Minor contribution of HLA class I-associated selective pressure to the variability of HIV-1 accessory protein Vpu

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ABSTRACT

Host HLA class I (HLA-I) allele-associated immune responses are major forces driving the evolution of HIV-1 proteins such as Gag and Nef. The viral protein U (Vpu) is an HIV-1 accessory protein responsible for CD4 degradation and enhancement of virion release by antagonizing tetherin/CD317. Although Vpu represents one of the most variable proteins in the HIV-1 proteome, it is still not clear to what extent HLA-I influence its evolution. To examine this issue, we enrolled 240 HLA-I-typed, treatment naïve, chronically HIV-infected subjects in Japan, and analyzed plasma HIV RNA nucleotide sequences of the vpu region. Using a phylogenetically-informed method incorporating corrections for HIV codon covariation and linkage disequilibrium among HLA alleles, we investigated HLA-associated amino acid mutations in the Vpu protein as well as in the translational products encoded by alternative reading frames. Despite substantial amino acid variability in Vpu, we identified only 4 HLA-associations in all possible translational products encoded in this region, suggesting that HLA-associated immune responses had minor effects on Vpu variability in this cohort. Rather, despite its size (81 amino acids), Vpu showed 103 codon–codon covariation associations, suggesting that Vpu conformation and function are preserved through many possible combinations of primary and secondary polymorphisms. Taken together, our study suggests that Vpu has been comparably less influenced by HLA-I-associated immune-driven evolution at the population level compared to other highly variable HIV-1 accessory proteins.

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1. Introduction

Immune-mediated adaptation occurs during an HIV-1 infection. The HLA class I (HLA-I)-restricted CD8^{+} cytotoxic T lymphocyte (CTL) response is one of the major forces driving HIV evolution, resulting in the selection of CTL escape mutants [1,2]. Despite the extensive genetic diversity of both HIV-1 and HLA-I alleles, escape pathways are reproducible and broadly predictable based on host HLA-I alleles [3–6]. Moreover, analysis of linked HLA-I and HIV datasets from large cohorts of HIV-infected subjects has facilitated our ability to map the landscape of immune escape mutations across HIV-1, identify immunogenic regions, and identify novel CTL epitopes [3,7].

Viral protein U (Vpu) is an accessory protein that is unique to HIV-1 and a subset of related simian immunodeficiency viruses.

The HIV-1 Vpu protein has two major functions: degradation of newly synthesized CD4 molecules in the endoplasmic reticulum and enhancement of the release of progeny virions from infected cells by antagonizing tetherin/CD317, a host restriction factor that directly binds and retains viral particles on the surface of infected cells (reviewed in [8,9]). As such, Vpu is thought to play a role in virus spread and pathogenesis in vivo. Interestingly, Vpu is the most variable protein among all HIV proteins as evidenced by a cross-sectional comparison of HIV-1 sequences isolated from HIV-infected individuals [10], raising the possibility that Vpu undergoes adaptation in response to host immune responses. However, Vpu has been shown to be a minor target for CTLs as revealed by IFN-γ Elispot assays with overlapping peptides based on the subtype B consensus sequence [11]. Considering the highly variable nature of Vpu, it is possible to miss responses if the autologous virus sequence is markedly different from the peptide sequence when using this Elispot assay system [12].

In the present study, we sought to identify HLA-associated polymorphisms in Vpu and alternate reading frames and examine to

what extent they are involved in Vpu amino acid variability at the population level. We utilize a published phylogenetic dependency network model [13], a comprehensive evolutionary model that considers all important confounding effects such as HIV phylogeny, HIV codon covariation, and linkage disequilibrium of HLA alleles.

2. Materials and methods

2.1. Patient samples

A total of 240 chronically HIV-1-infected, treatment-naïve subjects (CD4, median 237; IQR, 160-397; viral load, median 33,200; IQR, 222,000-55,400) followed at the AIDS Clinical Center, International Medical Center of Japan were enrolled in this study. All participants provided written informed consent. HLA-I typing was performed as previously described [14]. The most frequently observed *HLA-A, B*, and *C* alleles in this cohort were *HLA-A*24:02*, *HLA-B*52:01*, and *HLA-C*01:02*, respectively, consistent with HLA class I allelic frequencies of the Japanese people [14].

2.2. Sequence analysis of vpu

HIV-1 particles were precipitated by ultracentrifugation (50,000 rpm, 15 min) of patients' plasma, after which the viral RNA was extracted using standard methods. Following reverse transcription, DNA fragments encoding Vpu proteins were amplified by nested PCR, and gel purified as previously described [15,16]. The primers used were as follows: the primers for the first round of amplification were VVVa-F (5′-TTAAAAGAAAAGGGGG GATTGGGGG-3′) and VVVb-R (5′-ATTCCATGTGTACATT GTACTGT-3′); and those for the second round, VVVc-F (5′-AGATAATAGTGAC ATAAAAGTAGTGCCAAGAAG-3′) and VVVd-R (5′-CCATAATAGACT GTGACCCACAA-3′). The *vpu* sequence was then directly analyzed with an automated sequencer (Applied Biosystems 3500xL) and aligned to the *vpu* sequence of the HIV-1 subtype B reference strain HXB2 (Accession No. K03455). More than 90% of the subjects were infected with subtype B, as determined by phylogenetic analysis of concatenated sequences of *vif, vpr*, and *vpu* reading frames.

2.3. Analysis of amino acid sequence variability

A Shannon entropy score for each position in the Vpu protein was calculated and used to analyze amino-acid sequence variability, as described previously [10]. Entropy is a measure of the amino acid variability at a given position that takes into account both the number of possible amino acid residues allowed and their frequency.

2.4. Analysis of association between Vpu sequence polymorphisms and host HLA class I alleles

To identify HIV-HLA polymorphism associations, we employed a phylogenetically dependency network model [13], which comprehensively includes all confounding effects of the analysis, such as HIV founder effects, HIV codon co-variation, and linkage disequilibrium of HLA-I alleles. Multiple comparisons are addressed using q-values (refer the detailed methods given in refs. [4,5,13]); in the present study, a cutoff of $q < 0.2$ was used to denote statistically significant associations. HLA-associated polymorphisms were classified into two categories. "Nonadapted" amino acids are enriched in the absence of the restricting HLA of interest. Usually, "nonadapted" forms represent the consensus amino acid at that position, and they can be thought of as the "wild-type" or "susceptible" form particular to that allele. Conversely, "adapted" amino acids are those enriched in the presence of the HLA allele;

these can be thought of as the escape variant particular to that allele.

3. Results and discussion

3.1. Genetic variability of the vpu gene

We successfully amplified DNAs encompassing the *vpu* region from 216 of 240 samples (90%). Firstly, we analyzed the amino acid variability at each codon of Vpu by determining its Shannon entropy score. Two amino acid residues, Trp23 and Arg49, showed highly conserved (>98%) among individual sequences. Instead, most codons displayed substantial variability, with the average of the entropy score reaching 0.58 (Fig. 1A), confirming the findings by Yusim et al., which showed that Vpu is a highly variable protein [10]. Also, the amino acid variability of each codon in the present study correlated strongly with that of published subtype B sequence data from the Los Alamos database (Fig. 1B), suggesting that our observed pattern of amino acid variation in Vpu was generally representative of the variation observed in HIV-1 subtype B. In fact, the consensus amino acid sequences of subtype B and the present dataset were identical except for 3 amino-acid residues: positions 3, 5, and 24 (Fig. 1C). These amino acid residues were highly variable (Fig. 1A) and not directly associated with known Vpu functions (Fig. 1C).

3.2. HLA-associated polymorphisms in Vpu

As HLA-I-mediated selective contributes to HIV-1 sequence variability, especially the accessory protein Nef [4], we sought to examine whether HLA-I-mediated selective pressure substantially influenced the evolution of Vpu, another accessory protein. We applied a phylogenetic dependency network model [13], which adjusts for the confounding effects of HIV phylogeny, HIV codon covariation and linkage disequilibrium of HLA-I alleles.

In our dataset of 216 individuals, we identified only three HIV-HLA associations in Vpu: a nonadapted association between C*03 and Glu-5, a nonadapted association between A*33:03 and Arg-37, and an adapted association between A*33:03 and Lys-37. The presence of both nonadapted and adapted A*33:03-associated polymorphisms at Vpu codon 37 is consistent with an Arginine-to-Lysine escape mutation occurring at the C-terminus of the immunodominant HLA-A*33:03-restricted epitope in Vpu, [29]EYR-KILRQR[37] [11]. However, there was no HLA-restricted T cell epitopes around Vpu position 5 have been reported. Although we might have missed some polymorphisms due to the limited sample size in this study, these data suggest that HLA-I-mediated selective pressure toward Vpu does not substantially drive Vpu variability at the population level in this cohort.

3.3. HLA-associated polymorphisms in alternating reading frames

CTLs can recognize epitopes encoded by alternate reading frames including the antisense-strand sequences of HIV-1 *gag, pol*, and *nef* [17,18]. Therefore, we also investigated HIV-HLA polymorphism associations in peptide sequences encoded by alternative reading frames of the *vpu* gene. We observed no statistically significant HLA-associated polymorphisms in alternate or antisense reading frames, except for a single HLA-B*40:01 associated "adapted" lysine polymorphism at codon 2 of the overlapping Envelope reading frame which is initiated in the middle of the *vpu* gene (ORF + 2; Table 1, Fig. 2). Although this association was between Lys-2 of Env and *HLA-B*40:01*, no CTL epitopes have been reported in the context of HLA-B*40:01 in this region. Using bioinformatic prediction programs Epipred [19] and BIMAS [20] we
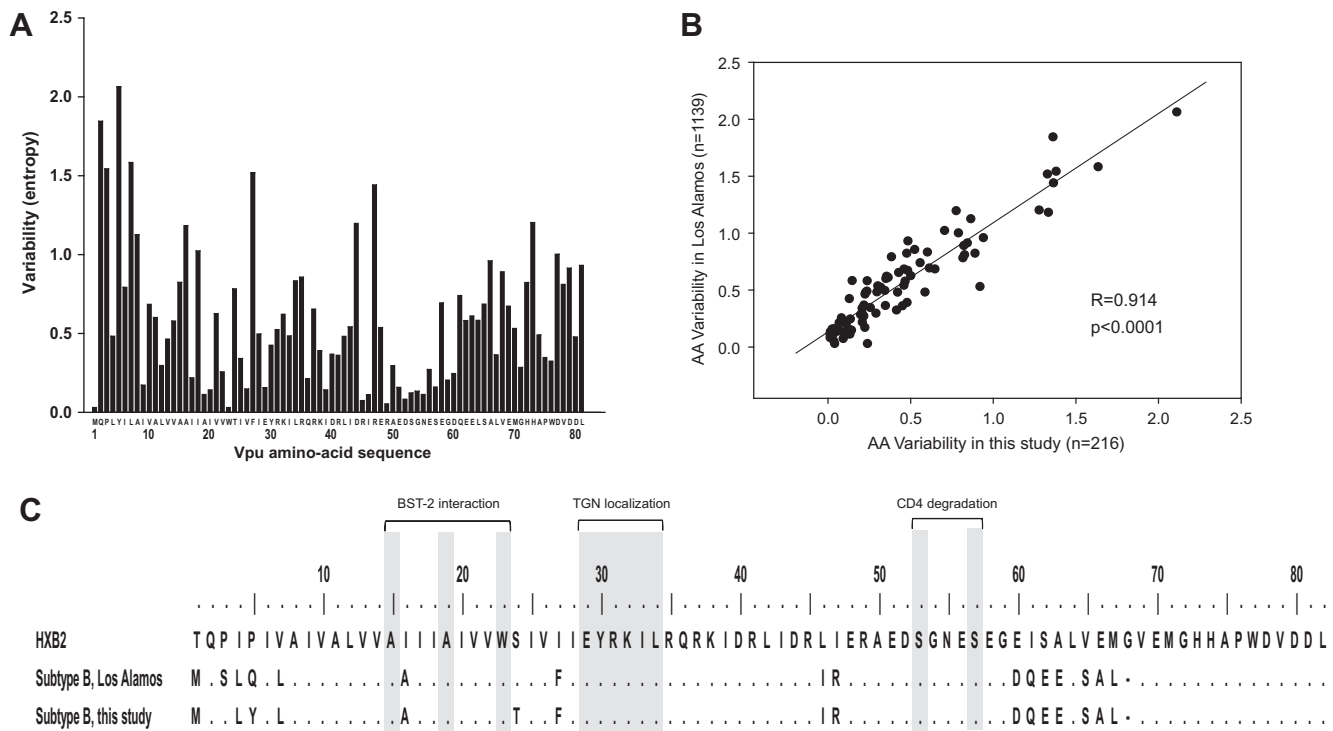
Fig. 1. Variability of the amino acid residues of HIV-1 Vpu. The amino acid sequence of Vpu was analyzed based on the cross-sectional studies on 216 HIV-infected subjects. The amino acid variability at each position of Vpu was analyzed by determining its Shannon entropy score (panel A). The Vpu sequence (subtype B, $n = 1139$) was retrieved from the Los Alamos HIV sequence database, analyzed for its amino acid variability, and compared with subtype B obtained from this study using Spearman Rank Order Correlation (panel B). The consensus sequences of Vpu obtained from Los Alamos database and this study were aligned with reference strain HXB2 and regions responsible for some key Vpu functions highlighted (panel C).

Table 1
Summary of HIV-HLA associations in the Vpu-encoded region.

| RF	Protein	Pos HXB2	aa	HLA	Association	p-Value	q-Value	Known epitope	
								Sequence	Reference
+1	Vpu	5	E	C*03	Nonadapted	2.13×10^{-5}	1.52×10^{-1}	none	–
		37	R	A*22:03	Nonadapted	3.40×10^{-6}	5.50×10^{-2}	²⁹EYRKILRQR³⁷	[11]
		37	K	A*33:03	Adapted	2.80×10^{-5}	1.52×10^{-1}	²⁹EYRKILRQR³⁷	[11]
+2	Env	2	K	B*40:01	Adapted	1.63×10^{-5}	1.67×10^{-1}	none	–

RF, reading frame; Pos HXB2, amino acid position when aligned to HXB2 sequence.

Fig. 2. The Vpu and a part of Env proteins and their associations with host HLA class I alleles. The nucleotide sequence and its deduced amino acid sequence of Vpu and of an overlapping part of Env with reference to the subtype B consensus sequence of Los Alamos database is shown. The amino acid residues associated with the indicated HLA class I alleles ($p < 0.05$, $q < 0.2$) are shown with adapted (red) and nonadapted (blue) residues. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

attempted to predict B*40:01-restricted CTL epitopes, but found none (data not shown). This failure is most likely due to the presence of several basic amino acids, such as Arg and Lys, in this region of Env (Fig. 2), as it has been shown that HLA-B*40:01
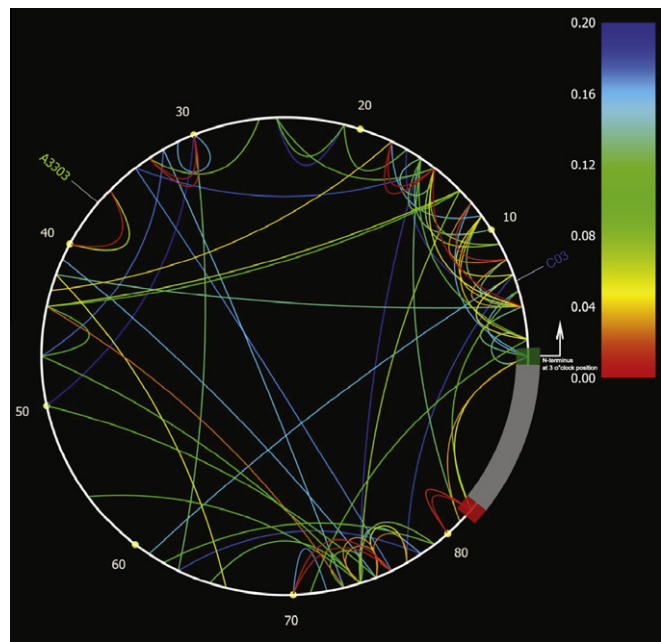
Fig. 3. Amino acid codon–codon covariation in Vpu. The circular map, generated by the PhyloDv software [13], shows Vpu codon–codon covariation associations as inner arcs connecting the association sites, with the HLA associations as tags pointing to their corresponding sites. Q values of individual codon pairs are represented as a heat map shown at the right.

preferentially binds peptides with acidic residues at their anchors [21]. This issue needs to be clarified in further studies using immunologic assays. Taken together, our results suggest that HLA-I-mediated selective pressure do not contribute to a large extent to population-level sequence variation in HIV-1 Vpu.

3.4. Codon–codon covariation of Vpu

Given that Vpu is functionally important in viral replication *in vivo*, the highly variable nature of Vpu amino acid sequences could be explained by complex networks of codon–codon covariation and/or secondary/compensatory mutation pathways. We therefore examined the codon–codon covariation of Vpu by using the phylogenetic dependency network model. Although Vpu consists of only 81 amino acids, we identified 103 covarying codon pairs in Vpu, displayed in Fig. 3. The covariation network in Vpu showed an uneven distribution, with a large number of codon–codon covariation networks at the N-terminal membrane-spanning region, a region responsible for BST-2 interaction [22]. Interestingly, the 3 HIV-HLA associations (Table 1, Fig. 2) were not significantly linked to any other amino acid residues. These data suggest that the conformation and function of Vpu may be preserved through many possible combinations of primary and secondary polymorphisms and that the HLA-I-associated immune-mediated selective pressure may have only a minor effect on such Vpu polymorphisms.

3.5. Association between Vpu polymorphisms and clinical parameters

Finally, we explored associations between Vpu polymorphisms and clinical parameters of HIV-infected patients (i.e., CD4 counts and plasma viral load). We observed no significant associations between Vpu polymorphisms and CD4 counts. However we identified a statistically significant association between amino acid residues at position 5 and viral load (Fig. 4). The patients harboring Val at Vpu-5 had significantly higher viral loads compared to those with amino acid residues other than Val at this position. Thus, amino acid residues at position 5 of Vpu showed several interesting features, i.e., the highest variability of all Vpu amino acids (Fig. 1A), nonadapted association of Glu-5 with *HLA-Cw*03*, and association of Val-5 with the increased viral load. Considering that the amino acid residue at this position is located in close proximity to the membrane-spanning region and that this region is functionally important for BST-2 binding, it would be interesting to examine functional effects of amino acid polymorphisms at position 5, whether they are mediated by host immune responses or otherwise.

In summary, we report here comparably fewer HLA-associated mutations in Vpu in this cohort although host HLA class I allele-associated immune responses are major forces driving the evolution of HIV-1 accessory proteins, such as Nef. Taken together, we conclude that the influence of immune selection on evolution of Vpu
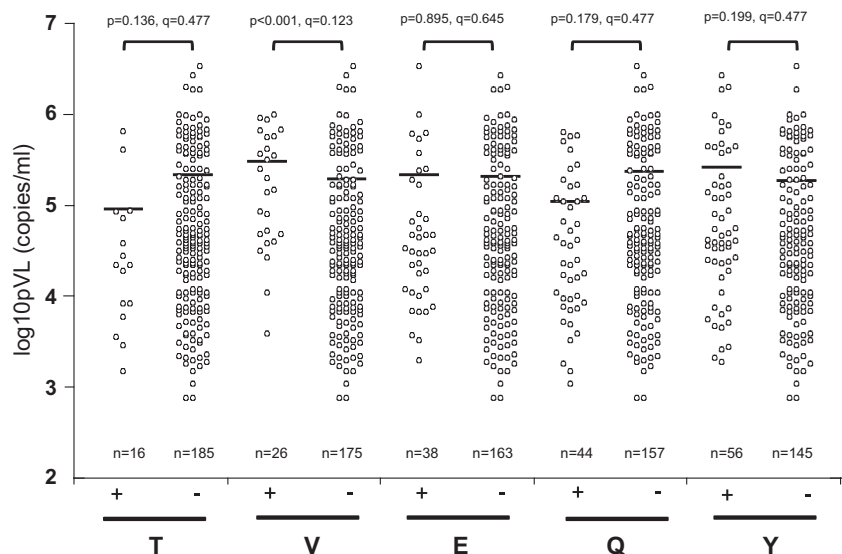
Fig. 4. Association between plasma viral load and amino acid polymorphism at position 5 of Vpu. HIV plasma viral loads, stratified by amino acid expression at Vpu codon 5, are shown. Vpu codon 5 exhibited 11 different amino acids positioning in our dataset; only those observed in >10 patients are shown here. Horizontal bars indicate medians. Statistical analysis was performed using the Mann-Whitney U-test.

at the population level may be reduced compared to other highly variable HIV-1 proteins, providing us with additional insight into differential evolutionary pathways among viral accessory proteins.

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