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Genetic Characterization of Herpes Simplex Virus Type 2 *UL23* Thymidine Kinase in Johannesburg, South Africa

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Abstract

Herpes simplex virus type 2 (HSV-2) is currently the leading cause of Genital Ulcer Disease (GUD) both globally and within South Africa. HSV-2 infections are most often treated with acyclovir (ACV), a guanosine nucleoside analogue that requires phosphorylation by virus-encoded thymidine kinase (TK). Resistance to ACV is mainly due to mutations in the viral *UL23* gene that codes for TK. ACV was added as part of the first-line syndromic management treatment algorithm for GUD in South Africa in late 2008. In order to assess the prevalence of TK-associated ACV resistance among HSV-2 virions detected in genital ulcer specimens, pre- and post-introduction of ACV, we amplified and fully sequenced the *UL23* gene of 254 HSV-2 positive specimens obtained from participants in GUD aetiological surveys conducted between 2007 and 2011 in Johannesburg, South Africa. We identified 63 nucleotide mutations in the *UL23* genes analysed, that resulted in 30 silent mutations and 32 amino acid changes. A large proportion (41%) of these amino acid changes were due to previously described natural polymorphisms that occur in both sensitive and resistant HSV strains. In addition, we identified 19 unknown amino acid changes in 30 samples that have not been described before. All mutations detected were outside the recognised TK conserved domains where ACV resistance mutations typically occur. No frameshift mutations or mutations causing stop codons were identified in those *UL23* genes analysed. Importantly, no evidence was found of known ACV resistance mutations in HSV-2 following the addition of ACV as first-line therapy for GUD.

Keywords: Herpes simplex virus; Acyclovir; Resistance mutation; Polymorphism; Thymidine kinase

Introduction

Herpes simplex virus type 2 (HSV-2) is the leading cause of genital ulcer disease (GUD) in sub-Saharan Africa where, in the general adult population, anti-HSV-2 IgG seroprevalence may range from 30 to 80% in women and from 10 to 50% in men [1]. After primary infection and replication, HSV-2 establishes latency in the sensory nerve ganglia, from where it can re-activate later in life [2]. HSV-2 infections are usually self-limited but can cause major complications in immunocompromised individuals [3]. Acyclovir (ACV), a nucleoside analogue of guanosine, was included in South Africa's national guidelines for first-line comprehensive management and control of sexually transmitted infections in August 2008 [4]. ACV, an inactive prodrug, is phosphorylated to its monophosphate form by virus-encoded thymidine kinase (TK) and then twice phosphorylated by cellular thymidilate kinases, resulting in the active triphosphate ACV form. Active ACV is a competitive inhibitor of viral DNA polymerase, competing with the natural nucleotide, deoxyguanosine triphosphate (dGTP), resulting in the cessation of viral replication by means of chain termination [5].

ACV-resistant HSV-2 can develop spontaneously due to the natural variability of the HSV population but can also emerge quickly during a course of ACV therapy, especially in immunocompromised individuals [6]. The prevalence of ACV resistant HSV-2 is much higher among immunocompromised individuals compared to those with a fully functional immune system, and is most likely due to prolonged virus replication in the presence of an impaired host immune response [7]. ACV resistance prevalence rates in immunocompromised individuals usually range between 2.5% and 10.9%, but can be as high as 36% among hematopoietic stem cell recipients [8,9]. The prevalence of drug resistant HSV-2 among immunocompetent patients is usually below 0.7% [8]. Despite the widespread use of ACV for almost 30 years, the prevalence of ACV-resistant HSV-2 has remained relatively stable [8]. HSV-2 resistance to ACV is either associated with a deficient phosphorylation by the viral TK or a reduction in the affinity of the DNA polymerase for ACV [3]. HSV-2 TK, encoded by the 1128 bp *UL23* gene, is a protein that consists of 376 amino acids [10]. Over 95% of cases of resistance emerging during ACV single-agent therapy are associated with defective phosphorylation by HSV TK [11]. This defective TK phosphorylation may result in TK deficiency or reduced TK activity [5]. Association with ACV resistance in HSV-2 is mostly due to mutations in the conserved domains of the *UL23* gene such as the ATP binding site (codons 51 to 63), the nucleoside binding site (NBS) (codons 169 to 177), six other conserved regions of the gene (positions 56 to 62, 83 to 88, 163 to 165, 172 to 174, 217 to 223 and 285 to 290) and the highly conserved cysteine at codon 337, which is responsible for sustaining the 3-dimensional structure of the active site (mutations cause a shifting of the lid domain that forms a lid enclosing the active site) [8]. Frameshift mutations, caused by insertions or deletions in the homopolymer guanosine (G) and cytidine (C) repeat regions of the *UL23* gene may also result in the synthesis of a nonfunctional truncated TK. Mutations that occur in the active

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and conserved sites therefore play a role in resistance due to possible changes in the structural integrity or catalytic functions of the enzyme [8]. In addition to these resistance-associated mutations, there are also a number of polymorphism mutations in the HSV-2 *UL23* gene, which are unrelated to resistance and usually occur outside the conserved domains of the gene. It remains a possibility that these polymorphism mutations could contribute to the creation of resistance in the presence of other mutations [12].

Direct detection of ACV resistance mutations in TK is very useful in settings where HSV-2 cell culture facilities are not available. However, the interpretation of DNA sequence-based results requires a clear distinction between resistance mutations and mutations caused by natural polymorphisms. The objective of this study was to obtain sequence-based data on the frequency of HSV-2 TK mutations in DNA extracted from HSV-2 positive genital ulcer swabs collected from GUD patients recruited to annual aetiological surveys conducted between 2007 and 2011. Given the relatively recent introduction of ACV as first-line treatment for GUD in the public sector, coupled with the facts that our GUD surveillance programme was initiated prior to this event and that the majority of our GUD patients are HIV-infected, we were in a unique position to determine the impact, if any, of widespread use of ACV to treat GUD on the emergence of ACV TK resistance mutations in HSV-2 isolates circulating in Johannesburg, South Africa.

Materials and Methods

Patients

Participants with GUD were enrolled with informed consent in five sequential annual aetiological surveys (2007-2011) undertaken in the first four months of each year in Johannesburg, South Africa. Paired genital ulcer swabs and sera were each labeled with a unique surveillance number and no patient identifying information was collected. Nurse-collected data on participants' gender and age, as well as laboratory-determined HIV serostatus data, were available for use with the HSV-2 specimens used in the current study. Both the annual surveillance programme and the current study received ethics clearance by the Human Research Ethics Committee (HREC) of the University of the Witwatersrand (protocols M051024 and M10258, respectively).

Specimens

All 314 ulcer specimens used in the present study had been shown to contain HSV-2 DNA using a commercial real-time HSV-1/2 typing PCR assay (Sacace Biotechnologies, Caserta, Italy) undertaken as part of the original laboratory testing procedures for each survey. The original genomic DNA, which had been stored at -70°C following extraction using the X-tractorGene automated DNA extractor (Qiagen, Valencia, CA, USA), was used as a template for ACV resistance testing.

Acyclovir resistance testing by *UL23* gene amplification and sequencing

The *UL23* gene was amplified by conventional PCR using the primer sequences described by Chibo et al. [12]. All PCRs were carried out using the Velocity DNA Polymerase kit (Bioline, London, UK) according to manufacturer's instructions. Amplification products were verified using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). PCR products were purified using the MSB Spin PCRapace kit (Stratec Molecular, Berlin, Germany) and sequenced in the forward and backward direction with the Big Dye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster City, CA, USA). The products were analyzed on a 16-capillary ABI 3130xl system (Applied Biosystems, Foster City, CA, USA). All contigs and sequences were edited, aligned and analyzed with Sequencing Analysis 5.2 (Applied Biosystems, Foster City, CA, USA), Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, MI, USA), BioEdit 7.0.9.0 (Ibis Biosciences, Carlsbad, CA, USA) and MEGA4 (The Biodesign Institute, Tempe, AZ, USA) software. The *UL23* gene sequence of HSV-2 strain HG52 (Accession no. Z86099) was used as a reference for sequencing alignment [13]. Sequencing results were interpreted by taking into account nucleotide substitutions, insertions, deletions, open reading framework shifts, and published results. All nucleotide sequences obtained in this study were compared to published strains on GenBank using standard nucleotide BLAST (http://www.ncbi.nlm.nih.gov/blast/). A χ^2-test of association was used to determine differences, if any, in the pre-ACV and post-ACV populations and to determine associations between the presence of one or more amino acid substitutions in *UL23* and HIV co-infection (STATA V.10.0, StataCorp, Texas, USA).

Results

A total of 314 HSV-2 positive genital ulcer specimens were available for testing. This number represented 123 specimens from the 2007 and 2008 surveys, conducted prior to the inclusion of ACV within national syndromic management treatment guidelines, and 191 specimens from the three surveys (2009-2011) conducted post-ACV introduction. Overall, 188/310 (60.6%) were male and 122/310 (39.4%) were female. Gender data for 4 patients were not recorded. The median age for both male and female patients was 29 years (range, 18-53 years). At the time of enrollment, 212/300 (70.7%) participants were HIV seropositive; HIV serostatus data for 14 patients were unavailable. The HIV prevalence was lower in the post-ACV group (79.0% pre-ACV vs. 65.8% post-ACV). There was no significant difference in the pre-ACV and post-ACV patient populations in terms of gender but a significant difference was observed for HIV serostatus (males vs. female, p=0.904; HIV serostatus p=0.018). We were able to amplify and fully sequence the *UL23* gene in 254/314 (80.9%) of specimens [104/123, (84.6%) pre-ACV introduction and 150/191 (78.5%) post-ACV introduction]. We were unable to amplify the *UL23* gene in 60/314 (19.1%) specimens. This was most likely due to very low HSV-2 viral loads which were below the detection threshold of the *UL23* PCR assay (determined at 5,000 copies/ml; data not shown), possibly accentuated by DNA degradation of stored DNA extracts over time.

Of the 254 specimens with fully sequenced HSV-2 *UL23* genes (1128 bp in all cases) we found 99.2-100% nucleotide identity with a total of 492 nucleotide changes in all specimens. Sixty six (66) specimens had a *UL23* gene with one nucleotide change (26.0%), 39 with 2 changes (15.4%), 68 with 3 changes (26.8%), 22 with 4 changes (8.7%), 10 with 5 changes (3.9%) and one with 6 changes (0.4%). These nucleotide changes occurred at 63 different nucleotide positions in the *UL23* gene (5.6% of total *UL23* gene sequence). This represented a nucleotide mutation frequency of 0 to 0.5 (mean 0.2) per 100bp of the *UL23* gene. Forty eight percent (48.0%) of these mutations were silent mutations and did not alter the amino acid composition of the protein. One specimen had two adjacent nucleotide changes at position 904 (A904T) and 905 (A905T) resulting in one amino acid change (N302F; N → F). A total of 54/254 (21.3%) specimens had a *UL23* gene with no nucleotide changes and was found to be identical to the *UL23* gene of HSV-2 reference strain HG52.

The 254 sequenced *UL23* genes shared 97.2-100% amino acid identity and represented 32 amino acid changes (Table 1), with a

Nucleotide change	Amino acid substitution	No. of specimens (%)
Known polymorphism mutations		
G77A	R26H (R → H)	12 (4.7)
G79A	A27T (A → T)	13 (5.1)
T85G	S29A (S → A)	5 (2.0)
G116A	G39E (G → E)	200 (78.7)
G199A	A67T (A → T)	1 (0.4)
T209G	M70R (M → R)	2 (0.8)
A232G	N78D (N → D)	122 (48.0)
G420T	L140F (L → F)	48 (18.9)
C476T	T159I (T → I)	2 (0.8)
G643A	A215T (A → T)	3 (1.2)
G659A	R220K (R → K)	13 (5.1)
A734G	N245S (N → S)	2 (0.8)
C850A	R284S (R → S)	3 (1.2)
Newly described mutations		
G53A	R18H (R → H)	1 (0.4)
C91T	P31S (P → S)	1 (0.4)
C113T	S38F (S → F)	1 (0.4)
G115A	G39K (E → K)	4 (1.6)
C131T	P44L (P → L)	1 (0.4)
C223T	P75S (P → S)	2 (0.8)
C392T	T131M (T → M)	3 (1.2)
G595A	A199T (A → T)	1 (0.4)
C671A	P224Q (P → Q)	1 (0.4)
G721T	D241Y (D → Y)	1 (0.4)
G730A	A244T (A → T)	1 (0.4)
C754T	R252C (R → C)	3 (1.2)
T788C	L263P (L → P)	1 (0.4)
C806T	A269V (A → V)	1 (0.40
G826A	E276K (E → K)	1 (0.4)
G889A	E297K (E → K)	1 (0.4)
A904T/A905T	N302F (N → F)	1 (0.4)
C1099T	R367C (R → C)	3 (1.2)
C1109T	A370V (A → V)	1 (0.4)

Table 1: Nucleotide and amino acid changes in the HSV-2 *UL23* gene detected in this study.

frequency of 0 to 1.6 (mean 0.5) changes per 100 amino acids. We therefore detected an overall amino acid variation of 8.5% of the total codons of the TK protein. We observed several polymorphisms that have been reported previously, specifically R26H, A27T, S29A, G39E, A67T, M70R, N78D, L140F, T159I, A215T, R220K, N245S and R284S (Table 1) [6,12,14]. Nineteen (59.4%) of the amino acid changes observed were new changes and have not been described before. All of these newly-described changes were detected outside conserved domains, and included R18H, P31S, S38F, G39K, P44L, P75S, T131M, A199T, P224Q, D241Y, A244T, R252C, L263P, A269V, E276K, E297K, N302F, R367C and A370V (Table 1). None of these nucleotide sequences have previously been submitted in GenBank as evident by a standard nucleotide BLAST search. All the amino acid changes detected were due to random nucleotide substitutions and no frameshift mutations (insertions or deletions) or mutations causing premature stop codons were detected.

The most common known amino acid variants, all with an allele frequency of >10%, included G39E (200/254; 78.7%), N78D (122/254; 48%) and L140F (48/254; 18.9%) and the most common newly described amino acid changes included G39K (4/254; 1.2%), T131M (3/254; 1.2%), R252C (3/254; 1.2%) and R367C (3/254; 1.2%). The two samples with the P75S variant also contained the G39E, N78D and R284S variants, while all samples with the R252C variant had no additional nucleotide changes. The nucleotide profiles were different in all other cases where there was more than one sample with the same newly described variant.

Among the 300 samples with linked HIV data, 70/88 (79.5%) HIV-negative and 174/212 (82.1%) HIV-positive samples could be sequenced. A total of 56/70 (80.0%) HIV-negative and 144/174 (82.8%) HIV-positive samples had amino acid changes in the *UL23* gene; there was no significant difference by HIV serostatus (p=0.960). Newly described variants R18H, P44L, L263P and A269V were only detected in HIV-negative samples, while variants P31S, S38F, A199T, P224Q, D241Y, A244T, E276K, E297K, N302F and A370V were found exclusively in HIV-positive samples. Only one HIV-positive sample contained more than one newly described variant (D241Y, E297K). No statistically significant association was found between HIV serostatus and the presence/absence of one or more amino acid changes in the *UL23* gene (p=0.587). The number of amino acid changes detected in the *UL23* gene in samples collected before and after the inclusion of ACV in the national treatment regimen were not significantly different (p=0.649).

Discussion

In this paper we described some of the known polymorphism mutations as well as novel mutations identified in the *UL23* gene of HSV-2 from HSV-2 positive ulcer swabs collected as part of the NMS programme. We did not study the DNA *pol* gene in these specimens due to the fact that the vast majority (95%) of cases of ACV resistance results from mutations in the *UL23* gene. Even though no clinical data were available about ACV resistance in these patients and exposure of patients to ACV remains purely potential, we found no evidence of known resistant mutations appearing in the specimens after ACV was introduced as first-line therapy for GUD. We identified 13 known and 19 unknown amino acid changes among 254 HSV-2 positive samples [6,12,14]. None of the unknown mutations detected in this study occurred in conserved areas of the HSV-2 *UL23* gene. Additionally, we identified 30 mutations that resulted in no amino acid change. One of the known polymorphism mutations detected, R220K, occurred in a conserved area of *UL23*, but this variant has been shown to occur in an ACV susceptible strain of HSV-2 [12]. Only one double nucleotide substitution (A904T/A905T) was observed resulting in the amino acid change N302F (N → F). Due to the number of mutations that have been described in the *UL23* genes of HSV isolates, it is sometimes difficult to differentiate between natural polymorphism and resistance mutations, especially when no previous isolate from the same individual was available for comparison after ACV therapy. Genotypic characterization of ACV resistance mutations in the *UL23* gene due to single or double nucleotide substitutions are diagnostically less conclusive and only frameshift mutations and mutations causing stop codons can be interpreted as resistance-causing mutations with

certainty. A number of studies have investigated correlations between phenotypes and genotypes of HSV-2 in order to establish a database of resistance-associated mutations in HSV-2 TK [12,14-18]. Although all the newly described amino acid changes were detected outside the TK resistance conserved domains and were most likely natural polymorphism mutations, their association with resistance should ideally be confirmed with phenotypic findings [19,20]. It is, however, unlikely that natural polymorphism mutations would affect active and conserved sites in HSV-2 TK [21]. There are currently limited HSV-2 ACV resistance data available from Africa. In agreement with our study findings, Watson-Jones et al. [6] failed to detect any ACV resistance mutations in the HSV-2 *UL23* gene of samples originating from South Africa, Zimbabwe, Zambia, Tanzania, Kenya and Uganda (HPTN 039 trial).

ACV-resistant HSV sometimes develop spontaneously, even in patients who had never been treated with ACV [22]. ACV resistance in HSV can also emerge very rapidly during the course of treatment in immunocompromised patients [23,24]. Given the fact that HSV infections with reduced susceptibility to ACV are usually higher in immunocompromised HIV-positive individuals, it was reassuring that we did not find any obvious ACV TK resistance mutations in our predominantly HIV seropositive study population. Even though the majority of the newly described TK mutations were found exclusively in HIV-positive samples this phenomenon most likely coincides with the increased frequency of TK resistance-associated mutations in immunocompromised individuals [14]. HIV viral load data and CD4 counts were not available for the samples analysed in this study and therefore it was not possible to assess the level of immunosuppression in these individuals. Possible explanations could be that these participants had not been sufficiently exposed to ACV, given the relative recent introduction of ACV for GUD treatment in South Africa's public sector clinics, or alternatively that only few of our population of HIV-infected patients were severely immunocompromised.

The present study had some limitations. Firstly, the study was undertaken in Johannesburg and is therefore not nationally representative. Secondly, as dry ulcer swabs were collected for the NMS programme, we were unable to isolate and culture the HSV-2 viruses and therefore we were unable to perform any phenotypic testing on the 19 HSV-2 positive samples with unknown mutations. Thirdly, for all samples collected between 2009 and 2011 the ulcer swabs were collected prior to patients receiving any ACV treatment. Due to the fact that the samples were initially collected for a GUD etiological survey no follow-up samples were collected after ACV therapy. We therefore were unable to determine if ACV resistant virions emerged during ACV therapy. Lastly, clinical isolates of HSV usually contain a mixture of wild-type and ACV-resistant viruses and it is possible that any minority resistant variants that were present were missed with conventional Sanger sequencing [25,26]. For future studies, ultra-deep next generation sequencing could be used to study these minority sequence variants.

In conclusion, our study demonstrated a high degree of polymorphism in the HSV-2 *UL23* gene encoding TK and the absence of mutations directly related to ACV resistance. It is important to report these new mutations to aid in the creation of an integrated database on HSV-2 TK mutations that will enable researchers to categorize and differentiate natural polymorphism mutations from resistance mutations and minimize the need to perform additional phenotypic analyses. Direct characterization of HSV-2 TK can thus be used as an indicator for possible ACV resistance in HSV-2. From the data presented in this study we can conclude that no evidence was found of known ACV resistance mutations in HSV-2 before and following the addition of ACV as first-line therapy for genital ulceration in Johannesburg, South Africa.

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