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Elevated hypermutation levels in HIV-1 natural viral suppressors☆

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Abstract

Mutations in the HIV-1 proviral genomes delay the progression of the disease. We compared the mutation status in full-length proviral genomes of 23 HIV-infected patients with undetectable viral loads in the absence of therapy named natural viral suppressors (NVS) or Elite Controllers with 23 HIV-infected controls (10 patients on HAART treatment and 13 untreated patients). Provirus DNA was extracted from PBMC for amplification and sequencing to determine the mutation status. Nine (39 %) of the 23 NVS patients had defective proviral genomes, compared to 4 of the treated controls (40%, p=0.96) and only one of the untreated controls (8%, p=0.059). Most of the defective genomes resulted from Gto-A hypermutation. Among patients with hypermutation, the rate ratio for mutation was significantly higher for the NVS compared to treated controls (p=0.043). Our data suggests that inactivation of the virus through the APOBEC3G system may contribute to the NVS phenotype.

Keywords

HIV-1; Natural viral suppressors (NVS); Hypermutation; Elite controllers

Introduction

In recent years, there have been important advances in the understanding of innate immunity and the role it plays in the control of HIV-1 infection. Various patterns of disease progression have been associated with host genetics, immunological and virological factors (Grabar et al., 2009; Okulicz et al., 2009). A subset of HIV-infected patients who are able to suppress circulating virus naturally, without the use of anti-retroviral drugs, have been studied extensively. Often referred to as “Elite Controllers” or “Elite Suppressors,” these individuals have very low to undetectable plasma HIV-1 RNA levels and relatively normal CD4+ T-cell counts (Wang et al., 2003; Walker, 2007; Sajadi et al., 2007; Blankson et al.,

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Authors’ contributions

LME carried out all the molecular genetic assays, sequence editing, sequence analysis, statistical analysis, and drafted the manuscript. MMS and JKC conceived the study, participated in its design and coordination, and helped to draft the manuscript. MC performed statistical analysis. RRR, WAB participated in the study design and manuscript oversight. All authors read and approved the final manuscript.

2007). In our Baltimore patient population, this phenotype is present in 1.5% of all HIV-1 seropositive individuals and they are referred to as “natural viral suppressors”, or “NVS” (Sajadi et al., 2009). Thus far there has been an intensive effort by us and others to identify the mechanisms by which these individuals suppress their virus and multiple host factors have been identified (Okulicz et al., 2009; Hunt, 2009; Salgado et al., 2010; Baker et al., 2009; Blankson, 2010).

In addition to host factors, viral factors that might be associated with the NVS phenotype have been investigated. In a recent study, viruses from controllers displayed significantly lower replication capacity compared to those from progressors ($p < 0.0001$) (Brumme et al., 2011). Others suggest that inhibitory mutations in *vpr, nef*, and *rev* could be associated with the NVS phenotype (Lum et al., 2003; Mologni et al., 2006; Iversen et al., 1995; Malim et al., 1991; Wang et al., 2003; Tolstrup et al., 2006; Caly et al., 2008). It has also been suggested that some individuals are infected with virions that are less capable of evading the host immunological response or are impaired. One common cause of impairment is due to viral genomes that contain APOBEC3G (A3G) or APOEC3F (A3F) induced hypermutation (Wang et al., 2003; Sandonis et al., 2009; Gandhi et al., 2008). In addition, mutations in epitopes for B27, B57, B58 recognition and the number of NFkappaB sites have also been correlated with long-term control (Migueles et al., 2000; Bailey et al., 2007; Goulder et al., 2001).

The current study investigates this unique group of NVS individuals to test the hypothesis that durable viral suppression may be caused in part by viral inactivation. Using both partial and full genome sequencing, others have found no significant mutations or deletions in the viruses with which these patients are infected (Blankson et al., 2007; Gandhi et al., 2008). This study presents 23 nearly full genome sequences of NVS patients, spanning most of the viral genome, for examination and comparison with genomes from two control groups. The control groups consist of 10 patients with undetectable viral loads on HAART treatment and 13 untreated patients with detectable viral loads.

Results

The NVS group consisting of 23 patients were 57% males, all African American, between the ages of 30 and 62 (mean 49.6; SD 9.1), with injecting drug use (IDU) as the main risk factor (57%), and viral loads ranging from <40 to 475 copies/mL. The control group on HAART consisting of 10 patients were 80% males, mostly African American (80%), between the ages of 27 and 52 years old (mean 39.5; SD 10.0), with heterosexual sex as their main risk factor (90%), and viral loads of less than 50 copies/mL. The treatment naïve controls consisting of 13 patients were 62% males, all African American, between the ages of 30 and 58 (mean 42.4; SD 9.0), with IDU as their main risk factor, and viral loads ranging from 2630 to <750,000 copies/mL (Table 1). Phylogenetic analysis of all 46 nearly full-length proviral genomes along with reference subtypes showed that all NVS all control are infected with subtype B, HIV-1 virus (phylogenetic tree not shown). A visual representation of the NVS samples, the controls, reference for subtype B (Ref.B. MN.M17449, Ref.B.FR. 83.HXB2, and Ref.B.US.AY331295) is shown in Fig. 1 where it can be observed that two control samples (07US.SAJ. C162.H2 and 06US.SAJ.C167.LH) are phylogenetically linked. No known epidemiological linkage was identified: both were female subjects who reported heterosexual sex as their risk factor.

From the nearly full-length sequences, we defined defective proviral genomes as having significant hypermutation rate ratio or having defective genes. Nine (39%) of 23 NVS patients had clearly defective proviral genomes based on sequence, compared to four (40%) from the control group on treatment (p=0.96) and only 1 (8%) from the untreated control

group (p=0.059). Most of the defective proviral genomes (78.6%) resulted from G-to-A hypermutation. There were no differences in the proportion of patients with hypermutations between NVS (26%), treated controls (40%), and untreated controls (8%). However, among patients that did have hypermutations, the median rate ratio for hypermutation was significantly higher for NVS compared to treated controls (4.87 vs. 3.11, Wilcoxon's two sample test p=0.043, respectively). The mean values of the hypermutation rate ratio were also comparable to the median values and were also significantly different between the two groups (4.84 vs. 3.07, p=0.029, respectively). Overall, the majority of the hypermutated genomes observed in all three groups were in an APOBEC3G context (G-to-A mutations in a GG context) with a hypermutation ratio ranging from 2.09 to 7.04 (Table 1).

In addition to hypermutation, proviruses were also defective due to frame shifts, incorrect start codons, and deletions. In the NVS group, all six hypermutated samples had incorrect start codons in several genes (ATG to ATA) and two of them also had frameshift mutations in the *nef* gene. Additionally, the NVS group included three samples that were clearly defective based on sequence alone; they had significant deletions that caused frameshifts in essential genes (Table 1). The controls on HAART also showed similar defects: all four hypermutated samples had incorrect start codons at different genes and two of them also had frameshifts in the *nef* gene. The one untreated control that was hypermutated had an incorrect start codon in the *nef* gene; however, about 44% of the genome was not hypermuted and it had an APOBEC Ratio of 3.4, a value lower than all other hypermutated samples (Fig. 2). In this paper, we define APOBEC Ratio as the total sum of the GG to GA substitutions, which are caused by APOBEC3 activity, in relation to GC to GT substitutions, which are background mutations. Hypermutation throughout the genomes of both control groups was notably lower than the NVS group, as can be observed on Table 1: not all genes were hypermutated. The naïve control group had the least number of hypermutated genes compared to the NVS group (mean number of 0.4 vs. 2.4, respectively, p=0.03). There was no difference in the mean number of hypermutated genes between naïve controls and controls on HAART. The degree of hypermutation can also be observed in Fig. 2: the mean APOBEC Ratio for the NVS group was 3.1 (SD, 3.0). The untreated controls had significantly lower APOBEC ratios than the NVS group (mean ratio of 1.7 vs. 3.1, respectively, p=0.04) but marginally lower when compared to the controls on HAART (mean ratio of 1.7 vs. 2.8, respectively, p=0.09).

In the literature, various mutations have been identified and associated with the long term non-progressors phenotype (Poropatich and Sullivan, 2011; O'Connell et al., 2010; Bailey et al., 2007; Deeks and Barre-Sinoussi, 2012). The following such mutations were found not to be associated with the NVS phenotype in this study: *vpr R77Q* and *F72L, rev L78I*, and *nef T138Q*. The number of NF-kappaB sites was not significantly different between the groups. Epitopes recognized by HLA Type I haplotypes, B27, B57 and B58, were identified in the NVS group but there was no significant enrichment for these. The diversity of *env* was analyzed, specifically the length and potential N-linked glycosylation sites of the V1–V2 loop and they were not found to be significantly different between the groups (Mens et al., 2010). Analysis of the pairwise genetic distances in each population revealed that the NVS patients' pairwise genetic distance over the entire genome was 7.0% (± 0.002%) compared to 9.2% (± 0.002%) among the controls. No significant differences were identified between the two control groups.

Discussion

The use of nearly full genome sequencing enabled the entire HIV genome to be characterized; therefore, defective viruses of multiple types could be detected. In this study, the NVS group had more defective genomes than the untreated HIV cohort, while no

difference was seen between the NVS and the control group on HAART. However, the degree of hypermutation was much greater in the NVS than the two control groups.

In the human host, the gene apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like editing complex 3 (APOBEC3) specifically targets retroviruses. This family of cytidine deaminases, specifically APOBEC3G (A3G) and APOBEC3F (A3F), was previously found to be responsible for hypermutation in HIV-1 (Harris and Liddament, 2004; Lecossier et al., 2003). In the absence of virally encoded virus infectivity factor (*Vif*), the A3G protein is incorporated into virions and causes hypermutation usually during reverse transcription of the genome (Liu et al., 2005). Defective viral genomes result from this, due to GG-to-GA mutations of the HIV-1 proviral DNA (Liu et al., 2005). Via a similar mechanism, A3F mediates GA-to-AA mutations but at a rate that is 10 times lower than that of A3G (Liu et al., 2005; Holmes et al., 2007).

Individuals able to control virus, whether by treatment or without, have a higher proportion of the proviral genomes that are defective. In chronic HIV infection, the proviral reservoir, which is stable in number, is in equilibrium between the number of newly infected versus dying cells, each of which may harbor defective or functional provirus. Previous studies have shown that patients harboring hypermutated sequence had viral loads significantly lower than those with nonhypermutated sequence (Pace et al., 2006).

In this study, 40% of HAART treated controls and 26% of NVS had evidence of hypermutation compared to 8% (or one) of the controls not on treatment. Even this one untreated control may be an exception: this individual not only had a relatively low viral load (2630 copies/mL) but was also infected with a virus that had a lower hypermutation rate ratio, lower APOBEC ratio, and about half of the genes analyzed were not hypermutated. The finding of a higher rate of defective viruses in patients with lower viral load suggests that the ratio of defective to functional proviruses increases when the viral load is lowered by therapy or the host immune system.

Although patients with low viral load (NVS or HAART treated) have a higher degree of hypermutation than naïve controls, the level of hypermutation was significantly higher in the NVS compared to the HAART group. The degree of hypermutation observed in the NVS samples and their low genetic diversity suggests that one possible mechanism for this phenotype is inactivation of the virus at initial infection by blocking HIV-1 replication directly or through inactivation by hypermutation.

Interestingly, while patients similar to the NVS are known to have higher viral loads than HAART treated patients (Hatano et al., 2009), in this study NVS individuals had more defective genomes than the treated controls (Table 1). Although this result was not statistically significant, it suggests that the number of circulating virus may not be the only factor affecting the prevalence of defective viruses, and other factors such as length of HIV infection, duration of therapy/viral control may also be important. In addition, for individual NVS patients, either infected with a defective virus or with strong host immunity may lead to the observation of frequent defective genomes.

One limitation of our study is that our technique of DNA extraction includes both integrated and unintegrated DNA. Although Elite Controllers have been shown to have large amounts of unintegrated DNA compared to integrated DNA (Graf et al., 2011; Buzon et al., 2011) mutations into the genome occur at reverse transcription (before this stage). Post-reverse transcription mutations are known to occur at the junction of the 2-LTR circle (Whitcomb et al., 1990; Pauza, 1990), but this would have no bearing on env, gag, and pol. Furthermore, it has been shown that sequences derived from PBMC-DNA (integrated and unintegrated) are phylogenetically similar to sequences derived from cell-free virus in serum (unintegrated)

when obtained from the same time point (Edo-Matas et al., 2010). Another limitation to our study is the number of NVS patients and the number of sequences analyzed. Patients who are able to suppress circulating virus naturally are rare; thus, establishing a cohort with a large number of these unique subjects is very difficult. The very low number of HIV-1 proviral DNA in the NVS patients is an obstacle in obtaining full-genome amplification. It has been reported that the lower boundary for full-genome amplification is around 300 proviral DNA copies per PCR reaction (Simmonds et al., 1990b). The proviral DNA previously reported in our NVS patients ranges from 1 to 74 copies per 10\widehat6 PBMC (Sajadi et al., 2007). Thus, the NVS patients in our study had such small quantities of proviral DNA that prevented us from replicating studies where they ran large number of PCR reactions per sample such is the case of single-genome amplification. The single-genome amplification method is widely used to identify founder viruses and viral populations. Using this method DNA is titrated by end-point dilution where a DNA concentration yields no more than 30% positive PCRs this is conformed to a Poisson distribution where a single template is present per reaction (Keele et al., 2008; Salazar-Gonzalez et al., 2008, 2011; Simmonds et al., 1990a). As mentioned above we were restricted to smaller number of PCR reactions since our target (9000 kb fragment) requires more DNA than PCR of small fragments. However, only about 1–2% of all reactions performed per sample resulted in a positive PCR. Due to the complexity of amplifying a 9000 base fragment and the 1–2% positive PCR success rate our sequences should be representative of the provirus population within each patient sample.

Method

Study subjects

The enrollment and data collection procedures for subjects in this prospective study have been described in detail elsewhere (Sajadi et al., 2007, 2009). Briefly, the case definition is as follows: patients must be drug naïve, be confirmed HIV-1 positive by Western blot and proviral DNA, and have viral loads of <400 copies per milliliter for a minimum 2-year period (one viral load >400 copies/mL in a 2-year period was allowed). The median of years of HIV-1 viral suppression was 6.7 (Sajadi et al., 2009). Two control groups of HIV-1 infected individuals were selected. One group had undetectable viral loads and was on HAART treatment (n=10) while the other had detectable viral loads and was treatment naïve (n=13). This study had IRB approval, and all individuals provided informed consent.

Extraction, amplification and sequencing

Peripheral blood mononuclear cells (PBMCs) from NVS were obtained. The cells were separated by the Ficoll–Hypaque technique and were used for DNA extraction by the QIAmp DNA extraction kit (QIAgen, Valencia, CA, USA). Due to the small quantity of proviral DNA in some of the samples, minor changes were made to the manufacturer QIAmp DNA extraction protocol. The extracted DNA was reconstituted in 40 μL of Buffer AE that was diluted in water (1:4) instead of the directed 200 μL. To further concentrate the extracted proviral DNA the 40 μL of DNA were reduced to ~10 μL using a speed vacuum. Using limiting template dilution nearly full-length proviral genomes (about 9000 kb) were amplified. Multiple reactions were carried out to achieve an end-point dilution with a positive PCR following previously described methods (Salminen et al., 1995; Carr et al., 1996, 2001 Arroyo et al., 2004). To minimize the chances of producing heterogeneous DNA targets and approximate amplification of a single copy of the target DNA the lowest possible dilution template was used in the first round PCR. Furthermore, to overcome the problem of amplifying rare templates sequences nested primers (internal primers) were used to ensure that only the targeted sequences were further amplified (Simmonds et al., 1990b). Primers MSF12/OFMR1 were used for the first round primers and F2NST/UNINEF7 were used as

the internal primers in a second-round amplification (Salminen et al., 1995; Carr et al., 2001; Arroyo et al., 2004). The potential recombination between DNA were addressed by using PCR conditions with an increased elongation time and using the lowest possible number of PCR cycles (Meyerhans et al., 1990; Judo et al., 1998). These fragments were then sequenced with Big Dye terminators using an ABI 3130 automated sequencer (Applied Biosystems Inc, Foster City CA), as previously described (Carr et al., 2001).

Analysis

Sequences were analyzed, edited, and assembled with Sequencher 3.0 (Ann Arbor, MI). All genes were analyzed for open reading frames and hypermutation was assessed using the software HYPERMUT 2.0 (Rose and Korber, 2000) along with a non-hypermutated reference isolate Ref.B.MN.M17449 (Accession number M17449). The rate ratio used in HYPERMUT software is defined as the number of nucleotide guanine (G) to adenine (A) substitutions divided by the number of adenine (A) to guanine (G) substitutions, adjusting for the adenine-rich nature of the HIV genome. In order to only quantify the substitutions caused by APOBEC3 activity we compared the total sum of the GG to GA substitutions compared to GC to GT substitutions, which are background mutations. This value provided us with a degree of hypermutation that we refer to as the APOBEC ratio. Phylogenetic analysis was performed by aligning the sequences obtained with reference sequences using the program MacGDE (Linton) followed by a Neighbor-joining method with Kimura's two-parameter model of distance calculation; bootstrap analysis was performed with 100 replicas. Phylogenetic trees were constructed using MEGA3 (Kumar et al., 2004; Saitou and Nei, 1987). In addition, the program SimPlot v.3.5.1 was used to explore recombination events on all sequences obtained (Lole et al., 1999). Point mutations associated with long term non-progressors found in the literature were sought and their significance was determined using fisher's exact test for categorical comparisons. Major mutations that have been associated with reduced susceptibility to protease and RT inhibitors were analyzed using the Stanford University HIV Drug Resistance Database: this online tool generated a genotypic antiretroviral drug resistance profile (Rhee et al., 2003; Shafer, 2006 Liu and Shafer, 2006). All sequences have been submitted to GenBank and can be found under accession numbers JF689852 to JF689897.

Conclusion

The current data, in which the NVS patients have higher hypermutation rate ratios than the controls, supports the hypothesis that their viruses were more heavily hypermutated by the ABOBEC3G system than viruses in the controls. It is clear that there are conflicting results about the types and the frequencies of mutations in non-progressors; thus, more research needs to be done to study the viral populations within each infected individual. Future studies could include performing pyrosequencing to fully understand the virological diversity and identifying genetic polymorphisms in the APOBEC3G gene (~10 kb) of the NVS subjects to identify factors that contribute to the NVS ability to suppress their infection naturally.

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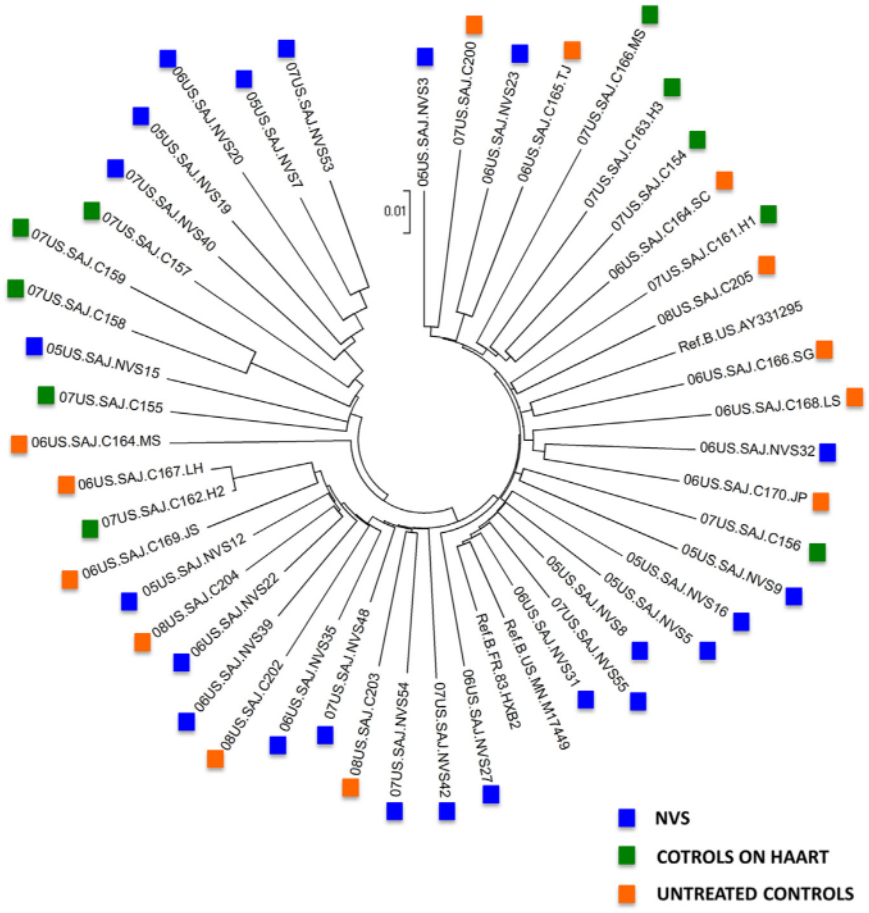

Fig. 1.

Phylogenetic tree of non-hypermutated HIV-1 near-full length sequences. NVS samples (blue squares), controls on HAART (green squares), untreated controls (orange squares), and the reference sequences (Ref.B.MN.M17449, Ref.B.FR.83.HXB2, and Ref.B.US.AY331295). The phylogenetic analysis has been performed on near-full length sequences and is based on neighbor joining method. They were constructed using MEGA3 (Kumar et al., 2004; Saitou and Nei, 1987).

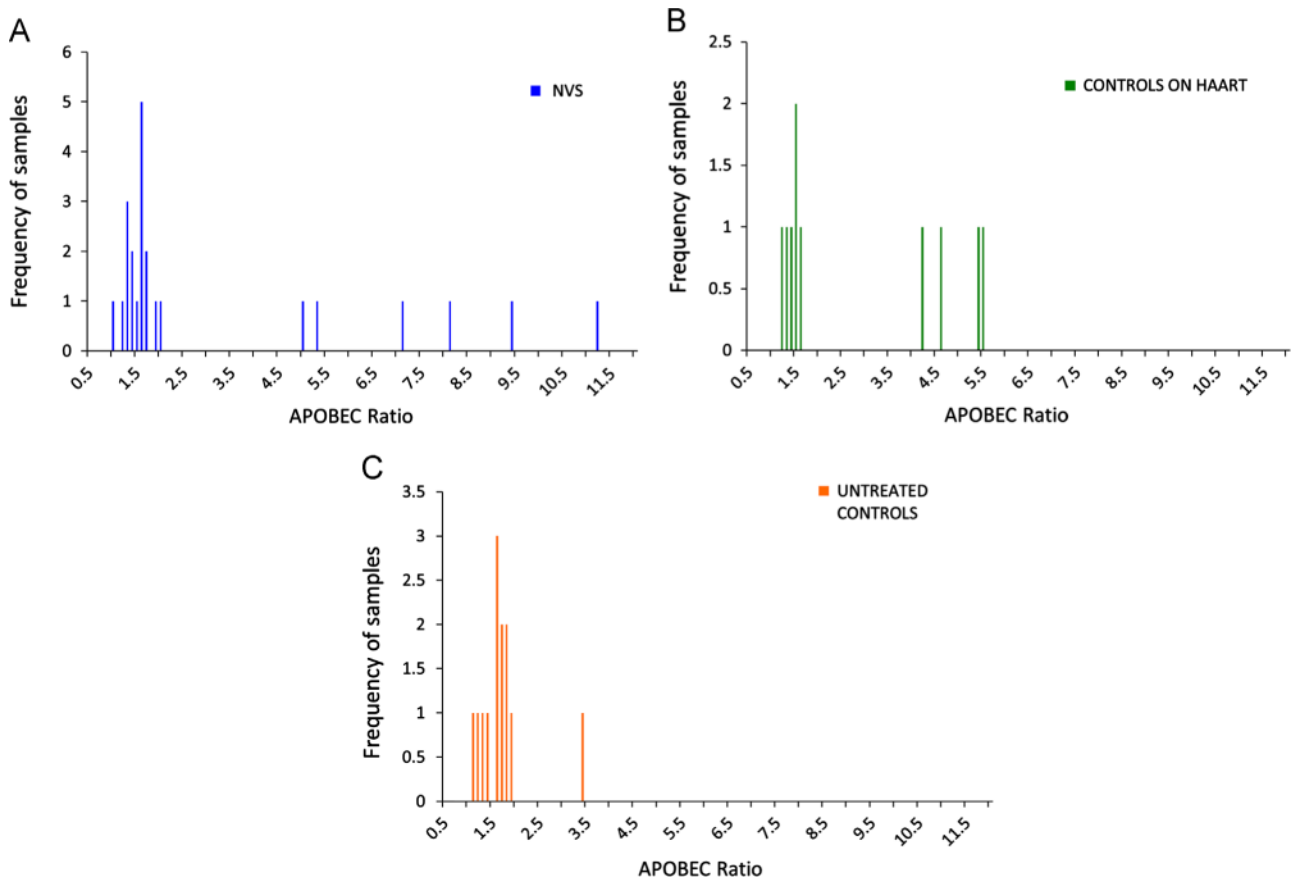

Fig. 2.

Levels of hypermutation in HIV-1 proviral genomes using the APOBEC Ratio. (A) Hypermutation ratio of NVS patients, (B) hypermutation ratio of controls on HAART, and (C) hypermutation ratio of untreated controls. The APOBEC Ratio is defined as all GG and GA substitutions compared to all GC and CT substitutions.

Table 1

Characteristics of NVS patients, controls on HAART, and untreated controls.

Subject ID	Age	Sex	Race	Year of HIV Diagnosis	Year of Sample	Risk Factor	Last Viral Load (copies/ml)	ART Treatment	REV (L78I)	Hypermutation Identified	Hypermutation Rate Ratio ^	APOBEC Ratio	Genes Analyzed: Gag	Pol	Vif	Vpr	Vpu	Env	Nef	Rev	Tat
05US.SAJ.NVS3	62	M	AA	1991	2005	IDU	<75	no	L	no		1.0									
05US.SAJ.NVS5	50	M	AA	1991	2005	MSM	93	no	L	no		1.9									
05US.SAJ.NVS7	60	M	AA	1994	2005	IDU	<75	no	L	yes	4.88 [p=7.68e-60]	8.1									
05US.SAJ.NVS8	58	M	AA	1989	2005	IDU	<40	no	L	no		1.3									
05US.SAJ.NVS9	53	M	AA	2003	2005	IDU	<40	no	L	no		1.4									
05US.SAJ.NVS12	40	M	AA	1997	2005	IDU	<40	no	L	no		1.3									
05US.SAJ.NVS15	36	F	AA	2002	2005	S	<50	no	L78I	yes	2.97 [p=1.18e-20]	5.0									
05US.SAJ.NVS16	43	F	AA	1988	2005	S	<40	no	L	no		1.7									
05US.SAJ.NVS19	58	M	AA	1997	2005	S	359	no	L	yes	7.04 [p=7.08e-69]	11.2									
06US.SAJ.NVS20	54	F	AA	1995	2006	IDU	120	no	N/A	yes	4.86 [p=4.88e-51]	7.1									
06US.SAJ.NVS22	60	M	AA	1994	2006	S	163	no	L	no		1.6									
06US.SAJ.NVS23	36	F	AA	1995	2006	S	<75	no	L	no		1.6									
06US.SAJ.NVS27	55	M	AA	1989	2006	IDU	475	no	L	no		1.7									
06US.SAJ.NVS31	49	M	AA	1992	2006	S	<48	no	L	no		1.5									
06US.SAJ.NVS32	50	F	AA	2000	2006	IDU	<48	no	L	no		2.0									
06US.SAJ.NVS35	53	M	AA	1997	2006	IDU	<75	no	L	no		1.6									
06US.SAJ.NVS39	30	F	AA	1995	2006	S	184	no	L78I	no		1.6									
07US.SAJ.NVS40	35	F	AA	1995	2007	IDU	<48	no	L	yes	3.66 [p=3.85e-36]	5.3									
07US.SAJ.NVS42	57	M	AA	1990	2007	IDU	<400	no	L	no		1.3									
07US.SAJ.NVS48	44	F	AA	1986	2007	S	328	no	L78I	no		1.4									
07US.SAJ.NVS53	52	F	AA	1986	2007	IDU	256	no	L	yes	5.64 [p=3.01e-71]	9.4									
07US.SAJ.NVS54	57	F	AA	2004	2007	S	<48	no	L	no		1.6									
07US.SAJ.NVS55	49	M	AA	1994	2007	IDU	<48	no	L	no		1.2									
CONTROLS_ON HAART																					
07US.SAJ.C154	40	M	AA	1998	2007	S	<50	yes	L	no		1.5									
07US.SAJ.C155	45	M	AA	1996	2007	S	<50	yes	L	yes	2.90 [p=1.50e-18]	4.2									
07US.SAJ.C156	23	M	AA	2005	2007	S	<50	yes	L	no		1.3									
07US.SAJ.C157	52	M	AA	2002	2007	S	<50	yes	L	yes	3.32 [p=9.08e-30]	5.5									
07US.SAJ.C158	44	F	AA	1998	2007	S	<50	yes	L	yes	3.53 [p=2.0e-39]	5.4									
07US.SAJ.C159	51	M	AA	2003	2007	IDU	<50	yes	L	yes	2.52 [p=1.19e-23]	4.6									
07US.SAJ.C161.H1	36	M	W	2003	2007	S	<50	yes	L	no		1.6									
07US.SAJ.C162.H2	46	F	W	2005	2007	S	<50	yes	L	no		1.5									
07US.SAJ.C.163.H3	27	M	AA	2004	2007	S	<50	yes	L	no		1.4									
07US.SAJ.C.166.MS	31	M	AA	2006	2007	S	<48	yes	L	no		1.2									
UNTREATED CONTROLS																					
06US.SAJ.C164.SC	30	F	AA	1999	2006	IDU	94,379	no	L	no		1.6									
06US.SAJ.C164.MS	48	M	AA	2006	2006	N/A	2,630	no	L	yes	2.19 [p=5.74e-10]	3.4									
06US.SAJ.C165.TJ	34	F	AA	1995	2006	N/A	>750,000	no	L	no		1.7									
06US.SAJ.C166.SG	45	F	AA	2003	2006	S	>750,000	no	L	no		1.6									
06US.SAJ.C167.LH	45	F	AA	1990	2006	S	>750,000	no	L	no		1.4									
06US.SAJ.C168.LS	39	F	AA	2004	2006	IDU	164,000	no	L	no		1.8									
06US.SAJ.C169.JS	52	M	AA	2004	2006	IDU	94,000	no	L	no		1.1									
06US.SAJ.C170.JP	58	M	AA	1996	2006	IDU	99,000	no	L	no		1.7									
07US.SAJ.C200	24	M	AA	2005	2007	S	208,000	no	L	no		1.3									
08US.SAJ.C202	42	M	AA	1992	2008	IDU	57,800	no	L	no		1.9									
08US.SAJ.C203	46	F	AA	1997	2008	IDU	43,000	no	L	no		1.8									
08US.SAJ.C204	45	F	AA	2007	2008	IDU	>100,000	no	L	no		1.2									
08US.SAJ.C205	43	F	AA	2007	2008	IDU	205,418	no	L78I	no		1.6									

^ Fisher Exact *p*-value where less than 0.05 is indicative of a hypermutant. The APOBEC Ratio is defined as all GG and GA substitutions compared to all GC and CT substitutions. Analysis of all genes: color grey shows defective due to hypermutation; orange defective due to incorrect start amino acid and hypermutation, yellow defective due to incorrect start amino acid, blue defective due to deletion/frameshift, red defective due to frameshift, incorrect starting codon and hypermutation.