Table 1. Allele frequencies of TRIM5α-exon 2 sequence variations and associations of them with HIV-1/AIDS susceptibility.

	Japanese			Indian			White	African–American
Sequence variations[a]	Control ($n=487$)	HIV-1-infected patients ($n=94$)	Odds ratio (95% confidence interval)	Control ($n=99$)	HIV-1-infected patients ($n=101$)	Odds ratio (95% confidence interval)	Control ($n=96$)	Control ($n=96$)
Gly31Ser	0.000	0.000	ND	0.000	0.000	ND	0.000	0.032
His43Tyr	0.184	0.133	0.67 (0.42 – 1.05)	0.227	0.134****	0.52 (0.31 – 0.89)	0.115	0.068
Cys58Tyr	0.000	0.000	ND	0.000	0.000	ND	0.000	0.011
G176del	0.005	0.000	ND	0.000	0.000	ND	0.000	0.000
Asp109Asp	0.000	0.000	ND	0.000	0.000	ND	0.000	0.005
Gly110Arg	0.002	0.021***	13.14 (2.53 – 68.21)	0.000	0.000	ND	0.000	0.000
Gly110Glu	0.000	0.000	ND	0.000	0.000	ND	0.005	0.005
Val112Phe	0.052	0.043	0.80 (0.37 – 1.70)	0.192	0.198	1.04 (0.63 – 1.71)	0.052	0.021
Thr128Thr	0.000	0.011**	ND	0.000	0.000	ND	0.000	0.000
Arg136Gln	0.105	0.144	1.48 (0.94 – 2.32)	0.177	0.173	0.98 (0.58 – 1.64)	0.349	0.177

ND, not defined.
[a]The numbers of sequence variations, except for G176del, are referenced by the amino acid coding position of TRIM5α. G176del is a deletion of a G at the coding nucleotide position 176 from the initiation site of translation.
**$P<0.05$ in Fisher's exact test, when compared with control.
***$P<0.01$ in Fisher's exact test, when compared with control.
****$P<0.05$ in χ^2 test with Yates correction, when compared with control.

Table 2. Haplotype frequencies of four common haplotypes for TRIM5α-exon 2 and association of them with HIV-1/AIDS susceptibility.

	Japanese				Indian			
Haplotype (His43Tyr-Gly110Arg-Val112Phe-Thr128Thr-Arg136Gln)	Control ($n=487$)	HIV-1-infected patients ($n=94$)	P value in permutation test	Odds ratio (95% confidence interval)	Control ($n=99$)	HIV-1-infected patients ($n=101$)	P value in permutation test	Odds ratio (95% confidence interval)
43His-110Gly-112Val-128Thr-136Arg	0.659	0.721	0.129	1.35 (0.96 – 1.91)	0.404	0.495	0.076	1.45 (0.97 – 2.15)
43Tyr-110Gly-112Val-128Thr-136Arg	0.184	0.089	0.001*	0.44 (0.26 – 0.75)	0.227	0.134	0.008*	0.52 (0.31 – 0.89)
43His-110Gly-112Val-128Thr-136Gln	0.103	0.090	0.645	0.87 (0.51 – 1.49)	0.177	0.173	0.909	0.98 (0.58 – 1.64)
43His-110Gly-112Phe-128Thr-136Arg	0.051	0.031	0.251	0.61 (0.26 – 1.44)	0.192	0.198	0.802	1.04 (0.64 – 1.71)

*$P<0.01$ in permutation test.

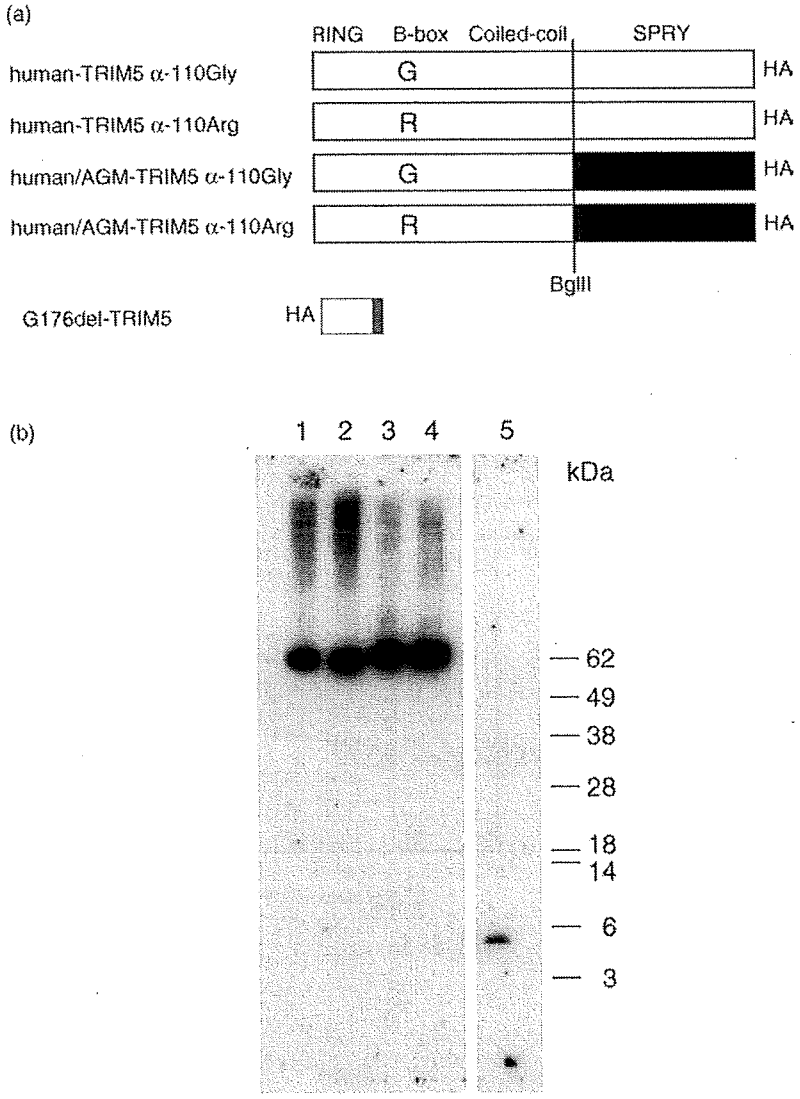

Fig. 1. Expression of TRIM5α constructs used in this study. (a) Schematic representation of TRIM5α fused with a hemaglutinin (HA)-tag. Domain structures of TRIM5α are shown at the top. White and black bars denote human and African green monkey (AGM) sequences, respectively. Gray bar denotes the G176del-specific 16 amino acid residues generated by the frameshift. A BglII site was used to exchange carboxy-terminal B30.2 (SPRY) domains between human and AGM TRIM5α. 'G' or 'R' denotes the amino acid residue at the 110th position. WT denotes wild type. (b) Western blot analysis of TRIM5 protein expressed by recombinant Sendai virus (SeV). MT4 cells were infected with a SeV containing a HA-tagged variant (110Arg) human TRIM5α (lane 1), wild-type human TRIM5α (lane 2), human/AGM chimeric 110Arg-TRIM5α (lane 3), human/AGM chimeric wild-type-TRIM5α (lane 4) and G176del-TRIM5 (lane 5). Sixteen hours after the infection, cells were lysed and subjected to SDS-PAGE. HA-tagged proteins were detected by anti-HA antibody.

110Arg-TRIM5α was similar to that of human/AGM chimeric wild-type-TRIM5α.

These TRIM5α constructs were tested for their ability to restrict the X4-tropic HIV-1 strain NL43 and HIV-2 strain GH123. MT4 cells infected with recombinant SeV expressing each of the TRIM5α constructs were super-infected with HIV-1 NL43 or HIV-2 GH123. We used SeV expressing cynomolgus monkey TRIM5α lacking the SPRY domain CM-TRIM5α-SPRY(−) as a negative control for functional TRIM5α, as overexpression of TRIM5α lacking the SPRY domain exerted a dominant negative effect on the endogenous human TRIM5α [24]. We also used SeV expressing AGM-TRIM5α lacking the coiled-coil domain AGM-TRIM5α-CC(−) as a non-interfering control [24]. As shown in Fig. 2a, both wild-type (110Gly) and variant (110Arg) human/AGM chimeric TRIM5α strongly restricted HIV-1 NL43. On the other hand, both wild-type and variant human TRIM5α showed only weak anti-HIV-1 activity. There was, however, a small increase of HIV-1 in cells expressing the human/AGM chimeric 110Arg-TRIM5α than the cells with the human/AGM chimeric TRIM5α. In the case of HIV-2, virus grew to higher titers in cells

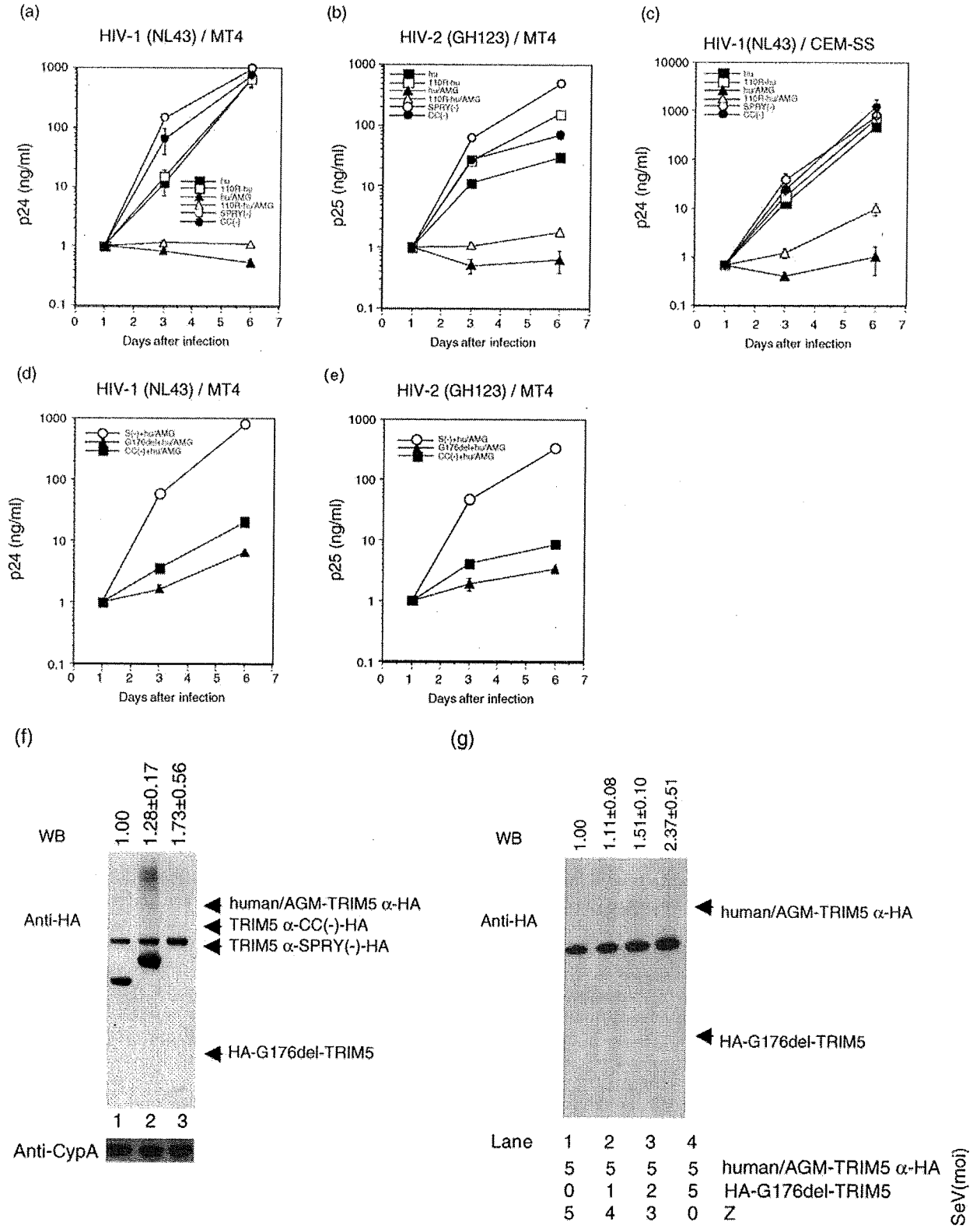

Fig. 2. Effect of TRIM5α variants on the anti-HIV-1 and anti-HIV-2 activities. Human MT4 (a, b) or CEM-SS (c) cells were infected with recombinant Sendai virus (SeV) carrying human wild-type TRIM5α (■; hu), human 110Arg-TRIM5α (□; 110R-hu), human/African green monkey (AGM) chimeric TRIM5α (▲; hu/AGM), human/AGM chimeric 110-ArgTRIM5α (△; 110R-hu/AGM), CM-TRIM5α-SPRY(−) (○; SPRY(−)) or AGM-TRIM5α-CC(−) (●; CC(−)). Nine hours after infection, cells were inoculated with HIV-1 NL43 (a and c) or HIV-2 GH123 (b). Culture surpernatants were periodically assayed for levels of p24 (a and c) or p25 (b). MT4 cells were simultaneously infected with two recombinant SeVs at 5 plaque-forming unit (PFU) per cell for

expressing dominant negative TRIM5α-SPRY(−) than in cells with noninterfering TRIM5α-CC(−), demonstrating the anti-HIV-2 activity of endogenous human TRIM5α (Fig. 2b). Both wild-type and variant human TRIM5α exhibited weak but apparent anti-HIV-2 activity, and HIV-2 grew to higher titers in cells expressing the human 110Arg-TRIM5α than in cells with the human wild-type-TRIM5α (Fig. 2b). In human/AGM chimeric version, wild-type TRIM5α completely restricted HIV-2 (Fig. 2b). In contrast, HIV-2 grew to slightly higher titers in cells expressing the human/AGM chimeric 110Arg-TRIM5α than in cells expressing the wild-type human/AGM chimeric TRIM5α (Fig. 2b). These results indicated that the Gly110Arg variant weakened the anti-HIV-1 and anti-HIV-2 activities of human TRIM5α in MT4 cells.

We recently found that the expression of TRIM5α protein introduced by SeV varied depending on cell types, that is, it was much lower in CEM-SS than in MT4 cells [25]. To evaluate the anti-HIV-1 activity of variant TRIM5α at more physiological levels of expression, we performed experiments using CEM-SS (Fig. 2c). Neither wild-type nor variant human TRIM5α exhibited anti-HIV-1 activity, probably due to the low level expression of TRIM5α in CEM-SS cells. However, HIV-1 grew to approximately 10 times higher levels in cells expressing the human/AGM chimeric 110Arg-TRIM5α than in cells with the wild-type chimeric TRIM5α, suggesting that the anti-HIV-1 activity of TRIM5α in CEM-SS cells was also reduced by the Gly110Arg substitution. Therefore, we concluded that the Gly110Arg polymorphism affected both the anti-HIV-1 and anti-HIV-2 activities of human TRIM5α.

Truncated G176del-TRIM5 enhanced antiviral activity of coexpressed TRIM5α

To express the G176del-TRIM5, we added an HA-tag at its N-terminus, because the expression of G176del-TRIM5 protein tagged with HA at the C-terminus could not be detected. Although the expression of HA-fused protein was clearly visualized by anti-HA antibody, its expression was much lower than the full-length TRIM5α (Fig. 1b). In cells infected with SeV expressing the G176del-TRIM5, HIV-2 grew to the same titers as those in cells infected with SeV expressing a nonfunctional mutant TRIM5α-CC(−), indicating that the G176del-TRIM5 lost the anti-HIV-2 activity (data not shown). We then investigated whether the G176del-TRIM5 showed any effects on the anti-HIV activity of coexpressed full-length TRIM5α, because all individuals carrying the G176del variant were in the heterozygous state. As shown in Fig. 2d and 2e, both HIV-1 and HIV-2 were restricted in cells simultaneously expressing the human/AGM chimeric TRIM5α and TRIM5α-CC(−). As expected, both HIV-1 and HIV-2 grew to high titers in cells expressing the human/AGM chimeric TRIM5α and the dominant negative mutant TRIM5α-SPRY(−) [24]. In contrast, both HIV-1 and HIV-2 were severely restricted in cells expressing the human/AGM chimeric TRIM5α and G176del-TRIM5 as compared within cells expressing the human/AGM chimeric TRIM5α and TRIM5α-CC(−). These results suggested that the G176del-TRIM5 enhanced the antiviral activity induced by the full-length TRIM5α.

Next, we investigated whether the truncated G176del-TRIM5 could affect the expression of TRIM5α. Expressions of the human/AGM chimeric TRIM5α in cells expressing either TRIM5α-SPRY(−), TRIM5α-CC(−) or G176del-TRIM5 are shown in Fig. 2f. Amount of human/AGM chimeric TRIM5α in cells coexpressing the G176del-TRIM5 was 1.7 times higher than that in cells coexpressing the TRIM5α-SPRY(−). When we infected a constant amount of SeV expressing the human/AGM TRIM5α in combination with the increasing amounts of SeV expressing the G176del TRIM5 variant, we found that the expression level of human/AGM TRIM5α was increased by the G176del TRIM5 (Fig. 2g).

Discussion

It is widely accepted that within host cells, there are restriction factors that oppose retroviral replication more effectively than the conventional arms of the immune

Fig. 2. *(continued)*
each SeV. CM-TRIM5α-SPRY(−) and human/AGM chimeric TRIM5α (○; S(−) + hu/AGM), AGM-TRIM5α-CC(−) and human/AGM chimeric TRIM5α (■; CC(−) + hu/AGM), or hemaglutinin (HA)-G176del-TRIM5 and human/AGM chimeric TRIM5α (▲; G176del + hu/AGM) were simultaneously inoculated. Nine hours after the infection, cells were superinfected with HIV-1 NL43 (d) or HIV-2 GH123 (e) and culture surpernatants were periodically assayed for levels of p24 (d) or p25 (e). The means with standard deviations of triplicate samples are shown. (f) Western blottings for TRIM5 protein and cyclophilin A from MT4 cells infected with SeV expressing the HA-tagged human/AGM chimeric TRIM5α (human/AGM-TRIM5α-HA) coexpressed with the AGM-TRIM5α-CC(−)-HA (lane 1; TRIM5α-CC(−)-HA), coexpressed with the CM-TRIM5α-SPRY(−)-HA (lane 2; TRIM5α-SPRY(−)-HA), or with the HA-G176del-TRIM5 (lane 3: HA-G176del-TRIM5). The relative amounts of human/AGM chimeric TRIM5α are shown on the top with the standard deviation of six independent samples. (g) MT4 cells were infected with SeV expressing the HA-tagged human/AGM chimeric TRIM5α coinfected with SeV expressing the HA-G176del-TRIM5 or an empty vector parental Z strain. The multiplicity of infection in each SeV is shown on the bottom. The relative amounts of human/AGM chimeric TRIM5α are shown on the top with standard deviation of triplicate samples.

system [1,2]. Because TRIM5α has crucial roles in the intracellular defense mechanisms against HIV-1 [2–4], sequence variations in TRIM5α might be associated with the susceptibility to HIV-1 infection and/or progression to AIDS. In this study, we demonstrated the association of 43Tyr-allele with the reduced susceptibility to HIV-1 infection in two ethnically distinct populations. In addition, we identified two novel rare variants, Gly110Arg and G176del, both of which had an impact on the anti-HIV-1 activity and susceptibility to HIV-1 infection.

The association of His43Tyr with the HIV-1 infection or AIDS progression has been tested in several studies, but the results were not consistent [16–20]. We found that the 43Tyr-allele was less frequent in the HIV-1-infected patients than in the ethnic-matched controls in both Japanese and Indian populations. The study sizes were not very large, but two independent ethnic populations did exhibit the same trends for the association with His43Tyr. We previously analyzed HIV-1-infected long-term nonprogressors and standard progressors in France and Japan for the TRIM5α polymorphisms and failed to find any differences in the frequency of 43Tyr-allele between these two HIV-infected groups both in France and Japan [19]. However, the allele frequency of 43Tyr in the Japanese HIV-1-infected patients we analyzed in the previous study was 0.143, which was similar to that in the present study (0.133, Table 1). Interestingly, several studies have reported that the anti-HIV-1 activity of TRIM5α with 43Tyr was lower than that with 43His [16,18]. In our previous study, we also showed that the anti-HIV-1 activity of TRIM5α with 43Tyr was lower than that with 43His, although the difference in anti-HIV-1 activity between the 43His-TRIM5α and 43Tyr-TRIM5α was very small [19]. In spite of the lower anti-HIV-1 activity of the 43Tyr-TRIM5α, several epidemiological studies have shown that the 43Tyr-allele was associated with the reduced susceptibility to HIV-1 infection [16,18], as demonstrated in this study. The reasons for the discrepancy between the epidemiological and functional effects of His43Tyr remain unclear at the moment. On the other hand, van Manen *et al.* [20] recently reported that homozygous status for 43Tyr was associated with the accelerated disease progression in white populations, which was consistent with the effect of His43Tyr variation on the anti-HIV-1 activity. Further epidemiological studies will be required to clarify the impact of His43Tyr on the susceptibility to HIV-1 infection and AIDS progression.

We also showed that the impact of His43Tyr on the susceptibility to HIV-1 infection was slightly different between Japanese and Indian. The frequency of 43Tyr-allele in the Indian HIV-1-infected patients was significantly lower than that in the Indian controls, but the significant difference was not found in Japanese. Different distribution of HIV-1 subtypes might be one of the reasons for the different contribution of 43Tyr-allele in the susceptibility, because all of the Indian patients examined in this study were infected with HIV-1 subtype C, whereas only subtype B was observed in our Japanese patients, as was found in the previous reports [26,27]. Kaumanns *et al.* [28] have reported that the antiretroviral activities of TRIM5α differed among the HIV-1 subtypes, although the differences in the in-vitro antiretroviral effect of TRIM5α between the subtypes C and B were not evident.

In this study, one focus was the functional impact of two rare TRIM5α variants found in our epidemiological studies. First, our findings indicated that the 110Arg variant weakened the anti-HIV-1 and anti-HIV-2 activities of human TRIM5α in human T-cell lines. This variant was observed more frequently in the Japanese HIV-1-infected patients than in the controls. This variation substitutes the smallest amino acid glycine with a positively charged amino acid arginine at the 110 amino acid position of TRIM5α and is located next to the amino acid residue 109Gly, which is suspected to be a zinc-coordinating residue in the B-box 2 domain [29]. This drastic change in amino acid character might change the structure of TRIM5α, in which an intact B-box 2 domain was essential for the antiretroviral activity of TRIM5α and disruption of the TRIM5α B-box domain by specific amino acid substitution resulted in loss of retroviral restriction [8,30–32]. The 3D structure of the amino acid residues 11–133 of TRIM5α was modeled by SWISS-MODEL, an Automated Comparative Protein Modeling Server (http://swissmodel.expasy.org/SWISS-MODEL.html) [33]. As shown in Fig. 3, residue 110 constituted one of the β sheets in the N-terminal half of TRIM5α. Interestingly, the location of residue 110 was close to residue 43 in the modeled 3D structure of TRIM5α (Fig. 3b and 3c). As described previously, His43Tyr was reported to affect antiretroviral activity. These data suggested that residue 110 might be one of the key amino acid residues in the TRIM5α structure like residue 43.

Second, we found that the truncated G176del-TRIM5 enhanced the antiviral activity of coexpressed full-length TRIM5α. Coinfection of SeVs expressing the G176del-TRIM5 and human/AGM-TRIM5α was accompanied by the increased protein level of full-length human/AGM-TRIM5α. The amount of human/AGM chimeric TRIM5α in cells coinfected with SeVs expressing the G176del-TRIM5 was 1.7 times higher than in cells coinfected with SeVs expressing the TRIM5α-SPRY(−). These data suggested that the truncated TRIM5α was degraded rapidly, resulting in a delay of the degradation process of full-length TRIM5α and leading to the augmentation of protein levels. Recently, we observed that coexpression of a splice variant of TRIM5, TRIM5γ, increased the amount of TRIM5α.

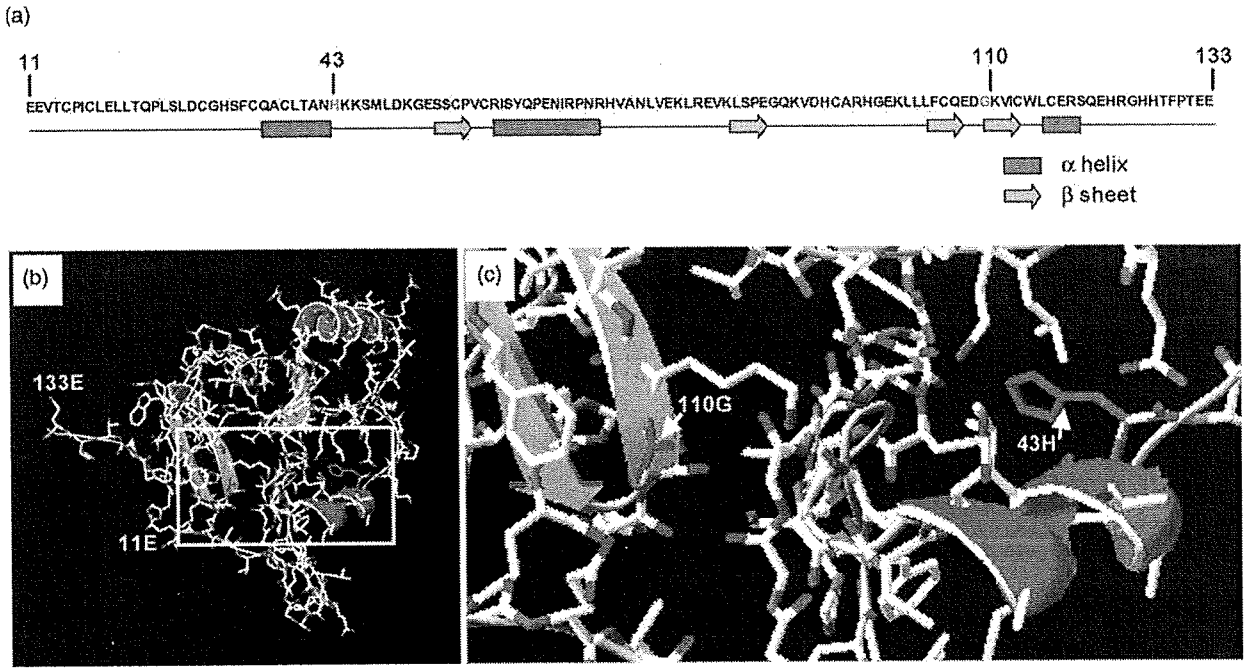

Fig. 3. Structural model of variant TRIM5α. (a) The primary structure is illustrated by the amino acid sequence of residues 11–133. One-letter amino acid code is used. The secondary structure is diagrammatically represented below the sequence showing the regions of the polypeptide chain, which are folded into the α helices (red boxes), the β sheets (green arrows) and random coils (black lines). (b) The 3D structure of amino acids 11–133 was modeled by SWISS-MODEL. (c) A magnified view, which is enclosed with a white square in (b), is shown. The arrows indicate the 43His and 110Gly residues.

TRIM5γ itself was unstable and its expression was lower than TRIM5α (Maegawa, unpublished data).

In this study, we identified three individuals harboring the G176del variant in the heterozygous state only in the Japanese controls, not in the HIV-1-infected patients. It appeared that the homozygous state for the G176del-allele would increase the susceptibility to HIV-1 infection, because it should result in null TRIM5α activity. It follows from the enhanced anti-HIV-1 activity of full-length TRIM5α by the truncated G176del-TRIM5 that the heterozygous state for the 176del-allele might mask the reduction in TRIM5α gene number and thus might not have a serious effect on the susceptibility to HIV-1 infection.

We demonstrated the association of common variant 43Tyr with the reduced susceptibility to HIV-1 infection in Japanese and Indian. We also identified two rare variants, 110Arg and G176del, which decreased and increased, respectively, the anti-HIV-1 activity in human cells expressing TRIM5α. We suggested that the sequence variations of TRIM5α were tightly linked to the susceptibility to or protection against the HIV-1 infection. However, further epidemiological studies using larger population samples will be required to clarify the impact of these rare variants on the HIV-1/AIDS susceptibility. In an effort to understand the genetic factors controlling the HIV-1 infection and AIDS progression, considerable attention should be paid to rare variants in addition to common variants in the candidate genes.

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*HLA-Cw*04* allele associated with nevirapine-induced rash in HIV-infected Thai patients

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Abstract

Background: A high incidence of rash has been reported in HIV-1 patients who received the anti-retroviral drug nevirapine. In addition, several studies have suggested that polymorphisms of human leukocyte antigen (*HLA*) genes may play important roles in nevirapine-induced rash. The aim of the present study was to evaluate the effects of different *HLA-C* alleles on rash associated with nevirapine in patients who started highly active anti-retroviral therapy (HAART) containing nevirapine in Thailand.

Results: A case-control study was carried out involving HIV-1 patients under treatment at Bamrasnaradura Infectious Diseases Institute, Nonthaburi, Thailand between March 2007 and March 2008. The study included all HIV/AIDS patients being treated with nevirapine-containing regimens. The study population comprised 287 HIV/AIDS patients of whom 248 were nevirapine-tolerant and 39 developed rash after nevirapine treatment. From the nevirapine-tolerant patients, 60 were selected as the control group on the basis of age, sex, and therapy history matched for nevirapine-induced rash cases. We observed significantly more *HLA-Cw*04* alleles in nevirapine-induced rash cases than in nevirapine-tolerant group, with frequencies of 20.51% and 7.50%, respectively (P = 0.009). There were no significant differences between the rash and tolerant groups for other *HLA-C* alleles except for *HLA-Cw*03* (P = 0.015).

Conclusion: This study suggests that *HLA-Cw*04* is associated with rash in nevirapine treated Thais. Future screening of patients' *HLA* may reduce the number of nevirapine-induced rash cases, and patients with alleles associated with nevirapine-induced rash should be started on anti-retroviral therapy without nevirapine.

Background

Highly active antiretroviral therapy (HAART) has significantly improved the prognosis of HIV-1-infected patients and prolonged AIDS-free survival[1]. HAART has also resulted in immune restoration and reduction of morbidity and mortality even for patients with advanced HIV-1 infection[1,2]. Nevirapine (NVP) is a non-nucleoside reverse transcriptase inhibitor (NNRTI) that has been shown to have high antiretroviral efficacy [3]. NVP-based HAART regimens have therefore been widely used in resource-limited countries because of their efficacy, availability and relatively low cost. In Thailand, the Government Pharmaceutical Organization (GPO) has produced GPOvir, a low cost (US$ 30 per month) and fixed-dose combination of NVP, stavudine (D4T), and lamivudine (3TC), which has been commercially available since March 2002.

However, NVP-associated rash has been reported to be as high as 48% after the start of treatment with this inhibitor [4]. Nearly 90% of the side effects of GPOvir are thought to be due to NVP hypersensitivity [5]. Skin rash is the most common adverse drug reaction associated with NVP, and hypersensitivity reaction to NVP is rapid and severe when drug administration is suspended and re-challenged [6]. Most patients develop rash between the first and third week of treatment [7], including the more severe forms of rash such as extensive maculopapular rash, serum sickness-like reaction, hypersensitivity syndrome, Steven-Johnson syndrome and toxic epidermal necrosis [7,8]. NVP-induced rash has been reported in 4.3-36% of adults [9,10] with the incidence for Thai patients ranging from 6 to 21% [5], reflecting the comparatively high incidence of rash in Asians [11]. Several features of NVP hypersensitivity suggest that genetic factors may play an important predisposing role in NVP hypersensitivity, in which NVP itself or NVP-induced antigens may trigger an immunological response that is dependent on CD4 T lymphocytes in susceptible hosts. This supports the hypothesis that the hypersensitivity reaction to NVP may be HLA-associated [9,12,13], while HLA-alleles have also been identified as clinically relevant susceptibility markers for hypersensitivity reaction to another antiretroviral drug [14]. Recent studies have shown that in Japan the *HLA-Cw*08* allele is associated with NVP hypersensitivity [15]. The objective of the study presented here was to compare allele frequency of *HLA-C* in Thai patients with rash who had to change from GPOvir to a regime containing efavirenz and those who were NVP-tolerant.

Results

A case-control study was carried out. The study population comprised 287 HIV/AIDS patients of whom 248 were nevirapine-tolerant and 39 developed rash. From the nevirapine-tolerant patients, 60 were selected as the control group on the basis of age, sex, and therapy history matched for nevirapine-induced rash cases. As shown in Table 1, the demographic and clinical characteristics of patients with NVP-induced rash were very similar to those of NVP-tolerant patients. The medians of CD4 cell counts of NVP-induced rash and NVP-tolerant group were comparable at both time points of immediately before NVP treatment and 6 month after treatment. It is known that HIV-infected patients frequently suffered from allergic drug reactions [16]. Nearly all of rash cases (37 out of 39) used steroids and more than half (35 out of 60 cases) of NVP-tolerant patients also used steroids. Four out of 35 NVP-tolerant steroid users had developed mild rash that could be controlled by steroid. Remaining 31 NVP-tolerant patients used steroid to suppress allergic reactions including chronic allergic skin, rhinitis, asthma, and drug reactions upon Pneumocystis carinii pneumonia treat-

Table 1: Demographics and immunological variables of the NVP-induced rash and NVP-tolerant groups

Variables	NVP-induced rash (n = 39)	NVP-tolerant (n = 60)	P value
Age (median IQR)	39.0 (34.0-44.0)	38.0 (35.0-41.75)	*0.71
Sex [n(%)]			**0.68
Male	22 (56.41%)	31 (51.67%)	
Female	17 (43.59%)	29 (48.33%)	
Duration of Treatment, year (median IQR)	1 (0-3)	1 (0-2.75)	*0.47
Pre-NVP-treatment CD4 T-cell count × 10^6/l (median IQR)	43.5 (19.50-135.50)	55 (28.25-137.50)	*0.71
Post-NVP-treatment CD4 T-cell count × 10^6/l (median IQR)	243 (155.00-328.00)	241 (185.00-312.25)	*0.98

*Mann-Whitney U-test
**Chi square test

ment, and immune reconstitution inflammatory syndrome. The NVP-induced rash cases manifested severe rash, which could not be suppressed by steroid, and had to change the regimen.

The frequencies of the *HLA-C* alleles identified in the 39 samples in the NVP-induced rash group and 60 samples in the NVP tolerant group are presented in Table 2. Frequency of *HLA-Cw*04* was approximately 21% for the patients with NVP-induced rash and 7.5% for the NVP-tolerant group, showing a statistically significant difference in *HLA-Cw*04* allele frequency (P = 0.0088, Fisher's exact test). The reported *HLA-Cw*04* allele frequency for the normal Thai population (0.102) [17] is higher than that of the NVP-tolerant group (0.075) and lower than that of the NVP-induced rash group (0.205). Although statistical significance of this difference was lost after stringent Bonferroni correction (Pc = 0.088), these results suggested that *HLA-Cw*04* was associated with NVP-induced rash in HIV-1 infected Thai patients. One-third of the patients with NVP-induced rash (13 out of 39) carried *HLA-Cw*04* in comparison with 15% of the NVP-tolerant patients (9 out of 60) (P = 0.047, Fisher's exact test; Pc = 0.47). When we compared 39 NVP-induced rash patients with 25 NVP-tolerant patients who did not receive steroid, the concentration of *HLA-Cw*04* allele in NVP rash cases was still apparent (0.205 vs 0.060, P = 0.04, Fisher's exact test).

In contrast to *HLA-Cw*04*, fewer *HLA-Cw*03* alleles were found in patients with NVP-induced rash than in NVP-tolerant ones (0.051 and 0.167) (P = 0.015, Fisher's exact test; Pc = 0.15). Approximately 7.7% of patients with NVP-induced rash (3 out of 39) carried *HLA-Cw*03* in comparison with 30% of the NVP-tolerant patients (18 out of 60) (P = 0.011 Fisher's exact test, Pc = 0.11). There were no significant differences between the NVP-induced rash and NVP-tolerant groups in allele frequencies of *HLA-Cw*01*, *HLA-Cw*05*, *HLA-Cw*06*, *HLA-Cw*07*, *HLA-Cw*08*, *HLA-Cw*12*, *HLA-Cw*14*, and *HLA-Cw*15*. Other *HLA-Cw** alleles were not detected in the tested samples. The NVP-tolerant group allele frequencies of *HLA-Cw*03* (0.167), *HLA-Cw*08* (0.125) and *HLA-Cw*12* (0.067) were very similar to those of the normal Thai population (0.174, 0.144 and 0.06, respectively) [17], which suggests that our genotyping was accurate.

*HLA-DRB1*01* was reported to be associated with NVP hypersensitivity in Australian [13] and French [18] cohorts. We therefore performed PCR-sequence specific oligonucleotide probe (SSOP) method to detect *HLA-DRB1*01* alleles. Contrary to our expectation, we failed to detect *HLA-DRB1*01* allele in 39 NVP-induced rash cases, while we detected five of this allele in 60 NVP-tolerant patients. This result suggested that the *HLA-DRB1*01* allele may not involved in NVP rash in Thai population.

The C allele of the SNP rs9264942, located in the 5'upstream region of the *HLA-C* gene, was reported to associate with higher levels of HLA-C expression and *HLA-Cw*04* allele [19]. To know whether or not higher levels of *HLA-C* gene expression associated with NVP-induced rash, we genotyped rs9264942 SNP by TaqMan SNP genotyping system. The C allele frequency in 39 NVP rash cases was 0.45, while it was 0.38 in 60 NVP-tolerant patients (P = 0.36, Chi square test). This result suggested that the *HLA-Cw*04* allele itself rather than the relative high levels of HLA-C expression was involved in NVP-induced rash.

Discussion

In the study reported here, we genotyped *HLA-C* alleles of 39 patients with NVP-induced rash and 60 NVP-tolerant Thai patients, and found that frequency of *HLA-Cw*04* was higher in NVP-induced rash Thai patients than in NVP-tolerant patients. While the number of samples in

Table 2: Occurrence of HLA-C alleles in the nevirapine (NVP)-rash cases and NVP-tolerant controls in Thailand

Allele	NVP-induced rash number (%)	NVP-tolerant number (%)	*P value
*HLA-Cw*01*	12 (15.38)	12 (10.00)	NS
*HLA-Cw*03*	4 (5.13)	20 (16.67)	0.01
*HLA-Cw*04*	16 (20.51)	9 (7.50)	0.009
*HLA-Cw*05*	2 (2.56)	3 (2.50)	NS
*HLA-Cw*06*	8 (10.26)	9 (7.50)	NS
*HLA-Cw*07*	19 (24.36)	39 (35.50)	NS
*HLA-Cw*08*	9 (11.54)	15 (12.50)	NS
*HLA-Cw*12*	6 (7.69)	8 (6.67)	NS
*HLA-Cw*14*	0	3 (2.50)	NS
*HLA-Cw*15*	2 (2.56)	2 (1.67)	NS
Total	78	120	

*Fisher's exact test
NS: P > 0.05

our study is small, the increased frequency of *HLA-Cw*04* in patients with NVP-induced rash suggested that this allele plays an important role in the development of rash after GPOvir treatment. *HLA-Cw*04* was found to be associated with rapid development of AIDS-defining conditions in Caucasians [20,21] but to have a protective effect in African Americans [22]. In hepatitis C virus infection cases, *HLA-Cw*04* was associated with viral persistence [23].

Previous studies [15,24] have suggested that *HLA-Cw*08* is associated with NVP hypersensitivity. Littera *et al.* studied 49 Sardinian HIV-1-positive patients treated with NVP and reported that *HLA-Cw*08* and/or *HLA-B*14(65)* is associated with NVP hypersensitivity [24]. Subsequently, Gatanaga *et al.* studied HIV-1 infected individuals in Japan [15]. In this study, 41 patients had a history of NVP treatment, 12 of whom showed NVP hypersensitivity. The frequency of *HLA-B*14* is nearly 0% in Japan. On the other hand, the frequency of *HLA-Cw*08*-positive patients in the NVP hypersensitive group was 42%, which was significantly higher than that the NVP tolerant group (10%) [15]. In our study, however, the frequencies of *HLA-Cw*08* in the NVP rash and tolerant groups were 0.115 and 0.125, respectively, without any significant difference between the two groups (P = 1.000, Fisher's test). Although the precise reason for the difference between the findings of these previous studies and ours is not clear at present, there were several differences between them. First, we genotyped approximately three times more patients with NVP-induced rash than was done in the previous studies. Second, our study focused on rash after NVP treatment but the other studies dealt with patients with rash and/or hepatotoxicity. Furthermore, it is possible that the levels of linkage disequilibrium between *HLA-C* alleles and those of other HLA locus and/or other genes differ among different ethnic groups. As described above, *HLA-Cw*04* was associated with rapid AIDS progression in Caucasians [20,21] but to have a protective effect in African Americans [22]. Thus, it is possible that other SNP(s) or HLA allele(s) responsible for NVP-induced rash is in a stronger linkage disequilibrium with *HLA-Cw*04* in Thais than the other ethnic groups tested previously.

Previous studies have also suggested that higher CD4 counts at baseline increased risk of NVP-induced rash [25-30]. In our study, however, the median baseline CD4 counts of NVP-induced rash cases (43.5 cells/μl) was very similar to that of 248 NVP-tolerant patients (43 cells/μl). The effects of high baseline CD4 counts on risk of NVP-induced rash were reportedly observed mainly in patients whose CD4 counts were over 250 cells/μl [29]. Accordingly, nearly all of patients in our study started NVP-containing regimen after their CD4 counts dropped below 250 cells/μl. Therefore, it is also possible that low baseline CD4 counts in our study limit the ability to detect an HLA association reported previously [15,24]. Nevertheless, our results are practically meaningful since most of HIV-1-infected individuals in Thailand start antiretroviral treatment after their CD4 counts drop below 250 cells/μl.

One report from Thailand demonstrated that the effects of high baseline CD4 counts on risk of NVP-induced rash were still observed even in patients whose baseline CD4 counts were below 250 cells/μl, although the levels of such effects was very small in patients whose baseline CD4 counts were below 200 cells/μl [30]. Therefore, we divided patients according to their baseline CD4 counts. When we picked up patients whose baseline CD4 counts were over 100 cells/μl, *HLA-Cw*04* allele frequency was 0.25 in 14 NVP-induced rash cases and 0.025 in 20 NVP-tolerant patients. Statistical significance of this difference rather increased in these groups (P = 0.0068). When we picked up patients whose baseline CD4 were less than 100 cells/μl, *HLA-Cw*04* allele frequency was 0.2045 in 22 NVP-induced rash cases and 0.0897 in 39 NVP-tolerant patients. Although there was an apparent trend towards high *HLA-Cw*04* frequency in NVP-induced rash cases, statistical significance of the difference greatly reduced in these groups (P = 0.094). Therefore, the difference in the allele frequency was more prominent in patients with higher CD4 counts even in our patient group.

During the preparation of this manuscript, Chantarangsu *et al.* reported a strong association between *HLA-B*3505* and NVP-induced skins rash in HIV-infected Thai patients [31]. Our results at least partly confirm theirs since *HLA-Cw*0401* showed the second highest levels of difference in allele frequency between NVP-rash and NVP-tolerant patients in their study [31]. It is known that *HLA-Cw*04* is in a linkage disequilibrium with *HLA-B*35* in Thais [32]. It is, thus, necessary to investigate whether or not *HLA-B*3505* is also overrepresented in NVP-rash cases in our study.

Conclusion

We observed higher frequency of *HLA-Cw*04* in NVP-induced skin rash than in NVP-tolerant patients in Thailand. In addition to *HLA-Cw*04* and/or *HLA-B*3505*, future screening of patients' *HLA* and genes involved in hypersensitive reactions may identify other alleles responsible for the incidence of NVP-induced rash. Patients possessing alleles responsible for NVP-rash should be started on anti-retroviral therapy without NVP.

Methods

Clinical specimens

A case-control study was carried out with HIV-1 infected patients who were under treatment at Bamrasnaradura Infectious Diseases Institute, Ministry of Public Health,

Nonthaburi, Thailand. The targeted study population comprised 672 HIV-1/AIDS patients and the study period ran from March 2007 to March 2008. Patients who developed apparent skin rash anywhere on the body after NVP containing HAART and had to change their NVP-containing regime to efavirenz-containing ones were diagnosed as rash. There were 39 patients who matched these criteria. Most of these 39 patients developed rash within two months after NVP treatment (NVP-rash), and none of them showed liver toxicity. On the other hand, 248 patients showed reasonably good adherence to NVP and did not develop rash at all or developed only mild rash that could be controlled by steroid within the observation period. The remaining 385 patients were excluded because of treatment without NVP (184 cases), incomplete clinical records (101 cases), treatment interruptions (62 cases), adverse drug effects other than rash (18 cases), and incomplete HIV-1 suppression (20 cases). From the 248 NVP tolerant patients, we first tried to have two control patients for each rash case matching age, sex, and duration of therapy before NVP containing HAART. However, some rash cases have only one control mainly due to the limitation of available reagents. Total of 60 samples were thus selected for the control group (NVP-tolerant). Age, sex, treatment history and CD4 cell counts were not different between test and control groups as shown in Table 1. Two hundred μl of whole blood was collected from each of those patients and kept at -20°C until DNA extraction with the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). All participants signed informed consent forms. The present study was approved by the institutional ethics committees of the Bamrasnaradura Infectious Diseases Institute and the Department of Diseases Control, Ministry of Public Health, Thailand.

HLA-C Typing

Medium-high resolution HLA-C typing was performed with an HLA-C typing kit (MPH-2 HLA-C typing kit, Wakunaga, Japan) according to the manufacturer's instructions. Any ambiguous results were checked by nucleotide sequence determination of PCR-amplified DNA fragments of HLA-C exons 2, and 3 [33]. GeneAmp® PCR system 9600 (Applied Biosystems, Foster City. CA) was used for all the PCR reactions and DNA sequencer 373 (Applied Biosystems) was used for determination of the nucleotide sequence of an amplified fragment.

HLA-DRB1*01 *detection*

We performed HLA class II DNA-based typing of DR1 as described in the 12th International Histocompatibility Working Group version 1.5. Amplified DNA with primer pair 2DRBAMP1 (5'-TTCTTGTGGCAGCTTAAGTT-3') and 2DRBAMP-B(5'-CCGCTGCACTGTGAAGCTCT-3') in exon 2 was treated with NaOH and the denatured DNA was loaded onto a nylon membrane manually using a milliblot system with a vacuum manifold. UV light was used to crosslink the DNA to the membrane. For hybridization with DR1 specific probe, we used DRB 1001w (5'-TGGCAGCTTAAGTTTGAA-3') digoxigenin-labeled SSOP for detection of *HLA-DRB1*01* positive sample. After stringent wash procedure, the membrane was incubated with an antibody to digoxigenin coupled with alkaline phosphatase. Addition of a substrate for alkaline phosphatase caused light to be emitted by the Lumiphos. This light was detected by exposure of X-ray film.

HLA-C 5' SNP genotyping

The rs9264942 SNP genotyping was performed by TaqMan SNP genotyping system with ABI real time PCR 7300. A validated primer and probe mix (C__29901957_10) were purchased from Applied Biosystems.

Statistical analysis

Differences in age, duration of therapy before start of NVP containing HAART, and pre- and post-therapy CD4 cell counts between case and control groups were evaluated by Mann-Whitney U test. A difference in proportion of sex was evaluated by Chi square test. Differences in the allele frequencies between the two groups were evaluated by Fisher's exact test. P values less than 0.05 were considered to be statistically significant. The corrected P (Pc) values were calculated by using Bonferroni's correction.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SL conceived of the study, participated in the design and coordination of the study, and drafted the manuscript, TR carried out genotyping and analysis of clinical data, SF carried out genotyping and analysis of clinical data, SU participated in coordination of the study and helped to draft the manuscript. WP participated in collection of clinical data and helped to draft the manuscript, PT participated in coordination of the study and helped to draft the manuscript, EEN supervised genotyping, participated in study design and helped to draft the manuscript, TS participated in the design of the study, performed the statistical analysis, and helped to draft the manuscript. All authors read and approved the final manuscript.

Author's information

SL is a chief of Immunology and Virology Laboratory, Bamrasnaradura Infectious Diseases Institute, which is a governmental institute with the largest infectious disease hospital in Thailand. TR and SF are research assistants of the study. SU is a sub-chief of Immunology and Virology Laboratory and working on HIV-1 diagnosis. WP is a clinician who is taking care of HIV-1 infected patients. PT is a director of Bamrasnaradura Infectious Diseases Institute.

EEN is an assistant professor of Osaka University, Japan. TS is a professor of Osaka University working on HIV-1 infection and host genome.

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HIV-2 capsids distinguish high and low virus load patients in a West African community cohort

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ABSTRACT

HIV-2 causes AIDS similar to HIV-1, however a considerable proportion of HIV-2 infected patients show no disease and have low plasma virus load (VL). An analysis of HIV-2 capsid (p26) variation demonstrated that proline at p26 positions 119, 159 and 178 are more frequent in lower VL subjects while non-proline residues at all three sites are more frequent in subjects with high VL. *In vitro* replication levels of viruses bearing changes at the three sites suggested that these three residues influence virus replication by altering susceptibility to TRIM5α. These results provide new insights into HIV-2 pathogenesis.

1. Introduction

HIV-2 was discovered in West Africa patients [1,2] shortly after the discovery of HIV-1, with HIV-2 entering humans via zoonoses distinct from the entry of HIV-1 some time in the early 20th century [3]. The two viruses have 60–80% sequence homology and have similar genomic organization, yet the viruses have distinct transmission rates and disease associations (reviewed in Ref. [4]). Although some HIV-2-infected patients progress to AIDS, the majority control infection [5–7] and patients with low VL survive longer [8]. Early descriptions of HIV-2 observed differences in the virus genetics [9] and noted that the HIV-2 epidemic behaved liked a mixture of pathological and non-pathological viruses [5,10]. Studying and understanding HIV-2 represents a possibility to discover how a potentially lethal lentivirus infection can be controlled by humans.

There are limited data on HIV-2 sequence variation associated with either long-term control or progression to disease [9,11,12]. Although some correlation of Nef variation with disease has been observed [13], most HIV-2 sequences in the public databases are derived from viruses isolated from AIDS patients where high VLs facilitate virus isolation and sequencing which could introduce bias in the data. Sequences derived from patients with low VL are essential for understanding disease non-progression in HIV-2 infection. A community cohort to study HIV was established in Caio, Guinea Bissau in 1988 [50], reviewed in Ref. [4]. HIV screening of the entire adult population in the Caio community cohort have identified HIV-2-positive subjects with a wide spectrum of clinical symptoms and survival [4,10,14]; a sequence analysis of the virus among these patients could identify virus variations that influence outcome.

The capsid (CA) protein (p24 in HIV-1; p26 in HIV-2) has a structure that is conserved among retroviruses [15,16]. p26 accumulates during replication as a Gag polyprotein, assembles into spherical structures that package viral RNA and is subsequently processed by the viral protease and reassembled into the mature virus cores [16–18]. The CA has a distinct amino-terminal domain (residues 1–145), that is exposed on the surface of cores, and a C-terminal domain (residues 146–230) which is required for oligomerization [19]. The 20 amino acid major homology region (MHR) in the C-terminal domain is highly conserved; changes in this motif can interfere with CA assembly, maturation and early stages of infec-

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Table 1
Comparison of the cohort p26 residues 119, 159 and 178 to HIV-2_{ROD} p26; variation with viral load.

ID	Source	VL	119	159	178	ID	Source	VL	119	159	178
ROD			P	P	P	ROD			P	P	P
CO310	RNA	<100	.	.	.	CO309	RNA	283	Q	S	.
CO315	DNA	<100	.	.	.	CO316	RNA	372	.	.	.
CO318	PRO	<100	.	.	.	CO364	RNA	387	A	.	.
CO319	RNA	<100	.	.	.	CO314	DNA	393	.	S	.
CO324	RNA	<100	.	.	.	CO354	DNA	413	.	.	.
CO326	RNA	<100	.	.	.	CO323	RNA	497	A	.	.
CO327	DNA	<100	.	.	.	CO365	RNA	523	A	S	A
CO339	RNA	<100	.	.	.	CO350	RNA	540	A	S	A
CO349	RNA	<100	.	.	.	CO322	RNA	610	.	S	A
CO359	RNA	<100	.	.	.	CO355	RNA	651	A	.	A
CO360	RNA	<100	.	.	.	CO357	RNA	813	A	S	Q
CO344	DNA	<100	A	.	.	CO332	RNA	1085	A	.	V
CO346	DNA	<100	A	.	.	CO305	RNA	1343	A	.	.
CO351	RNA	<100	A	.	.	CO340	RNA	1587	.	.	.
CO308	RNA	<100	Q	.	.	CO321	RNA	1608	.	.	.
CO337	DNA	<100	.	T	.	CO342	RNA	1907	A	S	.
CO361	RNA	<100	A	S	.	CO325	RNA	1999	.	.	.
CO366	RNA	<100	A	S	.	CO330	RNA	2653	A	S	.
CO367	DNA	<100	A	S	.	CO331	RNA	2949	A	S	A
CO363	DNA	<100	Q	S	.	CO320	RNA	3241	A	.	.
CO335	RNA	<100	A	.	A	CO307	RNA	3764	G	S	.
CO336	RNA	<100	A	.	A	CO368	RNA	6431	A	T	S
CO341	RNA	<100	A	.	A	CO345	RNA	9659	A	.	.
CO362	DNA	<100	A	.	A	CO312	RNA	9979	A	S	A
CO301	DNA	<100	A	S	.	CO334	RNA	10752	.	S	.
CO306	DNA	<100	A	S	A	CO311	RNA	14104	A	S	A
CO333	RNA	<100	A	S	A	CO313	RNA	17067	A	S	A
CO302	RNA	109	.	.	.	CO369	RNA	22446	Q	S	.
CO304	DNA	114	.	.	.	CO343	RNA	25836	A	S	.
CO303	RNA	154	A	S	A	CO338	RNA	28581	Q	S	A
CO356	DNA	182	.	.	.	CO317	RNA	37503	A	S	A
CO328	RNA	198	A	.	.	CO347	RNA	146284	A	.	.
CO353	DNA	234	.	.	.	CO348	RNA	148593	A	.	A
CO329	RNA	267	A	S	S	CO358	RNA	283542	A	S	.
CO352	RNA	275	.	S	.						

Samples from 69 HIV-2 singly-infected patients collected in 2006 were used for sequencing (see Section 2). Patient plasma VL was assayed by RT-PCR with a lower limit of detection of 100 virus copies/ml. For statistical analysis, undetectable viremia was given a value of 50 copies/ml. Viral loads (copies/ml) are indicated in second column. The source of template for the PCR reaction (circulation RNA (RNA) or proviral DNA (DNA)) is indicated. The reference sequence derived from HIV-2_{ROD} p26 is listed in the first row, in the subsequent rows identity with the HIV-2_{ROD} p26 sequence is indicated with a period (.).

tion [19,20]. The multiple functions impose strict constraints on the amino acid changes allowed in CA. Studying the limited variations that do occur in p26 can provide important information on both protein function and the selective forces acting on HIV-2. In this study, we have tested the hypothesis that HIV-2 p26 capsid variants modulate HIV-2 viral load.

2. Methods

2.1. Patient cohort.

Study subjects were recruited from the community-based cohort in Caio, Guinea Bissau (study subjects described in Ref. [14]). Briefly, HIV screening was performed with a Murex ICE HIV-1.2.0 immunoassay (Murex Diagnostics), confirmed and differentially diagnosed (HIV-1/HIV-2) using HEXAGON HIV (Human GmbH). Dually HIV-1+2 positive samples were subjected to peptide-based assays (Pepti-Lav 1-2, Sanofi Diagnostics Pasteur) and HIV-1 and HIV-2-specific polymerase chain reaction (PCR) with primers targeted to the long terminal repeats [7,8]. HIV-1+2 infected patients were excluded from the study. HIV-2 plasma viral load was quantified by PCR based method [7,21] with a lower limit of detection of 100 copies/ml, for analytic purposes, undetectable samples were assigned a value of 50 copies/ml. All participants were antiretroviral naïve at the time of the study and provided informed consent. The study was approved by the Gambian Government/MRC Ethics Committee, The Republic of Guinea Bissau Ministry of Health, and the Oxford Tropical Research Ethics Committee (OXTREC), UK.

2.2. p26 gene amplification strategy, optimization of primers.

p26 gene amplification primers were designed by selecting conserved sequences flanking the p26 coding region from all HIV-2 isolates in the Los Alamos HIV Database to be used as targets for PCR primers. Coupled reverse transcriptase and nested PCR reactions were performed with plasma-isolated virus RNA as template. The following primer sequences in various combinations were used for amplification:

MO017(OF, outer forward) GTCTGCGTCATTTGGTGCAT
MO018(IF, inner forward) CTGCAGAGAAAATGCCAAGCA
MO019(OR outer reverse) GGGCAGTTTGTCATGATGTGTCC
MO020(IR, inner reverse) GCCCTTCCTTTCCACAGTTCCA
MO021(IR) GCCCTTCCTTTCCACAATTCCA
MO030(OF)CACGCAGAAGAGAAAGTGAAAG
MO031(OR) CGGGGAAGTTGCGRGGCTT
MO032(IF) AGTAGACCAACAGCACCACC
MO036(OF) GTGGGCAGCGAATGAATTGG
MO037(OF) GTGGGCAGCGAACGAATTGG
MO038(OR) AAAGAGAGAATTGAGGTGCAGCA.

In case none of the primer combinations amplified a product from RNA, amplification of provirus was performed with the same

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series of primers using genomic DNA from the patients' peripheral blood mononuclear cells (PBMCs) as template. The origin of the sequence (viral RNA or proviral DNA) is indicated in Table 1. PCR products were excised from preparative gels, purified by QiaQuick columns, sequenced using the inner PCR primers from both directions and aligned using ClustalW; ambiguities were resolved by direct analysis of sequencing scans using a sequence alignment editor BioEdit [22].

The p26 sequences were monitored to rule out cross-contamination during sample handling or PCR. Contamination among different samples would generate multiple sequences with 100% identity. A phylogenetic analysis showed no identical sequences, with the closest pairs (CO336 and CO335) showing 0.58% difference (4 differences in 690 nucleotides). BLAST analyses revealed no homology greater than 94% with any sequence in the GenBank database (results not shown). All the p26 sequences were consistent with HIV-2 subtype A infections.

2.3. HIV-2 envelope gene amplification

Envelope sequences were obtained from 34 HIV-2 isolates selected randomly from the set of 69 Caio viruses (see below). The following PCR primers targeted to conserved envelope flanking sequences were used:

MO080 (outer forward) CAGTCATCACAGAGTCATGTG
MO076 (outer reverse) TCCTTGTGGATAYGAYCTGT
MO072 (inner forward) TCATGTGAYAAGCACTATTGGGA
MO077 (inner reverse) GGAAGAGAAAACAGGCCTATAGCC.

An approximately 1500 bp fragment (HIV-2_{ROD} C2 to gp41, positions 6783–8285) was amplified in a nested RT-PCR from plasma virus. PCR products were purified as described above, sequenced using inner PCR primers from both directions. If the initial four primers failed, these additional primers were used:

MO122a GTGGACTAACTGCAGAGGAGAATT
MO125 AGTTCTGCCACCTCTGCACT
MO125a AGAAAACCCAAGAACCCTAGCAC.

A fragment of approximately 1300 bp corresponding to HIV-2_{ROD} positions 6849–8210 was used for the phylogenetic analysis. The p26 and Env sequences described in this manuscript were submitted to GenBank (accession numbers GQ485448-GQ485550).

2.4. Molecular modelling

Three-dimensional (3-D) models of HIV-2 CAs were constructed by the homology-modelling technique using the Molecular Operating Environment (MOE) (Chemical Computing Group Inc., Quebec, Canada) as described in Refs. [23–26]. The two crystal structures of the HIV-1 CA proteins were used as templates for the modelling; a CA monomer at a resolution of 3.00 Å (Protein Data Bank (PDB) code: 1E6J [27]) and the dimer of CA C-terminal domain at a resolution of 1.70 Å (PDB code: 1A8O [19]). The amino acid sequence identity of HIV-1 (1E6J) and HIV-2 CA (CO310 in this study) is about 70.5%. The sequence similarity is sufficient to construct a structural model with an r.m.s. deviation of approximately 1.5 Å for the main chain between the predicted and actual structures [28]. The 3-D structures were optimized thermodynamically by energy minimization using MOE and an AMBER99 force field [29] and further refined the physically unacceptable local structures on the basis of evaluation of unusual dihedral angles, ψ and ϕ, by the Ramachandran plot using MOE.

The binding energies of the p26 dimer models, E_{bind}, were calculated as described elsewhere [25] [30], using the formula $E_{bind} = E_{dimer} - 2E_{monomer}$, where E_{dimer} is the energy of the p26 dimer; $E_{monomer}$ is the energy of the p26 monomer. Spearman's rank correlation coefficient for viral load and capsid binding energy and its statistical evaluation were calculated by SPSS ver 14 (SPSS Inc, Chicago).

2.5. Expression of TRIM5α

Construction of recombinant Sendai viruses (SeVs) carrying human MT4-TRIM5α-tag (Hu-TRIM5α-Sev) and cynomolgus monkey TRIM5α lacking the SPRY domain (CM-SPRY(-)-Sev) was described previously [23].

2.6. Construction of HIV-2 GH123 capsid variants

Mutant DNA constructs of infectious molecular clones of HIV-2 GH123 carrying alanine 119 (GH123/119A) was described previously [23]. Note that because of an insertion in the GH123 p26 protein, positions 120, 160 and 179 from Ref. [23] correspond to positions 119, 159 and 178 in this work. PCR-based mutagenesis with primer pairs P1, P2 and P3, P4 was used to generate mutant GH123 carrying alanine 178 (GH123/178A). Mutant GH123 carrying serine 159 and 178A (GH123/159S-178A) was then constructed using primer pairs P1, P5 and P4, P6 with GH123/178A as a template. Mutant GH123 carrying 119A, 159S and 178A (GH123/119A-159S-178A) was constructed with primer pairs P1, P5 and P4, P6; and both GH123/119A and GH123/178A as templates. Infectious viruses were prepared by transfection of 293T cells with mutant proviral DNA clones.

P1:CTTCCCTGTACAACAGACA, P2:TTTACTGCTGCATCTGTTTGTTCTG,
P3:CAGAACAAACAGATGCAGCAGTAAA,
P4:GTGCAGCAAGTCCTCTGTC
P5:TAGCTCTGGAATGATTCCTTTGGTCC,
P6:GGACCAAAGGAATCATTCCAGAGCTA

3. Results

3.1. p26 CA variation in the Caio cohort.

HIV-2 positive subjects were selected from the Caio community cohort to assess HIV-2 sequence variation among a wide range of VL (≤100 to >280,000 copies/ml). PCR was attempted from a total of 92 subjects and p26 sequences were obtained from 69 subjects; 53 sequences were derived from viral RNA and 16 were from proviral DNA. Additional clinical features of these patients were previously described [14].

Caio p26 sequences were compared to either the Caio 69 p26 consensus sequence (Fig. 1 upper) or to the reference strain HIV-2_{ROD} p26 [31] (Fig. 1 lower) to identify amino acid polymorphisms in p26. HIV-2_{ROD} was chosen as a reference because the virus was isolated early in the epidemic in Cape Verde, islands historically linked to Guinea Bissau. Both comparisons show a similar pattern with variant amino acid sites clustering outside of the alpha helices (marked in black) required for CA folding (Fig. 1). Variations that associate with high or low VL (Mann–Whitney test, $p < 0.05$, except for position 178) are highlighted with a dotted line. The polymorphisms at position 119, 159 and 178 were of special interest because they showed 35–70% variation from HIV-2_{ROD} and involved changes from the reference sequence proline, a change expected to strongly alter protein structure. The non-proline residues were either alanine (38 cases), glutamine (5), or glycine (1) at position 119, serine (29) or threonine (2) at 159, and alanine (18), serine (2), gultamine (1), or valine (1) at 178. Viruses in low VL patients often had proline at these three positions while in higher VL samples these sites were frequently occupied by

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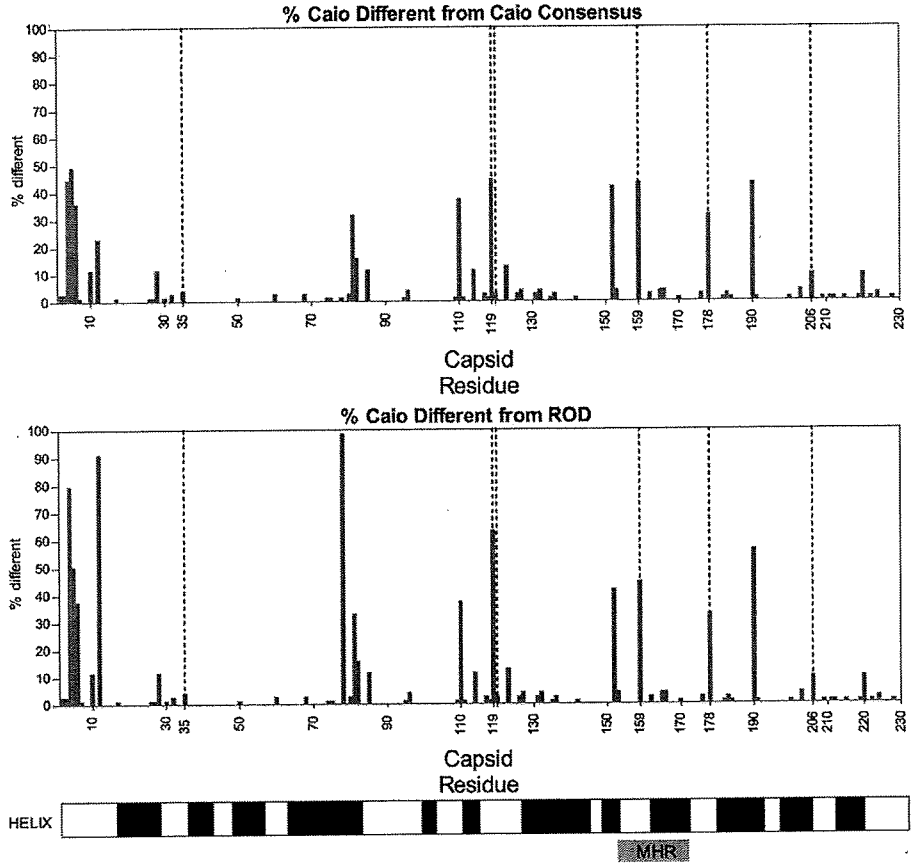

Fig. 1. Amino acid polymorphisms in the Caio HIV-2 p26. Polymorphisms in the p26 coding sequence are displayed as the percentage of the 69 sequences that differ at each position from either the Caio p26 consensus sequence (determined as the majority amino acid at each position, upper panel) or from the HIV-2_{ROD} p26 sequence (middle panel). Conserved alpha helices (in black) and the Major Homology Region (MHR) are indicated in the lower panel. Sites of variation that were associated with VL are indicated with dotted lines (Mann–Whitney test) ($p \leq 0.05$). [NB $p = 0.07$ for P178].

non-proine residues (Table 1). Three of the significantly varying positions (35, 120 and 206) were too infrequent for further study.

3.2. p26 CA variation correlating with VL

The association of proline 119, 159 and 178 with reduced VL becomes apparent when the log-transformed VL for each sample is plotted as a function of the total number of prolines at these three sites Fig. 2A. A Tobit regression analysis showed a clear relationship of increased VL with decreasing prolines in these three positions ($p = 0.003$).

To examine proline variation in more detail, CA types were grouped according the residue at each of the three positions using the code P (proline), N (not proline), or the wild card * (any amino acid) and ordered by increasing VL (Fig. 2B). Among the progression of median viral loads, there was a pattern of decreasing prolines, starting with the PPP group, through the intermediate forms to the complete non-proline NNN group (Fig. 2B). The two exceptions to this trend (the single proline NPN group had a lower median VL than the PNP and NPP groups) indicated that there may be interactions between specific combinations of these prolines in their effects on viral load but the number of examples was too small to demonstrate such effects statistically.

Considering the effect of single proline changes, proline 119 (P119) CAs (the P group) were isolated more frequently from patients with low VL with a 4.9-fold difference in the median VL of P** group compared to the N** group ($p = 0.0205$; Fig. 2B).

Proline 159 (P159) had a stronger effect on VL with at least 6.1-fold difference in the median VL of the *P* group compared to the *N* group ($p = 0.0075$; Fig. 2B). This site is of special interest being within the highly conserved MHR (Fig. 1) [20], essential for virion assembly.

Proline 178 (P178) showed modest variation with VL with only 3.5-fold difference in the median VL of the **P group compared to the **N group ($p = 0.0709$; Fig. 2B,). However, the presence of P178 was linked to the other two prolines: all CAs with both P119 and P159 had P178 (i.e. the PPP group = the PP* group, Fig. 2B). This may be due to a p26 folding requirement and/or genetic linkage.

Stronger associations were observed when the positions were analyzed in combination. The median VL in subjects with PP* viruses differed from NN* viruses by at least 13.6-fold ($p = 0.0028$, Fig. 2B). Importantly, median VL in subjects with viruses having all three prolines (PPP) compared to those lacking a proline at the sites (NNN) differed by at least 18.8-fold ($p = 0.0013$, Fig. 2B).

There are practical difficulties of sequencing circulating RNA genomes, especially from patients with <100 copies viral RNA per ml of plasma. Sixteen of the 69 sequences were derived from proviral DNA (listed as DNA in Table 1) because multiple attempts to obtain RNA sequence failed. To test if proline/VL associations were biased by the inclusion of these proviral sequences, the analysis was repeated after excluding the data from these 16 samples. The association of higher VL with non-proline residues remained significant at positions 119 ($p = 0.0123$) and 159 ($p = 0.0043$), and for the combined positions 119 + 159 ($p = 0.0012$) and 119 + 159 + 178

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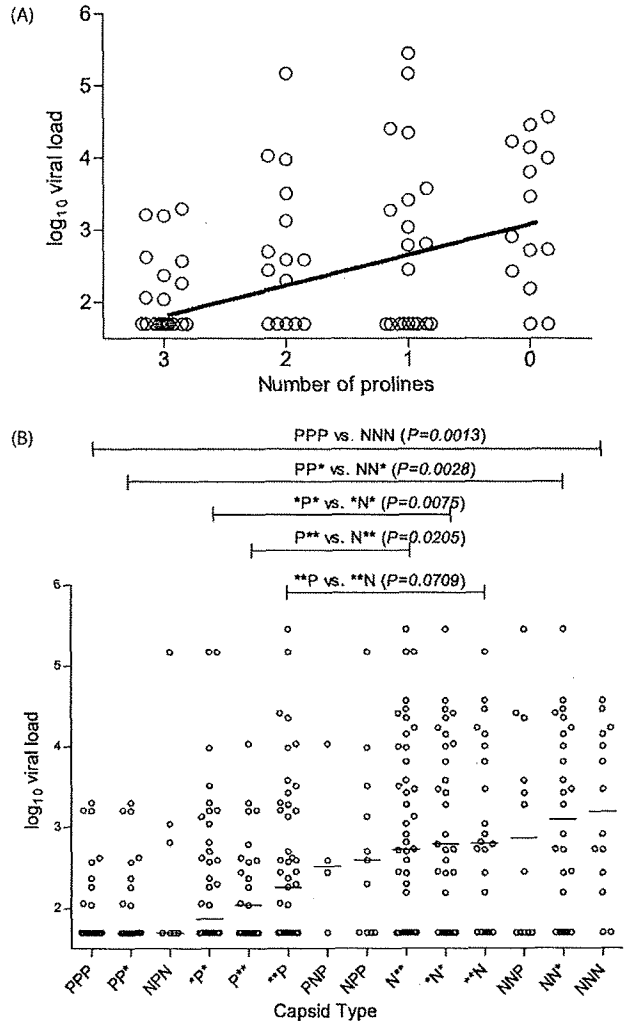

Fig. 2. HIV-2 VL correlates with amino acid variation at three p26 sites. (A) Relationship between VL and the number of prolines. Log-transformed VL for each sample was plotted as a function of the total number of prolines at p26 positions 119, 159 and 178. Tobit regression (Stata10, StatCorp TX, USA) was used to investigate the relationship between VL and the number of prolines at positions 119, 159 and 178. This form of regression is able to allow for censoring of viral loads below 100 in the dataset. The regression line was drawn with the equation $\log_{10}VL = 3.15 - 0.46 \times$ No. of prolines. (B) Relationship between VL and type of PPP motif. Log-transformed VL for each sample is plotted as a function of amino acid variation at p26 position 119, 159 and 178. Median VL are indicated by horizontal bars. The patients were stratified by the presence of proline (P) no proline residue (N) or any amino acid (*) at each of the positions 119, 159 and 178. For example, PPP = proline resides at positions 119, 159 and 178, NNN = no proline at the three positions. Comparisons of plasma VLs for different amino acid polymorphisms of particular interest were made using the non-parametric Mann–Whitney test (GraphPad Prism 5). *p*-values for these are shown above the figure.

($p = 0.0024$) (Table S1, lower panel). We conclude that independent of the origin of the sequences, there exists an association between low VL and proline residues at positions 119 and 159 independently, and with 119, 159 and 178 combined.

3.3. p26 CA variation influences susceptibility to TRIM5α

The TRIM5α was identified as a limit to cross-species retroviral infection [32–35]. This has led to a model of TRIM5α blocking retroviral infection by binding to the CA during entry, accelerating virus uncoating, and limiting subsequent steps in the infection process [32,34,36,37]. P119 was recently identified as a determinant of TRIM5α restriction [23] with HIV-2 CAs derived from TRIM5α sensitive viruses bearing a proline, and resistant strains having an alanine or glutamine at this site. It is possible that the reduced replication of the PPP viruses we observed in patients was part of the same phenomenon. Accordingly, the contribution of all three proline residues to TRIM5α restriction of replication was directly examined *in vitro*. Starting with the HIV-2 strain GH-123 as a PPP virus, P119, P159 plus P178, or all three prolines were altered to alanine or serine using site-directed mutagenesis. The growth of these variant viruses was compared in cells modified to express human TRIM5α; a parallel cell line expressing TRIM5α missing the SPRY domain, essential for p26 interaction, was used as a control to determine if these p26 changes altered virus replication independent of TRIM5α function [23]. Altering P119 (to produce APP) or P159 + P178 (to produce PSA) allowed 3-fold greater virus replication Fig. 3A. Alteration of all three prolines to ASA resulted in a 6-fold increase in virus replication. Thus the GH-123 variants displayed replication *in vitro* that closely mimic the behavior of the HIV-2 variants *in vivo*.

3.4. Phylogenetic analysis of p26 CA variation

One possible origin of the PPP form of p26 is that such a CA was encoded by a founder variant of HIV-2. The reduced growth of PPP viruses could be due to the p26 itself or to other shared and co-evolved features in these viruses. Alternately, changes at these three codons could occur more frequently. A phylogenetic analysis of Caio HIV-2 was performed to distinguish the PPP founder virus model from a multiple occurrence model. A founder effect with the appearance and spread of a PPP virus would appear as phylogenetic clustering of these variants. Ongoing selection for or against prolines in p26 would result in a phylogeny lacking PPP clustering. Because results derived from the 960 bp containing p26 coding could be dominated by variation in codons 119, 159 and 178, phylogeny was also inferred from a larger sequence spanning approximately 1300 bp of the envelope gene and including the highly variable V3 and V4 loops. The inferred phylogenies show that HIV-2 isolates encoding PPP p26 are distributed throughout the p26 and envelope trees (Fig. S1), supporting the conclusion that the occurrence of PPP p26 is not associated with a specific phylogenetic branch of Caio HIV-2 and is unlikely to be associated with a single occurrence of this CA motif. This conclusion is also supported by the high bootstrap values for some of the branches. These results are consistent with selection for and multiple appearance of the PPP p26 in the Caio population.

3.5. Modelling p26 CA sequence variation on structure and dimer formation

The bulky and constrained structure of proline strongly influences protein secondary structure; proline is inimical to alpha-helices and can kink otherwise flexible loops [38]. Thus polymorphisms involving prolines residues could alter the p26 structure. Using homology modelling, three-dimensional structures of six of the Caio HIV-2 p26 molecules (2 PPP, 2 ASA and 2 intermediate forms, APP and ASP) were constructed. The thermodynamically optimized 3-D structure models showed that the HIV-2 p26 consists of two packed core structures of N-terminal and C-terminal domains, a similar conformation to HIV-1 p24 [39]. Superimposition of the six HIV-2 p26 models showed that the overall 3-D structures of the variants were very similar with an exception: the amino acid substitution at position 119 from proline to alanine induced marked changes in the configuration of the loop between helices 6 and 7, as found previously with HIV-2 p26 N-terminal domain model [23]. In contrast, the substitution

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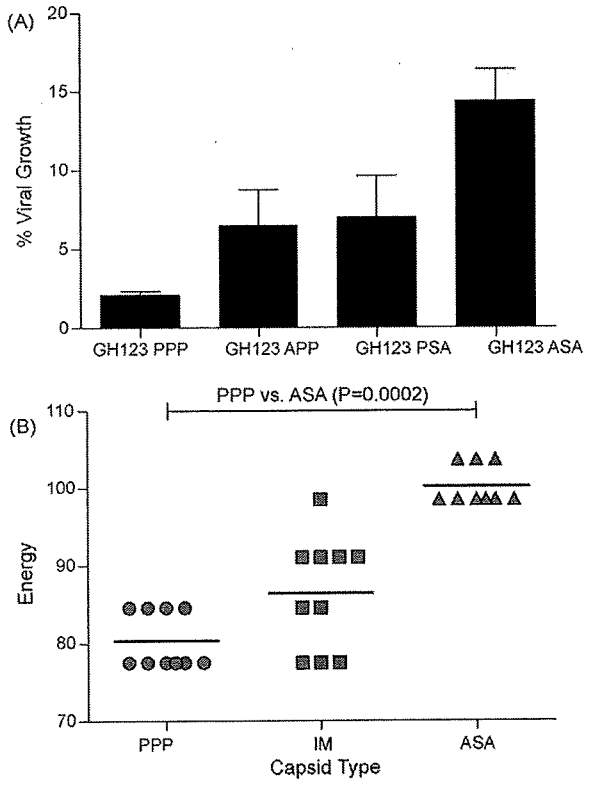

Fig. 3. HIV-2 capsid changes alter TRIM5α susceptibility and capsid stability. (A) Growth of HIV-2 GH123 (PPP) and its mutant viruses HIV-2 GH123/119A (APP), HIV-2 GH123/159S-178A (PSA), and HIV-2 GH123/119A-159S-178A (ASA) in the presence of human TRIM5α. MT4 cells (10^5) were infected with Hu-TRIM5α-SeV or CM-SPRY(-)-SeV at a multiplicity of infection of 10 plaque forming units per cell. 9 h after infection, the cells were superinfected with 20 ng of p26 of HIV-2 GH123, HIV-2 GH123/119A, HIV-2 GH123/159S-178A, or HIV-2 GH123/119A-159S-178A viruses. The culture supernatants were collected 6 days after infection, and the level of p26 was measured by using a RETROtek antigen ELISA kit (ZeptoMetrix Corp., Buffalo, NY). Ratios of HIV-2 CA levels of Hu-TRIM5α-SeV-infected cells to those of CM-SPRY(-)-SeV-infected cells are shown as percent growth. Error bars denote standard deviations in quadruplicate samples. A Kruskal–Wallis test for the entire data set clearly detected the difference of viral growth among those four viruses ($p = 0.006$). Furthermore, comparison of each mutant virus with GH123(PPP) using the Dunnett test also showed statistically significant differences of PPP vs. APP $p < 0.05$, PPP vs. PSA $p < 0.05$, and PPP vs. ASA $p < 0.01$. (B) Dimer binding energies as a function of capsid type. Samples were grouped into PPP, IM (intermediate, with proline at one or two of the three sites) or ASA, based on the amino acid at position 119, 159 and 178. The capsid dimer binding energies (absolute value) for each sequence were determined by homology modelling (see Section 2); the mean values for each group are indicated. Comparison between the PPP and the ASA group using the non-parametric Mann–Whitney test (GraphPad Prism 5) provided the indicated *p*-value.

at positions 159 or 178 induced no major changes in the main-chain backbone of HIV-2 CA (data not shown), suggesting that the structure of the CA can accommodate alternative residues at these sites.

Positions 159 and 178 are located in the C-terminal portion of p26 required for dimer formation as well as virion shell assembly [18]. Homology modelling of the p26 dimer was used to calculate binding energy for dimer formation. Since the C-terminal sequences used to prepare the initial six structures were common to a larger set of p26 sequences, dimer binding energies for 29 of the Caio CAs could be calculated. These calculations revealed that the PPP p26 dimers had weaker binding energies, the ASA dimers had stronger binding energies and the intermediate forms with only one or two of the prolines altered (IM) had intermediate binding energies (Fig. 3B). In addition, the viral loads of the 29 patients were modestly correlated with the absolute values of the binding energy of the viral CA ($r_S = 0.383$, $p = 0.040$). We conclude that the amino acid changes at positions 159 and 178 influence p26 dimer stability, with ASA CA dimers having a higher stability than PPP or APP CA dimers and the increased stability may be related to elevated VL in these patients.

4. Discussion

This study represents a detailed examination of HIV-2 p26 sequence variation within a set of 69 sequences isolated from subjects with both high and low VL. Disease progression after HIV-2 infection is highly dependent on VL [40] with subjects who control HIV-2 replication continuing to do this over a period of many years, suggesting that virus-host interactions result in a stable set-point of virus replication. The current study identified three sites of p26 variation correlating with VL. This study reveals a previously undescribed pattern of variation in the highly conserved p26 and indicates that the outcome of HIV-2 infection is partially predicted by the form of the p26 carried by the virus. In addition to confirming the importance of P119 as a determinant of TRIM5α restriction, the current study identified two additional amino acid positions (159 and 178) whose identities correlate with virus load. Although these associations were not significant after stringent adjustment for multiple comparisons, HIV-2 encoding p26 specifically modified at these three positions showed *in vitro* replication levels consistent with the *in vivo* VL data and further supported the conclusion that the three residues 119, 159 and 178 are important determinants of virus growth and influence TRIM5α restriction (Fig. 3).

Virus replication *in vivo* is influenced by a large number of host and viral factors and it would be naïve to conclude that these three residues are the sole determinants. That additional factors influence the course of infection is reflected in the VL data and the exceptions from the pattern (e.g. the three PPP viruses with greater than 10e3 VL and the 2 NNN viruses with undetectable VL). However, the power of such a population study is that patterns of HIV-2 behavior appear when large numbers of infection are monitored. As shown in Fig. 2, the substantial and statistically significant change in VL that accompanies the variation from PPP to NNN viruses strongly supports our conclusions that this p26 motif is an important determinant of the course of infection.

How might the PPP motif function? Our data support a destabilization of the CA by the three proline residues. P119 may directly form a recognition signal for TRIM5α binding, and the three proline residues may result in less tightly packed core that is more readily dismantled and processed after TRIM5α recognition. Our preliminary immunological studies show that patients with PPP virus mount stronger T cell responses to p26 and to the entire HIV-2 proteome (A.L., S.R.J. unpublished results) and this increased immune exposure might be a consequence of TRIM5α recognition and more efficient antigen presentation.

These results are consistent with TRIM5α restriction playing a direct role in limiting HIV-2 replication and a more indirect role in enhancing the immune response to the virus. The *in vitro* studies (Fig. 3A), although using manipulated cells and monitoring virus replication only over a short period of replication, demonstrated that variation in these three CA residues influence the susceptibility of HIV-2 replication to TRIM5α. *In vivo*, it is likely that TRIM5α effects are both manifested over multiple rounds of infection and TRIM5α may cooperate with other processes such as the adaptive immune response; *in vitro* cell culture conditions and growth in immortalized cell lines are unlikely to fully recreate these processes. We believe that our *in vivo* virus load data are the strongest support for the hypothesis that the PPP motif modulates VL.

If our hypothesis is correct, incident infections in Caio, infected patients that progress to require anti-retroviral therapy and moth-

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