Lack of Association between Intact/Deletion Polymorphisms of the APOBEC3B Gene and HIV-1 Risk

Mayumi Imahashi1,2, Taisuke Izumi3,4, Dai Watanabe5, Junji Imamura1, Kazuhiro Matsuoka1, Hirotaka Ode1, Takashi Masaoka1, Kei Sato6, Noriyoko Kaneko7, Seiichi Ichikawa7, Yoshio Koyanagi6, Akitumi Takaori-Kondo3, Makoto Utsumi1, Yoshiyuki Yokomaku1, Takuma Shirasaka8, Wataru Sugiura1,8, Yasumasa Iwatani1,8, Akifumi Takaori-Kondo3, Makoto Utsumi1, Yoshiyuki Yokomaku1, Takuma Shirasaka8, Wataru Sugiura1,8, Yasumasa Iwatani1,8

1 Department of Infectious Diseases and Immunology, Clinical Research Center, National Hospital Organization Nagoya Medical Center, Nagoya, Japan, 2 Department of Hematology and Oncology, Graduate School of Medicine, Nagoya University, Nagoya, Japan, 3 Department of Hematology and Oncology, Graduate School of Medicine, Kyoto University, Kyoto, Japan, 4 Japanese Foundation for AIDS Prevention, Chiyoda-ku, Tokyo, Japan, 5 Clinical Research Center, National Hospital Organization Osaka Medical Center, Osaka Japan, 6 Center for Human Retrovirus Research, Institute for Virus Research, Kyoto University, Kyoto, Japan, 7 Department of International Health Nursing, Graduate School of Nursing, Nagoya City University, Nagoya, Japan, 8 Department of AIDS Research, Graduate School of Medicine, Nagoya University, Nagoya, Japan

Abstract

Objective: The human APOBEC3 family of proteins potently restricts HIV-1 replication APOBEC3B, one of the family genes, is frequently deleted in human populations. Two previous studies reached inconsistent conclusions regarding the effects of APOBEC3B loss on HIV-1 acquisition and pathogenesis. Therefore, it was necessary to verify the effects of APOBEC3B on HIV-1 infection in vivo.

Methods: Intact (I) and deletion (D) polymorphisms of APOBEC3B were analyzed using PCR. The syphilis, HBV and HCV infection rates, as well as CD4+ T cell counts and viral loads were compared among three APOBEC3B genotype groups (I/I, D/I, and D/D). HIV-1 replication kinetics was assayed in vitro using primary cells derived from PBMCs.

Results: A total of 248 HIV-1-infected Japanese men who have sex with men (MSM) patients and 207 uninfected Japanese MSM were enrolled in this study. The genotype analysis revealed no significant differences between the APOBEC3B genotype ratios of the infected and the uninfected cohorts (p = 0.66). In addition, HIV-1 disease progression parameters were not associated with the APOBEC3B genotype. Furthermore, the PBMCs from D/D and I/I subjects exhibited comparable HIV-1 susceptibility.

Conclusion: Our analysis of a population-based matched cohort suggests that the antiviral mechanism of APOBEC3B plays only a negligible role in eliminating HIV-1 in vivo.

Citation: Imahashi M, Izumi T, Watanabe D, Imamura J, Matsuoka K, et al. (2014) Lack of Association between Intact/Deletion Polymorphisms of the APOBEC3B Gene and HIV-1 Risk. PLoS ONE 9(3): e92861. doi:10.1371/journal.pone.0092861

Copyright: © 2014 Imahashi et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was partly supported by a grant from the National Hospital Organization Network (grant NHO H23-AIDS) and by grants for HIV/AIDS research from the Ministry of Health, Labor, and Welfare of Japan (grants H22-AIDS-004 and H24-AIDS-005). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscripts.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: iwataniy@nh.hosp.go.jp

Introduction

Human APOBEC3 proteins are cellular cytidine deaminases that play crucial roles in the inhibition of retroviral replication, including that of HIV-1 [1–3]. The molecular mechanisms underlying APOBEC3-mediated HIV-1 restriction are primarily dependent on the editing [1,2] and/or non-editing activities [4,5] of these enzymes. The family of genes encoding the seven APOBEC3 proteins (APOBEC3A, B, C, DE, F, G, and H) is positioned in a tandem array on human chromosome 22 [6]. HIV-1 produces an accessory protein, Vif, that invalidates the antiviral functions of the APOBEC3 proteins by mediating the ubiquitination-proteasomal degradation of APOBEC3 in virus-producing cells [7]. APOBEC3C, DE, F, G, and H (haplotype II) are vulnerable to HIV-1 Vif-mediated degradation, whereas APOBEC3A and B are resistant [9–12].

Among the members of the APOBEC3 family, APOBEC3G has been consistently shown to possess powerful anti-HIV-1 activity in cell-based systems [1,2], and this protein may affect the pathogenesis of HIV-1 infection in vivo [13–19]. However, there is little consensus regarding the degree to which the other APOBEC3 family members, especially APOBEC3B, are able to restrict HIV-1 replication in vivo and in vitro. The anti-HIV-1 activity of APOBEC3B is undetectable when this gene is stably expressed in a human T cell line [20] and is detected only weakly after the transient transfection of HEK 293T or HeLa cells [20–22]. Because these findings have varied according to the experimental conditions employed, there is a fundamental
question whether the expression of human APOBEC3B, DE, and F plays a critical role in HIV-1 restriction in vivo. The potential role of APOBEC3B in modulating HIV-1 replication in vivo is of particular interest because this protein is resistant to HIV-1 Vif-mediated degradation [20,22–24].

A polymorphic deletion of a 29.5-kb segment between APOBEC3A exon 5 and APOBEC3B exon 8 has been identified in human populations; this polymorphism causes the loss of the entire APOBEC3B coding region [25]. A particularly high frequency of APOBEC3B deletion has been found among Asians [25]. According to Kidd et al., the deletion allele is rare in Africans (1%) and Europeans (6%), more common in East Asians (36%) and Amerindians (38%), and almost fixed in Oceanians (93%) [25].

Two independent groups have reported contrasting findings concerning the effects of the APOBEC3B gene deletion on HIV-1 acquisition and disease progression [26,27]. An et al. determined that the deletion allele genotype correlated with a higher risk of HIV-1 infection, whereas a study conducted by Iwaya et al. concluded that the deletion polymorphism had no effect on HIV-1 acquisition and the rate of disease progression to AIDS. An et al. included 4 patients with homozygous deletions of APOBEC3B in their HIV-1-seropositive cohorts of 656 European and 296 African-American individuals but no homozygotes for the deletion in their seronegative groups, which prevented a proper evaluation of the impact of the deletion polymorphism on HIV-1 acquisition and pathogenesis [26]. In contrast, the study conducted by Iwaya et al. in Japan utilized inappropriate enrollment [27], because the enrolled patients were all hemophiliacs who had survived HIV-1 infection for at least 10 years prior to the study and the information for individuals who had progressed to AIDS and death before the enrollment date was excluded.

To examine the impact of the APOBEC3B deletion polymorphism on HIV-1 infection risk in vivo, this study enrolled a matched cohort in Japan and investigated the impact of APOBEC3B gene intact/deletion polymorphisms on HIV-1 susceptibility and pathogenesis. In addition, we analyzed the effects of different APOBEC3B genotypes on HIV-1 replication kinetics in vivo.

Materials and Methods

Sample Collection

A total of 248 Japanese HIV-1-positive men who have sex with men (MSM) who were patients at Nagoya Medical Center (n = 203) and Osaka Medical Center (n = 45) were enrolled in this study from November 2011 to February 2013. The control group comprised 207 Japanese HIV-1-negative MSM who were recruited at the Nagoya Lesbian & Gay Revolution Plus (NLGR+) festival in June 2012. The study protocol was approved by the ethics committees of Nagoya Medical Center (registration number 2011-430) and Osaka Medical Center. Written informed consent was obtained from all the participants. The control subjects recruited at the NLGR+ festival provided anonymous consent. To collect information regarding their sex, nationality, age, and sexuality, anonymous questionnaires collated with linked numbers were obtained.

Genotyping

The APOBEC3B intact (I) and deletion (D) alleles were genotyped using a previously reported polymerase chain reaction (PCR) method [26] with slight modifications. Of note, the “intact (I)” in this study is used for the “insertion” that originally reported by Kidd et al. [25]. Briefly, the primer sets for amplifying the

Deletion and Insertion 2 fragments were the same as those previously described [26], although one additional set of primers for the Insertion 1 fragment was replaced by the two following oligonucleotide primers: Insertion3_F: 5'-GAGTGG-GAACGGCTCTCC-3' and Insertion3_R: 5'-CTCCCTGCCCAGCTTAGC-3’. The QiAamp DNA Blood Mini Kit (Qiagen, Valencia, USA) was used according to the manufacturer’s protocol to extract genomic DNA from whole blood (patients) or from buccal mucosa (controls).

Analysis of Viral Replication Capacity and Infectivity

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood samples from different HIV-1-negative donors with the I/I and D/D APOBEC3B genotypes (n = 5 for each) using Ficoll-Hypaque density gradient centrifugation (Pharmacia, Uppsala, Sweden). The PBMCs were then subjected to negative selection with the MACS CD4 T Cell Isolation Kit (Miltenyi Biotec, Cologne, Germany) to purify primary CD4 T cells. The cells were activated with 1 µg/ml of phytohemagglutinin (PHA) (Pharmacia) for 72 hours, infected with HIV-1 NL4-3 for 24 hours with a multiplicity of infection (MOE) of 0.01, washed twice, and maintained in RPMI-1640 medium with 20% fetal bovine serum (GBS), penicillin (50 U/ml)/streptomycin (50 µg/ml) (In-vitrogon, Carlsbad, USA), and 20 U/ml interleukin-2 (IL-2) (Roche Applied Science, Mannheim, Germany). The culture supernatants were assayed for the p24 antigen using the HIV-1 p24 Antigen Assay Kit (Coulter Corporation, Fullerton, USA) on the day of infection and on days 2, 4, 6, 8, 10, and 13 after infection. To analyze the viral infectivity of the infected PBMCs, culture supernatants were harvested six days post-infection and inoculated into TZM-bl cells [29] in black 96-well plates. The viral infectivity was assessed 48 hours post-infection by detecting β-galactosidase activity using the Galacto-Star System (Applied Biosystems, Foster City, USA).

Quantification of APOBEC3 mRNA

To analyze the mRNA expression levels of members of the APOBEC3 family, unstimulated CD4+ cells from three different genotyped subjects were prepared for RNA isolation. The induction rates of mRNA transcription for APOBEC3A or APOBEC3G were analyzed using monocyte-derived macrophages (MDMs). Briefly, monocytes were isolated from PBMCs from each genotyped healthy donor using CD14 MicroBeads (Miltenyi Biotec). The enriched CD14+ cells were plated at a cell density of 1 × 10^6/ml in 12-well plates in RPMI-1640 medium (Sigma, St. Louis, USA) with penicillin (50 U/ml)/streptomycin (50 µg/ml) for three hours, followed by the addition of 10% FBS and 10 ng/ml macrophage colony stimulating factor (M-CSF) (Peprotech, Rocky Hill, USA). Adherent cells were cultured for eight days to facilitate their differentiation into MDMs. Differentiated MDMs either received no stimulation or were stimulated with 100 U/ml of recombinant human interferon (IFN)-γ (Sigma) for six hours and were then lysed for RNA isolation. As previously described [14,30], total RNA isolated using the QiAamp RNA Blood Mini Kit (Qiagen) was used to synthesize cDNA with the SuperScript III First-Strand Synthesis System (Invitrogen) using random hexamers. The cDNA levels were quantified using real-time PCR in a Thermal Cycler Dice Real Time System (TP800) (Takara Bio, Shiga, Japan). The real-time PCR was employed to analyze the levels of APOBEC3, β-actin, and GAPDH mRNA, and the assays were performed according to the manufacturer’s protocol using SYBR Premix DimerEraser (Takara Bio, Shiga, Japan). The primer sets for the real-time PCR were purchased from FASMAC Co., Ltd. (Atsugi, Japan) and the oligonucleotide sequences are
shown in Table S1. The gene expression levels were calculated using the ΔΔCt (Ct; cycle threshold) and are presented as the ratio of APOBEC3 mRNA to β-actin or GAPDH mRNA.

Statistical Analysis

The relationships between APOBEC3B genotype and baseline characteristics were assessed using the Fisher exact test for categorical variables. The Mann-Whitney U-test was used for continuous variables. All the statistical analyses were performed with the statistical software EZR (Saitama Medical Center, Jichi Medical University), which is a graphical user interface for R (The R Foundation for Statistical Computing, version 2.13.0). More specifically, this software is a version of R commander (version 1.6–3) modified to add statistical functions that are frequently used in biostatistics [31]. All the p values were two-tailed. The effects of APOBEC3B gene deletion on the disease progression of HIV-1 were evaluated based on the CD4+ T cell counts and log10 HIV-1 viral load (RNA copy number/ml) at more than two time points before the start of antiretroviral therapy (ART). Patients whose CD4+ T cell counts and HIV-1 viral loads were measured at fewer than two time points were excluded from the statistical analyses of these factors. Other related infectious diseases were identified in the patients using the following definitions. If the rapid plasma reagin test and/or the Treponema pallidum latex agglutination (TPHA) test were positive, the patient was considered positive for syphilis. Patients were considered hepatitis B virus (HBV)-positive if either hepatitis B surface antigen (HBsAg) or hepatitis B core antibody (HBcAb) was present. In addition, patients were considered hepatitis C virus (HCV) carriers if they tested positive for HCV antibodies.

Results

APOBEC3B Genotype Frequencies in the Cohorts

The demographics of the HIV-1-positive and HIV-1-negative cohorts are shown in Table 1. A total of 248 HIV-1-infected Japanese MSM patients and 207 uninfected Japanese MSM were enrolled and analyzed in this study. To conduct a matched cohort study, all the participants were recruited from Nagoya and Osaka in the central area of Japan. First, a comparative analysis of the APOBEC3B genotype among the participants indicated that there were no significant differences in APOBEC3B genotype frequency between the HIV-1-positive (D/D 7.7%, I/D 44.0%, and I/I 48.4%) and HIV-1-negative (D/D 8.7%, I/D 39.6%, and I/I 51.7%) cohorts (p = 0.66) (Table 1). A comparison of the distributions of the APOBEC3B deletion allele in the HIV-1-positive and HIV-1-negative cohorts revealed that the D allele occurred in the HIV-1-positive (29.6%) and HIV-1-negative subjects (28.5%) at comparable rates (p = 0.71). We also analyzed the cDNA sequences of APOBEC3B I allele isolated from the Japanese healthy donors with the I/I or I/D genotypes (Table S1). There was one variant (rs#2076109; K62 (allele frequency, or AF = 0.4)) (E62 as the reference) although we could not detect any other variants changing the amino acid sequences within the 15 alleles. According to the 1000 Genome database, the variant (AF = 0.373) appears globally distributed but not limited in Japan or Asia. In addition, we tested the antiviral effect of APOBEC3B E62 and the variant with an overexpression system using 293T cells (Figure S1). The results demonstrated that the E62 variant had equivalent antiviral activity to APOBEC3B K62 in vitro. These data suggest that the I alleles in our Japanese cohorts are not strongly biased in terms of genetic and functional features.

Next, we analyzed the HIV-1-positive individuals for the prevalence of HBV, HCV, and syphilis, as well as for HIV-1 disease progression at a minimum of two time points before the commencement of ART. The prevalence of each infectious disease is presented in Table 2. The frequencies of the three APOBEC3B genotypes (D/D 7.6%, I/D 45.4%, and I/I 47.0%) among the 192 HBV-positive patients were not significantly different from those of the 94 HBV-negative individuals (D/D 9.6%, I/D 41.5%, and I/I 49.9%) (p = 0.69). In addition, the APOBEC3B genotype distributions did not differ significantly between the HCV-positive and HCV-negative patients (p = 1.00) or between the syphilis-positive and syphilis-negative patients (p = 0.62) (Table 2).

We also assessed the rates of both CD4+ T cell decline and plasma viral load increase at different time points after the first patient visit to the hospital prior to ART treatment. As shown in Figure 1, the changes in the CD4+ T cell counts (cells/µl/day) and viral loads (log10 copies/ml/day) did not differ significantly according to APOBEC3B genotype (CD4: p = 0.054; viral load: p = 0.96). The data from the 46 patients (D/D 6.5%, I/D 41.3%, and I/I 52.2%) who began ART before the second measurement of the CD4+ T cells and viral loads were excluded from the analysis. Of these 46 patients, 32 (D/D 3.1%, I/D 50.0%, and I/I 49.6%) began ART shortly after their first hospital visit due to AIDS onset; this decision was based on the domestic clinical guidelines of the Ministry of Health, Labor, and Welfare of Japan. There were no significant differences in the proportions of the APOBEC3B genotypes between the patients with CD4+ T cell count and viral load data from at least two time points and the 46 patients without complete data (p = 0.91). Detailed demographic information on the HIV-1 (+) patients is shown in Table 3. Moreover, we analyzed the non-ART periods from the first diagnosis through the ART introduction and set two groups: longer and shorter than median days from diagnosis to ART. As the genotype frequencies were compared (Table 4), the results showed no significant difference in the APOBEC3B genotype between the two groups (p = 0.96).

Moreover, we performed deep sequencing of the HIV-1 proviral DNAs that were isolated from the I/I, I/D or D/D patients’ PBMCs, and then analyzed the hypermutation rates on APOBEC3-preferred dinucleotide sequences: GG>AG and GA>AA mutations. The results showed that the hypermutation frequencies vary among different individuals although the levels of GA>AA hypermutation relative to the GG>AG are comparable among the three APOBEC3B genotypes (Figure S2). The data suggest that the APOBEC3B is not likely a major contributor to introduce hypermutations on the proviral DNAs in HIV-1(+) patients’ PBMCs.

The Effects of APOBEC3B Genotype on Other APOBEC3 Expression Profiles

To assess whether the APOBEC3B gene deletion altered the expression of the other proximal APOBEC3 genes, we compared mRNA expression profiles in fresh, unstimulated primary CD4+ cells of each APOBEC3B genotype: D/D, I/D, and I/I. As shown in Figure 2A, the mRNA expression levels of APOBEC3A, which is the APOBEC3 family member located closest to the APOBEC3B gene, were not significantly different between the I/I and D/D genotype groups (p = 0.63), although the levels would likely vary considerably among individuals. As expected, APOBEC3B mRNA expression levels were not detected in the D/D subjects (Figure 2A). The APOBEC3B mRNA levels in the I/D subjects were somewhat lower than in the I/I subjects, although this difference was not statistically significant (Figure 2A, p = 0.12). Moreover, the relative levels of APOBEC3C, DE, F, G, and H mRNA were comparable among the I/I, I/D, and D/D subjects (Figure 2A). We also analyzed APOBEC3B mRNA levels in...
PBMCs isolated from healthy donors and HIV-1 seropositive patients with or without the ART. Similar to the pattern of APOBEC3B mRNA levels in the CD4+ T cells of three genotyped subjects (Figure 2A), the mRNA expression is slightly lower in the I/D genotyped PBMCs than in the I/I whereas no detectable level of APOBEC3B mRNA in the D/D PBMCs (Figure S3). The different expression levels between the I/D and I/I PBMCs were not statistically significant (Figure S3). Moreover, comparative analysis showed that the APOBEC3B mRNA level of each I/I or I/D genotype appears relatively higher in the HIV-1 (+) patients, regardless the ART-treatment, than in the uninfected donors. However, the difference was not statistically significant (Figure S3).

In the APOBEC3B D allele, the APOBEC3A mRNA contains a 3′-untranslated region of APOBEC3B's and is subject to the upstream regulatory elements of the APOBEC3A. Thus, we further assessed whether the degrees to which APOBEC3A and APOBEC3G mRNA expression was stimulated by IFN-α in MDMs differed between the APOBEC3B I/I and D/D genotypes. The APOBEC3G mRNA expression was used as a control because the gene is distal to the APOBEC3B loci on the genome. As shown in Figure 2B, IFN-α stimulation resulted in APOBEC3A mRNA increases in APOBEC3A mRNA levels in the I/I and D/D MDMs of 1,999±1,190-fold and 1,251±264-fold, respectively. The APOBEC3G mRNA levels increased upon IFN-α stimulation by 28.6±41.8-fold (I/I) and 38.9±18.0-fold (D/D). A comparison of the mRNA expression magnitudes between the two homozygous APOBEC3B genotypes revealed no significant differences (p=0.4 and p=0.4 for APOBEC3A and APOBEC3G, respectively).

The Effects of APOBEC3B Genotype on HIV-1 Susceptibility in Vitro

We further analyzed the viral replication kinetics in primary PBMCs isolated from D/D or I/I donors. At an MOI of 0.01, the efficiency of HIV-1 replication was comparable between the D/D and I/I genotypes (Figure 3A). The p24 antigen levels in the culture supernatant from the I/I and D/D PBMCs were 8.7±3.0×10⁵ pg/ml and 1.3±0.2×10⁶ pg/ml, respectively, on day 8 (p=0.31) and 8.4±0.2×10⁵ pg/ml and 1.3±0.3×10⁶ pg/ml, respectively, on day 6 (p=0.13). At the peak of infection (day 6), the virus-containing supernatants derived from D/D and I/I PBMCs exhibited comparable levels of infectivity (p=0.86) (Figure 3B). These data suggest that the different APOBEC3B deletion genotypes are not associated with significantly different levels of HIV-1 susceptibility in vitro.

### Table 1. APOBEC3B genotype frequency in HIV-1-positive patients and HIV-1-negative controls.

|                | HIV-1 |          |          |        |
|----------------|-------|----------|----------|--------|
|                | Negative (%) | Positive (%) |        | p*     |
| Genotype       |        |          |----------|--------|
| D/D            | 18/207 (8.7) | 19/248 (7.7) | 0.66   |
| I/D            | 82/207 (39.6) | 109/248 (44.0) |       |
| I/I            | 107/207 (51.7) | 120/248 (48.4) |       |
| Allele         |        |          |----------|--------|
| D              | 118/414 (28.5) | 147/496 (29.6) | 0.71   |
| I              | 296/414 (71.5) | 349/496 (70.4) |       |

*Determined using the Fischer exact test. doi:10.1371/journal.pone.0092861.t001

### Table 2. APOBEC3B genotype frequency and clinical parameters in HIV-1-positive patients.

| APOBEC3B genotype | D/D (%) | I/D (%) | I/I (%) | p* |
|-------------------|---------|---------|---------|----|
| HBV               |         |         |         |    |
| Positive          | 10/132 (7.6) | 60/132 (45.4) | 62/132 (47.0) | 0.69 |
| Negative          | 9/94 (9.6) | 39/94 (41.5) | 46/94 (48.9) |       |
| Unknown           | 0/22 (0) | 10/22 (45.5) | 12/22 (54.5) |       |
| HCV               |         |         |         |    |
| Positive          | 0/7 (0) | 3/7 (42.9) | 4/7 (57.1) | 1.00 |
| Negative          | 19/241(7.9) | 106/241 (44.0) | 116/241 (48.1) |    |
| Syphilis          |         |         |         |    |
| Positive          | 9/116 (7.8) | 47/116 (40.5) | 60/116 (51.7) | 0.62 |
| Negative          | 10/131 (7.6) | 61/131 (46.6) | 60/131 (45.8) |       |
| Unknown           | 0/1 (0) | 1/1 (100) | 0/100 (0) |       |

*Determined using the Fischer exact test. doi:10.1371/journal.pone.0092861.t002
Discussion

There is only limited information about the roles played by APOBEC3 family members in vivo, with the exception of APOBEC3G. Previously, two independent groups reported conflicting conclusions regarding the impact of the APOBEC3B gene deletion on human HIV-1 infection in vivo, and this issue remains unclear [26,27]. Therefore, to determine the effects of different APOBEC3B genotypes on HIV-1 infection in vivo and in vitro, we investigated the frequencies of intact and deletion polymorphisms of the APOBEC3B gene in a matched cohort in Japan.

The comparison of APOBEC3B genotypes in HIV-1-infected patients and HIV-1-negative controls revealed similar APOBEC3B genotype distributions in the two groups: D/D 7.7%, I/D 44.0%, and I/I 48.4% in the infected cohort versus D/D 8.7%, I/D 39.6%, and I/I 51.7% in the uninfected cohort (p = 0.66). In addition, no significant associations between the APOBEC3B genotype and the subclinical parameters of disease progression were observed among the HIV-1-positive patients. We also found no differences between the mRNA expression profiles of other APOBEC3 family members in PBMCs. Furthermore, the IFN-α-stimulated mRNA induction rates for APOBEC3A and APOBEC3G in MDMs did not differ between the D/D and I/I genotypes. Moreover, the HIV-1 susceptibility levels in PBMCs were comparable between the two genotypes. Considered together, our findings suggest that the loss of APOBEC3B is not significantly associated with HIV-1 acquisition and pathogenesis in vivo and with HIV-1 susceptibility in vitro, which fully supports the results of the cohort study conducted by Itaya et al [27].

There are two possible explanations for the lack of APOBEC3B involvement in HIV-1 restriction. First, the APOBEC3B protein cannot be incorporated into viral cores. Efficient HIV-1 restriction requires that APOBEC3 family proteins are packaged into virions through associations with viral and/or nonviral RNA [1,2,28–30] and that the proteins are localized to the plasma membrane in virus-producing cells [31]. APOBEC3G colocalizes with HIV-1 RNA and cellular RNA in P bodies [32] and are dispersed throughout the cytoplasm that facilitate interactions with HIV-1 Gag proteins and their incorporation into nascent virions [1,2]. In contrast, APOBEC3B predominantly localizes to the nucleus [20,21,33], which may prevent its incorporation into virions.

The second possible explanation is that the low expression level of APOBEC3B in PBMCs [22,34,35] is insufficient to block HIV-

Table 3. Demographics of the cohorts.

|                          | HIV-1 Negative (n = 207) | HIV-1 Positive (n = 248) |
|--------------------------|--------------------------|--------------------------|
| Age (years), median [IQR] | 33 [26–39]               | 40 [36–51]               |
| Year of diagnosis, median [IQR] | NA                     | 2008 [2005–2010]         |
| ART naïve at entry, n (%) | NA                       | 20 (8%)                  |
| CD4⁺ cell count at entry (cells/mm³), median [IQR] | NA | 451 [294–534] |
| HIV-1 viral load at entry (copies/mL), median [IQR] | NA | 61 [≤40–410] |
| History of AIDS, n (%)   | NA                       | 32 (13%)                 |
| Days from diagnosis to entry, median [IQR] | NA | 1470 [539–2256] |
| Observation periods for disease progression (days), median [IQR] | NA | 56 [28–88] |
| Days from diagnosis to ART, median [IQR] | NA | 88 [38–599] |

aIQR denotes interquartile range.

bNA, Not applicable.

doi:10.1371/journal.pone.0092861.t003

Figure 1. Analysis of effects of genotype on parameters of HIV disease progression in the HIV-1-infected cohort. (A) Changes in CD4⁺ T cell counts (cells/µl/day) (n = 202). (B) Changes in HIV-1 RNA levels (log₁₀ copies/ml/day) in plasma (n = 202). The box plots show data between the 25th and 75th percentiles with central horizontal lines representing the median, and with whiskers showing the 10th and 90th percentiles. The open circles represent outliers with data >1.5-fold of the interquartile range. All the p values were determined using the Kruskal-Wallis test.
doi:10.1371/journal.pone.0092861.g001
1 replication, as shown in Figure 3. Similar to the HIV-1 results, overexpressed APOBEC3B potently suppresses HBV replication in vitro [36]. However, a study by Abe et al. on the frequency of the D/D genotype in HBV carriers demonstrated that the APOBEC3B gene deletion was not responsible for chronic HBV infection [37]. These data suggest that the high expression of APOBEC3B in vitro may produce exaggerated effects on both HIV-1 and HBV infection in vitro.

All the participants enrolled in this study were Japanese MSM, according to the information provided on anonymous questionnaires. Because approximately 80% of the HIV-1-positive patients in Japan are MSM [38], we investigated the effects of APOBEC3B deletion polymorphisms on this major mode of HIV-1 transmission rather than on the two other major modes (injection drug use and heterosexual intercourse). However, the effect of APOBEC3B genotype is less likely to be dependent on the mode of HIV-1 transmission because APOBEC3B mRNA expression in hematopoietic cells is lower and less tissue-specific than that of most of the other APOBEC3 family members [22,34,35].

| Genotype | 88 days> (%) | 88 days< (%) | p* |
|----------|--------------|--------------|----|
| D/D      | 9 (3.7)      | 10 (4.1)     | 0.961 |
| I/D      | 49 (19.9)    | 59 (24.0)    |    |
| I/I      | 57 (23.2)    | 62 (25.2)    |    |

*Median days from diagnosis to ART = 88 days.*

**Table 4. APOBEC3B genotype frequency on the days from diagnosis to ART (n = 246).**

---

(A) Comparison of mRNA expression levels of APOBEC3 in CD4+ cells isolated from intact (I/I), hemizygous (I/D) and deletion (D/D) individuals of healthy donors. The relative mRNA expression levels of APOBEC3A (I/I, n = 8; I/D, n = 4; D/D, n = 4), APOBEC3B (I/I, n = 11; I/D, n = 7; D/D, n = 5), APOBEC3C (I/I, n = 12; I/D, n = 7; D/D, n = 5), APOBEC3DE (I/I, n = 11; I/D, n = 9; D/D, n = 5), APOBEC3F (I/I, n = 12; I/D, n = 7; D/D, n = 5), APOBEC3G (I/I, n = 12; I/D, n = 7; D/D, n = 5), and APOBEC3H (I/I, n = 6; I/D, n = 7; D/D, n = 4) were determined using quantitative RT-PCR and were normalized to GAPDH. The red (I/I) or gray (D/D) dots represent the expression levels of donors whose PBMCs were used for the in vitro kinetics of HIV-1 replication and infectivity in Figure 3. The p values were calculated using Welch’s t-test. The error bar represents the standard error of the mean (SEM). (B) APOBEC3A (A3A) and APOBEC3G (A3G) mRNA expression levels under basal conditions (Ctrl) and after stimulation with 100 U/ml (+IFN-α) of interferon (IFN)-α in CD14+ MDs isolated from healthy control subjects. The black and white bars indicate D/D (n = 3) and I/I (n = 4) individuals, respectively. The p values were calculated with the Mann-Whitney U-test. The error bars represent the standard deviation. n.d., not detected. Ct, cycle threshold. n.s., not significant (p = 0.4 for both cases).

doi:10.1371/journal.pone.0092861.t004
doi:10.1371/journal.pone.0092861.g002
In the D/D genotype, APOBEC3A mRNA expressed from the genome has a 3'-untranslated region corresponding to that of APOBEC3B. In addition, the genomic location of the APOBEC3G coding region is closer to the highly IFN-responsive transcriptional element of APOBEC3A in the D/D genome than to in the I/I. Therefore, we evaluated whether APOBEC3B gene deletion altered the IFN-stimulated gene induction of the other APOBEC3 family members. Our results suggest that the 29.5-kb genomic deletion of APOBEC3B does not significantly affect the expression profiles of the proximal APOBEC3 family genes. Therefore, it is unlikely that the loss of the APOBEC3B gene in the D/D population leads to functional compensation via the mRNA expression modulation of the other APOBEC3 family members. Interestingly, Basin et al. have demonstrated that increased levels of APOBEC3G mRNA in PBMCs, (primarily CD14+ MDMs) following exposure to IFN-α correlated with HIV-1 susceptibility both in vivo and in vitro [13]. Our results showed that the induction magnitude of APOBEC3G mRNA upon the IFN-α stimulation was similar between the I/I and D/D genotypes (Figure 2B). This suggests that different HIV-1 susceptibility observed by Basin et al. is unlikely linked to the APOBEC3B intact/deletion genotypes.

Recent studies of tumors such as breast cancers [39–41] and lymphomas [42] have shown that increased expression of APOBEC3 in vivo was linked to the chronic induction of mutations and/or instability in genomic DNA. We did not observe any significant diagnosable HIV-associated cancers in our short-term cohort study. It may be necessary to continue our prospective studies for a longer period. In addition, because other studies have suggested that APOBEC3 gene deficiency is associated with higher susceptibility to two other ancient pathogens, human T-cell leukemia virus type 1 [43,44] and Plasmodium falciparum (the causative agent of malaria) [45], it would be beneficial to further investigate the correlations between APOBEC3B genotype and susceptibility to unknown pathogens.

Conclusions

Our analysis of a population-based matched cohort provided important evidence that the loss of the APOBEC3B gene is not associated with risk of HIV-1 infection and disease progression. In addition, the in vitro kinetics of HIV-1 replication and the infectivity of the virus in PBMCs were comparable between the D/D and I/I subjects. These results suggest that the APOBEC3B antiviral mechanism plays only a negligible role in eliminating HIV-1 in vivo. This finding may explain why HIV-1 has not evolved a Vif-based strategy to counteract APOBEC3B restriction. Further analyses to explore the role(s) of APOBEC3B in human are also required in other cohorts with diverse genetic backgrounds in Asia.

Supporting Information

Figure S1 Overexpression of two APOBEC3B variants and the antiviral effect of the variants in vitro. (A) A DNA fragment of the complete APOBEC3B open reading frame was amplified by RT-PCR from each RNA sample of healthy donors with APOBEC3B K62 (A3B K62) and E62 (A3B E62). Each of the fragment was replaced into the APOBEC3G gene position of the pcDNA A3G (Myc-His) WT (A3G WT) plasmid as previously described [8]. The primer sets for amplification of APOBEC3B cDNA were used as follows: the 1st PCR, 5'-gacgagcagcagacaagccag and 5'-aaccaggtctctgccttcc; the 2nd PCR, 5'-tcgagcgggacaggga-tcagatcagaaatccg and 5'-cgatacgtgcttcctgattctggagaatggc. The resultant APOBEC3B expression plasmids, pcDNA APOBEC3B K62 and pcDNA APOBEC3B E62, contain a C-terminal Myc/His tag (consisting of Myc and hexa-histidine epitopes). The sequences of both the insert and the boundary regions for the APOBEC3B expression plasmids were verified by DNA sequencing. The expression or control (Vector) plasmids were transfected into human embryonic kidney cells (HEK 293T) by using FuGENE HD (Promega, Madison, USA). At 48 hr after transfection, cell lysates were prepared with Laemmli buffer containing 2.5% 2-Mercaptoethanol and analyzed by western blot. Protein bands were probed with anti-β-tubulin rabbit polyclonal antibody (1/2,500) (ab6046, Abcam, Cambridge, USA) or anti-His mAb (1/3,000) (D291-3, Medical & Biological Laboratories Co., Nagoya, Japan) as previously reported [8]. (B) The effect of two APOBEC3B variants on HIV-1 infectivity in vitro was analyzed. For virus production, 293T cells were cotransfected with 1 μg of pNL-A-3 WT (HIV-1 WT) or pNL-A-3-vif-(HIV-1 vif-(-) plus 1 (black) or 0.1 (gray) μg of pcDNA APOBEC3B K62, pcDNA APOBEC3B E62, pcDNA 3.1 (+) (Vector), or pcDNA A3G (Myc-His) WT. Because it has been reported that the antiviral effect of APOBEC3B on HIV-1 in vitro can be observed when overexpressed in 293T cells but not T cell lines [20], we used 293T cells for the virus production. Virus infectivity was determined using TZM-bl cells [29]. Relative infectivity as relative light units (RLU) was calculated by normalizing for the amount of input CA, determined by p24 antigen ELISA (ZeptoMetrix, Buffalo, USA). Three independent experiments were performed. Results from one representative experiments are shown. A3G, APOBEC3G. (TIF)
gaggtt) and DRINR (5’-ctgtatcagaccccaatag; 

*vif*, DRVF1F (5’-gggttaaagacgcagcag) and 

*vif*, DRVF1R (5’-gtccttgcttctctgctag). For the nested PCR, a 2,735-bp (bp, nt 2,483–5,219) and an 859-bp (nt 4,953–5,812) fragment were generated using primer sets, DRRT1L (5’-gacatccctgcttacca-

taatgg)/DRRT1R (5’-ctggagttgctgctgacta) and 

DRVF2R (5’-ctggagttgctgctgacta)/DRVF2R (5’-gta-

taatggcttctctgctag), respectively. The resulting PCR products were purified with the QuIAquick PCR Purification kit (Qiagen) and quantified with the Quant-iT dsDNA BR kit (Life Technologies). Paired-end DNA libraries were prepared using the Nextera DNA sample prep kit (Illumina, San Diego, USA) according to the manufacturer’s protocol. The DNA libraries were sequenced on a MiSeq (Illumina) using the MiSeq reagent kit v2 to produce 250 bp x 2 paired-end reads. The reads generated by deep sequencing were mapped onto the reference sequence of HXB2 strain by BWA 0.7.3a program (http://bio-bwa. sourceforge.net). Then, sequences of a 150-base pairs-long region were extracted and the sequences containing bases with quality scores under 30 were omitted by our in house program. (B) Among the extracted sequences, the hypermutation types and the numbers of the dinucleotide sequences, GG>AG (red) and GA>AA (blue), were analyzed. In order to detect hypermutations, the unique sequences with >5-fold coverage depth were used. The frequency (%) of hypermutation is shown as mutation rates per dinucleotide sequence with .

The cumulative histograms represent number of hypermutated positions (y-axis) for GG

hypermuated positions (y-axis) for GG.

The cumulative histograms represent number of hypermutated positions (y-axis) for GG

Table S1 Oligonucleotide primers used for real-time PCR of *APOBEC3* and control. A real-time PCR assay was performed for *APOBEC3* and control genes (Gene symbol) using each of forward primer (S) and reverse primer (AS) sets.

Table S2 APOBEC3B variations in the I/D and I/I genotyped healthy donors. APOBEC3B cDNAs from I/D and I/I-genotyped healthy donors (n = 5 each) were amplified by the nested RT-PCR and cloned into pUC118 plasmids. The APOBEC3B cDNA sequences were determined by DNA sequencing. The individual APOBEC3B variants analyzed are shown. A3B, APOBEC3B.

Acknowledgments

The authors thank all of the patients and volunteers who agreed to participate in this study. We also thank the NLGR organization for cooperating in the recruitment of the MSM volunteers in Nagoya. The TZM-bi 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.

Author Contributions

Conceived and designed the experiments: MI TI NK SI YK ATK WS YI TY TN. Performed the experiments: MI TI KI TM KS YI. Analyzed the data: MI TI DW JI KS HO YK ATK YY WS YI. Contributed reagents/materials/analysis tools: MI DW JI SI MU YY TS. Wrote the paper: MI TI YK ATK WS YI TN.

References

1. Albin JS, Harris RS (2010) Interactions of host APOBEC3 restriction factors in HIV-1: implications for therapeutic cancer. Expert Rev Mol Med 12: e4. 2. Goila-Gaur R, Strebel K (2008) HIV-1 Vif, APOBEC, and intrinsic immunity. Retrovirology 5: 51.

3. Sheehy AM, Gaddis NC, Choi JD, Malim MH (2002) Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. Nature 394: 666–670.

4. Bishop KN, Verma M, Kim EY, WoIński SM, Malim MH (2008) APOBEC3G inhibits elongation of HIV-1 reverse transcripts. PLoS Pathog 4: e1000231.

5. Iwatani Y, Chan DS, Wang F, Maynard KS, Sugiura W, et al. (2007) Deaminase-independent inhibition of HIV-1 reverse transcription by APOBEC3B, APOBEC3C, and APOBEC3DE. J Virol 81: 1902–1911.

6. Zhen A, Wang T, Zhao K, Xiong Y, Yu XF (2010) A single amino acid difference in human APOBEC3H variants determines HIV-1 Vif sensitivity. J Virol 84: 12599–12608.

7. Chuan Y, Chang D, Wang F, Maynard KS, Sugiura W, et al. (2007) Deaminase-independent inhibition of HIV-1 reverse transcription by APOBEC3G. Nucl Acids Res 35: 7966–7986.

8. LaRue RS, Andrés-Vottier L, Blanchard Y, Coutelico SG, Dese D, et al. (2009) Guidelines for naming nonprimate APOBEC3 genes and proteins. J Virol 83: 494–497.

9. Yu X, Yu Y, Liu B, Luo K, Kong W, et al. (2005) Induction of APOBEC3G ubiquitination and degradation by an HIV-1 Vif-Cul5-SSCF complex. Science 302: 1056–1060.

10. Russell RA, Smith J, Barr R, Bhattacharyya D, Pathak VK (2009) Distinct domains within APOBEC3G and APOBEC3F interact with separate regions of human immunodeficiency virus type 1 Vif. J Virol 83: 1992–2003.

11. Smith JL, Pathak VK (2010) Identification of specific determinants of human APOBEC3F, APOBEC3G, and APOBEC3DE and African green monkey APOBEC3F that interact with HIV-1 Vif. J Virol 84: 12599–12608.

12. Zhen A, Wang T, Zhao K, Xiong Y, Yu XF (2010) A single amino acid difference in human APOBEC3H variants determines HIV-1 Vif sensitivity. J Virol 84: 1902–1911.

13. Biasin M, Piacentini L, Lo Caputo S, Kanari Y, Magri G, et al. (2007) Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G: a possible role in the resistance to HIV of HIV-exposed seronegative individuals. J Infect Dis 195: 960–964.

14. Chaipan C, Smith JL, Hu WS, Pathak VK (2013) APOBEC3G restricts HIV-1 to a greater extent than APOBEC3F and APOBEC3D in human primary CD4+ T cells and macrophages. J Virol 87: 444–453.

15. Do H, Vasilescu A, Dhop G, Hirtzhug A, Heath SC, et al. (2005) Exhaustive genotyping of the CEM15 (APOBEC3G) gene and absence of association with AIDS progression in a French cohort. J Infect Dis 191: 159–163.

16. Kourteva Y, De Pasquale M, Allos T, McMunn C, DaAquila RT (2012) APOBEC3G expression and hypermutation are inversely associated with human immunodeficiency virus type 1 (HIV-1) burden in vivo. Virology 430: 1–9.
17. Mulder LC, Ooms M, Majdak S, Smedresman J, Linscheid C, et al. (2010) Moderate influence of human APOBEC3F on HIV-1 replication in primary lymphocytes. J Virol 84: 9613–9617.

18. Uelenga NK, Sarr AD, Thakore-Meloni S, Sankale JL, Eisen G, et al. (2008) Differences in APOBEC3G expression in CD4+ T helper lymphocyte subtypes modulate HIV-1 infectivity. PLoS Pathog 5: e1000292.

19. Vetter ML, Johnson ME, Antons AK, Unutmaz D, D’Aquila RT (2009) Human and rhesus APOBEC3D, APOBEC3F, APOBEC3G, and APOBEC3H demonstrate a conserved capacity to restrict Vif-deficient HIV-1. J Virol 83: 11230–11234.

20. Pak V, Heidecker G, Pathak VK, Derse D (2011) The role of amino-terminal sequences in cellular localization and antiviral activity of APOBEC3B. J Virol 85: 5539–5547.

21. Yu Q, Chen D, Koni R, Mariani R, Unutmaz D, et al. (2004) APOBEC3B is a potent inhibitor of simian immunodeficiency virus replication. J Biol Chem 279: 53379–53386.

22. Bologn KN, Holmes RK, Soreby AM, Davidson NO, Cho SJ, et al. (2004) Cytidine deamination of retroviral RNA by diverse APOBEC proteins. Curr Biol 14: 1392–1396.

23. Doehle BP, Scharfer A, Cullen BR (2005) Human APOBEC3B is a potent inhibitor of HIV-1 infectivity and is resistant to HIV-1 Vif. Virology 309: 201–206.

24. Kidd JM, Newman TL, Tuzun E, Kaul R, Eichler EE (2007) Population stratification of a common APOBEC gene deletion polymorphism. PLoS Genet 3: e63.

25. An P, Johnson R, Phair J, Kirk GD, Yu XF, et al. (2009) APOBEC3B deletion and risk of HIV-1 infection. J Infect Dis 200: 1054–1058.

26. Itaya S, Nakajima T, Kaur G, Terumuma H, Ohtani H, et al. (2010) No evidence of an association between the APOBEC3B deletion polymorphism and susceptibility to HIV infection and AIDS in Japanese and Indian populations. J Infect Dis 202: 815–816; author reply 816–817.

27. Bower M, Cullen BR (2006) Single-stranded RNA facilitates nucleocapsid: plasmamembrane for packaging into human immunodeficiency virus type 1 virus-like particles in an RNA-dependent process requiring the NC basic linker. J Virol 81: 5000–5013.

28. Wicherowski MJ, Robb GB, Rana TM (2006) Human retroviral host restriction factors APOBEC3G and APOBEC3F localize to mRNA processing bodies. PLoS Pathog 3: e41.

29. Mistry MJ, Harris RS (2006) APOBEC3B and APOBEC3F inhibit L1 retrotransposition by a DNA deamination-independent mechanism. J Biol Chem 281: 16837–16841.

30. Ulenga NK, Sarr AD, Thakore-Meloni S, Sankale JL, Eisen G, et al. (2008) Differences in APOBEC3G expression in CD4+ T helper lymphocyte subtypes modulate HIV-1 infectivity. PLoS Pathog 5: e1000292.

31. Burnett A, Spearman P (2007) APOBEC3G multimers are recruited to the plasma membrane for packaging into human immunodeficiency virus type 1 virus-like particles in an RNA-dependent process requiring the NC basic linker. J Virol 81: 5000–5013.

32. Vetter ML, Johnson ME, Antons AK, Unutmaz D, D’Aquila RT (2009) Human and rhesus APOBEC3D, APOBEC3F, APOBEC3G, and APOBEC3H demonstrate a conserved capacity to restrict Vif-deficient HIV-1. J Virol 83: 11230–11234.

33. Pak V, Heidecker G, Pathak VK, Derse D (2011) The role of amino-terminal sequences in cellular localization and antiviral activity of APOBEC3B. J Virol 85: 5539–5547.

34. An P, Johnson R, Phair J, Kirk GD, Yu XF, et al. (2009) APOBEC3B deletion and risk of HIV-1 infection. J Infect Dis 200: 1054–1058.

35. Itaya S, Nakajima T, Kaur G, Terumuma H, Ohtani H, et al. (2010) No evidence of an association between the APOBEC3B deletion polymorphism and susceptibility to HIV infection and AIDS in Japanese and Indian populations. J Infect Dis 202: 815–816; author reply 816–817.

36. Bower M, Cullen BR (2006) Single-stranded RNA facilitates nucleocapsid: plasmamembrane for packaging into human immunodeficiency virus type 1 virus-like particles in an RNA-dependent process requiring the NC basic linker. J Virol 81: 5000–5013.

37. Vetter ML, Johnson ME, Antons AK, Unutmaz D, D’Aquila RT (2009) Human and rhesus APOBEC3D, APOBEC3F, APOBEC3G, and APOBEC3H demonstrate a conserved capacity to restrict Vif-deficient HIV-1. J Virol 83: 11230–11234.

38. Pak V, Heidecker G, Pathak VK, Derse D (2011) The role of amino-terminal sequences in cellular localization and antiviral activity of APOBEC3B. J Virol 85: 5539–5547.

39. Ulenga NK, Sarr AD, Thakore-Meloni S, Sankale JL, Eisen G, et al. (2008) Differences in APOBEC3G expression in CD4+ T helper lymphocyte subtypes modulate HIV-1 infectivity. PLoS Pathog 5: e1000292.