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Functional characterization of Vif proteins from HIV-1 infected patients with different APOBEC3G haplotypes

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Abstract

Objective—The human cytidine deaminase APOBEC3G (A3G) potently restricts HIV-1 but the virus, in turn, expresses a Vif protein which degrades A3G. A natural A3G-H186R variant, common in African populations, has been associated with a more rapid AIDS disease progression, but the underlying mechanism remains unknown. We hypothesized that differences in HIV-1 Vif activity towards A3G wild-type (WT) and A3G-H186R contribute to the distinct clinical AIDS manifestation.

Methods—Vif variants were cloned from plasma samples of 26 South African HIV-1 subtype C infected patients that either express WT A3G or A3G-H186R. The Vif alleles were assessed for their ability to counteract A3G variants using Western blot and single-cycle infectivity assays.

Results—We obtained a total of 392 Vif sequences which displayed an amino acid sequence difference of 6.2–19.2% between patients. The intra-patient Vif diversity from patient groups A3G^{WT/WT}, A3G^{WT/H186R} and A3G^{H186R/H186R} was similar. Vif variants obtained from patients expressing A3G^{WT/WT} and A3G^{H186R/H186R} were capable of counteracting both A3G variants with similar efficiency. However, the antiviral activity of A3G-H186R was significantly reduced in

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Author Contributions

KR, MO, VS, TN conceived and designed the experiments. KR, MO and ML performed the experiments. KR and MO analysed the data. NG heads and manages the study cohort and provided the samples. All the authors contributed to the manuscript writing and reviewed the final version.

both the presence and absence of Vif, indicating that the A3G-H186R variant intrinsically exerts less antiviral activity.

Conclusion—A3G WT and A3G-H186R are equally susceptible to counteraction by Vif, regardless of whether the Vif variant was obtained from A3G^{WT/WT} and A3G^{H186R/H186R} patients. However, the A3G-H186R variant intrinsically displayed lower antiviral activity, which could explain the higher plasma viral loads and accelerated disease progression reported for patients expressing A3G^{H186R/H186R}.

Keywords

HIV-1; HIV; Vif; subtype C; APOBEC3; APOBEC3G; Restriction factor

Introduction

Successful HIV-1 replication in the host depends on its ability to evade a myriad of innate and adaptive immune defences [1–7]. Among the innate immune factors that exert pressure on HIV-1 are host restriction factors such as the apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G protein (APOBEC3G, A3G), which belongs to the family of cytidine deaminases [8, 9]. A3G inhibits HIV-1 replication by deaminating single stranded viral DNA during reverse transcription resulting in guanine-to-adenine (G-to-A) mutations across the proviral genome [8, 10, 11]. Deaminase-independent mechanisms of A3G restriction have also been described [12–15]. HIV-1 viral infectivity factor protein Vif, promotes the proteasomal degradation of A3G allowing productive viral replication [16–18].

A natural A3G polymorphism H186R is frequent in African populations with minor allele frequencies ranging from 30% in South Africans [19], and between 25% to 51% in other African populations [20]. The polymorphism is rare in Caucasian (2% to 3%) and Asian (0% to 10%) populations [20]. This A3G-H186R variant has been associated with higher viral loads, decreased CD4+ T cell counts and a more rapid progression to AIDS in patients homozygous for A3G-H186R [19, 21–23]. However, other studies which mainly analysed the effect of the heterozygous A3G^{WT/H186R} variant found no association with disease progression [24–27]. Despite the clear correlation of A3G-H186R *in vivo*, the mechanism remains unknown [21, 28].

We recently showed that HIV-1 Vif adapts to different APOBEC3H (A3H) haplotypes in HIV-1-infected patients [29]. However, it remains unknown whether A3G haplotypes similarly select for specific Vif variants. If a given A3G variant is more or less susceptible to HIV-1 Vif mediated degradation it is conceivable that this altered viral host interaction could result in an altered HIV/AIDS disease presentation.

Of note, most Vif-A3G studies have focused on subtype B Vif variants, which only represent ~10% of all global infections [30]. However, the greatest burden of infections is in sub-Saharan Africa where HIV-1 subtype C predominates [30]. Only two studies, including one from our group, functionally analysed a limited number of subtype C variants. Both studies concluded that subtype C Vif had similar or enhanced activity against WT A3G [31, 32].

To our knowledge, the current study is the first to investigate the anti-A3G phenotype of subtype C Vif alleles obtained from patients with distinct A3G haplotypes.

Materials and Methods

Study participants

We selected 26 women from the “Centre for the AIDS Programme of Research in South Africa” acute infection study (CAPRISA 002) in Durban, South Africa [33] based on the A3G genotype information that was previously determined [19]. Plasma viremia and CD4+ T cell counts are regularly documented and samples are stored for future research. Participants provided written informed consent and ethical approval was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal.

We obtained plasma samples from 11 A3G^{WT/WT}, 10 A3G^{WT/H186R} and 5 A3G^{H186R/H186R} chronically infected, antiretroviral therapy-naïve study participants. The plasma viremia and absolute CD4+ T cell counts were obtained approximately 36 months post-infection and were comparable between patients with different A3G haplotypes.

HIV-1 Vif Amplification and Cloning

Viral RNA was extracted from plasma using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and cDNA synthesized using ThermoScript RT PCR System (Invitrogen, ThermoFisher Scientific, Massachusetts, USA). The HIV-1 Vif coding region was amplified by nested PCR with the Expand High Fidelity PCR System (Roche, Penzberg, Germany) using the primers: Vif1-forward 5' AAAATTAGCAGGAAGATGGCCAGT3' and Vif1-reverse 5' CTCCGCTTCTTCCTGCCATAGGAGAT3', and Vif2-forward 5' TACTCTGGAAAGGTGAAGG3' and Vif2-reverse 5' CTTCTGCCATAGGAGATGCCTAA3'. Five separate PCR reactions were performed per patient and gel purified amplicons were cloned into the pCR2.1 TOPO cloning vector (Invitrogen). Two to four clones of each PCR were sequenced resulting in 10 to 20 sequences for each patient. The GenBank accession numbers for the *vif* sequences generated in this study are KT881902 - KT882293.

Vif and A3G Expression Plasmids

One representative HIV-1 Vif variant from each of the 11 A3G^{WT/WT} and 5 A3G^{H186R/H186R} donors was selected for functional characterization. The Vif ORF was carboxy-terminal FLAG tagged and cloned into the mammalian expression plasmid pCRV1 as previously described [34, 35]. Carboxy-terminal Hemagglutinin (HA)-tagged WT A3G and A3G-H186R were cloned into the mammalian expression plasmid PTR600 as previously described [36].

Cell Culture

TZM-bl cells were provided by J. C. Kappes and X. Wu through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health, NIH Reagent Program. HEK-293T and TZM-bl were maintained at 37°C in a humidified atmosphere of 5% CO₂ in Dulbecco's high-glucose modified Eagle's medium (CellGro,

Corning, New York, USA), supplemented with 10% fetal bovine serum (FBS) and Penicillin/Streptomycin.

A3G Degradation and Single-Cycle Viral Infectivity Assays

HEK-293T cells were co-transfected with 500 ng of HIV pNL4-3 *vif*, 50 ng of each Vif expression plasmid and 20 ng of WT or A3G-H186R with 4 mg/ml of polyethylenimine as previously described [36]. The replication-competent molecular clone NL4-3 *Vif* was provided by the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health. After 48 hours, viral supernatants were collected and the cells were lysed and analysed by western blot as previously described [36]. Viral supernatants were used to infect TZM-bl cells and β -galactosidase activity was measured 48 hours post-infection as previously described [29].

Statistical Analysis

GraphPad Prism version 5.01 was used for statistical analyses (paired and unpaired t tests). P-values less than 0.05 were considered significant. Average relative infectivity values and their standard deviations were calculated from representative triplicate transfections.

Results

Phylogenetic Analysis of Vif sequences

Despite the availability of HIV-1 subtype C *vif* sequences [37–39], functional data regarding their anti-A3G activity remains very limited [31, 32]. We therefore cloned, sequenced and analysed HIV-1 subtype C *vif* alleles from patients homozygous for WT A3G, homozygous for A3G-H186R and from heterozygous patients [19]. We generated 392 full length HIV-1 subtype C *vif* clonal sequences. Phylogenetic analysis confirmed that all sequences were subtype C (data not shown) and *vif* clonal sequences from each patient clustered independently (Figure 1A). Intra-patient sequences differed between 0.1% and 4.9% and inter-patient diversity ranged from 6.2% to 19.2% at the protein level. We observed no significant correlations between intra-patient sequence diversity and viral loads or CD4+ counts (data not shown).

An alignment of each patient's consensus sequences is shown in Figure 1B. Putative sites of interaction with A3G or Cullin 5 and the E3 ubiquitin ligase complex are indicated by different colors (Figure 1B). The sites of potential interaction with A3G include amino acids at position 9, 22, 45 and 48 [34, 40]; the YRHHY motif (40–44) [41]; the VHIPLx4-5Lx2YWGL motif (positions 55–59, 64, 69–72) [42]; tryptophans at positions 5, 21, 38 and 89 [43], were not different between sequences. Similarly the HCCH and SLQYLA motifs, which are binding sites of Cullin 5 [44, 45] and Elongin C (144–149) were conserved [16, 46, 47].

Phenotypic characterization of HIV subtype C Vif variants

There is evidence that natural Vif variants differ in their ability to neutralize A3G [31, 32, 34] and we speculate that Vif diversity may emerge as it adapts to an individual's A3G repertoire. We functionally characterized the patient-derived subtype C Vif panel for A3G

degradation and counteraction in single-cycle infectivity assays. We co-transfected HIV Vif with individual Vif expression plasmids, WT A3G or A3G-H186R. HIV-1 infectivity was subsequently analysed by infecting TZM-bl reporter cells with viral supernatants collected two days post-transfection. Infectivity values were plotted relative to HIV-1 in the absence of A3G, which was set to 100%.

We first looked at whether Vifs obtained from A3G^{WT/WT} or A3G^{H186R/H186R} patients would differ in their ability to counteract A3G. All patient-derived Vif variants counteracted WT A3G and A3G-H186R to similar levels, irrespective of the patient's A3G variant. (Figure 2, compare within A and B, NS; not significant, unpaired t test). This indicates that HIV-1 Vif does not adapt to the different A3G variants *in vivo*. Western blot analysis showed that the level of A3G degradation (compare to the No Vif control) was similar among Vifs (compare between 2A and 2B), which is in agreement with the infectivity data. Additionally, we observed that the expression levels of the individual Vif variants was highly variable, but was independent of both the patient's A3G genotype from which they were derived as well as of their activity to degrade and counteract A3G (Figure 2A and B).

Intrinsic antiviral activity of WT A3G and A3G-H186R

To determine whether the A3G variants differ in the restriction activity, we compared viral infectivity in the presence of WT A3G or A3G-H186R (Figure 2, compare between A and B). The infectivity values in the presence of A3G-H186R were always significantly higher compared to WT A3G ($p < 0.0001$, paired t test), indicating that A3G-H186R restricts HIV less efficiently than WT A3G (Figure 2C). This difference is likely attributable to the lower basal antiviral activity of A3G-H186R, because the same result is also apparent in the absence of Vif (Figure 2D, 4.32 ± 0.21 for WT A3G versus 6.9 ± 0.28 for A3G-H186R, $p = 0.0007$, unpaired t test). This small but significant difference suggests that A3G-H186R intrinsically has reduced antiviral activity.

Taken together, we observed no evidence for functional adaptation of Vif to the different A3G variants *in vivo*. Importantly, our data show that the WT A3G was more efficient at restricting HIV compared to A3G-H186R variant, which may explain the accelerated HIV-1 disease progression in A3G^{H186R/H186R} patients.

Discussion

Several studies showed that A3G^{H186R/H186R} patients experience accelerated AIDS disease progression compared to individuals expressing A3G^{WT/WT} or A3G^{WT/H186R} [19, 21–23]. We observed that A3G-H186R restricts HIV-1 less potently than WT A3G both in the presence as well as in the absence of Vif. This novel finding indicates that A3G-H186R intrinsically has less antiviral activity, which is in agreement with a previous study which used cell free biochemical approaches which shows that A3G-H186R has reduced deaminase activity [48]. It is also possible that the H186R substitution may affect its packaging into virions, RNA binding or protein oligomerization and further mechanistic studies are required to elucidate the reduced antiviral activity of A3G-H186R. We speculate that A3G is not always fully counteracted by Vif *in vivo* and that the reduced restriction of

A3G-H186R leads to higher viral loads and a more rapid disease progression in patients expressing A3G^{WT/H186R}.

Studies show that SIV Vif adapts to polymorphisms in A3G [4, 49, 50]. We therefore anticipated that Vif would reduce its activity to degrade A3G-H186R because it poses less of a threat than A3G WT. However, Vif variants from A3G^{WT/WT} or A3G^{H186R/H186R} showed no differences in activity, indicating that Vif does not adapt to A3G-H186R. It is conceivable that the difference in anti-HIV activity of the A3G variants is too small to create sufficient selective pressure for Vif adaptation given that A3G-H186R has less antiviral activity than the WT counterpart.

Together, polymorphisms in multiple APOBEC3 proteins such as A3G and A3H in combination with a HIV strain encoding a specific Vif variant could have profound effects on HIV replication and HIV-disease progression in patients [19, 21–23, 28, 29, 51]. Indeed, the time to progression to AIDS varies widely in patients of which only a small percentage could be attributed to natural polymorphisms in genes commonly associated with differential outcome such as CCR5 or HLA [52–58].

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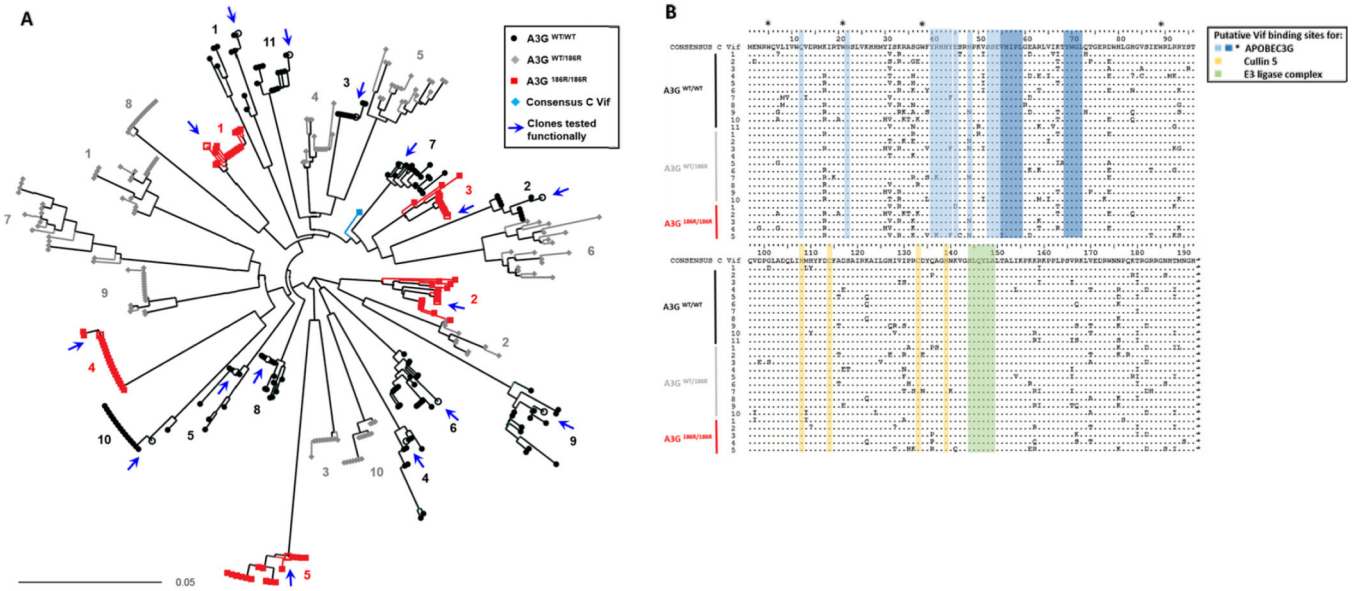


Figure 1. Sequence analysis of patient-derived HIV-1 Vif sequences

(A) Neighbour joining phylogenetic tree of 392 full length HIV-1 vif clonal sequences shows HIV-1 vif clonal sequences from each of 26 participants forming independent clusters. The patient’s A3G genotype from which Vif clones were derived are represented by the indicated symbols and colours. Vif clones that were functionally tested are represented by open symbols and arrows. Patient samples were assigned numbers that correspond in later figures. (B) Alignment of Vif amino acid consensus sequences of 26 study samples. Sequences are compared to a consensus subtype C reference sequence (obtained from the Los Alamos National Laboratory HIV database (<http://www.hiv.lanl.gov>)). Protein domains putatively involved in interactions that lead to proteasomal degradation of A3G are indicated; blue indicates I9, N22, E45 and N48, YRHYY (40–44), amino acids 52 to 72 including the highlighted VHIPLx4-5Lx2YWGI motif which are important for binding to A3G. * indicates tryptophan residues important for A3G binding; yellow indicates the HCCH motif important for binding to Cullin 5 and green shows SLQYLA motif important for recruitment of ubiquitin-ligase (E3) complex containing elongin –B and –C, cullin-5 and Rbx.

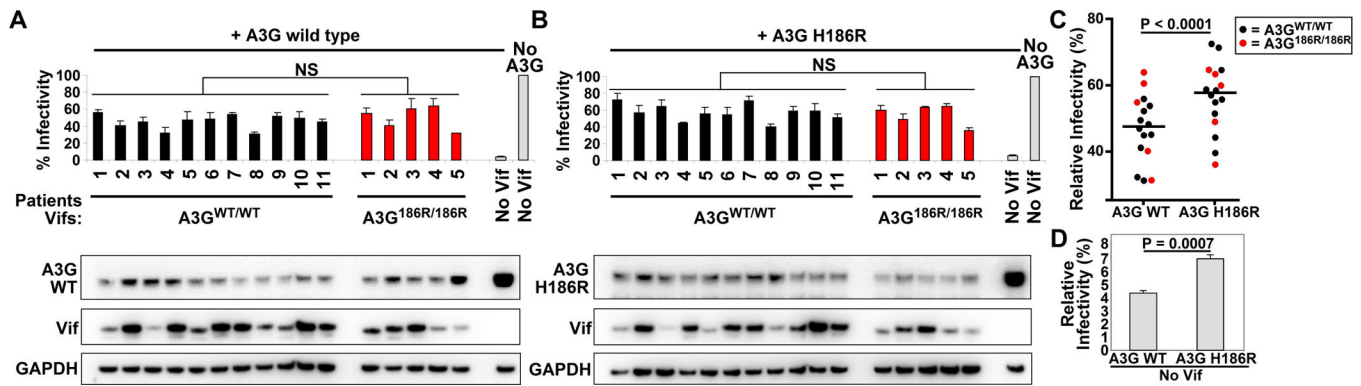


Figure 2. Activity of patient-derived subtype C Vifs against the WT A3G and A3G-H186R variants

Patient-derived Vifs were cloned into an expression vector and co-transfected with NL4-3 Vif and WT A3G (A) or A3G-H186R (B) expression plasmids and infectivity of the produced viruses was tested on TZM-bl reporter cells. NS; not significant, unpaired t test. 293T cell lysates were analysed by western blot and probed for HA, Vif and GAPDH serves as a loading control. Vif variants derived from homozygotes WT A3G are represented by black bars while Vifs derived from A3G-H186R homozygous carriers are represented by red bars. Error bars represent standard deviations from triplicate transfections. (C) Dot plot comparing the infectivity levels in the presence of WT A3G or A3G-H186R, $P < 0.0001$, paired t test. (D) Comparison of infectivities in the presence of WT A3G and A3G-H186R in the absence of Vif (also shown in 2A). $P = 0.0007$, unpaired t test. Error bars represent standard deviations from triplicate transfections.