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Ovis aries Papillomavirus 3: A prototype of a novel genus in the family *Papillomaviridae* associated with ovine squamous cell carcinoma

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ABSTRACT

Papillomaviruses play an important role in human cancer development, and have been isolated from a number of animal malignancies. However, the association of papillomaviruses with tumors has been poorly investigated in sheep. In this study, a novel ovine Papillomavirus, OaPV3, was cloned from sheep squamous cell carcinoma. Unlike the already known ovine papillomaviruses, belonging to the *Delta* genus, OaPV3 lacks the E5 open reading frame and maintains the conserved retinoblastoma motif in the E7 gene. OaPV3 infects exclusively epithelial cells, and was found in skin of healthy sheep of geographically separated flocks located in Sardinia (Italy). This new virus is transcriptionally active in tumors and shares low homology with all the other papillomaviruses, establishing a new genus. Taken together, the co-occurrence of OaPV3 and tumors, its cell and tissue tropism, and its gene repertoire, suggests a role for this virus in development of sheep squamous cell carcinoma.

Introduction

Papillomaviruses (PVs) are a diverse group of small, non-enveloped, double stranded DNA viruses that cause proliferations of the stratified squamous epithelium of the skin and of the mucosa in a wide variety of host species (Bernard et al., 2010; zur Hausen and de Villiers, 1994). A large number of different PV types have been genetically characterized and defined as genotypes, most of them being highly species-specific, and some being associated to defined degrees of pathogenicity (de Villiers et al., 2004; Van Ranst et al., 1992). More than 100 human genotypes (HPVs) are implicated in both benign (warts, papillomas or condylomas) and malignant lesions (zur Hausen, 2009). The *Alpha* genus contains predominantly mucosal HPVs, among which HPV16 and HPV18 are involved in the development of about two thirds of all cervical carcinomas worldwide. Moreover, several cutaneous HPVs of the *Beta* genus (types 5, 8, 9, 12, 14, 15, 17, 19–25) and of the *Nu* genus (type 41), have been frequently reported in association with squamous cell skin carcinomas of immunosuppressed but also of immunocompetent patients (zur Hausen, 2009), and in patients with the rare hereditary disease epidermodysplasia verruciformis (Kim et al, 2010; Nuovo and Ishag, 2000). Similarly, several animal genotypes have been isolated from canine, feline, rodents, primate, and bovine squamous cell carcinomas, and from other malignancies (see Table S1) (Borzacchiello et al., 2003; Campo, 2002; Chambers et al., 2003; Munday et al., 2009; Nasir and Campo, 2008; Yuan et al., 2007). Squamous cell carcinoma (SCC) is the most common form of skin cancer in sheep (*Ovis aries*), and it has been reported in Australia (Hawkins et al, 1981; Swan et al, 1984; Tilbrook et al., 1992), South Africa (Tustin et al., 1982), France (Lagadic et al., 1982), Spain (Méndez et al., 1997), Saudi Arabia (Ramadan et al., 1991), and Brazil (Del Fava et al., 2001). Papillomavirus DNA was identified by means of "in situ" hybridization techniques in pre-cancerous ear lesions (Trenfield et al., 1990), and in sheep perineal tumors (Tilbrook et al., 1992). Additionally, papillomavirus particles were identified by electron microscopy in pre-tumoral and tumoral lesions of ewes (Vanselow and Spradbrow, 1983). However, the two papillomavirus genotypes OaPV1 and OaPV2 isolated so far in sheep seem to associate only to fibropapillomas and have never been observed in precancerous lesions and skin tumors. Based on these preliminary data, we hypothesized that unknown epidermotropic ovine papillomaviruses (OaPVs) may exist, and that they may be detected in cases of ovine SCC. Consequently, we extracted DNA from cases of well differentiated ovine SCCs, and amplified circular DNA by the recently described multiple-primed rolling-circle amplification (RCA) method, which has been shown to

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be useful for the cloning of entire papillomavirus genomes (Dean et al., 2001; Rector et al, 2004; Rector et al, 2005). Indeed, papillomavirus-like DNA was amplified from two ovine SCCs, cloned, and sequenced. Sequence determination and analysis resulted in the detection and primary description of a third OaPV (OaPV3). The new isolate meets the majority of criteria needed to declare detection of a novel genus among the papillomaviruses (Bernard et al., 2010; de Villiers et al., 2004). In situ hybridization combined to immunohistochemistry revealed that OaPV3 infects exclusively epithelial cells. Finally PCR and RT-PCR allowed establishing the presence of OaPV3 in geographically separated flocks and of OaPV3 early transcripts in tumors, respectively.

Results

RCA, cloning and sequencing of the OaPV3 genome

DNA samples representative of 4 SCC biopsies were amplified using multiply primed rolling circle amplification (RCA). RCA generates linear, double-stranded tandem-repeated copies of circular template DNAs (Dean et al., 2001; Nelson et al., 2002). After denaturation of circular DNA templates, exonuclease-protected random hexamer primers anneal to the template DNA at multiple sites, and are extended by a phi 29 DNA polymerase. When this DNA polymerase reaches a downstream extended primer, strand displacement occurs. Newly synthesized strands can subsequently be available as targets for additional priming events, thus resulting in exponential isothermal amplification. The obtained double-stranded, high molecular-weight, tandem-repeated copies of the template DNA are then digested with a panel of restriction enzymes, and the presence of a papillomavirus genome is established by the appearance of a band (or multiple bands) consistent with the size of a papillomavirus genome (about 7–8 kb) in agarose electrophoresis.

After digestion with a panel of restriction endonucleases, RCA products obtained from 2 of the 4 SCCs generated an invariant pattern consistent with the size of a papillomavirus genome (Fig. 1A). In particular, *Eco*RI and *Stu*I produced a single band of about 7.5 Kb. Digestions with *Hind*III resulted in 3 DNA fragments of approximately 1, 2.1, and 4.2 kb, respectively. The use of *Nru*I, *Mlu*I, and *Kpn*I did not result in the appearance of detectable DNA fragments. The restriction pattern associated with the 2 SCC samples was compared to that of the fully sequenced ovine papillomaviruses OaPV1 and OaPV2, obtained *in silico* with the same restriction endonucleases (Table S2). Based on RCA results, we hypothesized the presence of an unknown papillomavirus in the 2 SCC samples. According to the guidelines for Papillomavirus taxonomy (Bernard et al., 2010; de Villiers et al., 2004), this putative novel papillomavirus was named OaPV3. The 7.5 Kb *Sac*I fragments obtained from the 2 RCA-amplified SCC DNA samples were successfully cloned into pUC19. When the two SCC clones were independently sequenced by primer walking, a 100% identity was observed, confirming that both SCC carcinomas contained the same virus. In order to establish that *Sac*I was indeed a single cutter of the OaPV3 genome, and to confirm that the full genome of OaPV3 was properly cloned into pUC19, a 0.8-kb region surrounding the *Sac*I site was amplified from SCC DNA and from a non-digested RCA product obtained from the same sample. Comparison of the nucleotide sequences obtained from the amplicons resulted in 100% identity, confirming that the complete OaPV3 genome was correctly cloned into pUC19 (data not shown).

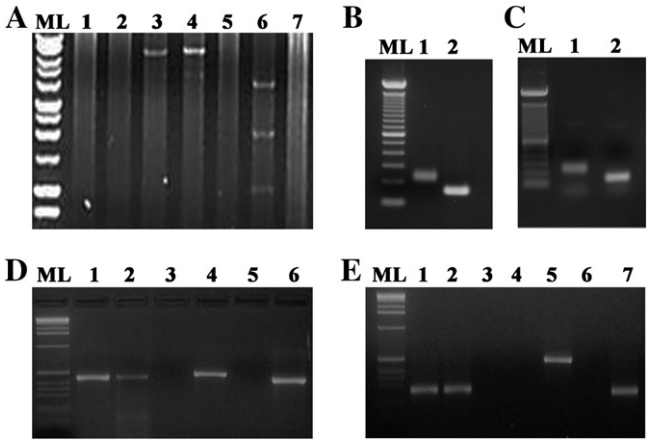

Fig. 1. Rolling circle amplification and restriction analyses. (A). ML: 1 kb DNA ladder; 1 to 6, RCA digestions with *Nru*I, *Mlu*I, *Sac*I, *Eco*RI, *Kpn*I, *Hind*III, respectively; 7, undigested RCA product. Production of Dig-labeled E6/E7 probes (B and C, respectively). ML, 100 bp DNA ladder; 1, digoxigenin-labeled probes; 2, unlabeled controls. Transcription of OaPV3 E6 from sSCC (D). ML: 1 kb DNA ladder; amplification of E6 from cDNA generated by using: 1, primer OaPV3/E6/START, and 2, random hexamers; 3, DNase-treated RNA extractions; 4, vendor positive control; 5, negative control; 6, Total OaPV3 DNA. Transcription of OaPV3 E7 from sSCC (E). ML: 1 kb DNA ladder; amplification of E7 from cDNA generated by using: 1, primer OaPV3/E7/START, and 2, random hexamers; 3, DNase-treated RNA extractions; 4, vendor negative control; 5, vendor positive control; 6, water; 7, Total OaPV3 DNA.

The complete OaPV3 genome sequence

The complete nucleotide sequence of the *Ovis aries* papillomavirus type 3 genome (OaPV3, GenBank accession number FJ796965) counts 7344 bp, and has a GC content of 46.8%. The OaPV3 genome contains the classical PV major ORFs E6, E7, E1, E2, L2, L1. A putative E4 is also present as a result of a spliced message. Notably, OaPV3 lacks the E5 ORF, which is constantly present in the ovine Papillomaviruses OaPV1 and OaPV2. The exact locations of the ORFs and comparisons with related papillomaviruses are shown in Fig. 2 and Table 1, respectively.

The noncoding region (NCR)

The classic noncoding region, located between the stop codon of L1 and the start codon of E6, counts 635 bp in OaPV3 (nt 1 to 635). NCR usually contains an E1 recognition site (E1BS) flanked by two E2-binding sites (E2BS), for binding of an E1/E2 complex in order to activate the origin of replication. OaPV3 NCR contains 6 typical E2-binding sites (E2BS) with the consensus sequence ACC-N6-GGT at nt 107–118, 152–163, 190–201, 234–245, 314–325, and 367–378. We failed to locate an E1BS. A double polyadenylation signal (AATAAATAAA, nt 7336–2) required for the processing of the L1 and L2 capsid mRNA transcript is present upstream of a CA dinucleotide and of a G/T cluster. The OaPV3 NCR contains the tata box (TATAAAT, nt 604–609) of the E6 promoter, located 25 nucleotides upstream the E6 start codon.

OaPV3 early region

The putative OaPV3 E6 contains two conserved zinc-binding domains (C-X-X-C-X29-C-X-X-C), separated by 36 amino acids. The E7 ORF contains one such zinc-biding domain and the conserved retinoblastoma tumor suppressor binding domain (LYCDE). The E1 ORF codes for the largest OaPV3 protein (607 amino acids), and contains the conserved ATP-binding site for the ATP dependent helicase (GPSDTGKS) in its carboxy-terminal part. It also contains a cyclin interaction RXL motif (the native KRRLF recruitment motif) which mediates E1 interaction with and phosphorylation by cyclin/Cdk complexes and is required for efficient HPV replication *in vivo* (Ma et al., 1999). Moreover, the open reading frame E4 was identified as a result of a spliced message unifying the first few codons of E1 (nt 1303–1315) with a downstream ORF in the +1 frame of the E2 ORF (nt 3676–4013). Remarkably, the typical open reading frame E5 could not be identified.