

Short Communication

Presence of bovine papillomavirus type 2 DNA and expression of the viral oncoprotein E5 in naturally occurring urinary bladder tumours in cows

Giuseppe Borzacchiello,¹ Giuseppe Iovane,¹ Maria Luisa Marcante,² Federica Poggiali,² Franco Roperto,¹ Sante Roperto¹ and Aldo Venuti²

Correspondence
Giuseppe Borzacchiello
borzacch@unina.it

¹Department of Pathology and Animal health, Faculty of Veterinary medicine 'FEDERICO II' Naples University, Via F. Delpino, 1 80137 Naples, Italy

²Regina Elena Cancer Institute, Laboratory of Virology, Via delle Messi d'Oro, 156 00158 Rome, Italy

Received 11 June 2003
Accepted 21 July 2003

Samples of neoplastic and normal urothelium were obtained from cows originating from areas of southern Italy, a region in which chronic enzootic haematuria is endemic and bracken fern infestation is widespread. Specimens were analysed for bovine papillomavirus type 2 (BPV-2) DNA, BPV-2 E5 expression and telomerase activity. A total of 46 of 60 tumours and 17 of 34 normal bladder mucosa samples harboured BPV-2 DNA. Analysis of a subset of samples showed E5 protein expression and telomerase activity in tumour tissue only. No normal samples positive for BPV DNA showed E5 protein expression or telomerase activity, suggesting the presence of DNA in a latent state. Taken together, these data on naturally occurring bovine bladder tumours corroborate the hypothesis of their virus origin.

In cattle, tumours of the bladder are commonly associated with a syndrome known as chronic enzootic haematuria (CEH), which is caused as a result of prolonged ingestion of bracken fern (Confer & Panciera, 2001).

Bracken fern (genus *Pteridium*) is believed to be the only higher plant proven to cause cancer naturally in animals (Smith, 1997). The fern contains immunosuppressive, mutagenic, clastogenic and carcinogenic chemicals. Previous studies have pointed out a strong relationship between bovine papillomavirus (BPV) and bracken fern. BPV-2 DNA was found in 69% of experimental bladder cancers and in 7 of 15 naturally occurring ones. This high degree of association between bladder cancers and BPV-2 suggested that this virus could play a role in bladder oncogenesis (Campo *et al.*, 1992).

A possible aetiopathogenic mechanism is that BPV-1/2 infect the epithelium of the bladder and produce an abortive or latent infection, as already hypothesized for BPV-4 in gastrointestinal tumours (Borzacchiello *et al.*, 2003; Campo *et al.*, 1994). The immunosuppressants and chemical carcinogens in bracken act synergistically with the virus to induce the formation of pre-neoplastic lesions. During neoplastic progression, the *ras* gene is activated (Campo *et al.*, 1990) and expression of the tumour suppressor fragile histidine tetrads locus is downregulated (Borzacchiello *et al.*, 2001).

Most previous studies have been performed under experimental conditions and in few cases of naturally occurring

bladder cancer; studies on large populations of cows are lacking.

In an attempt to validate the aetiopathogenic role of the association between BPV and bovine bladder cancers, our study was carried out on a large population of cattle from southern Italy, a region in which bracken fern is widespread and CEH is endemic. Samples were analysed for the presence of BPV DNA, E5 protein and telomerase activity. Telomerase is an enzyme that induces immortalization by preventing the progressive loss of telomere length during DNA replication (Bodnar *et al.*, 1998).

Examination of bladders from 4- to 24-year-old cows was performed at public slaughterhouses in the south of Italy. Tumour samples from all animals affected and from a number of healthy cows were collected for both histological and molecular analysis.

PCR amplification of viral sequences using consensus primers was done according to methods described previously (Borzacchiello *et al.*, 2001). These primers allow the amplification of a 307 bp DNA amplicon encompassing the BPV E5 sequence. A fragment corresponding to the expected size was amplified in 46 of 60 samples (77%), from which DNA of PCR-quality was obtained. To determine the papillomavirus serotype, PCR amplicons were sequenced and proven to correspond to the expected region of BPV-2. An example of this analysis is shown in Fig. 1. Lower positivity (17 of 34) for BPV-2 sequences was detected in the

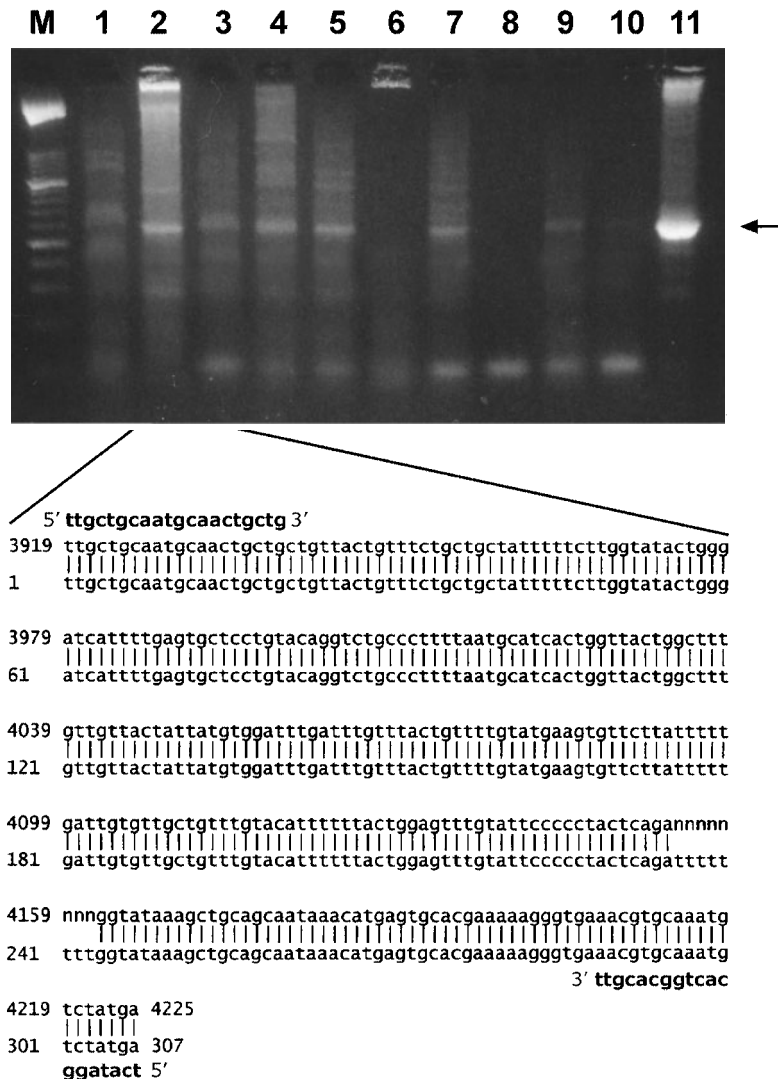


Fig. 1. PCR amplification of urinary bladder samples. Lanes: M, molecular mass marker type VI (Roche Diagnostics); 1–7, tumour samples; 8, negative control with no DNA added; 9 and 10, normal mucosa samples; 11, positive control of BPV-2 plasmid. The arrow indicates the position of the 307 bp BPV-2 PCR product. The lower part of the figure shows the sequence similarity between the sequence of the amplicon in lane 2 (top line) and the sequence of prototype BPV-2 (GenBank NC_001521) (bottom line). Primer sequences are in bold.

bladder mucosa of healthy cows as a control. The high incidence of BPV-2 DNA in all samples, both abnormal and normal mucosa, indicates clearly a high circulation of this virus in the areas examined. However, the difference between groups (pathologic versus normal samples) was statistically significant ($P < 0.01$, Fisher's exact test and χ^2), strongly suggesting an association of BPV-2 with cellular abnormalities of the urinary bladder. There was no association between the presence of viral DNA and a particular histological type of tumour (data not shown), indicating that the different histological features may be related to other factors. Viral DNA was also detected in pre-neoplastic lesions, supporting its role in tumour development. The high incidence of BPV-2 DNA in normal bladder possibly reflects the presence of an abortive or latent infection that may be activated by the immunosuppressive and/or carcinogenic chemicals in bracken fern.

To verify this hypothesis, the expression of E5, the major oncogenic protein of BPV-1/2, was analysed in different urinary bladder samples. E5 is a small hydrophobic protein

(44 aa) that is capable of inducing *in vitro* cell transformation through the activation of several kinases, from growth factor receptors to cdk cyclins (Venuti & Campo, 2002). E5 interacts physically with the cellular protein 16k ductin/subunit c, a component of the gap junction and of the V0 sector of vacuolar H^+ -ATPase (Conrad *et al.*, 1993; Faccini *et al.*, 1996; Finbow *et al.*, 1991; Goldstein *et al.*, 1991), causing the downregulation of gap junction communication (Ashrafi *et al.*, 2002; Faccini *et al.*, 1996; Oelze *et al.*, 1995) and the lack of acidification of endosomes and Golgi apparatus (GA) (Schapiro *et al.*, 2000; Straight *et al.*, 1995). However, its function *in vivo* is still unknown. Recent reports show E5 expression in both equine and bovine BPV-infected cancers (Bohl *et al.*, 2001; Carr *et al.*, 2001), suggesting a possible role of this viral protein in malignant transformation *in vivo*. A total of 36 BPV-positive (including pre-neoplastic lesions) and 10 BPV-negative tumours were analysed together with 15 BPV-positive and 10 BPV-negative normal mucosa samples. Briefly, paraffin sections were deparaffinized, rehydrated and heated in a microwave oven twice for 5 min each at 750 W to allow

antigen unmasking. Slides were then incubated with a 1 : 50 dilution of rabbit anti-E5 antiserum (kindly provided by R. Schlegel, Georgetown University, USA) and thereafter with FITC-conjugated secondary antibody (Chemicon). Immunofluorescence was analysed with a confocal laser scanning microscope (LSM 510, Zeiss). The pattern of immunostaining demonstrates clearly the presence of the E5 protein in BPV-positive tumours. The typical immunostaining pattern for the E5 protein is shown in Fig. 2. Almost all neoplastic cells from BPV DNA-positive tumour samples displayed cytoplasmic E5 immunoreactivity. In both pre-neoplastic and neoplastic lesions, a clear immunopositive signal for E5 was observed (Fig. 2a, b). E5 was located mostly within the cytoplasm of basal and suprabasal cells. In particular, a typical juxtannuclear immunoreactivity was also observed. No E5 immunostaining was detected in BPV-negative tumours, nor was it detected in normal urinary bladder mucosa positive for BPV DNA (Fig. 2c, d).

This is the first demonstration of E5 expression in naturally

occurring BPV-associated urinary bladder tumours. The localization of E5 in the basal layer of the transformed urothelium is similar to that reported for papillomas during early stages of infection in the deep layers of the infected epithelium (Anderson *et al.*, 1997; Burnett *et al.*, 1992; Chang *et al.*, 2001). It has been shown *in vitro* that, in agreement with its hydrophobic nature, E5 is localized in the endomembrane compartments of the endoplasmic reticulum and GA of the host cell (Burkhardt *et al.*, 1989; Pennie *et al.*, 1993). The cytoplasmic reactivity that is evident in pre-neoplastic and neoplastic samples is consistent with those findings, suggesting that *in vivo* membrane association is also likely. Moreover, some of the cells show accentuated staining in a perinuclear region, which could be the GA. It has been suggested that cancer cells may escape host immune control by E5-mediated downregulation of MHC class I molecules. This downregulation seems to be related to the interaction of E5 with 16k ductin/subunit c, which may lead to improper glycosylation and processing of MHC class I molecules (Marchetti *et al.*, 2002). Since the cows from

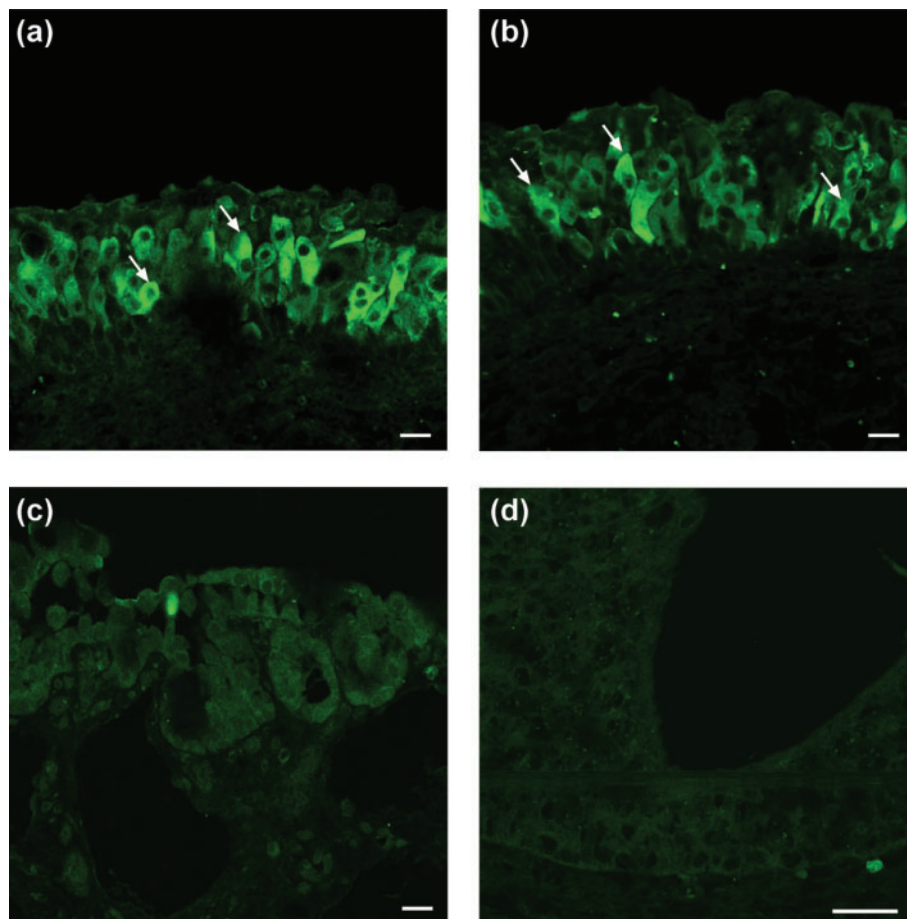


Fig. 2. E5 expression in urinary bladder mucosa. (a) BPV-2-positive severe dysplasia. Most dysplastic cells express a strong cytoplasmic E5 immunoreactivity (white arrows). (b) BPV-2-positive urothelial carcinoma grade I. Scattered neoplastic cells showing a juxtannuclear E5 staining are shown (white arrows). (c) BPV-2-negative urothelial infiltrating carcinoma grade II. No E5 immunoreactivity is seen. (d) BPV-2-positive normal urothelium. No E5 immunoreactivity is seen. Bar, 10 μ m.

which we collected the samples had been grazing on bracken fern-infested pasture, the immunosuppression caused by the plant could enhance the escape of the E5-expressing tumour cells from the immune system.

The absence of E5 staining in tumours that lacked BPV-2 DNA ruled out artefactual results, such as cross-reactivity with cellular proteins.

Interestingly, no normal urinary bladder with BPV DNA sequences showed positivity for E5 protein. These data demonstrate that the viral DNA is transcriptionally active only in abnormal tissue and reinforce the hypothesis that BPV is capable of infecting the urinary mucosa and remaining latent in normal tissue until some factors trigger viral gene expression. It is not known how latent viral DNA is activated but the chemicals of bracken fern are strong candidates. The flavonoid quercetin is present in high concentrations in the fronds of the fern. Quercetin is mutagenic and clastogenic, leading to single-strand DNA breaks (Campo, 1997; Plaumann *et al.*, 1996) and chromosomal rearrangements (Leal *et al.*, 2003). It promotes the full *in vitro* transformation of primary bovine cells infected with BPV-4 DNA (Cairney & Campo, 1995; Pennie & Campo, 1992) and upregulates viral DNA transcription via a *cis*-acting element in the viral transcriptional promoter (Connolly *et al.*, 1998). The consequent increased expression of the viral oncoproteins forces the cells to proliferate, despite quercetin-induced DNA damage (Beniston *et al.*, 2001). These findings have led to the suggestion that quercetin is a co-carcinogen of BPV-4 *in vivo*. However, the actual role of quercetin in bracken-related carcinogenicity is still an open question (Shahin *et al.*, 1999).

The hypothesis of a role of BPV in the pathogenesis of urinary bladder tumours seems also to be suggested by the analysis of telomerase activity in the urothelial tumour samples. The increased activity of cellular telomerase, a ribonucleoprotein enzyme that synthesizes telomeric DNA, is well documented in various immortalized cell lines. Telomerase activity is also detected in various animal and human cancers, and telomerase activation is thought to be a crucial factor in immortalization of cells and tumourigenesis (Argyle & Nasir, 2003; Kim *et al.*, 1994).

Recent reports indicate that papillomaviruses may also affect telomerase activity: the E6–E7 genes of human papillomavirus type 16 (HPV-16) increase telomerase activity during epithelial cell immortalization (Baeger *et al.*, 2002), whereas the E2 gene downregulates the human telomerase reverse transcriptase promoter (Lee *et al.*, 2002).

Telomerase activity was analysed in bladder samples using a commercial telomerase detection kit (Trapeze, Intergen) that utilizes the telomeric repeat amplification, whose sequence is highly conserved through mammalian species (Moyzis *et al.*, 1988). Briefly, specimens from a subset of tumours and controls, from which protein could be extracted, were lysed in CHAPS buffer, and after protein

content normalization, were subjected to PCR amplification according to the manufacturer's instructions. Analysis of each sample consisted of two assays: one with a protein extract and one with an extract that had been heat-treated at 80 °C. Heat treatment inactivates the telomerase and serves as a negative control. Aliquots of the reactions were subjected to electrophoresis on a non-denaturing 10% polyacrylamide gel. After electrophoresis, the gel was stained with ethidium bromide. A representative assay of positive tumour samples is shown in Fig. 3.

Telomerase activity was detected in 4 of 12 BPV-2-associated urinary bladder cancer. This is the first report of the detection of telomerase activity in bovine cells. Moreover, this activity was detected only in BPV-positive samples and not in normal specimens (0 of 10) or BPV-negative tumours (0 of 7). Although the number of samples examined is rather limited, this result seems to indicate that telomerase activity is upregulated at least in some BPV-associated cancers. However, further investigations are needed.

The presence of viral DNA, of endocytosolic E5 protein and the alteration of telomerase activity only in BPV-associated bladder tumours strongly supports that, in areas of CEH and of bracken fern infestation, more than 70% of tumours are caused by BPV infection. The virus and chemicals in bracken fern appear to act synergistically in inducing cell transformation and immune escape.

These results open the perspective to start a vaccination programme to eradicate infection. Since E5 seems likely to play a role in urinary bladder carcinogenesis, it would

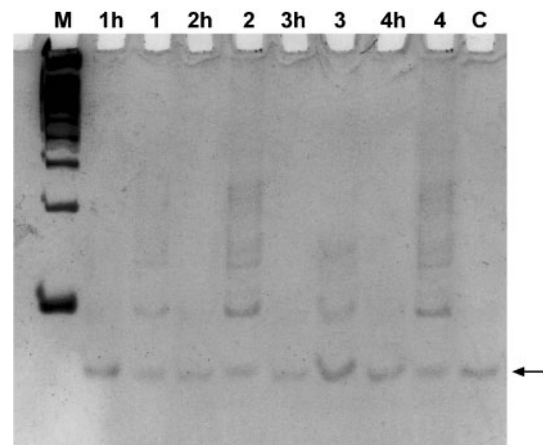


Fig. 3. Telomerase activity in urinary bladder samples. Protein extracts from urinary bladder tumours (lanes 1–4) and heat-inactivated samples (lanes 1h–4h) were analysed for the presence of telomerase activity using the detection kit Trapeze. The arrow indicates the 36 bp amplification product of the internal control. The DNA ladder over the 50 bp template indicates telomerase activity (lanes 1–4). C, negative control with no protein extracts added; M, 50 bp marker (Roche Diagnostics).

represent a target for immunomediated therapies. Recently, it has been reported that mouse vaccination with HPV-16 E5 reduced the growth of tumours induced by E5-expressing cells (Liu *et al.*, 2000). The potential for E5 vaccination should be validated in naturally occurring animal models and, moreover, the development of therapeutic/preventive immunoprophylaxis for these mucosal tumours would represent a model for human pathologies and could add precious indications for their treatment.

ACKNOWLEDGEMENTS

This work was supported partially by the Ministero dell'Istruzione, Università e Ricerca (MIUR) (PRIN 2000, 2002), Ministry of Health, Associazione Italiana Ricerca Cancro, Compagnia di San Paolo. We thank Professor M. Saveria Campo for her critical reading of the manuscript. We are indebted to Dr Richard Schlegel for his kind gift of the anti-E5 antibody and for his critical reviewing of the immunofluorescence data.

REFERENCES

- Anderson, R. A., Scobie, L., O'Neil, B. W., Grindlay, G. J. & Campo, M. S. (1997). Viral proteins of bovine papillomavirus type 4 during the development of alimentary canal tumours. *Vet J* **154**, 69–78.
- Argyle, D. J. & Nasir, L. (2003). Telomerase: a potential diagnostic and therapeutic tool in canine oncology. *Vet Pathol* **40**, 1–7.
- Ashrafi, G. H., Tsirimonaki, E., Marchetti, B., O'Brien, P. M., Sibbet, G. J., Andrew, L. & Campo, M. S. (2002). Down-regulation of MHC class I by bovine papillomavirus E5 oncoproteins. *Oncogene* **21**, 248–259.
- Baega, A. C., Berger, A., Schlegel, R., Veldman, T. & Schlegel, R. (2002). Cervical epithelial cells transduced with the papillomavirus E6/E7 oncogenes maintain stable levels of oncoprotein expression but exhibit progressive, major increases in hTERT gene expression and telomerase activity. *Am J Pathol* **160**, 1251–1257.
- Beniston, R. G., Morgan, I. M., O'Brien, V. & Campo, M. S. (2001). Quercetin, E7 and p53 in papillomavirus oncogenic cell transformation. *Carcinogenesis* **22**, 1069–1076.
- Bodnar, A. G., Ouellette, M., Frolkis, M. & 7 other authors (1998). Extension of life-span by introduction of telomerase into normal human cells. *Science* **279**, 349–352.
- Bohl, J., Hull, B. & Vande Pol, S. B. (2001). Cooperative transformation and coexpression of bovine papillomavirus type 1 E5 and E7 proteins. *J Virol* **75**, 513–521.
- Borzacchiello, G., Ambrosio, V., Galati, P., Poggiali, F., Venuti, A. & Roperto, F. (2001). The pagetoid variant of urothelial carcinoma *in situ* of urinary bladder in a cow. *Vet Pathol* **38**, 113–116.
- Borzacchiello, G., Ambrosio, V., Roperto, S., Poggiali, F., Tsirimonakis, E., Venuti, A., Campo, M. S. & Roperto, F. (2003). Bovine papillomavirus type 4 in oesophageal papillomas of cattle from the South of Italy. *J Comp Pathol* **128**, 203–206.
- Burkhardt, A., Willingham, M., Gay, C., Jeang, K. T. & Schlegel, R. (1989). The E5 oncoprotein of bovine papillomavirus is oriented asymmetrically in Golgi and plasma membranes. *Virology* **170**, 334–339.
- Burnett, S., Jareborg, N. & DiMaio, D. (1992). Localization of bovine papillomavirus type 1 E5 protein to transformed basal keratinocytes and permissive differentiated cells in fibropapilloma tissue. *Proc Natl Acad Sci U S A* **89**, 5665–5669.
- Cairney, M. & Campo, M. S. (1995). The synergism between bovine papillomavirus type 4 and quercetin is dependent on the timing of exposure. *Carcinogenesis* **16**, 1997–2001.
- Campo, M. S. (1997). Bovine papillomavirus and cancer. *Vet J* **154**, 175–188.
- Campo, M. S., McCaffery, R. E., Doherty, I., Kennedy, I. M. & Jarrett, W. F. (1990). The Harvey *ras* 1 gene is activated in papillomavirus-associated carcinomas of the upper alimentary canal in cattle. *Oncogene* **5**, 303–308.
- Campo, M. S., Jarrett, W. F. H., Barron, R., O'Neill, B. W. & Smith, K. T. (1992). Association of bovine papillomavirus type 2 and bracken fern with bladder cancer in cattle. *Cancer Res* **52**, 6898–6904.
- Campo, M. S., O'Neil, B. W., Barron, R. J. & Jarrett, W. F. (1994). Experimental reproduction of the papilloma-carcinoma complex of the alimentary canal in cattle. *Carcinogenesis* **15**, 1597–1601.
- Carr, E. A., Thèon, A. P., Madewall, B. R., Hitchcock, M. E., Schlegel, R. & Schiller, J. T. (2001). Expression of a transforming gene (E5) of bovine papillomavirus in sarcoids obtained from horses. *Am J Vet Res* **62**, 1212–1217.
- Chang, J. L., Tsao, Y. P., Liu, D. W., Huang, S. J., Lee, W. H. & Chen, S. L. (2001). The expression of HPV-16 E5 protein in squamous neoplastic changes in the uterine cervix. *J Biomed Sci* **8**, 206–213.
- Confer, A. W. & Panciera, R. J. (2001). The urinary system. In *Thomson's Special Veterinary Pathology*, 3rd edn, pp. 235–277. Edited by W. W. Carlton & J. F. Zachary. St Louis, Missouri: Mosby.
- Connolly, J., Morgan, I. M., Jackson, M. E. & Campo, M. S. (1998). The BPV-4 co-carcinogen quercetin induces cell cycle arrest and up-regulates transcription from the LCR of BPV-4. *Oncogene* **16**, 2739–2746.
- Conrad, M., Bubb, V. J. & Schlegel, R. (1993). The human papillomavirus type 6 and 16 E5 proteins are membrane-associated proteins which associate with the 16-kilodalton pore-forming protein. *J Virol* **67**, 6170–6178.
- Faccini, A. M., Cairney, M., Ashrafi, G. H., Finbow, M. E., Campo, M. S. & Pitts, J. D. (1996). The bovine papillomavirus type 4 E8 protein binds to ductin and causes loss of gap junctional intercellular communication in primary fibroblasts. *J Virol* **70**, 9041–9045.
- Finbow, M. E., Pitts, J. D., Goldstein, D. J., Schlegel, R. & Findlay, J. B. (1991). The E5 oncoprotein target: a 16-kDa channel-forming protein with diverse functions. *Mol Carcinog* **4**, 441–444.
- Goldstein, D. J., Finbow, M. E., Andresson, P., McLean, P., Smith, K., Bubb, V. & Schlegel, R. (1991). Bovine papillomavirus E5 oncoprotein binds to the 16K component of vacuolar H⁺-ATPases. *Nature* **352**, 347–349.
- Kim, N. W., Piatyszek, M. A., Prowse, K. R. & 7 other authors (1994). Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**, 2011–2015.
- Leal, A. M., Ferraz, O. P., Carvalho, C., Freitas, A. C., Beniston, R. G., Campo, M. S., Beçak, W. & Stocco dos Santos, R. C. (2003). Quercetin induces structural chromosome aberrations and uncommon rearrangements in cells transformed by the E7 protein of BPV-4. *Vet Comp Oncol* (in press).
- Lee, D., Kim, H. Z., Jeong, K. W., Shim, Y. S., Horikawa, I., Barrett, J. C. & Choe, J. (2002). Human papillomavirus E2 down-regulates the human telomerase reverse transcriptase promoter. *J Biol Chem* **277**, 27748–27756.
- Liu, D. W., Tsao, Y. P., Hsieh, C. H., Hsieh, J. T., Kung, J. T., Chiang, C. L., Huang, S. J. & Chen, S. L. (2000). Induction of CD8 T cells by vaccination with recombinant adenovirus expressing human papillomavirus type 16 E5 gene reduces tumor growth. *J Virol* **74**, 9083–9089.

- Marchetti, B., Ashrafi, G. H., Tsirimonaki, E., O'Brien, P. M. & Campo, M. S. (2002).** The bovine papillomavirus oncoprotein E5 retains MHC class I molecules in the Golgi apparatus and prevents their transport to the cell surface. *Oncogene* **21**, 7808–7816.
- Moyzis, R. K., Buckingham, J. M., Cram, L. S., Dani, M., Deaven, L. L., Jones, M. D., Meyne, J., Ratliff, R. L. & Wu, J. R. (1988).** A highly conserved repetitive DNA sequence, (TTAGGG)_n, present at the telomeres of human chromosomes. *Proc Natl Acad Sci U S A* **85**, 6622–6626.
- Oelze, I., Kartenbeck, J., Crusius, K. & Alonso, A. (1995).** Human papillomavirus type 16 E5 protein affects cell–cell communication in an epithelial cell line. *J Virol* **69**, 4489–4494.
- Pennie, W. D. & Campo, M. S. (1992).** Synergism between bovine papillomavirus type 4 and the flavonoid quercetin in cell transformation *in vitro*. *Virology* **190**, 861–865.
- Pennie, W. D., Grindlay, G. J., Cairney, M. & Campo, M. S. (1993).** Analysis of the transforming functions of bovine papillomavirus type 4. *Virology* **193**, 614–620.
- Plaumann, B., Fritsche, M., Rimpler, H., Brandner, G. & Hess, R. D. (1996).** Flavonoids activate wild-type p53. *Oncogene* **13**, 1605–1614.
- Schapiro, F., Sparkowski, J., Adduci, A., Supryniewicz, F., Schlegel, R. & Grinstein, S. (2000).** Golgi alkalization by the papillomavirus E5 oncoprotein. *J Cell Biol* **148**, 305–315.
- Shahin, M., Smith, B. L. & Prakash, A. S. (1999).** Bracken carcinogens in the human diet. *Mutat Res* **443**, 69–79.
- Smith, B. L. (1997).** The toxicity of bracken fern (genus *Pteridium*) to animals and its relevance to man. In *Handbook of Plant and Fungal Toxicants*. Edited by F. J. P. D'Mello. Boca Raton, FL: CRC Press.
- Straight, S. W., Herman, B. & McCance, D. J. (1995).** The E5 oncoprotein of human papillomavirus type 16 inhibits the acidification of endosomes in human keratinocytes. *J Virol* **69**, 3185–3192.
- Venuti, A. & Campo, M. S. (2002).** The E5 protein of papillomavirus. In *Human Papillomavirus: Perspectives in Medical Virology*, vol. 8, pp. 143–164. Edited by D. McCance. Amsterdam: Elsevier Science.