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CRISPR/Cas9-mediated genome editing of the thymidine kinase gene in a clinical HSV-1 isolate identifies F289S as novel acyclovir-resistant mutation

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ABSTRACT

Herpes simplex virus type 1 (HSV-1) is a neurotropic alphaherpesvirus that establishes a lifelong infection in sensory neurons of infected individuals, accompanied with intermittent reactivation of latent virus causing (a) symptomatic virus shedding. Whereas acyclovir (ACV) is a safe and highly effective antiviral to treat HSV-1 infections, long-term usage can lead to emergence of ACV resistant (ACV^R) HSV-1 and subsequently ACV refractory disease. Here, we isolated an HSV-1 strain from a patient with reactivated herpetic eye disease that did not respond to ACV treatment. The isolate carried a novel non-synonymous F289S mutation in the viral *UL23* gene encoding the thymidine kinase (TK) protein. Because ACV needs conversion by viral TK and subsequently cellular kinases to inhibit HSV-1 replication, the *UL23* gene is commonly mutated in ACV^R HSV-1 strains. The potential role of the F289S mutation causing ACV^R was investigated using CRISPR/Cas9-mediated HSV-1 genome editing. Reverting the F289S mutation in the original clinical isolate to the wild-type sequence S289F resulted in an ACV-sensitive (ACV^S) phenotype, and introduction of the F289S substitution in an ACV^S HSV-1 reference strain led to an ACV^R phenotype. In summary, we identified a new HSV-1 TK mutation in the eye of a patient with ACV refractory herpetic eye disease, which was identified as the causative ACV^R mutation with the aid of CRISPR/Cas9-mediated genome engineering technology. Direct editing of clinical HSV-1 isolates by CRISPR/Cas9 is a powerful strategy to assess whether single residue substitutions are causative to a clinical ACV^R phenotype.

Herpes simplex virus type 1 (HSV-1) is an endemic human pathogen that impacts global populations with mild to severe health complications. Herpes simplex viruses are neurotropic alphaherpesviruses that establish life-long latency in the trigeminal (HSV-1) or sacral ganglia (HSV-1 and HSV-2) of infected individuals (Liu, 2014). Whereas primary infections are generally asymptomatic, recurrent HSV infections can result in vesicular eruptions, primarily in the orolabial and genital mucosa. Severe infections can cause sight-threatening inflammation of the eye and even life-threatening infection of the brain (Ludlow et al., 2016).

The treatment of choice for HSV-1 infection is the antiviral agent acyclovir (ACV), a highly selective drug that inhibits alpaherpesvirus infections with limited side effects (Elion et al., 1977). ACV is a prodrug converted by the viral thymidine kinase (TK, *UL23*) to ACV-monophosphate, which is subsequently converted by cellular kinases to the active compound ACV-triphosphate (Gentry et al., 2011; Shiraki, 2018). ACV-triphosphate acts as a substrate for the viral DNA polymerase (DNA *pol, UL30 gene*) resulting in termination of viral DNA synthesis (Reusser, 1996). Long-term therapy with ACV may lead to the emergence of ACV resistant (ACV^R) HSV-1, especially in immunocompromised individuals and patients with herpetic eye diseases, causing ACV refractory disease (Piret and Boivin, 2011; van Velzen et al., 2013).

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Over 95% of ACV^R HSV-1 cases are caused by mutations in TK, while mutations in the DNA *pol* account for the remaining 5% (Burrel et al., 2010). As an alternative treatment, foscarnet (FOS) is the approved second-line antiviral drug to treat HSV-1 infection (Verdonck et al., 1993). FOS directly inhibits the viral DNA polymerase by binding close to the pyrophosphate binding site that is required for polymerase activity (Deray et al., 1989). Besides antivirals that act on the viral DNA polymerase, sulfonamides that target the viral helicase-primase complex are also promising anti-HSV-1 compounds (Kleymann et al., 2002).

Antiviral resistance analysis is conventionally carried out by phenotypical analysis using *in vitro* culture assays in the presence/absence of antiviral compounds (Sauerbrei et al., 2015). Sequencing of the coding regions of the HSV-1 *UL23* and *UL30* genes and subsequent comparison with databases of known inter-strain variability (not causing antiviral resistance) and mutations causing drug resistance allows for linking of HSV-1 genotypes with drug resistance phenotypes. Web based mutation resistance analyzers, such as the open platform mutation resistance analyzer (MRA) hosted by the University of Ulm (Chevillotte et al., 2010) aid in these studies. However, presence of novel non-synonymous mutations requires phenotypical analysis to differentiate between natural occurring inter-strain variability and drug resistance mutations. *In vitro* genetic evaluation of novel ACV^R mutations is typically carried out by generating recombinant viruses with e.g. Bacterial Artificial Chromosome technology (Brunnemann et al., 2018;

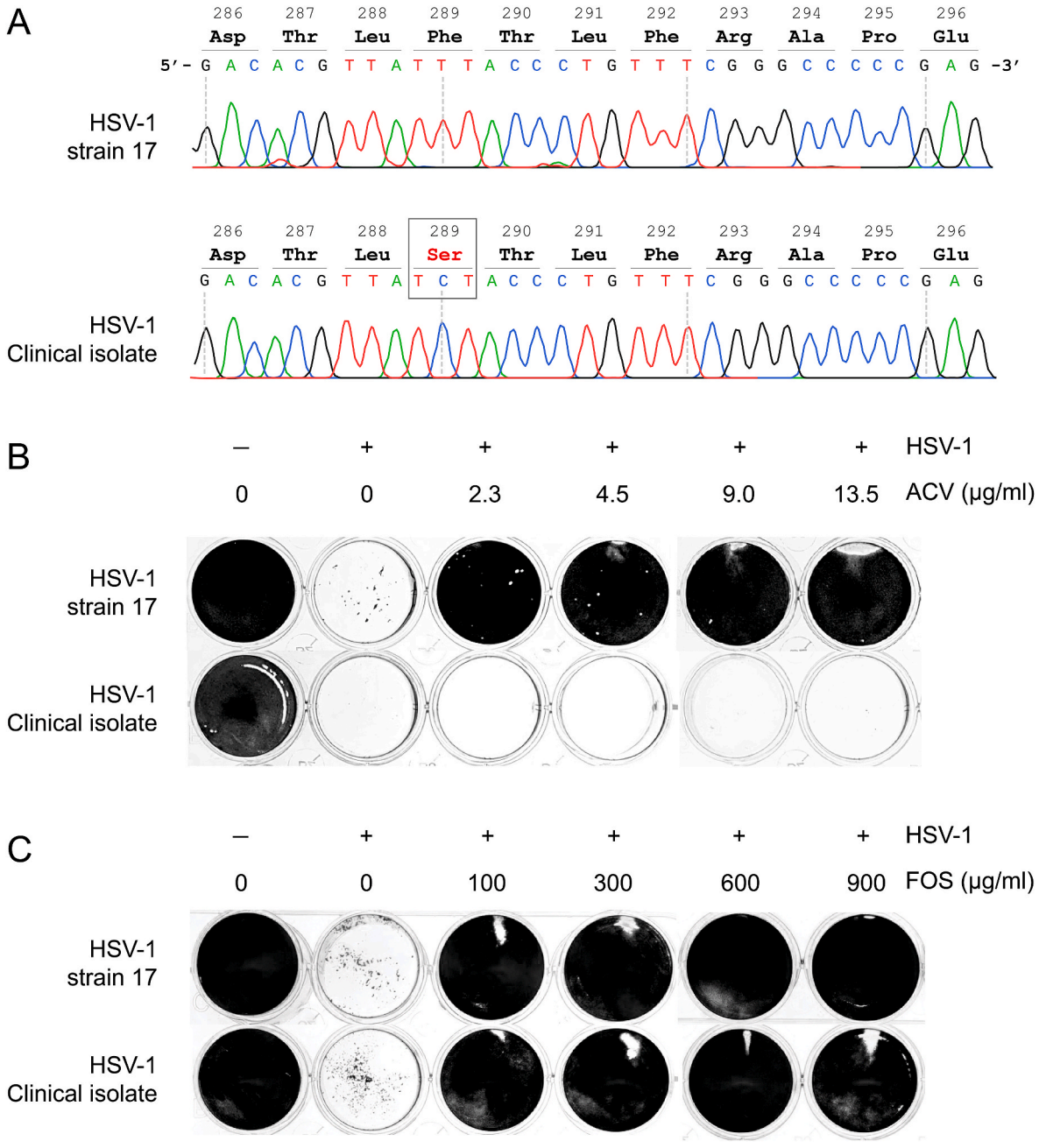

Fig. 1. The clinical HSV-1 isolate is resistant to Acyclovir and harbors a novel F289S mutation in the TK protein.
(A) Sanger sequencing traces of the clinical HSV-1 isolate and the reference HSV-1 strain 17. (B) Visualization of plaque reduction assay. HSV-1 strain 17 and HSV-1 clinical strain were cultured in Vero E6 cells in the presence of various concentrations of acyclovir (0, 2.3, 4.5, 9.0, 13.5 μg/ml). Cells were fixed and stained with crystal violet to visualize surviving cells at 6 dpi. Negative control: Vero E6 cells in absent of HSV-1 and ACV (0 μg/ml). Positive control: Vero cells in the presence of HSV-1 (MOI 0.01) only. (C) Same as in B), yet Foscarnet (0, 100, 300, 600, 900 μg/ml) was used instead of acyclovir.

Frobert et al., 2005; Sergerie and Boivin, 2006). Although powerful, this technology is time consuming and limited by the availability of BAC clones for a given virus, impeding its use to engineer mutations in clinical isolates. In comparison, recent development of the clustered regularly interspaced short palindromic repeats (CRISPR)–associated (Cas) system (CRISPR/Cas9) has allowed for directed mutagenesis of HSV-1 genomes (Oh et al., 2019; Van Diemen et al., 2016; Yin et al., 2021; Zhen et al., 2014) and could serve as a technique to directly engineer desired mutations in virus isolates that lack BAC clone availability. By reverting individual (or combinations of) non-synonymous mutations in HSV-1 *UL23* and *UL30* genes to their wildtype sequence, one can directly assess the contribution of these mutations to the clinical isolate's ACV^R phenotype in the context of an clinical isolate infection.

Here, we report on a 75 year old male who presented at an ophthalmology clinic with HSV-1-induced keratouveitis of the right eye. The patient experienced recrudescent herpetic eye infections in the previous years, which were successfully treated with topical ACV ointment in combination with oral valacyclovir. In this new episode of active disease, however, the patient developed a ACV refractory herpetic keraouveitis. A corneal swab was obtained for drug resistance testing. Sanger sequencing identified G6C, P42L, D77N, G240E, C251G, R281Q, and S321P substitutions in the isolate's *UL23* gene, which are described as natural polymorphisms not related to ACV resistance (Bestman-Smith et al., 2001; Chibo et al., 2004; Morfln et al., 2000). In addition, we identified a novel F289S substitution in *UL23* (Fig. 1A). The F289S substitution maps to a conserved region in HSV-1 thymidine kinase where several drug-resistance substitutions have been described (Kaspar et al., 2017). No drug resistance substitutions were identified in the isolate's *UL30* gene by Sanger sequencing.

Phenotyping of the clinical isolate showed resistance to acyclovir but not to foscarnet (Fig. 1B and C respectively), whereas the HSV-1 reference strain 17 was sensitive to both antiviral compounds (Fig. 1B and C). These observations were confirmed at RegaVir (Leuven, Belgium) where the clinical isolate was phenotyped on human embryonic lung fibroblasts using cytophatic effect (CPE) as read-out. Subsequently, a fold-change was calculated by comparing the effective concentration at 50% (EC_{50}) of the clinical isolate to the HSV-1 reference strain KOS. The clinical isolate tested resistant to acyclovir (fold-change (FC) in EC_{50} > 426], penciclovir (FC > 240), brivudine (FC > 662) and ganciclovir (FC > 643), and susceptible to foscarnet (FC 0.69), cidofovir (FC 0,69), adefovir (FC 1.06), and trifluridine (FC 1,71) (see Table 1).

To confirm whether the F289S substitution is the amino-acid change that causes the isolate's ACV^R phenotype, the mutation was reverted to the wildtype sequence by means of CRISPR/Cas9 genome editing. Vero E6 cells were transfected with CRISPR/Cas9 ribonucleoproteins (RNPs) that target *UL23* close to the site of the F289S substitution in the presence of a double-stranded DNA repair template (Fig. 2A). The RNP-transfected Vero E6 cells were subsequently infected with the clinical HSV-1 isolate, and progeny virus was cloned by two rounds of plaque purification. The *UL23* gene of two clones derived from independent targeting reactions were analysed by Sanger sequencing to confirm correct editing of *UL23* (Fig. 2B, and data not shown). Additionally, besides Sanger sequencing, the plaque-purified viruses were subjected to Illumina next-generation sequencing (NGS) of the TK gene to ensure clonalilty of the virus preparations at this locus. The viruses were clonal with >99,6 of Illumina sequence reads in agreement with the expected sequence at the TK gene encoding the amino acid position 289. Subsequent phenotypic characterization of one of the mutated isolates, hereafter referred to as 'clinical isolate S289F', by plaque reduction assay confirmed that the S289F revertant was ACV sensitive, whereas the parental isolate was ACV resistant (Fig. 2C). Additionally, the revertant strain regained sensitivity to drugs that act via TK activity (penciclovir, brivudine, and ganciclovir; Table 2).

Conversely, we used CRISPR/Cas9 genome editing of the ACV^S HSV-1 reference strain 17 to introduce the F289S TK mutation (HSV-1 strain 17 F289S; Fig. 2B). Besides the HSV-1 strain 17 F289S variant, the editing process also generated a mutant HSV-1 strain 17 variant that harbored an 11bp deletion which we identified during the plaque purification procedure. This deletion caused a premature stop-codon at position 302 (HSV-1 strain 17 d11; Fig. 2B), and we included this strain as control in our experiments. The UL30 gene from both strains were identical as compared to the parental virus. Whereas the parental HSV-1 strain 17 was ACV^S (Fig. 2C), introduction of either the F289S TK mutation or the premature TK stop-codon variant induced ACV-resistance (Fig. 2C). Additionally, both mutant strains gained resistance to drugs that act via TK activity (penciclovir, brivudine, and ganciclovir), but not to antivirals that bypass TK activity (foscarnet, cidofovir, adefovir, and trifluridine; Table 2).

To evaluate whether CRISPR/Cas9 editing of the viral genomes resulted in a differential viral fitness, we performed dual infection competition assays in the absence and presence of acyclovir (Fig. 2D). For this we mixed the clinical isolate with the CRISPR/Cas9-edited clinical isolate S289F and infected Vero E6 target cells in the absence and presence of acyclovir. Without antiviral pressure, both viruses replicated equally well, suggesting that CRISPR/Cas9 editing of the TK gene did not significantly impact viral fitness. However, the clinical isolate showed increased replication fitness under pressure of acyclovir (40,07 (±1,83)% to 99,16 (±0,22)%, p = 0,0021), which is agreement with our previous experiments. Conversely, dual infection competition assays with wildtype strain 17 and CRISPR/Cas9-edited strain 17 F289S showed increased replication fitness of the F289S mutant in the presence of acyclovir (78,08 (±2,58)% to 99,77 (±0,66)%, p = 0,029), whereas in the absence of antiviral pressure both viruses replicated equally well. In summary, we demonstrate that the TK F289S mutation in the clinical HSV-1 isolate is the amino-acid change responsible for the observed ACV^R phenotype.

In the current study, we illustrate that the CRISPR/Cas9 system is a powerful technique to direct specific mutagenesis in the genome of a clinical HSV-1 strain. By reverting candidate non-synonymous mutations of *UL23* or *UL30* in ACV^R HSV-1 isolates by usurping CRISPR/Cas9 technology, one can subsequently assess whether these are causative for the observed ACV^R phenotype. We believe that this technology could be broadly applied to experimentally validate candidate drug resistance mutations for HSV-1, but also other dsDNA viruses, thereby expanding

Table 1
Resistance properties of the clinical isolate F289S compared to KOS reference strain.

	Clinical isolate	Kos	Fold change (Clinical/Kos)
ACV (Acyclovir)	≥18.2 (±2,9)	0,043 (±0,021)	**≥426,7**
PCV (Penciclovir)	≥15.8 (±6,6)	0,066 (±0,027)	**≥240,4**
BVDU (Brivudine)	≥7.3 (±4,23)	0,011 (±0,011)	**≥662,1**
GCV (Ganciclovir)	≥1.64 (±0,60)	0,0025 (±0,0015)	**≥643,2**
PFA (Foscarnet)	20,04 (±7,9)	28,96 (±9,69)	0,69
CDV (Cidofovir)	0,34 (±0,19)	0,49 (±0,48)	0,69
PMEA (Adefovir)	17,46 (±8,2)	16,51 (±5,30)	1,06
TFT (Trifluridine)	1,04 (±0,93)	0,61 (±0,29)	1,71

EC_{50} (μg/ml) values ± SD are presented as measured by viral cytopathic effect (CPE) reduction assays from 7 independent assays.

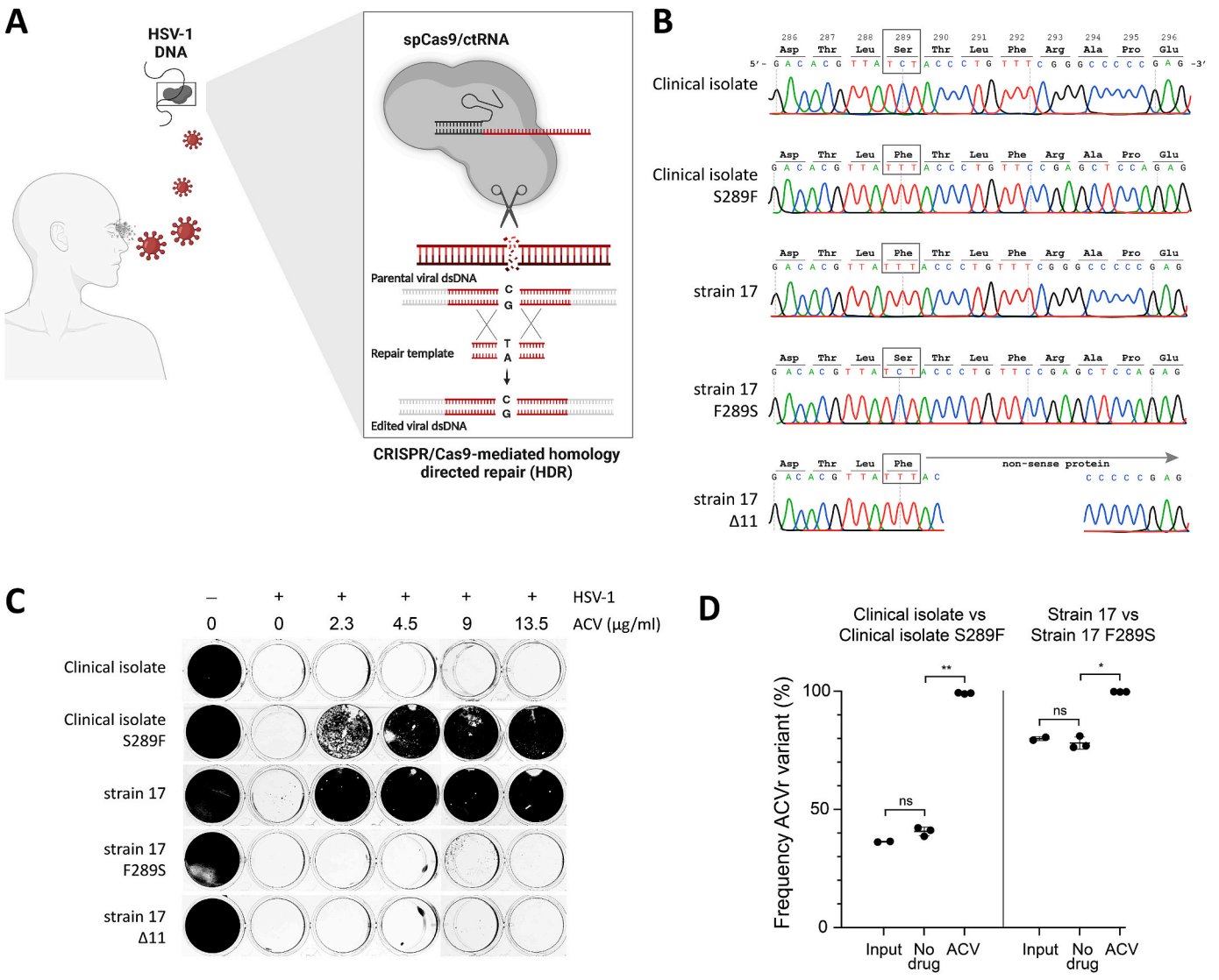

Fig. 2. CRISPR/Cas9-mediated genome editing of the clinical isolate identifies TK F289S as a ACV^R substitution.
(A) Schematic overview of the CRISPR/Cas9-mediated genome editing procedure of the clinical HSV-1 strain. CRISPR/Cas9 RNPs are introduced in Vero E6 cells together with dsDNA repair templates carrying the desired mutations. Upon infection by HSV-1, Cas9 cleaves the viral TK gene close at the F289S locus, triggering homologous recombination thereby incorporating the dsDNA repair template at the lesion. ctRNA: complex of crRNA = tracrRNA (B) Sanger sequencing traces of the parental and CRISPR/Cas9-aided generated HSV-1 strains. The amino-acid changes at position 289 are indicated; additional silent mutations were engineered in the viral genomes to prevent CRISPR/Cas9-mediated genome cleavage upon successful recombination. The HSV-1 strain 17 Δ11 strain carries an 11 bp deletion, resulting in a non-sense protein sequence downstream of the 289 position and yielding a stop codon at the 302 position. (C) Visualization of plaque reduction assay. HSV-1 clinical strain, clinical strain_S289F, strain 17, strain 17_F289S, and strain 17_d11 (MOI 0.01) were cultured in Vero E6 cells in the presence of various concentrations acyclovir (0, 2.3, 4.5, 9.0, 13.5 μg/ml). Cells were fixed and stained with crystal violet to visualize surviving cells at 6 dpi. Negative control: Vero cells in absent of HSV-1 and ACV (0 μM). Positive control: Vero cells in the presence of HSV-1(MOI 0.01) only. (D) Dual infection competition assays of parental HSV-1 versus CRISPR/Cas9-edited viruses. Competition of clinical isolate versus the clinical isolate S289F is presented on the left; strain 17 isolate versus the strain 17 F289S is presented on the right. Mixtures of parental vs edited virus were sequenced by NGS prior infection (input) and three separate cultures assessed at 3 dpi in the absence (no drug) and presence of 1 μg/ml ACV. All values represent the means ± SDs of the frequency of the acyclovir-resistant (ACVr) strain over the ACV-sensitive strain. Therefore, for the clinical HSV-1 strain, this denotes the original parental strain, while for strain 17, this indicates the strain carrying the TK F289S mutation. Statistical significance was calculated using a Brown–Forsythe and Welch ANOVA test (**$P < 0.01$; *$P < 0.05$).

the depth of existing mutation resistance databases.

In our hands, CRISPR/Cas9-mediated homologous recombination of HSV-1 was relatively inefficient, yielding the exact desired edit in ±3% of plaque-purified viruses. By further optimizing the design of the target-specific CRISPR RNA (crRNAs), delivery of CRISPR/Cas9 RNP and repair-template, and timing of the HSV-1 infection and harvest, one may increase the efficiency of correct homology directed repair (HDR) in viral genomes. Also, HDR efficiency may be enhanced by transiently inhibiting proteins central to NHEJ, as was described previously by multiple labs (Liu et al., 2018). Besides usurping spCas9 to drive the formation of double-strand DNA breaks, alternative CRISPR/Cas9 approaches could be used to direct specific genome editing of viral DNA genomes, such as prime-editing and DNA base-editing (Villiger et al., 2024).

As a consequence of the CRISPR/Cas9-mediated HDR process, we observed the emergence of viruses harboring insertions or deletions (indels) at the sgRNA target locus, attributable to non-homologous end joining-induced mutagenesis, rather than homologous recombination events. Furthermore, we identified a virus carrying an additional mutation outside of the UL 23 gene in a genomic region lacking a

Table 2
Resistance properties of the CRISPR/Cas9-mediated edited clinical isolate and Strain 17 reference strains.

	Clinical isolate			Strain 17				
	Parental	S289F	Fold change (parental/S289F)	Parental	F289S	Fold change (F289S/parental)	Δ11	Fold change (Δ11/parental)
ACV (Acyclovir)	≥17.8 (±4,4)	0,076 (±0,022)	**≥233,4**	0,023 (±0,012)	≥13.7 (±4,3)	**≥601,5**	≥17,3 (±5,5)	**≥759**
PCV (Penciclovir)	≥20 (±0,0)	0,103 (±0,054)	**≥193,4**	0,051 (±0,019)	≥13,4 (±6,6)	**≥265,3**	≥17,1 (±5,8)	**≥338,7**
BVDU (Brivudine)	≥10 (±0,0)	0,026 (±0,010)	**≥387,6**	0,041 (±0,013)	≥10 (±0,0)	**≥245,4**	≥8,6 (±2,9)	**≥209,9**
GCV (Ganciclovir)	≥1.76 (±0,48)	0,0031 (±0,0012)	**≥575,2**	≤0.0015 (±0,0010)	≥1.61 (±0,39)	**≥1093,5**	≥1.67 (±0,41)	**≥1136,1**
PFA (Foscarnet)	24,95 (±5,56)	24,28 (±4,93)	1,03	37,45 (±15,11)	21,21 (±0,74)	0,57	25,1 (±5,08)	0,67
CDV (Cidofovir)	0,78 (±0,51)	0,37 (±0,023)	2,12	0,20 (±0,12)	0,11 (±0,04)	0,53	0,18 (±0,10)	0,91
PMEA (Adefovir)	32,98 (±12,5)	16,71 (±3,64)	1,97	14,49 (±2,75)	10,11 (±2,26)	0,7	18,23 (±7,40)	1,26
TFT (Trifluridine)	4,22 (±3,30)	0,69 (±0,01)	6,10	1,08 (±0,28)	1,16 (±0,19)	1,07	2,46 (±2,14)	2,27

EC_{50} (μg/ml) values ± SD are presented as measured by viral cytopathic effect (CPE) reduction assays from 4 to 7 independent assays.
*This strain harboured an additional mutation (V521M) in the UL30 gene.

(degenerate) sgRNA target site, suggesting occurrence of a spontaneous mutation during replication or the preexistence of a mixed-strain infection within the clinical sample. This underscores the importance of conducting thorough genetic characterization of isolated viruses post-genetic manipulation to confirm the presence of the intended genetic modifications without unintended alterations. While deep sequencing of viral genes linked to drug resistance is highly advised, complete genome sequencing is preferable to eliminate the possibility of genetic alterations at other loci within the viral genome. Additionally, re-introducing the original genetic edit into a previously modified strain could serve as a crucial control. This reversion helps rule out phenotypic changes due to unintended mutations obtained in the initial modification attempt. In this regard, BAC technology offers an advantage, as cloned viral strains offer a stable DNA construct that can undergo rigorous sequence-checking and be utilized to generate infectious virus, faithfully representing the cloned genome upon initial growth. However, the necessity for cloned viral genomes in BAC vectors restricts the utility of BAC technology, while CRISPR/Cas9-mediated genome engineering offers a versatile method applicable to any dsDNA virus replicating under tissue culture conditions. This substantially broadens the potential for genetic modifications, encompassing those of novel clinical isolates.

To conclude, we have identified a new mutation (F289S) in the HSV-1 TK protein in a patient with recurrent herpetic eye disease who was refractory to ACV therapy. This amino acid substitution was identified as the causative ACV^R mutation with the aid CRISPR/Cas9-mediated genome engineering of the clinical isolate. Direct editing of clinical HSV-1 isolates by CRISPR/Cas9 is a powerful strategy to assess whether single amino-acid substitutions are causative to ACV resistance.

Consent for publication

The authors have obtained consent from the patient to publish individual patient data.

CRediT authorship contribution statement

Shuxuan Zheng: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Georges M.G.M. Verjans:** Conceptualization, Resources, Supervision, Writing – original draft, Writing – review & editing. **Anouk Evers:** Formal analysis, Investigation, Methodology, Validation. **Ellen van den Wittenboer:** Investigation, Methodology, Validation. **Jeroen H.T. Tjhie:** Formal analysis, Investigation, Methodology, Validation. **Robert Snoeck:** Methodology, Resources, Supervision, Writing – review & editing. **Emmanuel J.H.J. Wiertz:** Conceptualization, Supervision, Writing – review & editing. **Graciela Andrei:** Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – review & editing. **Jeroen J.A. van Kampen:** Resources, Writing – review & editing. **Robert Jan Lebbink:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2024.105950.

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