Papillomaviruses in Multiple Tumours of Twin Goats

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Abstract: *In situ* hybridisation, polymerase chain reaction as well as L1 antigen immunolabelling indicated the presence of Papillomavirus in tumour samples of twin goats. These findings suggest that caprine PVs may play a role in cutaneous and ocular tumour development, similarly to what has been suggested for humans.

Keywords: Papillomavirus, goat tumour, L1, RCA, squamous carcinoma, caprine.

Papillomaviruses (PVs), recently classified as *Papillomaviridae* family with different genera [1], are small, non-enveloped viruses which infect both humans and various animals from birds to mammals, usually causing benign proliferative epithelial lesions. Nevertheless, certain types of human PVs (HPVs), called "high-risk" or "oncogenic", such as HPV-16, HPV-18 and HPV-31, have been recognized as causative agents of cervical and anal cancers [2]. In addition, the involvement of bovine PV type 2 (BPV-2) in carcinogenesis of urinary bladder in cattle has also been demonstrated [3-5]. At the same time, it is becoming increasingly evident that other types of HPVs, particularly those belonging to genus beta, could be involved in nonmelanoma skin cancer, mainly squamous cell carcinoma (SCC), even though their putative etiologic role still remains unclear [6-9]. PV-associated basosquamous carcinoma has also been reported in an Egyptian fruit bat [10]; likewise, PV DNA sequences have been found in feline Bowenoid *in situ* carcinoma, a rare disease in cats that presents as multiple discrete plaques of epidermal hyperplasia and dysplasia [11]. Furthermore, current findings strongly support a potential active role of HPVs in the development of malignant melanoma [12-13]. As far as ocular tumours are concerned, they have also been aetiologically linked to PV infection both in humans and in cattle [14-17]. Recently, Van Doorslaer *et al*. [18] have determined the complete nucleotide sequence of *Capra hircus* PV-type 1 (ChPV-1), after amplification from healthy goat skin. However, PV-like DNA sequences have rarely been detected in caprine neoplasms [19], whereas they have not yet been identified in multiple coexisting tumours of such species.

In our study, we attempted to detect the presence of PV in multiple ocular and cutaneous neoplastic lesions spontaneously occurring in two adult Maltese twin goats, one male and one female, by means of immunohistochemical and biomolecular techniques. One year after the onset of ocular SCCs, which had been previously described [20], multiple cutaneous and additional ocular neoplasms were identified in both animals. Such tumours were histologically classified as multiple cutaneous fibropapillomas and SCCs (particularly located in periocular and perianal skin of both animals), one malignant melanoma of periocular skin, as well as one ocular fibrosarcoma. The immunohistochemical evaluation of paraffin-embedded tissue sections was carried out using a pre-diluted anti-PV polyclonal rabbit antibody (Ab) (Code No: N1547, Dako, Carpinteria, CA, USA), raised against chemically disrupted bovine PV-1 (BPV-1) and specifically reacting with L1 capsid protein of most known PVs (Dako specifications). Antigen retrieval was performed by heat-treating sections in citrate buffer at pH6 in a microwave oven for 15 min. Immune complexes were treated with secondary biotinylated Goat anti-Mouse&Rabbit antibody (ready-to-use, Biospa, Milan, Italy) and subsequently were detected by a peroxidase-labelled polymer method (envision+ Dual Link System Peroxidase, Dako). Immunohistochemical results showed positive intranuclear (Fig. **1a**, arrows) and perinuclear (Fig. **1a**, arrowhead) staining for BPV-1 L1 antigen only in a few squamous cells within ocular SCC of female goat. We performed negative controls by omitting primary antibody (Fig. **1b**) and by using irrelevant antibodies like polyclonal rabbit anti-human von Willebrand factor (Dako, Code n. A 0082) or polyclonal rabbit anti-human CD3 (Dako, Code n. 0452).

The very low number of positive cells may also explain the previous negative results on ocular neoplasms from the same animals [20].

Biomolecular studies were carried out by means of polymerase chain reaction (PCR), multiply-primed rolling-circle amplification (RCA), as well as *in situ* hybridisation (ISH).

Genomic DNA was extracted from formalin-fixed, paraffin-embedded tumour samples [21]. Ten microlitres of each samples were used as template for PCR amplification with the consensus primers MY09/MY11 that are able to amplify part of the L1 region of a large number of PV, as previously reported [20]. In all neoplastic tissue samples from both

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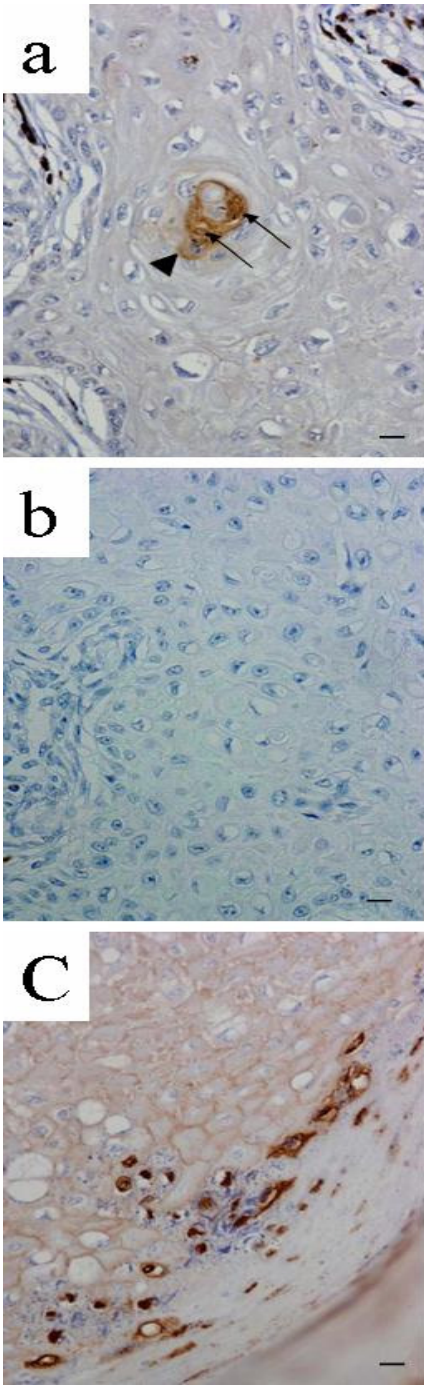

Fig. (1). Immunohistochemical staining for papillomavirus L1 capsid protein. Brown positive squamous cells within the ocular SCC (**a**). No stain within the ocular squamous cell carcinoma with the immuno-reaction lacking the primary antibody (**b**). Positive stain in the control tissue from a bovine wart (**c**). Scale bar 15μm.

animals a product of the predicted length (450 bp) was detected in ethidium bromide-stained 3% Nu-Sieve agarose gel. Direct sequencing of the fragment by an automated apparatus (Biogen srl., Rome, Italy) yielded sequences that were compared with those present in data bank by BLAST program (NCBI). A sequence homology ranging from 40 to 70 % with some known papillomaviruses, including BPV-6, HPV6 and ChPV-1, was revealed.

In the region of the highest homology between our sequence and that of ChPV-1 [18] we designed two primers that allowed the amplification of a 237 bp fragment (forward 5'-ACCCCAAAGCAAATTCAAATG-3'; reverse 5'- CTC AGCAACTATGTCTAAGC-3') (Fig. **2**). This amplified product was detected in all the lesional samples and the analysis of the sequence (GenBank accession no. **EU167550**) confirmed a 82% homology in the L1 region of ChPV-1.

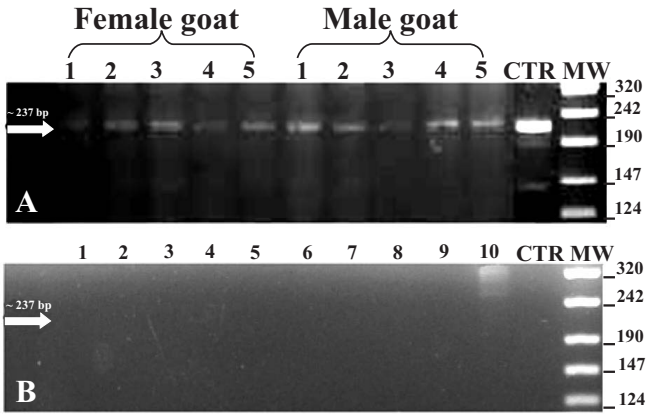

Fig. (2). Caprine papillomavirus DNA amplification.

Panel A. DNA samples from different areas and lesions (1-3 from ocular carcinomas; 4-5 from perianal warts) of twin goats was subjected to PCR amplification with specific primers as described in the text. An amplified product of approximately 237 bp was detected in all the samples. Panel B. DNA samples from normal skin of other healthy goats (1-6) and from normal tissues of the female (7-8) and male (9-10) goat. Molecular weight marker (MW) type VIII (Roche Diagnostics, Germany), the numbers represent the band length in basepair (bp) CTR, positive control consisting of recombinant plasmid DNA from HPV-8 which shares homologies with the used primers. Same sequence was revealed from all the amplified products. This sequence was not detected in normal samples, demonstrating that they are from exogenous organism and not from goat genomic sequences (Fig. **2**, Panel B).

In order to investigate the presence (and the sequence) of the entire genome of the papillomavirus under study and in the attempt to clone other sequences, the multiply-primed RCA reaction was performed using the TempliPhi 100 Amplification kit (Amersham Biosciences, Roosendaal, The Netherlands). In nature, replication of circular DNA molecules such as plasmids or viral genomes frequently occurs through a rolling circle mechanism. Rolling-circle amplification (RCA) is a novel technology mimicking this molecular amplification machinery. RCA has been used as a laboratory method for the amplification of small circular DNA templates via prolonged extension of exonuclease-resistant random hexamers. The method utilizes bacteriophage Φ 29 DNA polymerase, a high-fidelity enzyme with a strong strand-displacing capability, high processivity and proofreading activity. By this method, we have already revealed the presence of episomal HPV-16 DNA in head and neck tumours [22]. Briefly, one μg of extracted DNA was transferred into a 0.2-ml tube with 10 μl of TempliPhi sample buffer, containing exonuclease-protected random hexamers. The samples were denatured at 95°C for 5 min and after-

wards were placed on ice. A premix was prepared on ice by mixing, for each sample, 10µl of TempliPhi reaction buffer, containing salts and deoxynucleotides (dNTPs), and 1µl of TempliPhi enzyme mix, containing the Φ 29 DNA polymerase and exonuclease-protected random hexamers in 50% glycerol and 450 µM of extra dNTPs per sample. After mixing by vortexing, 5 µl of premix was added to the cooled samples. The reaction mixtures were incubated overnight (approximately16 h) at 30°C. Afterwards, the reaction mixtures were put on ice and subsequently heated to 65°C for 10 min to inactivate the Φ 29 DNA polymerase.

All the RCA products were digested with the rare cutting enzymes EcoRI, HindIII and BamHI. Only the digestion with the BamHI demonstrated the presence of circular DNA sequences, compatible with the double-stranded circular DNA genome of Papillomaviruses. Unfortunately, the attempts to clone this sequence to obtain plasmids carrying the entire or a partial sequence of this putative PV genome failed. Such negative result may be explained by the low number of viral sequences. Indeed, semi-quantitative analysis by scanning densitometry of the PCR product in agarose gel indicated the presence of few copies of viral DNA per cell (data not shown). To localize the presence of this papillomavirus within the lesions ISH was performed on paraffin-embedded tissue samples (Fig. **3**).

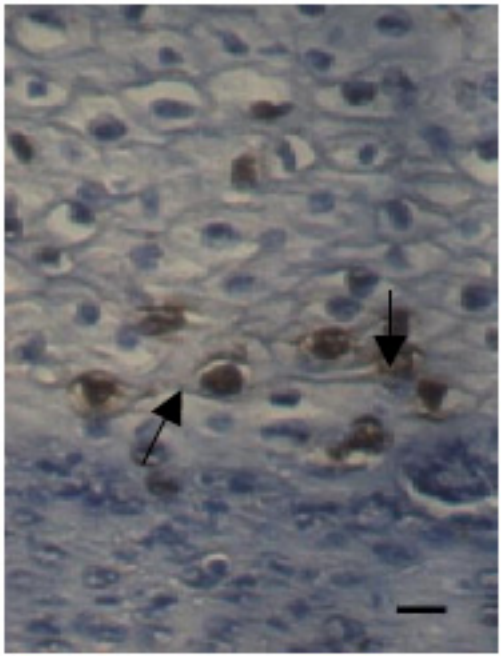

Fig. (3). *In situ* hybridization of perianal lesion of female goat.

Slides were counterstained with haematoxylin. The arrows indicate some positive nuclei with diffuse staining. Scale bar 10µm

The probes were made with the amplified products labelled with biotin d-UTP. Each probe was hybridized on thin slices of samples previously treated with H_2O_2 in order to inactivate endogenous peroxidase. The hybridization was carried out at 37°C overnight in an hybridization mix containing 50% of formamide. After washings, the hybridization was revealed by a tyramide signal amplification system for biotinylated probes (GenPoint, Dako). The slides were counterstained with haematoxylin. ISH showed the presence of the amplified DNA sequences in the nucleus of some neoplastic cells and the diffuse staining of the nuclei indicated that PV sequences were in episomal form, confirming the RCA results.

Taken together, the above data clearly indicate the presence of PV DNA sequences, as well as focal L1 capsid protein expression within the nucleus of neoplastic cells in multiple cutaneous and ocular tumours of the twin goats under study. Despite the failure in isolating and sequencing the entire genome, the presence of episomal DNA, together with the expression of capsid protein, strongly suggest the possible presence of papillomavirus particles.

These findings open the question about a possible involvement of PV infection in the development of such malignancies in the caprine species. The amplified nucleotide sequence also appeared to be rather different from the recently characterized sequence data of ChPV-1 [18], thus suggesting the existence of various types of caprine PVs, which could be differently implicated in malignant transformation.

The potential oncogenic role of papillomaviruses is currently investigated in human skin cancer. However, the etiologic role of HPVs in skin cancer still remains poorly defined, since HPV DNA is also frequently detectable in healthy skin or plucked hairs from individuals with and without skin cancer [23-26]. In addition, a low copy number of HPV DNA is usually associated with skin cancer, thus suggesting that it could be contained only in a minority of tumour [7]. The data so far obtained are more compatible with cutaneous HPVs being possibly involved in tumour initiation and progression, with a so-called "hit and run" mechanism of carcinogenesis, as already demonstrated for the BPV-4-associated oesophageal carcinoma [27]. In this respect, our biomolecular and immunohistochemical findings, indicative of low levels of PV DNA sequences and viral antigens expression, are in agreement with such hypothesis.

PVs could act as cofactors of other established risk factors in skin carcinogenesis, such as sunlight exposure or immunosuppression [7]. In this respect, promoter stimulation of certain HPV types by ultraviolet (UV) radiation has also been described [28-30]. Finally, PV infection could also represent an auxiliary factor for development of skin cancer in genetic disorders, since an association between EV-HPV types and SCCs has been observed in young xeroderma pigmentosum patients, a rare autosomal recessive disease, characterized by deficient DNA repair and hypersensitivity to UV irradiation [31].

In conclusion, the present study represents the first report on PV-associated multiple cutaneous and ocular tumours in twin goats. Even though the etiopathogenetic role of PV infection remains undefined, a cooperation between the viral agent and genetic predisposition or environmental factors could be supposed. Along with the putative role played by a papillomaviral agent in the etiology of the different tumour lesions which were observed in the two goats under study, also their "gemellarity" condition should be adequately taken into account. As a matter of fact, the study of twins may be of value in cancer epidemiology since twins are genetically identical, or share half of their segregating genes; in this re-

spect, while familial clustering has been observed for human neoplasms occurring at specific body sites, on one side, an increased risk of developing certain cancer types in man has been reported in monozygotic rather than in heterozygotic twins, on the other [32]. Nevertheless, further studies are needed to evaluate the prevalence of PV infection both in healthy and neoplastic goat skin. In addition, since detection of PV DNA in cancers could be a remnant of past viral activities or evidence of transient infections of tumors, it will be most important to evaluate type-specific immune response against early and late viral proteins as a reflection of viral activities over years [7]. Future investigations carried out both in humans and animals will probably contribute to clarify the role of PV infection in skin carcinogenesis.

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