Genetic variability in E6, E7 and L1 protein of HPV81 from HIV-1 positive women in Italy

Claudia Minosse, Anna R. Garbuglia, Daniele Lapa, Catia Sias, Maria S. Zaniratti, Maria R. Capobianchi

Laboratory of Virology, "L. Spallanzani" National Institute for Infectious Diseases, Rome Italy

SUMMARY

The genetic variability of E6, E7 and L1 of HPV81 from HIV-1 positive women carrying multiple HPV infections was investigated by clonal analysis for E6 and E7. The range of maximal divergence from the prototype was 0.6%-2.6% for E6 and 1.0%-3.1% for E7. Compared to prototype HPV81, 13 and 10 mutations were identified in E6 and E7, respectively. In the pRB binding domain of E7, all HPV81 clones showed D21, as reported for prototype HPV81 and for HPV16 and 18, while G22 is reported in HPV6 and 11. In the CR3 region, CxxC motif was conserved in all but one clone.

The L1 sequence of a single clone from 5 study patients was also established. The range of similarity with prototype HPV81 was 97.8%-99.2%, with 25 polymorphic sites. Two substitutions (R492K and T493S) were observed in 5/5, one (T287N) in 4/5 patients. Among L1 immune-related regions, BC loop presented T56N in 1/5, while FGb loop presented T287N in 4/5 patients.

Our data indicate the presence of polymorphisms in all 3 HPV81 genes analyzed, with a certain degree of intra-patient diversity. The importance of polymorphisms on HPV81 persistence and pathogenicity needs to be addressed in longitudinal studies involving larger patient numbers.

KEY WORDS: Human papillomavirus, PCR sequencing, L1 gene, E6 gene, E7 gene, Variants

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INTRODUCTION

Cervical carcinoma is the second most common malignancy among women worldwide and human papillomaviruses (HPV) are associated with cancer of the uterine cervix, as well as with squamous intraepithelial lesions (SIL) (Bosch *et al.*, 1995; Walboomers *et al.*, 1999; Bosch *et al.,* 2002; Lillo, 2005; Menzo *et al.,* 2007). To date, more than 100 HPV genotypes have been classified. Of these, up to 40 different types infecting the genital tract are classified as "Low Risk" (LR) and "High Risk" (HR) on the basis of their oncogenic potential (Lorincz *et al.,* 1992; Muñoz *et al.,* 2003; Muñoz *et al.,* 2006). E6 and E7 proteins, which interact with p53 and pRB, respectively, (Weinberg, 1991; Farthing and Vousden, 1994), are considered the main agents responsible for the oncogenic properties of HPV.

Several studies have recently addressed the prevalence of HPV types in immunocompetent and immuno-suppressed patients both in the presence and absence of cervical lesions (Ellerbrock *et al.,* 2000; Riva *et al.,* 2005, Tornesello *et al.,* 2008a and b). However, genotyping of HPV-positive samples is achieved by a variety of methods, which may be more or less comprehensive in detecting the various HPV types. This can lead to underestimates of prevalence and miss-classification of multiple infections involving some HPV types. In particular, HPV81, included in commercial typing methods only recently, appear to be more frequent than HPV11 and 6 (Ronco *et al.,* 2005; Cerqueira *et al.,* 2007) and is often detected in multiple infections (Cerqueira *et al.,* 2007). In a recent study

Corresponding author
Maria Rosaria Capobianchi, PhD
Laboratory of Virology
National Institute for Infectious Diseases "L. Spallanzani"
Via Portuense, 292 - 00149 Rome, Italy
E-mail: capobianchi@inmi.it

it is the second most frequent type in HIV-infected Italian women (Tornesello *et al.*, 2008a). Although HPV81 is considered LR, few studies mention the possible association between this type and pre-cancerous or cancerous lesions (Cerqueira *et al.,* 2007; Zeng *et al.,* 2008).
A growing number of epidemiological, etiological and molecular data suggest that variants of the same HPV type are biologically distinct and may confer different degrees of pathogenic risk (Bernard *et al.,* 2006). However, little is known concerning the molecular variants of LR-types with limited diffusion in the world, since most data concern E6 and E7 variants in HPV16 and 18 (as HR) and HPV6 and 11 (as LR). Similarly, intratypic sequence heterogeneity has been extensively studied only in HPV16 and, to a lesser extent, in HPV18, while few studies have addressed other HPV types (Stewart *et al.,* 1996; Gagnon *et al.,* 2005; Bernard *et al.,* 2006; Garbuglia *et al.,* 2007; Tornesello *et al.*, 2008b). In particular, we have shown that genetic diversity in E6 and E7 of oncogenic types frequently harboured by HIV-positive women in Italy (i.e. HPV16, 18, 31, and 33) is variable (range: 0.6-6.0% for E6 and 0.0-5.1% for E7, respectively) HPV31, i.e. the second most common HPV type, being the most variable in both regions (*Garbuglia et al.,* 2007).
HPV81 is the sixth most common HPV type in the cohort of HIV-positive women followed at the National Institute for Infectious Diseases, Rome, Italy, and is frequently found in association with other HR of LR HPV types. This study investigated the genetic variability in E6, E7 and L1 proteins of HPV81 in 6 HIV positive women coinfected with other HR and LR genotypes.

MATERIALS AND METHODS

A total of 810 samples were collected from HIV-positive women attending the outpatient clinic at the "L. Spallanzani" National Institute for Infectious Disease Rome between 2004 and 2008 (08/01/04-08/11/05 and 11/02/07-12/09/08). The median [IQR] age of these women was 39.7 years [34.3-44.9]. The cervical cytobrush-Ayres spatula sample was used to prepare the Pap smears, immersed in a tube that contained 1 mL of PBS, and gently agitated. The liquid was then aliquoted in 2 tubes and stored at -80°C until use. One aliquot was used for the detection and typing of HPV, and the remaining aliquot was used for the present study. All patients provided signed, informed consent for the use of their samples for research purposes. The collection of personal data and behavioural information was obtained using a questionnaire. The study was authorized by the institutional ethical committee.
The samples were screened with a PCR protocol for detecting HPV DNA by using one set of L1 general primers (MY09/MY11) previously described, targeting a highly conserved 450 bp fragment (Manos *et al.*, 1989). The samples underwent DNA automated-extraction by QIAamp Blood kit (Qiagen, Chatsworth, CA, USA) using the automated BioRobot MDx Workstation (MDx, Qiagen). Control DNA (β-globin) was tested to assess the integrity of DNA.

TABLE 1 - *Primers used for the amplification of E6, E7 and L1 of HPV 81.*

Primer	*Location, nt**	*Sequence, 5'→3'*	*Amplicon length, bp*
HPV81-E6E7 OS	65-85	CGGTCGACCGGGAAGGATACA	750 bp
HPV81-E6E7 OA	924-903	AGCCTCCACCATAAACCACCCT	
HPV81-E6E7 IA	862-845	TGTACCTTCCACATCAGCCA	
HPV81-L1 OS	5647-5663	ACCACCGTTCCTTTGTC	1537 bp
HPV81-L1 OA	7478-7458	CAATACACATATAAATACAAC	
HPV81-L1 IA	5724-5745	AGCCCCTTCTATAGTCCCTTCG	

*Nucleotide positions refer to the prototype AJ620209.

TABLE 2 - *Anagraphic and clinical characteristics of HIV-positive women showing HPV81 as either single, or multiple co-infection.*

*Patients**	*Age (years)*	*Additional HPV types*	*Cytology/ histology*
P211	Not available	18, 31, 58, 61	High-grade SIL**
P363	29	18, 31, 58, 61	NA
P627	51	None	Low-grade SIL**
P644	28	18, 62, 52/33/ 35/58***	NA
P798	26	33	Low-grade SIL**
P802	31	18, 54, 62, 52/ 33/35/58***	inflammation
P822	34	None	inflammation
P1372	28	None	inflammation
P422	31	58, 66	NA
P574	35	18, 33, 59, 61, 72, 83	NA
P1796	34	None	Normal
P1815	31	None	Normal
P1898	45	None	Normal
P441	47	None	NA
P635	41	None	NA
P1053	24	None	NA

*Patients in bold were selected for this study. **SIL: squamous intraepithelial lesion. ***The characterization method used did not distinguish between the HPV types separated by a dash. ****NA: not available.

The positive samples were typed by restriction fragment length polymorphism (RFLP) analysis. Multiple infections were resolved by a commercial hybridization-based method (LINEAR ARRAY HPV Genotyping, Roche).

HPV81-specific primers to amplify E6-E7 and L1 (Table 1) were designed and applied to samples selected to include multiple HPV infections (Table 2). Optimal PCR conditions included each specific primer at 0.5 µM in 50 µl final reaction volume, containing 1.5 U of TaqGold DNA polymerase, 200 µM each dNTP, 10 mM Tris-HCl (pH 8.8), 75 mM potassium chloride and 1.5 mM Magnesium Chloride. Ten µl extracted DNA was added to each PCR tube. Amplification was performed using the GeneAmp PCR System 2700, programmed for TaqGold activation at 94°C for 15 min; followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 30 s, and extension at 72°C for 1 min, to amplify E6 and E7 region, while for L1 region the extension condition was 72°C 1 min 45 s. The last cycle included a final extension at 72°C for 7 min. A 5 µl aliquots of the first amplification round was again amplified in a 100 µl reaction mixture containing 0.5 µM of outer-sense primer and 0.5 µM of inner-antisense primer (Table 1) with the same cycling profile as above.

The E6-E7 hemi-nested amplicons from each patient were cloned as follows. Amplified products were purified using the QIAquick PCR Purification kit (Qiagen). For L1 region, a 70 µl aliquot from the final hemi-nested amplification was resolved by electrophoresis on a 1.5% agarose gel to screen the appropriate-sized product. The correct DNA fragment was excised from the gel, purified by MinElute Gel extraction Kit (Qiagen). The purified PCR products of E6-E7 regions were ligated into the pGEM®-T Easy vector (Promega, USA) and transformed in competent Escherichia coli cells using the One Shot TOP10 System (Invitrogen Life Technologies). Plasmid DNA was extracted using the QIAprep Miniprep kit (Qiagen). A total of 79 clones (for E6 4-10 clones/sample, from 6 samples; for E7 4-8 clones/sample, from 6 samples. For L1, one single clone/sample was analyzed, starting from the amplicons obtained from 5 patients.

Sequencing was performed with ABI Prism 3100, using the BigDye Terminator cycle sequencing kit (Applied Biosystems). Amino acid sequence data were aligned to HPV81 prototype (GenBank accession number AJ620209) using CLUSTALW algorithm. Base positions were numbered according to the 1997 sequence database (Los Alamos National Laboratory Bioscience, 1997, Los Alamos, NM, USA, information available on line at http://hpv-web.lanl.gov).

To identify the sequence variations of those L1 regions (loops) considered to be involved in immune recognition, the loop positions of HPV81 were deduced from the corresponding positions

in HPV16 L1, according to CLUSTALW alignment.

RESULTS

Of the 810 samples tested, 383 (47.3%) were found HPV-positive. Of these 16 (4.2%) harboured HPV81 (7 in coinfection with other viral types, see Table 2). Six specimens (in bold in Table 2) containing HPV81 in multiple infection were selected for this study.

E6 sequence

HPV81 E6 sequence variations are shown in Figure 1. On the whole, 13 mutated sites were identified in the E6 analyzed region of 154 amino acids, with a resulting mean genetic variability of 0.4%, compared to prototype HPV81 sequence. Of those sites, one (position 16 in Fig. 1) showed 2 substitutions in 2 different patients; two positions (present in the same clone) were changed to stop codons.

On the whole, each clone showed >90% identity with prototype sequence; at least one clone har-

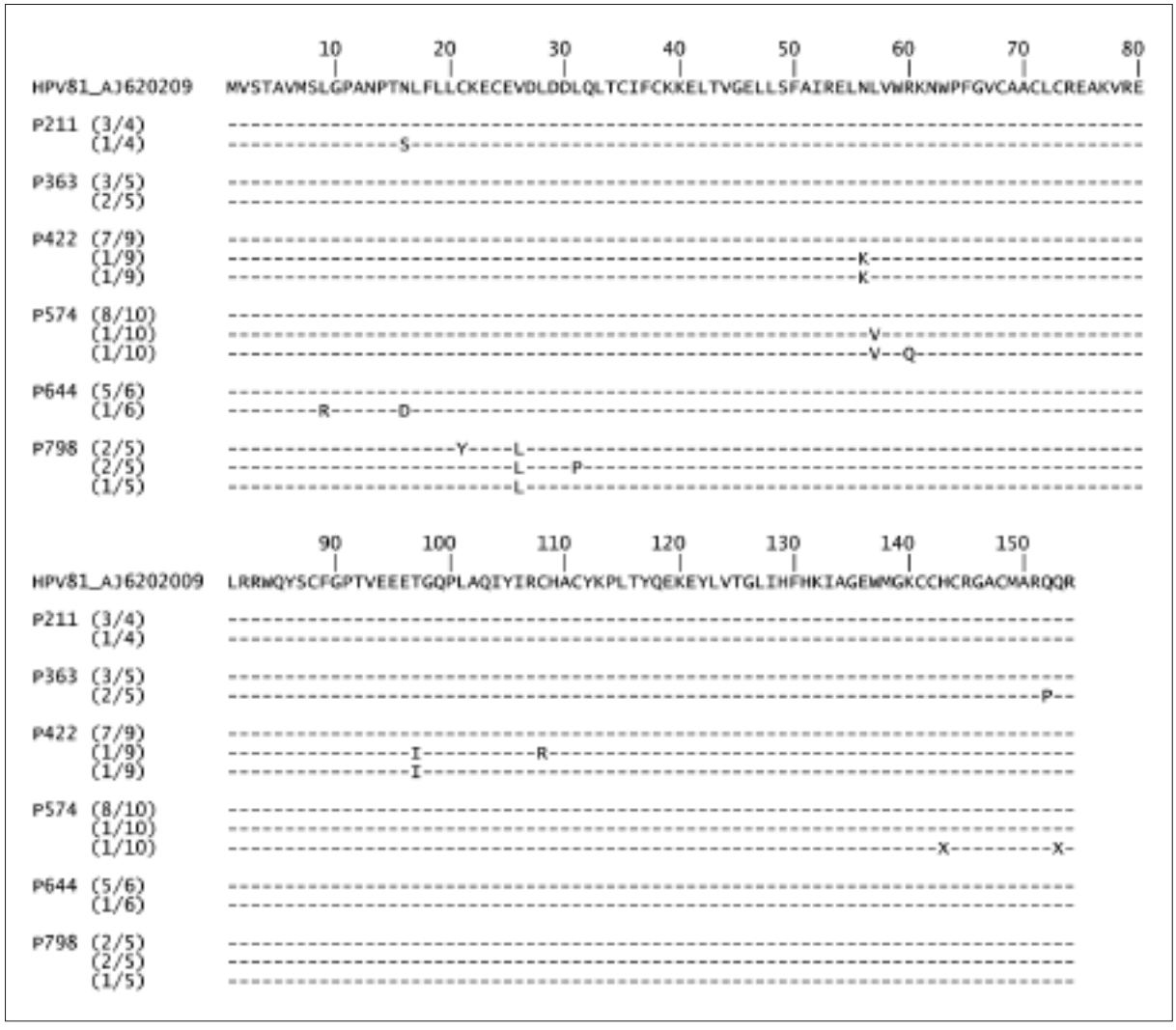

FIGURE 1 - *Alignment of deduced amino acid sequence of HPV81 E6 clones from 6 patients with mixed HPV infections (see Table 2 for patients' details). The patients from whom the sequences originated are indicated with the identification code. Relative frequencies of clones are shown in brackets. Dashes indicate amino acid sequence identity respect to the reference sequence. The nucleotide HPV81 E6 sequences of clones from the 6 patients have been lodged in the GenBank sequence database under accession number: GQ288717-GQ288754.*

bouring one or more amino acid changes in E6 protein was observed in all patients, with the highest number of amino acid changes in P798, who showed none of the clones identical to the prototype. For E6, 13 of 39 (33.3%) clones presented 1 to 4 amino acid variations, whose position was distributed according to a patient-specific pattern, resulting in maximal divergence with respect to the prototype ranging from 0.6% (observed in P211 and P363) to 2.6% (observed

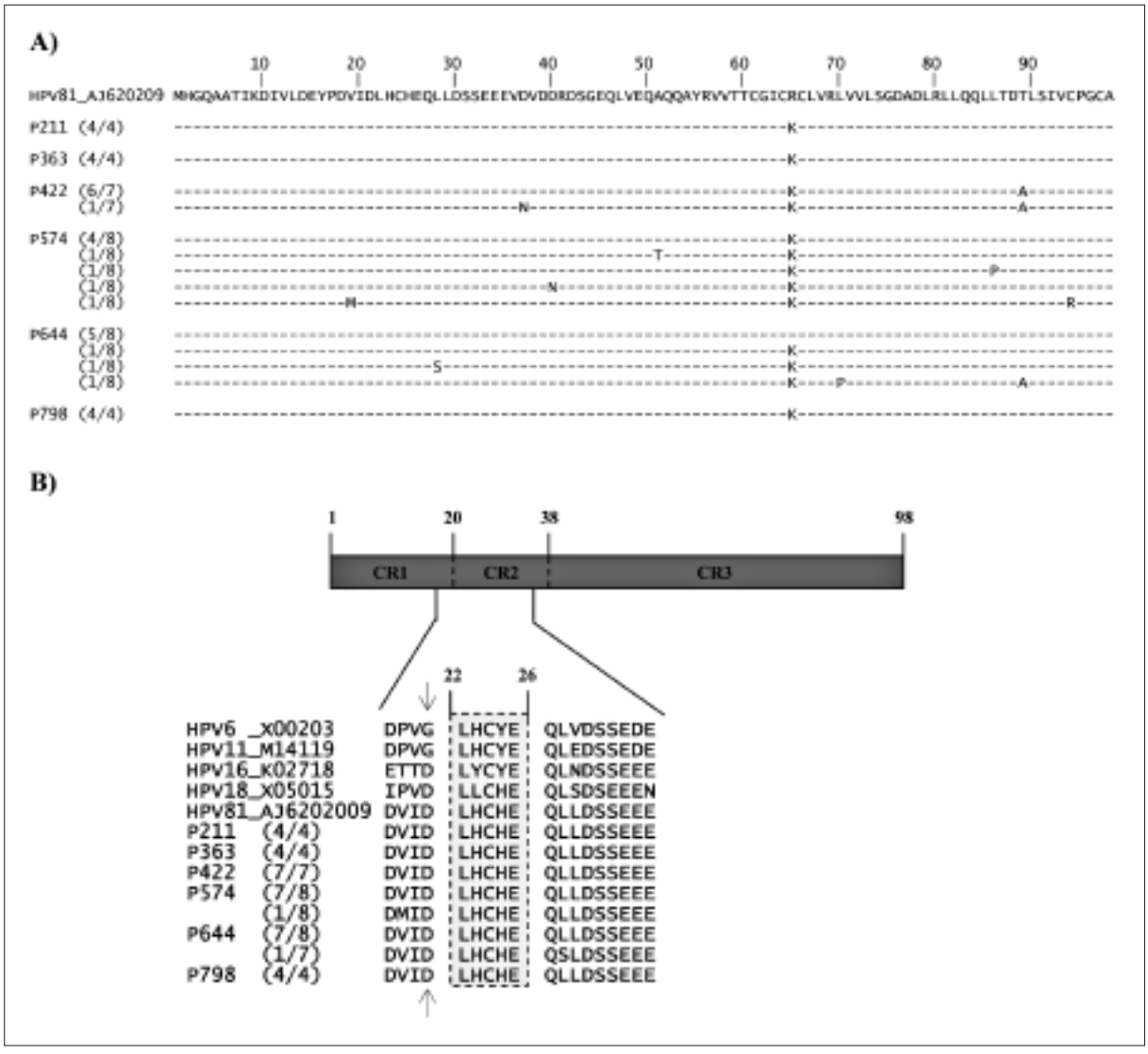

FIGURE 2 - *A) Alignment of deduced amino acid sequence of HPV81 E7 clones from 6 patients with mixed HPV infections (see Table 2 for patients' details). Relative frequencies of clones are shown in brackets. Dashes indicate amino acid sequence identity with respect to the sequence reference. The nucleotide HPV81 E7 sequences of clones from the 6 patients have been lodged in the GenBank sequence database under accession number: GQ288755-GQ288788. B) Individual sequences of the LXCXE domain (in the dashed box) in E7 conserved region 2 (CR2) of HPV81, from 6 patients with mixed HPV infections (see Table 2 for patients' details). For comparison, the amino acid sequences from reference HPV types, namely HPV6, 11, 16, 18 and 81, are shown. The numbering of the amino acid position is according to HPV16. The patients from whom the sequences originated are indicated with the identification code. The HPV prototypes used for the comparison are indicated with GenBank accession number. In the upper part of the figure, the position of the motif in the context of conserved regions 1, 2 and 3 (CR1, 2, and 3) of E7 is shown. The numbers indicate the borders of each conserved region.*

in P574). The detailed list of the observed mutations is the following: L9R (1/6 clones of P644), N16S (1/4 clones of P211), N16D (1/6 clones of P644), C21Y (2/5 clones of P798), V26L (5/5 clones of P798), L31P (2/5 clones of P798), N56K (2/9 clones of P422), L57V (2/10 clones of P574), R60Q (1/10 clones of P574), T97I (2/9 clones of P422), C108R (1/9 clones of P422), H143X (1/10 clone of P574), Q152P (2/5 clones of P363) and Q153X (1/10 clones of P574).

E7 sequence

HPV81 E7 sequence variations are shown in Figure 2A. The mean genetic variability in E7 was 1.3%, compared to reference sequence. Altogether, 10 amino acid changes were identified in the 98 amino acid-long E7 sequence, of which R65K was detected in all patients, with a clonal prevalence of 30/35 clones (85.7%). Five out of 8 clones of P644 showed 100% identity with the prototype; all clones from P211, P363,

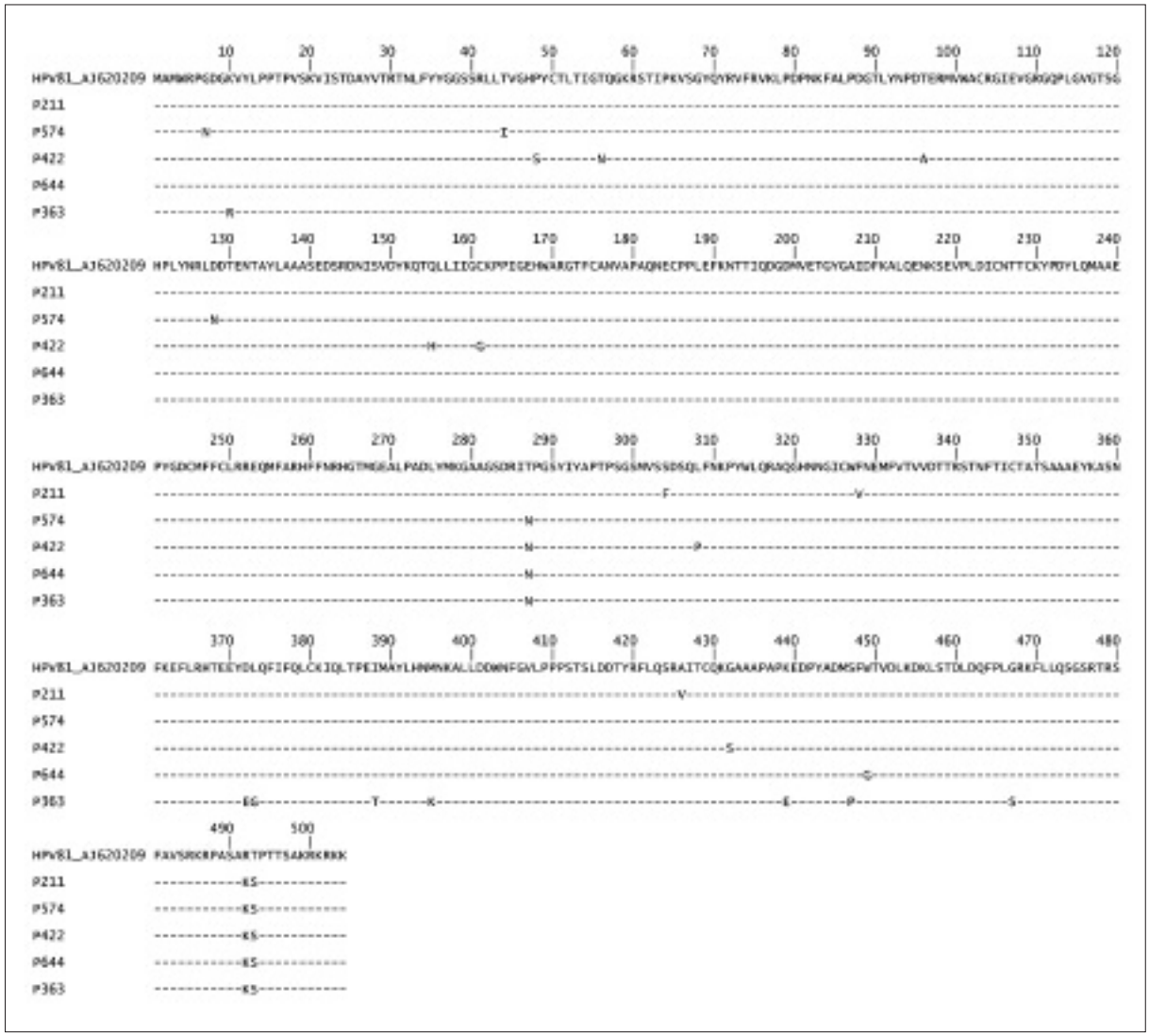

FIGURE 3 - *Alignment of deduced amino acid sequence of HPV81 L1 from 5 patients with mixed HPV infections (see Table 2 for patients' details). The patients from whom the sequences originated are indicated with the identification code. Sequences were aligned with HPV81 prototype AJ620209. Dashes indicate amino acid sequence identity respect to the reference sequence. Sequences were obtained from one cloned L1 fragment from each patient (see methods). The nucleotide HPV81 L1 sequences from the 5 patients have been lodged in the GenBank sequence database under accession number: GQ288789-GQ288793.*

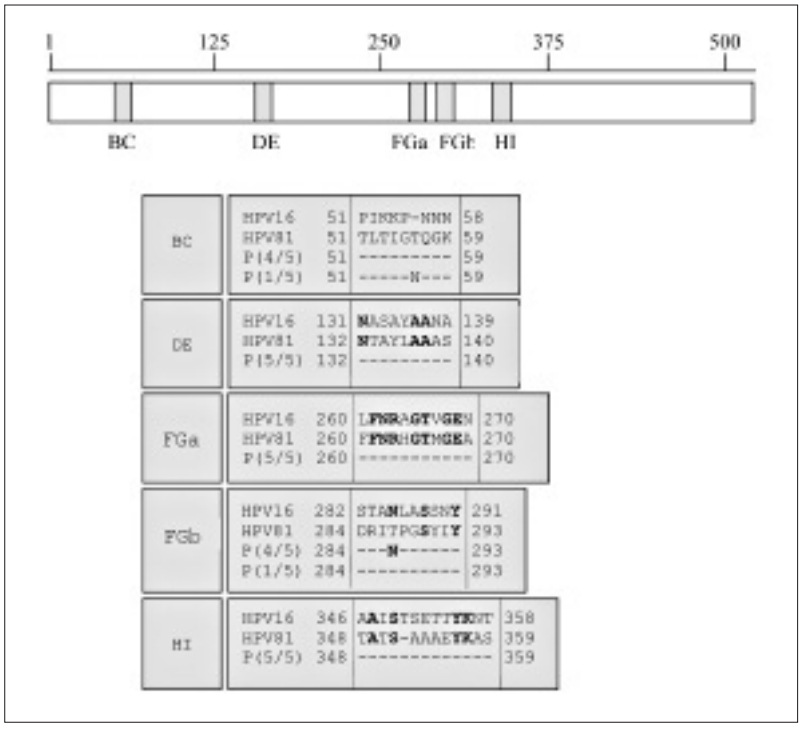

FIGURE 4 - *Variations in amino acid positions of surface-exposed loops of L1 protein of HPV81 in 5 patients with mixed HPV infections (see Table 2 for patients' details). The upper part shows the position of the loops in the context of the whole L1 protein. The boxes show the individual loops with patient sequences compared to prototype HPV81 and to the corresponding sequence of prototype HPV16. The identity of residues across patients and prototype HPV81 sequences are indicated by dashes. The conserved amino acid residues between HPV16 and HPV81 sequences are shown in bold.*

P798 E7 were identical, all showing only the R65K substitution (99.0% identity with respect to the prototype).

On the whole, each clone showed >90% identity with prototype sequence; 30 of 35 (85.7%) clones presented 1 to 3 amino acid variations, resulting in maximal divergence with respect to the prototype from 1.0% (P211, P363 and 798) to 3.1% (P422, P574 and P644).

The detailed list of the observed mutations is the following: R65K was observed in all clones from all patients, with the exception of P644, where it was observed in 3/8 clones; T89A (7/7 clones in P422 and 1/8 clones in P644), V19M (1/8 clones in P574), L28S (1/8 clones in P644), D37N (1/7 clones in P422), D40N (1/8 clones in P574), A51T (1/8 clones in P574), L70P (1/8 clones in P644), L86P (1/8 clones in P574) and C94R (1/8 clones in P574).

Figure 2B shows the comparison of the amino acid residues of HPV81 clones at position 22-26 with the homologous sequence or CR1/2 region of HPV16 and 18, corresponding to the pRB binding domain (LXCXE, amino acid residues 22-26 in HPV16). As in HPV81 prototype, all the clonal sequences analyzed in the present study confirmed the presence of D21, also shared by HPV 18 and 16 HR types. For reference, the corresponding position of LR HPV6 and 11 is represented by a glycine.

L1 sequence

The 504 amino acid sequence of L1 was obtained as a single clone sequence from 5 patients. It should be noted that none of the L1 sequences from the study patients were identical to those previously described. Comparison with the prototype L1 sequence revealed a mean similarity ranging from 97.8% (P363) to 99.2% (P644), with a total of 25 mutated positions over the 504 amino acid-long L1 sequence, with a resulting mean genetic variability of 1.4% compared to the reference sequence.

Among the 25 observed amino acid substitutions, 2 (R492K and T493S) were observed in 5/5 patients, and were also observed in 2 patients with HPV81 single infection (data not shown); one (T287N) was observed in 4/5 patients.

In addition, in the study patients we analyzed the sequence variations of those L1 surface-exposed regions considered, on the basis of the HPV16 information, to be involved in immune recognition. As shown in Figure 4, one mutation (T56N) with respect to prototype HPV81 was detected in the

BC loop from 1/5 patients, and one mutation (T287N) in the FGb loop from 4/5 patients.

DISCUSSION

To determine the prevalence and the genotype of HPV in HIV positive women attending the "L. Spallanzani" National Institute for Infectious Disease, we carried out a comprehensive study following a PCR protocol by using MY09/MY11 primers for L1 region. The HPV81 genotype resulted the sixth most prevalent HPV type (4.18%), according to previous data on HIV-coinfected women in Italy (Ronco *et al.*, 2005), and was associated with other LR and HR HPV types in a large proportion of cases (7/16, 43.7%).

Amino acid substitutions in viral genomes may affect virus assembly, carcinogenic potential and host immunologic responses. Moreover, it is still not known whether immunity to one HPV variant can protect against infection by another variant. Thus, identification of HPV genetic diversity in specific clinical settings may be important for the rational design of diagnostic, therapeutic and vaccine strategies (Stewart *et al.*, 1996). Very few studies have focussed on the genetic variability of HPV, mostly regarding HPV16, 18 (Bernard *et al.,* 2006; Garbuglia *et al.,* 2007; Tornesell*o et al.,* 2008b), and few regarding HPV31, 33, 35 (Calleja-Macias *et al.,* 2005a and b, Gagnon *et al.,* 2005; Garbuglia *et al.*, 2007). Gagnon and colleagues (*Gagnon et al.,* 2005) showed that the amino acid variants of HPV31 may be as frequent as 4.0% and 5.1% in E6 and E7, respectively. Calleja-Macias *et al.* (2005b) showed that the amino acid variability of HPV31 and HPV35 is 1.3% and 0.7%, respectively, in E6.

Recently, we showed that the amino acid variability of HPV16, 18, 31 and 33 varied between 0.6 and 5.7%, and between 0.0% and 5.1% in E6 and E7 respectively (Garbuglia *et al.,* 2007). In the present study we analyzed the genetic variability of E6, E7 and L1 regions of HPV81 harboured by patients infected with several HPV types. The sequence of E6 ORF revealed that it was highly conserved. The majority of the analyzed clones (26/39) showed no amino acid changes compared with the reference sequence. A single specimen (from P798) had clones with at least one amino acid variation. One clone of P574 presented two stop codons in the COOH end. Although our PCR protocol is optimized for high fidelity amplification of fragment with 2000 bp in length and the mutations are confirmed by sequencing both strands, PCR errors leading to artefact clonal diversity cannot be excluded. However, mere artefact results seem to be ruled out by the observation that only one amino acid mutation was found in 5/13 (38.5%) of mutated clones, 2 amino acid mutations in 6/13 (46.1%) clones, 3 changes in one clone and 4 in another clone.

The E7 sequences of 5/8 clones of P644 were identical to prototype sequence. The remaining clones had at least the R65K mutation. Moreover in some patients minor variants were also present, suggesting some degree of intra-patient polymorphism: V19M, L28S, D37N, D40N, A51T, L70P, L86P, C94R. T89A amino acid substitution was present in all clones analyzed of P422 and in 1 clone of P644.

The observation that in the motif LXCXE, considered fundamental for the interaction with the pRb, the sequences of all HPV81 clones were identical to that of the prototype, suggests low variability in this region. In particular, we confirmed the presence of aspartic acid (D) in position 21, that is also displayed by the HR HPV16 and 18, while in the corresponding position the LR HPV6 and 11 show glycine (G). Although this observation is far from elucidating the potential oncogenicity of HPV81 E7 protein, it emphasizes the need for further studies to evaluate the biological properties of this putative oncogene, particularly considering that HPV81, although considered LR, has been associated with pre-cancerous or cancerous lesions (Cerqueira *et al.,* 2007; Zeng *et al.,* 2008).

By sequence analysis of multiple clones for each patient, we detected the concomitant presence of several variants for both E6 and E7. Again we cannot rule out that the observed intra-patient variability may be due to artefacts during the PCR amplification, but we are rather confident that the phenomenon is real, as the presence of multiple intra-patient HPV variants has already been described, although its correlation with the persistence and progress of cytological damage has not been established (Bernard *et al.,* 2006; Sycuro *et al.,* 2008). The small size of samples and the non clonal analysis reported in literature consti-

tute the main obstacles to assessing whether the minor variants are the product of immune pressure or rather a genetic drift element. It would be necessary to study the persistence of the variants over time, and to correlate their presence and dynamics along with the clinical follow up to elucidate the pathogenetic relevance of intra-patient variability in E6 and E7. Thus conclusive consideration will emerge from studies when more detailed and wider molecular investigations are considered.

The data concerning the region L1 show that this region has a greater genetic variability than previously anticipated on the basis of published reports. This might be due to a lower immunological pressure in the HIV positive women analyzed or might be caused by a natural major variability of HPV81. All the isolates analyzed differ from the reference sequences in the amino acid positions 492 and 493 (arginine to lysine; threonine to serine). Those polymorphisms can represent signature substitutions in European specimens. In fact, each HPV type can be considered an isolated taxonomic unit, represented by a small number (up to 100) of closely related molecular variants. Related variants of any HPV type often cluster in specific parts of the world (Bernard *et al.,* 2006). Moreover we believe that these results may represent a starting point to study co-evolution HPV/host and to evaluate viral polymorphism in relation to geographical distribution. In fact, the distribution of divergent variants has been found to correlate frequently with geographic origin and ethnicity (Bernard, 1994).

The comparison of HPV81 L1 protein present in single infection with those found in multiple infections disclosed that the immunodominant epitopes were preserved both in the samples with single infections and in those with mixed infections (data not shown). This indicates that the high incidence of the HPV81 in multiple infections is not attributable to particular amino acid variations not neutralized by the antibodies toward other genotypes, but that in general the HPV81 needs a genotype-specific immune response to be neutralized. Currently the vaccines based on the virus-like particles (VLPs) composed of L1 protein target only few HPV types (Koutsky *et al.,* 2002; Harper *et al.,* 2004; Villa *et al.,* 2005), and the cross-neutralization among the HPV types has been observed only between those closely related (Christensen *et al.,* 1996; Combita *et al.,* 2002). Therefore, extended knowledge of the variability of neutralizing epitopes would be useful to deduce the possible cross-protecting activity of existing vaccines, and, eventually, to help in the design of more broadly protecting vaccines. This is particularly relevant for HPV81, that is very common among HIV-positive women, namely in Italy (Tornesello *et al.,* 2008a), and may be associated with pre-cancerous or cancerous lesions (Cerqueira *et al.,* 2007; Zeng *et al.,* 2008), particularly in immuno-suppressed patients.

In conclusion, our report shows that HPV81 exists in this Italian cohort in the form of intra-typic and intra-patient variants. Although it is generally recognized that patients can be infected by multiple genital HPV types, it is sometimes not appreciated that they can also carry concomitant multiple variants of the same HPV type (Ho *et al.,* 1991; *Xi et al.,* 1995). For this reason, further studies are needed to assess the persistence during the time could represent a factor influencing HPV oncogenic activity.

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The Authors declare the absence of any conflicts of interest.

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