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Expression of Platelet-derived Growth Factor-β Receptor and Bovine Papillomavirus E5 and E7 Oncoproteins in Equine Sarcoid

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Summary

Equine sarcoids are benign fibroblastic skin tumours that are recognized throughout the world. Infection with bovine papillomavirus (BPV) types 1 and 2 has been implicated as a major factor in disease development; however, the cellular mechanisms underlying fibroblast transformation remain poorly defined. The present study further characterizes aspects of the association with BPV in 15 equine sarcoids. BPV DNA was demonstrated in 12/15 tumours collected from different areas of Italy. Nine of these 12 tumours expressed the BPV oncoproteins E5 and E7, but these oncoproteins were not expressed by normal equine cells. The BPV E5 protein is known to bind to the platelet-derived growth factor-β receptor (PDGF-βR) and this molecule was expressed by 11 of the 12 sarcoids in which E5 was demonstrated. These findings add further weight to the theory that BPV and the PDGF-βR may have a role in the pathogenesis of this disease.

Keywords: bovine papillomavirus; horse; platelet-derived growth factor-β receptor; sarcoid

Introduction

Sarcoids are benign tumours of fibroblastic origin affecting the skin of horses, mules and donkeys and are considered to be the most common equine cutaneous neoplasm. The tumours most frequently arise from the skin of the head, ventral abdomen, legs and the paragenital region (Ragland *et al*., 1970). Sarcoids are locally invasive and often occur at sites of previous injury or scarring.

Bovine papillomavirus (BPV) is considered to be the aetiological agent of this tumour (Borzacchiello, 2008). Both BPV-1 and -2 have been detected in sarcoids with BPV-1 being predominant (Lancaster *et al*., 1979; Amtmann *et al*., 1980; Chambers *et al*., 2003). Equine sarcoid is an important tumour since it provides the only known example of natural cross-species infection by a papillomavirus. Moreover, while BPV infection in cattle produces benign lesions that may regress, the behaviour of the same virus in the horse is distinctly different as sarcoids are non-permissive for virus production, locally aggressive and rarely show regression (Borzacchiello, 2007).

Candidate molecules involved in BPV oncogenesis have been characterized. The BPV-1 *E5* oncogene encodes a 44 amino acid protein that is considered to be the major BPV oncoprotein (Schiller *et al*., 1986; Schlegel *et al*., 1986). E5 is a type II transmembrane protein that is expressed in the deep layers of infected epithelia (Burnett *et al*., 1992; Anderson *et al*., 1997; Venuti and Campo, 2002) and is largely localized to the membranes of the endoplasmic reticulum (ER) and Golgi apparatus (GA) of these cells (Burkhardt *et al*., 1989; Pennie *et al*., 1993). BPV E5 is expressed in the cytoplasm of both basal and suprabasal transformed epithelial cells (Bohl *et al*., 2001; Araibi *et al*., 2004) with a typical juxtanuclear pattern due to its localization in the GA (Borzacchiello *et al*., 2003).

The mechanism underlying BPV carcinogenesis is thought to relate to the binding (both *in vitro* and

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in vivo) of the E5 oncoprotein to the platelet-derived growth factor-β receptor (PDGF-βR) (DiMaio *et al.*, 2000; DiMaio and Mattoon, 2001; Borzacchiello *et al.*, 2006). This mechanism has been largely investigated *in vitro* and in naturally occurring bovine bladder cancer. However, in the latter situation, synergism between the virus and carcinogenic principles from bracken fern is required for full transformation (Campo *et al.*, 1992; Borzacchiello *et al.*, 2003).

The BPV-1 *E7* gene encodes a 127 amino acid zinc-binding protein that co-operates with E5 and E6 in inducing cell transformation. In natural infection, BPV-1 E7 expression occurs in the cytoplasm and nucleoli of cells of the basal and lower spinous layers of squamous epithelium. Once E7 is co-expressed with E5 and E6, the transformation capacity of the virus increases many fold (Bohl *et al.*, 2001). Such co-expression may also occur in bovine tumours of mesenchymal origin (Borzacchiello *et al.*, 2007). Mutants lacking the *E7* open reading frame are still able to induce transformation but at a lower efficiency, and produce transformants with altered characteristics (Sarver *et al.*, 1984).

The mechanisms described above have been largely investigated in bovine tumours and there are no studies published to date concerning the molecular mechanisms underlying BPV transformation in equine sarcoids. The aim of the present study was therefore to determine whether the mechanisms described in bovine carcinogenesis might also occur in equine sarcoid by examining the expression of key oncogenic molecules in the equine tumour.

Materials and methods

Tumour Samples

Examples of equine sarcoid ($n = 15$) were derived from the archives of the Department of Pathology and Animal Health, Naples University, and the Department of Comparative Biomedical Sciences, University of Teramo. Formalin-fixed tissue embedded in paraffin wax was available from each case. Sections taken from these blocks and stained by haematoxylin and eosin (HE) were re-evaluated by two observers (GB and VR) and the original diagnosis of sarcoid was confirmed. The 15 samples were derived from 13 different animals. The breeds of these animals included: Arabian thoroughbred ($n = 3$), Maremmano ($n = 1$), draft horse ($n = 6$), thoroughbred ($n = 3$), standard bred ($n = 2$), and one donkey and one mule. Seven were stallions, five were geldings and three were mares. The median age of the animals was 6 years old.

Detection of Viral DNA

DNA was extracted from the tissue embedded in paraffin wax with the DNeasy Tissue Kit™ (Quiagen, Milan, Italy) according to the manufacturer's protocol. Samples were lysed using proteinase K. Lysates were loaded onto DNeasy spin columns and, after two washes, pure DNA was eluted in low salt buffer.

Amplification of the *E5* open reading frame (ORF) was carried out by polymerase chain reaction (PCR) using a BPV-1 and -2 consensus primer pair, BPVE5F (TTGCTGCAATGCAACTGCTG corresponding to BPV nucleotides 3915–3934) and BPVE5R (TCATAGGCACTGGCACGTT corresponding to BPV nucleotides 4208–4225) amplifying a fragment of 311 bp from nucleotide 3915 to 4225. To evaluate the adequacy of the DNA, a control PCR for equine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequence was performed using the primers published by Yuan *et al.* (2008).

Aliquots (200–300 ng) of purified DNA were amplified in a 50 μl reaction system containing 1× *Pfx* amplification buffer, 0.3 mM dNTP mixture, 1 mM $MgSO_4$, 0.3 μM primer mixes and 1 unit platinum *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA, USA). Cycling conditions were as follows: denaturation for 2 min at 94°C, followed by 28 cycles of PCR amplification comprising denaturation at 94°C for 15 s, annealing at 50°C for 30 s and extension at 68°C for 50 s, followed by a final extension at 68°C for 7 min. Detection of the amplified products was carried out by electrophoresis in an ethidium bromide-stained agarose gel. Each experiment included a blank sample consisting of reaction mixture without DNA. PCR products were purified by SwitchCharge™ beads (Invitrogen) and sequenced using the Applied Biosystems (Foster City, CA, USA) Big Dye™ terminator cycle sequencing reagents and sequences were obtained by the use of an ABI 3100 automated sequencer. Sequence analysis was performed with Basic Local Alignment Search Tool (BLAST).

Immunohistochemistry

Sections were prepared from the 12 sarcoids in which BPV DNA had been detected. Briefly, the sections were de-waxed and then endogenous peroxidase activity was blocked by incubation in H_2O_2 0.3% in methanol for 20 min. Antigen retrieval was performed by pretreating with microwave heating (twice for 5 min each at 750 W) in citrate buffer pH 6.0. The primary antibodies were applied overnight at 4°C in a humid chamber. The polyclonal rabbit anti-E7 (a gift from Professor P. Howley, Harvard Medical School, Boston, USA) was applied at a dilution of 1 in 2000; the polyclonal rabbit anti-PDGF-β receptor

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(a gift from Professor DiMaio, Yale Medical School, New Haven, USA) was applied at a dilution of 1 in 1000; and monoclonal antibody against the proliferation marker Ki 67 (Clone MIB-1, Dako, Glostrup, Denmark) was applied at a dilution of 1 in 100.

Following incubation with primary antisera, the slides were washed three times with phosphate-buffered saline (PBS), then incubated for 30 min with the appropriate biotinylated secondary antibody (labelled streptavidin–biotin [LSAB] Kit; Dako). Sections were washed three times with PBS and then incubated with streptavidin-conjugated to horseradish peroxidase (LSAB Kit; Dako). Colour development was obtained by treatment with diaminobenzidine (Dako) for 5–20 min. Sections were counterstained with Mayer's haematoxylin. Negative controls included omission of the primary antiserum or replacement of these by appropriate normal sera. A sample of normal equine skin was also included as a further control.

The scoring of the immunoreactivity was determined in blinded fashion by two observers (GB and SM). The intensity of labelling in each specimen was scored as follows: (−) absent or very weak labelling, (+) weak, (++) moderate, or (+++) strong labelling. Proliferation index (PI) was derived from the Ki67 labelled sections by counting 1000 cells over 10 fields (examined with the 40× objective).

Immunofluorescence and Confocal Laser Scanning Microscopy

Briefly, sections from the paraffin wax embedded tissue were de-waxed, rehydrated and heated in a microwave oven in citrate buffer (as above) to allow antigen unmasking. Slides were then incubated with polyclonal sheep anti-E5 antiserum (a gift from Professor M.S. Campo, University of Glasgow, Scotland, UK) at a dilution of 1 in 50, and thereafter with secondary sheep antibody conjugated to fluorescein isothiocyanate (FITC; Chemicon, Billerica, MA, USA). For observation and photography, a confocal laser scanning microscope LSM-510 (Zeiss, Göttingen, Germany) was used as previously reported (Borzacchiello *et al.*, 2003).

SDS PAGE and Western Blotting

Two fresh unfixed tumour samples and one sample of normal skin were available for molecular analysis. These were snap frozen in liquid nitrogen and homogenized in 7.5 ml JS buffer (5 ml 1 M Hepes pH 7.5, 3 ml 5 M NaCl, 1 ml glycerol, 10 ml 10% Triton, 0.15 ml 1 M $MgCl_2$, 5 ml 0.1 M EGTA) with the addition of 2 ml 0.1 M sodium pyrophosphate, 0.2 ml aprotinin 5 mg/ml, 0.2 ml 0.1 M phenylmethylsulfonyl fluoride (PMSF), 0.2 ml 500 mM sodium orthovanadate (Na_2VO_3) and 0.25 ml 2 M NaF. Protein concentration was determined by use of a protein assay kit (Bio-Rad, Hercules, CA, USA). The protein extracts were loaded onto a 7.5% polyacrylamide gel and electrophoresed. The proteins were transferred from the gel onto polyvinylidene fluoride (PVDF) membranes by western blotting.

The membranes were blocked with 5% non-fat milk powder in Tris-buffered saline (TBS) at room temperature, washed with TBS-0.1% Tween-20 and incubated with polyclonal antibody against PDGF-βR at a 1 in 250 dilution overnight at 4°C. After washing, peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at a dilution of 1 in 1000 was applied to the blots for 2 h. Following further washing, bound antibody was visualized on enhanced chemiluminescence (ECL) film (Amersham Pharmacia Biotech, Uppsala, Sweden). The blots were stripped and re-probed with anti-β actin antibody (Calbiochem, Darmstadt, Germany) at a dilution of 1 in 5000 to confirm equal loading of proteins in each lane.

Results

Detection of Bovine Papillomavirus DNA

DNA of PCR-quality was recovered from all of the samples. A fragment of the expected size was amplified from 12 of the 15 tumour samples (80%) (Fig. 1). In order to determine the papillomavirus type the PCR amplicons were sequenced. Two of the 12 positive samples (numbers T3 and T7) were shown to contain BPV-2 DNA (17%), whereas the remaining 10 tumours contained BPV-1 DNA (83%). No association was found between the presence of BPV type-1 or -2 DNA and a particular type of tumour (data not shown).

Expression of E5, E7 and PDGF-βR

E5 protein was identified in nine of the 12 BPV-positive tumours (75%). Almost all neoplastic cells from BPV DNA-positive tumour samples displayed cytoplasmic E5 immunoreactivity. E5 was found mostly within the cytoplasm of neoplastic fibroblasts and particularly in a juxtanuclear location (Fig. 2). Normal fibroblasts in an area adjacent to the tumour did not express E5 (data not shown).

E7 expression was detected in nine of the 12 BPV-positive tumours (75%). Almost all neoplastic fibroblasts were positively labelled. The labelling was cytoplasmic, but in some samples intranuclear labelling was also recorded. The intensity of labelling

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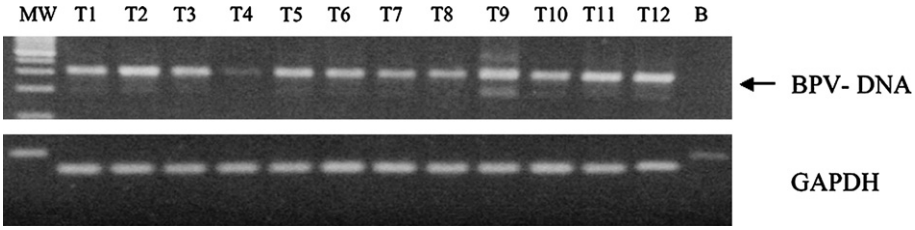

Fig. 1. PCR amplification of BPV DNA from 12 equine sarcoids. The arrow indicates the position of the 311 bp BPV PCR product. The tumour samples are numbered T1–T12, which corresponds with the numbering in Table 1. MW, molecular mass marker type VI (Roche Diagnostics); B, negative control with no DNA added. Amplification of GAPDH from all the samples is shown in the corresponding gel below.

ranged from weak ($n = 4$) to moderate ($n = 4$) or strong ($n = 1$) (Fig. 3; Table 1). Normal fibroblasts and other mesenchymal cells did not express E7.

Eleven of the 12 samples expressing E5 also expressed the PDGF-βR (91%). The positive labelling ranged from very weak ($n = 1$) to moderate ($n = 4$) or strong ($n = 6$) (Table 1). The receptor was also expressed by normal blood vessels as previously described (Borzacchiello *et al.*, 2006) (Fig. 4A).

To further confirm receptor expression a western blot analysis was performed on two tumour samples and one normal equine skin. The antibody demonstrated a band of the expected molecular weight in the neoplastic tissues (Fig. 4B).

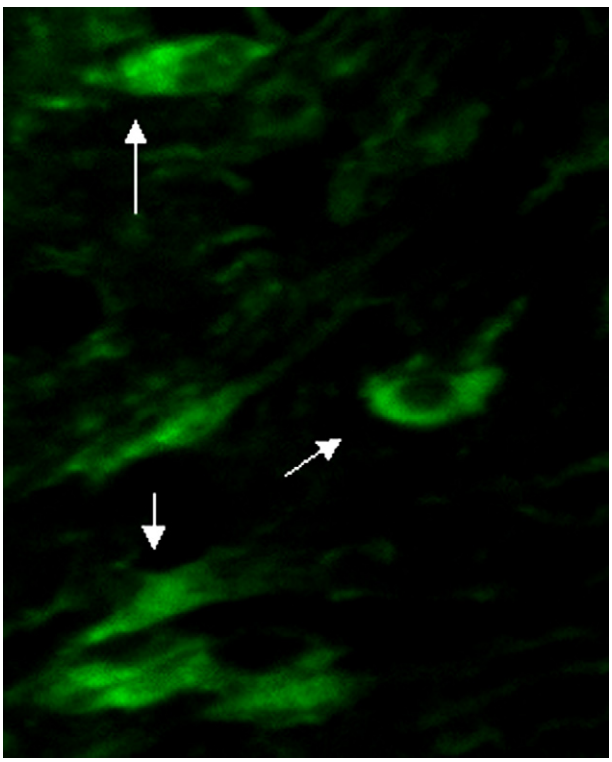

Fig. 2. E5 expression in equine sarcoids. Most neoplastic fibroblasts show cytoplasmic immunoreactivity with typical juxtanuclear labelling (arrowed). IF. 240×.

Proliferation Index

Ki67 immunoreactivity was detected in all 12 BPV DNA-positive tumour samples. The percentage of labelled cells ranged from 1.5 to 12.7, with a mean PI of 5.7%. PI in normal equine skin was recorded as 0.

Discussion

The results of the present study clearly show an association between BPV and equine sarcoid. Both BPV DNA and the two transforming oncoproteins E5 and E7 were expressed by the majority of tumours examined. To our knowledge, this is the first demonstration of the presence of BPV DNA in sarcoids collected from animals living in different areas of Italy. The 80% positivity for BPV DNA in this series is similar to the reported incidence of detection of viral DNA in these tumours in other geographical areas, which ranges from 73% to 100% (Otten *et al.*, 1993; Bloch *et al.*, 1994; Carr *et al.*, 2001a,b; Martens *et al.*,

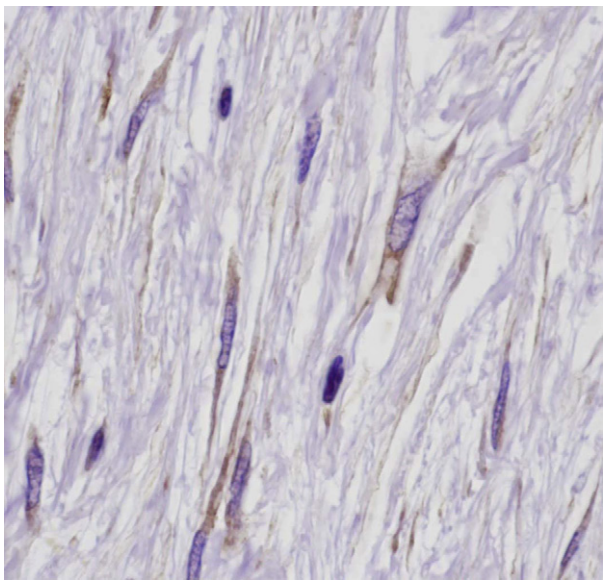

Fig. 3. E7 expression in equine sarcoids. Most neoplastic fibroblasts show cytoplasmic immunoreactivity. IHC. 240×.

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Table 1
Expression of BPV-1 or -2 DNA, oncoproteins E5 and E7, and PDGF-βR in equine sarcoid

Tumour number	*Viral DNA*	*E5*	*E7*	*PDGF-β receptor*
T1	BPV-1	+	+ +	+ +
T2	BPV-1	+	+ + +	+ +
T3	BPV-2	+	+ +	+
T4	BPV-1	+	+ +	+ + +
T5	BPV-1	+	+ +	+ + +
T6	BPV-1	0	+	+ + +
T7	BPV-2	+	+	+ + +
T8	BPV-1	+	0	+ + +
T9	BPV-1	0	+	+ +
T10	BPV-1	+	+	+ + +
T11	BPV-1	+	0	0
T12	BPV-1	0	0	+ +
T13	None	n/a	n/a	n/a
T14	None	n/a	n/a	n/a
T15	None	n/a	n/a	n/a

Expression of E5 was determined by immunofluorescence and scored as present (+) or absent (−). The scoring of the intensity of immunolabelling for E7 and PDGF-βR was: (−), absent or very weak signal; (+), weak signal; (+ +), moderate signal; (+ + +), strong signal. Tumours T13–T15 did not contain BPV DNA and were not assessed (n/a) for the remaining parameters.

2001a,b; Bogaert *et al*., 2007). The variation in detection rate may in part reflect differences in tumour fixation, as in our experience a prolonged period of storage in formalin reduces the likelihood of isolation of BPV DNA (data not shown). Both BPV-1 and -2 types were detected, with the former being the predominant type. However, there was no association between the virus type and the histological type of the tumour or expression of BPV oncoproteins or PDGF-βR. This observation suggests that the formation of tumours of differing histological appearance is related to factors other than the type of BPV within the lesion.

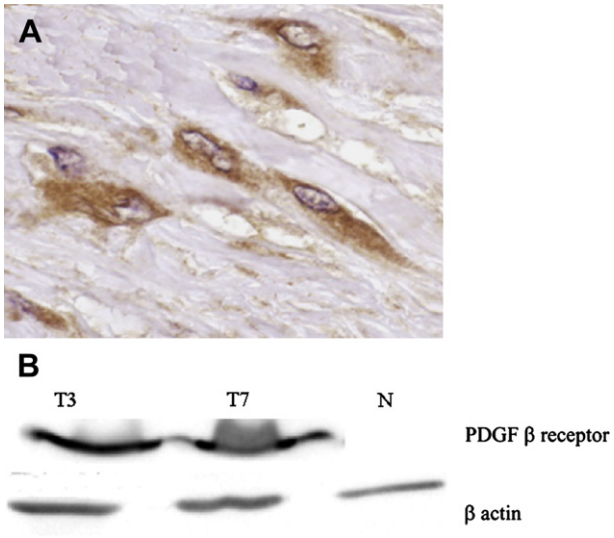

Fig. 4. PDGF-β receptor expression in equine sarcoids. (A) PDGF-βR is expressed within the cytoplasm of neoplastic fibroblasts. IHC. 240×. (B) Homogenized samples from two equine sarcoids (T3 and T7) and one sample of normal skin (N) were analysed by western blotting with rabbit anti-PDGF-βR. The blots were then stripped and re-probed with mouse anti-β actin antibody. The receptor is expressed in sarcoids and not in the normal skin.

E5 and E7 are the transforming proteins of the BPV. The amino acid sequence of E5 is identical in BPV-1 and -2 (Schlegel *et al*., 1986), so that a single polyclonal antiserum may be used to evaluate tumours containing both virus subtypes. This was confirmed by the results of our study, where E5 was expressed in sarcoids containing BPV-1 or -2 DNA without apparent difference. The expression of E5 in these tumours suggests that this oncogene has a role in fibroblastic transformation. BPV E5 is known to induce cell transformation of cultured rodent fibroblasts and keratinocytes by binding to and activating the PDGF-βR, a type I transmembrane receptor tyrosine kinase. By contrast, E5 does not directly activate either the α subunit of the PDGF receptor or the epithelial growth factor (EGF) receptor (Leptak *et al*., 1991; Petti and DiMaio, 1994; DiMaio *et al*., 2000). Each E5 dimer binds to two molecules of the PDGF-βR, forming a stable complex with these receptors and resulting in their activation. This activation involves tyrosine phosphorylation of each receptor molecule in the complex, and activation of the cellular SH2 domain-containing signalling proteins (Lai *et al*., 1998, 2000; DiMaio and Mattoon, 2001). This mechanism occurs *in vitro* as well as in naturally occurring bovine bladder epithelial cancer (Borzacchiello *et al*., 2006). The present study has shown that the PDGF-β receptor is also expressed in E5-positive equine sarcoids, suggesting that similar molecular mechanisms may be at play in the development of this tumour.

The BPV E7 oncoprotein is not thought to be directly involved in transformation, but to act by enhancing the transforming activity of the E5 and E6 oncoproteins (Bohl *et al*., 2001). However, it has recently been shown that BPV E7 binding to p-600 can lead to, at least in part, cellular transformation (Huh *et al*., 2005). We found E7 expression in the cytoplasm of fibroblasts in almost all sarcoids and E5 and E7 were co-expressed in some tumour samples, suggesting that these molecules may co-operate in tumour formation.

The PI of the equine sarcoids examined in this study was low. This finding is in accordance with other studies where the observed PI ranged from 0% to 11% (Martens *et al*., 2000; Nixon *et al*., 2005). This low rate of proliferation is reflected in the clinical observation that sarcoids are slow growing tumours.

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In conclusion, we have shown that a subset of equine sarcoids harbouring BPV DNA expresses the viral oncoproteins E5 and E7 together with the PDGF-βR. Ongoing studies are investigating possible receptor activation and downstream signalling leading to cellular transformation in order to gain further insight into the pathogenesis of formation of this neoplasm.

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