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Congenital papillomavirus infection in cattle: Evidence for transplacental transmission

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ABSTRACT

Vertical transmission of bovine papillomavirus (BPV) infection was investigated on livers and kidneys of four foetuses from cows suffering from BPV-2-associated urothelial cancers of the urinary bladder. PCR analysis revealed the presence of BPV-2 E5 DNA in the livers and kidneys of two foetuses. Amplified DNA fragments, composed of 502 bp, showed a 100% homology with BPV-2 sequences (GenBank accession number: M20219.1). BPV-2 was found to be transcriptionally active. Indeed, reverse transcriptase (RT)-PCR showed BPV-2 E5 transcripts. Sequencing of amplified cDNA, composed of 154 bp, showed a 100% identity with BPV-2 E5 sequences (GenBank accession number: M20219.1). Western blot analysis revealed the presence of dimers of E5 oncoprotein. Furthermore, a statistically significant increase of the phosphorylated (activated) form of the platelet-derived growth factor ß receptor (PDGFßR) was also detected in the fetal tissues. PDGFßR is believed to form the most important interaction with the E5 oncoprotein, thus regulating biological activity of virus protein. The strong concordance between virus found in fetal organs with virus detected in infected mothers provides evidence that BPV-2 can spread through blood and vertical infection occurs via transplacental transmission. Finally, molecular findings of this study raise unsolved questions about the potential role of BPVs in reproductive disorders. The presence of E5 oncoprotein, as in adult organs, may also activate the constitutive receptor PDGFßR in foetal organs, which plays a pivotal role in angiogenesis and embryonic development. Therefore, abnormal phosphorylation of PDGFßR may be involved in vascular and organogenesis abnormalities other than cancer.

1. Introduction

Papillomaviruses (PVs) are small, non-enveloped, double-stranded DNA viruses that infect mucosal and cutaneous epithelia of vertebrates, resulting in benign and malignant lesions of skin and mucosa (International Agency for Research on Cancer, 2007).

Bovine papillomaviruses (BPVs), are the second most studied PVs following human PVs. The BPV family comprises 24 fully sequenced and characterised members classified into five genera. BPV-2 belongs to Deltapapillomavirus (δPV) genus, all members of which are considered to be high-risk virus in association with neoplastic transformation (Daudt et al., 2018).

δPVs recognise *Bos taurus* as their classical host. However, δPVs are the only PVs known to be characterised by natural cross-species transmission and infection (International Agency for Research on Cancer, 2007). δPVs cause cutaneous tumours in horses (Lancaster et al., 1979), buffaloes (Silvestre et al., 2009; Pangty et al., 2010; Somvanshi, 2011), cats (Munday and Knight, 2010), African lions (Orbell et al., 2011), Cape mountain zebras, giraffes and sable antelopes (Williams et al., 2011; van Dik et al., 2012). BPV DNA has been identified in a squamous cell carcinoma involving the head and neck of a mare and in cutaneous wart lesions from ovines (Kainzbauer et al., 2012; Mazzucchelli-de-Souza et al., 2018). Furthermore, δPVs, especially BPV-2 and BPV-13, play a central role in bovine and bubaline bladder carcinogenesis (Campo et al., 1992; Roperto et al., 2013b, 2016). Recently, congenital infection by BPV-2 and BPV-13 has been reported in newborn lambs (Roperto et al., 2018b).

E5 oncoprotein, the best studied protein (DiMaio and Petti, 2013), is believed to be the major oncoprotein of δPVs. It displays transforming activity via numerous pathways. Its interaction with the platelet-derived growth factor β receptor (PDGFβR) represents the most important pathway responsible for cell transformation in spontaneous

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carcinogenesis of cattle. Indeed, it has been shown that E5 oncoprotein is responsible for PDGFβR phosphorylation and binds to the activated form of PDGFβR, which, in turn, results in AKT phosphorylation, a downstream mediator that appears to play a crucial role in cell transformation in naturally occurring urothelial cancer of cattle (Borzacchiello et al., 2006; Roperto et al., 2013b; Russo et al., 2016).

δPV E5 DNA was found in peripheral blood mononuclear cells (PBMCs) of cattle suffering from BPV infection (Campo et al., 1992; Stocco dos Santos et al., 1998) and equine with sarcoids (Brandt et al., 2008). BPV-1 and BPV-2 DNA was also found in bloodstream of cattle affected by papillomavirus co-infection (Santos et al., 2016). BPV-1, BPV-2 and BPV-13 DNA have also been found in PBMCs of sheep (Roperto et al., 2018a). Furthermore, the E5 oncoprotein of BPV-2 was detected for the first time in PBMCs of cattle suffering from urothelial tumours (Roperto et al., 2008). PBMCs and placentas of cows are believed to be additional sites for BPV-2 productive infection as L1 protein was found in CD4(+) and CD8(+) lymphocytes and placentomes (Roperto et al., 2011, 2012; Melo et al., 2015).

The primary mode of PV transmission is the horizontal route by sexual activity through direct contact with infected cervical, vaginal, vulvar, penile or anal epithelium (International Agency for Research on Cancer, 2007). However, epidemiological and clinical data support several possible non-sexual modes of transmission including vertical transmission from an infected mother to the offspring (Freitas et al., 2013; Koshimaa et al., 2012, 2017; Sabeena et al., 2017). Much controversy still exists related to vertical transmission of PV DNA especially about the magnitude of the risk and the route and timing of such transmission (Lee et al., 2013).

The aim of this paper was to report, for the first time, the presence of BPV-2 E5 oncoprotein in the liver and kidneys of two bovine foetuses from infected cows with urinary bladder tumours associated with BPV-2 infection.

2. Materials and methods

2.1. Ethics statement

In this study we did not perform any animal experiments. All samples were collected post-mortem from slaughterhouses, and no ethics approval was required.

2.2. Animals and tissue samples

Liver and kidney samples of four bovine foetuses, between days 210 and 240 of gestation, were retrieved from the archive of our department. Samples from two foetuses were stored at −80 °C since they had been frozen in nitrogen liquid; samples from other two foetuses were from foetal tissues fixed in formalin. Foetuses were from cows, all of which were slaughtered after a mandatory clinical ante-mortem examination as required by the European Union legislation. All four cows had suffered from chronic enzootic haematuria. Post-mortem examination revealed the presence of bladder tumours in all animals. Molecular virological investigations performed on neoplastic bladder samples detected the presence of BPV-2 E5 oncoproteins. In one of these pregnant cows, a BPV-2 productive infection was also detected in blood and placentomes (Roperto et al., 2011, 2012). In the remaining three pregnant cows, the presence of BPV-2 E5 oncoprotein was unveiled in the bladder and blood only (Roperto et al., 2013a). Healthy and pathological tissues (bladder and liver) used as control in this study were also retrieved from our archive.

2.3. DNA extraction and polymerase chain reaction (PCR) amplification

Total DNA was extracted from samples of livers and kidneys of four foetuses, neoplastic bladder samples from an infected mother and liver samples from a healthy cow as a negative control using a DNeasy Blood & Tissue Kit (Qiagen TM, ME, DE) according to the manufacturer's instructions. PCR was performed with 100 ng of DNA. Specific primer sets for PCR was performed with a specific primer set designed by the Primer-BLAST online tool for BPV-2 E5 DNA composed of 502 bp, using β-actin as a control. The following primers were used: BPV-2 E5 forward 5′- TCATAGACATTTGCACGTT -3′, reverse 5′- TCAGGCACAGAT CTTGATCA -3′; β-actin forward 5′- GAGCGTGGCTACAGCTTCA-3′, reverse 5′- CATTGCCGATGGTGATGA -3′. Conditions for PCR were: 94 °C for 10 min, 40 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s.

2.4. RNA extraction and reverse transcription (RT)-PCR

Total RNAs were extracted from the frozen liver and kidney tissues of two positive foetuses and from bladder tumour samples of two infected cows, including an infected mother, by RNeasy Mini Kit (74134, Qiagen TM, ME, DE), according to the manufacturer's instructions. All RNA samples were analysed spectrophotometrically on a Nanodrop (Nanovue plus, GE healthcare, IL, USA). Five hundred nanograms of the total RNA was used to generate the first strand of cDNA by the QuantiTect Reverse Transcription Kit (205311, Qiagen TM, ME, DE) according to the manufacturer's instructions. PCR was performed with a specific primer set designed by the Primer-BLAST online tool for BPV-2 E5 cDNA composed of 154 bp, using β-actin as a control. The following primers were used: forward 5′- CACTGCCATTTGTTTTTTTC-3′, reverse 5′- GGAGCACTCAAAATGATCCC -3′; BPV-13 E5: forward 5′- CACTGC CATTTGGTGTTCTT -3′, reverse 5′- AGCAGTCAAAATGATCCCAA-3′, β-actin forward 5′-TAGCACAGGCCTCTCGCCTTCG-3′, reverse 5′-GCACA TGCCGGAGCCGTTGT-3′. Conditions for PCR were: 94 °C for 10 min, 40 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s. For each sample, RT-PCR was repeated in triplicate to validate the accuracy of the obtained data.

2.5. Sequence analysis

PCR products from DNA and cDNA were purified using a Qiaquick PCR Purification Kit (Qiagen TM, ME, DE) and bidirectionally sequenced using a BigDye_Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) following the manufacturer's recommendations. Sequences were dye-terminator removed by DyeEx_ 2.0 spin kit (Qiagen TM, ME, DE) and run on a 3500 Genetic Analyser (Applied Biosystems, CA, USA). Electropherograms were analysed using Sequencing Analysis v5.2 and Sequence Scanner v1.0 software (Applied Biosystems, CA, USA). The sequences obtained were compared to others in GenBank using the BLAST programme.

2.6. Western blot analysis for BPV-2 E5 oncoprotein and platelet-derived growth factor receptor (PDGFR)

Western blot analyses were performed on BPV-2 E5 cDNA-expressing liver and kidneys of the two foetuses, from neoplastic bladder and liver samples of an infected mother showing BPV-2 E5 oncoprotein in blood and from healthy liver tissue samples used as negative control. PDGFR immunoblotting was performed on liver and kidney samples of two foetuses, (one infected and one non-infected) and on neoplastic bladder samples of an infected mother. All samples were lysed in radioimmunoprecipitation assay-morpholinepropanesulfonic acid (RIPA-MOPS) buffer (20 mM MOPS, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% deoxycholate, and 0.1% SDS) containing protease and phosphatase inhibitors and extracted proteins were quantified by Bradford assay. Sixty micrograms of extracted proteins were boiled and electrophoresed for 1.5 h at 150 V on a 15% (wt/vol) polyacrylamide/sodium dodecyl sulphate (SDS) gel. Samples were transferred for 1.5 h at 100 V at polyvinylidene difluoride (PVDF) membranes in transfer buffer (25 mM Tris base, 192 mM glycine, and 20% (vol/vol) methanol). Membranes were blocked in 5% (wt/vol) nonfat dry milk in TBST (10 mM Tris · HCl (pH 7.4), 167 mM NaCl, 1% Tween-20) for 1 h

and incubated overnight at 4 °C with an anti-E5 rabbit polyclonal antiserum recognising the C-terminal 14 amino acids of the BPV E5 protein (a kind gift provided by Prof. DiMaio, Yale University, New Haven, USA), diluted 1:1,250, in 5% (wt/vol) milk/TBST, an anti-pPDGFR-β, a rabbit polyclonal antibody (sc-1209-R), an anti-PDGFR-β, a rabbit polyclonal antibody (sc-432) and an anti-β actin mouse monoclonal antibody (sc-47778), Santa Cruz Biotechnology, TX, USA. Blots were washed 3 times in TBST and subsequently incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit or donkey anti-mouse secondary antibody diluted 1:3000, in 5% (wt/vol) milk/TBST. Blots were then washed and visualised by enhanced chemiluminescence.

2.7. Statistical analysis

Results are presented as means ± SD. The different expression of PDGFβR and PDGFβR phosphorylated protein content in neoplastic bladder and liver and kidney bovine foetus samples was assessed by one-way analysis of variance (ANOVA), followed by Student's *t*-test for significant differences between the mean values of protein content using GraphPad PRISM software version 5 (GraphPad Software, CA, USA). A P value ≤ 0.05 was considered to indicate statistical significance.

3. Results

In liver and kidneys of two foetuses as well as in the neoplastic bladder of an infected mother, PCR analysis detected amplicons, sequencing of which identified a BPV-2 E5 DNA fragment composed of 502 bp. This DNA fragment showed an absolute homology (100%) with the known sequences of BPV-2 E5 DNA. (GenBank accession number: M20219.1) (Fig. 1).

Next, to evaluate whether BPV-2 was transcriptionally active, the possible transcript presence of E5 oncoprotein was investigated in two foetuses and in an infected mother. RT-PCR showed BPV-2 E5 mRNA amplicons. The amplified mRNA was sequenced and it appeared to be composed of 154 bp having a 100% identity with BPV-2 E5 sequences (GenBank accession number: M20219.1) (Fig. 2).

Immunoblotting performed on foetal liver and kidney samples as well as in neoplastic bladder and liver samples of infected mothers revealed the presence of dimers of E5 oncoprotein (Fig. 3), which corroborated that E5 oncoprotein can spread via blood.

Since it has been shown that platelet-derived growth factor ß receptor (PDGFßR) is activated by binding of a dimer of BPV E5 oncoprotein to the transmembrane domain of the receptor and this

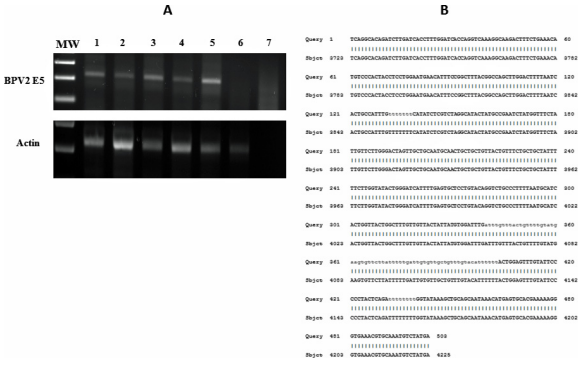

Fig. 1. PCR analysis in foetal organs. (A) Lane MW = DNA molecular weight marker (100-base pair (bp) ladder), lane 1: bladder tumour sample from a BPV-2-infected mother; lane 2: foetus #1: liver tissue; lane 3: foetus #1: kidney tissue; lane 4: foetus #2: liver tissue; lane 5: foetus #2: kidney tissue; lane 6: healthy liver (negative control); lane 7: no template control (no DNA added). (B) The amplicon sequences, composed of 502 bp, showed 100% identity with BPV-2 E5 (GenBank accession number: M20219.1).

interaction is the most important in modulating biological activity of the oncoprotein (DiMaio and Petti, 2013), western blot analysis unveiled a statistically significant increase of phosphorylated (activated) PDGFßR both in liver and kidney tissues from foetuses infected by BPV-2 in comparison with liver and kidney tissues from non-infected foetuses (Fig. 4). Similar results were also seen in neoplastic bladder samples from infected mothers (Fig. 4).

4. Discussion

This study reported the expression of BPV-2 E5 oncoprotein in tissues of two foetuses from cows with naturally occurring urinary bladder cancers associated with bovine BPV infection. To our knowledge, this report represents the first case of spontaneous intrauterine bovine δPV infection of the conceptus. Intrauterine PV infection can be spread via the maternal urogenital tract or transplacentally (Zouridis et al., 2018). Perfect BPV genotype concordance between mothers and foetuses suggests that BPV infection in the foetuses was acquired from infected mothers via a transplacental route. The δPV viraemic phase may be associated with infection of the placenta and infected white cells may pass the maternal-foetal barrier as other viruses (Silasi et al., 2015). Similar mechanisms have been suggested for transplacental transmission of HPVs (Lee et al., 2013; Zouridis et al., 2018). In naturally occurring bladder tumours of cows associated with δPV infection, BPV-2 E5 infection is known to occur in blood and placentomes (Melo et al., 2015; Roperto et al., 2011, 2012), where foetal villous trees interdigitate with maternal crypts, thus providing the continuously increasing substances between the dam and the foetus. Furthermore, experimental studies appear to corroborate our suggestions. Indeed, Stocco dos Santos et al. (1998) documented transplacental transmission of BPV-2 in cows as they showed BPV-2 DNA-harbouring PBMCs in neonatal calves from experimentally infected mothers. Furthermore, transplacental transmission of bovine BPVs resulting in congenital disease has recently been documented in newborn lambs from ewes that harboured δPV DNA in their PBMCs but did not suffer from any apparent neoplastic disease (Roperto et al., 2018a,b). Both experimental and natural δPV infection data support the idea that vertical transmission to the foetus via a haematogenous route is a viable mechanism for BPV intrauterine infection during pregnancy.

Western blot analysis detected E5 oncoprotein dimers, which showed that the oncoprotein is biologically active in foetal organs. Dimerisation of E5 is required for its activity, including activation of PDGFßR (DiMaio and Petti, 2013). Furthermore, as in adult organs, we have shown that the activated PDGFßR is increased in E5-expressing foetal livers and kidneys but not in livers and kidneys of foetuses not suffering from any papillomavirus infection. The simultaneous presence of an active form of E5 oncoprotein and an increased form of phosphorylated PDGFßR in foetuses infected by BPV-2 raised many intriguing questions. It is well-known that the PDGFßR constitutive activity has many physiological functions. In particular, it promotes angiogenesis and plays an important role in embryonic development and organogenesis (Heldin and Westermark, 1999; Hoch and Soriano, 2003; Van den Akker et al., 2008; Chen et al., 2015). Aberrant PDGFßR activation may result in vascular, cardiac, renal, placental, pulmonary abnormalities (Heldin and Westermark, 1999; Bjarnegård et al., 2004; Magnusson et al., 2007). E5 oncoprotein is known to phosphorylate PDGFßR in naturally occurring bladder tumours of cattle (Borzacchiello et al., 2006; Roperto et al., 2013b).

Although BPV E5 and abnormal phosphorylation of PDGFßR are known to be involved in congenital neoplastic oral lesions of lambs (Roperto et al., 2018b) and foetal membranes of buffalo (Roperto, manuscript in preparation), congenital tumours are extremely rare in cattle (Yeruham et al., 1999; Misdorp, 2002; Winslow et al., 2017). Therefore, the presence of abnormally activated PDGFßR by E5 oncoprotein in bovine foetal organs may contribute to the development of congenital abnormalities and even neoplasia.

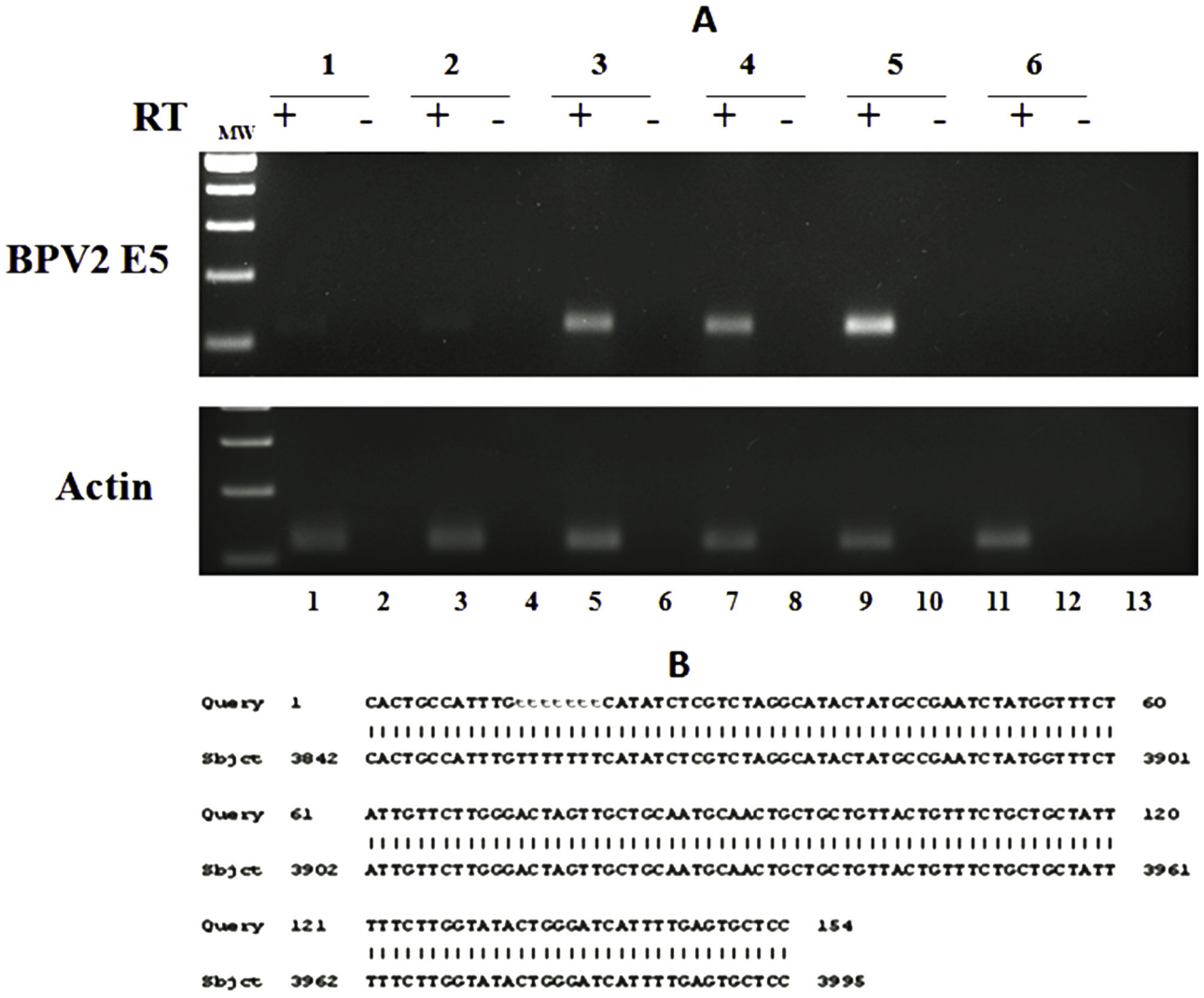

Fig. 2. RT-PCR with reverse transcriptase enzyme (RT+) (lanes 1, 3, 5, 7, 9, 11) or without (RT-) (lanes 2, 4, 6, 8, 10 and 12) in foetal organs. (A) Lane MW = DNA molecular weight marker (100-base pair (bp) ladder), sample 1: foetus #1: liver tissue; sample 2: foetus #1: kidney tissue; sample 3: foetus #2: liver tissue; sample 4: foetus #2: kidney tissue; sample 5: bladder tumour sample from a BPV-2-infected mother; sample 6: healthy liver (negative control); lane 13: no template control (no cDNA added). (B) The amplicon sequences, composed of 154 bp, showed 100% identity with BPV-2 E5 (GenBank accession number: M20219.1).

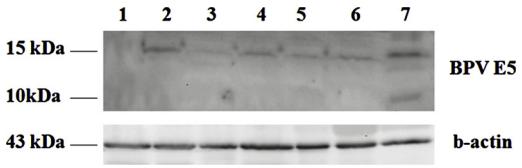

Fig. 3. Western blot analysis performed on foetal tissues from a BPV-2-infected mother 1: healthy bovine liver tissue (negative control); 2: liver tissue from a BPV-2-infected mother; lane 3: foetus #1: kidney tissue; lane 4: foetus #1: liver tissue; lane 5: foetus #2: kidney tissue; lane 6: foetus #2: liver tissue; 7: BPV-2-associated neoplastic bladder as positive control. Actin protein levels were detected to ensure equal protein loading.

As this is the first study reporting the overexpression of phosphorylated PDGFßR in foetal organs infected by BPVs, additional studies are necessary to evaluate these congenital risks. It should be noted that intrauterine HPV transmission is believed to be a significant risk factor for spontaneous abortion and preterm delivery (Ambühl et al., 2016) as well as for intrauterine growth restriction (Ford et al., 2017). Although controversial, it has also been suggested that HPV infection may be responsible for both cancer and subfertility (Depuydt et al., 2016). We speculate that similar risks may exist for bovine foetuses and cows infected with BPVs.

5. Conclusion

Despite the growing number of pathogens associated with *in utero* foetal disease, the mechanistic basis of vertical transmission of infection across the placental barrier remains largely unknown (Arora et al., 2017).

To date, BPV infection has not been considered to be associated with reproductive disorders in cattle and/or with quality of semen although BPV DNA has been found in semen and spermatozoa of bulls (Lindsey et al., 2009; Silva et al., 2011, 2014). However, it has been shown that papillomavirus infection in semen may be associated with poor sperm progressive motility and may cause a high sperm DNA fragmentation (Boeri et al., 2019). Our findings provide evidence for further investigations of foetal risks associated with BPV infection in pregnant cows. It is noteworthy that the reported incidence of infertility and abortions in cattle associated with infectious agents is increasing (Anderson, 2007; Sordillo, 2016), thus representing major problems in the bovine industry as the actual causes are identified quite rarely (Yoo, 2010).

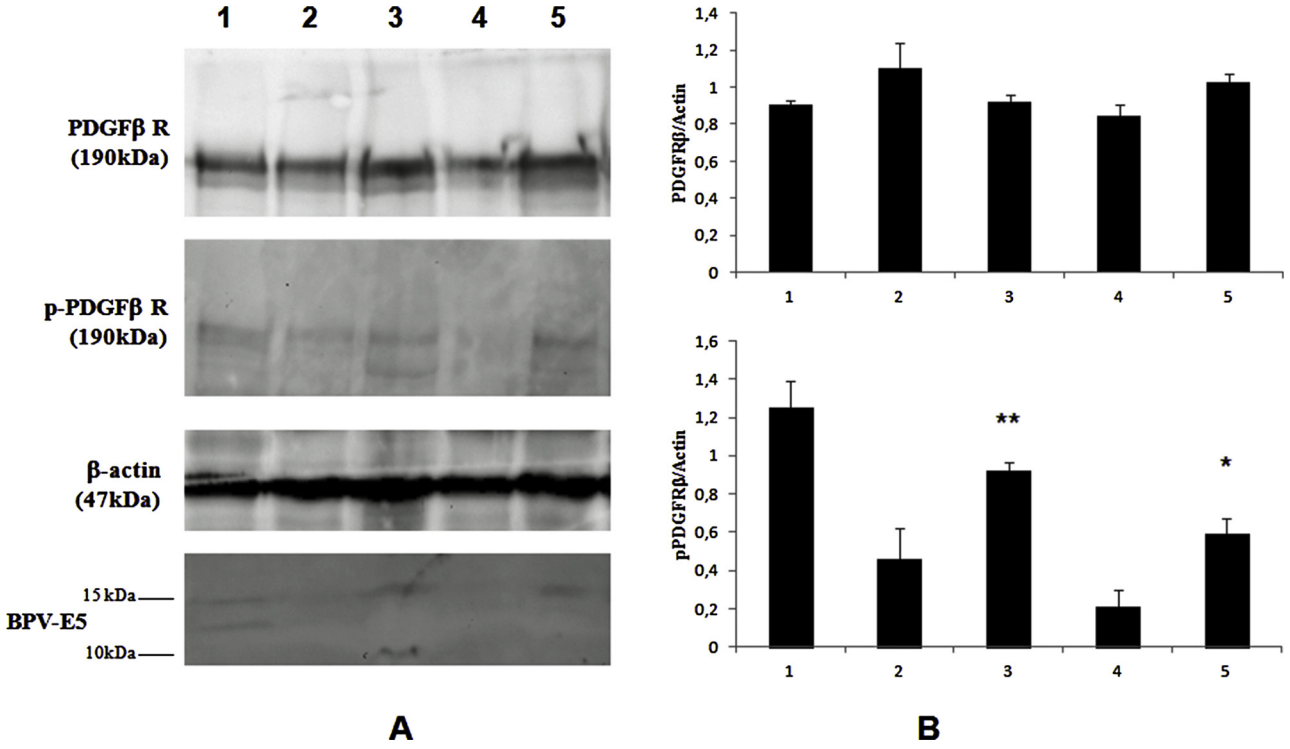

Fig. 4. Total and phosphorylated PDGFßR expression. (A) Western blot analysis. Lane 1: BPV-2 associated bladder tumour tissue expressing E5 oncoprotein; lane 2: liver tissue from non-infected foetus negative for E5 protein; lane 3: bovine liver tissue from BPV-2-infected foetus positive for E5 oncoprotein; lane 4: bovine kidney tissue from non-infected foetus negative for E5 protein: lane 5: bovine kidney tissue from BPV-2-infected foetus showing E5 expression. Actin protein levels were detected to ensure equal protein loading and to determine densitometric analysis. (B) Densitometric analysis for total and phosphorylated PDGFβR was performed in comparison with actin protein levels. Total PDGFßR in all analysed tissues showed no statistical significant differences; phosphorylated PDGFßR content levels were increased in bladder tumour tissue and in the livers and kidney of two foetuses positive for E5 protein expression in a statistically significant manner compared with livers and kidneys from non-infected fetuses negative for E5 protein. Quantitative densitometric analysis of the filters was performed with Image Lab software (ChemiDoc; Bio-Rad Laboratories), and significance was determined by one way ANOVA; E5-positive liver from lane 2 *vs* E5-negativeliver from lane 3 (**, $P \leq 0.001$); E5-positive kidney from lane 4 *vs* E5-negative kidney from lane 5 (*, $P \leq 0.05$).

Declaration of conflicting interests

The author(s) declared no potential conflicts of interests with respect to the research, authorship, and/or publication of this article.

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