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SHORT COMMUNICATION

In-depth analysis of compartmentalization of HIV-1 matrix protein p17 in PBMC and plasma

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SUMMARY

HIV-1 p17 plays an important role in the virus life-cycle and disease pathogenesis. Recent studies indicated a high heterogeneity of p17. A high number of insertions in the p17 carboxy-terminal region have been more frequently detected in patients with non-Hodgkin lymphoma (NHL), suggesting a role of altered p17 in lymphomagenesis.
Based on p17 heterogeneity, possible PBMC/plasma compartmentalization of p17 variants was explored by ultra-deep pyrosequencing in five NHL patients.
The high variability of p17 with insertions at the carboxy-terminal region was confirmed in plasma and observed for the first time in proviral genomes. Quasispecies compartmentalization was evident in 4/5 patients. Further studies are needed to define the possible role of p17 quasispecies compartmentalization in lymphomagenesis.

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The human immunodeficiency virus 1 (HIV-1) matrix protein p17 (p17) is a 17-kDa myristoylated protein derived from the extreme N-terminal region of the Gag precursor protein p55. This protein plays important functions including regulation of viral entry, intracellular trafficking and replication (Freed, 1998). For these reasons, p17 has long been considered a highly conserved primary amino acid sequence.
However, several studies have shown that consequences of mutations depend on where they are located. In fact, virions carrying mutations within the p17 amino(N)-terminal domain displayed severe impairments in infectivity, fusion, and envelope incorporation, whereas HIV-1 variants presenting mutations on the carboxy (C)-terminal region have unaltered fitness (Bhatia *et al.*, 2007; Casella *et al.*, 1997). On the other hand, recent findings from our group indicate that sequence variations occurring on the C-terminal region may confer functional properties of potential pathogenic relevance to p17 proteins (Giagulli *et al.*, 2011).
Furthermore, using high-resolution analysis performed with ultra-deep pyrosequencing (UDPS) we showed that the intra-patient diversity of p17 quasispecies is much higher than predicted previously. In particular, an unexpectedly high frequency of deletions and insertions was observed, all located in a restricted area of the C-terminal region (Giombini *et al.*, 2014).
Intriguingly, plasma virions from patients with non-Hodgkin lymphoma (NHL) showed a significantly higher p17 heterogeneity than those from patients without NHL, with a much higher frequency of variants with C-terminal insertions (Dolcetti *et al.*, 2015).
Compartmentalization of HIV-1 genomes between peripheral blood mononuclear cells (PBMC, proviral DNA sequences) and plasma (viral RNA sequences) is well-documented, and has generally been based on the analysis of env (Rozera *et al.*, 2014) and pol (Wang *et al.*, 2000) genome regions. In addition, compartmentalization between plasma and liver tissue has been described for gag and env (Blackard *et al.*, 2011). For all the genomic regions considered in these studies, three distinct patterns can be observed. In the first, plasma virionic RNA and cell-derived proviral DNA variants are completely intermingled; in the second, the quasispecies derived from two compartments are mixed but at least one statistically significant cluster of sample-associated variants is observed: in the third pattern, a complete or near-complete separation of plasma- and cell-derived variants is observed (Rozera *et al.*, 2014; Wang *et al.*, 2000; Blackard *et al.*, 2011).
However, no information is currently available on possible compartmentalization of the p17 quasispecies. The present study aimed to investigate by UDPS the possible compartmentalization of the p17 quasispecies in HIV-1 ge-

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nomes present in circulating (i.e. replicating) virions and in the proviral sequences archived in PBMC from patients with chronic HIV-1 infection not undergoing antiretroviral therapy.

The study samples were obtained from five HIV-1 patients enrolled at the Centro di Riferimento Oncologico (CRO), Aviano, Italy, and previously included in our recent study on the possible role of HIV-1 matrix protein p17 variants in lymphomagenesis (Dolcetti *et al.*, 2015). Subjects were selected based on the availability of paired samples of PBMC and plasma. Three were male, and the median age was 35.0 years (range: 31.0-43.0 years). All patients had diffuse large B-cell lymphoma (DLBCL) and were off antiretroviral therapy for various reasons at the time of sampling. Overall, the median viral load (range) was 85,163 (30,244-223,926) copies/mL and the median CD4 count (range) was 157.0 (16.0-560.0) cells/mm. A more detailed patient description was reported in Dolcetti *et al.*, 2015 (correspondence of patient's ID: pt 1, c104; pt 2, c105; pt 3, c106; pt 4, c112; pt 5, c120).

Total DNA was extracted from PBMC using the 'DNA blood' extraction kit (Qiagen). To maximize the genetic heterogeneity to be amplified and sequenced, for each DNA sample the amplicons from four replicate PCR were pooled, representing the content from $2x10^6$ to $9x10^6$ PBMC. Plasma HIV-1 RNA extraction, quantification and UDPS, carried out with the 454 Life Sciences platform (GS-FLX, Roche Applied Science, Monza, Italy), were performed as previously described (Giombini *et al.*, 2014).

The pipeline adopted for the bio-informatic analysis was the same as that described in Dolcetti *et al*. (Dolcetti *et al.*, 2015); only sequence clusters with a frequency >0.4% of p17 were considered. The evolutionary distance was calculated by determining the Hamming distance of amino acid sequences. For a good graphic visualization of phylogenetic trees, all reads with ambiguous portions were excluded from the dataset used for tree construction, and only sequences represented by at least two reads were considered. The maximum likelihood phylogenetic trees were then inferred with the MEGA program (v6) using the Jones-Taylor-Thornton (JTT) amino acid substitution model plus gamma; gaps were treated considering all sites. After the correction pipeline, the median number of sequences (coverage/sample) was 9,926 (range: 7,725-15,729) for plasma RNA and 10,977 (range: 7,878-15,194) for PBMC DNA. The intra-sample diversity of p17 protein, calculated by Hamming distance, was lower in circulating genomic sequences than PBMC proviral sequences [median (range): 385.0 (257.7-422.0) $x10^{-4}$ vs 434.2 (313.9-508.3) $x10^{-4}$ substitutions per site], although the difference was not statistically significant (p=0.22).

As illustrated in *Table 1*, one or multiple amino acid insertions were observed even at the same position in the C-terminus region of p17 from all samples, with frequencies ranging between 0.46% and 87.74%. Furthermore, in all patients, the insertions were observed at one or maximum two positions and, except for pt 2, they were detected either at different frequencies in viral RNA and proviral PBMC DNA, or only in one of the two samples, suggesting compartmentalized distribution. Positions 117-118 and 125-126 were the most affected by insertions and, in particular, insertion of multiple Ala residues at 117-118 was observed in proviral DNA, in agreement with what has been already reported for viral RNA (Dolcetti *et al.*, 2015). Interestingly, for three patients the cumulative frequency of all the insertions in a given position accounted for almost all the quasispecies components both in viral RNA and in proviral DNA (pt 1: 95.81% for RNA and 99.26 for PBMC; pt2: 100% for RNA and 98.78 for PBMC; pt5: 90.43% for RNA and 77.78 for PBMC).

In the light of these findings, phylogenetic trees were constructed to analyze viral compartmentalization between proviral DNA and circulating RNA genomes, and Mantel's test was performed for statistical support of the compartmentalization. Specifically, two dissimilarity matrices were compared: the first was based on Hamming genetic distance calculated between all plasma and PBMC variants for each individual patient; in the second matrix, the Hamming distances were replaced with 0 if the sequences were from the same compartment (e.g., plasma versus plasma or proviral DNA versus proviral DNA) and with 1 if the sequences were from distinct compartments (e.g., plasma versus PBMC or vice versa). In this test, *r*

Table 1 - *Intra-patient frequency of amino acid insertions in p17MA sequence.*

Patient ID	*Amino acid positiona*	*Insertion*	*Intra-patient frequency (%)*	
			RNA	*PBMC*[b]
Pt1	117-118	ATA		0.46
		QAAA	0.52	
		AAT	0.67	
		AATA		1.18
		AAAAA	1.27	
		AA	6.31	10.88
		AAA	32.50	86.74
		AAAA	54.54	
		Totalc	95.81	99.26
Pt2	114-115	AQP	7.52	5.53
		AQQAQQ	16.54	15.44
		AQQ	75.94	77.81
		Total	100	98.78
	125-126	S		2.70
Pt3	117-118	AAA	0.55	
		AA	3.96	24.92
		A	18.86	2.86
		Total	23.37	27.78
	125-126	TGNS	0.54	
Pt4	114-115	T		0.96
	117-118	A	40.09	
Pt5	115-116	Q	2.46	
	125-126	NS		0.47
		S	2.69	
		SS	87.74	76.78
		Total	90.43	77.25

aAmino acid positions are referred to the prototype genotype B strain BH10 (UniProtKB P04585), adopted as reference for this analysis.
bPBMC: peripheral blood mononuclear cell.
cTotal intra-patient frequency (%), calculated for each position, is shown.

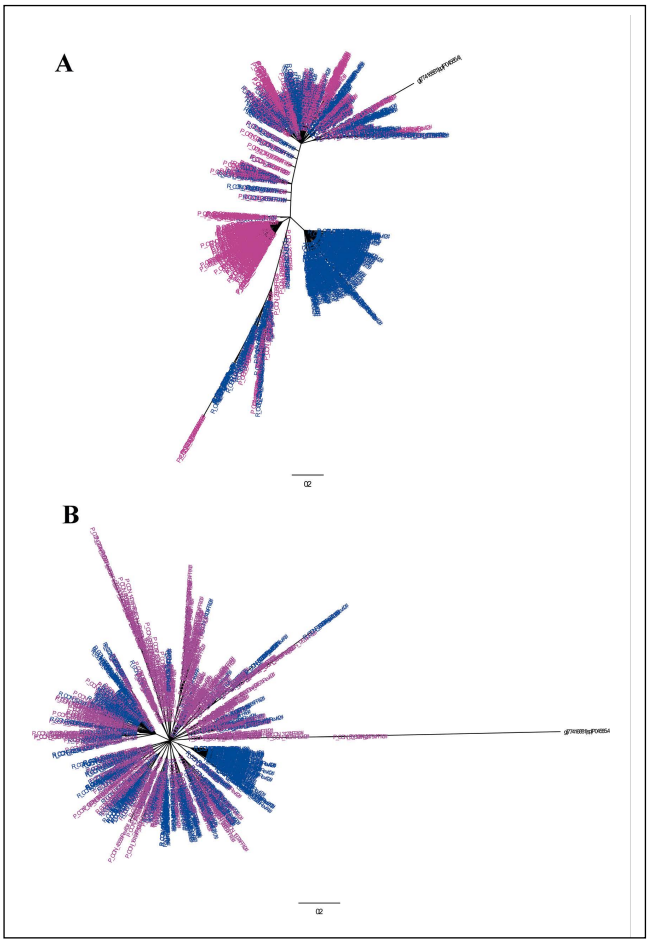

Figure 1 - *Phylogenetic trees built with p17 sequences obtained from plasma and PBMC of two representative patients.*
Panel A shows an example (pt1) of compartmentalization between RNA plasma sequences and PBMC DNA proviral sequences. Panel B shows phylogenetic tree from pt2 where the sequences from the two compartments are completely intermingled.
Colour code: HIV-1 plasma RNA (Blue); HIV-1 PBMC proviral DNA (Pink); prototype genotype B strain BH10 (UniProtKB P04585, Black) was adopted as reference for this analysis.

is the correlation coefficient between the two dissimilarity matrices. To assess the p-value of any apparent departure from r=0, the rows and columns of the first matrix were subjected to 1,000 random permutations, with the correlation coefficient r being recalculated after each permutation. The p-value of the observed correlation is the proportion of such permutations that lead to a higher correlation coefficient. Mantel test was computed with R software version 3.1.1 using Package Ecodist (Goslee and Urban, 2011).
Observing phylogenetic trees, for 4 out of 5 patients (i.e. pt 1, pts 3-5) the majority of quasispecies of the two compartments were intermingled, although distinct clusters could be distinguished. For these patients, the results of Mantel's test were consistent with a significant compartmentalization (pt 1, p=0.004; for pts 3-5 p=0.001). Instead, the phylogenetic tree of patient 2 showed plasma and PBMC variants completely intermingled, and lack of compartmentalization was confirmed by Mantel's test (p=0.11). *Figure 1* shows the phylogenetic trees of patient 1, representative of the first pattern (*panel A*), and of patient 2 (*panel B*).
This study, based on ultrasensitive evaluation of p17 quasispecies in HIV-1 infected patients with non-Hodgkin lymphomas confirms and extends the results of our previous studies (Giombini *et al.*, 2014; Dolcetti *et al.*, 2015), showing the presence of p17 quasispecies with multiple C-terminal insertions not only in the circulating plasma but also in proviral genomes harbored by PBMC. For both compartments, one or multiple amino acids were inserted, but in all patients but one the frequency and type of variants differed between two compartments, supporting compartmentalized p17 quasispecies.
HIV-1 compartmentalization has been shown for several genome regions, and is associated with clinical stage and pathogenetic events (Rozera *et al.*, 2014; Wang *et al.*, 2000; Blackard *et al.*, 2011). In the present study, we show that p17 quasispecies are also significantly compartmentalized between viral RNA circulating in plasma and proviral DNA archived in PBMC. This is in line with the concept that, while plasma harbors recently produced virus variants, PBMC harbor a combination of both recently produced and archived virus variants in integrated proviral DNA (Chun *et al.*, 1995; Chun *et al.*, 1997; Chun *et al.*, 1998).
Recently, p17 variants with insertions in the C-terminus portion were shown to possess enhanced B-cell growth-promoting and clonogenic activity (Dolcetti *et al.*, 2015), and this capability has been correlated to the pathogenesis of non-Hodgkin lymphomas, raising the hypothesis that p17 may act as a microenvironmental factor promoting lymphoma development, progression and metastasis.
Considering that the frequency of HIV quasispecies components harboring p17 insertions could positively impact on the extent of B-cell growth and clonogenicity, the compartmentalized distribution of these variants in cell populations that shuttle proviral genomes between blood and lymph nodes may play a major role in HIV-related lymphomagenesis.
However, several other factors contribute to the increased risk of NHL development in this setting, including the ability of HIV virions and of other HIV-encoded proteins to trigger and sustain the aberrant activation of B lymphocytes, which may express activation-induced cytidine deaminase, a DNA editing enzyme that can induce chromosome translocations involving oncogenes critical for lymphoma development. Unlike what occurs in the general population (Koff *et al.*, 2015), HIV-mediated immunosuppression may also lead to the loss of immunoregulatory control of oncogenic herpesviruses, such as EBV and KSHV (Epeldegui *et al.*, 2010; Shiels *et al.*, 2011). It is worth mentioning in this respect that EBV infection renders B cells permissive to the biologic effects of p17 and its variants through up-regulation of CXCR2 receptor expression (Martorelli *et al.*, 2015). Our findings stimulate further studies involving longitudinal samples and also investigating lymph nodes in order to better understand the possible role of HIV compartmentalization in the complex pathogenesis of HIV-related lymphomas.

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