



Expression of the feline leukemia virus subgroup C receptors in normal and neoplastic urothelium of the urinary bladder of cattle associated with bovine papillomavirus infection

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ABSTRACT

The feline leukemia virus subgroup C receptors (FLVCRs) were originally cloned as virus receptors, but are now believed to function also as heme transporters and are expressed in a broad range of tissues in a wide range of mammalian species. Expression of FLVCR1 and FLVCR2 was investigated in 19 bovine papillomavirus-associated urinary bladder cancers and in 15 non-neoplastic samples of bladder from cattle. E5 oncoprotein of bovine Deltapapillomaviruses (δPVs) was detected in 17 of the 19 bladder cancers. Flvcr1 and Flvcr2 were amplified and sequenced both in neoplastic and non-neoplastic samples showing a 100% identity with bovine Flvcr1 and Flvcr2 mRNA sequences present in GenBank database (accession numbers: NM_001206019.1 and NM_001192143.1, respectively). Reverse transcription (RT)-PCR showed that Flvcr1 and Flvcr2 were over-expressed in 4 and 5 out of 19 urothelial cancers, respectively, but in none of the non-neoplastic samples. In addition, western blot analysis detected higher levels of FLVCR1 and FLVCR2 in samples in which transcripts were not increased, suggesting post-translational changes to these proteins. Increased FLVCR1 and FLVCR2 was also observed immunohistochemically in the neoplastic cells. Immunolabeling for FLVCR1 was seen in the cytoplasm and plasm membrane of urothelial cancer cells, whereas immunolabeling for FLVCR2 was present within the nucleus. This is the first time that FLVCR expression has been investigated in bovine tissues and the first to suggest that expression could be increased in cancers. Additional studies are required to define the role, if any, of FLVCR in papillomavirus-associated cancer cells.

1. Introduction

Feline leukemia virus subgroup C receptors (FLVCRs) are encoded by the SLC49 gene family (Khan and Quigley, 2013) and were first identified as the cell surface receptor for feline leukemia virus (Tailor et al., 1999; Quigley et al., 2000; Shalev et al., 2009). The initial studied receptor was designated FLVCR1 (Tailor et al., 1999; Quigley et al., 2000). Although a paralog of FLVCR1 was previously investigated as FLVCR14q (Lipovich et al., 2002), FLVCR2 was only identified as a second distinct feline leukemia virus receptor in 2009 (Shalev et al., 2009).

FLVCR1 has been identified in most animal species and is expressed ubiquitously with the highest expression observed in the liver,

duodenum, kidney, lung, spleen, brain, and placenta (Quigley et al., 2004; Keel et al., 2008). FLVCR1 was identified as a mammalian cell heme exporter that is required for iron homeostasis and red blood cell differentiation due to its ability to protect erythroid progenitors from potential heme toxicity during the heme synthesis phase of erythropoiesis (Quigley et al., 2004; Keel et al., 2008). FLVCR1 also appears to be required for T lymphocyte development and peripheral survival (Philip et al., 2015). Furthermore, FLVCR1 is crucial to control heme metabolism in hepatocytes (Vinci et al., 2014), duodenum (Fiorito et al., 2015), and in endothelial cells (Chiabrando et al., 2012; Petrillo et al., 2018). In humans, mutations in the Flvcr1 gene causes retinitis pigmentosa with and without posterior column ataxia (Rajadhyaksha et al., 2010; Yusuf et al., 2018) as well as sensory

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neurodegeneration with loss of pain perception (Chiabrando et al., 2016; Castori et al., 2017).

FLVCR2 is expressed in a broad range of tissues from animal species with expression in brain, placenta, lung, liver and kidney (Duffy et al., 2010). FLVCR2 protein shares about 60% amino acid sequence identity with FLVCR1. Like FLVCR1, FLVCR2 is believed to be important in cell heme transport (Duffy et al., 2010). FLVCR2 has been found to be closely correlated with nonheme iron import proteins such as transferrin receptor1 and divalent metal transporter 1 (Best et al., 2016). However, assumption that FLVCR2 is a heme importer is not definitive and the substrate specificity of this protein remains unconfirmed (Chiabrando et al., 2014; Ponka et al., 2017). In humans, mutations of Flvcr2 gene are associated with Fowler syndrome, a proliferative vascular disorder of the brain (Lalonde et al., 2010; Meyer et al., 2010).

Spontaneous neoplasms of the urinary bladder of cattle are very rare accounting for 0.01%–0.1% of all bovine malignancies (Pamukcu, 1974). However, cattle that are infected by bovine deltapapillomaviruses (δ PVs) (Roperto et al., 2013, 2016a; Roperto et al., 2016b), believed to be representatives of high-risk PVs (Daudt et al., 2018), and exposed to chemical carcinogens in bracken fern (*Peridium* spp) such as ptaquiloside develop very high rates of bladder tumors (Pamukcu et al., 1976; Carvalho et al., 2006; Roperto et al., 2010a,b).

Iron can contribute to both tumor initiation and growth; recent work has also shown that iron has a role in the tumor microenvironment and in metastasis. A plethora of iron-regulatory proteins have recently been associated with cancer development and growth and the role of these proteins in cancer biology is becoming increasingly recognized (Torti and Torti, 2013).

The aim of the present paper is to investigate the expression of FLVCR1 and FLVCR2 in healthy and in bovine papillomavirus-associated neoplastic bladders. To our knowledge, the expression of neither Flvcr1 nor Flvcr2, known to be located on bovine chromosomes 16 and 10 respectively (Zimin et al., 2009), has been previously described in bovine tissues. Additionally, there are no studies investigating a possible role of FLVCR1 and FLVCR2 in papillomavirus carcinogenesis of cattle.

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 is required.

2.2. Tumor samples

Nineteen cows with clinical chronic enzootic hematuria were slaughtered and urothelial samples were collected after bladder neoplasms had been identified during routine meat inspection. Fifteen mucosa samples of urinary bladder were also collected from healthy cows. Both neoplastic and non-neoplastic bladder samples were subdivided and either fixed in 10% buffered formalin for microscopical investigations or immediately frozen in liquid nitrogen, stored at -80°C for subsequent molecular biological analysis.

2.3. Histopathology and immunohistochemical procedures

Tissues were routinely processed for paraffin embedding. The neoplasms were classified from 5- μm -thick hematoxylin-eosin-stained sections using morphological criteria previously reported (Roperto et al., 2010a). Paraffin sections from non-neoplastic and neoplastic bladder samples were de-waxed in xylene and rehydrated through decreasing alcohols. Heat induced epitope retrieval (HIER) was performed by pretreating with microwave heating (twice for 5 min each at 750 W) in 10 mM sodium citrate buffer pH 6.0. All slides were allowed to cool

at room temperature and washed gently three times with phosphate buffered saline (PBS, pH 7.4, 0.01 M). Endogenous peroxidase activity was blocked by treatment with peroxidase blocking solution (Vector Laboratories Inc., CA, USA) for 20 min at room temperature. For blocking non-specific binding, the sections were incubated for 1 h with 2.5% normal goat blocking serum (Vector Laboratories, Inc. CA, USA). Polyclonal rabbit anti-FLVCR1 (LS-C291063, LSBio, LifeSpan Biosciences, Inc., WA, USA), and anti-FLVCR2 (HPA037984, Sigma-Aldrich, MO, USA) primary antibodies diluted at 1 in 300 and 1 in 100, respectively, in PBS, were applied for 1 h at room temperature in a humid chamber. The slides were washed gently three times with PBS, then incubated with ImmPRESS peroxidase polymer anti-rabbit IgG reagent made in goat (Vector Laboratories Inc., CA, USA). Color development was obtained by treatment with ImmPACT DAB peroxidase substrate (Vector Laboratories Inc., CA, USA) for 2–10 min. Sections were counterstained with Mayer's hematoxylin. Antibody specificity was demonstrated by using control sections from the same normal and pathological tissue samples where the respective primary antibodies were omitted and replaced by corresponding species- and isotype-matched immunoglobulins (IgG) (Bethyl Laboratories, Inc., TX, USA) at same concentrations.

2.4. Reverse transcription (RT)-PCR

Total RNA was extracted from 19 bovine urothelial tumor samples and 15 bladder samples from healthy cows by RNeasy Mini Kit (Qiagen TM, ME, DE), in according to the manufacturer's instructions. Genomic DNA was removed from RNA preparations using RNase-free DNase I Fermentas Life Sciences (Thermo Fisher Scientific, MA, USA). 1 μg of the total RNA was used to generate the single strand of cDNA by the QuantiTect Reverse Transcription Kit (Qiagen TM, ME, DE), according to the manufacturer's instructions. PCR was performed with a specific primer set designed by the Primer3 online tool for E5 gene of BPV-1, BPV-2, BPV-13 and BPV-14. Primers specific to bovine Flvcr1 and Flvcr2 were also designed. The following primers were used: BPV-1 E5 ORF forward 5'-GCCTTTTCTTCATCTGACTG-3', reverse 5'-GCCAGTGA TGTAAGGCATT-3'; BPV-2 E5 ORF forward 5'-CACTGCCATTTGTTTT TTTC-3', reverse 5'-GGAGCACTCAAATGATCCC-3'; BPV-13 E5 ORF forward 5'-CACTGCCATTTGGTGTTCCT-3', reverse 5'-AGCAGTCAAAA ATGATCCCAA-3'; BPV-14 E5 forward 5'-CTGCCGATTTCAAAGGT GCT-3', reverse 5'-ACAAGATACGCATTTAGAAGGGA-3'; Flvcr1 forward, 5'-GGAGAAGAAGTGAATGCTGG-3', reverse 5'-GAAACCAAGC AACCTCCAG-3'; Flvcr2 forward 5'-TGGTCTGGTGTTCAGCT-3', reverse 5'-GGAGCAGAGGGATGTAGGTC-3'. Conditions for PCR were: 94°C for 5 min, 40 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 30 s.

2.5. Sequence analysis

PCR products, obtained by RT-PCR, were purified by 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 manufacturer's recommendations. Sequences were dye-terminator removed by DyeEx_2.0 spin kit (Qiagen TM, ME, DE) and run on a 3500 Genetic Analyzer (Applied Biosystems, CA, USA). Electropherograms were analyzed using Sequencing analysis v5.2 and sequence scanner v1.0 softwares (Applied Biosystems, CA, USA). The sequences obtained were analyzed by BLAST program.

2.6. Real time PCR

To perform real time PCR analysis, total RNA and cDNA from 19 bovine urinary bladder tumor and 15 normal urothelium samples were generated as above reported. Real time PCR was carried on a Bio Rad CFX Connect™ Real Time PCR Detection System (Bio Rad Hercules, CA, USA) using iTaq Universal SYBR® Green Supermix (Bio Rad Hercules,

CA, USA). Each reaction was set in triplicate and the primers used for FLVCR1 and FLVCR2 were the same of RT-PCR. The thermal profile for the PCR was 95 °C for 10 min, 40 cycles of 94 °C for 15 s, 56 °C for 30 s, followed by melting curve. The relative quantification (RQ) was calculated by using CFX Manager™ software, based on the equation $RQ = 2^{-\Delta\Delta Cq}$, where Cq is the quantification cycle to detect fluorescence. Cq data were normalized to the reference β -actin gene (forward: 5'-TAGCACAGGCTCTCGCCTTCGT-3', reverse 5'-GCACATGCCGGAGCCGTTGT-3').

2.7. Statistical analysis

Results are presented as means \pm SE. The FLVCR1 and FLVCR2 expressions were assessed by one-way ANOVA, followed by Tukey's test for multiple comparisons of means using GraphPad PRISM software version 5 (GraphPad Software, San Diego, CA). A P value \leq 0.05 was considered to indicate statistical significance.

2.8. Western blot analysis

Healthy and neoplastic bovine urothelial samples were lysed in RIPA buffer (50 mM Tris-HCl pH 7.5, 1% Triton X-100, 400 mM NaCl, 1 mM EDTA, 2 mM PMSF, 1.7 mg/ml Aprotinin, 50 mM NaF, and 1 mM sodium orthovanadate). They were clarified by centrifugation, separated by SDS-PAGE and transferred onto nitrocellulose membranes (GE Healthcare, UK, RPN303D). Membranes were blocked with TBST (TBS and 0.1% Tween 20) containing 5% no fat dry milk for 1 h at room temperature, being subsequently incubated overnight at 4 °C with an anti-FLVCR1 (LS-C291063, LSBio, LifeSpan BioSciences, Inc., WA, USA), anti-FLVCR2 (HPA037984, Sigma-Aldrich, MO, USA) and anti-actin (Santa Cruz Biotechnology, TX, USA) primary antibodies. Membranes were washed three times with TBST, incubated for 1 h at room temperature with goat anti-rabbit (Bio-Rad, CA, USA) and goat anti-mouse (Bio-Rad, CA, USA) HRP-conjugated secondary antibodies diluted at 1:2000 in TBST, and washed three times with TBST. Immunoreactive bands were detected using Western Blotting Luminol Reagent (Santa Cruz Biotechnology, TX, USA) and ChemiDoc XRS Plus (Bio-Rad, CA, USA). Images were acquired with Image Lab Software version 2.0.1 (Bio-Rad, CA, USA).

3. Results

3.1. Microscopical patterns of tumors and virological findings

Histology revealed that 14 of the 19 neoplasms were papillary carcinomas (10 high-grade and four low-grade). The remaining 5 were invasive carcinomas (3 high-grade and 2 low-grade).

Either BPV-2 E5 or BPV-13 E5 transcripts were detected in 17 cancer samples with just BPV-2 E5 detected in 5 of 19 tumor samples, BPV-13 E5 in 5 of 19 cancer samples and BPV-2 and BPV-13 E5 in 7 of 19 cancer samples (Table 1). Sequencing of the amplicons showed a 100% identity to either BPV-2 E5 or BPV-13 E5 sequences deposited in GenBank (accession number: M20219.1 and JQ798171.1, respectively). Neither BPV-1 E5 nor BPV-14 E5 transcripts were detected by RT-PCR in any cancer samples. E5 oncoprotein expression was detected in 17 out of 19 bladder cancers (~89%) by western blot (Fig. 1). E5 oncoprotein immunolabeling was evident in the cytoplasm and on the cell membrane of urothelial cancer cells from bladder cancer samples only (Fig. 2). Neither BPV E5 transcripts nor E5 protein expression were detected in any of the non-neoplastic bladder samples.

3.2. Molecular and immunohistochemical detection of FLVCR1 and FLVCR2, novel proteins involved in iron metabolism

In the context of an ongoing project to study iron metabolism in bovine papillomavirus-associated urothelial cancer cells, we

investigated FLVCR1 and FLVCR2 that, though originally cloned as virus receptors, are novel proteins known to be heme transporters. Using primers specific to bovine Flvcr1 and Flvcr2, RT-PCR detected transcripts in healthy bladder urothelium as well as in all neoplastic bladder samples. A 219 bp amplicon for Flvcr1 and a 156 bp amplicon for Flvcr2 were amplified by PCR. Sequencing revealed a 100% identity with bovine Flvcr1 and Flvcr2 mRNA sequences deposited in GenBank (accession number: NM_001206019.1 and NM_001192143.1, respectively) (data not shown). Quantitative real time PCR showed that Flvcr1 and Flvcr2 mRNA levels were increased in 4 and 5 out of 19 neoplastic bladder samples, respectively, in a statistically significant manner ($p \leq .05$) (Fig. 3). Western blot analyses performed on bladder samples detected the presence of a band with ~60 kDa molecular weight consistent with FLVCR1 and FLVCR2 expression in all neoplastic and non-neoplastic samples (Fig. 1). Densitometric analysis revealed that FLVCR1 and FLVCR2 expression levels were significantly higher in the samples with increased transcripts than in those without increased transcripts. However, FLVCR1 and FLVCR2 were significantly overexpressed in additional 3 cases, respectively, that did not have increased transcripts suggesting that post-translational changes occur. Altogether, FLVCR1 and FLVCR2 were overexpressed in 7 (~41%) and 8 (~47%) out of 17 neoplastic samples that were infected by bovine papillomavirus, respectively (Fig. 4). All FLVCR1-overexpressing cancers showed BPV abortive infection through E5 oncoprotein detection. Furthermore, E5 protein was detected in 7 out of 8 FLVCR2-overexpressing cancers. In contrast, neither BPV E5 oncoprotein nor overexpression of the viral receptors was present in any non-neoplastic samples.

Immunohistochemistry revealed FLVCR1 immunolabeling of the cytoplasm and cell membrane within urothelial cancer cells (Fig. 2). In contrast, immunolabeling for FLVCR2 was predominantly nuclear (Fig. 2).

4. Discussion

This study shows that both FLVCR1 and FLVCR2 are constitutively expressed in urothelial cells of the urinary bladder of cattle. Their expression was statistically higher in neoplastic than in non-neoplastic urothelial tissues. As these receptors have not previously described in bladder mucosa of any animal species, the roles of these receptors are unknown in cattle.

Even if still poorly characterized, FLVCR1 and FLVCR2 are novel proteins that appear to be involved in the metabolism of iron. As FLVCR1 and FLVCR2 are ubiquitously expressed, it is conceivable that their heme transporter activity may be relevant in different tissues. In the present study, both FLVCR1 and FLVCR2 were overexpressed in close to half percentage of papillomavirus-associated bladder cancers of cattle. Interestingly, all cancers showing overexpression of FLVCR1 also contained the BPV E5 oncoprotein. BPV E5 oncoprotein was also detected in all except for one of the cancers showing overexpression of FLVCR2. While additional studies are required, our results are consistent with the assumption that the BPV E5 oncoprotein could promote the expression of FLVCR1 and FLVCR2, thus showing a correlation between virus infection and FLVCR expression levels. There was a difference between transcript expression and FLVCR levels, which confirms that post-translational mechanisms regulating FLVCR protein expression also occurs in bovine cells.

The frequent detection of altered expression of these proteins in the neoplasms suggests that the neoplastic cells may differ from their non-malignant counterparts in the levels of proteins that are involved in iron metabolism. Iron metabolism is disordered in tumors compared with normal tissues (Kazan et al., 2017). Alteration of iron metabolism is thought to influence the development and behavior of the human cancers (Torti and Torti, 2013).

It is conceivable that both receptors have an important role in the maintenance of vesical heme homeostasis just they do in other organs.

Table 1Histologic diagnosis of δ PV-associated urothelial carcinomas. LG = Low-grade; HG = High-grade; + = presence of E5 cDNA; – = absence of E5 cDNA.

Case Number	Microscopic patterns	BPV-1 E5 cDNA	BPV-2 E5 cDNA	BPV-13 E5 cDNA	BPV-14 E5 cDNA
1	HG Papillary urothelial carcinoma	–	+	+	–
2	HG Papillary urothelial carcinoma	–	–	+	–
3	LG Papillary urothelial carcinoma	–	+	–	–
4	HG Papillary urothelial carcinoma	–	+	–	–
5	HG Papillary urothelial carcinoma	–	+	–	–
6	HG Papillary urothelial carcinoma	–	+	+	–
7	HG Invasive urothelial carcinoma	–	+	+	–
8	LG Invasive urothelial carcinoma	–	–	–	–
9	LG Invasive urothelial carcinoma	–	+	+	–
10	HG Invasive urothelial carcinoma	–	+	+	–
11	HG Invasive urothelial carcinoma	–	–	+	–
12	LG Invasive urothelial carcinoma	–	–	–	–
13	HG Invasive urothelial carcinoma	–	+	+	–
14	HG Papillary urothelial carcinoma	–	+	+	–
15	LG Papillary urothelial carcinoma	–	–	+	–
16	HG Papillary urothelial carcinoma	–	–	+	–
17	LG Papillary urothelial carcinoma	–	–	+	–
18	HG Papillary urothelial carcinoma	–	+	–	–
19	HG Papillary urothelial carcinoma	–	+	–	–

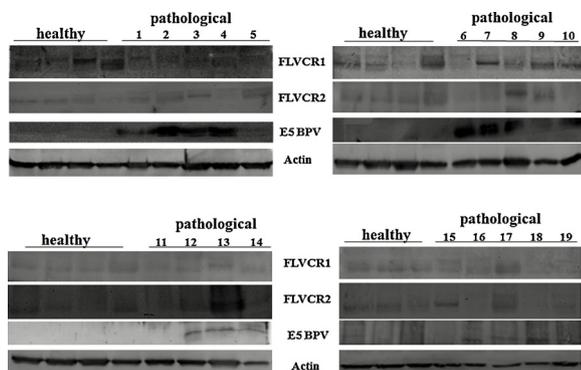


Fig. 1. Western blot analysis showed expression of FLVCR1 and FLVCR2 in 15 healthy bladder urothelium and in 19 bladder cancers. BPV E5 oncoprotein was detected in 17 of 19 cancer samples. Actin protein levels were detected to ensure equal protein loading.

It is worthwhile remembering that homeostatic intracellular levels are essential for cell survival including cancer cells (Baukman et al., 2013, 2015) and are achieved by a network of iron-dependent proteins (Manz et al., 2016; Shen et al., 2018). Deregulation of bladder iron homeostasis in urothelial cells has been associated to development of bladder disorders in people (Verma et al., 2015).

FLVCR1 plays a central role in cellular heme homeostasis as it controls the intracellular heme pool (Mercurio et al., 2015). Furthermore, FLVCR1 modulates cell proliferation of mucosa thus contributing to the maintenance of its integrity (Fiorito et al., 2015). FLVCR2 is believed to be a heme transporter and it has been found to be over-expressed in astrocytes and precursor cells of pericytes being able to play a role in neoangiogenic events (Sharma et al., 2012.). FLVCR1 and FLVCR2 may play an important role in host defense against pathogens. It has been shown that regulation of iron availability in the bladder is crucial to control uropathogenic *Escherichia coli* (UPEC), the predominant cause of urinary tract infections (UTIs) and urothelial cell death (Baukman and Mysorekar, 2016).

There is a very scant information about FLVCR1 in comparative oncology, including human oncology. However, studies in humans suggest that FLVCR1 is important for the survival of neuroblastoma cells (Chiabrando et al., 2016). Furthermore, overexpression of FLVCR1 has been detected in hepatitis C virus-associated hepatocellular carcinoma (Nakano et al., 2018). It has been suggested that FLVCR1 may be involved in coproporphyrin III transport system leading to porphyria cutanea tarda, an extrahepatic manifestation of the virus

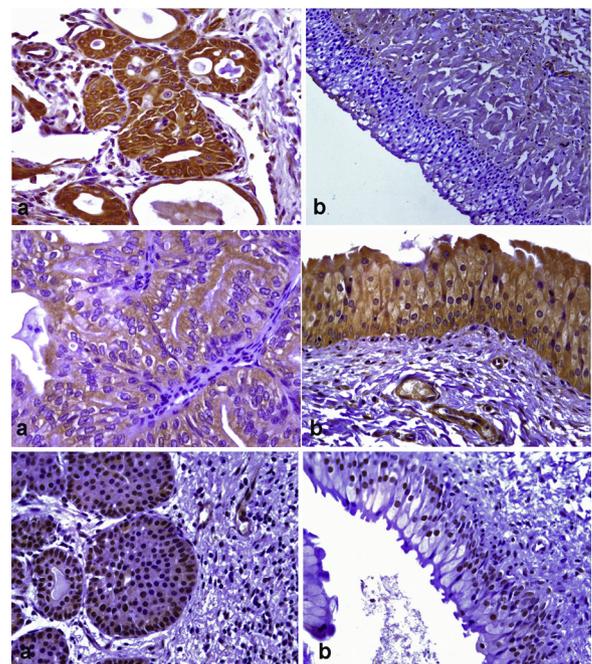


Fig. 2. Upper row - a) Immunohistochemical detection of E5 oncoprotein in the urothelial cancer cells; b) Urothelial mucosa from healthy cattle; middle row: a) Immunohistochemical detection of FLVCR1 in urothelial cancer cells; b) FLVCR1 in normal urothelial mucosa; lower row: a) Nuclear immunodetection of FLVCR2 in urothelial cancer cells; b) Nuclear expression of FLVCR2 in normal urothelial mucosa.

infection (Nakano et al., 2018) Recently, upregulation of FLVCR1 was detected in synovial sarcoma (SS) cells both in *in vivo* and *in vitro* studies (Peng et al., 2018). Although the biological role of the over-expression of FLVCR1 in SS remains to be elucidated, it has been suggested that FLVCR1 may act as an oncoprotein thus contributing to promote proliferation and tumorigenicity of SS (Peng et al., 2018).

To the authors' knowledge, FLVCR2 involvement in neoplastic events is entirely unknown as it has never previously been investigated.

It is conceivable that the expression of FLVCR1 and FLVCR2 could be related to bladder iron status and be associated with bladder iron content. FLVCR1 has been shown to be associated with placental iron concentration (Jaacks et al., 2011). FLVCR2 has been found to be expressed in astrocytes from brain of a rat model of stroke with an

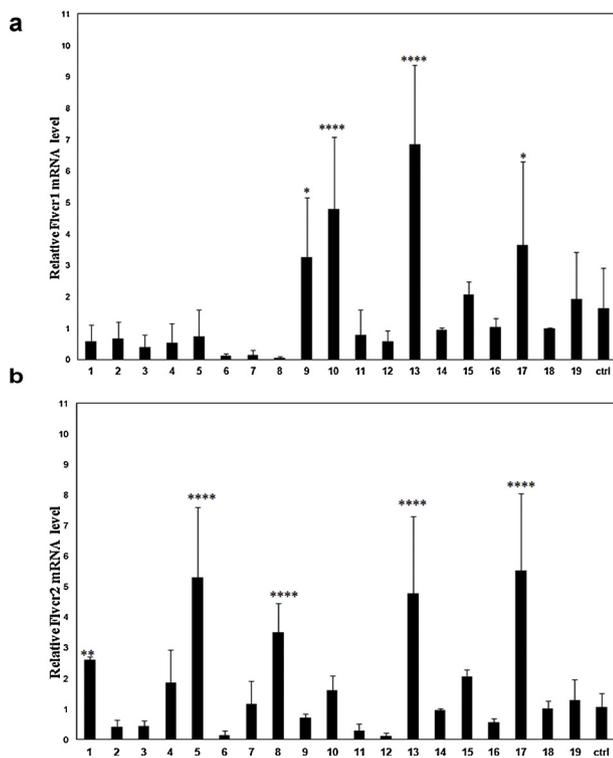


Fig. 3. a) Real time RT-PCR. FLVCR1 mRNA levels in the 15 normal control (CTRL) and in the 19 neoplastic bladder samples. In particular, it was shown that in 9, 10, 13 and 17 bladder samples there was a statistically significant difference in expression of FLVCR1 mRNA compared to normal bladders ($p \leq 0.05$). Data are expressed as mean \pm S.E.M. of three separate experiments performed in triplicate * $p < 0.05$; **** $p < 0.001$. b) Real time RT-PCR. FLVCR2 mRNA levels in the 15 normal control (CTRL) and in the 19 neoplastic bladder samples. In particular, it was shown that in 1, 5, 8, 13 and 17 bladder samples there was a statistically significant difference in expression of FLVCR2 mRNA compared to normal bladders ($p \leq 0.05$). Data are expressed as mean \pm S.E.M. of three separate experiments performed in triplicate ** $p < 0.01$; **** $p < 0.001$.

accentuated heme content (Sharma et al., 2012).

In our study, it is speculated that high levels of FLVCR1 might be involved in an increase in iron efflux and overexpression of FLVCR2 in a dysregulation of heme intake. Recently, it has experimentally been shown that FLVCR2, when overexpressed, may decrease heme transport (Ponka et al., 2017). Therefore, iron levels that could occur in bovine urothelial cancer cells may ultimately be involved in regulating proteins that control the cell cycle. Phosphorylation of Rb protein, which in turn releases the transcription factor E2F from Rb, is a well-known cell cycle mechanism regulated by iron (Malumbres and Barbacid, 2009; Torti and Torti, 2013). Similar mechanisms have been shown to take place in urothelial cancer cells of cattle (Roperto et al., 2010b).

5. Conclusion

FLVCR1 and FLVCR2 are novel proteins involved in the metabolism of iron, the role of which has not been well defined in viral infections. Further studies are required to gain insights into relationship between iron and viral infections that will be a crucial topic of future research. Many virus use proteins of iron metabolism as target for infection (Wessling-Resnick, 2018). Understanding how iron metabolism and viral infection interact might suggest new methods to control disease (Drakesmith and Prentice, 2008). In particular, we need to improve our knowledge about the association between iron status and papilloma-virus infection since iron could be an important factor that may influence duration of PV infection (Siegel et al., 2012).

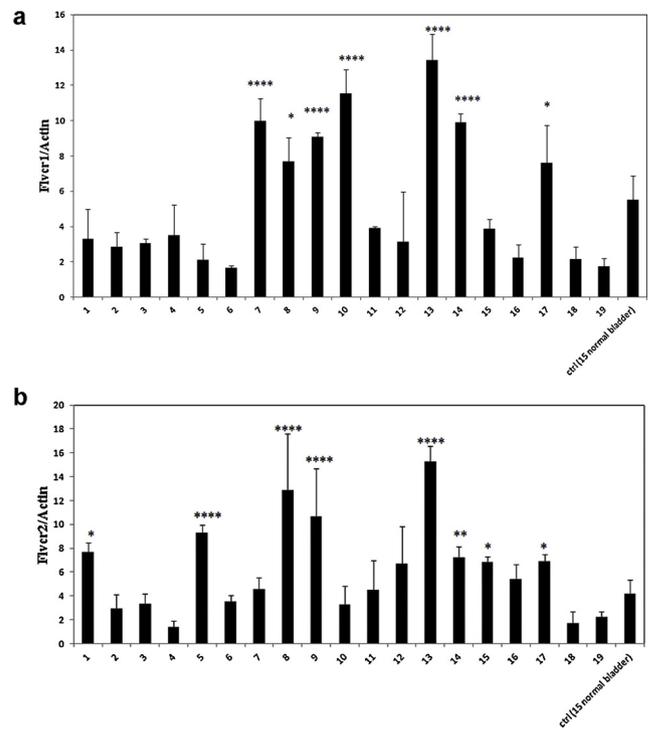


Fig. 4. Quantitative densitometric analysis of FLVCR western blot. a) A statistically significant overexpression of FLVCR1 protein is evident in 7, 8, 9, 10, 13, 14 and 17 cancer samples; b) FLVCR2 overexpression is evident in 1, 5, 8, 9, 13, 14, 15, 17 bladder cancer. All cancer samples were compared with control bladders composed of 15 healthy urothelial mucosa. Data are expressed as mean \pm S.E.M. of three separate experiments performed in triplicate * $p < 0.05$; **** $p < 0.001$. Quantitative densitometric analysis of the filters was performed with Image Lab Software (ChemiDoc; Bio-Rad Laboratories) and significance determined by the Student T-test.

Declaration of conflicting interests

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