Genetic variability in E6 and E7 genes of human papillomavirus -16, -18, -31 and -33 from HIV-1-positive women in Italy

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SUMMARY

Among 544 HIV-positive women screened for HPV-DNA between 2003 and 2005, 265 (48.7%) were HPV-positive: 24 (9.1%) harboured HPV-16, 21 (7.9%) HPV-31, 12 (4.5%) HPV-18, 7 (2.6%) HPV-33. E6 and E7 of these HPV types were sequenced to assess their diversity. Ranges of inter- and intra-variant diversity were 1.2-3-3%, and 0.8-1.8 for E6 and 0.6-2.7%, and 0.6-2.0% for E7, respectively. HPV-31, the second most common HPV type, showed the highest diversity for both regions. On the whole, 26 out of 59 mutations were non-synonymous. The variability of these proteins may have implications in HPV vaccine strategies.

KEY WORDS: Human Papillomavirus (HPV), Genotypes, Variants, HIV-1, Women

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Human papillomavirus (HPV) is the main aetiological factor in the development of squamous neoplasia of cervix (Walboomers *et al.,* 1999; Lillo, 2005; Menzo *et al.,* 2007).

On the basis of their oncogenic potential, HPV types that infect the genital tract are classified as low risk (LR) and high risk (HR). HR-HPV types (including HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -68) account for 93% of infections in squamous intraepithelial lesions (SIL) and invasive cervical cancers (Walboomers *et al.,* 1999; Franco *et al.,* 1997). The E6 and E7 proteins, able to bind tumour suppressor gene products, namely p53 and retinoblastoma (pRb), are the major oncogenic proteins involved in HPV carcinogenesis (Wainberger *et al.,*1991; Farthing *et al.,* 1994).

Despite abundant data on the molecular mechanisms of HPV-associated carcinogenesis *in vitro*, the pathways leading to the development of cervical neoplasia *in vivo* are not fully understood. It is generally assumed that the immune system represents an important factor in the control of HPV-related cervical neoplasia. In fact HPV-associated cervical and anal cancer rates are elevated in patients with AIDS (Einstein, 2004). Several studies showed the association between HIV-related disease progression markers, i.e. HIV RNA level and CD4+ T cell count, and neoplasia development (Harris *et al.,* 2005; Strickler *et al.,* 2005). However, very little information is available on HPV oncoprotein variants and their association with discariosis onset (ASCUS, L-SIL, H-SIL). To date, the most extensive studies on HPV variants in HIV-infected women focussed on HPV-16 (Chaturvedi *et al.* 2004; Schlecht *et al.*, 2005; Gheit

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et al., 2006), while data on the prevalence of the other HR HPV types and their variants, particularly in the Italian population, are lacking. In this study we describe the prevalence of HPV-16, HPV-18, HPV-31, HPV-33 in HIV positive women cared for at the L. Spallanzani National Institute of Infectious Disease (INMI). In addition we analyzed the genetic variability inside E6 and E7 in these HR HPV genotypes. With this purpose 544 HIV-positive women over 18 years of age (median 36.8, IQR 32.2-42.3) were screened at INMI between 2003 and 2005. The study was approved by the ethical committee of L. Spallanzani INMI and all individuals signed an informed consent form and underwent a structured interview.

Exfoliated cervico-vaginal cells were tested for the presence of HPV DNA using a well-established PCR protocol that amplifies a highly conserved 450bp segment in the L1 viral gene (Manos *et al.*, 1989). Two hundred and sixty five women (48.7%) were found HPV-positive, including 45 with multiple HPV and six with undetermined HPV types. Among single infections, 24 (9.1%) harboured HPV-16, 12 (4.5%) HPV-18, 21 (7.9%) HPV-31, 7 (2.6%) HPV-33. These data confirm that HPV-31 is the second most prevalent HPV type in Italian women (De Francesco *et al.*, 2005; Rassu *et al.*, 2005), as reported in other European countries (Johnson *et al.*, 2003; Speich *et al.*, 2004; Beerens *et al.*, 2005). Among women with typed HPV, 37 (18 HPV-16, 3 HPV-18, 12 HPV-31, 4 HPV-33) had a stored sample in sufficient amount to perform sequence analysis for at least one oncoprotein. The median (IQR) age of these women was 38.6 [33.4-43.3] years, and the median (IQR) CD4+ T-cell count was 280.5 (159.9-529.3) cells/µl. The entire region of E6 and E7 of 16, 18, 31, 33 HR genotype were amplified and sequenced on both strands, using Big Dye fluorescent dye-terminator chemistry (Applied Biosystems). The PCR was performed using GenAmp PCR System 2700 with AmpliTaqGold DNA polymerase (Applied Biosystems) according to the manufacturer's instructions, using 50 µl reaction volume. The reactions for HPV-16/HPV-18 and HPV-31 were the following: an initial step for the Taq ac-

TABLE 1 - *Primers used for the amplification of E6 and E7 of human papillomavirus (HPV)-16, -18, -31 and -33.*

Primer	*Location, nt*	*Sequence, 5'→3'*	*Amplicon length, bp*
HPV-16F-E6	41-55	ATCGGTGAACCGAA	539
HPV-16R-E6	579-563	AGGTGTATCTCCATGCA	
HPV-18F-E6	69-100	TGTATATAAAAGATGTGAGAAACACACCACAA	632
HPV-18R-E6	700-672	TTCTTCCTTGAGTCGCTTAATTGCTCGT	
HPV-31F-E6	50-80	GAACCGAAAACGTTGGTATATAAAGCACT	561
HPV-31R-E6	610-580	AGGTTGCAAATCTAACACATAGTCTTGCAAC	
HPV-33F-E6	49-68	GTTCAACCGAAAACGGTGCA	529
HPV-33R-E6	577-556	CTCATGGCGTTTTTACACGTCA	
HPV-16F-E7	483-500	ATATAAGGGGTCGGTGGA	429
HPV-16R-E7	911-895	TTACATCCCGTACCCTC	
HPV-18F-E7	528-551	TGCAACCGAGCACGACAGGAACGA	504
HPV-18R-E7	1031-1009	CATTTTCGTCCTCGTCATCTGAT	
HPV-31F-E7	509-637	ACGTTGCATAGCATGTTGGAGAAGACCTC	398
HPV-31R-E7	906-879	ATTGCATCCCGTCCCCTCCCCATCTGTA	
HPV-33F-E7	536-558	GACGTAGAGAAACTGCACTGTGA	372
HPV-33R-E7	907-887	GCCCCATTTGTACCTTCAGGA	

Nt = nucleotide. The nucleotide positions refer to the corresponding prototype.

tivation: 94°C for 17 min, 35 cycles (94°C for 1 min, 58°C for 50 s and 72°C for 1 min), and an extension step of 72°C for 7 min. For the HPV-33 amplification the unique variation was an annealing temperature of 45°C. For HPV-31 a nested PCR was focused. Primers were designed using the *genefisher* utility (http://bibiserv.techfak.uni.bielefeld.de/genfischer). The GeneBank reference sequences used were: HPV-16: NC001526 (Seedorf *et al.,* 1985), HPV-18: NC_001357 (Cole *et al.,* 1987), HPV-31: NC_001527 (Goldsborough *et al.*, 1989) and HPV-33:M12732 (Cole *et al.,* 1986). The primer sequences and the relative positions are listed in Table 1.

Compared to prototype HPV sequences, neither frame shifts, nor premature stop codons, nor insertions or deletions were observed in all analyzed sequences. The detected mutations are summarized in Table 2.

For HPV-16, 8/16 patients (50.0%) harboured viruses containing at least one nt variation in E6. Maximal divergence from the prototype was 8 (1.7%), and maximal intervariant diversity was 15 nucleotides (3.1%). Variant 350G (L83V) was the most frequent (n=5), and was not associated only with HSIL or cervical cancer. No base changes were identified in the splice donor or in the two splice acceptor sites (Los Alamos National Laboratory Bioscience, 1997, information available on line at http://hpv-web.lanl.gov). A synonymous variation at nt 325 (T→C), not previously described, was observed in one patient.

E7 sequences identical to prototype were found in 12/13 isolates analyzed, while one patient showed a variant with two synonymous nucleotide substitutions (intravariant and intervariant divergence =0.6%).

For HPV-18 E6, maximal divergence from the prototype and maximal intervariant diversity were represented by 7 nucleotides (1.5%), only one leading to aminoacid substitution (N129K). In E7, maximal divergence from prototype was 4 (1.2%) and maximal intervariant diversity was 5 nucleotides (1.5%). Only two non-synonymous mutations (H2Y and N92S) were observed, both in the same patient (P629) with normal pap smear.

For HPV-31, only 3 out of 12 had nucleotide sequences identical to the reference in E6. Maximal divergence from the prototype was 8 (1.8%) and maximal intervariant diversity was 15 nucleotides (3.3%), 8 of which were non-synonymous. Multivariants were found in 9 cases, seven with normal cytological pattern and only 2 presenting with L-SIL (P78, P1160). The K123R substitution, located in one of the putative sites for p53 binding (Gagnon *et al.,* 2005), was found in 4 patients, two of them (P322, P726) with a normal cervical smear, one with ASCUS (P724) and one with L-SIL (P78). In E7 a polymorphic site (nt 573) not previously described, was found. Maximal divergence from the prototype and maximal intervariant diversity were 6 (2.0%) and 8 nucleotides (2.7%) respectively. In particular, one aminoacid substitution was observed at position 23 (H23Y), leading to a complete homology with the DLYCYE motif of HPV-16, the putative binding domain of retinoblastoma protein (Gagnon *et al.,* 2005). The extent of variability of HPV-31 in E6 and E7 was similar to that described among Canadian women (Gagnon *et al.,* 2005). We found 2 variants in E6 (R8S; R144L), and one in E7 (T5P), not shown in the women considered in the Canadian study.

For HPV-33 E6, all 4 samples had nucleotide sequence variations, as compared to the prototype. Maximal divergence from the prototype was 4 (0.8%) and maximal intervariant diversity was 5 nucleotides (1.2%). E7 region was studied in two samples (P232 and P72), both showing 2 nucleotide changes, both synonymous (intravariant and intervariant divergence =0.7%). E6 and E7 polymorphism was not associated with H-SIL, as previously described (Khouadri *et al.,* 2006).

In the overall distribution of HPV variants, dyscaryosis was present in 12/15 (80.0%) cases with E6 and E7 wild type, and in 11/21 (52.3%) cases with non-synonymous mutations in E6 and/or E7 (odds ratio =3.6), but due to the small number of cases this difference was not statistically significant (p=0.159 in Fisher's exact test). The highest intervariant diversity value was found in E6 HPV-31 coding region. Due to the limited number of samples considered in this study, it is possible that the entire spectrum of possible viral variants were not analyzed, therefore further investigation is needed to appreciate the real extent of HPV variability and variant frequency in Italy. On the whole, our data indicate that genetic diversity in E6 and E7 of oncogenic HPV types frequently harboured by HIV-positive women in Italy is rather high. About half (i.e. 26/59) mutations were non-synonymous. HPV-31, the second

TABLE 2 - *Nucleotide and aminoacid changes identified in E6 and E7 from HPV-16, -18, -31 and -33.*

HPV-16			***E6***															***E7***	
		nt position	*82*	*83*	*84*	*132*	*143*	*145*	*241*	*256*	*261*	*286*	*289*	*307*	*325*	*335*	*350*	*789*	*795*
		aa position				*R10T*	*Q14D*				*V53A*					*H78Y*	*L83V*		
Patient number	*cytology*	*nucleotide in prototype NC_001526*	*G*	*A*	*A*	*G*	*C*	*G*	*T*	*C*	*T*	*T*	*A*	*G*	*T*	*C*	*T*	*T*	*T*
P343	L-SIL		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P403	H-SIL		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P385	H-SIL		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P223	Neopl		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	-	-
P56	L-SIL		-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-
P170	L-SIL		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P410	Norm		-	-	-	C	G	T	-	-	-	A	G	-	-	T	-	C	G
P41	L-SIL		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	-	-
P596	Norm		C	-	-	-	-	-	-	-	C	-	-	-	-	-	-	nd	nd
P600	H-SIL		-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-
P769	L-SIL		-	-	-	-	-	-	-	-	-	-	-	-	C	-	G	-	-
P799	H-SIL		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	nd	nd
P1161	L-SIL		-	C	C	-	-	-	G	-	-	-	-	-	-	-	-	-	-
P415	H-SIL		-	-	-	-	-	-	-	T	-	-	-	A	-	-	G	nd	nd
P318	Norm		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	nd	nd
P209	H-SIL		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P393	H-SIL		-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-
P42	nd		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

HPV-18						***E6***					***E7***			
Patient number	*cytology*	*nt position* / *aa position*	*251*	*266*	*374*	*485*	*491* N129K	*548*	*549*	*593* H2Y	*640*	*666*	*751*	*864* N92S
		nucleotide in prototype NC_001357	*T*	*G*	*G*	*T*	*C*	*A*	*C*	*C*	*C*	*C*	*C*	*A*
P299	L-SIL		-	-	-	C	-	-	A	-	-	-	-	-
P428	L-SIL		-	-	-	C	-	-	A	-	-	-	T	-
P629	Norm		C	A	A	C	A	G	A	T	T	T	-	G

HPV-31						***E7***																		
		nt position	*131*	*248*	*261*	*276*	*285*	*297*	*320*	*394*	*428*	*475*	*520*	*533*	*538*	*539*	*553*	*570*	*572*	*573*	*580*	*626*	*670*	*695*	*743*
		aa position	*R8S*		*I52L*	*D57N*	*H60Y*	*T64A*				*K123R*	*A138V*		*R144L*	*R144L*		*E4N*		*T5P*		*H23Y*		*E46K*	*K62E*
Patient number	*cytology*	*nucleotide in prototype NC_001527*	*A*	*T*	*A*	*G*	*C*	*A*	*A*	*G*	*A*	*A*	*A*	*A*	*G*	*T*	*A*	*G*	*A*	*A*	*G*	*C*	*C*	*G*	*A*
P355	Norm		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G
P366	ASCUS		-	-	-	-	-	G	-	-	-	-	T	-	-	-	G	-	-	-	A	-	T	A	G
P78	L-SIL		-	-	-	A	-	-	T	-	-	G	T	-	-	-	-	-	-	-	-	T	T	A	G
P378	H-SIL		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	T	A	G

P176	L-SIL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P45	Norm	-	-	-	-	T	-	T	A	G	-	T	-	-	-	-	-	-	-	A	-	T	A	G	
P253	Norm	-	-	-	-	T	-	T	A	G	-	T	-	-	-	-	-	-	-	A	-	T	A	G	
P322	Norm	-	-	C	-	-	G	T	A	-	G	T	-	-	-	-	-	-	-	-	-	T	A	G	
P1160	L-SIL	-	-	-	-	T	-	T	A	G	-	T	G	T	G	-	-	-	-	A	-	T	A	G	
P726	Norm	-	C	-	-	-	G	T	-	-	G	T	-	-	-	G	-	-	-	-	T	T	A	G	
P724	Ascus	-	C	-	-	-	G	T	-	-	G	T	-	-	-	-	A	C	-	-	T	T	A	G	
P1300	Norm	C	-	-	-	T	-	T	-	G	-	T	-	-	-	-	-	-	C	A	-	T	A	G	

HPV- 33			***E6***				***E7***		
		nt position	*181*	*213*	*283*	*364*	*480*	*737*	*862*
		aa position	*N25H*	*K35N*	*P59S*	*N86H*			
Patient number	*cytology*	*nucleotide in prototype M12732*	*A*	*A*	*C*	*A*	*A*	*A*	*A*
P101	L-SIL		C	-	-	-	-	nd	nd
P72	Norm		C	-	-	C	T	G	T
P396	Norm		-	-	-	-	-	nd	nd
P232	Norm		-	C	T	C	T	G	T

n.d.: not done. First row: HPV type and sequenced regions. Second row: nucleotide positions referred to prototype positions. Third row: aminoacid changes. Fourth row: nucleotides in prototype sequences. Only the mutated nucleotides are shown, whereas the invariant positions are marked with a dash. Cytology results are reported according to the 2001 Bethesda classification system (Solomon et al., 2002). ASCUS, Atypical squamous cells of undetermined significance; SIL, squamous intraepithelial lesion; L-SIL, High-grade SIL; Low-SIL, Low-grade SIL. GenBank accession number: EF422093-422127 for E6 region sequences; EF422128-422158 for E7 region sequences.

most common HPV type, showed the highest diversity for both regions.

It is still not known whether the immunity to one HPV variant can protect against infection from the other variants, thus recognition of HPV genetic diversity, particularly in specific clinical settings such as HIV infection, may have potential implications for vaccine strategies.

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