL23 gene in HSV1 isolates. However, resistant mutation D672N (nucleotide change G2014A) was found inside the UL30 gene in seven of the isolates. One mutation associated with resistance to acyclovir (M183stop) was found inside the UL23 gene in one HSV2 isolate. Resistant mutation E678 G (nucleotide change A2033G) was identified inside the UL30 gene in six of the HSV2 isolates.

Conclusions: This study confirmed the presence of resistance mutations within the Czech population, but it will be necessary to examine a higher number of isolates for further conclusions.

1. Introduction

Human herpetic infections have been known since antiquity, but the first in vitro cultivation was performed in 1925 [1]. In 1968, two different types were distinguished: herpes simplex virus type 1 (HSV1) and herpes simplex virus type 2 (HSV2), with different clinical manifestations and tropism [2]. Despite high seroprevalence in the population, HSV infections mostly run a severe course only in cases of patients with immunosuppression [3].

Herpes simplex virus resistance mechanisms, by which the virus blocks the effect of antiherpetics, were first mentioned relatively soon after the introduction of acyclovir (ACV) into routine treatment. One of the mechanisms of resistance, a mutation causing non-functioning or malfunctioning of the gene UL23 for the production of HSV thymidine kinase, has been known since 1987. In the case of resistance due to mutation in the UL23 gene, ACV can be replaced with other antivirals such as foscarnet (FOS) or cidofovir (CDV). Later, a second important mechanism of resistance to ACV was discovered, a mutation in the gene for herpes virus DNA polymerase (UL30 gene). This pattern confers resistance to FOS, and thus eliminates ACV and FOS from therapy [4].

A total of six conserved regions have been identified in the UL23 gene. Changes in the regions called the 'ATP-binding site' (the part of the TK enzyme binding ATP), the 'nucleoside-binding site' (the part of the TK enzyme binding nucleosides), and Cys336 (HSV1) and Cys337 (HSV2) are hot spots for formation of ACV-resistant mutants [4,5]. According to earlier studies, the mutations leading to ACV resistance were in about 50% of cases associated with deletion/insertion (a frame shift

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mutation), and in about 50% of cases with a single amino acid substitution. According to recent studies, a deletion/insertion [4,6] prevails in 62–80% of cases. To date, 134 (for HSV1) and 72 (for HSV2) UL23 mutations associated with resistance to antiherpetics have been described. The number of natural polymorphisms (i.e. gene sequence changes) not related to resistance is also very high: 109 for HSV1 and 23 for HSV2 [7].

Mutations in the viral polymerase gene (UL30) are less frequent but of great importance because the mutations are very often associated with resistance to other antiherpetics interfering with viral polymerase activity [5,7]. Mutations associated with resistance to ACV and its ester derivatives are mostly described in regions II, III and VI, and more rarely in δ-region C. Resistance to FOS is similarly often described in regions II, III and VI, and less often in the non-conserved region between regions VI and I. Therefore, mutations in the common regions (mostly II and VI) can cause combined resistance to both ACV and FOS. The mutations S724N (region II) and L778M (region VI) are then associated with resistance to ACV (and its ester derivatives) and FOS, and decreased sensitivity to CDV [5,7]. Unlike mutations in the UL23 gene, there are mostly single amino acid substitutions in the UL30 gene, and less often insertion/deletion [5]. A total of 64 (HSV1) and 18 (HSV2), mutations associated with resistance to antiherpetics have been described. Natural polymorphisms with no relationship to resistance have been more frequent: 205 for HSV1 and 78 for HSV2 [7].

Even though ACV resistance in the immunocompetent population is very low (<1%) [8], it is already about 6% in human immunodeficiency virus (HIV)-positive patients [9]. Similar results have also been described in other studies: the prevalence of ACV resistance varied from 0.1–0.7% in immunocompetent individuals. In the case of HIV-positive patients, however, this prevalence was 3.5–7.0%, and the highest resistance level was postulated in patients after stem cell transplantation: 4.1–10.9% [4].

In the Czech Republic, data on resistance are sparse. In work from authors dealing with the treatment of eye infections caused by HSV1, treatment failure was described in 14% of patients, albeit without confirmation of genotyping or EC_{50} determination [10]. The main aim of this retrospective study analysing the association of relevant mutations and antiviral resistance in the Czech Republic was to verify the presence of HSV-resistant isolates in the Czech Republic.

2. Materials and methods

2.1. Materials

This study used various samples from patients hospitalised in the University Hospital in Hradec Kralove, Czech Republic in the 8 years from 2009–2016. The samples were gathered from patients examined for HSV1 or HSV2 viral load in various areas of the body. Samples were selected exclusively from patients with an unusual reaction to antiviral therapy (no clinical or laboratory improvement after 7 days of therapy) or with unusual clinical signs (generalised infection or repeated/recurrent infection). Fifteen isolates of HSV1 were collected (10 isolates from buccal or lesion swabs, three from the lower respiratory tract, one from blood, and one from cerebrospinal fluid), mostly from patients in intensive care units (n = 7) or standard wards (n = 5); seven isolates of HSV2 were collected (four isolates from lesion swabs, two from cerebrospinal fluid, and one from blood) from outpatients (n = 4) and patients in standard hospital wards (n = 3).

The history of any known previous ACV treatment before sample collection is shown in Tables 1 and 2. In general, outpatients had mostly been treated with topical antiherpetics and there was no information on therapy length in this patient group. Hospitalised patients with severe infections such as meningitis or herpetic pneumonia were under treatment with ACV, according to the Summary of Product Characteristics. The minimum treatment length was 10 days in this patient group.

After viral load examination, all DNA isolate samples that fitted the criteria were deep frozen at −80 °C until analysed for detection of resistance. All DNA isolates were obtained using QIAamp™ DNA Mini Kit (QIAgen®, Germany) or MagCore Viral Nucleic Acid Extraction Kit (MagCore®, UK) according to the manufacturer's instructions. The viral load was from 8.6×10^3 copies/mL to 2.07×10^8 copies/mL. The commercial kit GeneProof Herpes Simplex Virus (HSV-1/2) PCR Kit (GeneProof®, Czech Republic)

Table 1
Results of UL23 and UL30 HSV1 Sanger sequencing, with basic information about samples.

Patient number	Sample collected	Sample type	Previous acyclovir treatment	UL23		UL30	
				Resistance mutation	Polymorphism without resistance	Resistance mutation	Polymorphism without resistance
1	2016	BS	LT	NO	NO	D672N	T566A, P920S, A1208T
2	2016	LS	NO	NO	NO	D672N	T566A, A1208T
3	2016	LS	LT	NO	Q89R	NO	N425T, T566A, M905V
4	2013	LS	1 W SC	NO	Q89R, A192V, G251C, V267LP268T, D286E	NO	T566A
5	2013	LS	LT	NO	G240E	NO	T566A
6	2013	LS	NO	NO	NO	NO	T566A
7	2009	TA	17 D SC	NO	Q89R, G240E, V267L, P268T, D286E	D672N	A562T, T566A, A1208T
8	2009	LS	NA	NO	NO	D672N	T566A, A1208T
9	2012	BL	NO	NO	NO	D672N	T566A, P920S, A1208T
10	2009	BA	7 D SC	NO	Q89R, G240E, V267L, P268T, D286E	D672N	A562T, T566A, E688K, E1085K, A1208T
11	2013	BS	NO	NO	Q89R	NO	T566A
12	2009	CF	NA	NO	Q89R, A192V, D215A, G251C, P268T, D286E	NO	T566A
13	2012	BA	NO	NO	NO	NO	T566A, A1203T
14	2016	LS	LT	NO	V348I	NO	T566A, N425T, P875S
15	2016	LS	LT	NO	Q89R	D672N	T566A, E688K

Abbreviations: BS, buccal swab; LS, lesion swab; TA, tracheal aspirate; BL, blood; BA, bronchoalveolar lavage; CF, cerebrospinal fluid; NA, previous treatment by acyclovir is unknown; SP, standard prophylaxis dosing; SC, standard curative dosing; LT, local treatment with topical acyclovir; D, day; W, week; M, month.

Table 2
Results of UL23 and UL30 HSV2 Sanger sequencing, with basic information about samples.

Patient number	Sample collected	Sample type	Previous acyclovir treatment	UL23		UL30	
				Resistance mutation	Polymorphism without resistance	Resistance mutation	Polymorphism without resistance
1	2012	LS	NA	NO	P31R, G39E, N78D, L140F	E678G	NO
2	2013	CF	SC	NO	G39E, N78D,	E678G	NO
3	2013	LS	NA	NO	G39E, N78D, L140F	E678G	NO
4	2013	LS	NO	NO	G39E, N78D, L140F	E678G	NO
5	2016	CF	14 D SC	NO	G39E, N78D, L140F	E678G	NO
6	2017	LS	LT, SC	NO	NO	NO	NO
7	2017	BL	SP 12 M, [a]	M183Stop	L140F	E678G	NO

Abbreviations: BS, buccal swab; LS, lesion swab; TA, tracheal aspirate; BL, blood; BA, bronchoalveolar lavage; CF, cerebrospinal fluid; NA, previous treatment by acyclovir is unknown; SP, standard prophylaxis dosing; SC, standard curative dosing; LT, local treatment with topical acyclovir; D, day; W, week; M, month.
[a] Patient treated by foscarnet, repeatedly.

was used for HSV 1/2 diagnostic and viral load examination. The detection limits for used PCR method were 122 copy/mL for HSV1 and 194 copy/mL for HSV2.

The entire study design was in accordance with the Declaration of Helsinki Protocol, and the Medical Ethics Committee of the University Hospital in Hradec Kralove approved the study design (Opinion of the Ethics Committee No. 201707 S03 P). All the participants gave written informed consent before integration into the study.

2.2. Sequencing design

Identification of areas to be sequenced was based on literature review [7,8,11,12]. Parts of the UL23 and UL30 genes have been identified where the changes associated with the development of resistance to antiherpetics have most frequently been reported to date. For HSV1, the UL23 and UL30 genes are composed of linear DNA of 1131 and 3708 bp. The segments of the genes that were selected as the most variable with respect to resistance were identified as an area with length approximately 1050 bp for UL23 and 2700 for UL30. For HSV2, the UL23 and UL30 genes are composed of 1195 and 3762 bp linear DNA. Selected sections for current sequencing were an area approximately 1100 bp for UL23 and two segments for UL30 of 600 bp and 1100 bp.

2.3. Sanger sequencing

This study designed its own Sanger sequencing method, which was able to include all chosen areas. It was necessary to divide the sections into several parts to amplify such long areas. They were then individually sequenced. For HSV1, the UL23 gene segment was divided into one fragment (during protocol evaluations a variant with division into two fragments was also tested), and the UL30 gene was separated into five fragments. For HSV2, two fragments were needed for UL23 and three fragments for UL30. Sets of primers were designed to amplify the required regions (Table 3). The amplification products were purified by magnetic particles on the Biomek 4000 device and sequenced using the BigDye Terminator v3.1 (Applied Biosystems) kit and ABI PRISM 3500xL Genetic Analyzer (Applied Biosystems). The acquired data were analysed with Seqscape v3.0 (Applied Biosystems) software. The Sanger sequencing method design for HSV1/2 resistance mutation identification was verified by the international quality control organisation QCMD (panel HSV-DR) with full success. The average sample quality score was generated from quality values for each base evaluated by the KB Basecaller (DNA Sequencing Analysis Software, version 6, Applied Biosystems).

Table 3
Selected fragments of genes UL23 and UL30 used for amplification before sequence analysis.

HSV1 Gene	Region	Primer	Product length
UL23	A	R101/F594	494 bp
	B	R576/F1130	555 bp
or			
UL23	A + B	R101/F1130	1030 bp
UL30	A	F863/R1838	976 bp
	A/B	F1529/R2130	601 bp
	B	F1819/R2801	983 bp
	C	F2505/R3262	758 bp
	D	F2919/R3262	1100 bp
HSV2 Gene	**Region**	**Primer**	**Product length**
UL23	1	F36/R740	705 bp
	2	F370/R1098	728 bp
UL30	1	F603/R1188	586 bp
	2	F1759/R2284	526 bp
	3	F2260/R2816	557 bp

2.4. Evaluation of sequencing results

Free on-line mutation resistance analyser software (available on web site: https://www.informatik.uni-ulm.de/ni/mitarbeiter/HKestler/mra/app/index.php?plugin=form) (Ulm University, Germany) was used to evaluate HSV1 results. As there is no such software for HSV2, all sequencing results were aligned to sequence without mutations by ClustalW application and all mutations found this way were manually annotated, and mutations associated with resistance were identified.

3. Results

3.1. HSV1

The discovered mutations were divided into two groups: 1) consisting of so-called natural polymorphisms not associated with resistance to antivirals; and 2) consisting of genome changes associated with antiviral resistance. Natural polymorphisms were relatively frequent in the isolates. Six isolates were detected without mutation in UL23 and four with multiple mutations in UL23 (the most frequent amino acid changes being Q89R, V267 L and P268 T). All isolates had genetic polymorphisms in UL30 (the most frequent being A562 T, T566A and A1208 T). Complete information about natural polymorphisms can be seen in Table 1.

No mutations associated with antiviral resistance were observed in UL23. Such mutations were detected in UL30 in seven isolates (46.7%), and the same mutation D672 N (nucleotide change G2014A) was detected in all these samples. This mutation was identified by mutation resistance analyser software as a mutation associated with resistance to ACV, penciclovir (PEN), FOS, and brivudine, and susceptibility to CDV [7].

3.2. HSV2

As with HSV1, frequent natural polymorphisms were found in the UL23 gene in six of the isolates. The most frequent amino acid changes were G39E, N78D and L140 F. So-called silent mutations (nucleotide changes without changes in amino acids) in the UL30 gene were detected in four isolates. One mutation in the UL23 gene associated with resistance to ACV was found in one isolate. This mutation is called M183stop and is associated with resistance to ACV, PEN and brivudine, but with retained susceptibility to FOS and CDV [7]. This result was in concordance with results from the HSV2 isolates of the same patients from the laboratory of University Hospital La Pitié-Salpêtrière-Charles Foix (Paris, France), where suspicious samples were examined before this study developed its own method. Six isolates (85.7%) were positive for resistance mutation in the UL30 gene. The E678G mutation (nucleotide change A2033G) associated with resistance to ACV and retained susceptibility to FOS was found in all of these six isolates [7]. Complete information about all found mutations can be seen in Table 2.

No correlation was found between previous ACV treatment and resistant mutations in either HSV1 or HSV2. The resistant isolates were from patients with per oral/intravenous or topical ACV administration as well as from patients without previous ACV treatment.

4. Discussion

Resistance of HSV to ACV leads to the highest mortality in cases of severe infection. According to a Japanese study of patients after haematopoietic stem-cell transplantation, 15% of patients developed HSV1 infection within 100 days after transplantation, and the authors reported that 28% of these HSV1 infections were resistant to ACV. The authors also reported that the 100-day death rate was significantly higher in patients with ACV-resistant HSV1 than those with susceptible HSV1 (64% vs. 39%) [13]. The importance of HSV resistance evaluation is supported by new publications, which show increasing evidence of resistant isolates. A large French study of 1425 patients showed an increase in the level of resistance in the immunocompromised population over 10 years from 3.8% (samples obtained from 2002–2006) to 15.7% (samples collected from 2007–2011) [8]. Another recent publication from Japanese authors presents the first described breakthrough infection of HSV1 despite the use of ACV prophylaxis in a patient after haematopoietic stem cell transplantation with T-cell depletion. Infection caused by resistant HSV1 isolates had developed by day 20, with further (secondary) lesions by day 33 [14]. Furthermore, according to these data, methods of detection of HSV mutations causing antiviral-resistant infections will be necessary for effective treatment of HSV infections.

Phenotypic and genotypic methods can be used to detect resistance. The first group includes cultivation-based methods and determination of IC_{50} (the concentration for 50% inhibition) by plaque reduction assay. This is considered to be the gold standard. The method is rather labour-intensive and time-consuming and requires infectious viral particles in the sample of biological material (HSV cultivation is thus impossible from the blood of a seropositive patient). These methods are thus unsuitable for routine use, but serve as confirmation of genetic analysis. Accordingly, genetic diagnostics of resistance to antivirals are of paramount importance. The current main method for resistance detection is genome sequencing. Sequencing methods have the potential to capture new polymorphisms associated with resistance that are not detectable by previous methods [7]. Other experimental methods used at the boundary between phenotype and genotype methods are the use of mass spectrometry or high-performance liquid chromatography, in which the virus is cultured in the presence of the antiviral and the subsequent degradation product is detected. This product is different in the cases of resistant virus and susceptible virus [15,16]. There are two workplaces in the Czech Republic capable of routinely detecting resistant HSV1 and HSV2, namely: the National Reference Laboratory for Herpes Viruses (the method of plaque reduction assay and Sanger sequencing) and the University Hospital in Hradec Kralove (both Sanger sequencing and massive parallel sequencing).

The current findings confirmed the occurrence of HSV isolates resistant to antiherpetics in the Czech Republic, both for HSV1 and HSV2. The high numbers of resistant isolates from this study were caused by the examination of pre-selected isolates from patients with a suspicion of clinical resistance. A very interesting finding was the presence of the same resistance profile in all positive samples, both HSV1 and HSV2. The most frequent mutations associated with resistance to antivirals have predominantly been published in the UL23 gene rather than in the UL30 gene (about 95% vs. 5%) [4,5,7,8]. Therefore, it was very interesting that the resistant mutation from current isolates was predominately in the UL30 gene, which is strictly in contrast with literature data. One mutation was found in the UL23 gene; the reason for this discrepancy is unknown. All positive samples were over a long time period and from both intensive care unit and outpatient departments, and there were no epidemiological connections found among any positive results during the epidemiological survey. These findings confirmed the presence of resistant HSV mutants in the population, and not only in immunosuppressed patients.

The mutations associated with resistance to antivirals from the current isolates have previously been well described. Sauerbrei et al. first described mutation D672 N of the HSV1 isolates in 2010 [17]. This mutation was located outside of the conserved regions of the HSV viral polymerase gene and the resistance was evaluated for ACV, PEN, FOS, and brivudine [7]. The mutation E678G of the HSV2 isolates was first described in 2000 by Sarinsky et al. and in 2004 by Chibo et al. [18,19]. The mutation is located in non-conserved regions of the HSV2 DNA polymerase gene and associated with resistance to ACV [7]. The other discovered mutation is M183stop. It is located in a non-conserved region of the HSV2 thymidine kinase gene; it was first described in 2001 by Sarisky et al. [20]. This mutation was associated with resistance to ACV, brivudine and PEN [7].

The correlation between the ACV dosing regimen and development of ACV resistance in the case of HSV is not as clear as in the treatment of human cytomegalovirus with ganciclovir. According to several studies, prophylactic application of ACV did not cause the increased level of HSV resistance [21,22]. On the other hand, there have been reports that ACV prophylaxis may lead to increased ACV resistance [23], or that prophylactic use could select the resistant mutant [14]. Some studies have also presented that some resistant mutations did not need the selection pressure of pre-applied ACV [7,20,24]. The current study also confirmed these data because it found the mutation in patients both after previous treatment with ACV and also without previous treatment.

The current study confirmed relatively high genetic variability in both genes (UL23 and UL30), as evidenced by the high number of

polymorphisms, more of which were found in HSV1 isolates than in HSV2 isolates. The same results have been discussed in previous publications [7,24]. The observation that some polymorphisms seem to be frequent in the majority of current isolates is very interesting. Although very similar results have also been published in German and French studies [8,21], the polymorphisms in the current study were different. This finding suggests the possibility of population/region-specific polymorphisms. However, the relatively low number of isolates in the current study may account for the different findings. The current study only evaluated the presence of known mutations associated with resistance to antivirals. In the absence of phenotypic testing it was not possible to differentiate non-annotated natural and antiviral resistance-associated polymorphisms.

This study had some limitations. Since it only used stored DNA isolates, phenotypic methods could not be used for confirmation of sequencing results. The number of isolates was too low to assert a definite conclusion about the resistance level in the Czech population.

5. Conclusions

These results confirmed the existence of antiviral resistance polymorphisms in isolates of both HSV1 and HSV2 in the Czech Republic, suggesting antiviral resistance in these isolates. However, the study was not prospective and further analyses in a larger patient cohort are necessary for broader validation of the conclusions.

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Competing interests

The authors declare that they have no competing interests.

Ethical approval

The medical ethics committee of the University Hospital in Hradec Kralove approved the study design (Opinion of the Ethics Committee No. 201707 S03P)

Authors' contributions

Fajfr M - designed the research, defined the research aim, performed the research, analysed the data and interpreted the results; Pliskova L - performed the research (laboratory work), analysed the data and interpreted the results; Bolehovská R - performed the research (laboratory work), analysed the data and interpreted the results; Uhlířová Z - performed the research (laboratory work); Vrbacký F - analysed the data and interpreted the results.

All authors contributed to the writing of the manuscript and have read and approved the final version.

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