Input of recombinant phenotyping for the characterization of a novel acyclovir- resistance mutation identified in a patient with recurrent herpetic keratitis

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Abstract

We report here a case of an immunocompetent patient suffering from recurrent epithelial herpetic keratitis associated with the emergence of antiviral resistance. Indeed, the not previously described amino acid change L340R within herpes simplex virus thymidine kinase, was shown to confer acyclovir-resistance by recombinant phenotyping using bacmid technology.

Abstract - word count: 48

Ocular manifestations related to herpes simplex virus 1 (HSV-1) are a major cause of visual morbidity worldwide. HSV-1 infection can involve both anterior and posterior eye segments, but most commonly, it consists in a corneal epithelial dendritic ulcer named herpetic keratitis (HK). Recurrent episodes frequently occur in the majority of patients after the first episode of HK and can lead to visual loss, due to corneal scarring and neovascularization. HK process manifests clinically as a painful infection with raised dendritiform lesions easily visible using fluorescein staining (Tsatsos et al., 2016). Therapeutic intervention of HK involves systemic or topical treatments with antivirals. Antiviral combination regimens or corticosteroid adjuncts may be used to enhance healing and improving outcomes. The use of an antiviral therapy for management of HK recurrent episodes is based on acyclovir (ACV) or its prodrug, valacyclovir (VACV). Trifluridine (TFT) is the most widely used topical antiviral agent. An ophthalmic gel preparation of ganciclovir (GCV) is also approved as a topical antiviral treatment, both safe and equally effective as ACV. Topical GCV could be used as an alternative in ACV-resistance cases. However, GCV, as ACV, is primo-activated by the viral thymidine kinase (TK) and, unfortunately, ACV-resistant isolates show frequently cross-resistance to GCV. Foscarnet (FOS) is an alternative antiviral agent for treating HK. In association with topical antiviral therapy, the HK medical care may also include corneal debridement (Tsatsos et al., 2016).

Management of patients who experience HK recurrences despite antiviral prophylaxis is challenging, as these recurrences may be due to viral resistance, pharmacokinetics, poor drug absorption, or poor patient compliance. Long-term antiviral exposure to (V)ACV may facilitate the selection of resistant HSV-1 (Rousseau et al., 2017; van Velzen et al., 2013). Initially mainly reported in immunocompromised patients, HSV-1 resistance to antivirals was

subsequently reported with high prevalence in immunocompetent patients (Burrel et al., 2013b; Duan et al., 2009).

ACV requires activation by the virus-encoded TK (UL23 gene) and targets the viral DNA polymerase (UL30 gene) to hinder viral genome replication by a chain termination mechanism. FOS directly inhibits the viral DNA polymerase (UL30). Mutations conferring resistance to antivirals have been mapped both in UL23 and UL30 genes. However, TK alterations account for 95% of ACV-resistance cases. Genotypic assays, based on UL23 and UL30 gene sequencing, allow the determination of HSV resistance to antivirals in a clinically relevant time frame (Burrel et al., 2010; Piret and Boivin, 2016). If the viral isolate can be recovered in cell culture from the clinical sample, a phenotypic assay (i.e., plaque reduction assay [PRA] with the determination of efficient concentration 50% [EC_{50}]) may be performed. Briefly, the susceptibility of HSV-1 clinical isolates to antiviral drugs was determined by PRA in Vero cell culture. Laboratory strain KOS (HSV-1) was used as antiviral susceptible control. HSV-1 susceptibility was tested towards ACV (Merck, Lyon, France) and FOS (AstraZeneca, Rueil-Malmaison, France). HSV isolates were considered to be resistant at EC_{50} values ≥ 7 µM and 330 µM for ACV and FOS, respectively (Burrel et al., 2010). PRA is a second-line phenotypic assay that is performed when the results of the genotypic assay are not conclusive due to not previously described or undefined mutations (Burrel et al., 2013a). However, diagnosis of HSV resistance to antivirals is challenging since the contribution of many mutations remains to be determined.

Case report

We report here a case of a 43-year-old man with a long history of recurrent epithelial HK successfully cured over past decades. Noteworthy, the patient has a corneal graft in 2006.

The detailed timeline of clinical evolution, successive preventive and curative treatments, and results is presented in Figure 1. On October 23rd, 2017 (day 0), he presented with ocular redness and decreased vision. Dendritic HK was diagnosed and corneal swabs collected for virological investigation were positive for HSV-1 by real-time PCR. After corneal epithelial debridement, he received oral VACV and topical corticosteroids. Since clinical symptoms remained unchanged, antiviral therapy was modified with successive adjunction of topical GCV and TFT. Despite well-conducted treatments, new dendritic lesions appeared (day 8 to day 49), raising the suspicion of resistance to ACV. As corneal scrapping was still positive for HSV-1 by real-time PCR, full-length TK and DNA polymerase viral genes were amplified and sequenced for genotypic antiviral resistance testing, as previously described [6]. Apart from natural polymorphisms (N23S, K36E R89Q, G240E, and A265T within viral TK, and S33G, A330R, V905M, and T1208A within viral DNA polymerase), viral TK harbored the not previously described amino acid change L340R potentially conferring ACV-resistance as this is closely located to C336Y, a well-known amino acid change accounting for ACV-resistance (day 49). As the genotypic assay was not conclusive, due to this undefined mutation, a phenotypic assay was performed. Indeed, this novel L340R change was associated with resistance to ACV according to PRA (EC_{50} > 7µM) whereas FOS susceptibility was not affected (EC_{50} < 66 µM) (Table 1). Intravenous FOS treatment was started on day 50 and healing rapidly occurred after 4 days. TFT regimen was reintroduced at discharge in association with topical antibiotics and corticosteroids to ensure complete healing. Retrospective genotypic analysis showed that L340R change appeared under antiviral selection pressure. Indeed, only natural polymorphisms were detected in samples collected on day 0 and the clinical isolate recovered at that time was sensitive to ACV (EC_{50} = 1.3 µM) (Table 1). In hindsight, we used bacmid technology for recombinant phenotyping to characterize the contribution of L340R

change in HSV-1 TK in ACV resistance. In order to formally assess the role of L340R change, the entire TK gene with the not previously described mutation was amplified by PCR from the ocular sample and was introduced directly into HSV BAC vector (kind gift from J. Cohen, NIH/NIAID, US), previously deleted from the native TK gene, by homolog recombination and positive selection (Boutolleau D, 2017). The integrity of the entire TK gene including the desired nucleotide substitution was validated by sequencing UL23 TK gene of the modified-bacmid construction. Vero cells were used for BAC-modified transfection and generation of recombinant HSV. Then, the generated recombinant HSV was phenotyped for ACV and FOS susceptibility by PRA. Recombinant viruses with TKs conferring susceptibility and resistance to ACV were used as controls. Indeed, the recombinant virus with wild-type TK bearing only well-known natural polymorphisms was sensitive both to ACV (EC_{50} = 2 µM) and FOS (EC_{50} < 66 µM), whereas recombinant virus TK-deleted associated with a TK-deficient phenotype was resistant to ACV (EC_{50} > 50 µM) and sensitive to FOS (EC_{50} < 66 µM). L340R-TK recombinant isolate was shown to be resistant to ACV (EC_{50} > 7µM) and sensitive to FOS (EC_{50} < 66 µM), highlighting the role of L340R change in resistance to ACV (Table 1).

Discussion

HK is an important cause of visual impairment potentially leading to corneal blindness. Prophylaxis with (V)ACV significantly reduces the recurrence rate of HK. Combined therapies of anti-HSV drugs such as systemic and topical antivirals together with corticosteroids are associated with significant reduced disease burden and duration. This case emphasizes the possible emergence of HSV-1 resistance to ACV among immunocompetent patients with recurrent HK, as previously reported (Burrel et al., 2013b; Duan et al., 2009). Resistance to antiviral has to be promptly detected in order to switch antiviral treatment to avoid corneal morbidity associated with recurrent HK. Currently used method for the diagnosis of ACV

resistance mainly relies on genotypic assay using Sanger sequencing method for the detection of mutations conferring drug resistance in a clinically relevant time frame (Burrel et al., 2010; Piret and Boivin, 2016). However, an exhaustive database of drug-resistance mutations and natural polymorphisms has not been established so far for the interpretation of genotypic assay. Moreover, because of the presence of mutations of unknown role, less than 65% of HSV antiviral susceptibility profile could be determined readily by genotypic assay (Burrel et al., 2013a). The involvement of newly detected HSV TK mutations in resistance to ACV may be determined by functional assays with the measure of enzymatic activity of recombinant TKs. However, the roles of individual mutations in HSV drug resistance have to be formally assessed by recombinant phenotyping [7]. The use of BAC-cloned genomes of cytomegalovirus (CMV), varicella-zoster virus (VZV), and HSV constitutes the gold-standard strategy to assess the formal role of unknown mutations potentially implicated in drug resistance (Boutolleau D et al., 2017; Brunnemann et al., 2016, 2015; Lurain and Chou, 2010). Classically, each mutation of interest is generated by site-directed mutagenesis into a wild-type gene, previously cloned into a plasmid, from a susceptible viral reference strain. Modified gene is then transferred into a BAC vector. To our knowledge, this study reports for the first time the direct transfer of the entire TK gene from a HSV-1 clinical isolate into a BAC vector. This strategy has the advantage to take into account the overall phenotypic contribution of the entire profile of amino acid changes and to study the potential compensatory role of mutation towards antiviral resistance or replicative properties. In conclusion, the present study highlights an original recombinant phenotyping approach in order to assess the role of the novel amino acid change L340R identified in TK of HSV-1 clinical isolate recovered from a patient with recurrent HK.

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Transparency declaration

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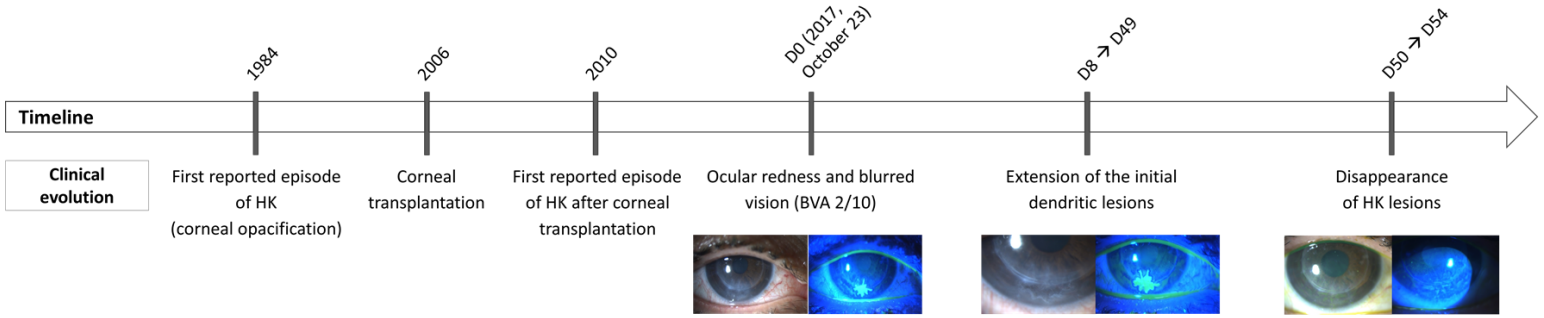
Figure 1



Clinical evolution	First reported episode of HK (corneal opacification)	Corneal transplantation	First reported episode of HK after corneal transplantation	Ocular redness and blurred vision (BVA 2/10)	Extension of the initial dendritic lesions	Disappearance of HK lesions
Treatments	No data	VACV prophylactic regimen (1 year)	VACV curative regimen	First corneal epithelial debridement Initial treatment: VACV 1g×3/day Artificial tears Antibiotics Topical CS	Second corneal epithelial debridement Successive regimens without efficacy: Oral ACV alone Oral ACV + topical GCV Oral ACV + topical TFT VACV + topical TFT Topical antibiotics and CS	Third corneal epithelial debridement Switch of antiviral treatment: Intravenous FOS Artificial tears Topical antibiotics and CS TFT regimen reimplemented at discharge associated with topical antibiotics and CS
Virological investigations	No sampling	No sampling	No sampling	HSV-1-positive corneal swab Genotypic assay*: No TK nor DNA polymerase mutation associated with antiviral resistance Phenotypic assay (PRA)*: ACV-S (EC_{50} = 1.3 µM) FOS-S (EC_{50} < 66 µM)	HSV-1-positive corneal swabs Genotypic assay*: - Newly identified mutation within TK (L340R) potentially associated with ACV-R - No DNA polymerase mutation associated with antiviral resistance Phenotypic assay (PRA)*: ACV-R (EC_{50} = 14.1 µM) FOS-S (EC_{50} < 66 µM)	HSV-1-positive corneal swab HSV recombinant virus: - Transfer of full-length TK (containing L340R mutation) recovered from clinical sample into a HSV BAC vector♦ - Recombinant virus susceptibility testing using PRA* ACV-R (EC_{50} = 31.4 µM) FOS-S (EC_{50} < 66 µM)

Figure 1 legend. Timeline of clinical evolution, treatments and virological investigations

The corneal ulcer (epithelial dendritiform lesion) evolution is visible with or without fluorescein staining on the presented pictures. * Genotypic HSV-1 resistance was tested by Sanger sequencing of TK and DNA polymerase genes, as previously described (Burrel et al., 2010). Phenotypic susceptibility to acyclovir (ACV) and foscarnet (FOS) was determined by plaque reduction assay (PRA). HSV-1 isolates were considered to be resistant at effective concentration 50% (EC_{50}) values ≥ 7 µM and ≥ 330 µM for ACV and FOS, respectively (Burrel et al., 2010). ♦ The entire TK gene with the not previously described amino acid change L340R was amplified by PCR from the ocular sample recovered from the patient and was introduced directly into HSV BAC vector (kind gift from J. Cohen, NIH/NIAID, US), previously deleted from the native TK gene, by homolog recombination and positive selection [9]. The integrity of the entire TK gene including the desired nucleotide substitution was validated by sequencing UL23 TK gene of the modified-bacmid construction. Vero cells were used for BAC-modified transfection and generation of recombinant HSV. Then, the generated recombinant HSV was phenotyped for ACV and FOS susceptibility by determining EC_{50} by PRA. ACV-R: acyclovir-resistant; BAC: bacterial artificial chromosome; BVA: binocular visual acuity; CS: corticosteroids; D: day; EC_{50}: antiviral 50% effective concentration; FOS-S: foscarnet-susceptible; GCV: ganciclovir; HK: herpetic keratitis; TFT: trifluridine; TK: thymidine kinase; VACV: valacyclovir.

Table 1. Summary of TK and DNA mutations within all tested isolates

Virus	Mutation in TK	Mutation in DNA polymerase	ACV phenotype (PRA)
Clinical isolate (day 0)	NP: N23S, K36E R89Q, G240E, A265T	NP: S33G, A330R, V905M, T1208A	Sensitive EC_{50} = 1.3 µM
Clinical isolate (day 49)	NP: N23S, K36E R89Q, G240E, A265T Unknown[a]: L340R	NP: S33G, A330R, V905M, T1208A	Resistant EC_{50} = 14.1 µM
Laboratory reference strain KOS	NP: N23S, K36E, R89Q, V138I, A265T	NP: S33G, A330R, V905M, P1124H, T1208A	Sensitive EC_{50} < 1 µM
L340R-TK recombinant virus	NP: N23S, K36E R89Q, G240E, A265T Unknown[a]: L340R	NA	Resistant EC_{50} = 34.1 µM

[a] Novel mutation L340R no previously reported as a natural polymorphism or a mutation associated with resistance to ACV. Viral isolates were considered to be resistant at effective concentration 50% (EC_{50}) values ≥ 7 µM and ≥ 330 µM for ACV and FOS, respectively (Burrel et al., 2010). ACV: acyclovir; EC_{50}: efficient concentration 50%; NA: not applicable; NP: natural polymorphism; PRA: plaque reduction assay; TK: thymidine kinase.