# The S68G polymorphism is a compensatory mutation associated with the drug resistance mutation K65R in CRF01_AE strains

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# Abstract

Background: The rate of S68G mutation in human immunodeficiency virus type 1 (HIV-1) reverse transcriptase has increased and is closely related to the K65R mutation among treatment-failure CRF01_AE-infected patients. We aim to explore the temporal association of S68G and K65R mutations among treatment-failure CRF01_AE-infected patients and disclose the role of the S68G mutation on nucleotide/nucleoside reverse transcriptase inhibitor (NRTI) susceptibility and viral replication with the K65R double mutation.

Methods: Occurrence of S68G and K65R mutations was evaluated among HIV-1 of various subtypes in the global HIV Drug Resistance Database. The temporal association of S68G and K65R mutations was analyzed by next-generation sequencing (NGS). The impact of the S68G mutation on NRTI susceptibility and replication adaptability was analyzed with pseudovirus phenotypic resistance assays and growth competition assays, respectively.

Results: The frequency of the S68G mutation increased significantly in all HIV subtypes and circulating recombinant forms in treatment-experienced patients (except for subtype F), as did the frequency of the K65R/S68G double mutation. NGS revealed that the S68G mutation occurred following K65R mutation among three out of four patients. No significant difference in fold-change for tenofovir, lamivudine or efavirenz was detected between K65R and K65R/S68G mutations in phenotypic resistance assays. The K65R/S68G double mutant outgrew the K65R mutant within 13 days of co-culture for any input ratio among all patients.

Conclusions: S68G might be a natural polymorphism and compensatory mutation of K65R selected by NRTIs among the CRF01_AE strain of HIV-1, which does not affect NRTI susceptibility, but improves the replication adaptability on K65R mutants.


# Background

Interpretation of Human immunodeficiency virus type 1 (HIV–1) drug resistance mutations (DRMs) arises mainly from the phenotype and genotype studies on subtype-B, which accounts for ~12.1% of cases of HIV infection worldwide [1]. Many non-B subtype HIV–1-infected patients in developing countries receiving ART need more precise interpretation of DRMs [2, 3]. The high genetic diversity of HIV–1 poses great challenges for interpretation of the genotyping of drug resistance.

Several studies have suggested that DRMs might not be fully consistent among different subtypes of HIV–1. For instance, HIV–1 strains of different subtypes can develop various “signature” DRMs under the same ART regimen [4, 5]. Some studies have suggested that natural polymorphisms of non-B subtypes of HIV–1 change the drug susceptibility to ART independently or join with other mutations [6, 7]. More studies are needed to elucidate the role of natural polymorphisms and some treatment-associated mutations for non-subtype B HIV–1.

CRF01_AE is the first HIV–1 circulating recombinant form identified worldwide. It originated from Central Africa but contributes to 4.6% of total HIV–1 infections worldwide [8, 9]. CRF01_AE might have some distinctive characteristics on pathways related to natural polymorphisms and DRM development [10–12]. A preliminary study on CRF01_AE-infected patients in China by our research team found that the frequency of the S68G mutation showed a 5.5% increase in treatment-failure patients. Moreover, the S68G mutation demonstrated a close link with the K65R mutation. The role of the S68G mutation on drug resistance and its relationship with the K65R mutation has yet to be elucidated. So, we conducted this study to explore how often the S68G mutation and K65R/S68G double mutation occurred among various HIV–1 subtypes worldwide; ascertain the temporal association of S68G and K65R mutations among treatment-failure CRF01_AE-infected patients; understand the role of the S68G mutation on NRTI susceptibility and viral replication when combined with the K65R mutation.

## Methods

# Occurrence of S68G and K65R/S68G mutations among HIV–1 of various subtypes

The prevalence of S68G mutation and K65R/S68G double mutation among various HIV–1 subtypes was analyzed in reverse transcriptase inhibitor (RTI)-naive and RTI-experienced individuals from the HIV Drug Resistance Database of Stanford University (https://hivdb.stanford.edu/cgi-bin/MutPrevBySubtypeRx.cgi; version of 19 April 2019). If a person had more than one isolate with a different mutation at the same position, only one mutation was calculated per person. Mutations occurring in ≥1% and ≥2 persons were included for analyses. All sequences with a mixture of mutations were excluded from analyses.

# Study participants and sample collection

Four treatment-failure CRF01_AE-infected patients were selected from the long-term ART cohort at the First Affiliated Hospital, China Medical University (Fig. S1). They had patient-identification numbers of 301635, 301770, 301844 and 302335. The treatment regimens were tenofovir (TDF), lamivudine (3TC) and efavirenz (EFV). Genotypic testing for drug resistance and viral load (VL) of plasma was tested at baseline as well as 6, 7, 10 and 12 months after treatment. Thereafter, all four patients changed treatment regimens. Among the four patients, 301635 and 302335 were followed up four times for 6–7 months, and 301770 and 301844 were followed up five times for 10–12 months. The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of China Medical University (Shenyang, China). Written informed consent was obtained from all participants.

# Extraction, amplification, and sequencing of HIV–1 DNA

Viral RNA was extracted from plasma using a QIAamp® RNA Blood Mini kit (Qiagen, Stanford, VA, USA). Partial pol gene were amplified using primers 503b-F (5'-CAAAAATTGGGCCTGAAAATCCATA–3') and 52r-R (5'-TGTGGTATTCCTAATTGAACTTCCCA–3') with a nested polymerase chain reaction (PCR). PCR products were purified with an Agencourt AMPure™ kit (Beckman Coulter, Fullerton, CA, USA) and quantified using a Qubit® dsDNA BR Assay kit (Life Technologies, Carlsbad, CA, USA). PCR products were evaluated with a Bioanalyzer (2100; Agilent Technologies, Santa Clara, CA, USA) to control the size of the amplified fragments, and a library was constructed based on an input of 130–150 ng. A TruSeq™ Nano DNA HT Sample Preparation kit was employed for construction of a DNA library (Illumina, San Diego, CA, USA). The quality of library construction was monitored using a DNA 7500 kit (Agilent Technologies). The standardized libraries were incorporated into 50% quality-controlled PHIX Libraries (Miseq phix control v3; Illumina). Sequencing was carried out using Miseq Reagent V3–600 cycles (Illumina) after all operations had been qualified.

First, FASTQC (version 0.11.5) was used to evaluate the sequencing quality of paired-end FASTQ sequence files. Then, FASTX-Tookit (version 0.0.13) was employed to remove reads with short reading lengths and low Q-SCORES. Trimmomatic was used to remove low-quality bases at the beginning or end of the sequence [13]. Finally, the data were uploaded to HyDRA online analysis software for statistical analyses of the coverage and mutation rate of DRMs at the target sequence of each sample [14].

# Site-directed recombinant viruses and Phenotypic resistance assay

PR genes (codons 1–99) and RT genes (codons 1–240) of CRF01_AE-infected persons were amplified with a nested PCR using primers Round2-F (5'-ATAGCCAAAAATTGCAGGGCCCCTAGRAAAAAG–3') and Round2-R (5'-GTCCTTCCTTTCCACATTTCCA–3'). The S68G mutation was reversed to wild-type by *in vitro* site-directed mutagenesis using primers G68S-F (5'-AAGAGAAAGGACAGTACCAAATGGAGAAAG–3') and G68S-R(5'-TCTCCATTTGGTACTGTCCTTTCTCTTTAT–3'). PR genes and RT genes of pNL4–3-ΔE-Luc plasmid were replaced by patients-derived genes containing the K65R/S68G double mutation and K65R mutation, then co-transfected with envelope plasmid VSVG for pseudovirus packaging. The titers of viral stocks were determined according to the Spearman–Karber method [15].

The susceptibility of the virus to AZT, 3TC and TDF were calculated as the concentration of drug required to inhibit virus growth by 50% (IC50). All experiments were conducted in triplicate. The IC50 of the mutant virus was compared with the IC50 of the fully susceptible, wild-type virus NL4–3-ΔE-Luc. The resistance level of the mutant was judged by fold change (FC) in IC50 compared with that of the wild-type virus.

# Growth competition assay

PR genes and RT genes of pNL4–3-wt plasmid were replaced by patients-derived genes containing a K65R mutation and K65R/S68G double mutation. Viral stocks of the K65R mutant and K65R/S68G double mutant were mixed at 40%:60%, 50%:50% and 90%:10% respectively. Peripheral-blood mononuclear cells ($3 \times 10^5$) from healthy donors were infected with the same virus titers (multiplicity of infection = 0.05) from each of the competing viruses. All experiments were conducted in triplicate. Viral fitness was determined by full pairwise competitions between all combinations of viruses. On days 5, 7, 10 and 13, half of the culture supernatant was harvested, and viral RNA was extracted. The viral pol gene was amplified and sequenced. The ChromatQuan Internet tool was used to calculate the viral ratio at each time point [16].

# Statistical analysis

The nonparametric t-test was used to analyze differences in the frequency of the S68G mutation between RTI-naive vs. treated persons, as well as the FC between K65R alone and K65R/S68G double mutant. The chi-squared test was employed to evaluate possible association of HIV–1 subtypes with the K65R/S68G double mutation. $P < 0.05$ was considered significant.

## Results

# Frequency of the S68G mutation increased significantly in RTI-experienced patients and commonly co-occurred with the K65R mutation among CRF01_AE strains

A total of 172639 RTI-naive and RTI-experienced people infected with HIV–1 were analyzed in the HIV Drug Resistance Database of Stanford University: subtype A (n = 12415), subtype B (n = 83006), subtype C (n = 36436), subtype D (n = 3705), subtype F (n = 2418), subtype G (n = 4638), CRF01_AE (n = 20507) and CRF02_AG (n = 9514).

Before ART, the prevalence of the S68G mutation in the subtypes mentioned above was 1.3%–7.3%, with the highest prevalence being for CRF01_AE. Among RTI-experienced patients, the prevalence of the S68G mutation increased among almost all subtypes ($p < 0.05$), with the highest increase (10%) among CRF01_AE (except for a small decrease in subtype F) (Fig. 1). To our surprise, the S68G mutation showed a high tendency to coincide with the K65R mutation in various subtypes. The prevalence of the K65R/S68G double mutation among RTI-experienced patients with various subtypes accounted for

21.1%–61.7% (Table 1). CRF01_AE had the highest frequency for the K65R/S68G double mutation ($p < 0.05$).

# The S68G mutation is often selected on the basis of the K65R mutation

An average of 260,000 reads were obtained from each sample with high quality (>Q30), which could reflect the sequence characteristics of the mutant comprehensively. We looked at NGS data among the three CRF01_AE-infected patients in whom the natural polymorphism S68G was not detected before ART. The K65R mutation was detected in patient–302335 as the dominant mutant quasispecies 3 months post-ART, accounting for 92.99%, which increased to 94.63% at 6-months post ART, and the K65R/S68G double mutation was detected among 51.14% quasispecies at 6 months post-ART. The other two infected patients, 301770 and 301844, had the predominant quasispecies with the K65R mutation at 3–6 months post-ART, accounting for 95.38% and 92.51%, respectively, among which the K65R/S68G double mutation occured at 77.52% and 59.19% respectively; moreover, almost all quasispecies carried the K65R/S68G double mutation at 7–12 months post-ART in these two patients. In patient–301635, the S68G mutation was detected as the predominant natural polymorphism, appearing in >90% quasispecies before ART. However, the K65R mutation was not detected until 7 months post-ART, when only 11.28% of quasispecies were found to have the K65R/S68G double mutation (Fig. 2).

# The S68G mutation does not decrease the drug susceptibility of the K65R mutation to AZT, TDF or 3TC

The frequency of the K65R mutation in patient–301635 was too low to select clones of the K65R/S68G double mutation, so it was not included in subsequent analyses. Therefore, clones of the K65R/S68G double mutation and clones of the K65R mutation from the other three patients were used for analyses of phenotypic drug resistance. The virus harboring the K65R/S68G double mutation in patients 301770, 301844 and 302335 had FC to AZT of 0.562±0.067, 1.483±0.529 and 1.359±0.137, which were not significantly different from the K65R mutation with FC of 0.925±0.214, 1.262±0.618 and 0.793±0.09, respectively ($p > 0.05$). Moreover, no significant changes in resistance levels were observed for 3TC or TDF (Fig. 3).

# The S68G mutation compensated for the replication adaptability of the K65R mutation

Virus stocks of the K65R and K65R/S68G mutations were mixed with different input ratios and cultured for ≤13 days. At the end of culture, the K65R/S68G double mutation outgrew irrespective of the input ratio. In the 40%:60% input group, the composition of K65R/S68G clones increased from 60% to 88%. In the 50%:50% input group, the composition of K65R/S68G clones increased from 50% to 84%. In the 90%:10% input group, the composition of K65R/S68G clones increased from 10% to 62%(Fig. 4).

# Discussion

We found that the S68G mutation was a common natural polymorphism in various HIV–1 subtypes, predominant among CRF01_AE strains. The S68G mutation often followed occurrence of the K65R mutation in treatment-failure CRF01_AE-infected patients. However, the preexisting of S68G natural polymorphism might not promote evolution of the K65R mutation. S68G did not change the drug susceptibility of the virus directly, but instead compensated for the replication deficiency associated with K65R mutation.

Our results suggest that the mutation rate of S68G increased in almost all HIV–1 subtypes in drug-experienced populations. Moreover, over 20% of the K65R mutation was accompanied by the S68G mutation, among which CRF01_AE strains showed the highest rate of the K65R/S68G double mutation, up to 61.7%, suggesting that S68G might be new drug resistance-associated mutation. This finding is supported by several studies on non-B-subtype HIV–1[17, 18]. S68G linked with K65R have been also observed in some virologic failure subjects cases [18–22]. However, the unexpected high frequency of the S68G mutation in combination with the K65R mutation in the CRF01_AE strain has not been reported previously.

We discovered, for the first time, the temporal association of the K65R mutation and S68G mutation *in vivo.* The NGS data of longitudinal samples demonstrated clearly a time lag between occurrence of the S68G and K65R mutations, and the frequency of the K65R/S68G double mutation was lower than that of the K65R mutation *in vivo.* Hence, the K65R mutation might to be selected first as the major mutation under the pressure of ART, and S68G might be an accessory mutation of K65R that is selected subsequently on the basis of the K65R mutation. However, whether the preexisting of S68G natural polymorphism before treatment could help development of the K65R mutation? We noticed that in one patient with over 90% quasispecies carrying the S68G mutation before treatment, the K65R mutation was not detected until 7 months post-ART, which was even later than other three patients without the S68G polymorphism before treatment. Hence, the S68G mutation might not promote development of the K65R mutation.

Several scholars have studied evolution of K65R and S68G mutations using standard testing for HIV drug resistance or molecular clones, but the conclusions have been controversial. Ross reported that mutations at position of 68 followed the initial selection for K65R and M184V mutations [23]. However, Wirden found the S68G mutation was the dominant quasispecies, whereas K65R and S68G mutations were the minor quasispecies, and suggested that the S68G mutation might have no relationship with the

K65R mutation [24]. Because of the limitation of Sanger sequencing, molecular clones and PCR bias, variants of less than 20% of the viral population can barely be detected [25]. However, the NGS used in our study could identify viral variants with frequencies as low as 1%, and generate data of magnitude 1000s to 10000s compared with Sanger sequencing [26]. Longitudinal samples from a single patient can enable study of the evolution of combined mutations [27]. Therefore, we discovered clearly, for the first time, the relationship between S68G and K65R mutations.

The K65 residue is located in the key position of the deoxy-nucleoside triphosphate binding pocket and associated with resistance to nucleoside analogs and polymerase fidelity [28]. This residue is an important site for DRMs, and confers resistance through reduced efficiency of NRTI incorporation and reducing the sensitivity of HIV–1 to many NRTIs. It has also been speculated that the K65R substitution improves the fidelity of the polymerase, thereby reducing many types of RT errors, including substitution and frameshift mutations [29–31]. It has been reported that the increase in polymerase fidelity may make the viral population less able to adapt to drug treatment and immune surveillance, and reduce replication adaptability [32]. Given the importance of K65 residues in the RT function of HIV–1 and the observed reduced adaptability of the K65R mutant, the virus must evolve compensatory mutations to recover this disadvantage. With regard to patients that develop the K65R/S68G double mutation, the replication adaptability of the virus restored by a compensatory mutation can help the virus survive under drug pressure, which may lead to disease progression [33, 34]. Besides, this adaptive evolution could shape HIV–1 at the population level, when these viruses contribute to epidemics through transmission; the resulting, potentially more virulent virus, could eventually modulate disease progression in newly infected hosts [35].

One limitation of this study is that because of the high success rate of antiviral therapy in our cohort, the number of subject is relatively small. Nevertheless, this study provides evidence to aid interpretation of drug-resistant mutations on CRF01_AE strains.

# Conclusions

We deciphered the role of the S68G mutation on the HIV reverse transcriptase of CRF01_AE strains. Our study provides new evidence to improve interpretation of drug-resistant mutations on the CRF01_AE strain as well as non-B subtypes of HIV–1. The present study also suggests that there are more potential DRMs in non-B subtypes of HIV–1, which calls for more studies on the HIV phenotypes involved in drug resistance.

# Abbreviations

ARV, antiretroviral; ART, antiretroviral treatment; DRMs, drug resistance mutations; HIV, human immunodeficiency virus; NGS, next generation sequencing; NRTI, nucleos(t)idic reverse transcriptase inhibitor; VL, viral load; 3TC, Lamivudine; AZT, Zidovudine; TDF, Tenofovir; EFV, Efavirenz.

## Declarations

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# Authors' contributions

LSJ, OYJM designed and performed the experiments, analyzed data, and wrote the manuscript. ZB, AMH, WL, DHB and ZM provided the data and the information of participants. SH and HXX conceived and designed the experiment, revised the manuscript. All authors read and approved the final manuscript.

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# Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

# Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of China Medical University (Shenyang, China). Written informed consent was obtained from all participants.

# Consent for publication

Not applicable.

# Competing interests

There is not competing interest in the present research.

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# Tables

Table 1. Frequency of mutation on site 68 of reverse transcriptase between different subtypes in K65R mutants

| Mutation | Subtype (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | A (n = 214) | B (n = 748) | C (n = 1345) | D (n = 55) | F (n = 19) | G (n = 147) | CRF_01AE (n = 230) | CRF_02AG (n = 171) |
| G | 41.6 | 38.9 | 27.9 | 27.3 | 21.1 | 29.3 | 61.7 | 43.3 |
| N | 3.7 | 7 | 19.4 | 18.2 | 15.8 | 6.8 | 6.1 | 3.5 |
| D | 1.4 | 3.2 | 2.8 | 1.8 | – | – | 2.2 | 1.2 |
| K | 1.9 | 2.8 | 2.4 | 3.6 | – | 1.4 | 1.3 | 1.8 |
| R | 0.5 | 2.7 | 0.8 | 1.8 | – | 2.0 | 0.9 | 0.6 |
| Total | 49.1 | 54.6 | 53.3 | 52.7 | 36.9 | 39.5 | 72.2 | 50.4 |

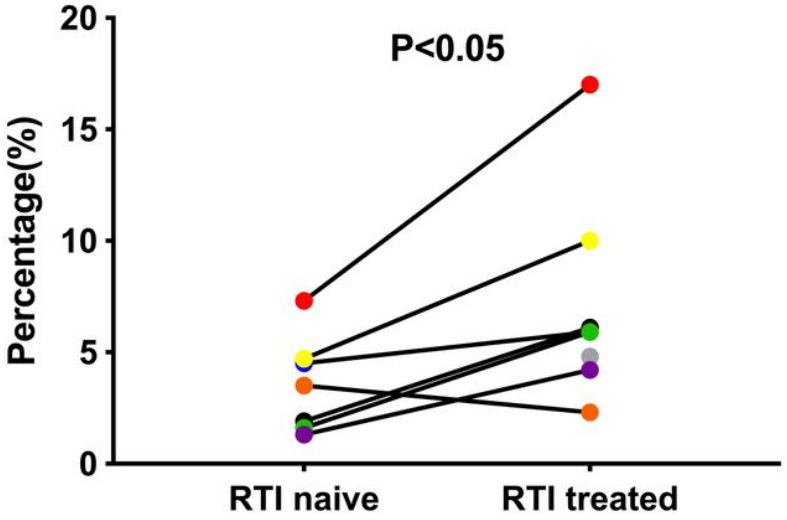
n, number of isolates according to subtype and drug class exposure; G, glycine; N, asparagine; D, aspartic acid; K, lysine; R, arginine.

* All data derived from the Stanford University HIV Drug Resistance Database.

# Supplemental Figure Legend

Supplemental Fig 1. Flowchart of study participants.

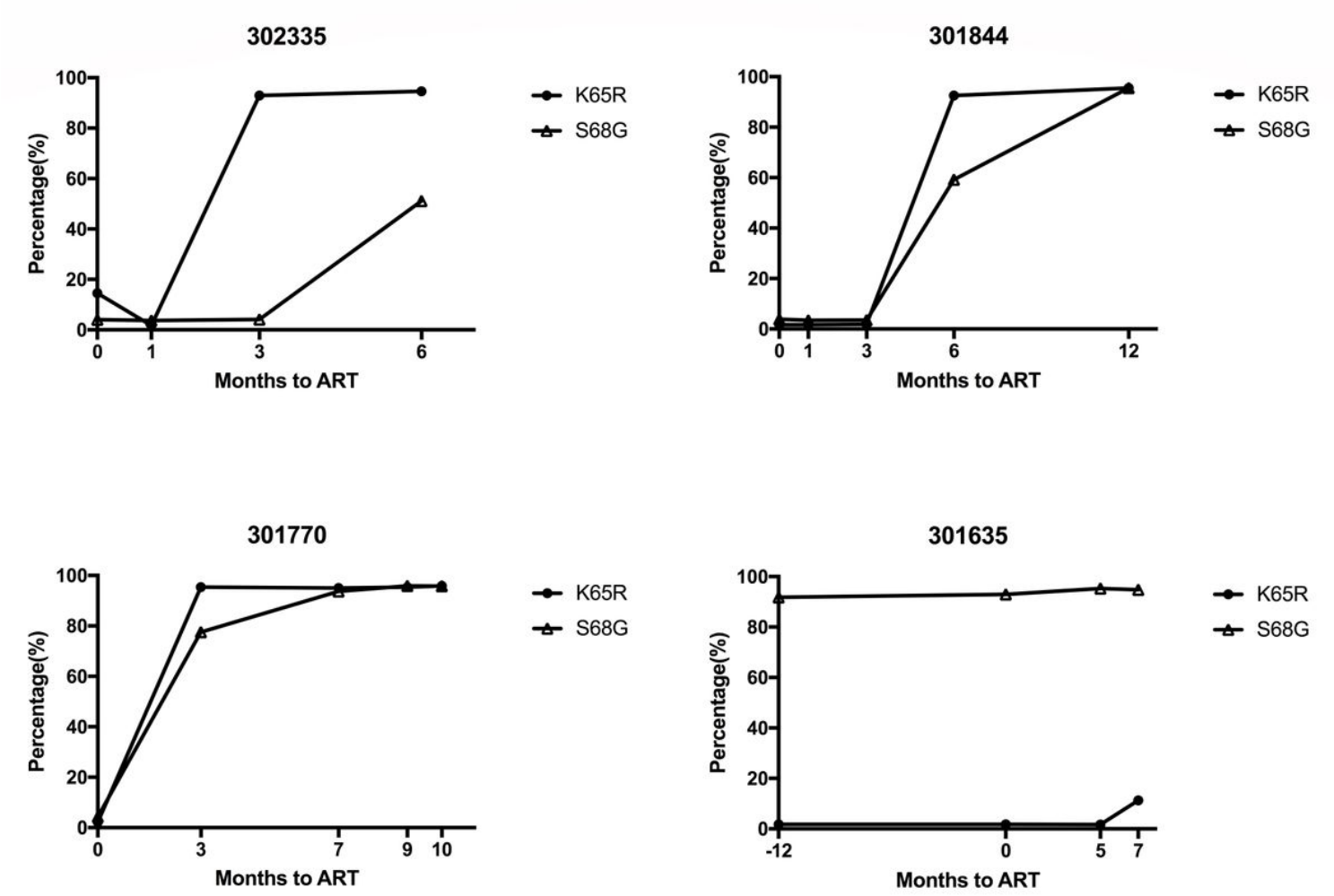
# Figures




| | Naive | Treated |
|---|---|---|
| A | 7089 | 5326 |
| B | 46589 | 36417 |
| C | 15809 | 20627 |
| D | 2023 | 1682 |
| F | 1532 | 886 |
| G | 1564 | 3074 |
| CRF01_AE | 14336 | 6171 |
| CRF02_AG | 5175 | 4339 |

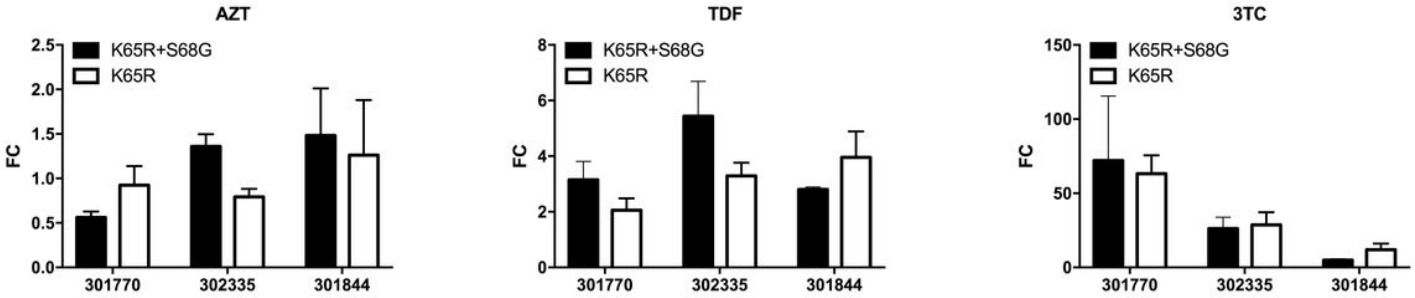
**Figure 1**

Prevalence of the S68G mutation among HIV-1 strains of various subtypes in the Stanford University HIV Drug Resistance Database. RTI, Reverse transcriptase inhibitors; RTI-naive patients had never been exposed to antiretroviral drugs, whereas RTI-treated patients were receiving RTI; the table on the right contains the number of persons with isolates according to subtype and drug-class exposure.




## Figure 2

The temporal association of S68G and K65R mutations in CRF01_AE-infected individuals during antiretroviral treatment. 302335, 301844, 301770 and 301635 were four CRF01_AE-infected individuals in which both S68G and K65R mutations were detected after treatment failure. Longitudinal plasma samples were studied with NGS on pol sequences. The black circle represents the percentage of K65R quasispecies , and the black triangle represents the percentage of S68G quasispecies .




## Figure 3

Pseudoviruses-based phenotypic resistance assay on three patients to AZT, TDF and 3TC. Black columns and white columns represent the K65R/S68G double mutation and K65R mutation pseudoviruses with mutant pol gene sequences from patients, respectively. FC, fold change in IC50 compared with the wild-type virus; AZT, zidovudine; TDF, tenofovir; 3TC, lamivudine. Data were averaged from ≥3 independent experiments.

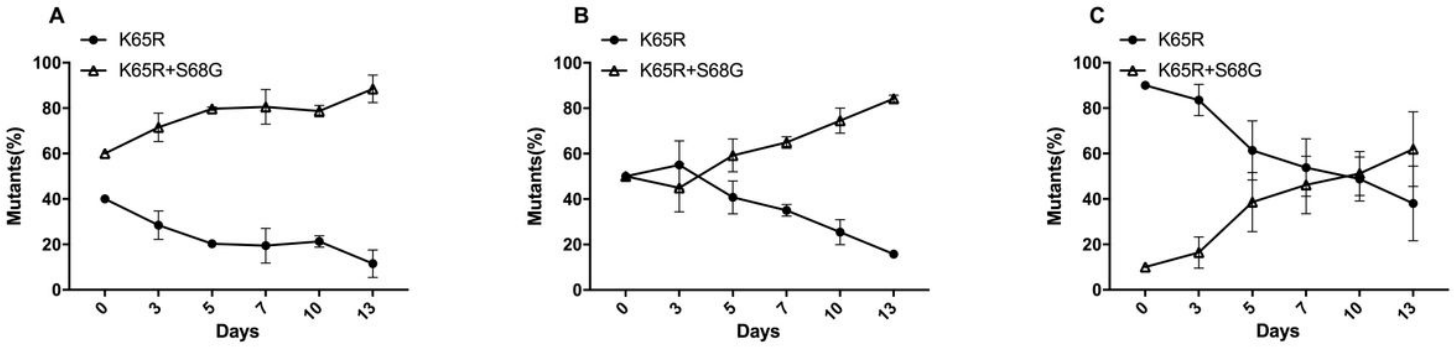



**Figure 4**

Growth competition assay of K65R and K65R/S68G infectious clones. The infectious clones of K65R and K65R/S68G infectious clones from patients were co-cultured for 13 days in PBMCs with input ratios for K65R vs. K65R/S68G: 40%:60%(A), 50%:50% (B) and 90%:10% (C). Viral RNA from culture supernatants was used to amplify target regions of pol genes and were sequenced. ChromatQuan (http://indra.mullins.microbiol.washington.edu/cgi-bin/chromatquant.cgi) was used to calculate viral percentages at each time point. An average of three independent experiments are shown with error bars representing the SD.

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementalfigure.tif