

# HIGH FREQUENCY OF STOP CODONS IN THE HUMAN IMMUNODEFICIENCY VIRUS-1 PROTEASE GENE FRAME IN HUMAN IMMUNODEFICIENCY VIRUS+ INDIVIDUALS WITH BELOW DETECTABLE LEVELS OF PLASMA VIREMIA DURING HIGHLY ACTIVE ANTIRETROVIRAL THERAPY

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## ABSTRACT

We performed sequence analysis of HIV-1 proviral protease gene fragment (560 base pairs) amplified from *ex-vivo* peripheral blood mononuclear cells of 83 HIV+ individuals with Below Detectable Levels (BDL) (<20-40 RNA copies/ml plasma) and detectable levels of plasma HIV viremia while on HAART. Noteworthy was the systematic presence of stop codons identified only in the BDL group and not in individuals with detectable plasma viremia ( $p < 0.0001$ ). The stop codons dominated positions 16 and 157 in the protease gene. This suggests that specific mutations in the protease gene possibly provide transitory molecular control of viral replication to below detectable levels in plasma during HAART. Thus, these mutations could potentially be exploited for long-term control of HIV.

**Keywords:** Human Immunodeficiency Virus (HIV), Highly Active Antiretroviral Therapy (HAART), Stop Codons, Gag Gene, Protease Inhibitors

## 1. INTRODUCTION

How antiretroviral drugs shape the architecture of HIV over time is visible at the level of emerging drug resistance mutations and viral evolution during Highly Active Antiretroviral Therapy (HAART) (Potter *et al.*, 2003; 2004; 2006). It is unknown if mutations emerging in the presence of NRTI, NNRTI and protease inhibitors can provide complete but transitory control of HIV replication *in vivo* to Below Detectable Levels (BDL) of plasma HIV (<20-40 copies of HIV RNA/ml plasma). Even though HIV can be suppressed by HAART to below detectable levels in plasma, low-level HIV

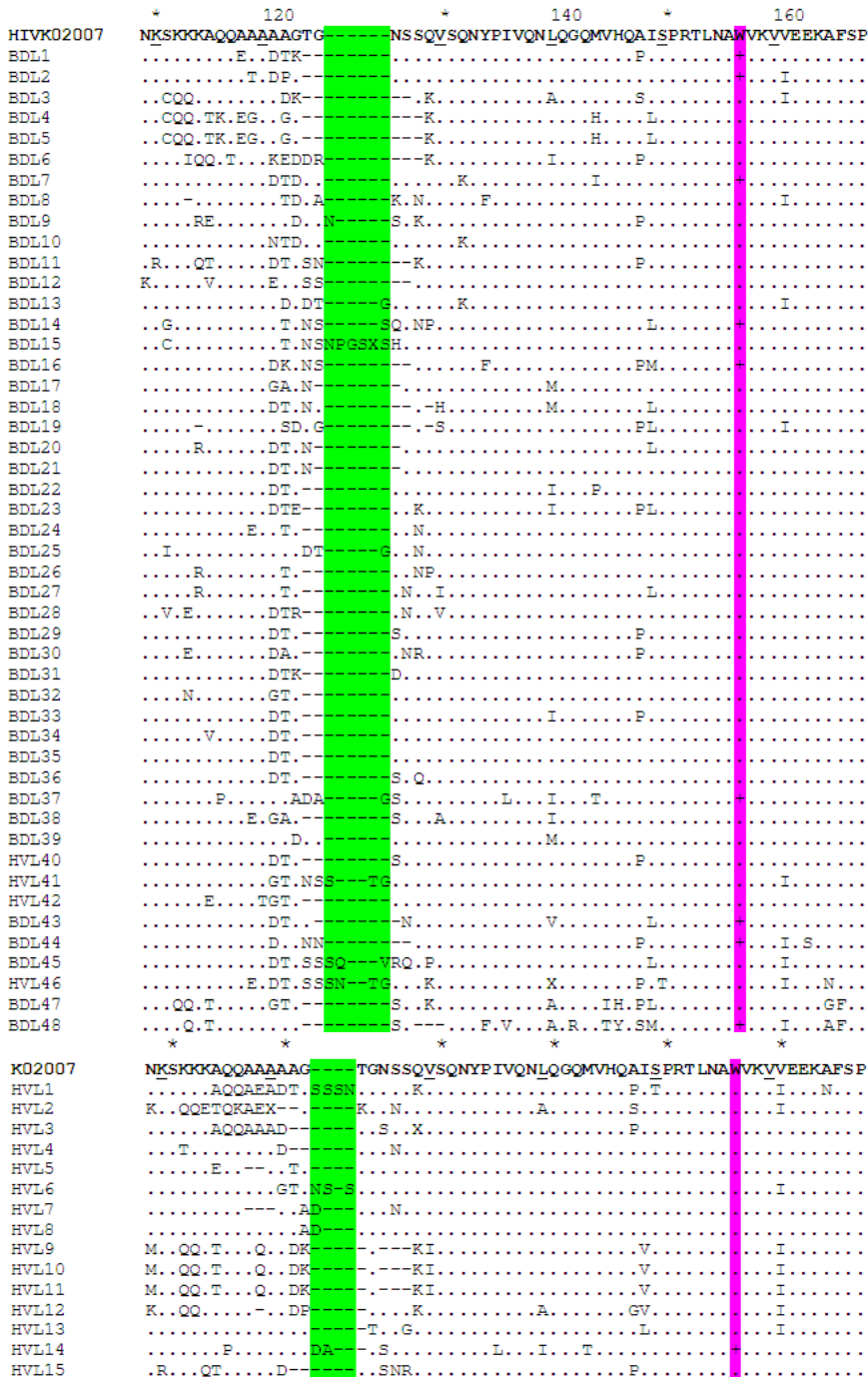
replication continually occurs in cellular reservoirs despite HAART (Potter *et al.*, 2003; 2004). PCR amplification of HIV from plasma cannot be achieved successfully when the virus reaches below the limit of detection (<20-40 copies of HIV/ml plasma), although can be detected in the Peripheral Blood Mononuclear Cells (PBMC) as integrated provirus. Therefore, we analyzed this HIV provirus in the *protease* gene derived from *ex-vivo* obtained PBMC from HIV+ individuals who were able to achieve complete control of plasma HIV by HAART and compared them against individuals who displayed varying ranges of plasma viremia while on HAART.

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**Table 1.** Details of positions of stop codons, insertions and deletions in the gag gene derived from HIV-strains from HIV patients showing complete control of viremia while on HAART and HIV patients failing HAART. The positions of the codons is compared to HIVK2007 (subtype B) as shown in **Fig. 1**

Patient IDs	Corresponding patient number in the figure	STOP CODON POSITION	Insertions	Deletions
1	BDL1	16, 157		124-125
2	BDL2	157		124-125
3	BDL3	13		124-127
4	BDL4			124-128
5	BDL5			124-128
6	BDL6			126-128
7	BDL7	157		
8	BDL8	13,55,73		113
9	BDL9	74	1 in 126	
10	BDL10	15,76,103		
11	BDL11	69,74,		127-128
12	BDL12			127-128
13	BDL13		1 in 126	
14	BDL14	21,40,72,157	1 in 126	
15	BDL15		6 in 126	
16	BDL16	157		127-128
17	BDL17			125-126
18	BDL18			127-128,130
19	BDL19			114,127-128,130
20	BDL20			125-126
21	BDL21			125-126
22	BDL22			124-125
23	BDL23			124-125
24	BDL24	16		124-125
25	BDL25	16	1 in 126	
26	BDL26			124-125
27	BDL27			124-125
28	BDL28			124-125
29	BDL29			124-125
30	BDL30			124-125
31	BDL31	28		124-125
32	BDL32			124-125
33	BDL33	16		124-125
34	BDL34			124-125
35	BDL35			124-125
36	BDL36	16		124-125
37	BDL37	157		
38	BDL38	16		124-125
39	BDL39			
40	BDL40			124-125
41	BDL41		3 in 126	
42	BDL42			124-125
43	BDL43	16,36,157		125-126
44	BDL44	157		126-127
45	BDL45		3 in 126	
46	BDL46		4 in 126	
47	BDL47			124-125
48	BDL48	16,157		124-125, 128-130
49	not shown	157		
50	not shown	157		
51	not shown	13,74,157		
52	not shown	15,74		
53	HVL1		4 in 124	
54	HVL2			121-122
55	HVL3			122-123
56	HVL4			122-123
57	HVL5			118-119
58	HVL6		3 in 124	
59	HVL7		1 in 124	118-120
60	HVL8		1 in 124	
61	HVL9			
62	HVL10			107,124,126-128
63	HVL11			107,124,126-128
64	HVL12			119,124
65	HVL13			124
66	HVL14	157		124
67	HVL15			122-123
68	not shown			
69	not shown			
70	not shown			
71	not shown			
72	not shown			
73	not shown			
74	not shown			
75	not shown			
76	not shown			
77	not shown			
78	not shown			
79	not shown			
80	not shown			
81	not shown			
82	not shown			
83	not shown			

	*	20	*	40	*	60	*	80	*	100
HIVK02007	MGARASVLSGGELDK	VEKIRLRPGGKKYK	LKHIVWASRE	LERFAVNPGL	LETSEGC	QILGQLQPSL	QTGSEEL	RLSYNTVAT	LYCVHQR	IDVDRDTKEALEKIEEBEQ
BDL1	.....Q.R	.....Q.R	.....A.G	.....R	.....A.G	.....R	.....R	.....R	.....R	.....D
BDL2	I.....C.K	.....R	.....A	.....P	.....V	.....G	.....E	.....D	.....D	.....D
BDL3	IPARAS.FR.RK	T.....H.MI	L.....L	.....L	.....D.K	I.....A.K	T.....T	.....KN	E.R	.....D
BDL4	.....L.R	K.H.....R	RM.L.....L	.....L	.....K	IQ.....A.K	T.....T	.....K	E.R	.....D
BDL5	.....I.R	K.S.....R	RM.L.....L	.....L	.....K	IQ.....A.K	T.....T	.....K	E.R	.....D
BDL6	.....I.R	K.....Q	R.L.....L	.....K	I.....K	IN.....A	T.....T	.....F	.....TG	R.....D
BDL7	.....V	FL.D.R	.....R	.....F	.....R	.....G	.....I	.....D	.....D	.....D
BDL8	.....I	S.....R	.....Q	.....V	.....K	.....K	.....V	.....K	.....V	.....V
BDL9	.....I	SA.....R	.....A	S.K.R	.....LI	.....YSI	.....A	K	.....IE	.....K
BDL10	.....X	GGD.....R	.....R	.....W	.....T	.....S	.....E	V	.....I	.....F
BDL11	.....I	.....Q	R.....L	.....L	.....M	EL	.....T	.....A	.....IK	.....F
BDL12	.....I	.....G	R.....R	.....L	.....A	.....E	.....A	.....T	.....F	.....NK
BDL13	V.V.....I	.....F	.....N	.....P	.....K	.....K	.....K	.....K	.....K	.....D
BDL14	.....WI	.....R	.....K	.....E	.....F	.....F	.....I	V	.....W	.....E
BDL15	.....R	.....R	.....V	.....F	.....K	.....R	.....K	.....R	.....V	.....D
BDL16	.....I	.....R	.....Q	R.....L	.....L	.....F	.....I	.....V	.....K	.....D
BDL17	.....R	.....R	.....R	.....R	.....I	.....F	.....I	.....N	.....E	.....D
BDL18	I.....R	.....R	.....D	.....K	.....E	.....K	.....E	.....L	.....D	.....D
BDL19	.....R	.....R	.....V	.....K	.....E	.....L	.....D	.....D	.....D	.....D
BDL20	.....A	.....R	.....I	.....K	.....D	.....D	.....D	.....D	.....D	.....D
BDL21	.....R	.....R	.....A	.....K	.....F	.....K	.....E	.....D	.....D	.....D
BDL22	.....R	.....Q	.....S	.....R	.....H	.....V	.....K	.....R	.....D	.....D
BDL23	.....R	.....Q	.....E	.....Q	.....D	.....TG	.....K	.....E	.....A	.....K
BDL24	.....R	.....Q	.....E	.....Q	.....K	.....V	.....K	.....R	.....D	.....D
BDL25	.....I	.....Q	.....Q	.....K	.....V	.....K	.....R	.....D	.....D	.....D
BDL26	.....R	.....Q	.....Q	.....E	.....F	.....V	.....EI	.....D	.....L	.....D
BDL27	.....Q	.....Q	.....K	.....V	.....K	.....V	.....D	.....D	.....D	.....D
BDL28	.....A	.....R	.....L	.....D	.....R	.....V	.....I	.....V	.....K	.....EI
BDL29	.....R	.....R	.....Q	R.....L	.....D	.....K	.....V	.....K	.....EI	.....D
BDL30	.....R	.....R	.....Q	.....K	.....K	.....F	.....V	.....G	.....D	.....D
BDL31	.....I	.....R	.....S	.....R	.....L	.....K	.....M	.....K	.....T	.....EI
BDL32	.....I	.....G	.....R	.....AG	.....E	.....A	.....F	.....T	.....E	.....D
BDL33	.....E	.....R	.....QR	.....R	.....L	.....V	.....I	.....V	.....EI	.....D
BDL34	.....R	.....R	.....Q	.....R	.....R	.....E	.....D	.....D	.....D	.....D
BDL35	.....K	.....Q	.....D	.....D	.....K	.....EI	.....D	.....D	.....D	.....D
BDL36	.....R	.....R	.....E	.....A	.....F	.....EI	.....D	.....D	.....D	.....D
BDL37	.....R	.....R	.....Q	.....L	.....A	.....K	.....R	.....D	.....D	.....D
BDL38	.....I	.....R	.....R	.....Q	.....LM	.....D	.....T	.....A	.....K	.....V
BDL39	.....K	.....T	.....K	.....M	.....L	.....Q	.....A	.....K	.....F	.....EI
HVL40	.....K	.....R	.....Q	.....A	.....A	.....T	.....I	.....V	.....I	.....D
HVL41	.....R	.....R	.....D	.....E	.....I	.....D	.....D	.....D	.....D	.....D
HVL42	.....R	.....R	.....A	.....D	.....A	.....K	.....R	.....D	.....D	.....D
BDL43	.....K	.....R	.....T	.....Q	.....A	.....K	.....G	.....E	.....R	.....D
BDL44	.....I	.....KS	.....L	.....A	.....K	.....F	.....A	.....V	.....N	.....E
BDL45	.....I	.....X	.....K	.....L	.....A	.....K	.....F	.....A	.....V	.....N
HVL46	.....I	.....K	.....R	.....K	.....A	.....V	.....K	.....A	.....V	.....N
BDL47	.....T	.....K	.....A	.....L	.....L	.....F	.....SA	.....Q	.....L	.....E
BDL48	.....I	.....K	.....A	.....R	.....L	.....L	.....A	.....Q	.....IE	.....A
	*	*	*	*	*	*	*	*	*	*
K02007	MGARASVLSGGELDK	VEKIRLRPGGKKYK	LKHIVWASRE	LERFAVNPGL	LETSEGC	QILGQLQPSL	QTGSEEL	RLSYNTVAT	LYCVHQR	IDVDRDTKEALEKIEEBEQ
HVL1	.....IL	.....R	.....K	.....V	.....D	.....D	.....D	.....D	.....D	.....D
HVL2	.....T	.....K	.....A	.....RM	.....L	.....L	.....Q	.....MI	.....T	.....K
HVL3	.....K	.....R	.....Q	.....R	.....A	.....A	.....T	.....K	.....I	.....V
HVL4	.....R	.....R	.....R	.....G	.....E	.....A	.....K	.....K	.....V	.....D
HVL5	.....R	.....R	.....A	.....D	.....E	.....I	.....D	.....D	.....D	.....D
HVL6	.....R	.....R	.....R	.....Q	.....L	.....M	.....S	.....D	.....K	.....FK
HVL7	.....K	.....R	.....AD	.....KR	.....V	.....K	.....G	.....EI	.....D	.....D
HVL8	.....V	.....K	.....R	.....Q	.....L	.....M	.....S	.....D	.....K	.....FK
HVL9	.....S	.....I	.....R	.....EK	.....N	.....K	.....K	.....C	.....M	.....L
HVL10	.....S	.....I	.....R	.....EK	.....N	.....K	.....K	.....C	.....M	.....L
HVL11	.....S	.....I	.....R	.....EK	.....N	.....K	.....K	.....C	.....M	.....L
HVL12	.....I	.....R	.....K	.....T	.....R	.....H	.....Q	.....L	.....L	.....S
HVL13	.....I	.....R	.....R	.....R	.....K	.....T	.....K	.....T	.....K	.....E
HVL14	.....R	.....R	.....Q	.....R	.....L	.....A	.....K	.....F	.....A	.....V
HVL15	.....I	.....W	.....QS	.....R	.....R	.....L	.....ME	.....KA	.....IK	.....F



**Fig. 1.** Alignment of the deduced Gag amino acid from Below Detectable Levels patients (BDL) and patients with detectable plasma viremia (HVL). K02007 sequence serves as HIV-1 Gag reference sequence. Matching amino acids are denoted by dots (.), + stands for stop codons. Pink color indicates positions of G→A hypermutation hotspots 16 and 157, remaining stop codons are colored turquoise; bright green represents regions with deletions and/or insertions of amino acids into the sequences.

**Table 2.** Summary showing total number of stop codons between BDL and viremic groups

	BDL	Viremic
Number of samples	52.0	31.0
Total	83.0	
Samples with stop codon(s)	24.0	1.0
Percentage (%)	46.2	5.9
<b>Amino acid number\stop codon number</b>		
1 to 80 (excl. 16)	18.0	0.0
81 to 168 (excl. 157)	1.0	0.0
16	8.0	0.0
157	12.0	1.0
Total number of stop codons	39.0	1.0
p value		

The objective was to determine if mutations other than drug resistance ones, such as single nucleotide changes and/or frame-shift mutations that emerge during HAART, could segregate HIV individuals with or without detectable plasma. Since the majority of antiretroviral drugs target the gag and pol genes of the HIV life cycle, we focused only on mutations that emerge during complete control of plasma viremia with HAART, as they may have the potential to explain how viral fitness and replicative ability is altered transiently during HAART. We analyzed 83 HIV+ individuals by sequencing the 560bp gag gene fragment derived from primary PBMCs of the two HIV+ groups discussed above.

Our data demonstrates that in addition to other pharmacological and virological aspects of drug-mediated control of viremia in HIV+ individuals, viral control was defined by the preponderance of inactivating mutations at position 16 and 157 in the gag gene ( $p < 0.0001$ ) (**Fig. 1 and Table 1-2**). These comprised of frame interrupting stop codons that were unique to individuals showing BDL of plasma viremia on HAART. These mutations were lost as viremia emerged during therapy, which was apparent from viremic patients, suggesting a possible role in transitory viral inactivation *in vivo* during HAART. Thus, it is apparent that the gain of these mutations leads to viral control *in vivo*, whereas the loss of these mutations leads to restoration of viral replication, which is evident from the complete and systematic absence of these mutations at position 16 and 157 of the gag gene derived from viremic patients on HAART.

## 2. MATERIALS AND METHODS

Blood samples from 83 HIV sero-positive individuals were obtained from the Department of

Virology, Centre for Infectious Diseases and Microbiology Laboratory Services, ICPMR, Westmead Hospital and the Australian Red Cross, following informed consent. This study was approved by the Human Ethics Committee of the Sydney West Area Health Service. Prior written consent of patients was obtained for obtaining whole blood. Whole blood (10-20 mL) from individuals was collected in heparinised or EDTA vacuum tubes, centrifuged at 800x g for 10 min and the cellular fraction collected. This fraction was diluted in sterile PBS define to 35 mL and carefully layered over 15 mL of Ficoll Hypaque in a 50 mL centrifuge tube (Becton Dickinson, Franklin Lakes, NJ, USA). After centrifugation at 800x g for 20 min, PBMCs were carefully removed from the interface with a transfer pipette and then pelleted by further centrifugation at 400x g for 10 min. The supernatant was discarded and the pellet resuspended in PBS, then spun again at 400x g for 10 min. DNA extractions were performed using QIAGEN DNeasy Blood + Tissue Kit (Qiagen, Germany), according to the manufacturer's protocol. DNA was visualized on an agarose gel under UV light.

The proviral DNA was amplified using the HIV-specific gag gene primer pairs in a nested Polymerase Chain Reaction (PCR). Double-nested PCRs were used to amplify HIV-1 target templates in all cases and significant care was taken to avoid PCR contamination and carryover problems by conducting PCR in designated and remotely located facility from the DNA laboratories and sterilizing the PCR laboratory with UV irradiation. Negative controls were included in both rounds of amplification in all cases, using material derived from template-free (PBS) DNA extractions. For the gag gene amplification, one primer set was used for external amplification (gag2, MSF12) and a second pair for internal amplification (gag1, gag583R). PCR products were subjected to clean-up prior to sequencing and purified using the 96-well PCR purification plates on a vacuum manifold. Sequencing was performed using the ABI PRISM BigDye Terminator V3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, CA, USA). Reactions were carried out in a total of 12  $\mu$ L using 1  $\mu$ L of purified PCR product. Unincorporated dye terminators were removed from sequencing reactions by Sephadex purification. For sequence analysis, multiple sequence alignments of the viral sequences were performed using CLUSTALW program (Thompson *et al.*, 1994). Analysis of the frequency of the number of codons differing between BDL and viremic groups was



performed by Mann-Whitney non-parametric test in the SPSS software package.

### 3. RESULTS AND DISCUSSION

Due to undetectable plasma virus in BDL patients and the limitation it poses in amplifying HIV from plasma of these patients, we took the advantage of HIV-integrated provirus in ex-vivo-derived uncultured PBMCs for DNA amplification. Analysis of the proviral DNA sequences from PBMCs of 83 HIV+ individuals on HAART clearly demonstrated that stop codons in the protease gene are prevalent in HIV-1 strains *in vivo* when plasma HIV load is at below detectable levels (<20-40 copies/ml plasma). This is in contrast to viremic individuals, where no stop codons were detected ( $p < 0.0001$ ). These observations clearly show an unusual aspect of molecular control of HIV during HAART, which is possibly achieved transiently via the inactivating mutations at positions 16 and 157 of the protease gene unique to BDL HIV+ patients on HAART. Alternatively, these mutations were also reliable in predicting undetectable plasma viremia in each case. The sequence analyses showed that even though the occurrence of stop codons was a significant feature of HIV+ BDL individuals, both variants (with and without stop codons) prevailed *in vivo* in most cases, with the higher preponderance of mutant variant at all times in BDL individuals. Although this virus population modulation may be one indication of viral control during HAART, the existence of two populations with and without stop codons in BDL individuals is also suggestive of low-level HIV replication in some cellular reservoirs. Since total PBMCs were analyzed for sequencing, the exact cell type could not be identified. Previously resting memory CD4+ T cells have been shown to harbor defective viral populations during HAART-induced HIV control (Kieffer *et al.*, 2005). The co-existence of defective and non-defective populations in the blood stream of BDL individuals also suggests that effect of these mutations on viral replication control may be transitory, which we believe is the case. This is further reflected in the comparison against individuals with plasma viremia, where no interrupting stop codons were found and invariably only intact viral genomes were observed. It may also suggest that the absence of stop codons in virus during the viremic phase may signal breakthrough viral population with drug resistance mutations and antiretroviral drug treatment failure, a fact

not dissected in this study. Thus, the presence of these inactivating stop codons may harbor some prognostic value.

Of note were the stop codons, which were created via systematic elimination of tryptophan residues (W) by a process of "G-to-A hypermutation" (Pathak and Temin, 1990) in which G-to-A transitions far exceeded all other mutations in viral sequences (Borman *et al.*, 1995). G-to-A hypermutation has been described in about 38% of asymptomatic individuals and 50% of seroconverters (Janini *et al.*, 2001), although the functional relevance remains poorly understood. Further, it is unclear whether hypermutation is influenced by viral escape or if it is a function of the cell population harboring the virus. The loss of coding potential of these hypermutated HIV sequences strongly suggests that they are incapable of generating progeny virions and cannot contribute to the HIV gene pool (Janini *et al.*, 2001). Consistent with these findings, we have previously reported a case of a long-term non-progressor where G-to-A hypermutation was significantly higher in the gag and pol genes, some of which resulted in stop codons (Wang *et al.*, 2003). It is believed that the PBMC-derived proviral sequences may display a higher incidence of G-A hypermutation (Janini *et al.*, 2001). However, a recent longitudinal analysis of full-length HIV-1 genomes from an American cohort of 20 slow progressors and non-progressors (Wang *et al.*, 2000) did not show a predominance of G-A hypermutation in the non-progressors, thereby suggesting a minimal role of this phenomenon in defining the rate of disease progression. Nonetheless, from our analyses it appears that the G-A changes resulting in stop codons in the protease gene correlate with low or non-replicative virus. This comparative observation that HIV strains from viremic individuals being completely devoid of stop codons suggests that G-A hypermutation may play a significant role in rendering HIV genomes defective and may be important in containing HIV disease progression in some cases. Nonetheless, the comparison of BDL and viremic patients clearly show that this is a transitory phenomenon and further investigations are needed to dissect the exact relevance of these mutations in the context of HIV disease progression. HIV culture studies have previously shown that G-A hypermutation may be transitory and can be induced by mitogens (Janini *et al.*, 2001). This *in vitro* situation appears to be consistent with the *in vivo* situation we have observed in our study. But the only difference is that these G-A changes are taking place in HIV patients while on HAART. Overall, supporting our

observations, Kieffer *et al.* (2005) also showed interrupting protease gene stop codons in the gag region in 9/9 individuals from the resting memory CD4<sup>+</sup> T cells in individuals with below detectable levels of plasma HIV. This transitory control of plasma HIV to BDL during HAART may provide insights into a more durable HIV control once the biological role and mechanism of the emergence of these novel mutations is more clearly understood.

#### 4. CONCLUSION

Overall, these analyses are the first to point out that the occurrence of transitory stop codons in HIV strains during HAART are able to sustain below detectable levels of HIV for a certain period of time and there is quasispecies modulation *in vivo* between mutant and wild-type populations within the gag gene, which eventually defines the predominant variant *in vivo*. Our data suggest that HAART is able to contain HIV in plasma to below detectable levels by impairing the ability of HIV to replicate due to stop codons, we have reported herein. It should be emphasized that HIV protease can tolerate substantial amount of mutations; at least one third of its 99 amino acids can deviate from the wild-type sequence without altering function, but the mutations at positions 16 (aa residue 5) and 157 (aa residue 52) as noted in this study are unique and have not been reported before. In addition, these mutations lie outside active-site triad (Asp25, Thr26, Gly27) of HIV protease, which is located in a loop whose structure is stabilized by a network of hydrogen bonds similar to that in the eukaryotic enzymes (Wlodawer and Erickson, 1993). Further functional studies are needed to clarify the role of these mutations in transitory viral replication control *in vivo* and also possibly in predicting fluctuating plasma concentrations of the drugs and emergence of drug resistance.

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