Short Communication

Bovine papillomavirus type 2 infects the urinary bladder of water buffalo (Bubalus bubalis) and plays a crucial role in bubaline urothelial carcinogenesis

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Bovine papillomavirus type 2 (BPV-2) has been shown to infect and play a role in urinary bladder carcinogenesis of buffaloes grazed on pastures with ferns from the Marmara and Black Sea Regions of Turkey. BPV-2 DNA has been found in both neoplastic and non-neoplastic lesions of the urinary bladder. Furthermore, this virus may be a normal inhabitant of the urinary bladder since BPV-2 DNA has also been detected in clinically normal buffaloes. The viral activation by fern immunosuppressant or carcinogen may trigger the urothelial cell transformation. The E5 oncoprotein was solely detected in urothelial tumours and appeared to be co-localized with the overexpressed and phosphorylated platelet derived growth factor (PDGF) β receptor in a double-colour immunofluorescence assay. Our results indicate that the E5–PDGF β receptor interaction also occurs in spontaneous tumours of the bubaline urinary bladder, revealing an additional role of BPV-2 in bladder carcinogenesis of buffaloes.

Papillomaviruses (PVs) are small, non-enveloped, dsDNA viruses with a well-defined tropism for cutaneous and mucosal epithelia in a wide variety of animals and humans (IARC, 2007; Moody & Laimins, 2010). PVs are involved in the pathogenesis of animal and human tumours (zur Hausen, 2009). The increasing interest to investigate PVs has led to the discovery of novel PV types. Therefore, the classification of papillomaviruses (de Villiers et al., 2004) has been updated including 189 types after the incorporation of 28 novel human PV (HPV) and 48 novel animal PV types (Bernard et al., 2010). Meanwhile, further new PV types have been described previously (Bottalico et al., 2012; Rogovskyy et al., 2012; Zhu et al., 2012).

Thirteen bovine papillomavirus (BPV) types (BPV-1 to -13) have been completely described and assigned to four genera (Lunardi et al., 2012). Bovine papillomavirus types 1 and 2 (BPV-1/-2) belong to the genus Deltapapillomavirus, species 4 (de Villiers et al., 2004) just like the recently detected BPV-13 (Lunardi et al., 2012). BPV-1/-2 are the only papillomaviruses known to infect both epithelial and mesenchymal cells and show an interspecies-transmission (Campo, 2006; IARC, 2007). It was so far believed that BPV-1/-2 replication and virion production, similar to other PVs, were confined to stratified epithelium (Campo, 2006; IARC, 2007; Shafti-Keramat et al., 2009). Recently, an in vivo productive infection of BPV-2 has also been reported in peripheral blood mononuclear cells (PBMCs) and placental trophoblastic epithelium (Roperto et al., 2011; Roperto et al., 2012).

BPV-1/-2 cause cutaneous tumours in horses (Lancaster et al., 1979), buffaloes (Silvestre et al., 2009; Pangty et al., 2010; Somvanshi, 2011), cats (Munday & Knight, 2010), African lions (Orbell et al., 2011), Cape mountain zebras, giraffes and sable antelopes (Williams et al., 2011; van Dyk et al., 2012). In addition, BPV DNA has been identified in a squamous cell carcinoma of head and neck in a mare (Kainzbauer et al., 2012).
BPV-2 is known to play a central role in bladder carcinogenesis of adult cattle reared on pasturelands rich in bracken fern (*Pteridium aquilinum*) (Campo et al., 1992; Borzacchiello et al., 2003; Wosiaki et al., 2006; Roperto et al., 2008). As a matter of fact, tumours of the urinary bladder are very common in cattle grazing on pastures containing bracken fern and their incidence varies but may be more than 90% in adult animals (Pamukcu et al., 1976; Ozkul & Aydin, 1996; Roperto et al., 2010a).

The activation of the platelet derived growth factor (PDGF) β receptor and c-Src proteins is known to occur in E5-dependent epithelial carcinogenesis (Suprynowicz et al., 2002; Borzacchiello et al., 2006, 2007). New molecular pathways have been found in BPV-2-associated bladder carcinogenesis of cattle. BPV-2 has been shown to be responsible for an overexpression of E2F3 growth factor via the activation of the proteolytic form of Calpain 3 (Capn 3), leading to urothelial cell proliferation (Roperto et al., 2010b).

Tumours of the urinary bladder of buffaloes have only sporadically been reported (Pamukcu, 1957; Ozkul & Aydin, 1996), thus making this a totally uncovered area of investigation (Somvanshi, 2011; Somvanshi et al., 2012).

Here, we describe the presence of BPV-2 DNA, the expression of E5 oncoprotein and its interaction with the PDGF β receptor that appears to be overexpressed and phosphorylated in 21 urinary bladder tumours in buffalo from the Marmara and Black Sea Regions of Turkey. This study is the first to reveal an association between BPV-2 infection and tumours of the urinary bladder in buffaloes reared on lands rich in bracken fern, demonstrating that BPV-2 is involved in buffalo bladder carcinogenesis too.

Histological examination of the bladder neoplastic lesions detected microscopic patterns consistent with the diagnosis of papilloma (two cases), papillary urothelial neoplasms of low malignant potential (PUNLMP - two cases), papillary carcinoma (12 cases) and invasive carcinoma (five cases) following morphological parameters recently suggested for urinary bladder tumours of cattle (Roperto et al., 2010a). Reactive urothelial atypia (two cases) and chronic cystitis (three cases) characterized by the presence of so-called tertiary lymphoid follicles were also seen.

PCR yielded BPV-2 DNA identical fragments of 503 bp in all neoplastic as well as in three of five non-neoplastic bladder samples (Fig. 1). PCR analysis was also performed on 11 urinary bladder samples from apparently healthy buffaloes. The BPV-2 DNA fragment was also detected in more than 50% being found in six of them, thus indicating the presence of a possible latent infection.

The presence of BPV-2 DNA was also confirmed by sequencing (Fig. 1), which revealed a perfect homology (100%) with bovine BPV-2 DNA sequences repeatedly found in Italy (Borzacchiello et al., 2003). GenBank accession number of our representative BPV-2 sequence is NC_001521.

Immunohistochemically, the expression of the E5 oncoprotein was detected in bladder tumours only (Fig. 2a). No E5 immunostaining was seen in both non-neoplastic lesions and normal mucosa containing BVP-2 DNA (Fig. 2b). Just like in bladder cancers of cattle, E5 oncoprotein showed a strong interaction with the activated PDGF β receptor in cancer cells thus appearing to be responsible for bubaline urothelial cell transformation. In fact, the expression and phosphorylation status of the receptor were investigated in 12 tumours (seven papillary carcinomas, three invasive carcinomas, one papilloma and one PUNLMP). PDGF β receptor was found to be over-expressed and phosphorylated in the urothelial tumours versus non-neoplastic as well as normal urothelium of buffaloes, suggesting that the receptor is activated in the tumour cells more than in non-neoplastic and normal cells (Fig. 2c, d). Furthermore, E5 expression and PDGF β receptor were detected by immunofluorescence (Fig. 3a, b), and their cytoplasmic co-localization was clearly revealed by laser scanning confocal microscopy (LSCM) as judged by the yellow fluorescence of the merged image (Fig. 3c).

On the contrary, a few cells, showing the PDGF β receptor constitutively expressed and phosphorylated were detected by LSCM both in control and pathological non-neoplastic tissues; furthermore, no E5 expression was seen; consequently, the fluorescence of the merged image failed to show any co-localization of these proteins (Fig. 3d).

To our knowledge, no association of BPV-2 infection and urinary bladder tumours has been reported for buffaloes thus far. Here, we provide evidence for the expression of the E5 oncoprotein in neoplastic lesions solely within the buffalo urinary bladder. However, BPV-2 DNA was also found in a rather high number of non-neoplastic bladder lesions as well as in some apparently healthy urinary bladder. Our results indicate that a latent infection of BPV-2 may occur in bubaline normal urothelium. This correlates with a recent report regarding the detection of BPV-2 DNA in the normal urinary bladder of a buffalo from a herd kept very close to cattle (Pathania et al., 2012). Furthermore, our findings indicate that the E5 oncoprotein is responsible for urothelial cell transformation via the activation of the PDGF β receptor also in buffaloes.

The Marmara and Black Sea Regions of Turkey are geographical areas where temperate weather and field pH ranging from 5.5 to 7.5 are considered favourable ecological conditions for luscious growth of ptaquiloside-containing ferns (Alonso-Amelot & Avendaño, 2002; Vetter, 2009). Cattle and buffaloes are the backbone of the rural economy of these regions, being the main source of milk, meat, work power and manure. Due to local cattle and buffalo breeding techniques, buffaloes graze daily on these fern-infested lands. We suggest that the carcinogenic and/or immunosuppressive chemicals of fern cause and/or contribute to urothelial cell transformation acting synergistically with BPV-2 infection. Where animal husbandry based on intensive breeding techniques does not allow the animals to feed on contaminated pastures, the bladder
disease is unknown despite the fact that clinical cases of BPV infection can be seen, i.e. papillomatosis of the skin. Similar findings are known to occur also in some cases of cancer of the upper GI tract in humans. A geographical overlap between consumption of bracken fern, infection of the alimentary canal mucosa by HPV and oesophageal cancer in the Minais Gerais region of Brazil has been reported (Campo, 2006).

BPV-2 infection is becoming more important from an economic point of view in large animals. In fact BPV-2 infection takes place also in the placenta of aborted buffalo (S. Roperto, unpublished data) and of cows, leading to severe reproductive disorders (Roperto et al., 2012). Further detailed epidemiological and virological studies are needed to better understand the incidence of BPV-2 infections in buffaloes and its emerging role in neoplastic and non-neoplastic diseases.

Cattle and buffalo breeding techniques used in many world regions, i.e. in Turkish and Indian regions, make these investigations rather urgent since cattle and buffalo herds are kept together increasing the risk of cross-infection.

Bladder tissue specimens were sampled in public slaughter-houses (Bafra, Coskun and Istanbul) in the Marmara and Black Sea Regions (Turkey). All buffaloes were from herds grazing daily on bracken and other fern pastures. Samples from 21 urinary bladders with severe neoplastic and five with non-neoplastic lesions as well as from 11 normal urinary bladders were collected.

Fig. 1. PCR amplification of BPV-2 DNA. The amplified 503 bp DNA fragment. Lane 1: M, molecular mass marker (1 kb DNA Ladder; Microtech). Lanes 2–4 are representative of two papillary carcinomas (PC) and of one invasive carcinoma (IC). Lanes 5–7 are representative of two chronic cystites (CC) and of one reactive atypia (RA). Lane 8: DNA from urinary bladder of healthy ovine (OVI). Lane 9: a normal urinary bladder without BPV-2 DNA. Lane 10: a normal urinary bladder containing BPV-2 DNA. Lane 11: positive (Pos) control containing a cloned BPV-2 DNA; lane 12: negative (Neg) control (no DNA added). The lower part of the figure shows 100 % homology between the sequence of the amplicons in lanes 2–4 and the sequence of BPV-2 found in Italy (GenBank accession no. M20219.1).
For histological investigations, 5 μm thick sections from paraffin-embedded specimens were stained with haematoxylin and eosin (HE). For immunohistochemical analysis, the sections were deparaffinized and endogenous peroxidase activity was blocked by incubation with 0.3 % H2O2 in methanol for 20 min. Antigen retrieval was performed by heat treatment (twice for 5 min each at 750 W) in citrate buffer pH 6.0. Slides were washed three times with PBS, pH 7.4, 0.01 M, and then incubated for 1 h at room temperature with donkey serum (Sigma-Aldrich) diluted at 1 : 10 in PBS. A polyclonal sheep anti-BPV-2 E5 primary antibody (courtesy of Dr M. S. Campo, University of Glasgow, UK) diluted at 1 : 40 000 in PBS, was applied for 1 h at room temperature in a humid chamber. The sections were rinsed three times for 5 min with PBS before application of the donkey anti-sheep biotinylated secondary antibody (Santa Cruz Biotechnology), diluted at 1 : 100 in PBS for 40 min at room temperature. Colour development was obtained by incubation with diaminobenzidine (DakoCytomation). DNA was extracted from frozen representative pathological and control normal bladder samples using the DNeasy Tissue kit (Qiagen). PCR compatibility of thus obtained DNA was assessed by standard β-actin PCR by using specific primers designed by Primer BLAST software. To amplify the entire BPV-2 genome, the purified DNA was submitted to the multiply primed rolling-circle amplification technique using a mix with 20 ng sample DNA, allow antigen unmasking. Slides were then pre-incubated with normal donkey serum diluted at 1 : 20 in PBS for 30 min, and overlaid with polyclonal sheep anti-BPV-2 E5 diluted at 1 : 25 in PBS at 4 °C, overnight, in a humid chamber. Then, a polyclonal goat anti-p-PDGF β receptor antibody (Santa Cruz Biotechnology) diluted at 1 : 25 in PBS was applied overnight. A secondary antibody Alexa Fluor 488 donkey anti-sheep (green) (Invitrogen, Molecular Probes) and a secondary antibody Alexa Fluor 546 donkey anti-goat (red) (Invitrogen, Molecular Probes), diluted at 1 : 50 in PBS were applied for 2 h at room temperature. The slides were washed three times with PBS and mounted under aqueous medium (Sigma-Aldrich). For observation and photography, a laser scanning confocal microscope (LSM-510; Zeiss) was used.

Fig. 2. Papillary carcinoma. (a) Immunohistochemical detection of cytoplasmic E5 in neoplastic urothelial cells. Magnification, ×550. Chronic cystitis. (b) Immunohistochemical analysis failed to show the E5 oncoprotein in BPV-2 DNA-containing urothelial cells. Magnification, ×550. Papillary carcinoma. (c) Overexpression and phosphorylation status of the PDGF β receptor detected immunohistochemically. Magnification, ×550. Normal urothelium from healthy buffaloes. (d) A few urothelial cells are seen to express an activated PDGF β receptor. Magnification, ×550.

Fig. 3. Papillary carcinomas. (a) LSCM investigation: red immunofluorescence showing the overexpression and phosphorylation status of the PDGF β receptor. Magnification, ×550. (b) Green immunofluorescence detecting the expression of the E5 oncoprotein. Magnification, ×550. (c) Co-localization of the receptor and the oncoprotein documented by yellow immunofluorescence of the merged image. Arrows show the frequent juxtaglomerular localization of these proteins. Magnification, ×550. (d) Normal urothelium from healthy buffaloes. Immunofluorescence of the merged image failed to show any co-localization of the E5 oncoprotein with PDGF β receptor. Magnification, ×550.
12.5 μM of each primer, 4 mM dNTPs and 10 U phi 29 DNA polymerase (Fermentas) (Rector et al., 2004). The resulting linear dsDNA product was purified using MinElute PCR Purification kit (Qiagen). For the detection of BPV-2 DNA, specific primers for a 503 bp DNA amplicon encompassing the BPV-2 E5-L2 ORF sequence (nt 3723–4225) were designed by Primer BLAST software. Aliquots 50–100 ng each sample were amplified in 25 μl of reaction mixture containing 4 mM MgCl₂, 2.5 μl 10 × Gold buffer, 5 U AmpliTaq Gold DNA Polymerase (Applied Biosystems), 480 nM each primer and 200 µM each dNTP. The reaction was carried out in a thermocycler (Veriti; Applied Biosystems) using the 5′-TCAGGCACAGATCTTGATCA-3′ forward primer and the 5′-TCATAGACATTTGCACGTT-3′ reverse primer. PCR conditions were as follows: denaturation for 3 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 1 min. A final extension step at 72 °C for 5 min was performed in each PCR assay. Amplification products were visualized by gel electrophoresis using ethidium bromide staining. In each experiment we included a no template control and cloned BPV-2 DNA as a positive control (a kind gift by Dr A. Venuti, Istituto dei Tumori ‘Regina Elena’, Roma). The amplified DNA was subjected to direct sequencing in an automated apparatus (ABI Prism 3100 Genetic Analyzer; Applied Biosystems).

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