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► To cite this version:

Marie-Liesse Pacreau, Olivier Bomme, Sonia Burrel, David Boutolleau. High conservation of varicella-zoster virus helicase-primase complex, the target of the new antiviral drug amenamevir. *Antiviral Research*, 2021, 195, pp.105189. 10.1016/j.antiviral.2021.105189 . hal-03390674

HAL Id: hal-03390674

<https://hal.sorbonne-universite.fr/hal-03390674>

Submitted on 21 Oct 2021

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High conservation of varicella-zoster virus helicase-primase complex, the target of the new antiviral drug amenamevir

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Abstract

Varicella-zoster virus (VZV) resistance to current antiviral drugs, that all target the viral DNA polymerase, represents a growing concern, notably among immunocompromised patients. Amenamevir, a novel antiviral that inhibits the VZV helicase-primase (HP) complex, is approved in Japan for the treatment of herpes zoster. In this study, we describe the low natural polymorphism of VZV HP complex (interstrain identity >99.7% both at nucleotide and amino acid levels) among 44 VZV clinical isolates. This work enabled to settle the maps of natural polymorphisms of VZV HP complex and to provide the genotypic tools for the monitoring of the emergence of VZV resistance to amenamevir in patients.

Keywords: Varicella-zoster virus; Helicase-primase complex; Natural polymorphism; Antiviral resistance testing

Varicella-zoster virus (VZV) is a ubiquitous alphaherpesvirus that causes varicella (chickenpox), as a primary infection, and herpes zoster (shingles), as a localized reactivation (Kennedy and Gershon, 2018). VZV infections are associated with significant morbidity and mortality, especially in immunocompromised populations, due to disseminated and chronic infections (Sauerbrei et al., 2011). The mainstay antiviral therapy for VZV infections over the past decades relies on nucleoside analogues that target the viral DNA polymerase (encoded by open reading frame 28 [ORF28]): acyclovir and penciclovir, and their oral prodrugs valaciclovir and famciclovir (Andrei and Snoeck, 2021; Piret and Boivin, 2016; Sauerbrei et al., 2011). Nucleoside analogues require phosphorylation by VZV thymidine kinase (TK, encoded by ORF36) and cellular kinases before being incorporated into the growing DNA chain by the viral DNA polymerase and inhibiting DNA replication by chain termination mechanism. The alternative option is intravenous foscarnet that inhibits directly the viral DNA polymerase without the need of phosphorylation by viral TK. However, the therapeutic management of VZV infections may be challenged by the emergence of VZV resistance to antivirals. To date, VZV resistance to acyclovir and/or foscarnet has been reported almost exclusively in case-reports of immunocompromised patients who require prolonged or repeated therapies, such as transplant recipients and HIV-infected individuals (Mercier-Darty et al., 2018; Piret and Boivin, 2016; Sauerbrei et al., 2011), and in rare cases of immunocompetent individuals with recurrent VZV keratitis (Boutolleau et al., 2019; Gueudry et al., 2013). To our knowledge, only one published study reported a prevalence of VZV resistance to antivirals of 27% in a cohort of hematological patients with persistent VZV infection (van der Beek et al., 2013). Moreover, in the National Reference Center for Herpesvirus in Pitié-Salpêtrière Hospital (Paris), we observed a prevalence of VZV resistance to antivirals of 11% during the period 2010-2018 among patients with no significant regression of clinical lesions associated with VZV infection despite antiviral treatment for at least 10 days (Boutolleau et al., 2019). VZV resistance to acyclovir is mostly conferred by mutations in TK and, less frequently, in DNA polymerase (Piret and Boivin, 2016; Shiraki et al., 2021). Moreover, the nephrotoxicity of foscarnet may limit its use. Therefore, novel therapeutic options with novel viral targets are highly needed for the management of severe and drug-resistant

VZV infections. The VZV helicase-primase (HP) complex constitutes a promising target for novel antiviral agents inhibiting DNA replication. This heterotrimeric complex consists of 2 essential proteins, ORF55 helicase and ORF6 primase, interacting with ORF52 accessory protein, which are well conserved among *Herpesviridae* (Klinedinst and Challberg, 1994; Ligat et al., 2018). The VZV HP complex possesses multiple enzymatic activities that are essential for DNA replication and viral growth: DNA helicase and single-stranded DNA-dependent ATPase localized within ORF55 helicase, and primase localized within ORF6 primase (Chono et al., 2010). HP inhibitors (HPis) represent a breakthrough in the development of anti-herpesvirus agents. Conversely to classical antivirals, they do not target the viral DNA polymerase and do not need activation by the viral TK: they inhibit directly the viral HP complex and, therefore, remain active against viruses that are resistant to acyclovir and/or foscarnet (Andrei and Snoeck, 2021; Shiraki et al., 2021). The oxadiazolephenyl derivative amenamevir (ASP2151) is an HPI that possesses antiviral activity against not only herpes simplex viruses 1 and 2 (HSV-1 and HSV-2) but also VZV, setting it apart from pritelivir (AIC316) which inhibits only HSV replication and demonstrated clinical efficacy in patients with genital herpes (Chono et al., 2010; Wald et al., 2016). Additionally, amenamevir is active against acyclovir-resistant VZV in vitro (Chono et al., 2010). Amenamevir appeared to be effective and well tolerated in the treatment of herpes zoster in immunocompetent patients in a randomized, double-blind, valaciclovir controlled phase 3 study and was therefore approved in Japan in September 2017 (Kawashima et al., 2017). To date, one single mutation conferring resistance to amenamevir has been identified in VZV ORF55 helicase (Chono et al., 2010). Moreover, poor is known regarding the natural polymorphism of VZV HP complex. In this context, the aims of this work were to develop novel techniques for the sequencing of VZV HP complex of 44 VZV clinical isolates and one VZV laboratory strain to investigate the genetic variability and to provide a precise map of VZV HP complex natural polymorphism in order to facilitate the interpretation of VZV genotypic resistance testing toward amenamevir.

The present study included the analysis of the VZV laboratory strain Ellen (ATCC-VR-1367) and 44 VZV clinical isolates recovered in cell culture from mucocutaneous swabs during the period 2012-2019, as

previously described (Perrier et al., 2016). Clinical samples were collected from 44 unrelated patients (21 males, 23 females, median age, 57 years) experiencing VZV infection (varicella, n = 4; herpes zoster, n = 38; unspecified, n = 2). Patients were mainly immunocompromised: solid organ transplantation (n = 7), hematopoietic stem cell transplantation (n = 5), solid cancer (n = 4), hemopathy (n = 2), HIV infection (n = 3), auto-immune disease (n = 10), unknown immune status (n = 13). None of the patients received prior amenamevir therapy. Novel methods were developed for the amplification and sequencing of VZV ORF55 and ORF6 according to previously described procedures (Collot et al., 2016; Perrier et al., 2016). Briefly, VZV DNA extraction was performed using the MagNA Pure Compact Instrument (Roche Diagnostics, Meylan, France). The full-length ORF55 (2646 base pairs [bp]) and ORF6 (3252 bp) were amplified by nested PCR using the proofreading enzyme Expand High Fidelity (Roche Diagnostics). Thereafter, amplification products were sequenced by Sanger method using 4 overlapping primer pairs and analyzed with the automated capillary sequencer ABI 3730XL DNA Analyzer (Applied Biosystems) (Table 1). All nucleotide and amino acid sequences obtained were compared with that of VZV reference strain Dumas with GenBank accession number **X04370** (Davidson and Scott, 1986) using SeqScape v2.5 software (Applied Biosystems). All VZV ORF55 and ORF6 sequences determined in this study have been deposited in the GenBank database under accession numbers **MZ152705** through **MZ152790**.

Our results showed a very low natural polymorphism of VZV HP complex. At the nucleotide level, the interstrain identity of ORF55 and ORF6 ranged from 99.8% to 100% among the 44 clinical isolates and the laboratory strain Ellen investigated (Table 2). In comparison with Dumas reference sequences, 23 and 21 nucleotide substitutions were identified within ORF55 and ORF6, respectively, corresponding to a total number of nucleotide substitutions per strain ranging from 0 to 6 within both genes. The majority (>61%) of those nucleotide substitutions were silent. At the amino acid level, the interstrain identity of VZV HP complex ranged from 99.7% to 100%. Five and 8 different amino acid changes were identified in ORF55 helicase and ORF6 primase, respectively, corresponding to 0.57% and 0.74% of the

total codons of the proteins. Each VZV strain harbored 0 to 2 amino acid changes in ORF55 helicase and 0 to 3 in ORF6 primase (Table 2). Overall, the frequency of those amino acid changes related to natural polymorphism of VZV HP complex ranged from 2.2% to 22.2% (Table 3). The most frequent changes were K616R (17.8%) in ORF55 helicase and G496V and H575Q (22.2%) in ORF6 primase. All natural polymorphisms described in this study were evenly distributed alongside VZV helicase and primase, and all but one of them were located outside conserved domains of the proteins. Thus, A1053T change, identified only in the laboratory strain Ellen, was located in the conserved zinc finger motif. The new data obtained in this work together with the data reported so far in the literature enabled to provide the maps of natural polymorphisms of VZV ORF55 helicase and ORF6 primase (Fig. 1) (Chono et al., 2010; Tyler et al., 2007).

The emergence of VZV resistance to standard antiviral drugs, especially acyclovir, represents a growing concern, notably among immunocompromised patients (Piret and Boivin, 2016; Sauerbrei et al., 2011; van der Beek et al., 2012), but also in some rare cases of immunocompetent patients with recurrent VZV keratitis (Boutolleau et al., 2019; Gueudry et al., 2013). For those patients and other vulnerable ones with severe VZV infections, HPis, alone or in combination, constitute an alternative possibility of therapeutic management. In the present study, we developed novel techniques for the sequencing of VZV full-length ORF55 helicase and ORF6 primase. Those techniques will be useful for the rapid VZV genotypic resistance testing to HPis, since molecular approach significantly shortens the time for the detection of drug resistance in a clinically relevant time frame (Burrel et al., 2010).

This work enabled to describe extensively the natural polymorphism of VZV HP complex in the era of HPis. Despite the lack of phenotypic test of VZV isolates towards amenamevir, the mutations identified in the present study are most certainly natural polymorphisms since (i) they were detected in VZV clinical isolates recovered from patients with no prior amenamevir therapy and (ii) they were not located within or downstream motif IV of the helicase, where almost all mutations conferring HSV resistance to HPis have been previously identified (Collot et al., 2016; Sato et al., 2021). Moreover,

some authors showed that minor pre-existing HPI resistant mutations could be detected in HSV-1 clinical isolates at a frequency of 10^{-4} to 10^{-5} plaque forming units (pfu), but those isolates remained susceptible to pritelivir (Biswas et al., 2007; Sukla et al., 2010). However, it is noteworthy that those low-frequency mutations cannot be detected by the Sanger method used in the present study. Our results obtained from the VZV laboratory strain Ellen and 44 VZV clinical isolates demonstrated a very low natural polymorphism of VZV HP complex. Thus, the interstrain identity was >99.7% at the nucleotide and the amino acid levels for both helicase and primase. This high conservation of VZV HP complex is similar to the high conservation (>99%) that we have previously reported for other viral targets of anti-VZV drugs (ORF36 TK and ORF28 DNA polymerase), and anti-HSV drugs (UL23 TK and UL30 DNA polymerase; UL5 helicase and UL52 primase) (Burrell et al., 2010; Collot et al., 2016; Perrier et al., 2016). These results are also in accordance with the overall nucleotide identity of >99.7% obtained by the comparison of 18 complete VZV genomes (Tyler et al., 2007). Among all herpesviruses, VZV is known to display the lowest nucleotide variation within its genome, estimated at 0.05-0.06% (Quinlivan and Breuer, 2006).

In this study, we identified 5 and 8 amino acid changes related to natural polymorphism in VZV helicase and primase, respectively, corresponding to 0.57% and 0.74% of the total codons of the proteins. Only 3 of them, namely K316R in ORF55 helicase, and G496V and H575Q in ORF6 primase, were detected with a frequency over 15%; each of the 9 other polymorphisms was detected in a single VZV strain. Few previous studies reported natural polymorphisms of VZV HP complex: Tyler et al. performed the genomic cartography of 18 complete VZV genomes and Chono et al. sequenced the helicase and primase subunits from an amenamevir-resistant VZV strain (Chono et al., 2010; Tyler et al., 2007). In this study, all 5 amino acid changes identified in the ORF55 helicase were described for the first time, and 2 out of the 8 amino acid changes identified in the ORF6 primase, G496V and H575Q, were previously reported in a VZV strain from Canada (Tyler et al., 2007). The present data together with previous ones enabled to provide the maps of natural polymorphisms of VZV ORF55 helicase and ORF6 primase (Chono et al., 2010; Tyler et al., 2007) (Fig. 1). All but one (20/21) were located outside the

conserved motifs of the viral enzymes previously defined (Ligat et al., 2018; Klinedinst and Challberg, 1994). The remaining change A1053T, which was detected only in the laboratory strain Ellen, was located in the conserved zinc finger motif of ORF6 primase. However, this polymorphism does not concern the 4 conserved residues involved in the formation of the zinc finger motif (Ligat et al., 2018). The use of HPIs in medical practice, a novel class of anti-herpesvirus agents with a different mechanism of action from classical anti-herpesvirus agents used so far, should significantly improve the therapeutic management of HSV and VZV infections. First, HPIs will permit the treatment of infections due to viruses that are resistant to acyclovir and/or foscarnet (Andrei and Snoeck, 2021; Shiraki et al., 2021). Moreover, multidrug combination therapies will now be possible. Thus, the combination of HPIs with the nucleoside analogues acyclovir and penciclovir showed a synergistic antiviral effect against HSV and VZV in vitro (Chono et al., 2013). Therefore, combination therapies may constitute a potential approach for treating severe, sometimes life-threatening, diseases due to HSV or VZV, such as encephalitis or infections in immunocompromised patients.

To date, no emergence of HSV or VZV resistance to HPIs has been detected in patients treated with those novel drugs. All mutations conferring resistance to HPIs described so far have been selected in cell culture through serial passages of virus in presence of HPIs. For HSV, resistance mutations are mainly located immediately downstream of motif IV of UL5 helicase, but few mutations have also been identified in UL52 primase (Collot et al., 2016; Sato et al., 2021). For VZV, only one mutation conferring resistance to amenamevir has been identified (Chono et al., 2010). After the selection of a VZV resistant mutant in cell culture in the presence of increasing concentrations of amenamevir, sequence analysis identified the N336K change in ORF55 helicase of VZV, corresponding to the N342K change in UL5 helicase of HSV-1 that was previously shown to confer resistance to amenamevir (Biswas et al., 2009). Further in vitro studies are needed to extend the catalog of VZV resistance mutations to amenamevir. In conclusion, this work enabled to settle a valuable baseline of VZV ORF55 helicase and ORF6 primase natural polymorphisms. The novel genotypic techniques developed will be useful for the monitoring

of the emergence of VZV resistance to amenamevir and potential other antiviral compounds that inhibit VZV HP complex in patients included in clinical trials or treated in medical practice.

Acknowledgements

This work was supported in part by the Association pour la Recherche sur les Infections Virales (ARIV).

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Table 1

Primers used for amplification and sequencing of VZV full-length ORF55 helicase and ORF6 primase.

Gene	Function	Name	Sequence (5' → 3')
ORF55	First-round PCR (outer primers)	VZORF55-F1	F: GGGGCGTACTCATTCCAGTA
		VZORF55-F1	R: GTCTTCACCGCCATTGATCTT
	Second-round PCR (inner primers)	VZORF55-F2	F: ACTTCCATTCAAGCCCTGGT
		VZORF55-R2	R: CAGTGAAGAACCCGCCTAAC
	Sequence reaction	VZORF55-A	R: CGGGAAGATTAGCAGGATTG
		VZORF55-B	F: GTTTTCGCGGAAATCACATT
		VZORF55-C	R: TCCAGGGTTAAGGCAGCTAA
		VZORF55-D	F: AAACGCTTGAGTACGGGCTA
		VZORF55-E	R: ATCGACATACGCGCTAAAGG
		VZORF55-F	F: CCAAAGTAACGGCCTCCATA
+ VZORF55-F2/VZORF55-R2			
ORF6	First-round PCR (outer primers)	VZORF6-F1	F: GCGTGACAATTACGTGTTCC
		VZORF6-F1	R: GGACCAAACGACGGGTATTA
	Second-round PCR (inner primers)	VZORF6-F2	F: AACCGGTCCACCATTAATCA
		VZORF6-R2	R: CACAGCGGTTAAAGCCTCTT
	Sequence reaction	VZORF6-A	R: TGCCGCTTCACTACGAGATA
		VZORF6-B	F: GTCCCGTAAATACGGACCAA
		VZORF6-C	R: GCCATTACGAACGTCAAGT
		VZORF6-D	F: TACCCAATGGGATGGGTTT
		VZORF6-E	R: CCACTCGCTGTTGTTGCTTA
		VZORF6-F	F: ACCCGATATACAGGCATTGG
+ VZORF6-F2/VZORF6-R2			

F: forward; R: reverse

Table 2

Variations of VZV helicase-primase complex, both at nucleotide and amino acid levels, among 44 clinical isolates and the laboratory strain Ellen (ATCC-VR-1367) in comparison with reference strain Dumas (GenBank accession number [X04370](#)).

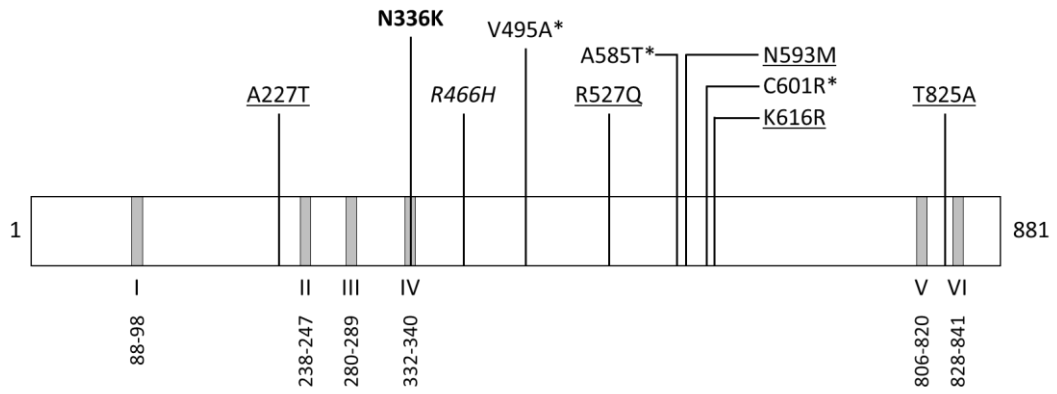
Parameter	ORF55 helicase	ORF6 primase
Nucleotide identity (%)	99.85 – 100	99.82 – 100
Nucleotide mutations (no.)	23	21
Strains with nucleotide mutations (%)	17 (37.78)	31 (68.89)
Frequency per strain (median)	0 – 6 (0)	0 – 6 (1)
Silent mutations (%)	17 (73.91)	13 (61.91)
Amino acid identity (%)	99.77 – 100	99.72 – 100
Amino acid changes (no.)	5	8
Frequency per strain (median)	0 – 2 (0)	0 – 3 (0)
Variation of the total codons (%)	0.57	0.74

Table 3

Frequencies of amino acid changes related to natural polymorphism of VZV ORF55 helicase and ORF6 primase identified in this study in comparison with reference strain Dumas (GenBank accession number X04370).

	Amino acid changes	No. of VZV strains (%)
ORF55 helicase	A227T	1 (2.2)
	R527Q	1 (2.2)
	N593M	1 (2.2)
	K616R	8 (17.8)
	T825A	1 (2.2)
ORF6 primase	I139V	1 (2.2)
	M161L	1 (2.2)
	C280R	1 (2.2)
	G496V	10 (22.2)
	T518A	1 (2.2)
	H575Q	10 (22.2)
	A894T	1 (2.2)
	A1053T	1 (2.2)

A



B

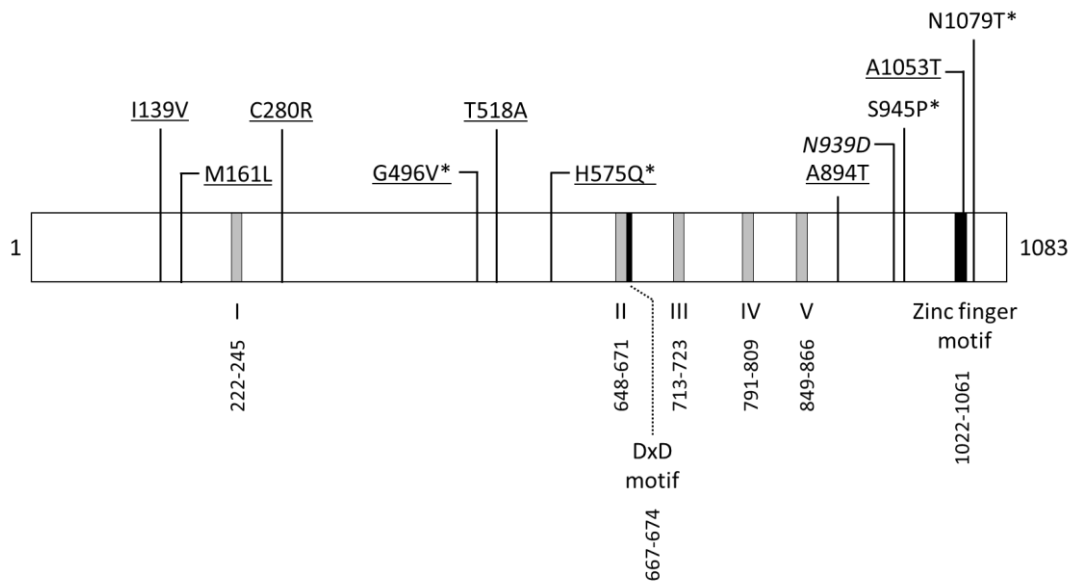


Fig. 1. Polymorphism map of VZV helicase-primase complex.

Figure legends

Fig. 1. Polymorphism map of VZV helicase-primase complex.

(A) ORF55 helicase is a 881-amino-acid protein. The 6 conserved motifs (I to VI) are represented by the grey boxes with the positions (codon numbers) indicated below (Ligat et al., 2018). All amino acid positions related to natural polymorphism reported so far in the literature are indicated: those described in this study are underlined, those reported by Tyler et al. (2007) are represented with an *, and the one reported by Chono et al. (2010) in an amenamevir-resistant VZV strain is indicated in italic. The amino acid change conferring resistance to amenamevir described by Chono et al. (2010) is in bold.

(B) ORF6 primase is a 1083-amino-acid protein. The 5 conserved motifs (I to V) and the DxD and zinc finger motifs are represented by the grey and the black boxes, respectively, with the positions (codon numbers) indicated below (Ligat et al., 2018; Klinedinst and Challberg, 1994). All amino acid positions related to natural polymorphism reported so far in the literature are indicated: those described in this study are underlined, those reported by Tyler et al. (2007) are represented with an *, and the one reported by Chono et al. (2010) in an amenamevir-resistant VZV strain is indicated in italic.