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Assay of HIV gp41 amino acid sequence to identify baseline variation and mutation development in patients with virologic failure on enfuvirtide☆

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Abstract

In this study, we retrospectively assessed a gp41 genotypic assay in 404 enfuvirtide-naïve individuals (340 clade B, 64 non-B clade) to determine the prevalence of baseline polymorphisms and in 41 patients virologically failing enfuvirtide to determine correlates of resistance to this agent. Conserved and polymorphic regions of gp41 were identified in clade B isolates, with 127 of 328 codons (38.7%) being highly conserved (<1.0% variation) and 74 of 328 codons (22.6%) being partially conserved (1.0–5.0% variation). Polymorphisms were observed throughout gp41 in non-B clade virus sequences compared to the clade B reference strain, ranging from 53 natural substitutions in clade D to 76 in clade A. Insertions were common at positions 3, 105, 215 and 276. In the patients failing enfuvirtide, mutations were detected in the 10 amino acid region at positions 36–45 in all plasma virus sequences. Six additional mutations were selected outside of the common region which may be clinically significant at positions 33, 73, 75, 126, and 138. Two or three mutations at positions 36–45 were observed in the majority of plasma virus sequences from patients with virologic failure following the use of enfuvirtide. Further study is required to determine the clinical relevance of the clade related polymorphisms and the new mutations identified in the patients with virologic failure.

Keywords: HIV; Enfuvirtide; gp41; Genotyping assay; Polymorphism; Resistance

1. Introduction

Despite the significant reduction in human immunodeficiency virus (HIV) related morbidity and mortality associated with combination antiretroviral (ARV) therapy (cART), successful therapy remains complicated by drug interactions, metabolic adverse effects and extensive cross resistance among agents with intracellular targets, particularly in heavily treatment experienced individuals (Palella et al., 1998; Department of Health and Human Services, 2006). Enfuvirtide is therefore a welcome addition to the ARV armamentarium available for the management of these patients, given the low potential for drug

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interactions and novel mechanism of action relative to existing drug classes (Oldfield et al., 2005). Enfuvirtide is a synthetic 36 amino acid peptide homologous with the C-terminal residues 109–144 of the heptad repeat 2 (HR-2) region of the HIV gp41 transmembrane subunit (Wild et al., 1994). By competitively binding to the HR-1 domain of gp41, the interaction of HR-1 and HR-2 that is necessary for successful viral-CD4+ cell fusion is disrupted (Kilby et al., 1998). Administered at a dose of 90 mg twice daily by subcutaneous injection, enfuvirtide-based therapy resulted in significantly improved virologic and immunologic outcomes relative to regimens without enfuvirtide in controlled trials of heavily treatment experienced patients (Lazzarin et al., 2003; Nelson et al., 2005). However, despite these positive clinical data, therapy with enfuvirtide can be readily compromised by the appearance of mutations in the gene encoding for gp41 if concomitant background therapy is suboptimally active against HIV. Specifically, mutations mapped to the glycine–isoleucine–valine (GIV) sequence of the HR-1 domain of gp41 as well as single or multiple amino acid changes within residues 36–45 of HR-1 have been associated with reduced susceptibility to enfuvirtide in both in vitro studies and clinical trials (Sista et al., 2004; Rimsky et al., 1998; Derdeyn et al., 2001; Wei et al., 2002; Mink et al., 2005; Poveda et al., 2002, 2004). Although additional mutations outside of these regions have been reported in small published and unpublished studies, the clinical importance of these substitutions remains largely unclear (Poveda et al., 2005; Roman et al., 2003; Xu et al., 2005).

Primary resistance to enfuvirtide in patients naïve to this agent appears to be uncommon. The majority of data presented thus far suggests that the 36–45 and GIV domains of gp41 are highly conserved in enfuvirtide-naïve patients, with the exception of polymorphisms at codon 42, which have been observed in approximately 16% of isolates at baseline (Sista et al., 2004; Roman et al., 2003; Xu et al., 2002). This pattern appears similar regardless of HIV subtype, although data are limited in this regard (Aghokeng et al., 2005). These findings are in keeping with the data and sequences in the Los Alamos Database (http://hiv-web.lanl.gov). However, there exists conflicting data demonstrating a relatively high frequency of baseline enfuvirtide mutations in patients naïve to this agent and evidence of a higher frequency of baseline mutations in codons 36–45 in non-B subtype isolates relative to subtype B, highlighting the need for further study of enfuvirtide resistance (Carmona et al., 2005; Xu et al., 2005).

In this study, we report on the genotype sequence of gp41 in 404 enfuvirtide-naïve patients and on the appearance of gp41 sequence mutations in plasma virus sequences from 41 patients with virologic failure following the use of enfuvirtide. Knowledge of the baseline sequence variations and polymorphisms in gp41 and the clinical correlation of mutations that develop once failing enfuvirtide will be important to clinicians who will be using this drug. Further, new agents that inhibit gp41 fusion are under development and gp41 resistance conferred by using enfuvirtide may lead to some degree of decreased susceptibility to these agents. Therefore, in the future, it will be essential that resistance testing for this class of agents be incorporated into currently used assays to optimized the use of this agent in ARV-experienced patients (Hirsch et al., 2003).

2. Materials and methods

2.1. Plasma HIV RNA extraction and genotyping

The development of the assay and gp41 sequencing was carried out at the B.C. Centre for Excellence in HIV/AIDS, which routinely conducts RT and PR gene sequencing procedures on HIV-1 isolates for most Canadian centres. To investigate gp41 sequence variation, standard RT/PR amplification and sequencing procedures were modified to target codons 1–326 of the gp41 region of HIV-1 (Alexander et al., 2001). Following automated or manual RNA extraction from plasma, a 978 base pair nested RT-PCR product generated from forward and reverse overlapping gp41-specific primers was generated for each isolate. Sequences were resolved on an ABI Prism 3100 or 3700 DNA Analyzer and compared to HXB2 wild-type clade B. The majority of sequences in this study encoded for 326 amino (acid) AA residues, although gaps were inserted in approximately 10% of sequences to maintain alignment.

2.2. Patient populations

The gp41 genotype assay was tested in two independent patient populations. To assess the sequence variation in the gp41 region between HIV-1 clades, the assay was tested on a cross section of 404 frozen, archived plasma viral sequences from enfuvirtide-naïve patients enrolled in the B.C. Centre for Excellence in HIV/AIDS Drug Treatment Program.

To assess the development of new gp41 mutations, the assay was used on baseline and serial plasma viral sequences from 41 Canadian patients who had virologic failure on an enfuvirtide-containing regimen. Virologic failure was defined as the failure to achieve full virologic suppression with a plasma viral load <50 copies/ml on an enfuvirtide-containing regimen, or virologic rebound to >500 copies/ml following full virologic suppression despite drug continuation. To be included in the analysis, patients must have had a baseline viral load, fulfil the criteria for virologic failure and have a minimum of 1 month follow-up while receiving enfuvirtide. The patients were identified from 13 centres in 10 Canadian cities and had received enfuvirtide either from the phase III TORO-1 trial (Lalezari et al., 2003) or from the enfuvirtide expanded access program. Patient follow-up occurred approximately 1 month after starting the new enfuvirtide-containing regimen and then every 3 months thereafter, as long as enfuvirtide was continued.

2.3. Statistical analysis

Statistical analysis was performed using SAS Version 8.2 statistical software (SAS Institute, Cary, NC, USA). Baseline and plasma virus sequence characteristics were summarized with medians and interquartile ranges for continuous variables and proportions for categorical variables. In the enfuvirtide-naïve

Table 1
Baseline characteristics of enfuvirtide-naïve patients and patients failing enfuvirtide

Covariate	Enfuvirtide-naïve patients ($N=404$)	Patients failing enfuvirtide ($N=41$)
Age[a]	37 (32, 43)	44 (40, 50)
Male	84%	95%
MSM	Unknown	85% (33/39)
Previous ARV treatment	25%	100%
Previous PI treatment	19%	100%
Previous NNRTI treatment	3.6%	100%
Number of previous ARV drugs[a]	Not available	12 (11, 15)
Number of previous ARV regimens[a]	Not applicable	6 (5, 9)
Baseline pVL $\log_{10}$/ml[a]	5.1 (4.7, 5.6)	5.1 (4.7, 5.5)
Baseline CD4/μl[a]	270 (100, 420)	52 (24, 122)

MSM, men who have sex with men; ARV, antiretroviral; PI, protease inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitors; pVL, plasma viral load.
[a] Median (interquartile range).

population, differences at AA residues, as well as multiple base pair insertions were compared between clades, with particular attention to the fusion peptide, HR-1, HR-2 and cytoplasmic domain regions of gp41. Mutations detected in three or more patients (≥7.3%) are reported and considered potentially clinically significant that developed in the population experiencing virologic failure on ENF were significant, we defined significant mutations as those that were found in three or more patients (≥7.3%). Sequence changes with a prevalence >5% in the enfuvirtide-naïve population and sequence changes present at baseline in the patients experiencing treatment failure were considered polymorphisms and not significant mutations. Finally, box plots of viral load were used to describe the distribution of $\log_{10}$ viral load after acquiring increasing numbers of gp41 mutations. Since individuals often had multiple viral load measurements at the same number of mutations, the mean $\log_{10}$ viral load was calculated for each individual for each number of mutations before creating the box plots.

3. Results

3.1. Variation of gp41 sequence in enfuvirtide-naïve patients

The assay was robust, reproducible and valid. Baseline gp41 sequence was obtained for 404 enfuvirtide-naïve patients. The baseline characteristics of these patients are listed in Table 1. The majority of these patients were ARV-treatment naïve and were male (Table 1). Of the 404 plasma viral sequences, 340 contained clade B virus and 64 were typical of non-B clade, including clades C ($n=20$), A ($n=18$), D ($n=10$), AG ($n=8$), AE ($n=4$), G ($n=2$), F ($n=1$) and J ($n=1$).

The 10 amino acid region comprising positions 36–45 and GIV regions of gp41 were highly conserved in the viral sequences derived from the naïve patients relative to the HIV HXB2 reference strain. Only mutations N42S (11.3%) and V38G (0.6%) were detected at a prevalence above 0.5% within this motif.

Sequence variations occurred across the entire gp41 region both within and across various HIV clades. In the clade B sequences, 38.7% (127/328) of the codons were highly conserved with <1% variation, 22.6% (74/328) were partially conserved with 1–5% variation and 38.7% (127/328) were variable with >5% variation. Polymorphisms were observed to varying degrees among non-B clade plasma virus sequences, ranging from a low of 53 natural substitutions in clade D to a high of 76 in clade A. Among clade B plasma virus sequences, 32 substitutions qualified as natural polymorphisms. Overall, amino acid substitutions were observed with greater than 50% frequency in clades A, C, and D, compared with <5% frequency in clade B, with much of the clade specific variation occurring in the cytoplasmic domain.

Amino acid insertions occurred at positions 3, 105, 215, and 276 with variable prevalence across the clades (range 0–90%). In particular, a seven amino acid insertion at position 276 of the Helix 2 region of the cytoplasmic domain which would result in an additional two "turns" of a helix conformation was identified in 90%, 71% and 13% of subtypes A, C and D plasma virus sequences, respectively. In contrast, this substitution was not observed in any clade B isolates. The significance of this observation is unclear, but is perhaps notable that there may be an association between substitutions at this position in clade B virus (usually H for R) and gp-120 mutations.

3.2. Gp41 mutations in patients with virologic failure to enfuvirtide

Of 90 patients who started enfuvirtide-containing ARV regimens in Canada during the study period for whom clinical data and viral sequences were forwarded to the study centre, 49 did not meet the inclusion criteria of the study. Specific reasons for exclusion included no baseline samples for sequence analysis ($n=23$), did not meet the pre-specified criteria of virologic failure ($n=18$) and did not have follow-up for 1 month or longer ($n=8$). The baseline characteristics of the remaining 41 patients are summarized in Table 1. All 41 plasma sequences contained

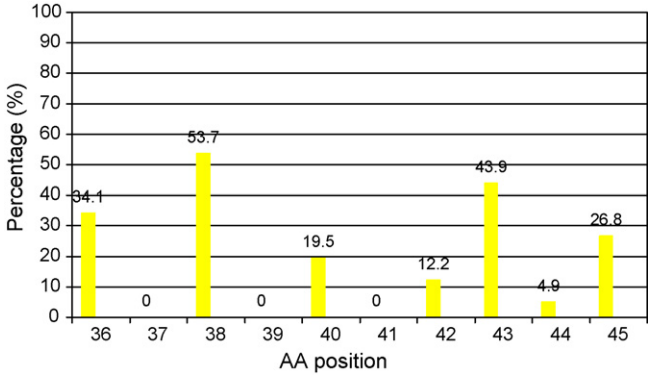

Fig. 1. Distribution of gp41 mutations at 36–45 after virologic failure. Mutations in amino acids 36–45 were identified in plasma virus from all 41 patients, particularly at positions 36D, 38A, 38M and 43D.

Table 2
New gp41 mutations selected after virologic failure on ENF present in >3 patients

gp41 codon	Number of patients failing ENF with substitutions	Number of ENF-naïve patients with substitutions[a]
24	6 (14.6%)	294 (72.8%)
33	**4 (9.8%)**	5 (1.2%)
73	**3 (7.3%)**	3 (0.7%)
75	**3 (7.3%)**	14 (3.5%)
90	3 (7.3%)	38 (9.4%)
113	4 (9.8%)	285 (70.5%)
126	**7 (17.1%)**	13 (3.2%)
135	3 (7.3%)	157 (38.9%)
137	3 (7.3%)	137 (33.9%)
138	**6 (14.6%)**	18 (4.5%)
140	3 (7.3%)	88 (21.8%)
321	6 (14.6%)	204 (50.5%)
322	5 (12.2%)	235 (58.2%)

ENF, enfuvirtide. Bolded are the likely clinically significant mutations.
[a] Represents the number of patients of the 404 ENF-naïve population that had this substitution present; we assume that a substitution is a polymorphism if it is present >5% in this ENF-naïve population

clade B virus. The median duration of follow-up and median number of viral load samples per patient were 14.5 months (interquartile range, 11, 22) and 11 months (interquartile range, 7, 17), respectively. Fig. 1 shows the frequency of observed 10 AA substitutions at positions 36–45. Mutations in amino acids 36–45 were identified in all 41 plasma virus sequences, particularly at positions 36D, 38A, 38M, 42T and 43D (Table 2). The most common observed change was observed at position 38. Other amino acid substitutions of unknown clinical significance within this domain were identified in plasma virus derived from 13 patients relative to baseline sequences, including 36A, 36V, 38E, 38V, 40H, 40P, 40T, 42D, 43H, 43K, 43S, 44M and 45M. Furthermore, specific amino acid substitutions that were potentially significant according to our definition were observed at positions 33, 73, 75, 126 and 138. A summary of previously identified mutations known to reduce viral susceptibility to enfuvirtide and potentially significant mutations identified in our study is provided in Table 3 (Mink et al., 2005).

Table 3
Summary of previously described enfuvirtide mutations (Mink et al., 2005) and new gp41 mutations selected after virologic failure on enfuvirtide present in >3 patients

Amino acid substitutions	Fold change in enfuvirtide EC_{50}[a]
G36D	7.6
G36E	39.3
G36S	7.3
V38A	16
Q40H	21
N42T	3.8
N42S	0.5
N43D	18
N43S	5.6
N43K	5.3
L44M	1.8
L45M	1.4
G36S + L44M	15
N42T + N43K	32
N42T + N43S	61
V38A + N42D	140
V38A + N42T	149
V38E + N42S	513
L33V/F/M	
E73D	
Y75F/H	
N126K/S	
S138A/M	

Substitutions in bold are those that were observed in our study to be of potential clinical significance, for whom phenotypic correlation is required to determine extent of impact on viral susceptibility to enfuvirtide.
[a] Changes in EC_{50} reported in previously published studies.

Table 4
Median time to development of sequential gp41 mutations (AA36–45)

Number of mutations	Proportion of patients with this number of mutations	Median time to developing the subsequent mutation (days)
1	40 (97.6%)	63
2	24 (58.5%)	21[a]
3	12 (29.3%)	49[a]
4	3 (7.3%)	28[a]
5	1 (2.4%)	0[a]

[a] Time from previous mutation.

The distribution of the number of cumulative mutations that were observed in the 36–45 amino acid domain while continuing a failing enfuvirtide-containing regimen, and the time to the appearance of subsequent mutations is summarized in Table 4. In the majority of cases, two or three mutations were observed in this area following virologic failure with enfuvirtide. The median time to the appearance of the first mutation was 2 months following the start of the enfuvirtide-containing regimen and subsequent failure; however, this was influenced by the timing of testing viral loads which is often done in 1 month intervals.

The box plots of mean $\log_{10}$ viral load after acquiring increasing numbers of gp41 mutations are shown in Fig. 2. The median viral load was 4.4 $\log_{10}$ copies/ml (IQR 3.8, 5.0), 4.2 $\log_{10}$ copies/ml (IQR 3.7, 4.9), 4.7 $\log_{10}$ copies/ml (IQR 4.2,

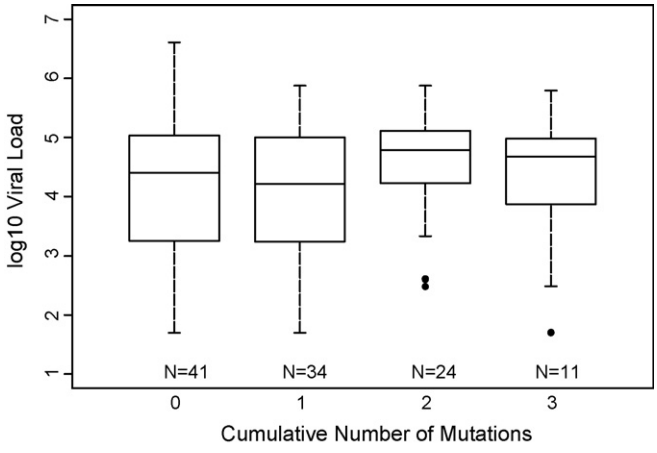

Fig. 2. Box plots of mean $\log_{10}$ viral load after acquiring increasing number of gp41 mutations. Median viral load for 0, 1, 2 and 3 mutations were 4.4 $\log_{10}$ copies/ml (IQR 3.8, 5.0), 4.2 $\log_{10}$ copies/ml (IQR 3.7, 4.9), 4.7 $\log_{10}$ copies/ml (IQR 4.2, 5.0) and 4.9 $\log_{10}$ copies/ml (IQR 4.5, 5.2), respectively.

5.0) and 4.9 $\log_{10}$ copies/ml (IQR 4.5, 5.2) for 0, 1, 2 and 3 mutations, respectively. For the 18 participants with viral load measurements after both 1 and 2 mutations, the median change in $\log_{10}$ viral load copies/ml was 0.2 (0, 0.5) (Wilcoxon signed rank $p = 0.02$).

4. Discussion

The efficacy and safety of enfuvirtide in the management of treatment experienced HIV-infected patients has been well described. However, as with other classes of ARVs, knowledge of baseline polymorphisms and mutations associated with acquired resistance to enfuvirtide will be essential to both the optimal use of this agent and similar derivatives that may become available at a later time. Early in vitro observations associated loss of enfuvirtide susceptibility with mutations at positions 36–38 of the GIV amino acid sequence found in the HR-1 region of gp41 (Rimsky et al., 1998). Subsequent in vitro and in vivo data expanded the spectrum of amino acid substitutions that could elicit enfuvirtide resistance. Specifically, single and multiple amino acid substitutions in positions 36–45 have conferred an average 5–10 and 100-fold decrease in enfuvirtide susceptibility, respectively, with certain substitutions resulting in >400-fold changes (Rimsky et al., 1998; Mink et al., 2005; Sista et al., 2004). The role of the 36–45 amino acid region in the development of enfuvirtide resistance has been further confirmed by an analysis of 292 patients experiencing virologic failure while receiving enfuvirtide in phase II and III clinical trials, in which 98.8% of viral isolates with greater than fourfold decreases in susceptibility had substitutions in this region (Melby et al., 2006).

Our findings corroborate the aforementioned data, in that plasma virus from all 41 patients failing enfuvirtide-based therapy had mutations in the key 36–45 amino acid sequence of gp41, with the majority of sequences demonstrating two or three mutations in this region. The sequential accumulation of gp41 mutations coupled with the observed increase in plasma viral load suggests that enfuvirtide continues to exert a selective pressure after the initial selection of a single key mutation. In addition, plasma virus from some patients developed mutations in other areas of gp41 both within and outside the common 10 amino acid region. Specifically, mutations at positions 33, 73, 75, 126 and 138 were observed and may be clinically significant, although phenotypic characterization of virus harbouring these mutations will be necessary to confirm their relevance to patient care. These novel mutations may have been observed in our cohort since patients remained on failing therapy for a median of 14.5 months. Finally, the results of our box plots of mean $\log_{10}$ viral load after acquiring increasing numbers of gp41 mutations would suggest the viral load increased by a median of 0.2 (0, 0.5) ($p = 0.02$) after the acquisition of 1 and 2 mutations, revealing that this development of gp41 mutations while receiving enfuvirtide is clinically significant.

Consistent with previous findings and available data from the Los Alamos Database (http://hiv-web.lanl.gov), primary mutations in the amino acid motifs critical for the development of enfuvirtide resistance were uncommon in patients naïve to this agent, further highlighting the important role played by this area in successful viral fusion. Since our assay was designed to evaluate most of the gp41 amino acid sequence, we were able to identify polymorphisms throughout gp41 outside the 36–45 amino acid region in both clade B and non-B clade plasma virus sequences, thereby adding to data describing gp41 sequence variation in non-B clade virus (Aghokeng et al., 2005; Roman et al., 2003). The clinical significance of these baseline substitutions, however, merits further study. For instance, while Chinnadurai et al. (2005) demonstrated a fivefold reduced sensitivity to enfuvirtide in non-B clade isolates with the single or multiple substitutions L33V, L54M/Q56K or L34M/L54M/Q56R, differences in baseline susceptibility to enfuvirtide did not correlate with clinical outcome in phase II and III clinical trials of this agent (Melby et al., 2006). Although there was no difference in response between clade B versus non-B virus in these trials, these data were limited by the comparatively small number of patients with non-B virus enrolled, rendering it difficult to draw firm conclusions about the impact of baseline substitutions on non-B virus susceptibility to enfuvirtide.

Our study is limited by the relatively small number of patients included in the analysis and lack of phenotypic corroboration of the observed mutations, making it difficult to draw firm conclusions regarding the clinical significance of the findings. More research is needed in the area of gp41 resistance especially correlating the observed genotypic mutations with phenotypic and observed clinical responses. Identification of these mutations is an important first step in the evaluation of resistance to the fusion inhibitors and in the development of reliable resistance assays that can be incorporated into clinical practice. An understanding of the genetics of resistance will also be important as new agents in this class are developed.

Conflict of interests

Some authors have received educational and/or research grants and have acted as consultants and on speaker bureaus for Hoffman LaRoche Canada (MRL, JSM, SLW).

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