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UL23, *UL30*, and *UL5* characterization of HSV1 clinical strains isolated from hematology department patients

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ABSTRACT

Genotypic diagnosis of HSV drug resistance can be performed routinely in a clinically relevant time. Nevertheless, data about HSV mutations (polymorphism or resistance) is not exhaustive which hinders the interpretation of such tests. The *UL23, UL30*, and *UL5* genes are of greatest interest as these encode, respectively, thymidine kinase, DNA polymerase, and helicase, which, if mutated may affect the effectiveness of acyclovir, foscarnet, cidofovir, and helicase-primase inhibitors. The present study aimed to extensively characterize *UL23*, *UL30*, and *UL5* genes. A total of 239 clinical HSV1 recovered from patients admitted to the hematology departments of the Lyon teaching hospitals were included in this single-center retrospective study. Drug resistance was evaluated using the neutral red dye-uptake assay, and sequencing using the Sanger method. Additional information on HSV1 natural polymorphism and resistance is now available. Twenty-two amino acid substitutions related to polymorphism were described on UL23 (E43A, L50M, L68R, Q109K, A133V, A136N, S150L, D258N, S263L, P280S, N301S, A316S, M322L, I326V, D330A, D338H, Q342H, T344I, Q349R, V352L, R370W, E371D), and 6 amino acid substitutions on UL30 (G641R, G645D, E649G, G679D, R681L, I966M). Moreover, the UL23 substitution L242P was added to ACV resistance-related mutations. There were 12 substitutions on UL23 (A37S, V70M, S74L, H151N, P154S, P155Q, L159R, E225L, Y248H, Q270R, N303Y, M372I), and 8 on UL5 (L49I, L138V, S173L, A280T, A575V, V600A, A602T, D862N) that remain of unclear significance with regards to drug resistance. The mean (± standard deviation, SD) number of natural polymorphisms in UL23 was 2.53 (± 2.55), in UL30 it was 0.83 (± 1.02), and in UL5 it was 5.00 (± 1.59) There was no association between HSV1 phenotype and the frequency of substitutions. The results reported herein provide valuable new information concerning HSV1 mutations that will assist the interpretation of genotypic assays.

1. Introduction

All currently marketed anti-herpetic drugs target the viral DNA polymerase (DNA pol). Acyclovir (ACV), an acyclic guanosine analogue, is the first-line treatment of herpes simplex virus (HSV) infections owing to its effectiveness and safety (Vere Hodge and Field, 2013). It acts selectively, as its initial phosphorylation is mediated by the viral thymidine kinase (TK) encoded by the *UL23* gene. Foscarnet (FOS) and cidofovir (CDV), used as second-line treatments in case of ACV resistance, do not require viral TK phosphorylation and directly inhibit DNA pol that is encoded by the *UL30* gene. More recently, viral helicase, encoded by *UL5* gene, has become a target for the development of alternative treatments, even on ACV-resistant HSV (Tyring et al., 2012); the helicase-primase inhibitors (HPI) are currently under phase II development (Wald et al., 2014).

Occurrence of ACV-resistant HSV type 1 (HSV1) remains a major

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concern in clinical hematology departments. The prevalence of ACV-resistant HSV1 strains can reach 30% among hematopoietic stem cell transplant (HSCT) recipients (Frobert et al., 2014). ACV resistance cases are mostly related to mutations in the *UL23* gene (90–95% of cases), whereas a minority is related to mutations in the *UL30* gene (5–10% cases) (Piret and Boivin, 2014). The *UL23* gene is not essential for viral replication and is permissive to mutations as compared to *UL30* gene (Burrel et al., 2010). However resistance mutations occurring within the *UL30* gene can induce resistance to both ACV and FOS, and also multi-resistance affecting CDV (Piret and Boivin, 2016). With regards to *UL5*, certain mutations inducing HPI resistance have already been described (Sukla et al., 2010; Field and Biswas, 2011).

Genotypic analyses, by classical Sanger sequencing or by next generation sequencing, allow detection of drug-resistant HSV within a clinically relevant timeframe (Frobert et al., 2008; Fujii et al., 2018). However, a robust and complete mutation database is essential to implement genotypic techniques. For HSV1, further characterization of the *UL23, UL30*, and *UL5* genes is required and this was the objective of the present study.

2. Materials and methods

2.1. Patients and clinical specimens

During the 2012–2016 period, patients tested HSV1-positive by real-time PCR and hospitalized in one of the three clinical hematology departments of the Lyon teaching hospitals were included in this single-center retrospective study. For each patient, only the first excretion of HSV1 was considered. Samples tested were oropharyngeal, cutaneous, nasopharyngeal, bronchoalveolar lavage fluids (BALF), anal, genital, or ocular. Briefly, nucleic acids were extracted using the automatic nucleic acid platform NucliSENS EasyMAG (BioMérieux, Marcy-l'Etoile, France) according to manufacturer's recommendations. Samples (200 µL) were eluted in 50 µL. Detection of HSV was performed using the HSV1 & HSV2 R-gene™ Quantification kit (Argène, BioMérieux, Marcy-l'Etoile, France), according to the manufacturer's recommendations.

2.2. Phenotypic analysis

Screening for ACV and FOS resistance was performed by the chessboard method using neutral red dye-uptake assay as previously described (Langlois et al., 1986). The inhibitory concentration 50 (IC50) cut-off values were 6.5 µM for ACV and 350 µM for FOS (Nugier et al., 1992). FOS resistance was tested in specific clinical and virological situations such as ACV resistance, recurrence of herpes under treatment, or severe clinical conditions. Resistance to CDV and HPI was not tested.

2.3. Genotypic analysis

Amplification of *UL23*, *UL30*, and *UL5* genes was carried out using Q5® Hot Start High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA, USA) as previously described (Burrel et al., 2010; Frobert et al., 2008). Primers for *UL5* amplification and sequencing are listed in Table 1. Amplicons were sequenced using the Sanger method (Genoscreen, Lille, France). Nucleotide sequences were then compared to the sensitive reference strain KOS (GenBank access number JQ763480) using SeqmanII software (DNAStar Inc., Madison, WI, USA) that also provided amino acid translation. Silent mutations were not taken into account. *UL23* and *UL30* genotypic analysis was performed for all specimens, whereas the UL5 gene encoding the helicase was only sequenced for ACV-resistant specimens. Substitutions that have no impact on drug susceptibility were classified as natural polymorphisms, whereas those that induced drug resistance were classified as resistance mutations; substitutions that may have an impact but that has yet to be clearly proven were classified as substitutions of unknown significance. All sequences have been submitted to the GenBank database under accession numbers MK895980 through to MK896218 for *UL23*, MK935191 through to MK935338 for *UL30*, and MK878593 through to MK878616 for *UL5*.

Table 1
Primers for *UL5* amplification and sequencing.

Function	Fragment	Name	Sequences (5′-3′)
Amplification	1	HSV1 UL5_33_F	ACCTTTACCCAGCCGTCCT
		HSV1 UL5_1628_R	CGTCCCACTAAACCCAAAA
	2	HSV1 UL5_1351_F	TACCTGAAGGTGACCCGTGA
		HSV1 UL5_2898_R	TCAAAAACATGCACCACCTG
Sequencing	1	HSV1 UL5_33_F	ACCTTTACCCAGCCGTCCT
		HSV1 UL5_336_F	ACGTGGCGGCCTTAGAGT
		HSV1 UL5_844_F	GCAGCAACGTGATCGTCAT
		HSV1 UL5_1628_R	CTGCCCACTAAACCCAAAAA
		HSV1 UL5_659_R	CGTGAGGTCCAAAATCACCT
	2	HSV1 UL5_1351_F	TACCTGAAGGTGACCCGTGA
		HSV1 UL5_1851_F	GGAGAACTAACGGCGGAGA
		HSV1 UL5_2318_F	ACACACGGGTGTTTGCCTTT
		HSV1 UL5_2160_R	TAAAGGGGGCGACTTCAAAT
		HSV1 UL5_2702_R	GCGCAGAGCCGATAGTATGT

2.4. Statistical analysis

Differences in frequencies of UL23, UL30, and UL5 mutations were evaluated using Student's t-test. A test was considered statistically significant when the p value was less than 0.05 (Prism v7.0, GraphPad Software, La Jolla, CA, USA).

2.5. Phylogenetic analysis of UL23 among ACV-sensitive and resistance HSV1

The *UL23* sequences were aligned using MUSCLE in SeaView 4.7 software. The maximum likelihood phylogenetic tree was inferred from the *UL23* alignment after model selection (best fitting substitution model K3Pu + F + R3 according to BIC score) using IQ-TREE. Statistical robustness and reliability of the branching order within the tree were assessed by ultrafast bootstrapping (10,000 replicates) with IQ-TREE. Trees were finally viewed and edited using FigTree v1.4.4 software (http://tree.bio.ed.ac.uk/software/figtree/).

2.6. Ethics statement

Samples were collected for regular clinical management during hospital stay. No additional samples were taken for the purpose of this study. Patient confidentiality was strictly protected. This study was approved by the ethics committee of the Hospices Civils de Lyon, France.

3. Results

3.1. Patients and clinical specimens

A total of 239 patients were included. Patients' characteristics are summarized in Table 2. Clinical samples were oropharyngeal (n = 126, 52.7%), cutaneous (n = 48, 20.1%), nasopharyngeal (n = 45, 18.8%), BALF (n = 12, 5.0%), anal (n = 5, 2.1%), genital (n = 2, 0.8%), and ocular (n = 1, 0.5%).

3.2. Phenotypic data

Among the 239 HSV1 PCR-positive samples, viral replication did not occur in 39; ACV sensitivity was thus investigated by the phenotypic method for 200 samples. Among these, 36 had an ACV IC50

Table 2
Clinical characteristics of patients.

	Adults		Children	
	HSCT + (n = 85)	HSCT-(n = 101)	HSCT + (n = 4)	HSCT-(n = 49)
Median age, years (range)	51 (19–68)	60 (19–83)	4.5 (2–18)	10 (1–18)
Male-to-female sex ratio	1.30	1.35	1.00	1.04
Infection, n (%)				
viral reactivation	66 (77.6)	32 (31.7)	3 (75.0)	23 (46.9)
primary infection	0	0	1 (25.0)	1 (2.0)
missing data	19.0 (22.4)	69 (68.3)	0	25 (51.1)
Antiviral prophylaxis (ACV or val-ACV), n (%)				
prophylaxis	40 (47.1)	4 (4.0)	1 (25.0)	1 (2)
no prophylaxis	33 (38.8)	52 (51.5)	2 (50.0)	41 (83.7)
missing data	12 (14.1)	45 (44.5)	1 (25.0)	7 (14.3)
Underlying hematological disease, n (%)				
Acute myeloid leukemia	46 (54.1)	55 (54.5)	2 (50.0)	4 (8.7)
Acute lymphoblastic leukemia	8 (9.4)	12 (11.9)	0 (0)	17 (34.7)
Hodgkin's lymphoma	1 (1.2)	0 (0)	1 (25)	3 (6.1)
Non-Hodgkin's lymphoma [a]	8 (9.4)	16 (15.8)	0 (0)	6 (12.2)
Other hematological disease [b]	22 (25.9)	16 (15.8)	1 (25)	7 (14.3)
Non-hematological pathology disease,[c] n (%)	0 (0)	2 (2)	0 (0)	12 (24.5)

HSCT: hematopoietic stem cell transplant; ACV: acyclovir; Val-ACV: valacyclovir.
[a] Burkitt's lymphoma, lymphoplasmacytic lymphoma, mantle cell lymphoma, follicular lymphoma, nodal marginal zone lymphoma, diffuse large B-cell lymphoma, angioimmunoblastic T cell lymphoma, peripheral T cell lymphoma or T cell prolymphocytic leukemia.
[b] Refractory anemia with excess blasts, refractory cytopenia with multilineage dysplasia, myelofibrosis, myelodysplastic syndrome, chronic myeloid leukemia, chronic lymphocytic leukemia, multiple myeloma, chronic myelomonocytic leukemia, primary polycythemia, sickle cell disease or hemolytic anemia.
[c] Amyloidosis, nephroblastoma, neuroblastoma, sarcoma, ependymoma, cavum cancer or testicular cancer.

beyond the 6.5 µM cut-off and were thus considered as ACV-resistant. FOS sensitivity was determined for 51 samples, among which 5 had an IC50 beyond the 350 µM cut-off, and were thus considered as FOS-resistant. All FOS-resistant strains were ACV cross resistant.

3.3. Genotypic data

3.3.1. UL23 analysis

The *UL23* gene could be amplified and sequenced for the 239 HSV1-positive samples; 36 sequences were identical to the KOS reference strain and 164 had only natural polymorphisms. Among these 164 HSV1, 22 substitutions never previously described were identified in ACV-sensitive strains (Table 3; Fig. 1).

A total of 32 HSV1 (13.4%) harbored a previously described resistance mutation (Gaudreau et al., 1998; Saijo et al., 2002; Chibo et al., 2004; Duan et al., 2009); these were a single-nucleotide substitution for 20 sequences (62.5%), a single-nucleotide deletion for 9 sequences (28.1%), and a single-nucleotide insertion for 3 sequences (9.4%). The amino acid L242P substitution, previously described as unclear significance substitution (Frobert et al., 2014), was the only unknown mutation identified within the *UL23* gene of an ACV-resistant and FOS-sensitive HSV1 and could thus be classified as a resistance mutation (Table 3).

Seven HSV1 had a never previously described substitution; as no phenotypical data was available, these were thus considered as of unclear significance. Among the 32 with resistance mutations, 5 mutations (S74L, P154S, L159R, E225L, and Y248H) were considered as of unclear significance. Substitutions at 151, 316, and 352 reported herein have previously been described but for other amino acids (H151Y, Frobert et al., 2008; A316V and V352I,Schmidt et al., 2015).

3.3.2. UL30 analysis

The *UL30* gene could be amplified and sequenced for 148/239 HSV1. A total of 67 sequences were identical to the KOS reference strain and 77 sequences had only natural polymorphisms; among the latter, 6 substitutions never previously described were identified in ACV and FOS -sensitive strains (G641R, G645D, E649G, G679D, R681L, and I966M; Table 4; Fig. 2).

Three HSV1 had a previously published substitution conferring both

Table 3
Previously unreported UL23 amino acid substitutions.

Substitutions	ACV IC50, µM	FOS IC50, µM	Classification
A37S	NA	NA	Unknown
E43A	1.0	NP	Polymorphism
L50M	< 0.8	NP	Polymorphism
L68R	1.2	NP	Polymorphism
V70M	NA	NA	Unknown
S74L	31.0	< 62.5	Unknown
Q109K	< 0.8	101.0	Polymorphism
A133V	< 0.8	NP	Polymorphism
A136N	< 0.8	NP	Polymorphism
S150L	< 0.8	NP	Polymorphism
H151N	NA	NA	Unknown
P154S	67.0	70.0	Unknown
P155Q	NA	NA	Unknown
L159R	45.0	88.0	Unknown
E225L	20.0	136.0	Unknown
L242P	14.0	< 62.5	Resistance
Y248H	42.0	125.0	Unknown
D258N	1.0	NP	Polymorphism
S263L	1.3	NP	Polymorphism
Q270R	NA	NA	Unknown
P280S	1.0	NP	Polymorphism
N301S	< 0.8	NP	Polymorphism
N303Y	NA	NA	Unknown
A316S	< 0.8	NP	Polymorphism
M322L	< 0.8	NP	Polymorphism
I326V	1.0	NP	Polymorphism
D330A	< 0.8	NP	Polymorphism
D338H	< 0.8	NP	Polymorphism
Q342H	< 0.8	NP	Polymorphism
T344I	< 0.8	NP	Polymorphism
Q349R	< 0.8	NP	Polymorphism
V352L	1.5	NP	Polymorphism
R370W	1.3	NP	Polymorphism
E371D	1.5	NP	Polymorphism
M372I	NA	NA	Unknown

ACV: acyclovir; FOS: foscarnet; NP: not performed; NA: not assessable.
The inhibitory concentration 50 (IC50) cut-off values were 6.5 µM for ACV and 350.0 µM for FOS.

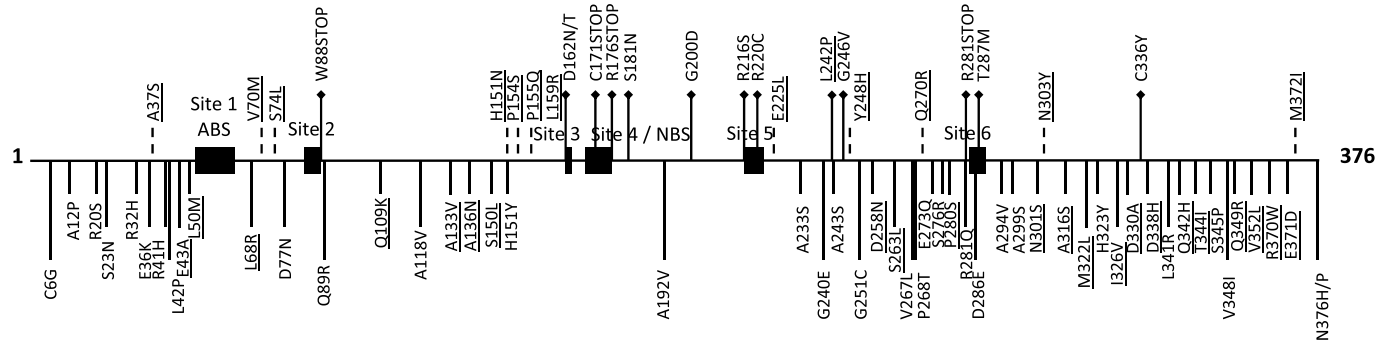

Fig. 1. Schematic representation of the UL23 substitutions (amino acids) reported among the 239 HSV1. The black boxes represent the 6 conserved/active regions: ABS is the ATP binding site and NBS is the nucleoside binding site. Natural polymorphisms are indicated below the horizontal line. Above the horizontal line, resistance mutations are schematized by a black line and a rhombus and mutations of unclear significance by a dashed line. Previously unreported amino acid substitutions are underlined. Substitution frequency of the present study is represented by three sizes of line: small line for mutations detected in less than 1% of the strains, intermediate line for a frequency of detection between 1% and 5%, and long line for those detected in more than 5% of the strains.

Table 4
Previously unreported UL30 amino acid substitutions.

Substitutions	ACV IC50, μM	FOS IC50, μM	Classification
G641R	< 0.8	107.0	Polymorphism
G645D	< 0.8	110.0	Polymorphism
E649G	1.0	101.0	Polymorphism
G679D	< 0.8	125.0	Polymorphism
R681L	< 0.8	104.0	Polymorphism
I966M	2.0	226.0	Polymorphism

ACV: acyclovir; FOS: foscarnet.
The inhibitory concentration 50 (IC50) cut-off values were 6.5 μM for ACV and 350.0 μM for FOS.

Table 5
Previously unreported UL5 amino acid substitutions.

Substitutions	IC50 ACV (μM)	IC50 FOS (μM)	Classification
L49I	16.0	638.0	Unknown
L138V	15.0	538.0	Unknown
S173L	42.0	92.0	Unknown
A280T	31.0	< 62.5	Unknown
A575V	31.0	< 62.5	Unknown
V600A	15.0	538.0	Unknown
A602T	15.0	538.0	Unknown
D862N	16.0	638.0	Unknown

ACV: acyclovir; FOS: foscarnet.
The inhibitory concentration 50 (IC50) cut-off values were 6.5 μM for ACV and 350.0 μM for FOS.

ACV and FOS resistance (A605V, S724N, and V813M). One ACV and FOS -resistant HSV1 harbored no resistance substitution but a combined substitution previously described as natural polymorphisms (D672N and N711K). One ACV and FOS resistant HSV1 could not have *UL30* amplified.

3.3.3. UL5 analysis

The *UL5* gene could be amplified and sequenced for 24/36 HSV1; 8 substitutions were not previously reported (Table 5; Fig. 3).

3.3.4. Comparison of UL23, UL30, and UL5

3.3.4.1. Frequency of amino acid substitutions. Among viruses with natural polymorphisms, UL23 presented a mean (± standard deviation, SD) 2.53 (± 2.55) natural polymorphisms per virus, UL30 a mean (± SD) 0.83 (± 1.02) natural polymorphisms per virus, and UL5 a mean (± SD) 5.00 (± 1.59) natural polymorphisms per virus. UL23 presented a significantly higher mean number of natural polymorphisms per virus than UL30 ($p < 0.001$). Given that UL5 was only sequenced from ACV-resistant specimens, comparison of the number of natural polymorphisms in UL5 to the number of natural polymorphisms in UL23 or UL30 was not possible. For UL23, among viruses with natural polymorphisms, ACV-sensitive HSV1 had a mean (± SD) 2.69 (± 2.62) natural polymorphisms per virus, and ACV-resistant HSV1 had a mean 1.78 (± 2.02) natural polymorphisms per virus ($p = 0.05$). Among the latter, this was 1.91 (± 1.97) for strains with frameshift mutations and 1.52 (± 2.12) for strains with substitution mutations. For UL30, among viruses with natural polymorphisms, ACV-sensitive HSV1 had a mean (± SD) 0.82 (± 1.02) natural polymorphisms per virus, and ACV-resistant HSV1 had a mean 0.84 (± 1.01) natural polymorphisms per virus ($p = 0.95$).

3.3.4.2. Localization maps of amino acid substitutions. Most natural polymorphisms were located outside active or conserved regions of proteins and most resistance mutations were located on highly-conserved parts or close to these. In the UL23, there was a D286E natural polymorphism within site 6 (conserved region), and G200D and

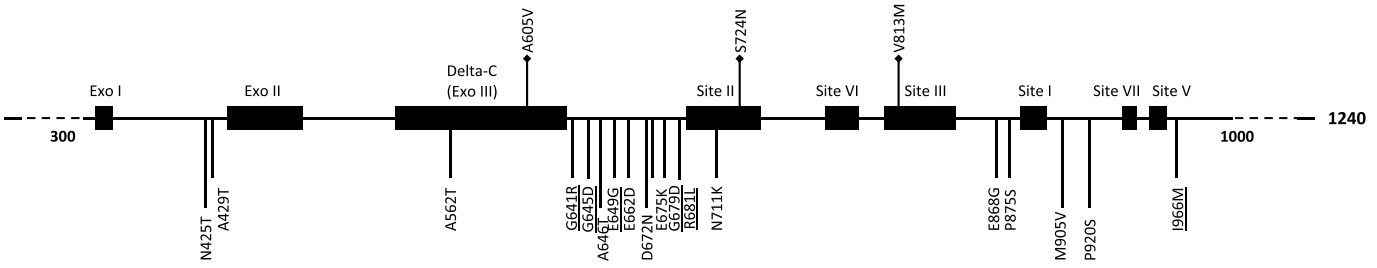

Fig. 2. Schematic representation of the UL30 substitutions (amino acids) among the 148 HSV1. Conserved regions and functional domains are indicated by black boxes. Natural polymorphism mutations are represented below the horizontal line. Resistance mutations are represented by a black line and a rhombus. Previously unreported amino acid substitutions are underlined. Intermediate lines are for mutations detected in less than 5% of the HSV1 of this study and long lines for those detected in more than 5%.

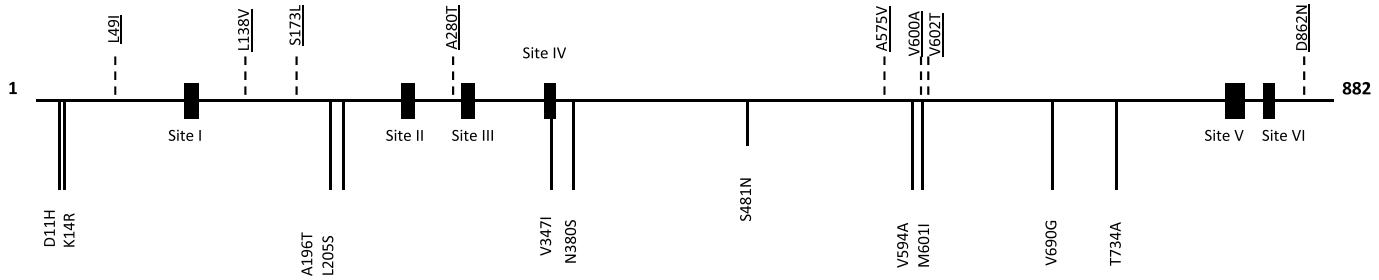

Fig. 3. Schematic representation of the UL5 substitutions (amino acids) among the 24 HSV1. Conserved regions and functional domains are indicated by the black boxes. Natural polymorphism mutations are indicated below the horizontal line. Unclear significant mutations are represented by dashed lines above horizontal line. Previously unreported amino acid substitutions are underlined. Intermediate lines are for mutations detected below 5%, and long lines for frequent mutations more than 5%.

G246V resistance mutations outside any active or conserved regions (Fig. 1); in the UL30, there was a A562T natural polymorphism within delta-C and N711K within motif II (conserved regions; Fig. 2). For the UL5, one natural polymorphism (V347I) was detected on the IV conserved motif (Fig. 3).

3.3.4.3. Local sequence variation according to ACV-sensitive and ACV-resistance HSV1. No significant difference in sequence variation and no clustering of ACV-sensitive or ACV-resistant HSV1 was found (Fig. 4).

4. Discussion

The present study provides additional information on HSV1 mutations; a total of 28 new natural polymorphisms, 1 new resistance mutation, and 20 mutations of unknown significance (12 were on UL23 and 8 on UL5) are described. Among the latter, A37S and Q270R on UL23 are presumably natural polymorphims as A37S is adjacent to E36K previously reported as polymorphim and Q270R is located in a high frequency area of natural polymorphims (Kudo et al., 1998; Frobert et al., 2008; Burrel et al., 2010; Bohn et al., 2011; Schmidt et al., 2015). In addition, five UL23 mutations of unknown significance (S74L, P154S, L159R, E225L, and Y248H) were found in ACV-resistant HSV1 harboring a known ACV resistance mutation, and therefore cannot be excluded as potentially related to drug resistance. In particular, E225L is located close to the 216–222 conserved site and Y248H is near the known T245M resistance mutation (Saijo et al., 2002). A further four UL23 mutations of unknown significance (V70M, P155Q, N303Y, and M372I) were neither located at “hot-spots” or an area of high polymorphims frequency, and for H151N, although H151Y has been reported as a natural polymorphim (Frobert et al., 2008), the impact of an asparagine instead of a tyrosine should be further explored as both the position and amino acid may be of importance. This is illustrated herein by the UL23 M322L mutation, located outside conserved regions, which was observed within two ACV-sensitive viruses (and therefore classified as natural polymorphim) although M322K is reported to lead to resistance (Bae et al., 2006). For UL5, the impact of the mutations of unknown significance could not be established as no phenotypic data regarding HPI was available. Nevertheless, none of the 8 mutations of unknown significance was located on the motif IV region (amino acids 341 to 356) where all mutations conferring HPI resistance have been described, and V600A and V602T are adjacent to M601I, previously published as polymorphims (Collot et al., 2016). Furthermore, as no patient was treated with HPI, in the absence of selection pressure, these mutations are likely to correspond to UL5 polymorphims. It is interesting to note that there were no mutations of unknown significance for UL30. However, 1 strain with a FOS resistance phenotype had 2 mutations on the UL30 (D672N and N711K) that were previously considered as natural polymorphims (Burrel et al., 2010). N711K is located within a conserved region of the gene, and D672N has been reported to be a resistance mutation in another study (Sauerbrei et al., 2010). Their impact on resistance must therefore be clearly established and using real-time culture assays could help to perform phenotypic analysis (Piret et al., 2016).

To the best of our knowledge, only Burrel et al. (2010) have reported the frequency of mutations in UL23 and UL30; they found a mean 1.6 substitutions per 100 aa for UL23 and 0.4 for UL30 (Burrel et al., 2010). Although slightly higher than that found herein, the results are within the same range. Burrel et al. (2010) also compared the variability of UL23 to that of UL30 and found a greater polymorphism (around 2.5 fold) for UL23 (Burrel et al., 2010), which is also within the same range as that observed herein. However, no published study has compared the frequency of mutations in ACV-sensitive to ACV-resistant HSV1. This comparison was made herein and the finding was that the frequency of natural polymorphism was not associated with acquisition of drug resistance. For UL5, the low natural polymorphism previously described by Collot et al. (2016) could not be confirmed herein due to the small number of HSV1 investigated for this gene (Collot et al., 2016). This point should be further studied, particularly with the perspective of HPI availability.

Although hypotheses about the impact of a specific substitution on acquisition of drug resistance are easy to formulate, mechanistic studies are necessary in order to classify mutation of unknown significance as either natural polymorphim or resistance mutation. Most UL23 studies rely on site-directed mutagenesis to reproduce, *in vitro*, substitutions observed in clinical viruses, and UL23 phosphorylation functionality can be measured by non-isotopic assays (Malartre et al., 2012; Sauerbrei et al., 2013). UL30 studies are more complex and may require generation of recombinant viruses, harboring each individual mutations, but also double substitutions to investigate effect of compensatory mutations (i.e. D672N and N711K of this study) (Drouot et al., 2013; Brunnemann et al., 2015). Recent helicase-primase complex studies are based on the synthesis of recombinant sub-unit by a baculovirus system (Bermek et al., 2017), and, although complex, this approach would be of great interest for UL23 and UL30. The functional characterization of all mutations would lead to the establishment of a complete and reliable database for HSV, as is currently the case for HIV, and HBV (HIV Drug Resistance Database, Stanford University; HBV Geno2Pheno Database, Max Planck Institut Informatik). The need for reliable data is underlined by the discrepancies between reports. For instance, Saeurbrei et al. report that the UL23 R41H substitution confers ACV resistance (Sauerbrei et al., 2016) while it has been previously classified as natural polymorphim by several teams (Chibo et al., 2004; Schulte et al., 2010). Some substitutions are reported as resistance mutations in the review by Sauerbrei et al., (2016) whereas they were found elsewhere associated with a mutation of unknown significance, for instance Q104H with L297S (Gaudreau et al., 1998), and I143V with A186P (Sauerbrei et al., 2010). In such cases, classifying both substitutions as resistance mutations might be hazardous as ACV resistance is most frequently associated with only one UL23 substitution. Some substitutions are reported in the review by Sauerbrei et al., (2016) as

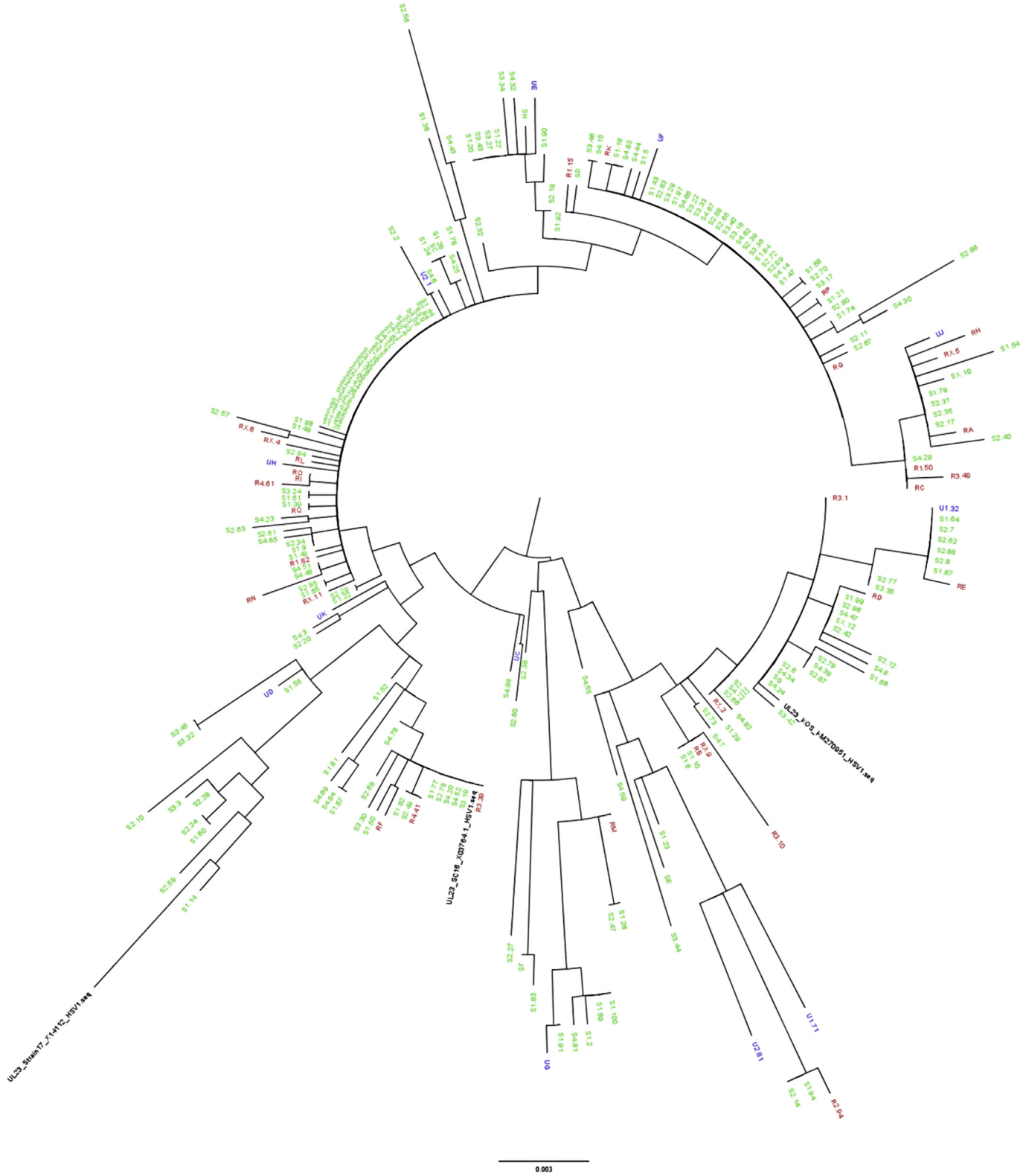

Fig. 4. Phylogenetic analysis of HSV1 *UL23* gene sequences among ACV-sensitive and ACV-resistant HSV1. Phylogenetic tree was constructed with all HSV *UL23* gene sequences and with those of 3 HSV1 reference (in black; GenBank accession number X14112 for strain 17, KM270051 for strain KOS and X03764 for strain SC16, respectively) using IQ-TREE software (model selection and tree inference). Scale (bottom) represents the number of nucleotide substitutions per site. The phenotypic ACV-resistance profile of HSV clinical isolates is indicated as follow: S for ACV-sensitive (green), R for ACV-resistance (red) and U for substitution of unknown significance (blue).

ACV resistance mutations but are not reported in the review by Boivin et al. who present only confirmed amino acid substitutions conferring ACV resistance (Piret and Boivin, 2016): A168T (Darby et al., 1986), L208F (Sauerbrei et al., 2011), M322K (Bae et al., 2006), A365T (Wang et al., 2011). Conversely, some resistance mutations are reported in the review by Boivin et al. (Piret and Boivin, 2016) but are not in the review by Saeurbrei et al. (Sauerbrei et al., 2016): T63S and Q125 E/L (Kussmann-Gerber et al., 1998), M128F (Kussmann-Gerber et al., 1999), L291P (Duan et al., 2009), and L364P (Morfin et al., 2000).

In conclusion, the results reported herein provide valuable new information regarding HSV1 mutations that will assist the interpretation of genotypic assays; a database of HSV mutations is more than ever required.

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