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HIV-1 Vif: a guardian of the virus that opens up a new era in the research field of restriction factors

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The research on virion infectivity factor (Vif) protein had started in late 1980s right after HIV-1 was cloned, and the function of Vif had been a mystery for a long time. However, the research on Vif has finally lead to the identification of APOBEC3G, which opens up a new era in the research field of host restriction factors in HIV-1 infection followed by TRIM5α, Tetherin/BST-2, and SAMHD1. This suggests that continuation of basic research on fundamental questions is quite important. We still have many questions on Vif and APOBEC3 and should continue to work on these proteins in the future in order to better regulate HIV-1. We will discuss not only the history but also recent advances in Vif research.

Keywords: HIV-1 Vif, restriction factor, ubiquitin ligase, cell cycle arrest, p53, MDM2

INTRODUCTION

HIV-1 virion infectivity factor (Vif) was identified as an accessory gene right after the HIV-1 genome was sequenced. It is well conserved among lentiviruses except in equine infectious virus and plays a crucial role in the viral life cycle to facilitate viral infectivity as its name indicates (Desrosiers et al., 1998). In the early reports, Strebel and collegues described that the mutant virus deficient in the *vif* gene produces virion particles normally; however, the particles are ~1000 times less infectious than the wild type (Fisher et al., 1987; Strebel et al., 1987). The underlying mechanism of Vif function had been unsolved and a mystery for a long time.

EARLY OBSERVATIONS OF Vif FUNCTION LEAD TO IDENTIFICATION OF APOBEC3G

Virion infectivity factor exerts its function in a cell-type-specific manner. Vif is dispensable for producing infectious viral particles in permissive cells such as all known adherent cells (e.g., HeLa and 293T cells) and some T cell lines (e.g., CEM-SS and SupT1 cells); in contrast, Vif is indispensable in non-permissive cells such as physiologically relevant $CD4^+$ T cells and macrophages, and other T cell lines (e.g., CEM and H9 cells; Gabuzda et al., 1992; Sakai et al., 1993; Simon et al., 1998b). These findings raise two possibilities; one is that permissive cells have a vif-like cellular factor which facilitates virion infectivity, another is that non-permissive cells possess an anti-HIV-1 host factor which is antagonized by Vif. Later studies using heterokaryon experiments have shown the latter possibility (Madani and Kabat, 1998; Simon et al., 1998a). In 2002, Malim's group identified this factor using very sophisticated subtraction cloning methods between non-permissive CEM cells and its derivative subclone permissive CEM-SS cells, which was first called as CEM15 and is now known as APOBEC3G (Sheehy et al., 2002). Details of functions of APOBEC3G and other APOBEC3 family members are described and discussed in many reviews and other chapters of this issue (Goila-Gaur and Strebel, 2008; Wissing et al., 2010; Kitamura et al., 2011).

In addition to the above described main function, early studies also revealed several important Vif functions including dimerization (Yang et al., 2001), virion incorporation (Camaur and Trono, 1996; Simon et al., 1997), and phosphorylation (Yang et al., 1996; Yang and Gabuzda, 1998); however, the significances of these functions are not discussed much recently. Recently, a novel Vif function on cell cycle has been reported, which is discussed in more detail later.

Vif ANTAGONIZES APOBEC3G

As described above, the main function of Vif is to antagonize APOBEC3G. Right after identification of APOBEC3G, many studies have shown that Vif inhibits the virion incorporation of APOBEC3G, which is mainly attributable to degradation of cellular APOBEC3G via the proteasomal pathway (Marin et al., 2003; Sheehy et al., 2003; Stopak et al., 2003; Mehle et al., 2004b). However, some studies have also shown that Vif directly inhibits the virion incorporation of APOBEC3G (Opi et al., 2007) or that Vif inhibits translation of APOBEC3G (Mariani et al., 2003; Stopak et al., 2003).

Yu et al. (2003) have independently shown that Vif forms E3 ligase complexes with cellular proteins including Cullin 5, Elongin B, and C (Vif–Cul5–EloB/C complex) using mass-spectrometry techniques. They and others have also shown that this complex works as the E3 ligase for APOBEC3G to induce polyubiquitination of APOBEC3G and direct it to the 26S proteasome for degradation (Mehle et al., 2004a; Yu et al., 2004; Kobayashi et al., 2005). Iwatani et al. (2009) have identified four critical lysine residues (K^{297}, K^{301}, K^{303}, and K^{334}) in APOBEC3G which are required for Vif-mediated degradation, although others have reported that Vif can ubiquitinate and degrade a lysine-free APOBEC3G

(Shao et al., 2010). Vif also antagonizes other APOBEC3 proteins from APOBEC3C to H by the same E3 ligase complex (Shirakawa et al., 2006).

Virion infectivity factor binds to the E3 ligase complex through two interaction sites; it binds to Elongin C through its suppressors of cytokine signaling (SOCS) box motif (Mehle et al., 2004a; Yu et al., 2004), $S^{144}LQYLA^{149}$, and to Cullin 5 through a zinc-binding motif (Luo et al., 2005; Mehle et al., 2006), $H^{108}x_5Cx_{17-18}Cx_{3-5}H^{139}$ (**Figure 1**). The SOCS box motif is well conserved among Vif proteins, indicating that this motif is crucial for Vif function, and mutation of S^{144}, a presumed phosphorylation site in Vif, affects binding of Vif to Elongin C (Mehle et al., 2004a). The zinc-binding motif is also important for Vif function to form the E3 ligase complex. Therefore, a zinc chelating agent can inhibit Vif function in infectivity assays (Xiao et al., 2007).

THE INTERACTION OF Vif WITH APOBEC3 PROTEINS

It is quite important to reveal the interaction sites between Vif and APOBEC3 proteins, because the regulation of this interaction may lead to the development of novel therapeutic strategies for HIV-1 infection. However, their structural information is not fully elucidated yet, because it is quite difficult to produce these proteins as soluble forms. Thus, the information described below is mainly obtained by many studies using site-directed mutagenesis, which sometimes shows different results.

First of all, the most important and confirmed evidence is that the interaction between Vif and APOBEC3G is critically dependent on $D^{128}PD^{130}$ in APOBEC3G (Huthoff and Malim, 2007). Many groups have simultaneously reported this evidence by comparing human and African green monkey (agm) APOBEC3G (Bogerd et al., 2004; Mangeat et al., 2004; Schrofelbauer et al., 2004; Xu et al., 2004). In detail, HIV-1 Vif binds and antagonizes human APOBEC3G, but not agm APOBEC3G. In contrast, SIVagm Vif antagonizes agm APOBEC3G, but not human APOBEC3G. By comparing amino acids residues and preparing chimeric APOBEC3G between human and agm APOBEC3G, they identified D^{128} as the determinant of the species-specific binding of Vif to APOBEC3G (Bogerd et al., 2004; Mangeat et al., 2004; Schrofelbauer et al., 2004; Xu et al., 2004). On the other hand, SIVmac and HIV-2 Vif can antagonize both human and agm APOBEC3G, indicating that the interaction between Vif and APOBEC3G is not restricted by D^{128}, in other words, D^{128} is not the sole determinant for species-specific target by Vif (Gaur and Strebel, 2012). Furthermore, the interaction between Vif and APOBEC3G is regulated by phosphorylation of APOBEC3G at T^{32} by protein kinase A (Shirakawa et al., 2008).

The interaction sites in Vif are reported by many groups and are much more complicated. The binding site only for APOBEC3G is $Y^{40}RHHY^{44}$ (Russell and Pathak, 2007), while that only for APOBEC3F is $D^{14}RMR^{17}$ (Russell and Pathak, 2007), and $T^{74}GERxW^{79}$ (He et al., 2008). The binding sites for both APOBEC3G and F are $W^{21}KSLVK^{26}$ (Chen et al., 2009; Dang et al., 2009), $V^{55}xIPLx_{4-5}Lx\Phi x_2YWxL^{72}$ (He et al., 2008), and $Y^{69}xxL^{72}$ (Pery et al., 2009; **Figure 1**). To identify the real interaction sites, we have to wait a little longer until we will get the structural information of these complexes.

Vif AND CBFβ

Recent mass-spectrometry screening of Vif-binding proteins has identified a T cell transcription factor, core-binding factor subunit

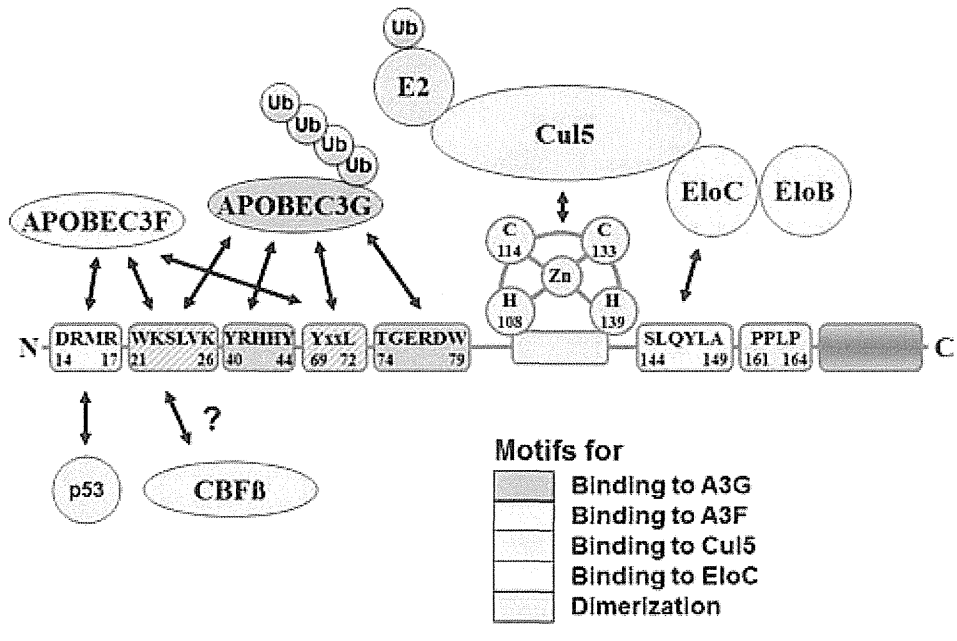

FIGURE 1 | Schematic figure of the virion infectivity factor (Vif) protein and amino acid motifs for binding to Vif-interacting proteins. Pink indicates binding motifs for A3G; light blue indicates binding motifs for A3F; light green indicates binding motifs for Cul5; yellow indicates binding motifs for EloC; light pink indicates motifs for dimerization. Vif binds to p53 and CBFβ in its N-terminal regions, but binding motifs were not elucidated yet.

beta (CBFβ), as an important Vif-binding protein (Jager et al., 2012; Zhang et al., 2012). CBFβ directly binds to Vif and plays a crucial role in forming a stable Vif–Cul5–EloB/C E3 ligase complex. Without CBFβ, the Vif–Cul5–EloB/C E3 ligase complex is not stable enough to polyubiquitinate APOBEC3G and its function is severely impaired. The binding sites of Vif with CBFβ are identified as W^{21} and W^{38} (**Figure 1**). However, the mechanisms by which CBFβ regulates the E3 ligase complex are still under investigation. Furthermore, since CBFβ is an important T cell transcription factor, it would be very interesting to determine whether Vif affects T cell differentiation.

Vif IS ALSO UBIQUITINATED

Fujita et al. (2004) have reported that expression of the Vif protein in virus-producing cells is maintained at very low levels, which is regulated by the ubiquitin–proteasome pathway. It is because its high expression inhibits viral infectivity by affecting proteolytic processing of Gag protein (Akari et al., 2004). We have identified the E3 ligase for Vif as mouse double minute 2 homolog (MDM2; Izumi et al., 2009; **Figure 2**). Since Vif is a component of a Cul5–EloB/C complex, one report showed that this complex ubiquitinated Vif (Mehle et al., 2004a). Another report showed that other E3 ligases such as neural precursor cell expressed developmentally down-regulated protein 4 (Nedd4) and atrophin-interacting protein 4 (AIP4) bound to Vif, however, it didn't show the direct evidence of Vif ubiquitination by these ligases (Dussart et al., 2004). The identification of the E3 ligase has lead to elucidation of the mechanisms of Vif-induced G2 cell cycle arrest described below.

A NOVEL Vif FUNCTION: G2 CELL CYCLE ARREST

In early 1990s, viral protein R (Vpr) had been shown to induce G2 cell cycle arrest in HIV-1-infected cells (He et al., 1995; Re et al., 1995; Roshal et al., 2003; Nakai-Murakami et al., 2006). Many groups have extensively worked on Vpr-induced G2 arrest in terms of its molecular mechanisms and published many papers. Although only one paper reported the virological significance of G2 arrest induced by Vpr (Goh et al., 1998), the basic and fundamental questions of why the virus needs to induce G2 arrest still remain unsolved. More than 10 years had passed since then, and two recent reports came out, describing that Vif as well as Vpr-induce G2 arrest in HIV-1-infected cells (Sakai et al., 2006; Wang et al., 2007). We have recently shown the molecular mechanisms by which Vif induces G2 arrest (Izumi et al., 2010; **Figure 2**). Vif activates p53, which is well known as a tumor suppressor gene and the regulator of cell cycle as "a guardian of the genome." Vif binds and activates p53 by stabilizing and sequestering it to the nucleus. Activation of p53 induces its downstream cascade such as activation of p21 and inactivation of Cdc2/CyclinB, resulting in G2 arrest. Furthermore, we identified the amino acid residues in Vif responsible for its interaction with p53 and a Vif mutant which does not induce G2 arrest. Using a mutant virus which possesses the *vif* mutant, we have demonstrated that Vif-induced G2 arrest facilitates viral replication (Izumi et al., 2010; **Figure 2**). Thus, HIV-1 needs to have G2 cell cycle arrest to efficiently replicate so that it possesses two accessory genes such as *vif* and *vpr*. Vif induces G2 arrest in a p53-dependent manner, while Vpr accomplishes the same goal in a p53-independent manner.

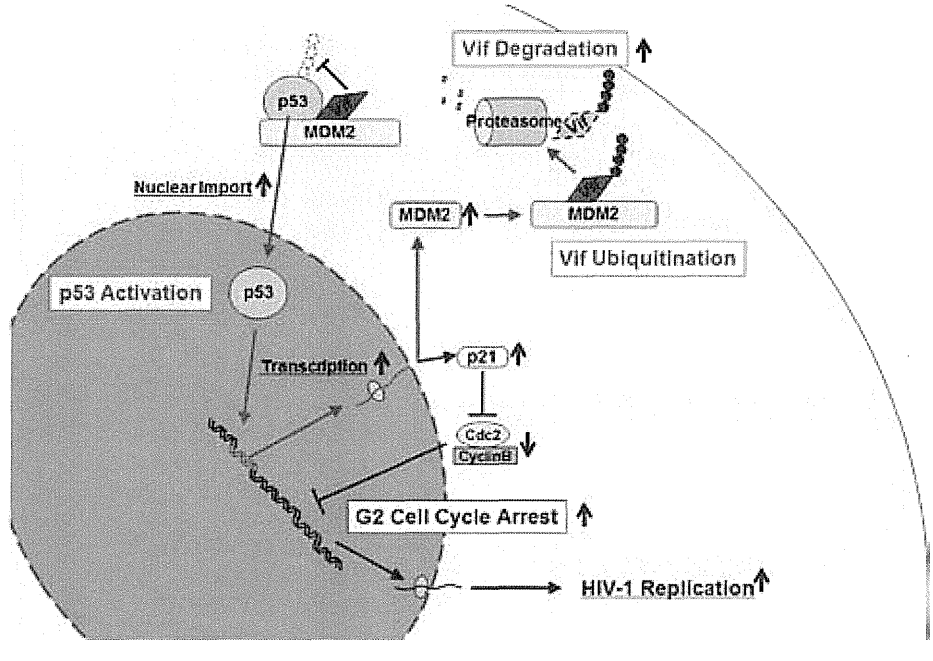

FIGURE 2 | The mechanisms how Vif is ubiquitinated and degraded and how Vif induces G2 cell cycle arrest. Vif is ubiquitinated and degraded by MDM2. On the contrary, Vif inhibits ubiquitination of p53 by MDM2 to induce activation and nuclear import of p53. Activated p53 induces transcription of several genes including MDM2 and p21. Enhanced expression of MDM2 may lead to more Vif ubiquitination and degradation, which forms the autoregulatory circuit of Vif expression. On the other hand, activation of p21 leads to G2 cell cycle arrest, resulting in more HIV-1 replication.

CONCLUSION

HIV Vif is an intriguing viral protein, not only because it opens up a new era in the research field of host restriction factors, but also because it has a variety of functions for the viral life cycle by interacting several cellular proteins. It suggests that it might be a good target for control of HIV-1 infection.

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A Naturally Occurring Single Amino Acid Substitution in Human TRIM5α Linker Region Affects Its Anti-HIV Type 1 Activity and Susceptibility to HIV Type 1 Infection

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Abstract

TRIM5α is a factor contributing to intracellular defense mechanisms against retrovirus infection. Rhesus and cynomolgus monkey TRIM5αs potently restrict HIV-1, whereas human TRIM5α shows weak effects against HIV-1. We investigated the association between a single nucleotide polymorphism in the TRIM5α linker 2 region (rs11038628), which substituted aspartic acid (D) for glycine (G) at position 249, with susceptibility to HIV-1 infection in Japanese and Indian subjects. rs11038628 is rare in Europeans but common in Asians and Africans. Functional analyses were performed by multiple-round replication and single-round assays, and indicated that the G249D substitution attenuated anti-HIV-1 activity of human TRIM5α. A slight attenuation of anti-HIV-2 activity was also observed in TRIM5α with 249D. The predicted secondary structure of the linker region suggested that the 249D substitution extended the α-helix in the neighboring coiled-coil domain, suggesting that human TRIM5α with 249D may lose the flexibility required for optimal recognition of retroviral capsid protein. We further analyzed the frequency of G249D in Japanese (93 HIV-1-infected subjects and 279 controls) and Indians (227 HIV-1-infected subjects and 280 controls). The frequency of 249D was significantly higher among HIV-1-infected Indian subjects than in ethnicity-matched control subjects [odds ratio (OR) = 1.52, $p = 0.026$]. A similar weak tendency was observed in Japanese subjects, but it was not statistically significant (OR = 1.19, $p = 0.302$). In conclusion, G249D, a common variant of human TRIM5α in Asians and Africans, is associated with increased susceptibility to HIV-1 infection.

Introduction

TRIM5α FROM RHESUS MONKEYS restricts human immunodeficiency virus-1 (HIV-1) replication at the postentry,[1] preintegration stage in the viral life cycle through rapid degradation of the HIV-1 core,[2] whereas human TRIM5α restricts HIV-1 only weakly but potently restricts N-tropic murine leukemia virus.[3,4] TRIM5α is a member of the tripartite motif-containing proteins and consists of RING, B-box 2, coiled-coil, and PRYSPRY (B30.2) domains. TRIM5α recognizes the multimerized capsid (CA) proteins of an incoming virus by its α-isoform-specific PRYSPRY domain. Studies of chimeric TRIM5αs have shown that the determinant of species-specific restriction against viral infection resides in the variable regions of the PRYSPRY domain.[5–11]

Infection by HIV-1 and progression to acquired immune deficiency syndrome (AIDS) vary among human individuals, and these phenomena are considered to be at least partially controlled by diversity in the human genome.[12,13] Two common TRIM5α functional polymorphisms, H43Y and R136Q, have been studied with regard to the association with HIV-1 infection.[14–21] Price *et al.* sequenced exon 2 of the *TRIM5* gene in 1,032 women enrolled in a long-term monitored Pumwani sex worker cohort, and found that women with the R136Q polymorphism were less likely to seroconvert despite heavy exposure to HIV-1 through active sex work.[15] Previous studies, including ours, showed the reduced antiviral activity of the H43Y substitution, but the associations with HIV-1 infection and disease progression were inconsistent among studies.[14,16–20] Javanbakht *et al.* reported a paradoxical

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protective effect of TRIM5α with 43Y against HIV-1 transmission in African-Americans.[14] Taken together, these findings indicate that anti-HIV-1 activity of human TRIM5α cannot protect humans from an HIV-1 pandemic, but may affect the rate of HIV-1 transmission.

In the present study, we investigated the association between a single nucleotide polymorphism (SNP) in the TRIM5α linker 2 region (rs11038628) between coiled-coil and PRYSPRY domains with susceptibility to HIV-1 infection. This SNP substituted aspartic acid (D) for glycine (G) at position 249. We show here that this SNP is associated with increased susceptibility to HIV-1 infection.

Materials and Methods

Cloning and expression of TRIM5α

The generation of recombinant Sendai viruses (SeVs) expressing human TRIM5α derived from MT4 cells, rhesus monkey TRIM5α derived from LLC-MK2 cells, and cynomolgus monkey TRIM5α lacking the PRYSPRY domain has been previously described.[9,22] All these TRIM5αs carried a hemagglutinin (HA) tag (YPYDVPDYAA) at the C-terminus. The D-to-G substitution at the 249th position was introduced into MT4 TRIM5α by polymerase chain reaction (PCR) site-directed mutagenesis. The resultant PCR fragment was cloned into pSeV18+b(+) as a vector. Recombinant SeVs expressing human TRIM5α carrying G at position 249 were recovered according to the previously described method.[23] The second passages in embryonated chicken eggs were used as stock virus for all experiments.

Western blotting analysis

MT4 cells (1×10^6) infected with recombinant SeVs expressing HA-tagged TRIM5α proteins were lysed in lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate). TRIM5α proteins in the lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins in the gel were then electronically transferred onto a membrane (Immobilon; Millipore, Billerica, MA). Blots were blocked and probed with anti-HA high-affinity rat monoclonal antibody (Roche, Indianapolis, IN) overnight at 4°C. Blots were then incubated with peroxidase-conjugated anti-rat IgG (American Qualex, San Clemente, CA), and bound antibodies were visualized with a Chemilumi-One chemiluminescent kit (Nacalai Tesque, Kyoto, Japan).

Viral infection

MT4 cells (1×10^6) were infected with SeVs expressing MT4-derived human TRIM5α (249D), human TRIM5α (249G), rhesus monkey TRIM5α, or cynomolgus monkey TRIM5α lacking the PRYSPRY domain [CM-TRIM5α-SPRY(−)] at a multiplicity of infection (MOI) of 10 plaque-forming units (PFU) per cell and incubated at 37°C for 9 h. Aliquots of 1×10^5 cells were then superinfected with HIV-1 NL43 or HIV-2 GH123. Each superinfection used a titer of virus corresponding to 7 ng of p24 of NL43 or 20 ng of p25 of GH123. Experiments were performed with triplicate samples. The culture supernatants were collected periodically and the level of p24 or p25 was measured using a RETROtek antigen ELISA kit (ZeptoMetrix, Buffalo, NY). For the single-round infection assay, hamster TK-ts13 cells were infected with SeV expressing TRIM5α as described above, and superinfected with a vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped HIV-1 vector expressing green fluorescence protein (GFP) under the control of the cytomegalovirus (CMV) promoter. The original HIV-1 vector was based on the BH10 strain.[24,25] To construct the lentivector possessing CA of NL4-3, we replaced the *Eco*RI–*Apa*I fragment corresponding to MA and CA of the pMDLg/p.RRE packaging vector with that of NL4-3.[26] In case of HIV-2, we used a canine cell line Cf2Th and VSV-G pseudotyped HIV-2 vector expressing GFP under the control of the LTR promoter.[27] Two days after infection, the cells were fixed with formaldehyde, and GFP-expressing cells were counted by a flow cytometer.

Human DNA subjects

The protocol for the present study was approved by the Ethics Review Board of the Medical Research Institute of Tokyo Medical and Dental University and that of the All India Institute of Medical Science. At setup of the cohort of HIV-1-infected Japanese subjects with hemophilia in 1995, all patients had been infected for longer than 10 years but were asymptomatic without any antiviral measures. Blood samples were collected from 93 well-characterized patients who were selected from the cohort after obtaining written informed consent.[28,29] Control DNA samples were prepared from Epstein–Barr virus-transformed human B cell lines established from randomly selected healthy donors ($n=279$) and obtained from the Japan Health Sciences Foundation. DNA samples from HIV-1-infected individuals were prepared from the blood samples using a QuickGene DNA whole blood kit S (Fujifilm, Tokyo, Japan). In addition, blood DNA samples were obtained from 227 HIV-1-infected Indian subjects and 226 healthy Indian volunteers with informed consent in related hospitals with the All India Institute of Medical Sciences, New Delhi.

Identification and genotyping of nucleotide variations in TRIM5α exon 5

Primer sets were designed to amplify the genomic segments covering the entire TRIM5α exon 5 as follows: sense primer (5′-GATGCGGTCATGCTATGTTG-3′) and antisense primer (5′-CGAATGCTGATTTATGACCATA-3′). Genomic DNA was subjected to PCR amplification followed by sequencing using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Polymorphisms were identified using the Sequencher program (Gene Code Co., Ann Arbor, MI).

Statistical analysis

All statistical analyses in this study were performed using GraphPad InStat version 3.06 for Windows (GraphPad Software, San Diego, CA). Pairwise linkage disequilibrium (LD) (r^2) was estimated using SNPAlyze version 6.0 standard (Dynacom Co., Ltd., Chiba, Japan).

Prediction of the peptide secondary structure

The Chou–Fasman methods were used to predict the secondary structure of TRIM5α using GENETYX-MAC version 15 software (Genetyx Corporation, Tokyo, Japan).

Results

Anti-HIV-1 activity of TRIM5α was attenuated by G249D substitution

We previously cloned human TRIM5α from the CD4-positive T cell line MT4 and noted that there is a G-to-D amino acid substitution (G249D) in comparison with the reference sequence (NM_033034).[9] This position is known as a polymorphic site in the human *TRIM5* gene (rs11038628) located in the linker 2 region between the coiled-coil and PRYSPRY domains (Fig. 1). Initially, we speculated that this polymorphism would have no effect on antiviral activity due to its presence in the linker 2 region. Goldschmidt *et al.*[18] reported that HeLa cells stably transduced with TRIM5α with 249D did not differ in susceptibility to HIV-1 infection. However, a tendency toward higher *in vitro* p24 production was observed at 7 days after infection in peripheral blood mononuclear cells from white subjects with the 249D allele, although the difference was not statistically significant mainly due to the limited number of subjects with the mutant allele.[18] In addition, Old World monkey TRIM5α, including those of African green monkey, rhesus monkey, and cynomolgus monkey, also bears G at this position (Fig. 1). The HapMap project showed the 249D allele to be rare in whites (allele frequency: 0.053) but common in Japanese (allele frequency: 0.343) and African populations (allele frequency: 0.367). These findings prompted us to reevaluate the effects of this SNP on HIV-1 infection in Asians in which the frequency of G249D is higher than in whites.

To investigate the functional significance of G249D on the anti-HIV activity of TRIM5α, we constructed SeV containing C-terminal HA-tagged human TRIM5α (249G) (Fig. 1) by site-directed mutagenesis on MT4 TRIM5α, which bears D at position 249. As shown in Fig. 2A, the expression level of TRIM5α (249G) was comparable to that of TRIM5α (249D) in recombinant SeV-infected MT4 cells.

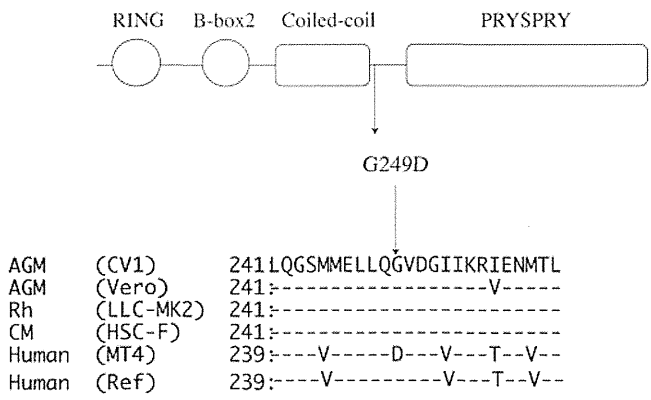

FIG. 1. Schematic presentation of TRIM5α structure. Circles and squares represent functional domains of TRIM5α. The position of the G249D polymorphism is shown by arrows. The amino acid sequences of African green monkey (AGM) TRIM5α from CV1[9] and Vero cells, rhesus monkey (Rh) TRIM5α from LLC-MK2,[10] cynomolgus monkey (CM) TRIM5α from HSC-F,[9] human TRIM5α from MT4 cells,[9] and the reference sequence (NM_033034) were aligned. Dashes denote an identical amino acid to AGM TRIM5α from CV1.

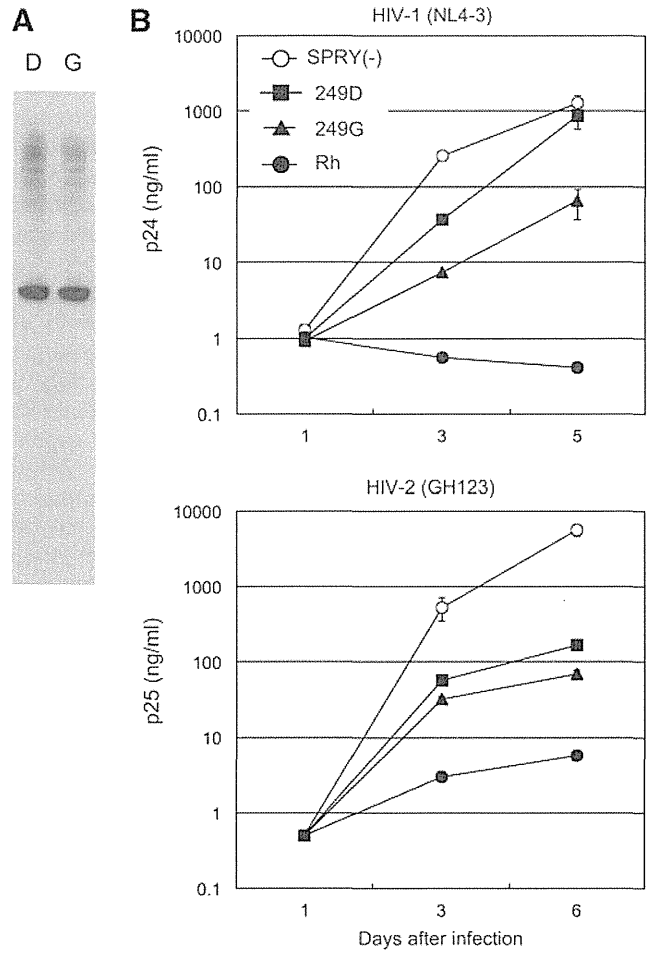

FIG. 2. **(A)** Lysates of MT4 cells infected with recombinant Sendai virus (SeV) expressing hemagglutinin (HA)-tagged human TRIM5α with 249D (lane D) and with 249G (lane G) were visualized by western blotting with an antibody against HA. Representative results of three independent experiments are shown. **(B)** MT4 cells were infected with SeV expressing TRIM5α lacking the PRYSPRY domain [SPRY(−); white circles], MT4-derived human TRIM5α (249D; black squares), human TRIM5α (249G; black triangles), or rhesus monkey TRIM5α (Rh; black circles). Nine hours after SeV infection, cells were inoculated with HIV-1 strain NL4-3 or HIV-2 strain GH123, and culture supernatants were periodically assayed for levels of p24 or p25, respectively. Data points are means for triplicate samples with SD. Three and six days after infection, statistically significant differences ($p < 0.05$) of HIV-1 and HIV-2 growth were observed between human TRIM5α (249D) and human TRIM5α (249G) by unpaired *t* test. Representative data of at least three independent experiments are shown.

These TRIM5α constructs were tested for their ability to restrict the X4-tropic HIV-1 strain NL4-3 and HIV-2 strain GH123. MT4 cells infected with recombinant SeV expressing each of the TRIM5α constructs were superinfected with HIV-1 NL4-3 or HIV-2 GH123. We used SeV expressing cynomolgus monkey TRIM5α lacking the PRYSPRY domain as a negative control for functional TRIM5α, as overexpression of TRIM5α lacking the PRYSPRY domain was shown to exert a dominant negative effect on endogenous human TRIM5α.[30] As shown in Fig. 2B, MT4-derived human TRIM5α (249D) showed only

weak anti-HIV-1 activity, as we demonstrated previously.[21] On the other hand, human TRIM5α (249G) showed stronger restriction activity to HIV-1 NL4-3 than human TRIM5α (249D). In the case of HIV-2, both human TRIM5α with 249G and 249D exhibited apparent anti-HIV-2 activity. The human TRIM5α (249G) showed stronger restriction activity to HIV-2 GH123 than human TRIM5α (249D), although the difference was very small (Fig. 2B, lower panel). These results indicated that the G249D variant weakened the anti-HIV-1 and anti-HIV-2 activities of human TRIM5α.

TRIM5α is known to restrict viral infection at the early steps of HIV replication. To evaluate the anti-HIV-1 activity of human TRIM5α at the early stages, we performed the single-round infection assay using a GFP expression vector (Fig. 3). The hamster cell line TK-ts13, which lacks endogenous TRIM5α expression, was infected with recombinant SeV expressing human TRIM5α. We superinfected cells with VSV-G pseudotyped lentivector expressing GFP under the control of the CMV promoter. We used HIV-1 vectors bearing CA derived from BH10 (Fig. 3A) and NL4-3 (Fig. 3B). Both HIV-1 GFP vectors were suppressed to a greater degree by human TRIM5α (249G) than by MT4-derived human TRIM5α (249D). A similar result was obtained when we used the HIV-2 GFP vector (Fig. 3C). Taken together, these observations indicated that the G249D polymorphism affected the anti-HIV-1 and anti-HIV-2 activities of human TRIM5α.

Associations of TRIM5α G249D polymorphism with susceptibility to HIV-1 infection

We sequenced TRIM5α exon 5 and found G249D in the populations tested. The associations of G249D polymorphism with susceptibility to HIV-1 infection are summarized in Table 1. The frequency of 249D was significantly higher in the HIV-1-infected Indian subjects than in the ethnicity-matched controls [odds ratio (OR) = 1.52, $p = 0.026$]. A similar tendency was also observed in the Japanese population, but did not reach statistical significance (OR = 1.19, $p = 0.302$).

Previously, we sequenced TRIM5α exons 2 of the same subjects as above and reported the association of H43Y with susceptibility to HIV-1 infection.[21] The levels of LD indicated that G249D in exon 5 and H43Y in exon 2 were not in tight linkage disequilibrium in either Japanese ($r^2 = 0.18$, $n = 188$) or Indian ($r^2 = 0.02$, $n = 96$) populations.

Discussion

The G249D polymorphism in TRIM5α is common in Asian and African populations. It was initially speculated that there was no functional effect of this SNP, as it is located outside of any functional domains of human TRIM5α. Contrary to our expectation, however, we observed attenuation of anti-HIV-1 and anti-HIV-2 activity of the G-for-D substitution with both multiround replication and single-round infection assays. Furthermore, we investigated two ethnic populations, Japanese and Indian, for the G249D polymorphism and found the association of the TRIM5α 249D allele with enhanced susceptibility to HIV-1 infection.

Amino acid position 249 of human TRIM5α lies within the linker region for which no three-dimensional structural data have yet been reported. Therefore, we performed secondary structure prediction by the Chou–Fasman method[31] to examine the possible effect of this SNP on the protein structure.

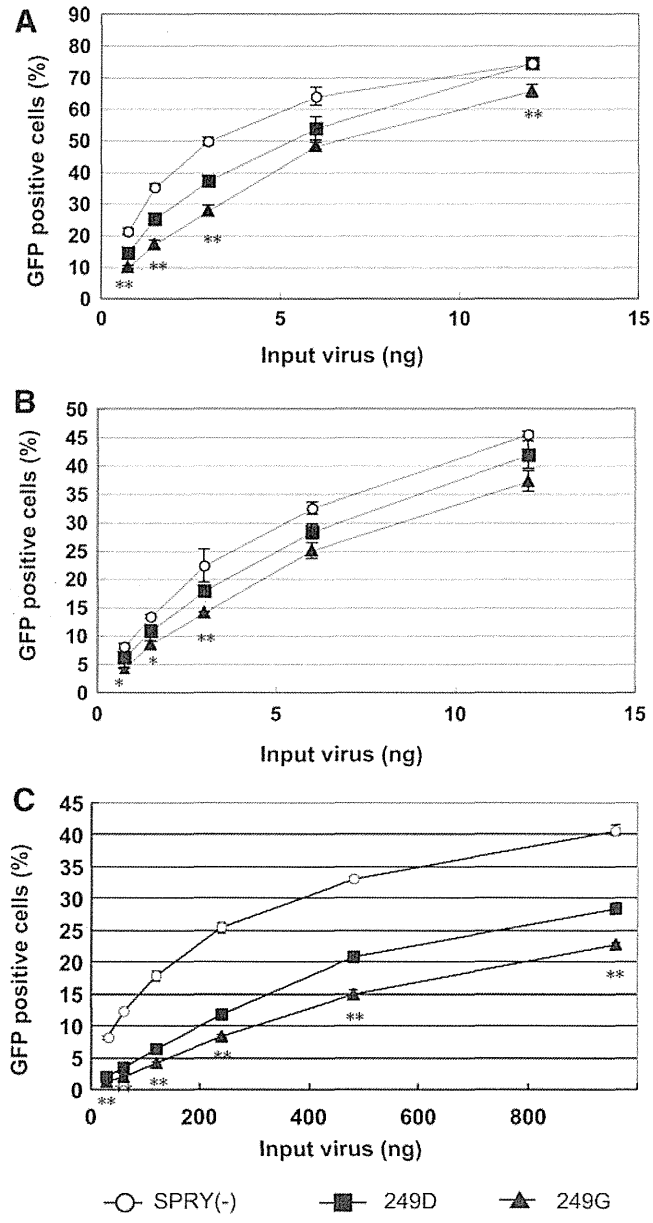

FIG. 3. TK-ts13 cells infected with SeVs expressing TRIM5α lacking the PRYSPRY domain [SPRY(−); white circles], MT4-derived human TRIM5α (249D; black squares), or human TRIM5α (249G; black triangles) were exposed to green fluorescence protein (GFP)-expressing HIV-1 vector based on BH10 **(A)** or NL4-3 **(B)**. **(C)** Cf2Th cells infected with SeVs were exposed to an HIV-2 vector based on ROD. GFP-positive cells were counted by a flow cytometer. Data points are means for triplicate samples with SD. */** The statistically significant differences, $p < 0.05$ and $p < 0.001$, respectively, in unpaired t test between human TRIM5α (249D) and human TRIM5α (249G). Representative results of two independent experiments are shown.

The G-to-D substitution increased the probability of α-helix formation and resulted in the extension of the α-helix from the coiled-coil region into the linker 2 region. Similar results were obtained by the PREDETOR in http://mobyle.pasteur.fr (data not shown). This suggested that TRIM5α with 249G would be more flexible than TRIM5α with 249D.

TABLE 1. ASSOCIATION OF rs10038628 (G249D) WITH SUSCEPTIBILITY TO HIV-1 INFECTION IN JAPANESE AND INDIAN POPULATIONS

	Japanese				*Indian*			
	HIV-1-infected (n=93)	*Control* (n=279)	*Odds ratio (95% CI)*	p-*value*	*HIV-1-infected* (n=227)	*Control* (n=280)	*Odds ratio (95% CI)*	p-*value*
rs10038628								
GG	28 (30%)	98 (35%)	0.80 (0.48–1.32)	0.376	161 (71%)	226 (81%)	0.58 (0.39–0.88)	0.010
DG	47 (51%)	137 (49%)			63 (28%)	49 (17%)		
DD	18 (19%)	44 (16%)	1.28 (0.70–2.35)	0.422	3 (1%)	5 (2%)	0.74 (0.17–3.12)	0.736[a]
Allele D	83 (45%)	225 (40%)	1.19 (0.85–1.67)	0.302	69 (15%)	59 (11%)	1.52 (1.05–2.21)	0.026

[a]Fisher's exact test.

Human TRIM5α was obviously not effective in protecting against HIV-1 infection compared with the strong Old World monkey TRIM5α, as only humans are susceptible and Old World monkeys are resistant to HIV-1 infection. With experimental overexpression of human TRIM5α, the anti-HIV-1 activity of human TRIM5α was variable among previous reports.[1,5,9,14,16,20,21] Our previous data showed the weakest anti-HIV-1 activity of human TRIM5α,[9,20,21] even though we used the SeV system, which allowed high expression levels of inserted genes. As described in the present study, the 249D substitution would explain why our human TRIM5α derived from MT4 showed little potency against HIV-1. We examined the G249D SNP in commonly used human cell lines, CEM, HeLa, Jurkat, and 293T, and found that these were all homozygous for 249G, but MT4 was homozygous for 249D. This is not surprising because the allele frequency of 249D is high in Japan but quite rare in whites and MT4 cells were established from Japanese donor blood.[32] On the other hand, MT4 is highly susceptible to HIV-1 infection,[33] which is in good agreement with the present data.

Previously, Goldschmidt *et al.* failed to observe the attenuation of antiviral activity by the 249D mutation.[18] One possible reason for the discrepancy between their results and ours is the difference in expression system used. Goldschmidt *et al.* used HeLa cells stably transduced with TRIM5α with various mutations.[18] Transduced cell lines sometimes develop unexpected phenotypic changes during the cloning procedure. In contrast, we used the SeV system, and the conditions of cells infected with different recombinant viruses were always comparable, especially among those expressing full-length TRIM5α. It should be noted that Goldschmidt *et al.* also reported a tendency toward higher *in vitro* p24 production 7 days after infection in peripheral blood mononuclear cells from individuals with the 249D allele, which is consistent with our present results.[18]

We clearly showed that the 249D allele was associated with increased susceptibility to HIV-1 infection in the Indian population. However, although a similar tendency was observed in the Japanese population, the association was not significant. The precise reason why the effect of G249D was unclear in the Japanese population is not yet clear. It should be noted that our Japanese patients were infected through contaminated blood products in the early 1980s. On the other hand, the Indian patients were infected through heterosexual contact after the HIV-1 pandemic in Asia after 1990. It is possible that the difference in route of HIV-1 transmission may be responsible for this difference between Japanese and Indian patients. Further studies in well-characterized cohorts are necessary to confirm our findings regarding HIV-1 transmission and the possible effects of this SNP on AIDS progression.

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Author Disclosure Statement

No competing financial interests exist.

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Polymorphisms in *Fas* Gene Is Associated with HIV-Related Lipoatrophy in Thai Patients

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Abstract

The present study aimed to evaluate the role of genetic polymorphisms in the emergence of lipoatrophy or lipodystrophy in HIV-infected patients with antiretroviral therapy (ART) in Thailand. Position 455 upstream of the *Apolipoprotein C3* gene (*ApoC3* T–455C, rs2854116), codon 64 of the *Beta3 adrenergic receptor* gene (*ARβ3* Tcod64C, rs4994), and position 670 upstream of the *Fas* gene (*Fas* A–670G, rs1800682) were genotyped in 829 HIV-infected Thai patients who had started ART. Crude and adjusted incidence rate ratios (IRR) were calculated using Poisson regression. The serum levels of cholesterol, triglycerides, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) were also analyzed. Multivariate analysis revealed an association between the *Fas* −670AA genotype, but not the *ApoC3* −455 or *ARβ3* cod64 genotypes, with the incidence of lipoatrophy after adjusting for gender and stavudine (d4T)-containing regimens (IRR = 1.72, 95% CI = 1.20–2.45, $p = 0.003$). However, *ApoC3* −455C homozygous patients showed elevated serum levels of triglycerides, while this genotype did not affect serum total cholesterol, HDL, or LDL levels in patients with lipoatrophy or lipodystrophy. In contrast, the *ARβ3* cod64 genotype did not show any significant association with the serum levels of cholesterol, triglycerides, HDL, or LDL. In conclusion, *Fas* −670AA affected the incidence of lipoatrophy in HIV-1-infected Thai patients, while the *ApoC3* −455C allele affected the serum levels of triglycerides. These results confirmed the role of genetics in the development of ART-related metabolic disorders.

Introduction

METABOLIC AND MORPHOLOGICAL alterations observed in human immunodeficiency virus (HIV)-infected patients represent a significant health problem related both to the possible long-term consequences and to the behavioral and psychological well-being of patients. Since the introduction of highly active antiretroviral therapy (HAART), the life expectancy of patients infected with HIV type 1 (HIV-1) has increased remarkably. Despite the clinical benefits, long-term HAART is associated with a complex spectrum of morphological alterations, characterized by body changes that may stigmatize patients and compromise their compliance with therapy, as well as metabolic effects, including dyslipidemia and insulin resistance, which lead to an elevated risk of developing diabetes mellitus and myocardial infarction.[1–3]

Morphological alterations are collectively named lipodystrophy (LD); however, in recent years it has been recognized that fat loss and fat gain represent distinct entities. Clinical features of fat loss include loss of subcutaneous fat in the face, arms, legs, and buttocks, while fat accumulation is characterized by excess fat deposition in visceral adipose tissue in the abdomen, breasts, dorsocervical region, and trunk.[4]

The underlying mechanism of antiretroviral therapy (ART)-related lipoatrophy (LA) has not been fully elucidated. Many mechanisms have been suggested, including mitochondrial toxicity by nucleoside reverse transcriptase inhibitors (NRTIs), inhibition of adipocyte differentiation by protease inhibitors (PIs), increased levels of inflammatory cytokines, and HIV itself.[5–7] Mitochondrial toxicity has been clearly shown to play a pivotal role; in particular, thymidine analogs are strong inhibitors of DNA polymerase-γ and lead

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to mitochondrial depletion and dysfunction[8]; mainly stavudine (d4T), but also zidovudine (AZT) and didanosine (ddI) are strongly related to LA.[9,10] In previous *in vitro* and clinical studies, newer nucleoside and nucleotide agents, such as lamivudine (3TC), emtricitabine, abacavir, and tenofovir, appear to be much weaker inhibitors of mitochondrial DNA polymerase-γ or other mitochondrial functions, and appear to be associated with a lower risk of events thought to be related to mitochondrial toxicity.[11] However, a certain degree of mitochondrial toxicity is still present and will probably still affect HIV patients under ART, although to a lesser extent.[12–14] In fact, switch studies from d4T to other NRTIs and complete switch off of thymidine analogs showed modest even if consistent increases in limb fat.[15–17]

The pathogenesis of lipohypertrophy appears to be multifactorial, with age, sex, HIV itself, immune depression, and duration and type of ART related to its appearance.[18–20] PIs were suggested to be involved in lipohypertrophy; however, visceral fat accumulation also occurs in the absence of PIs.[21,22] A compartment-specific effect of mitochondrial toxicity within the adipose tissue may be related to lipohypertrophy[23] as well as dysregulation of fatty acid metabolism and altered expression of adipokines.[24–26]

However, LA and lipohypertrophy do not occur in all treated patients, and there is a very large degree of interindividual variability in the timing of emergence and severity of symptoms. A study of identical twins showed that genetic factors are involved in the accumulation of visceral adipose tissue.[27] These data suggest that host genetic factors may play a role, and inherited predispositions may have a significant influence on the appearance of LD and metabolic alterations as well as on the viroimmunological response to the drugs.

Therefore, host genetic polymorphisms may have a significant effect on the response to ART in terms of metabolism and immune response.[28] A study of Italian HIV patients suggested that single nucleotide polymorphisms (SNPs) in genes involved in apoptosis and lipid metabolism mediate the development of LA.[29] In particular, the TT and CT genotypes at position −455 of the *Apolipoprotein C3* (*ApoC3*) gene (*ApoC3* T–455C, rs2854116), nonsynonymous (W to R) CC and CT genotypes at codon 64 of the *Beta3 adrenergic receptor* (*ARβ3*) gene (*ARβ3* Tcod64C, rs4994), and the AA genotype at position −670 of the *Fas* gene (*Fas* A–670G, rs1800682) were associated with increased incidence rates of LA.[29] ApoC3 protein, localized mainly in very low-density lipoprotein and high-density lipoprotein (HDL), is involved in fat metabolism and may delay the catabolism of triglyceride-rich particles. ApoC3 inhibits lipoprotein lipase and hepatic lipase and decreases the chylomicrons in hepatic cells.[30,31] ARβ3 protein, expressed mainly in visceral adipose tissue,[32,33] contributes to both lipolysis and the delivery of free fatty acids. *Fas* is the main gene that controls cell death by inducing apoptosis. A previous study indicated that Fas and Fas ligand influenced the immune reconstitution induced by ART.[34]

In Thailand, the National Access to Antiretroviral Care program started in 2002 using standard regimens with GPO-VIR (a combination of d4T, 3TC, and nevirapine) as the first line of ART. The present study was performed to determine whether these genetic polymorphisms found in the Italian cohort also mediate the emergence of LA or LD in HIV-infected patients starting ART in Thailand in consideration of the relevance of these side effects in the currently used regimens and of the differences in ethnicity, alimentation, and way of life of the Thai population.

Materials and Methods

Study design

HIV-1-infected individuals receiving ART at Bamrasnaradura Infectious Diseases Institute, Nonthaburi, Thailand were enrolled in this study. LA was defined as the presence of fat loss at any site of the body (face, arms, legs, or buttocks). LD was defined as LA combined with fat accumulation diagnosed at the same time. Fat accumulation was defined as the presence of at least one alteration, such as buffalo hump, or fat accumulation in the breast, abdomen, neck, or lipomas. To qualify, the alteration had to be recognized by both the patient and the physician.[35] Most patients who developed LA or LD changed regimen for those without d4T. A 200 μl sample of whole blood was collected from each patient and stored at −20°C until DNA extraction. The genotypes of *ApoC3* −455, *ARβ3* cod64, and *Fas* −670 were compared between patients who developed LA or LD and those who did not. Patients' age, gender, transmission route, CD4 counts at pre-ART and at 1 year post-ART (cells/μl), drug regimens, and duration of therapy were obtained from their medical records. Lipid profile data were obtained from patients' records of serum biochemical tests. Blood for this test was collected when patients were in the fasting state; however, not all patients had serum biochemical test data as the test incurs extra costs for patients. All participants signed an informed consent form. The study was approved by the institutional ethical committees at the Bamrasnaradura Infectious Diseases Institute and Department of Disease Control, Ministry of Public Health, Thailand.

DNA extraction

Genomic DNA was extracted from 200 μl of each whole blood sample using a QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.[36]

Genotyping

Genotyping of *Fas* gene promoter polymorphism, A–670G (rs1800682). Polymerase chain reaction (PCR) and restriction fragment length polymorphism analysis for *Fas* A–670G, modified from a previously published technique,[37] was conducted with the primers 5′-CTACCTAAGAGCTATCTA CCGTTC-3′ and 5′-GGCTGTCCATGTTGTGGCTGC-3′. The PCR product was digested with the restriction enzyme *Mva*I (Roche Biochemicals, Mannheim, Germany) at 37°C overnight. Two polymorphic alleles could be distinguished on the basis of restriction length polymorphisms (188 bp for G and 232 bp for A).

Genotyping of *ApoC3* gene promoter polymorphism, T–455C (rs2854116). TaqMan SNP assay was performed using the StepOne real-time PCR system (Applied Biosystems, Foster City, CA) with primers (5′-GAGCTCAGCCCCTG TAACCAG-3′ and 5′-ACACAGCCTGGAGTAGAGGG-3′) and TaqMan MGB probes (5′-VIC-CTCCAAACACCCC-MGB-3′ for the C allele and 5′-FAM-TTTACTCCAAA CATCC-MGB-3′ for the T allele).

Genotyping of *ARβ3* gene coding polymorphism, Tcod64C (rs4994). TaqMan SNP assay was performed with a TaqMan SNP assay kit (C___2215549_20; Applied Biosystems).

Statistical analysis

Person-year analysis was conducted to investigate a single endpoint: the incidence of LA or LD. If the patient was diagnosed as both LA and LD, the earlier date of the first diagnosis was used as the endpoint. We focused on the time to the occurrence of the first event. Person-years at risk were calculated from the start date of ART until the last available follow-up or development of the event, whichever occurred first. The Poisson regression univariate model was used to determine whether the genetic polymorphisms as well as patients' age, gender, transmission route, CD4 counts at pre-ART and at 1 year post-ART, and starting drug regimes were predictors of LA or LD. Associations were expressed as incidence rate ratio (IRR) and corresponding 95% confidence interval (CI). Factors showing significant association with LA or LD were then included as covariates in the Poisson regression multivariate model to adjust for their possible confounding effects. Starting drug regimens were grouped into either d4T-containing regimens or other backbone regimens. Continuous variables were compared using the Wilcoxon signed rank test for paired samples and Kruskal–Wallis test for unpaired samples. A variable was considered significant at $p < 0.05$.

Results

Incidences of LA and/or LD in HIV-1-infected Thai patients undergoing ART

A total of 829 HIV-1-infected individuals who had started ART were enrolled in this study at the Bamrasnaradura Infectious Diseases Institute, Nonthaburi, Thailand, from April 2008 to August 2009. Patient demographic data are shown in Table 1. The study population consisted of 478 males (57.7%) and 351 females (42.3%). Most of the patients contracted infection through sexual contact. Almost half of the patients started ART immediately after being diagnosed with HIV infection. Median CD4 count per microliter pre-ART was 45 and that post-ART (after 1 year) was 210.5. The mean follow-up period was 4.85 years (SD = 2.42).

Table 1. Demographic Data of All the Patients

Demographic data	n = *829*
Mean age (SD) years	41.12 (7.77)
Male (%)	478 (57.66)
Transmission	
Sexual (%)	806 (97.23)
Intravenous drug user (%)	8 (0.97)
Unknown (%)	15 (1.81)
Median baseline CD4 (cells/μl) (IQR)	45 (15, 127.5)
Median CD4 (cells/μl) after treatment (IQR)	210.5 (142, 305)
Median duration of therapy before onset of lipoatrophy or lipodystrophy (years, IQR) (n = 270)	2.63 (1.67, 4.00)
Median duration of therapy of nonlipoatrophy or nonlipodystrophy (years, IQR) (n = 559)	5.53 (4.10, 7.18)

SD, standard deviation; IQR, interquartile range.

We first combined patients with LA and those with LD for analysis, as fat loss was commonly observed in both LA and LD cases. Among the 829 patients, 270 (32.6%) were diagnosed with LA or LD within the observation period. We kept on monitoring the other 559 (67.4%) until the end of the observation period and found that they developed neither LA nor LD (non-LA/LD cases). The median duration of therapy until diagnosis was 2.63 years for the LA or LD cases and 5.53 years for the non-LA/LD cases. In a follow-up of 4019.8 person-years, the incidence rate of LA or LD was 6.70 per 100 person-years. LA or LD cases were found more frequently in females (40.7%) than in males (26.6%), and the incidence rate per 100 person-years for females (9.11) was significantly higher than that for males (5.18) (IRR = 1.76, 95% CI = 1.37–2.25, $p < 0.001$) (Table 2). Patients using d4T/3TC backbone regimens as starting drugs showed a significantly higher incidence rate per 100 person-years (7.71) than those with other non-d4T-containing (mostly AZT/3TC) regimens (3.49) (IRR = 2.21, 95% CI = 1.53–3.28, $p < 0.001$) (Table 2). These findings were consistent with those of previous studies in other ethnic groups.[9,20,29,38,39] In contrast, age, transmission route, and CD4 counts pre-ART and at 1 year post-ART did not significantly affect the incidence of LA or LD (Table 2).

Fas −670AA, but not ApoC3 −455 or ARβ3 cod64 genotype, affects incidence of LA or LD in HIV-1-infected Thai patients

To elucidate the possible effects of genetic polymorphisms on the development of LA or LD, we determined the genotypes of *Fas* −670, *ApoC3* −455, and *ARβ3* cod64 of 829 HIV-1-infected Thai patients. LA or LD cases were found significantly more frequently among patients with the *Fas* −670AA genotype than in those with other *Fas* −670 genotypes (IRR = 1.41, 95% CI = 1.06–1.86, $p = 0.015$, Table 2). The *Fas* −670AA genotype was found in 25.9% of the LA or LD cases and in 20.2% of the non-LA/LD cases during the observation period. Increased frequency of the *Fas* −670AA genotype in LA or LD cases was observed in both males and females (data not shown). In cases of *ApoC3* −455 and *ARβ3* cod64 genotypes, however, there were no statistically significant differences in the frequency of LA or LD between groups (Table 2). Adjustment for gender and starting drug regimens in multivariate Poisson regression analysis resulted in a slightly higher IRR for *Fas* −670AA (1.47, 95% CI = 1.12-194, $p = 0.005$, Table 2). This difference was statistically significant after correction for multiple testing of three SNPs ($p = 0.015$).

Fas–670AA genotype affects incidence of LA but not that of LD in HIV-1-infected Thai patients

Among 270 LA and/or LD cases, 148 were diagnosed as LA singly and 105 were diagnosed as LD singly. There were 17 cases diagnosed as LA first who then subsequently developed fat accumulation. These 17 cases were grouped as LD cases. The median durations of therapy until diagnosis were 2.58 years for LA and 2.83 years for LD cases. When we analyzed LA and LD separately, females showed a higher IRR in both LA (1.60, 95% CI = 1.14–2.24, $p = 0.005$) and LD (2.31, 95% CI = 1.59–3.38, $p < 0.001$) (Tables 3 and 4)., Similarly, d4T-containing regimens showed significantly higher IRR in both

TABLE 2. UNIVARIATE AND MULTIVARIATE ANALYSIS OF ALL PATIENTS (N=829)

Factors	Events[1]	Person-years	Events/100 person-years	Crude		Adjusted[2] (n=829)	
				IRR (95% CI)	p-value	IRR (95% CI)	p-value
Gender							
Male	127	2449.9	5.18	1			
Female	143	1569.9	9.11	1.76 (1.37–2.25)	<0.001	1.70 (1.33–2.16)	<0.001
Transmission							
Unknown[3]	7	95.9	7.3	1			
Sexual	263	3924.0	6.7	0.92 (0.44–2.31)	0.782		
NRTIs							
No-d4T	33[4]	945.8	3.49	1			
d4T	237[5]	3074.0	7.71	2.21 (1.53–3.28)	<0.001	2.05 (1.42–2.96)	<0.001
Age×10 (years)				0.87 (0.74–1.02)	0.090		
CD4 at baseline[5]				0.9988 (0.9973–1.0003)	0.107		
CD4 after therapy				0.9497 (0.9987–1.0005)	0.458		
Fas−670							
AG/GG	200	3221.6	6.21	1			
AA	70	798.2	8.77	1.41 (1.06–1.86)	0.015	1.47 (1.12–1.94)	0.005
ApoC3−455							
CT/TT	206	3139.0	6.56	1			
CC	64	880.9	7.27	1.11 (0.82–1.47)	0.474		
ARβ3 cod64							
CC/CT	58	815.8	7.11	1			
TT	212	3204.0	6.62	0.93 (0.69–1.27)	0.621		

[1]Diagnosis of lipoatrophy or lipodystrophy.
[2]Gender, NRTIs, and *Fas*−670 were included as covariates.
[3]Including eight intravenous drug users.
[4]No-d4T group mainly used AZT. One case had received ddI instead of AZT as an initial regimen.
[5]CD4 cells/μl.
IRR, incidence rate ratios; NRTIs, nucleoside reverse transcriptase inhibitors; d4T, stavudine.

LA (2.46, 95% CI=1.48–4.36, $p<0.001$) and LD (2.10, 95%CI=1.23–3.80, $p=0.003$) (Tables 3 and 4). In the case of the *Fas* −670AA genotype, however, significantly higher IRR was observed only in LA (1.68, 95% CI=1.15–2.41, $p=0.006$) but not in LD (1.19, 95%CI=0.74–1.83, $p=0.433$) (Tables 3 and 4). The statistical significance of higher IRR of LA for *Fas* −670AA did not change after adjustment for gender and starting drug regimens in multivariate Poisson regression analysis (1.72, 95% CI=1.20–2.45, $p=0.003$, Table 3). The difference was statistically significant even after correction for multiple testing of three SNPs in two groups (LA or LD) ($p=0.018$). These results suggested that the *Fas* −670 genotype affects LA without fat accumulation.

Lipid profiles of HIV-1-infected Thai patients

We analyzed the serum lipid profiles of the patients. From the records of 829 patients, serum cholesterol levels were obtained in 610 and 628 cases at the first and last measurement of the observation period, respectively. Serum triglyceride (TG) levels were obtained in 646 and 716 cases at the first and last measurement, respectively. Serum HDL levels were obtained in 393 and 367 cases at the first and last measurement, respectively. Serum low-density lipoprotein (LDL) levels were obtained in 382 and 438 cases at the first and last measurement, respectively. Serum cholesterol levels showed a normal distribution, while serum TG, HDL, and LDL levels did not. Therefore, we used a nonparametric method to evaluate the statistical significance of differences in patient lipid profiles. Slight increases in serum cholesterol levels were observed in the LA (from median 194.5 to 206 mg/dl, $p=0.016$), LD (from median 194 to 205 mg/dl, $p=0.001$), and non-LA/LD cases (from median 195 to 200 mg/dl, $p=0.033$) during ART (Table 5). Similarly, significant increases in TG were observed in the LA (from median 130 to 183.5 mg/dl, $p=0.019$) and LD (from median 122 to 165.5 mg/dl, $p<0.001$), but not in non-LA/LD cases (from median 138 to 148.5 mg/dl, $p=0.359$; Table 5). Accordingly, the LA and LD cases showed significantly higher TG levels than the non-LA/LD cases at the last measurement ($p=0.007$; Table 5). These results indicated that lipid metabolism in LA and LD cases was more severely damaged by ART than that in non-LA/LD cases. It should also be noted here that the LA and LD cases showed weak but clear trends toward lower TG levels than non-LA/LD cases at the first measurement ($p=0.091$; Table 5). In fact, when combined, the LA and LD cases showed significantly lower TG levels (median 122 mg/dl) than the non-LA/LD cases (median 138 mg/dl) at the first measurement ($p=0.049$). On the other hand, HDL and LDL levels remained almost unchanged during the treatment period in the LA and LD, but non-LA/LD cases showed a slight decrease in LDL (from median 137 to 132.5 mg/dl, $p=0.026$; Table 5).

As the LA and LD cases showed similar lipid profiles, we combined the LA and LD cases again and analyzed the effects of patients' genotypes on the lipid profile together. The results indicated that the *ApoC3* −455CC genotype was associated with decreased serum cholesterol levels in non-LA/LD cases at the last measurement ($p=0.035$; Table 6). However, this association was not observed in LA and LD cases ($p=0.393$;

TABLE 3. LIPOATROPHY: RATE OF INCIDENCE AND MULTIVARIATE ANALYSIS (N=707)

Factors	Events[1]	Person-years	Events/100 person-years	Crude IRR (95% CI)	Crude p-value	Adjusted[2] (n=707) IRR (95% CI)	Adjusted[2] (n=707) p-value
Gender							
Male	76	2282.1	3.33	1			
Female	72	1350.9	5.33	1.60 (1.14–2.24)	0.005	1.56 (1.13–2.16)	0.007
Transmission							
Unknown	3	85.2	3.52	1			
Sexual	145	3547.8	4.09	1.16 (0.39–5.69)	0.865		
NRTIs							
No-d4T	17	880.5	1.93	1			
d4T	131	2752.5	4.76	2.46 (1.48–4.36)	<0.001	2.32 (1.40–3.85)	0.001
Age×10 (years)				0.88 (0.71–1.08)	0.228		
CD4 at baseline[3]				0.9985 (0.9964–1.0006)	0.151		
CD4 after therapy[3]				0.9991 (0.9979–1.0003)	0.151		
Fas−670							
AG/GG	105	2920.4	3.59	1			
AA	43	712.6	6.03	1.68 (1.15–2.41)	0.006	1.72 (1.20–2.45)	0.003
ApoC3−455							
CT/TT	118	2866.1	4.12	1			
CC	30	766.9	3.91	0.95 (0.61–1.43)	0.818		
ARβ3 cod64							
CC/CT	38	748.0	5.08	1			
TT	110	2885.0	3.81	0.75 (0.51–1.12)	0.134		

[1]Diagnosis of lipoatrophy.
[2]Gender, NRTIs, and *Fas*−670 were included as covariates.
[3]CD4 cells/μl.
IRR, incidence rate ratios; NRTIs, nucleoside reverse transcriptase inhibitors; d4T, stavudine.

TABLE 4. LIPODYSTROPHY: RATE OF INCIDENCE AND MULTIVARIATE ANALYSIS (N=681)

Factors	Events[1]	Person-years	Events/100 person-years	Crude IRR (95% CI)	Crude p-value	Adjusted[2] (n=681) IRR (95% CI)	Adjusted[2] (n=681) p-value
Gender							
Male	51	2234.3	2.28	1			
Female	71	1346.3	5.27	2.31 (1.59–3.38)	<0.001	2.19 (1.53–3.15)	<0.001
Transmission							
Unknown	4	86.0	4.65	1			
Sexual	118	3494.5	3.38	0.73 (0.28–2.71)	0.508		
NRTIs							
No-d4T	16	860.3	1.86	1			
d4T	106	2720.2	3.90	2.10 (1.23–3.80)	0.003	1.82 (1.07–3.09)	0.027
Age×10 (years)				0.85 (0.67–1.07)	0.171		
CD4 at baseline[3]				0.9988 (0.9966–1.0010)	0.278		
CD4 after therapy[3]				1.0000 (0.9990–1.0014)	0.696		
Fas−670							
AG/GG	95	2888.0	3.29	1			
AA	27	692.6	3.90	1.19 (0.74–1.83)	0.433	1.27 (0.83–1.95)	0.278
ApoC3−455							
CT/TT	88	2781.9	3.16	1			
CC	34	798.7	4.26	1.34 (0.88–2.02)	0.148		
ARβ3 cod64							
CC/CT	20	714.5	2.80	1			
TT	102	2866.1	3.56	1.27 (0.78–2.17)	0.328		

[1]Diagnosis of lipoatrophy.
[2]Gender, NRTIs, and *Fas*−670 were included as covariates.
[3]CD4 cells/μl.
Lipodystrophy cases included 17 cases diagnosed with both lipoatrophy and lipodystrophy.
IRR, incidence rate ratios; NRTIs, nucleoside reverse transcriptase inhibitors; d4T, stavudine.

TABLE 5. LIPID PROFILE OF PATIENTS

	LA cases[1]			*LD cases*[2]			*Non-LA/LD cases*[3]			
Lipid profile	n	*Median*	*IQR*	n	*Median*	*IQR*	n	*Median*	*IQR*	p-*value*[4]
Cholesterol (mg/dl)										
First test[5]	96	194.5	165.25–231.25	77	194	159.00–216.50	437	195	160.00–224.00	0.507
Last test[6]	121	206	177.50–236.00	95	205	180.00–239.00	412	200	176.500–227.75	0.106
p-value[7]		0.016			0.001			0.033		
TG (mg/dl)										
First test[5]	98	130	92.25–190.25	89	122	88.00–164.50	459	138.0	95.00–201.00	0.091
Last test[6]	140	183.5	109.50–285.00	116	165.5	103.75–256.25	460	148.5	97.00–224.75	0.007
p-value[7]		0.019			< 0.001			0.359		
HDL (mg/dl)										
First test[5]	43	50	40.00–67.00	45	51	44.00–70.00	305	50	40.00–62.00	0.648
Last test[6]	63	52	42.00–66.00	51	52	42.00–63.00	253	50	40.00–62.00	0.419
p-value[7]		0.933			0.885			0.392		
LDL (mg/dl)										
First test[5]	41	136	117.00–174.00	51	133	116.00–168.00	290	137	108.00–167.00	0.731
Last test[6]	70	135.5	107.75–166.25	88	136.5	114.25–165.75	280	132.5	111.00–157.00	0.295
p-value[7]		0.777			0.735			0.026		

[1]Lipoatrophy cases.
[2]Lipodystrophy cases.
[3]Patients without lipoatrophy or lipodystrophy.
[4]Kruskal–Wallis test.
[5]The first measurement after patients started antiretroviral therapy.
[6]The last measurement of the observation period. For LA and LD cases, this corresponded to the diagnosis of LA and LD, respectively.
[7]Wilcoxon signed rank test.
LA, lipoatrophy; LD, lipodystrophy; IQR, interquartile range; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Table 6) or at the first measurement in both LA and LD cases and non-LA/LD cases ($p=0.254$ and $p=0.583$, respectively). The *ApoC3* −455CC genotype was also found to be associated with elevated serum TG levels in the LA and LD cases at the time of LA or LD diagnosis ($p=0.021$; Table 6). In the non-LA/LD cases, the *ApoC3* −455CC genotype had virtually no effect on serum TG levels. These results suggested that the *ApoC3* −455CC genotype promotes dysregulation of lipid metabolism in LA or LD cases, even though this allele had no deleterious effect on the onset of LA or LD (Table 2). On the other hand, the *ApoC3* −455CC genotype affected neither HDL nor LDL serum levels (data not shown). The *ARβ3* cod64 genotype had no effect on the serum lipid data of any patient group (Table 6). Similarly, *Fas* −670AA, which was found to be significantly associated with LA, did not show any effects on serum lipid levels (Table 6).

Discussion

In the present study, we evaluated the influence of SNPs involved in apoptosis and lipid metabolism on developing LA or LD in Thai HIV-1 patients. Specifically, we analyzed the SNPs of the *Fas* A–670G, *ApoC3* T–455C, and *ARβ3* Tcod64C in patients undergoing ART.

Of the SNPs investigated, our findings indicated that the AA genotype of *Fas* −670 was significantly associated with the risk of LA but not that of LD. This result is consistent with that of a previous study in Italian HIV patients by Zanone Poma *et al.*[29] It was suggested that adipocyte apoptosis induced by mitochondrial toxicity of NRTIs was the primary mechanism involved in LA.[15] Consistent with this suggestion, increased *Fas* gene expression levels were observed in adipose tissue of HIV-1 patients with NRTI-associated LA.[40] The *Fas* A–670G polymorphism is located in the γ-interferon activation site, to which transcription factors such as signal transducer and activator of transcription (STAT) bind.[41] Therefore, this SNP may affect the level of *Fas* gene transcription and may induce apoptosis, consequently increasing the risk of LA. The *Fas* A–670G polymorphism has been reported to be associated with several autoimmune diseases and inflammatory disorders,[41–43] which may involve a mechanism similar to LA. The frequency of the AA genotype of *Fas* −670 in 829 HIV-1-infected Thais was 22.1%, which is similar to the percentages in Europeans and Japanese but is markedly higher than that in Africans. As *Fas* −670AA caused a 70% increase in risk of LA, this genotype is a candidate for future pretherapy genetic screening, although it is still necessary to find other SNPs that increase the risk of LA for accurate screening. It is also necessary to confirm our findings on *Fas* −670AA in a larger patient cohort.

On the other hand, the frequency distribution of the *ARβ3* cod64 and *ApoC3* −455 genotypes was not significantly different between LA or LD cases and non-LA/LD cases. The *ARβ3* Tcod64C polymorphism failed to show any effect on lipid metabolism among Thai patients in this study. The results for *ARβ3* cod64 were consistent with a previous report that showed no association of this polymorphism with lipid metabolism during weight loss in obese subjects.[44] However, in the Italian cohort, this genotype was associated with the risk of developing LA.[29] Several studies have reported that people with this SNP showed an increased risk of developing obesity and glucose intolerance,[32,33] whereas other studies failed to find any correlation between this mutation and body mass index.[45,46]

The results for *ApoC3* −455 were also not consistent with those of the Italian cohort, which showed an association of the

Table 6. Patient Genotype and Lipid Profile

	Cholesterol (mg/dl)											
	LA and LD cases[1]						*Non-LA/LD cases*[2]					
	First test			*Last test*			*First test*			*Last test*		
Genotype	n	*Median*	*IQR*	n	*Median*	*IQR*	n	*Median*	*IQR*	n	*Median*	*IQR*
ApoC3−455 CC	42	202	172.50–225.00	51	214	183.00–239.00	86	196	154.75–220.25	86	193.5	164.50–220.50
ApoC3−455 CT/TT	131	191	159.00–218.00	165	204	179.50–240.00	351	195	161.00–224.00	326	202.5	177.75–229.00
p-value[3]		0.254			0.393			0.583			0.035	
ARβ3 cod64 TT	138	194	159.75–224.25	169	206	177.50–216.50	347	198	162.00–224.00	332	202	175.25–228.00
ARβ3 cod64 CT/CC	35	194	177.00–207.00	47	202	178.00–246.00	90	189	156.00–223.50	80	194	174.25–221.00
p-value[3]		0.806			0.658			0.360			0.328	
Fas−670 AA	39	192	158.00–217.00	53	211	182.50–240.00	87	197	158.00–223.00	85	201	176.50–232.50
Fas−670 AG/GG	134	195	165.75–223.50	163	205	173.50–236.75	350	195	161.00–224.00	327	199	175.50–226.00
p-value[3]		0.259			0.602			0.820			0.493	
	TG (mg/dL)											
	LA and LD cases[1]						*Non-LA/LD cases*[2]					
	First test			*Last test*			*First test*			*Last test*		
Genotype	n	*Median*	*IQR*	n	*Median*	*IQR*	n	*Median*	*IQR*	n	*Median*	*IQR*
ApoC3−455 CC	44	132	102.25–209.75	61	222	129.00–327.50	91	145	99.00–204.00	94	158	99.25–210.25
ApoC3−455 CT/TT	143	122	87.00–179.00	195	166	101.00–248.25	368	136	93.25–201.00	366	144	97.00–226.00
p-value[3]		0.277			0.021			0.771			0.945	
ARβ3 cod64 TT	150	126	93.00–185.75	198	175	77.50–281.00	363	140	97.00–199.00	360	144.5	93.25–218.75
ARβ3 cod64 CT/CC	37	108	82.50–160.00	58	167.50	109.00–274.50	93	124.5	77.75–206.75	100	154	107.00–232.50
p-value[3]		0.174			0.973			0.368			0.500	
Fas−670 AA	42	120	89.50–162.75	64	167	99.25–270.75	92	128	94.00–202.50	96	123.5	96.50–223.75
Fas−670 AG/GG	145	123	89.00–184.00	192	175	103.00–260.00	367	140	95.00–201.00	364	152	97.00–224.75
p-value[3]		0.580			0.530			0.655			0.498	

[1]Lipoatrophy and lipodystrophy cases.
[2]Patients without lipoatrophy or lipodystrophy.
[3]Kruskal–Wallis test.
LA, lipoatrophy; LD, lipodystrophy; IQR, interquartile range.

ApoC3 −455T allele with LA. However, it was also reported that the *ApoC3* −455C but not the *ApoC3* −455T allele was associated with metabolic syndrome.[47] In fact, a recent study in French HIV patients showed that the *ApoC3* −455C but not the *ApoC3* −455T allele is associated with the severity of ART-induced dyslipidemia and occurrence of LD.[48,49] Therefore, the precise role of the *ApoC3* T–455C polymorphism in LA or LD during ART should be carefully reevaluated in a larger cohort of HIV patients.

Nevertheless, our study also showed that the *ApoC3* −455CC genotype was associated with elevated serum levels of TG, which was consistent with the findings of a previous report showing that the *ApoC3* −455C allele was associated with metabolic syndrome.[47] These data suggest that the mechanism underlying the promotion of LA and LD in HIV-infected individuals receiving ART may be different from that promoting metabolic syndrome. However, the differences in results may be due to ethnic differences in the cohorts. It was reported that genetic polymorphisms in hepatic lipase showed different effects on plasma lipid levels in different ethnic groups.[50] Foulkes *et al.* found race- and ethnic-specific differences in plasma lipid levels under ART, as well as differences in the influence of *ApoC3* T–455C and the other SNP in *ApoC3* gene on the development of protease inhibitor-related hypertriglyceridemia.[51] Further studies including reevaluation of these SNPs in larger patient cohorts are therefore necessary to elucidate the precise mechanisms underlying LA and LD in HIV-infected individuals receiving ART.

In conclusion, the *Fas* −670AA genotype, but not the *ApoC3* −455 or *ARβ3* cod64 genotypes, affected the incidence of LA in HIV-1-infected Thai patients. None of these alleles affected the incidence of LD. On the other hand, the *ApoC3* −455CC, but not the *ARβ3* cod64 genotype, affected the serum levels of TG.

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