L1 gene sequence of a putative novel type human papillomavirus in an immunocompetent patient with glans lichen sclerosus

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SUMMARY

The identification of a putative novel type human papillomaviruses (HPV) strain related to HPV-RTRX3 in a subject with penile skin warts and glans lichen sclerosus is reported.
A beta-HPV-RTRX3-like strain was detected in a immunocompetent patient with glans lichen sclerosus. HPV screening was performed by PCR in L1 gene. The MY fragment showed 99% nt identity with HPV-RTRX3 and 64.5% nt identity with HPV-37. The remaining part of the L1 gene showed similarity with HPV 80, 15, 17, and 37. Based on the presence of penile lichen sclerosus and the HPV-RTRX3-like strain found in our patient, a potential correlation was hypothesized.

KEY WORDS: Human Papillomavirus, L1 gene, Phylogeny, Recombinant variant, Penile lichen sclerosus.

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Human papillomaviruses (HPV) are common infectious agents in humans, and more than 150 HPV genotypes have been fully characterized. HPV may be associated with specific cutaneous and mucosal manifestations at multiple anatomical sites (Kovanda *et al.,* 2011; Cardoso and Calonje, 2011). HPV genotypes are grouped into five HPV genera according to their tissue tropism, genetic homology and other biological and clinical properties (Cardoso and Calonje, 2011). The phylogenetic relationship between HPV genotypes is based on the analysis of the complete L1 gene sequence, and classification of new HPV types implies at least 10% nucleotide sequence dissimilarity (de Villiers *et al.,* 2004; Bernard *et al.,* 2010). Notably, infection with some alpha-HPVs is associated with the development of cervical carcinoma and other mucosal cancers in the anogenital region of both genders (IARC, 2007; Bouvard *et al.,* 2009). On the other hand, infection by some beta-HPV is associated with cutaneous lesions in humans (de Villiers *et al.,* 2004).
In more detail, beta-HPV genotypes 3, 5, 8, 9, 10, 12, 14, 15, 17, 19 to 25, 28, 29, 36 to 38, 46, 47, 49 to 51 and 59 have been associated with a rare cutaneous disorder (epidermodysplasia verruciformis) in patients with congenital immunodeficiency (Jablonska and Majewski, 1994; Berkhout *et al.,* 1995), HIV-infected individuals and transplant recipients (Cardoso and Calonje, 2011). Moreover, skin tumors associated with beta-HPV have been reported in both immunosuppressed and immunocompetent subjects (Soler *et al.,* 1993; Stark *et al.,* 1994; Shamanin *et al.,* 1994; Tieben *et al.,* 1994). Interestingly, among the beta-HPV types associated with epidermodysplasia verruciformis, some belong to a subgroup of incompletely characterized HPV sequences (RTRX1-RTRX10) ob-

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served in skin lesions of renal transplant recipients (Berkhout *et al.,* 1995; Bens *et al.,* 1998).
The identification of an HPV strain closely related to HPV-RTRX3, but also showing genetic similarity with other beta-HPV in an immunocompetent subject with glans lichen sclerosus is reported.
Glans lichen sclerosus was observed in a 38-year old male immunocompetent patient during an outpatient visit. The patient was monitored during the period November 2011 to July 2012, and sequential urine and genital swab samples were analyzed. The study was approved by the Institutional Review Board of the Fondazione IRCCS Policlinico San Matteo according to guidelines on the use of biologic specimens for scientific purposes in keeping with Italian law (art. 13 Law 196/2003), and after having obtained written informed consent from the patient.
Viral DNA was extracted from urine samples, urethral swabs and swabs of the lichen sclerosus lesions using the automatic Easy Mag extractor (Biomerieux, Lyon, France). In detail, 1 ml of the first urine sample collected in the morning was examined, along with 1 ml resuspended volume of urethral swab material (Copan, Brescia, Italy). Initial typing of HPV was performed by sequencing a 455bp PCR fragment (nt 6680 to nt 7134) of the L1 gene using reported MY09 and MY11 primers (Gravitt *et al.,* 1998; Qu *et al.,* 1997; Sotlar *et al.,* 2004; Barzon *et al.,* 2011) (Table 1).
The β_2-microglobulin housekeeping gene was amplified in parallel as a control (Watzinger *et al.,* 2004). Moreover, three sequential positive urine samples were analyzed over a six-month period to confirm the persistence of the HPV strain, while urethral and lesions swabs were tested only at the beginning.
Two distinct amplicons of the MY L1 fragment from two sequential urine samples were cloned into the pCR 2.1 vector (TA Cloning Kit, Invitrogen, Groningen, The Netherlands). Single positive clones, were directly sequenced (Paolucci *et al.,* 2009). Nucleotide sequences of the MY L1 region obtained from 30 clones per specimen were aligned with the gene Bank reference strains using the Sequencer 5.0 (Gene Codes Corp., Ann Arbor, MI, USA) software program.
The complete HPV L1 gene sequence (nt 5583 to nt 7356) was obtained from urine samples, by generating 5 amplicons (L1 A, Fap, L1 B, MY and L1 C) overlapping no less than 57bp (Table 1). The analysis was performed using the primer walking technique (Kovanda *et al.*, 2011) on the basis of nucleotide sequences of closely related genotypes (HPV-15, -17 and -37) in the MY region.
PCR reactions were carried out in a 100 µl reaction volume containing 10 µl extracted DNA, 2 µl AmpliTaq Gold enzyme and 200 µM (each) of dNTPs and 10 µl 10X PCR reaction buffer. The thermal profile was 10 min at 94°C, followed by 50 cycles consisting of 1 min at 95°C, 1 min at 55°C-62°C depending on primers, 1 min and 40 s at 72°C and a final 7 min extension at 72°C.
Direct sequencing of PCR products was performed using an automatic sequencer (ABI PRISM 3100 genetic analyzer DNA Sequencer, Applied Biosystems, Foster City, CA, USA) and the BigDye Terminator v1.1 Cycle Sequencing

TABLE 1 - *Primers used for L1 PCR amplification and sequencing.*

Primer	*Sequence 5'-3'*	*Nucleotide position*	*Amplicon, size*
L1 A Fwd	TTACCACAATCTGACACGCCAAC	5583-5605	L1 A, 339bp
L1 A Rew	ACCCTGAATTGATTTCCTGACACC	5899-5922	
Fap 59 Fwd[a]	TAACWGTIGGICAYCCWTATT	5835-5855	Fap, 483bp
Fap 64 Rew	CCWATATCWVHCATITCICCATC	6295-6318	
L1 B Fwd	GTCCCCCTTTAGAATTAAGGAACAC	6261-6285	L1 B, 484bp
L1 B Rew	GGTGTTGTCTGCTACAGTAATAAAC	6721-6745	
MY 09 Fwd[b]	CGTCCMARRGGAWACTGATC	7115-7134	My, 455bp
MY 11 Rew	GCMCAGGGWCATAAYAATGG	6680-6700	
L1 C Fwd	CATTTTGGAAGGTAGATCTTACAGA	7071-7095	L1 C, 285bp
L1 C Rew	TCAGGAATTTATAATCTGATTAACCG	7331-7356	

[a]Shen-Gunther *et al*. Gynecology Oncology 123: 263-71, 2011 [22]; [b]Gravitt *et al*., J. Clin. Microb. 36: 3020-27,1998 [14].

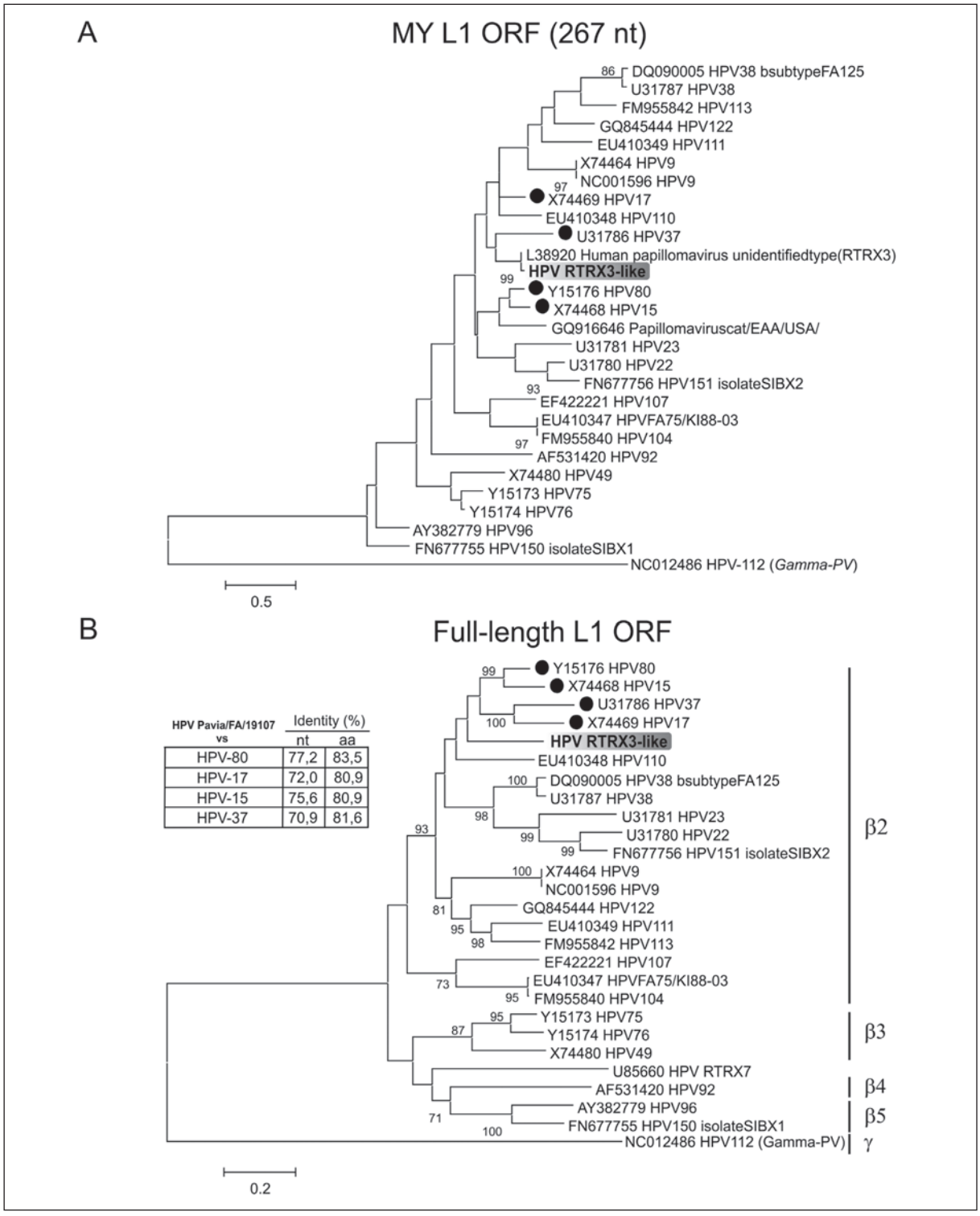

FIGURE 1 - *Maximum likelihood phylogenetic trees based on MY L1 and complete L1 sequences. Phylogenetic trees based on an alignment of the (A) partial L1 and (B) complete L1 sequences. The tree branches of HPV types closest to the HPV-RTRX3-like strain are reported with a bold line. Numbers at the nodes represent the percentage of bootstrap probabilities as determined by 1000 replications. Only bootstrap values greater than 70% are reported.*

kit (Applied Biosystems, Foster City, CA, USA). The sequences obtained were analyzed using the Sequencer 5.0 software program and compared with available HPV genome sequences utilizing the Blast program (http://blast.ncbi.nlm.nih.gov).

The sequences were aligned using the ClustalW program integrated within the MEGA version 5.0 package (Tamura *et al.*, 2011). The best-fitting nucleotide substitution model was estimated by means of a hierarchical likelihood ratio for each genome region analyzed (Tamura-Nei for L1 sequences alignment and Tamura-3-parameter for MY sequences alignment). Phylogenetic tree reconstruction was performed with the maximum likelihood method with parameters selected by a model test program implemented in the MEGA program. Branch support was assessed by bootstrap analysis with 1000 replicates. Bootstrap values of 70% were used as a cut off point for cluster analysis**.**

An HPV strain with 99% nt identity to HPV-RTRX3 and 64.5% nt identity to HPV-37 was identified in urine samples and urethral swabs (Figure 1A). Other HPV strains were absent in the urine and urethral samples, as demonstrated by the clonal analysis of the MY L1 fragment (30/30 clones, 100% HPV-RTRX3). To further characterize the HPV-RTRX3-like strain, the L1 gene was completely sequenced.

In the phylogenetic tree based on complete L1 sequences, the HPV-RTRX3-like strain (accession number KF922510) was positioned in a separate branch between the group of HPV types 80, 15, 37 and 17 and HPV-110 (Figure 1B). In the L1 region, nucleotide identity was less than 90% with respect to the closest HPV reference types. In detail, the nucleotide sequence of HPV-RTRX3-like L1 ORF shared 77.2% similarity with HPV-80, 75.6% with HPV-15; 72.0% with HPV-17 and 70.9% with HPV-37.

In a patient with glans lichen sclerosus, a putative novel type HPV strains was detected. Interestingly, in the L1 MY fragment, the HPV-RTRX3-like strain was similar to RTRX3 and to a lesser extent to HPV-37. When comparing the entire L1 gene, the patient's strain was related to HPV types 80, 15, 17, and 37. The reference sequence for HPV-RTRX3 (accession number L38920) available in GenBank includes only the MY fragment, while large portions of the HPV-RTRX3 genome are lacking. Taxonomic criteria proposed by de Villiers *et al*. in 2004 and revised by Bernard *et al*. (2010) have defined a L1 nucleotide identity lower than 90% (Bernard *et al.,* 2010) as a cut-off to define HPV types within a species. The results of this study showed that the HPV-RTRX3-like strain was related to a group of well-known HPV types. However, nucleotide identity was always < 80%. Recently, HPV types 6, 8, 16, 18, 23, 36 and 38 have been reported in childhood penile lichen sclerosus (Clouston *et al.,* 2011). Although, an association of penile lichen sclerosus in adults with HPV infection is still not demonstrated (Clouston *et al.,* 2011), the identification of a putative novel type HPV-RTRX3-like strain in penile lichen sclerosus could shed some new light on the pathogenesis of this disease.

In conclusion, the HPV-RTRX3-like strain of our patient showed a high similarity with HPV-RTRX3 strain in the MY L1 fragment (Berkhout *et al.,* 1995; Bens *et al.,* 1998), while the entire L1 gene sequence was similar to HPV 80, 15, 17, and 37. A potential etiological relation between penile lichen sclerosus and the HPV-RTRX3-like strain infection could be speculated, but additional investigations are needed to confirm this hypothesis.

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