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Takuya Inagaki\textsuperscript{1,2,3}, Masaaki Satoh\textsuperscript{1}, Hikaru Fujii\textsuperscript{1}, Souichi Yamada\textsuperscript{1}, Miho Shibamura\textsuperscript{1,4}, Tomoki Yoshikawa\textsuperscript{1}, Shizuko Harada\textsuperscript{1}, Haruko Takeyama\textsuperscript{2,3}, Masayuki Saijo\textsuperscript{1,2}

\textsuperscript{1}Department of Virology I, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan

\textsuperscript{2}Department of Life Science and Medical Bioscience, Waseda University, Wakamatsucho 2-2, Shinjuku-ku, Tokyo 162-8480, Japan

\textsuperscript{3}Computational Bio Big-Data Open Innovation Laboratory, AIST-Waseda University, Okubo 3-4-1, Shinjuku-ku, Tokyo 169-0072, Japan

\textsuperscript{4}Department of Pediatrics, The University of Tokyo Hospital, 7-3-1 Hongo, Bunkyo-ku,
Tokyo 105-8470, Japan
稲垣拓哉 $^{1,2,3}$, 佐藤正明 $^{1}$, 藤井ひかる $^{1}$, 山田壮一 $^{1}$, 柴村美帆 $^{1,4}$, 吉河智城 $^{1}$, 原田志津子 $^{1}$, 竹山春子 $^{2,3}$, 西條政幸 $^{1,2}$

$^{1}$国立感染症研究所ウイルス第一部, 162-8640 東京都新宿区戸山1-23-1.

$^{2}$早稲田大学先進理工学研究科生命医科学専攻, 162-8480 東京都新宿区若松町2-2.

$^{3}$産総研・早大 生体システムビッグデータ解析オープンイノベーションラボトリ, 169-0072 東京都新宿区大久保3-4-1.

$^{4}$東京大学病院小児科, 105-8470 東京都文京区本郷7-3-1.
Corresponding author: Masayuki Saijo

Tel: +81-3-4582-2660; Fax: +81-3-5285-1150

E-mail: msaijo@nih.go.jp

Keywords

Herpes simplex virus type 1 • Acyclovir • Resistance • Herpes simplex encephalitis • Thymidine kinase

Running head

ACV sensitivity and neurovirulence of HSV-1
Summary

Several cases of herpes simplex encephalitis (HSE) caused by acyclovir (ACV)-resistant herpes simplex virus type 1 (HSV-1) have been reported. Amino acid substitutions of R41H, Q125H, and A156V in the viral thymidine kinase (vTK) gene were reported to confer ACV-resistance. Recombinant HSV-1 clones with each amino acid substitution in the vTK were generated using the bacterial artificial chromosome (BAC) system. A recombinant HSV-1 with the substitution of Q125H showed ACV-resistance, while those of R41H or A156V were sensitive to ACV. Furthermore, a recombinant HSV-1 with the substitution of Q125H was less virulent than the repaired virus, but maintained neurovirulence in mice at relatively high level. Substitution of Q125H, which was detected in the neonatal HSE patient, conferred ACV-resistance, but the substitutions of R41H and A156V, which were detected in the immunocompetent adult HSE patients, did not. This suggests that HSE caused by ACV-resistant HSV-1 during the course of ACV-treatment might be a very rare event in immunocompetent patients. Showing resistance to ACV-treatment does not always indicates emergence of ACV-resistant HSV-1 in HSE patients.
Introduction

Herpes simplex virus type 1 (HSV-1) is a ubiquitous human pathogen that causes a wide spectrum of diseases, including encephalitis [herpes simplex encephalitis (HSE)]. HSE, which occurs relatively frequently in adolescents and elderly individuals, is one of the most common sporadic viral encephalitis (1–3). HSE patients are treated with administration of acyclovir (ACV) (1–3).

ACV is a guanosine analogue molecule that is phosphorylated by the thymidine kinase (TK) of HSV-1 to form ACV-monophosphate (4, 5). ACV-monophosphate is further phosphorylated by cellular kinases to form ACV-triphosphate (ACV-TP) (4, 5). ACV-TP inhibits the viral DNA polymerase activities and is incorporated into the viral DNA, resulting in the termination of viral DNA elongation, showing the inhibitory effect on HSV-1 replication (6).

HSV-1 acquires ACV-resistance due to mutations in the viral TK (vTK) gene or DNA polymerase gene; the former mutation is far more common, with an incidence of approximately 95% (5). ACV-resistant (ACVr) HSV-1 infections occur in immunocompromised patients, such as those who have undergone hematopoietic stem cell transplantation and those with congenital immunodeficiency (7–10).
Recently, there are several reports, in which ACVr HSV-1 were reported to be or suspected as a causative agents of intractable HSE (11–15). HSV-1, in which the glutamine of the 125th residue in the vTK was replaced with histidine (Q125H), appeared in the cerebrospinal fluid (CSF) of a neonate with HSV-1 encephalitis during the course of ACV-treatment, might be ACVr (11). The Q125H amino acid substitution in the vTK was confirmed to confer ACV-resistance by an indirect virological assay (11, 16). Schulte et al. and Bergmann et al., respectively, reported that the R41H and the A156V amino acid substitutions in the vTK, which were detected in the CSF of immunocompetent adult HSE patients, who were resistant to ACV-treatment, might confer ACV-resistance (12, 13). As the emergence of ACVr HSV-1 in those studies were simply confirmed either by the indirect assay or sequence analysis, there is no convincing evidence that the R41H, the Q125H, and the A156V amino acid substitutions in the vTK confer ACV-resistance (11–13).

To confirm whether or not these amino acid substitutions confer ACV-resistance, viruses with each mutation in the same genetic background should be generated and tested for ACV-sensitivities. This might be the only way to evaluate the impact of the mutations on ACV-resistance. In the present study, recombinant HSV-1 clones, which carried each of these amino acid substitutions, were generated using the
bacterial artificial chromosome (BAC) system (17). The ACV-sensitivity of each recombinant HSV-1 clone was measured with a plaque reduction assay (PRA) and the neurovirulence of the ACVr HSV-1 clone was determined in mice.

Materials and Methods

Cells

Vero cells were cultured in Dulbecco’s Modified Eagle Essential Medium (DMEM) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 5% calf serum (CS, Thermo Fisher Scientific, CA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific). COS7 cells were cultured under the same conditions except that the serum used for the culture medium was 10% fetal bovine serum (FBS, Biological Industries, Beit Haemek, Israel).

Viruses

Authentic HSV-1 strain F (HSV-1 F) (18), a kind gift from Dr. Y. Kawaguchi at The University of Tokyo, Japan under permission from Dr. B. Roizman at the University of Chicago, USA, ACVr HSV-1 strain TAR (HSV-1 TAR) (10, 19), which was isolated from an immunocompromised patient, and recombinant HSV-1 clones generated from
the whole genome of HSV-1 F cloned into a BAC (pYEbac102) (17) were propagated in
Vero cells cultured in DMEM supplemented with 1% CS (DMEM-1CS). Cells infected
with each HSV-1 clone were disrupted by sonication treatment on ice (once) or frozen
and thawed 3 times to obtain viral stocks. The viral stocks were obtained from the
supernatant fractions of the medium after centrifugation and were stored at -80°C. The
infectious dose of the viral stocks was determined with a standard plaque assay in Vero
cells and was expressed as plaque forming units (pfu)/ml. The authentic HSV-1 F, which
was used in the present study, had the nucleotide sequences of the vTK and viral DNA
polymerase coding region, both of which were identical to those of the reference
(GenBank accession number: GU734771.1). The nucleotide sequences of those regions
in the plasmid pYEbac102 used in this study and YK304—an HSV-1 clone generated
from the pYEbac102 (17)—were also identical to those of the reference (GU734771.1),
with the exception of the nucleotide substitutions of C25T and C165G in the vTK
coding region, which resulted in amino acid substitutions of H9Y and D55E,
respectively.

Plasmids

To generate a plasmid pBS-delTK, the nucleotide residues 387-749 in HSV-1 F TK gene

were amplified by a polymerase chain reaction (PCR) using the primer sets (Table 1) and cloned into the XhoI and KpnI sites of pBluescript II KS (+) (Stratagene, CA, USA).

To generate a plasmid pBS-delTK-KanR, the I-SceI restriction site and aphA1 gene of pEP-KanS (20)—kindly provided by Dr. Y. Kawaguchi under permission from Dr. N. Osterrieder at Freie Universität Berlin, Germany—were amplified by a PCR using the primer sets (Table 1) and cloned into the PspOMI site of pBS-delTK.

**Construction of recombinant HSV-1**

The recombinant viruses, rHSV-1-R41H, rHSV-1-Q125H, and rHSV-1-A156V, carrying the R41H, Q125H, and A156V amino acid substitutions in their vTK, respectively, were constructed by the two-step Red-mediated mutagenesis procedure using *Escherichia coli* GS1783 containing pYEbac102, a full-length infectious HSV-1 F clone (17)—a gift from Dr. Y. Kawaguchi, with permission for use from Dr. G.A. Smith of Northwestern University, USA and Dr. N. Osterrieder—as described previously with the use of the primer sets (Table 1, Fig. 1) (20, 21).

The recombinant viruses, rHSV-1-R41Hr and rHSV-1-A156Vr, in which the R41H and the A156V amino acid substitutions in rHSV-1-R41H and rHSV-1-A156V
were repaired, respectively, were constructed as described previously with the use of the primer sets (Table 1, Fig. 1) (20, 21).

The vTK activity-deficient recombinant virus, rHSV-1-delTK, in which the nucleotide residues 387-749 in the vTK gene of rHSV-1-Q125H were deleted according to the previous study (22) and in addition the Q125H substitution in that was repaired, was constructed as described previously with the use of the primer sets (Table 1, Fig. 1) (20, 21).

The recombinant virus rHSV-1-Q125Hr, in which nucleotide residues of the vTK gene deleted in rHSV-1-delTK were repaired, was constructed as described previously with the use of a DNA fragment, which was amplified by a PCR from pBS-delTK-KanR using the primer set (Table 1, Fig. 1) (20, 21, 23).

To generate recombinant viruses, COS7 cells were transfected with each BAC containing full-genome of the recombinant HSV-1 using FuGENE6 transfection reagent (Promega, WI, USA).

*In vitro viral replication*

Vero cells were inoculated in triplicate with each virus at a multiplicity of infection (moi) of 5 per cell and incubated for 1 hour at 37°C under 5% CO₂. The cells were then
washed 3 times with phosphate buffered saline solution (PBS) and cultured in DMEM-1CS. The cell cultures were harvested at designated time points and the viral infectious doses were measured by a standard plaque assay in Vero cells.

**Western blotting**

Vero cells were inoculated with mock inoculum or each virus at an moi of 1 per cell, harvested at 24 hours after inoculation, and subjected to Western blotting as described previously with some modifications (19, 24). Rabbit anti-HSV-1 TK antibody (19) and mouse anti-HSV-1 and herpes simplex virus type 2 ICP5 antibody (Abcam, Cambridge, UK), both of which were diluted 1,000 times with T-PBS (PBS with 0.1% Polyoxyethylene(20) Sorbitan Monolaurate (Wako Pure Chemical Industries)), were used as the primary antibodies to detect the vTK and ICP5 polypeptides expressed. Horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG antibody (SeraCare Life Sciences, MA, USA) and HRP-labeled goat anti-mouse IgG antibody (SeraCare Life Sciences), which were diluted 5,000 times and 3,000 times with T-PBS, respectively, were used as the secondary antibodies for detection of the vTK and ICP5 polypeptides, respectively.
The sensitivity of viruses to ACV and other vTK-associated compounds

The sensitivities of the viruses to antiviral compounds including ACV (Tokyo Chemical Industry, Tokyo, Japan), ganciclovir (GCV, Tokyo Chemical Industry) and penciclovir (PCV, Tokyo Chemical Industry) were evaluated in Vero cells with a PRA as described previously (10, 19, 24–27). The 50% inhibitory concentration (IC$_{50}$) of each antiviral compound to each virus was calculated graphically.

The neurovirulence of rHSV-1-Q125H in mice

The neurovirulence of the viruses was measured as described previously (25). In brief, 3-week-old female ICR mice (Japan SLC, Inc., Shizuoka, Japan) were intracerebrally inoculated with the designated infectious doses of rHSV-1-Q125H, rHSV-1-delTK, or rHSV-1-Q125Hr in 50 µl of DMEM under anesthesia with a combination of medetomidine, midazolam, and butorphanol. Three mice were inoculated with viral solution at each dilution level. Survival and the body weight of the mice were monitored daily for 14 days after inoculation and the 50% lethal dose (LD$_{50}$) of each virus was calculated by the Reed and Muench method. When the mice lost more than 20% of their initial body weight, they were euthanized by exposure to excess CO$_2$. The experiments were conducted twice, independently.
Ethical statement

The animal experiments were conducted in strict accordance with the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan and in strict compliance with animal husbandry and welfare regulations under the approval of Animal Care and Use Committee of the National Institute of Infectious Disease (No. 117026).

Results

Generation of recombinant HSV-1 clones with the amino acid substitutions in their vTK

The recombinant viruses were confirmed to not have any additional or unintended changes in their vTK or viral DNA polymerase genes from pYEbac102 and YK304. The 2 amino acid substitutions, H9Y and D55E, in the vTK polypeptide of the all recombinant viruses did not affect the phenotypes of these recombinant viruses with regard to the replication kinetics and the expression of vTK polypeptide in Vero cells, and ACV-, GCV-, and PCV-sensitivities (Fig. 2, Fig. 3, and Table 2).
The growth kinetics and the vTK expression of recombinant HSV-1 clones in Vero cells

All the recombinant HSV-1 clones replicated with the same growth capacity as HSV-1 F (Fig. 2). With the exception of rHSV-1-delTK, the vTK polypeptide expression levels of the recombinant viruses were in line with that of the authentic HSV-1 F (Fig. 3). In contrast with the intact vTK shown in the other lanes, rHSV-1-delTK showed a blurred band between 32 kDa and 27 kDa (Fig. 3).

The sensitivities of rHSV-1-R41H, rHSV-1-Q125H, rHSV-1-A156V, and their revertant viruses to vTK-associated antiviral compounds

The IC$_{50}$ values of ACV, GCV, and PCV to each recombinant HSV-1 clone are shown (Table 2). The IC$_{50}$ values of these antiviral compounds to rHSV-1-R41Hr, rHSV-1-Q125Hr, and rHSV-1-A156Vr were similar to those of HSV-1 F, indicating the two amino acid substitutions, H9Y and D55E in the vTK of all recombinant HSV-1 clones, did not confer ACV-, GCV-, and PCV-resistance. rHSV-1-Q125H, rHSV-1-delTK, and HSV-1 TAR showed ACV-resistance, while rHSV-1-R41H and rHSV-1-A156V did not. rHSV-1-Q125H showed resistance not only to ACV, but also to GCV and PCV.
The neurovirulence of rHSV-1-Q125H in mice

The neurovirulence of rHSV-1-Q125H, rHSV-1-delTK, and rHSV-1-Q125Hr in mice was determined by inoculating intracerebrally with each of the recombinant viruses. The LD$_{50}$ values of rHSV-1-Q125H, rHSV-1-delTK, and rHSV-1-Q125Hr in Experiment 1 were $10^{2.50}$, and $10^{4.25}$, $10^{1.75}$ pfu, respectively, while those in Experiment 2 were $10^{2.75}$, and $10^{4.25}$, $10^{1.75}$ pfu, respectively.

Discussion

The novelty of this study was that recombinant HSV-1 clones with each of the amino acid substitutions, which were suspected of conferring ACV-resistance in HSE patients, were generated using the BAC system and tested not only for ACV-sensitivity, but also for neurovirulence in mice.

All the recombinant viruses carrying each of the mutations in the vTK showed the same growth property as their repaired viruses and wild-type HSV-1 F (Fig. 2), as the vTK gene of HSV-1 is dispensable for viral replication in vitro (25, 28). No intact but probable truncated vTK polypeptide shown as a blurred band between 32 kDa and 27 kDa was expressed in the cells infected with rHSV-1-delTK (Fig. 3). These results indicate that recombinant viruses with each of vTK-mutations in the same genetic
background were generated successfully.

To the best of our knowledge, there are no case reports describing the isolation of infectious HSV-1 from the CSF of HSE patients, showing that the isolation of infectious HSV-1 is quite difficult (2). The difficulty might be due to the presence of neutralizing antibodies in CSF (1, 13), even though HSV-1 replicates in the CNS (11, 13). The difficulty makes it impossible to directly evaluate the sensitivities of the causative HSV-1 to ACV and other antiviral agents. Thus, in the previous reports, the nucleotide sequences of the vTK genes amplified from CSF of the HSE patients with a PCR were determined to speculate whether the causative HSV-1 was ACVr or not based on the previously reported ACV resistance-associated substitutions (11–15).

The patient information described in these reports is summarized in Table 3. The 2 cases of HSE were definitely caused by ACVr HSV-1, as frameshift mutations were detected in the vTK genes amplified from CSF samples collected from a patient with chronic lymphocytic leukemia (CLL) and from another patient receiving anti-tumor necrosis factor (TNF)-alpha monoclonal antibodies (14, 15).

The Q125H amino acid substitution in the vTK conferred ACV-resistance, consistent with the previous study (11). In the neonatal HSE case, HSV-1 genome with the Q125H amino acid substitution in the vTK increased during the course of ACV
therapy (11). The virus appeared in the neonate during the course of ACV-therapy was definitely ACVr HSV-1.

The other 2 amino acid substitutions, R41H and A156V, did not confer ACV-resistance. HSV-1 clones with each of the R41H and A156V amino acid substitutions in the vTK were reported to be ACV-sensitive in previous reports (7, 29–31). Brunnemann et al. generated recombinant viruses, which carried any one of 11 amino acid substitutions including R41H, and showed the R41H amino acid substitution did not confer ACV-resistance (29). This study supports the results that the R41H amino acid substitution did not confer ACV-resistance (29). There is no evidence, therefore, that ACVr HSV-1 had emerged in the immunocompetent adult HSE patients reported (12, 13). So far, the reports describing the emergence of ACVr HSV-1 in the CNS of HSE patients are restricted to the cases of the neonate and the immunocompromised patients (11, 14, 15).

vTK-impaired ACVr HSV-1 tends to reduce the neurovirulence in mice (25, 32, 33). Consistently, rHSV-1-Q125H was less neurovirulent than rHSV-1-Q125Hr; however, it was more virulent than the vTK-deficient and highly attenuated rHSV-1-delTK, suggesting that the ACVr HSV-1 with the Q125H substitution in the vTK that appeared in the neonate with HSE affected the disease at some degree. The result
indicates that the proper selection, modification, and change of antiviral agents are needed for the proper treatment of HSE patients, especially patients with immunodeficiency including neonates. The sequential quantification of the HSV-1 genome in CSF and the determination of the vTK gene sequence should be recommended for such purposes.

The Q125H substitution in the vTK conferred ACV-resistance, but not GCV- or PCV-resistance (11). However, rHSV-1-Q125H showed cross-resistance to GCV and PCV. The result was contradictory to the findings of the previous report (11). This indicates the limitation of indirect assays in measuring sensitivities to GCV and PCV without the use of infectious viruses.

vTK-deficient rHSV-1-delTK, in which nucleotide residues 387-749 in the vTK gene were deleted according to the previous study (22), lacked the expression of functional vTK (Fig. 3), and was expected to reduce the expression of UL24 gene (34, 35). It was reported that the impairment of UL24 polypeptide reduced the viral replication capacity in vitro and in the mouse trigeminal ganglia (28, 36). However, the replication capacity of rHSV-1-delTK was in line with the other viruses including rHSV-1-Q125Hr and HSV-1 F (Fig. 2). Although it was unclear whether reduction of UL24 polypeptide expression in rHSV-1-delTK affected its attenuation or not, the
attenuation of rHSV-1-delTK to neurovirulence was of the same magnitude with that of vTK-frameshifted and -deficient HSV-1 (25, 32, 33).

In summary, These results suggest that the HSE, which shows resistance to ACV-treatment differs from the HSE caused by ACVr HSV-1. HSE caused by ACVr HSV-1 might occur in neonates and immunocompromised patients. The emergence of ACVr HSV-1 in the CNS of immunocompetent adult HSE patients might be a rare event.

Acknowledgements

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Conflict of interest

None to declare.
References


**Figure legends**

**Fig. 1.** Schematic diagrams of the generation of recombinant HSV-1 with amino acid substitution and the repairs. The full-length genome of YK304—an HSV-1 clone generated from the pYEbac102 (17)—in the linear form is presented at the top. The only vTK gene (UL23) of each recombinant virus is depicted in the figure.

**Fig. 2.** The growth kinetics of recombinant HSV-1 clones and HSV-1 F. Vero cells were infected with each virus at an moi of 5 per cell and harvested at the designated time points. The data is shown as a mean and standard deviation calculated from the 2 independent triplicate reactions.

**Fig. 3.** The expression of vTK and ICP5 polypeptides of recombinant HSV-1 clones. Vero cells were infected with each virus at an moi of 1 per cell and harvested at 24 hours after infection for Western blotting. ICP5 was set as the control. The white arrowhead indicates the probable truncated vTK polypeptide.
Table 1. The primer sets used in the present study.

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Generation of rHSV-1-A156Vr

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Table 2. The 50% inhibitory concentration values of the antiviral compounds on each HSV-1 clone.

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<td></td>
<td>Q125H</td>
<td>delTK</td>
<td>Q125Hr</td>
<td>R41H</td>
<td>R41Hr</td>
<td>A156V</td>
<td>A156Vr</td>
<td>TAR</td>
</tr>
<tr>
<td>ACV*1</td>
<td>0.64 ± 0.07</td>
<td>8.5 ± 1.64</td>
<td>10 ± 0.50</td>
<td>0.51 ± 0.01</td>
<td>0.52 ± 0.02</td>
<td>0.51 ± 0.02</td>
<td>0.59 ± 0.02</td>
<td>0.49 ± 0.01</td>
<td>≥40</td>
</tr>
<tr>
<td>GCV*2</td>
<td>0.21</td>
<td>2.5</td>
<td>6.8</td>
<td>0.14</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>PCV*2</td>
<td>1.2</td>
<td>6.3</td>
<td>24</td>
<td>0.79</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

*1: The data indicate the mean ± standard deviation from 2 independent experiments.

*2: The data are representative of 3 independent experiments.

NT: Not tested.
Table 3. Summary of the cases of HSE reported to be due to ACVr HSV-1.

<table>
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<tr>
<td>Year reported</td>
<td>2013</td>
<td>2010</td>
<td>2017</td>
<td>2018</td>
<td>2014</td>
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<tr>
<td>Patient information</td>
<td>Age</td>
<td>Sex</td>
<td>Immunostatus</td>
<td>Mutations in the vTK</td>
<td>Immunostatus</td>
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<td>0 months</td>
<td>Male</td>
<td>Neonate</td>
<td>Q125H</td>
<td>Neonate</td>
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<tr>
<td></td>
<td>27 years</td>
<td>Female</td>
<td>Immunocompetent</td>
<td>R41H</td>
<td>Immunocompetent</td>
</tr>
<tr>
<td></td>
<td>45 years</td>
<td>Female</td>
<td>Immunocompetent</td>
<td>A156V</td>
<td>Immunosuppressed due to CLL</td>
</tr>
<tr>
<td></td>
<td>76 years</td>
<td>Female</td>
<td>Immunosuppressed</td>
<td>Frameshift</td>
<td>Immunosuppressed due to anti-TNF-alpha antibodies treatment</td>
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</table>
|                        | 50 years               | Female         | Immu...
<table>
<thead>
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<th>resistance</th>
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