Antiviral Research 90 (2011) 242–247

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Resistance testing of clinical varicella-zoster virus strains

Andreas Sauerbrei *, Johanna Taut, Roland Zell, Peter Wutzler

Institute of Virology and Antiviral Therapy, German Reference Laboratory for HSV and VZV, Jena University Hospital, Jena, Germany

ARTICLE INFO

Article history:
Received 7 March 2011
Revised 15 April 2011
Accepted 19 April 2011
Available online 23 April 2011

Keywords:
Varicella-zoster virus
Phenotypic resistance
Genotypic resistance
Thymidine kinase
DNA polymerase

ABSTRACT

Acyclovir resistance of varicella-zoster virus (VZV) has been reported in rare cases of immunocompromised patients. In this study, the natural polymorphism of the thymidine kinase (TK) and DNA polymerase (pol) genes was examined in 51 clinical VZV isolates sensitive to acyclovir (ACV). In addition, 16 VZV strains with clinical resistance to ACV were analyzed. None of the ACV-sensitive strains of the clades 1, 3 and 5 showed gene polymorphism of the TK. By contrast, the DNA pol gene exhibited polymorphism-related substitutions as a function of the VZV clade. The novel substitutions M286I, E824Q, R984H and H1089Y were detected in strains of clades 3 and 5. In the TK gene of 7 VZV strains with clinical ACV resistance, the novel substitutions L73I, A163stop, W225R, T256M, N334stop and the deletion of nucleotides 19–223 were found to be associated most likely with resistance. In one strain showing the substitution W225R, ACV resistance could be confirmed by the viral phenotype. In the DNA pol gene, the novel amino acid substitutions T237K and A955T could be detected, but their significance remains unclear. In conclusion, the characterization of resistance using genetic analysis of the TK and DNA pol genes has to be considered the method of choice for the determination of VZV resistance to antiviral drugs. In a considerable number of patients with clinical ACV-resistant VZV infections, resistance cannot be verified by virological methods.

1. Introduction

Varicella-zoster virus (VZV) is a member of the Herpesviridae family and is divided into the five major clades 1–5 (Breuer et al., 2010) showing a distinctive geographic distribution in temperate versus tropical regions (Loparev et al., 2007; Quinlivan et al., 2002). VZV causes two distinct diseases. During childhood, primary infection results in typical signs of varicella, and zoster is caused by endogenous reactivation after the virus has established lifelong latency. The occurrence of zoster is thought to be associated with waning VZV-specific T-cell-mediated immunity (Gershon et al., 1997), e.g. in the elderly or in patients with immunodeficiency. While complications of varicella are rarely observed in immunocompetent infants, patients with impaired cellular immune function, e.g. patients with oncological diseases, organ or bone marrow transplantation, autoimmunopathies, congenital immune defects or persons infected with the human immunodeficiency virus, have a special risk of severe courses of varicella (Arvin, 1999). Varicella pneumonia has been considered the most important complication in pregnant women. Furthermore, maternal infection during the first two trimesters or near term can be associated with a substantial risk of intrauterine infection or neonatal varicella (Sauerbrei and Wutzler, 2007). Zoster is often complicated by pain termed as post herpetic neuralgia if the pain persists after the rash healed (Gilden et al., 2009). Other important complications include neurological manifestations, hemorrhagic and necrotic alterations of the skin, bacterial super-infections and eye or ear involvement. In immunocompromised patients, zoster is associated with significant morbidity and mortality due to disseminated and chronic infections (Au et al., 2003; Gnann, 2002).

The current drug of choice for the antiviral treatment of VZV infections in patients at risk is the nucleoside analogue acyclovir (ACV) (De Clercq, 2004). Because of the low bioavailability, the drug has to be administered intravenously for 7–10 days (Gross et al., 2003). For oral treatment of VZV infections, especially of zoster, valyciclovir, the prodrug form of ACV, famciclovir, the prodrug of penciclovir (PCV) and (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU, brivudin) are available. The general mode of action of these nucleoside analogues is through inhibition of viral DNA polymerase (pol) by acting as competitive inhibitors and/or DNA chain terminators. This action requires three intracellular phosphorylation steps to convert the nucleoside analogues into their mono-, di- and triphosphates. The viral thymidine kinase (TK), which acts as thymidine and thymidylate kinase, is involved in the first phosphorylation step of ACV and PCV and equally in the second phosphorylation step of BVDU. Acyclovir resistance has to be assumed if clinical findings improve only slowly or not at all (Gross et al.,

* Corresponding author. Address: Institute of Virology and Antiviral Therapy, Jena University Hospital, Hans-Knoell-Strasse 2, 07745 Jena, Germany. Tel.: +49 3641 9395700; fax: +49 3641 9395702.
E-mail address: Andreas.Sauerbrei@med.uni-jena.de (A. Sauerbrei).

0166-3542/$ - see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2011.04.005

2003). In such cases, another treatment option would be intravenous foscarnet (FOS). This pyrophosphate analogue acts directly on the viral DNA pol by impeding pyrophosphate release from deoxynucleotides during DNA syntheses (Chrisp and Clissold, 1991). Likewise, the acyclic nucleoside phosphonate cidofovir (CDV) has been shown to be active against VZV and a broad range of DNA viruses (De Clercq, 2004). Following intracellular phosphorylation to the diphosphate form, CDV acts as chain terminator.

Acyclovir and/or FOS resistance of VZV has only been reported in rare cases of immunocompromised patients, such as in persons developing the acquired immune deficiency syndrome (AIDS) (Boivin et al., 1994; Bryan et al., 2008; Crassard et al., 2000; Fillet et al., 1998; Hatchette et al., 2008; Jacobson et al., 1990; Linnemann et al., 1990; Lyall et al., 1994; Morfin et al., 1999; Pahwa et al., 1988; Safrin et al., 1991; Saint-Léger et al., 2001; Snoeck et al., 1994; Talarico et al., 1993; Visse et al., 1998; Wunderli et al., 1996). Similarly to the herpes simplex virus (HSV), resistance to ACV is associated with deficient TK activity (Morfin et al., 1999) or, less often, with an alteration of substrate specificity (Boivin et al., 1994). Acyclovir or FOS resistance conferred by mutations in the DNA pol gene has also been described (Fillet et al., 1994; Pahwa et al., 1988).

The aim of this study was to characterize the natural polymorphism of both the TK and DNA pol genes of 51 clinical ACV-sensitive VZV isolates obtained from patients with varicella or zoster. In addition, 16 VZV strains with clinical resistance to ACV were analyzed genotypically for resistance. In 3 out of these strains, genotypic findings could be compared with phenotypic features.

2. Materials and methods

2.1. Viral strains and cell cultures

In this study, 51 ACV-sensitive VZV isolates from 29 patients with varicella and 22 patients with zoster were included. From the patients with varicella, aged between 1 and 37 years (mean 7.2 years), 17 were male and 12 female. The age of zoster patients, 11 were male and 11 were female, was between 3 and 87 years (mean 47.3 years). Strains were isolated in human embryonic lung fibroblasts (HELF) (Sauerbrei et al., 1999) between 2000 and 2008 from vesicle fluid and in one case from pharyngeal tissue after the patients' specimens were sent to the German reference laboratory for HSV and VZV for diagnosing VZV infection. There was no information about any antiviral therapy in these patients. No patient had received varicella vaccination. The reference strains parental Oka (pOka) and vaccine Oka (vOka), kindly provided by the Institute of Virology, Charité Medical School, Berlin, Germany, were used as controls. Testing of resistance phenotype (method described below) revealed inhibitory concentrations 50% (IC_{50}) of ACV between 0.4 and 1.3 μg/ml (1.8–5.8 mM). Considering the cut-off value for resistance (see also 2.3.), all strains were ACV-sensitive. The viral strains were genotyped using the scattered single nucleotide polymorphism (SNP) method on the basis of sequencing open reading frames 1, 21, 22, 37, 50, 54 and 60 (Sauerbrei et al., 2008) and classified into the major clades described recently (Breuer et al., 2010).

Furthermore, the study included 16 VZV strains that were obtained from 15 patients with zoster and one patient with VZV encephalitis for resistance testing because of clinical resistance against ACV. This means, there was no clinical improvement under administration of ACV for at least 10 days (Balfour et al., 1994; Safrin et al., 1991). However, detailed information about the administration of ACV including any alternative treatment with FOS was not available. Patients' data and clinical information are summarized in Table 1. There were 6 female and 8 male patients and their age was between 6 and 66 years (mean 38.4 years). Age and gender were not known in 2 patients. Strains were obtained from vesicle fluid in case of zoster and from cerebrospinal fluid in case of encephalitis. All clinical samples were submitted to the German reference laboratory for HSV and VZV to verify ACV resistance by laboratory methods. VZV strains could be isolated in HELF from the vesicle fluids of 3 patients with zoster (No. 2, 3 and 5), but in the remaining cases cell culture isolation of viruses was not successful.

Table 1
Clinical data of patients.

No. of viral strain	Patient		Clinical data (diagnosis)
	Age (years)	Gender	
1	n.a.	n.a.	Recurrent generalized zoster after therapy with ACV, NHL
2	57	m	Persistent zoster under therapy with valacyclovir, TCL
3	36	f	Persistent zoster under therapy with ACV after BMT
4	n.a.	n.a.	Persistent zoster under therapy with ACV, CLL
5	14	f	Persistent zoster trigeminus under therapy with ACV, AML
6	6	m	Persistent zoster under therapy with ACV, IS
7	66	f	Persistent zoster under therapy with ACV, IS
8	49	f	Persistent zoster under therapy with ACV, IS
9	18	f	Zoster under chemoprophylaxis with ACV, IS
10	71	m	Persistent zoster under therapy with ACV
11	11	m	Zoster under chemoprophylaxis with ACV after SCT
12	64	m	Persistent zoster under therapy with ACV after SCT
13	7	m	Persistent zoster under therapy with ACV, IS
14	31	m	Persistent VZV encephalitis under ACV, IS
15	43	m	Persistent VZV pneumonia under therapy with ACV, HCL
16	64	f	Persistent zoster under therapy with ACV, IS

ACV – acyclovir, AML – acute myeloid leukaemia, BMT – bone marrow transplantation, CLL – chronic lymphocytic leukaemia, HCL – hairy cell leukaemia, IS – immunosuppression, NHL – Non-Hodgkin's lymphoma, SCT – stem cell transplantation, TCL – T-cell lymphoma, f – female, m – male, n.a. – not available.

2.2. Antiviral test compounds

The following antiviral compounds were used for the phenotypic characterization of resistance to VZV: acyclovir (ACV: GlaxoSmithKline, Uxbridge, UK), brivudin (BVDU; Berlin-Chemie AG, Berlin, Germany), penciclovir (PCV; GlaxoSmithKline, Uxbridge, UK), foscarnet (FOS; AstraZeneca, Wilmslow, UK), and cidofovir (CDV; Vistide®, Pharmacia and Upjohn, Luxembourg).

2.3. Phenotypic characterization of resistance

VZV strains were grown and titrated in human Caucasian fetal lung fibroblasts of the cell line Wi 38 (European Collection of Cell Cultures, Salisbury, UK) using the method described previously (Sauerbrei et al., 2007). Antiviral testing was performed in 48-well flat-bottomed microtitre plates by means of plaque reduction assay. First, 200 μl of Wi 38 cell suspension per well were seeded at a density of 2×10^5 ml^{-1}. After addition of antiviral compounds at a final half log dilution over a range between 0.0625 and 8 μg ml^{-1} (ACV, PCV, CDV), between 0.0001 and 8 μg ml^{-1}

(brivudin) or between 4 and 512 μg ml^{-1} (FOS), cells were infected with about $10^{3.7}$ $TCID_{50}$ ml^{-1} of cell-free VZV corresponding to a multiplicity of infection of 0.12. It followed the incubation of plates for 3 days at 36 °C in a humid atmosphere containing 1% CO_2. During this interval, the virus-induced plaques became visible. The cells were fixed and stained with 0.3% crystal violet in 3% formalin per well for at least 1 h. Subsequently, the plates were washed under running tap-water at intervals of several hours and distilled water was used for the final wash. Finally, plaques were counted with the aid of a dissecting microscope (Carl Zeiss, Jena, Germany) with ×100 magnification and the 50% inhibitory concentration (IC_{50}) was estimated. Each experiment was performed at least twice and data points of IC_{50} were presented as the arithmetic means.

The VZV reference strain vOka served as sensitive virus control. A viral strain was regarded as resistant if the mean IC_{50} was calculated as four times the mean value of the VZV control strain proved to be susceptible to the corresponding antiviral drug (Morfin and Thouvenout, 2003). On the basis of the mean control values, following cut-off levels for drug resistance were defined: ACV 2.7 ± 0.6 μg ml^{-1} (11.9 ± 2.7 μM); BVDU 0.05 ± 0.04 μg ml^{-1} (0.16 ± 0.12 μM); PCV 5.3 ± 4.8 μg ml^{-1} (19.0 ± 17.4 μM); FOS 126 ± 53 μg ml^{-1} (658 ± 277 μM); CDV 2.7 ± 1.3 μg ml^{-1} (9.8 ± 4.4 μM).

2.4. Genotypic characterization of resistance

The genetic analysis of resistance of VZV was carried out by the amplification of DNA fragments of the viral TK and DNA pol genes as well as subsequent sequencing. The oligonucleotide primers were based on the reference strain Dumas (VZV clade 1, GenBank Accession No. X04370). The TK gene (UL 36) was amplified and sequenced in 2 fragments while the DNA pol gene (UL 28) was amplified as 4 fragments and used to generate up to 12 overlapping fragments for sequencing.

After isolation of DNA from virus-infected cells of the passage 1–3 or from patients' specimens by means of QIAamp® Blood Kit (Qiagen, Hilden, Germany), viral DNA was amplified by polymerase chain reaction (PCR). Fragments of the TK and DNA pol genes were amplified by the use of High Fidelity Enzyme Mix (Fermentas, St.-Leon-Rot, Germany). Standard PCR mixtures contained 10 μM of each primer (Table 2) plus approximately 50 ng template DNA in a volume of 50 μl. For amplification of TK gene, there was an initial denaturation step for 3 min at 95 °C and reaction mixtures were cycled 38 times through denaturation at 94 °C for 50 s, annealing at 55 °C for 50 s, and polymerization at 72 °C for 90 s followed by a final extension step at 72 °C for 5 min. The amplification of the DNA pol gene consisted of an initial denaturation step over 3 min at 95 °C, 10 cycles for 40 s at 95 °C, 15 s at 55 °C and 7 min at 68 °C, 25 cycles for 40 s at 95 °C, 45 s at 55 °C and 7 min at 68 °C and a final extension step for 10 min at 68 °C. Amplified DNA fragments were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Afterwards, viral DNA was quantified spectrophotometrically. An amount of 200–700 ng DNA μl^{-1} was used for sequencing.

Sequencing reactions of purified PCR products were performed by means of the cycle sequencing method. First, VZV subgenomic regions were sequenced using DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences Europe, Freiburg, Germany) and 10 μM oligonucleotide primers (Table 2). Sequencing reaction mixture with a total volume of 10 μl consisted of 2–7 μl of the purified viral DNA, 0.5 μl primer and 2 μl sequence reaction mix containing DNA polymerase and labeled ddNTPs. After initial incubation at 95 °C for 3 min to denature the template DNA, the thermal conditions of amplification followed 25 cycles of 95 °C for 20 s, 50 °C for 15 s, and 60 °C for 60 s. A final extension step was done at 60 °C for 10 min. After precipitation and washing with ethanol, the DNA fragments were analyzed on the ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, Darmstadt, Germany) using ABI PRISM™ DNA Sequencing Software Version 2.1.2 (Applied Biosystems). Sequencing was also performed by a second method using 2–6 μl of the purified DNA, 0.5 μl primer and the components of the Genome Lab™ Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit (Beckman Coulter, Krefeld, Germany) according to the manufactures instructions. The following conditions were applied: 1 min at 95 °C, 29 cycles for 20 s at 95 °C, 20 s at 50 °C and 3 min at 60 °C. Amplicons were precipitated, washed in ethanol and analyzed in the CEQ™ 8000 Genetic Analysis System (Beckman Coulter, Krefeld, Germany). Sequences of the PCR products were verified on both DNA strands. All data were compared with published sequences of the reference strain Dumas using the software MEGA 4.1.beta. The specified nucleotide sequences and amino acid positions correspond to sequences and positions in the reference strain Dumas.

Table 2
Primers for amplification and sequencing of thymidine kinase (TK) and DNA polymerase (pol) gene of VZV.

VZV DNA fragment (amplicon in bp)	Name	Sequence 5′⇒ 3′	Nucleotides of VZV genome
VZV TK	VZV-TK1	GGT CAC CAT TAT TTC ATC AGA TC	64322–64344
Fragment A (926 bp)	VZV-TK2R	CTC CCA CTA AGT ATC TGG ACA AG	65248–65226
VZV TK	VZV-TK3	CCT GAA GAC GCA CAA CGC CTC AC	65041–65063
Fragment B (864 bp)	VZV-TK4R	GAA AAA CAC GTA CAC GCG AGT ATG	65905–65882
VZV DNA pol	VZV-Pol1	CAA ACA GAG ACT GTG GTG CCA TC	46991–47013
Fragment A (1000 bp)	VZV-Pol2+	GTC AGA TAT TAT TAA CTT ACG C	47391–47412
	VZV-Pol2b	CAC ACC GTT TAC CTG CGG TAT TC	47423–47445
	VZV-PolRa	CTT AGT CGT CCA CCA TCC GCC TAC	47633–47610
	VZV-Pol1R	GTT TTG TCC TCC TAT AAA GTT GG	47991–47969
VZV DNA pol	VZV-Pol3+	CAT TCA GCA ATG GAA ACA CAC G	47749–47770
Fragment B (1251 bp)	VZV-Pol4+	GAA CGC TAC TTT CAA TGT CTG G	48125–48146
	VZV-PolRc	CAG ATG AAG CAG TGT TAT TAG AC	48355–48333
	VZV-Pol5+	CGG ATG GAT TCA AAC GTT TAA CC	48494–48516
	VZV-Pol2R	CTA GTG GAC CGA ATA CAC GAG G	49000–48979
VZV DNA pol	VZV-Pol5+	CGG ATG GAT TCA AAC GTT TAA CC	48494–48516
Fragment C (1420 bp)	VZV-Pol3	CTA GCT AGC CTT GCA ACC GCG G	48877–48898
	VZV-Pol8	GTA TAT AGA ATT AAG TTT CTC C	49245–49266
	VZV-Pol8a	CTC GGG AGC GTA CTG TTT TAC G	49314–49335
	VZV-PolRd	CAA TCC CTC GAC AGT CTT TAG AAC	49495–49472
	VZV-Pol3R	CTA CGA TAC ACA GCC ATG TGC G	49914–49893
VZV DNA pol	VZV-Pol9	CAT CGT TTG GTA TAG CCT GCA G	49625–49646
Fragment D (1120 bp)	VZV-Pol10	GGC AAG TAA TAC AGA TAA CTC GC	50055–50077
	VZV-PolRf	CTT TCG TGG GGA TAT GTT TGA CC	50328–50306
	VZV-Pol4R	GCG GAA ACC ACA ACA AAT CAC GTG	50745–50722

2.5. Statistical analysis

The association of amino acid substitutions in the TK and DNA pol gene in ACV-sensitive VZV isolates with the different clades was analyzed by means of the Fisher's exact test. The level of significance was 0.05 (two-sided).

3. Results

3.1. Acyclovir-sensitive varicella-zoster virus isolates

The data about the natural polymorphism of the TK and DNA pol genes of VZV as a function of the viral clade are summarized in Table 3. Eighteen ACV-sensitive viral isolates of clade 1 had the amino acid substitution S288L, caused by the nucleotide substitution C > T at position 863, in the TK gene when compared with the reference strain Dumas and there was no substitution in the DNA pol gene. Furthermore, 14 isolates of clade 3 were assigned to the same genetic profile of the TK and DNA pol genes. In addition to S288L, 2 isolates of clade 3 contained the amino acid substitution S863G which is located outside conserved regions of the DNA pol gene and one isolate exhibited the novel substitutions M286I and H1089Y which are also not situated in the conserved DNA pol gene regions. In comparison, 12 out of 14 VZV isolates of clade 5 had the combination of the substitutions S288L in the TK gene and S863G in the DNA pol gene. One strain each contained the novel substitutions E824Q and R984H which are located in and out the conserved regions of DNA pol gene, respectively, and one isolate did not show any substitution in addition to S288L. Two ACV-sensitive VZV isolates that could not be attributed to any of the major VZV clades had the same amino acid profile as the majority of clade 5 strains. The 2 clade 2 strains pOka and vOka showed a clearly different DNA pol profile containing the combination of the substitutions G186C, S863G and C1159R which are all situated outside conserved gene regions. The profile of amino acid substitutions detected in the DNA pol gene of clade 5 (S863G in 12 out of 14 viral strains) was significantly different from that of clade 1 (S863G in no one of 18 viral strains) and clade 3 (S863G in 2 out of 17 viral strains).

3.2. Varicella-zoster virus strains with clinical resistance to acyclovir

Table 4 summarizes findings of genotypic resistance by sequencing analysis of TK and DNA pol genes in 16 clinical VZV strains obtained with suspicion of clinical resistance to ACV. Out of these 16 strains, only 3 could be isolated in cell culture resulting in successful phenotypic analysis of resistance. One isolate (No. 3) revealed phenotypic resistance to ACV, BVDU, PCV and sensitivity to FOS and CDV. A mixture of two VZV strains with different TK polymorphism was found by direct amplification and sequencing from the vesicle fluid of this patient whereas only one phenotype was detected in the isolated viral population. The half of the viral population contained the novel polymorphism W255R that was found outside active or conserved regions of the TK gene. The remaining 2 isolates (No. 2 and 5) did not show any indication of resistance. In additional 6 strains (No. 1, 4, 6, 11, 13 and 15) analyzed only by sequencing, a genetic correlate of resistance could not be found in the TK gene and in the DNA pol gene. However, a limitation was that in 5 out of these 6 strains the DNA pol gene could not be sequenced since the available amount of viral DNA was limited. Five VZV strains (No. 7–9, 14 and 16) only analyzed genotypically, exhibited unambiguous genetic markers in the TK gene suggesting resistance even though in 2 of them the DNA pol gene could not be analyzed. These markers were A163stop, T256M, Q303stop, N334stop and deletion of the nucleotides at positions 19–223 resulting in a frameshift. Finally, one strain each had the substitution L73I (No. 12) in the TK gene and the substitutions T237K (No. 10) or A955T (No. 7) in the DNA pol gene.

All strains but one had the polymorphism-associated amino acid substitution S288L in the TK gene unlike the reference strain Dumas. In one strain (No. 16), this substitution could not be verified because of the presence of a deletion of nucleotides 19–223 leading to a frameshift mutation. One strain (No. 7) exhibited the substitution S863G and another strain had the novel substitution K103Q which is situated outside conserved or active regions of the TK gene.

Table 3
Natural polymorphism of the thymidine kinase (TK) and DNA polymerase (pol) genes in 51 acyclovir-sensitive clinical VZV isolates of the VZV clades 1, 3, 5 as well as the parental Oka strain (pOka) and vaccine Oka (vOka) of the VZV clade 2. Novel amino acid exchanges are in bold.

VZV clade	Number of strains	Amino acid substitutions	
		TK	DNA pol
1	18	S288L	–
3	14	S288L	–
	2	S288L	S863G[a]
	1	S288L	**M286I**[a], **H1089Y**[a]
5	11	S288L	S863G[a]
	1	S288L	–
	1	S288L	**E824Q**[b], S863G[a]
	1	S288L	**R984H**[a]
Not classified	2	S288L	S863G[a]
2 (pOka, vOka)	2	S288L	G186C[a], S863G[a], **C1159R**[a]

[a] Located outside conserved gene regions.
[b] Located in conserved gene regions.

Table 4
Amino acid substitutions in thymidine kinase (TK) and DNA polymerase (pol) genes of 16 VZV strains with clinical resistance to acyclovir. Strain No. 2, 3 and 5 were tested for phenotypic resistance. Novel amino acid exchanges are in bold.

No. of viral strain	TK		DNA pol	
	Polymorphism	Polymorphism associated with clinical resistance	Polymorphism	Polymorphism associated with clinical resistance
1	S288L	–	n.d.	n.d.
2[a]	S288L	–	–	–
3[b]	S288L	**W225R**	–	–
4	S288L	–	n.d.	n.d.
5[c]	S288L	–	–	–
6	S288L	–	n.d.	n.d.
7	S288L, S863G	Q303stop	**A955T**[d,e]	**A955T**[d,e]
8	S288L	**T256M**[d]	n.d.	n.d.
9	S288L, **K103Q**[e]	**N334stop**	–	–
10	S288L	–	**T237K**[e]	**T237K**[e]
11	S288L	–	–	–
12	S288L	L73I[e]		–
13	S288L	–	n.d.	n.d.
14	S288L	**A163stop**	n.d.	n.d.
15	S288L	–	n.d.	n.d.
16	–	**Deletion of nt 19–223**	–	–

n.d. – not done; nt – nucleotide.
[a] Phenotype (inhibitory concentration 50% – IC_{50}): acyclovir 0.3 μg ml^{-1}, brivudin 0.01 μg ml^{-1}.
[b] Phenotype (IC_{50}): acyclovir, penciclovir > 8.0 μg ml^{-1}, brivudin 2.8 μg ml^{-1}, foscarnet 8.3 μg ml^{-1}, cidofovir 0.2 μg ml^{-1}.
[c] Phenotype (IC_{50}): acyclovir 1.4 μg ml^{-1}, brivudin 0.002 μg ml^{-1}, penciclovir 4.0 μg ml^{-1}, foscarnet 23.2 μg ml^{-1}, cidofovir 0.4 μg ml^{-1}.
[d] Located in conserved gene regions.
[e] Relation to polymorphism or resistance is not clear.

4. Discussion

In contrast to the HSV, sensitivity of VZV isolation in cell culture is low and has been reported between 20% and 43% from vesicle samples (Beards et al., 1998; Dlugosch et al., 1991; Sauerbrei et al., 1999, 2011). Reasons for that are the extreme instability and cell association of the virus, the restricted spectrum of cell cultures and the prolonged incubation time for up to a few weeks required for developing cytopathic changes. Thus, the characterization of resistance genotype using genetic analysis of the TK and DNA pol genes has to be considered the method of choice for the determination of VZV resistance to antiviral drugs. However, there is little information in the literature about non-synonymous mutations which can be regarded as related to resistance or to the natural gene polymorphism.

In the first part of the present study, 51 ACV-sensitive clinical VZV isolates from patients with varicella or zoster were examined for the natural polymorphism of the TK and DNA pol genes. All strains of the clades 1, 3 and 5 showed no gene polymorphism of the TK apart from the amino acid exchange S288L which cannot be detected in the European reference strain Dumas. These findings confirm previous studies (Morfin et al., 1999) and are in contrast to the pronounced TK polymorphism of HSV-1 and HSV-2 (Bohn et al., in press; Burrel et al., 2010). The DNA pol gene exhibited polymorphism-related substitutions as a function of the VZV clade. Whereas the DNA pol gene of clade 1 and clade 3 strains did not contain typical polymorphism-related substitutions, there was a significant association of the clade 5 strains with the substitution S863G. In addition, individual strains of the clades 3 and 5 showed substitutions such as M286I, E824Q, R984H and H1089Y which have never been reported in the literature. One of them (E824Q) was located in the conserved gene regions but was not connected with any FOS resistance (data not shown). In the clade 2 strains pOka and vOka, the genetic pattern of the TK was equal to that of clade 1, 3 and 5, but the DNA pol revealed higher gene polymorphism which found expression in the substitutions G186C, S863G and C1159R located outside conserved regions (Kamiyama et al., 2001).

In the second part of this study, 16 VZV strains with clinical resistance to ACV were examined genotypically, 3 out of them were also characterized phenotypically. In the TK gene of 7 strains, the resistance-associated amino acid substitutions L73I, A163stop, W225R, T256M, Q303stop, N334stop and the deletion of nucleotides 19–223 resulting in a frameshift error were found. One out of them (Q303stop) has been reported previously in 2 patients with AIDS developing persistent zoster under treatment with ACV (Fillet et al., 1998; Saint-Léger et al., 2001) and the remaining 6 are novel. A limitation of this study is that most novel mutations could not be assigned to the viral phenotype. Only in one strain showing the substitution W225R, the resistance against ACV, BVDU and PCV could be confirmed phenotypically. However, the stop of translation (substitutions A163stop and N334stop) in 2 strains and the frameshift mutation (deletion of nucleotides 19–223) in one strain each are beyond any doubt about the relation to resistance. However, the replicative capacity of these strains could not be assessed because of the failure to isolate the viruses in cell culture. Since the substitution T256M is located in the conserved gene region and has been reported recently to be associated phenotypically with resistance to ACV (Bleymehl et al., 2011), its relation to resistance seems to be also verified. By contrast, the significance of the substitution L73I for resistance has to be confirmed either by correlation between phenotype and genotype or by site-directed mutagenesis experiments. This also concerns the novel amino acid substitution K103Q which is most likely a part of the natural gene polymorphism of the TK. The DNA pol gene could not be sequenced in 7 out of 16 VZV strains showing clinical resistance to ACV. The reason was a lack of viral DNA prepared from the patients' specimens. Nevertheless, the novel amino acid substitutions T237K and A955T could be detected in the DNA pol gene of 2 strains. The substitution A955T, which is situated in the conserved gene region V, was found in combination with Q303stop in the TK gene suggesting unclear significance of A955T. The substitution T237K was not combined with additional non-synonymous mutations in both the TK and DNA pol genes. However, since the clinical resistance could not be confirmed by the determination of the viral phenotype, the significance of this substitution also remains ambiguous.

In resistance testing of HSV, mixtures of viruses with different susceptibility to antiviral drugs can hamper phenotypic and genotypic test procedures. In one patient of this study, a mixed population with regard to polymorphism was detected by sequencing of the TK gene. The viral phenotype was proven to be ACV-resistant and did not change during several passages of the virus in cell culture without selective pressure of ACV. However, parallel genotyping studies revealed a reduction in the proportion of viruses with the polymorphism identified (data not shown). These findings suggest that the determination of IC_{50} using plaque reduction assay is very reliable to identify resistance although the great majority of the viral population has to be considered ACV-sensitive. For HSV, the plaque reduction assay has been described to identify viral isolates as ACV-resistant if >20% of the virus population is resistant (Bacon et al., 2003).

The 16 VZV strains with clinical resistance were mostly obtained from patients with persistent zoster under therapy with ACV. Recovery of ACV-resistant VZV strains was associated with profound immunosuppression in 7 (No. 3, 7–9, 12, 14, 16) out of 8 patients, whereas in one patient (No. 10) information about any immunodeficiency was not available. However, resistance could not be verified by the use of genotyping methods in 8 strains and by the additional use of phenotyping procedure in 2 out of these 8 strains. This means that in half of the patients with the administration of ACV for at least 10 days the resistance could not be confirmed by virological methods. On the one hand, ACV-resistant VZV infection has been defined clinically by the persistence of lesions despite 10 days of therapy (Balfour et al., 1994; Safrin et al., 1991). On the other hand, Bovin et al. (1994) reported that the mean time for progression from ACV susceptibility to ACV resistance in paired VZV isolates from 4 patients with AIDS was 7.5 weeks. Furthermore, Saint-Léger et al. (2001) suggested that persistent lesions at day 10 may simply indicate the need for prolonged ACV in immunocompromised patients and that treatment failure after 21 days of therapy is more predictive of ACV resistance in this setting. Even though this could partially be confirmed in this study, the impairment of cellular immunity seems to be a crucial factor for the development of ACV resistance in VZV.

References

Arvin, A.M., 1999. Chickenpox (varicella). In: Wolff, M.H., Schünemann, S., Schmidt, A. (Eds.), Varicella-Zoster Virus. Molecular Biology, Pathogenesis, and Clinical Aspects. Contrib. Microbiol. Karger, Basel, 3, 96–110.

Au, W.Y., Ma, S.Y., Cheng, V.C., Ooi, C.G., Lie, A.K., 2003. Disseminated zoster, hyponatraemia, severe abdominal pain and leukemia relapse: recognition of a new clinical quartet after bone marrow transplantation. Br. J. Dermatol. 149, 862–865.

Bacon, T.H., Levin, M.J., Leary, J.J., Sarisky, R.T., Sutton, D., 2003. Herpes simplex virus resistance to acyclovir and penciclovir after two decades of antiviral therapy. Clin. Mcrobiol. Rev. 16, 114–128.

Balfour Jr., H.H., Benson, C., Braun, J., Cassens, B., Erice, A., Friedman-Kien, A., Klein, T., Polsky, B., Safrin, S., 1994. Management of acyclovir-resistant herpes simplex and varicella-zoster virus infections. J. Acquir. Immune Defic. Syndr. 7, 254–260.

Beards, G., Graham, C., Pillay, D., 1998. Investigation of vesicular rashes for HSV and VZV by PCR. J. Med. Virol. 54, 155–157.

Bleymehl, K., Ciantl, J., Schmidt-Chanasit, J., 2011. Phenotypic and genetic characterization of varicella-zoster virus mutants resistant to acyclovir,

brivudine and/or foscarnet. Med. Microbiol. Immunol. <doi:10.1007/s00430-011-0191-4>.
Bohn, K., Zell, R., Schacke, M., Wutzler, P., Sauerbrei, A., in press. Gene polymorphism of thymidine kinase and DNA polymerase in clinical strains of herpes simplex virus. Antiviral Ther.
Boivin, G., Edelman, C.K., Pedneault, L., Talarico, C.L., Biron, K.K., Balfour Jr., H.H., 1994. Phenotypic and genotypic characterization of acyclovir-resistant varicella-zoster virus isolated from persons with AIDS. J. Infect. Dis. 170, 68–75.
Breuer, J., Grose, C., Norberg, P., Tipples, G., Schmid, D.S., 2010. A proposal for a common nomenclature for viral clades that form the species varicella-zoster virus: summary of VZV Nomenclature Meeting 2008, Barts and the London School of Medicine and Dentistry, 24–25 July 2008. J. Gen. Virol. 91, 821–828.
Bryan, C.L., Prichard, M.N., Daily, S., Jefferson, G., Hartline, C., Cassady, K.A., Hilliard, L., Shimamura, M., 2008. Acyclovir-resistant chronic verrucous vaccine strain varicella in a patient with neuroblastoma. Pediatr. Infect. Dis. J. 27, 946–948.
Burrel, S., Deback, C., Agut, H., Boutolleau, D., 2010. Genotypic characterization of UL23 thymidine kinase and UL30 DNA polymerase of clinical isolates of herpes simplex virus: natural polymorphism and mutations associated with resistance to antivirals. Antimicrob. Agents Chemother. 54, 4833–4842.
Crassard, N., Souillet, A.L., Morfin, F., Thouvenot, D., Claudy, A., Bertrand, Y., 2000. Acyclovir-resistant varicella infection with atypical lesions in a non-HIV leukemic infant. Acta Paediatr. 89, 1497–1499.
Chrisp, P., Clissold, S.P., 1991. Foscarnet. A review of its antiviral activity, pharmacokinetic properties and therapeutic use in immunocompromised patients with cytomegalovirus retinitis. Drugs 41, 104–129.
De Clercq, E., 2004. Antiviral drugs in current clinical use. J. Clin. Virol. 22, 115–133.
Dlugosch, D., Eis-Hübinger, A.M., Kleim, J.P., Kaiser, R., Bierhoff, E., Schneweis, K.E., 1991. Diagnosis of acute and latent varicella-zoster virus infections using polymerase chain reaction. J. Med. Virol. 35, 136–141.
Fillet, A.M., Dumont, B., Caumes, E., Visse, B., Agut, H., Bricaire, F., Huraux, J.M., 1998. Acyclovir-resistant varicella-zoster virus: phenotypic and genotypic characterization. J. Med. Virol. 55, 250–254.
Fillet, A.M., Visse, B., Caumes, E., Dumont, B., Gentilini, M., Huraux, J.M., 1994. Foscarnet-resistant multidermatomal zoster in a patient with AIDS. Clin. Infect. Dis. 21, 1348–1349.
Gershon, A., Mervish, N., LaRussa, P., Steinberg, S., Lo, S.H., Hodes, D., Fikrig, S., Bonagura, V., Bakshi, S., 1997. Varicella-zoster virus infection in children with underlying HIV infection. J. Infect. 176, 1496–1500.
Gilden, D., Nagel, M.A., Mahalingam, R., Mueller, N.H., Braszeau, E.A., Pugazhenthi, S., Cohrs, R.J., 2009. Clinical and molecular aspects of varicella-zoster virus infection. Future Neurol. 4, 103–117.
Gnann Jr., J.W., 2002. Varicella-zoster virus: atypical presentations and unusual complications. J. Infect. Dis. 186 (Suppl. 1), S91–S98.
Gross, G., Schöfer, H., Wassilew, S., Friese, K., Timm, A., Guthoff, R., Pau, H.W., Malin, J.P., Wutzler, P., Doerr, H.W., 2003. Herpes zoster guideline of the German Dermatology Society (DDG). J. Clin. Virol. 26, 277–289.
Hatchette, T., Tipples, G., Peters, G., Alsuwaidi, A., Zhou, J., Mailam, T.L., 2008. Foscarnet salvage therapy for acyclovir-resistant varicella zoster. Report of a novel thymidine kinase mutation and review of the literature. Pediatr. Infect. Dis. J. 27, 75–77.
Jacobson, M.A., Berger, T.G., Fikrig, S., Becherer, P., Moohr, J.W., Stanat, S.C., Biron, K.K., 1990. Acyclovir-resistant varicella zoster virus infection after chronic oral acyclovir therapy in patients with the acquired immunodeficiency syndrome (AIDS). Ann. Int. Med. 112, 187–191.
Kamiyama, T., Kurokawa, M., Shiraki, K., 2001. Characterization of the DNA polymerase gene of varicella-zoster viruses resistant to acyclovir. J. Gen. Virol. 82, 2761–2765.
Linnemann Jr., C.C., Biron, K.K., Hoppenjans, W.G., Solinger, A.M., 1990. Emergence of acyclovir-resistant varicella-zoster virus in an AIDS patient on prolonged acyclovir therapy. AIDS 4, 577–579.
Loparev, V., Martro, E., Rubtcova, E., Rodrigo, C., Piette, J.C., Caumes, E., Vernant, J.P., Schmid, D.S., Fillet, A.M., 2007. Toward universal varicella-zoster virus (VZV) genotyping: diversity of VZV strains from France and Spain. J. Clin. Microbiol. 45, 559–563.
Lyall, E.G., Ogilvie, M.M., Smith, N.M., Burns, S., 1994. Acyclovir resistant varicella zoster and HIV infection. Arch. Dis. Childh. 70, 133–135.
Morfin, F., Thouvenout, D., 2003. Herpes simplex virus resistance to antiviral drugs. J. Clin. Virol. 26, 29–37.
Morfin, F., Thouvenout, D., De Turenne-Tessier, M., Lina, B., Aymard, M., Ooka, T., 1999. Phenotypic and genetic characterization of thymidine kinase from clinical strains of varicella-zoster virus resistant to acyclovir. Antimicrob. Agents Chemother. 43, 2412–2416.
Pahwa, S., Biron, K., Lim, W., Swenson, P., Kaplan, M.H., Sadick, N., Pahwa, R., 1988. Continuous varicella-zoster infection associated with acyclovir resistance in a child with AIDS. JAMA 260, 2879–2882.
Quinlivan, M., Hawrami, K., Barrett-Muir, W., Aaby, P., Arvin, A., Chow, V.T., John, T.J., Matondo, P., Peiris, M., Poulsen, A., Siqueira, M., Takahashi, M., Talukder, Y., Yamanishi, K., Leedham-Green, M., Scott, F.T., Thomas, S.L., Breuer, J., 2002. The molecular epidemiology of varicella-zoster virus: evidence for geographic segregation. J. Infect. Dis. 186, 888–894.
Safrin, S., Berger, T.G., Gilson, I., Wolfe, P.R., Wofsy, C.B., Mills, J., Biron, K.K., 1991. Foscarnet therapy in fife patients with AIDS and acyclovir-resistant varicella-zoster virus infection. Ann. Int. Med. 115, 19–21.
Saint-Léger, E., Caumes, E., Breton, G., Douard, D., Saiag, P., Huraux, J.M., Bricaire, F., Agut, H., Fillet, A.M., 2001. Clinical and virologic characterization of acyclovir-resistant varicella-zoster viruses isolated from 11 patients with acquired immunodeficiency syndrome. Clin. Infect. Dis. 33, 2061–2067.
Sauerbrei, A., Eichhorn, U., Schacke, M., Wutzler, P., 1999. Laboratory diagnosis of herpes zoster. J. Clin. Virol. 14, 31–36.
Sauerbrei, A., Philipps, A., Zell, R., Wutzler, P., 2007. Genotyping of varicella-zoster virus strains after serial passages in cell culture. J. Virol. Methods 145, 80–83.
Sauerbrei, A., Stefanski, J., Philipps, A., Krumbholz, A., Zell, R., Wutzler, P., 2011. Monitoring prevalence of varicella-zoster virus clades in Germany. Med. Microbiol. Immunol. <doi:10.1007/s00430-010-0178-6>.
Sauerbrei, A., Zell, R., Philipps, A., Wutzler, P., 2008. Genotypes of varicella-zoster virus wild-type strains in Germany. J. Med. Virol. 80, 1123–1130.
Sauerbrei, A., Wutzler, P., 2007. Herpes simplex and varicella-zoster virus infections during pregnancy – current concepts of prevention, diagnosis and therapy. Part 2: varicella-zoster virus infections. Med. Microbiol. Immunol. 196, 95–102.
Snoeck, R., Gérard, M., Sadzot-Delvaux, C., Andrei, G., Balzarini, J., Reymen, D., Ahadi, N., De Bruyn, J.M., Piette, J., Rentier, B., Clumeck, N., De Clercq, E., 1994. Meningoradiculoneuritis due to acyclovir-resistant varicella zoster virus in an acquired immune deficiency syndrome patient. J. Med. Virol. 42, 338–347.
Talarico, C.L., Phelps, W.C., Biron, K., 1993. Analysis of the thymidine kinase genes from acyclovir-resistant mutants of varicella-zoster virus isolated from patients with AIDS. J. Virol. 67, 1024–1033.
Visse, B., Dumont, B., Huraux, J.M., Fillet, A.M., 1998. Single amino acid change in DNA polymerase is associated with foscarnet resistance in a varicella-zoster virus strain recovered from a patient with AIDS. J. Infect. Dis. 178 (Suppl. 1), S55–S57.
Wunderli, W., Miner, R., Wintsch, J., von Gunten, S., Hirsch, H.H., Hirschel, B., 1996. Outer retinal necrosis due to a strain of varicella-zoster virus resistant to acyclovir, gancyclovir, and sorivudine. Clin. Infect. Dis. 22, 864–865.