Imunohistochemical and molecular aspects and the use of predictive markers in urothelial cancer in cattle

SUMMARY OF THE PhD THESIS

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INTRODUCTION

Bladder injuries play an increasingly more prominent role in tumor pathology, and cattle can be an experimental animal model for studying the pathogenesis of bladder neoplasms. Within bovine urothelial pathology, the most common lesions are bladder mucosal inflammation. Cystitis have a varied factorial etiology, among the most common causes being bacterial infections, administration of drugs that induce antibiotic resistance, exposure to certain toxic chemical substances such as metabolites of cyclophosphamide or ingestion of toxic plants. Toxic cystitis (Chronic Enzootic Haematuria (CEH) / Haemorrhagic Cystitis) is given by the chronic ingestion of imperial bracken fern (*Pteridium spp.*) that causes chronic enzootic bovine haematuria syndrome and is manifested by acute bladder hemorrhages, chronic cystitis or lower urinary tract malignancies, particularly bladder tumors. Cattle fed small amounts of fern develop microscopic haematuria, followed by macroscopic haematuria. Microhaematuria is associated with bruising or suffusions in the urothelial lining of the renal calices, renal pelvis, ureter and bladder. Microscopical findings include capillary ectasia and congestion. These vascular alterations predispose to bleeding in the bladder wall and nodular, hemangioma type lesions in the affected regions. Haematuria installs insidiously, characterized by a slow but progressive course, with remissions of variable duration, from a few days to several months.

Neoplasms that develop in the urinary bladder are both epithelial and mesenchymal in nature and are both benign and malignant. The studies show that the benign, epithelial ones know a higher incidence among urothelial tumors, the most common of which are papillomas.

Of great interest for the study of preneoplastic lesions and bladder tumors is the analysis of another pathogen factor involved, namely bovine papilloma virus. Molecular studies have demonstrated the presence of viral DNA in urinary bladder biopsies, but the connection between this and the consumption of bracken fern is not fully elucidated.

Key words: Bovine enzootic haematuria, BPV, urothelial tumors
STRUCTURE OF THE THESIS

The paper entitled "Imunohistochemical and molecular aspects and the use of predictive markers in urothelial cancer in cattle" contains 124 pages and is written in compliance with current norms in two parts.

The first part contains 28 pages, divided into 7 chapters and summarizes the literature review related to the epidemiological aspects of urothelial lesions in cattle, histological classification of urethral and bladder tumors, etiology and pathogenetic mechanisms involved in the development of urothelial neoplasms and immunohistochemical markers used in the diagnosis of tumors.

The second part, extends over 76 pages and includes the personal researches conducted during 2011-2015. Each chapter is divided into subchapters describing the main objectives, materials and methods used, results obtained with discussion of their novelty compared to other studies and partial conclusions drawn after each study. The results of the research are illustrated in a number of 30 figures and synthesized in 10 tables.

RESULTS OF THE RESEARCH

Chapter 9, entitled "Macroscopical and histopathological evaluation of urothelial lesions" includes the evaluation from a macroscopic and histopathological point of view of urothelial biopsies taken from slaughtered cattle and classification of all changes found by performing an evidence with diagnosed lesions.

For this study, bladder samples were collected from a number of 475 cattle, males and females, aged between 3 and 15 years, originating from areas where the endemic character of chronic haematuria is known. Most of the cattle showed no clinical signs, pathological changes in the bladder being encountered as a slaughterhouse surprise. For the histopathological examination tissues were introduced into a fixing agent (10% formaldehyde, pH 7) for 24 hours, after that being processed by paraffin embeding and stained using usual hematoxylin-eosin technique. After macroscopical and histopathological examination, a number of pathological changes of the bladder were identified, such as circulatory, inflammatory, preneoplastic and neoplastic lesions. From the total number of biopsies studied, 370 were represented by normal mucosal epithelium (without pathological changes); 32 urothelial fragments had inflammatory lesions, in most cases chronic cystitis. In 6
cases, urothelial hyperplasia lesions were identified, without an inflammatory infiltrate. Tumoral lesions were benign neoplasms, represented in most cases by papillomas), 32 in number, and malignancies, 27. In 8 cases we encountered vascular lesions, including hemorrhages and subepithelial oedema, not accompanied by inflammation.

Although papillomavirus infection determines intranuclear inclusions in some epithelia, after histopathological examination we can say that in this study there were no such intranuclear inclusions identified in the transitional epithelium.

Therefore, we conclude that chronic bladder inflammation is associated with a number of hyperplasia, metaplasia, and dysplasia changes of the urinary bladder. Urothelial carcinomas are aggressive, causing the underlying tissue structures infiltration, tissue necrosis and vascular invasion.

Chapter 10, entitled "Morphological, histochemical and immunohistochemical characterization of Cystitis glandularis foci in inflammatory and neoplastic urothelial lesions" includes the evaluation of immunohistochemical expression of diagnostic and nuclear proliferation markers, in preneoplastic and neoplastic lesions of the bladder encountered in cattle. The biological material consisted of 50 pieces of urothelial biopsies assessed by histopathology using standard H-E staining and immunohistochemistry, using the following monoclonal antibodies: anti-panCK (clone AE1/AE3/ Leica, UK), anti-CK20 (clone PW31/Novocastra, UK), anti-E-cadherin (clone 36B5/ Leica, UK) and anti-Ki-67 (clone MM1/ Leica, UK).

In our study, panNCK expression, characterized by shades of brown marking the epithelial cell cytoplasm, was intensely expressed in normal mucosa (grade 3 intensity); we considered it positive control, and according to it we evaluated the expression of IHC at our other analyzed groups. An intense expression of panCK we observed in groups 3, 4 and 6, respectively hyperplasia lesions, epithelial dysplasia / in situ carcinoma and papillary urothelial neoplasms of low malignant degree. Regarding other lesions studied (inflammation and malignant tumors), this marker's expression was of moderate intensity. CK20 expression was negative in both normal mucosa (positive control) and in urothelial lesions. Cytokeratin expression has not presented a variation in relation to the severity of the neoplastic process, nor any variation in its cytoplasmatical distribution.

Normal transitional epithelium expressed a strong E-cadherin immunoreactivity at intercellular level. This was observed in all cases considered normal mucosa. In urothelial biopsies with chronic cystitis, outbreaks of lesions expressed a moderate and homogeneous immunoreactivity of E-cadherin compared with normal epithelium. In the tissues of neoplastic lesions (transitional cell carcinoma, squamous cell carcinoma), E-cadherin immunoreactivity was also moderate and homogeneous in the
dysplastic epithelium from the surface of the neoplastic processes. Malignant neoplastic areas expressed a weak and heterogeneous immunoreactivity of E-cadherin, in multiple microscopic fields being negative.

KI-67 expression in normal transitional epithelium was negative, we didn’t notice marked nuclei. In chronically inflamed mucosa group, the KI-67 immunopositive cells were represented by only a few inflammatory cells (lymphocytes), integral urothelial cells being immunonegative. The group with epithelial hyperplasia was also negative, there were no immunopositive cells noticed in any microscopic field. Regarding neoplastic lesions, we observed an increase in average expression of KI-67 index, not as much at papillomas, where it was 2.34%. There is a slight numerical increase of marked nuclei starting with the epithelial dysplasia / in situ carcinoma group, i.e. 5.40%. In papillary urothelial neoplasms of low malignancy grade, the mean KI-67 expression was 5.16%, thus demonstrating the need to differentiate this type of tumor from papillomas. The most intense immunohistochemical expression was observed in carcinomas, especially in infiltrative transitional cell carcinomas, 29%. Between all analyzed groups we identified a variation of the mean expression of KI-67 index, thus observing an increased expression in transitional cell carcinoma and PUNLMP. After using statistical analysis (Tukey-Kramer test for multiple comparisons), we obtained highly significant values (p<0.001) between all the groups studied, lees so between dysplasia and PUNLMP. The gradual increase in KI-67 expression according to the increase in cancer malignity demonstrates the usefulness of this marker in early and correct diagnosis of tumors.

Chapter 11, entitled "Morfological, histochemical and immunohistochemical characterization of Cystitis glandularis foci in inflammatory and neoplastic urothelial lesions", includes the evaluation and comparison of immunoexpression of CDX2, CK7, CK20, KI-67 and E-cadherin markers in intestinal metaplasia processes encountered in chronic cystitis and urothelial cancer in cattle.

For this study we used 24 urothelial mucosa biopsies, divided into 3 groups: group 1 – normal mucosa (5 cases), group 2 – chronic cystitis (6 cases) and group 3 (13 cases) – urothelial neoplasms. These samples were stained hematoxylin-eosin and AB-PAS (to identify Globet cells), then by immunohistochemistry using monoclonal antibodies anti-CDX2 (clone AMT28/ Novocastra, UK), panCK (clone AE1/AE3/ Leica, UK) CK7 (clone RN7/Novocastra, UK), CK20 (clone PW31/Novocastra, UK), Ki-67 (clone MM1/ Leica, UK) and E-caderină (clone 36B5/ Leica, UK).

CDX2 expression was not observed in normal urothelial mucosa. In group 2, with chronic cystitis, CDX2 expression has been identified in one case out of 6; it had a diffuse character, marking all areas with intestinal metaplasia morphology. Also, in group 3, from a total number of 13 cases with urothelial neoplasms, a positive CDX2
outbreak was observed in one case with infiltrative squamous cell carcinoma, the rest of the intestinal metaplasia areas remained unmarked.

PanCK, characterized by marking the epithelial cell cytoplasm in shades of brown, it has been intensely expressed both in normal mucosa and inflammatory and tumoral lesions. PanCK had a week expression (1+) in outbreaks of intestinal metaplasia associated with chronic cystitis compared with those present in urothelial tumors. The expression of CK20 and CK7 were negative both in normal intestinal mucosa (positive control) and in urothelial lesions.

Normal transitional epithelium expressed a strong E-cadherin immunoreactivity at intercellular level. In mucosal biopsies with chronic cystitis (group 2), intestinal metaplasia foci expressed a moderate and homogeneous immunoreactivity of E-cadherin compared with normal epithelium. The fragments of neoplastic lesions (transitional cell carcinoma, squamous cell carcinoma, adenocarcinoma), showed a moderate and homogeneous E-cadherin immunoreactivity both in the intestinal metaplasia foci and the dysplastic epithelium from the surface of the neoplastic processes.

Average Ki-67 expression in intestinal metaplasia foci of individuals with chronic cystitis was 20.8%, and in the case of individuals with urothelial neoplastic lesions of the average expression of Ki-67 in intestinal metaplasia foci was 23.15%, with no significant statistical differences between the two groups (p > 0.05). Areas of tumor malignancies expressed a Ki-67 immunoreactivity of 49.38%. After implementing multiple comparison test between outbreaks of intestinal metaplasia and neoplasia insignificant values were obtained (p > 0.05).

Based on morphological and immunohistochemical aspects evidenced, we can conclude that these intestinal metaplasia foci show no malignant characteristics and therefore they do not pose a risk in the development of malignant neoplasms, they coexist with tumoral lesions.

Chapter 12, entitled "Viral DNA extraction from urothelial biopsies", presents a correlation between different urothelial lesions caused by bovine papillomavirus infection subtypes BPV-1, 2 and 4, in cattle from geographical regions with chronic enzootic haematuria.

For this we used 19 biopsies (urothelial mucosa) out of which 14 presented with lesions (8 neoplastic and 6 non-neoplastic) and 5 without lesions. From the 14 samples with pathologic changes, one case was represented by follicular chronic cystitis, 5 cases consisted of epithelial and subepithelial haemorrhages, and the 8 cases of neoplastic lesions were: 3 cases of papillomas, two cases of infiltrative papillary carcinoma, one case of non-infiltrative papillary carcinoma, a case of infiltrative adenocarcinoma and one infiltrative transitional cell carcinoma. From these biopsies,
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total DNA was extracted and was amplified by polymerase chain reaction using specific primers for the bovine papillomavirus subtypes 1, 2 and 4.

Following PCR analysis of the studied samples, seven out of 8 cases presenting with neoplastic lesions and one without any lesions were positive for BPV-2. BPV-2 wasn’t identified in the samples with non-neoplastic lesions. For BPV-1 and BPV-4 all 19 samples were negative.

The presence of the virus in the normal urothelium, without pathological changes, demonstrates that, in some cases, the infection with BPV-2 may be latent, but in the case of a weakened immune system, or overlapping the action of environmental or alimentary carcinogenic factors (for example bracken fern - Pteridium spp.) it can activate, causing urothelial tumors.

Chapter 13, entitled "Fractal analysis – predictive marker to diagnose urothelial tumors in cattle", aims evaluating a new predictive marker, fractal dimension analysis, in pre-neoplastic and neoplastic urothelial lesions in cattle and demonstrating its usefulness as a predictive marker in tumor diagnosis in veterinary medicine.

To achieve this purpose we used histological slides (stained hematoxylin-eosin) from 40 individuals divided into 7 groups: group 1 – normal mucosa (without pathological changes); group 2 – chronic cystitis; group 3 – epithelial hyperplasia; group 4 – epithelial dysplasia/ in situ carcinoma (ISC); group 5 – urothelial papilloma; group 6 – papillary urothelial neoplasm with low malignancy potential; group 7 – carcinoma. Next, nuclei showing characteristics of each case were randomly chosen, imported into ImageJ™ software (Wayne Rasband (HHI), USA) and subjected to fractal analysis using the "box-counting" method. The results were recorded and subjected to statistical analysis using specialized programs MS Excel™ 2010 (Microsoft™, USA) and GraphPad InStat™ v3.05 (GraphPad™, USA).

Average degree of complexity of the internal structure of the cell nucleus ranged between all groups studied. The highest fractal dimension was found in normal cells, $DF = 1.617 \pm 0.039$ (average ± standard deviation), while the lowest value was recorded in group 7 (urothelial carcinomas), $DF = 1.550 \pm 0.042$. FD intermediate values were found in: group 2 - chronic cystitis (1.563 ± 0.042); group 3 - epithelial hyperplasia (1.612 ± 0.038); group 4 - epithelial dysplasia / ISC (1.580 ± 0.065); group 5 - papillomas (1.573 ± 0.050); group 6 - PUNLMP (1.559 ± 0.064). It can be seen that in PUNLMP and carcinomas, nuclear fractal dimension average was lower than others groups. Significantly lower values in these groups show that nuclear morphology is different from the nuclei of normal cells, the latter having a more organized layout and structure (degree of nuclear chromatin arrangement).

One Way ANOVA was used to compare the fractal dimensions between the group with normal mucosa and the mucosa showing pathological changes. It was found that
the nuclear fractal dimensions of urothelial cells followed a normal distribution. Differences with a p-value of 0.05 or less were considered significant statistically. There was a significant difference between the mean values of the fractal dimension of the cell nucleus in individuals with normal mucosa and those with pathological changes (p < 0.001). Following the completion of Tukey-Kramer multiple comparison test, there were highly significant values obtained (p < 0.001) between group 1 (the group with normal mucosa) and all the other groups. Regarding the comparison between groups with injuries, there were statistically significant differences only between groups 3 and 6, respectively 3 and 7 (3 - hyperplasia group, group 6 - PUNLMP, group 7 - carcinomas).

This study demonstrated that malignant urothelial cells lose their nuclear characteristics, aspect which may be quantified by analyzing the fractal dimension (FD).
OVERALL CONCLUSIONS

1. Cattle develop a varied neoplastic urothelial pathology, some injuries like papillary hyperplasia, in situ carcinoma and PUNLMP (papillary urothelial neoplasms of low malignancy potential) not being classified by WHO (2004), these lesions being found only in human pathology. The most important lesions are represented by chronic cystitis and urothelial neoplasms (transitional cell carcinoma, squamous cell carcinomas and adenocarcinomas).

2. The diversity of neoplasms found in urinary bladders taken from bovine affected by enzootic haematuria emphasizes the need to watch more closely the animals from areas reach in bracken fern.

3. Correct interpretation of non-neoplastic/preneoplastic lesions associated with chronic cystitis is an important factor in the early diagnosis of urothelial cancer.

4. Among immunohistochemical markers used, the one that has proved most effective in diagnosis and prognosis of tumors was Ki-67 (cell proliferation marker).

5. Cystitis glandularis lesions were identified in both chronic inflammation and urothelial neoplasms; the form of intestinal metaplasia was found more frequently than the typical form of Cystitis glandularis.

6. Intestinal metaplasia foci, initially identified through histopathological examination, then confirmed by histochemical technique (AB-PAS), were present in the surface epithelium, in the case of both chronic cystitis neoplasms. Morphologically, dysplastic aspects have not been observed, cells being well differentiated and resembling intestinal Goblet cells (complete intestinal metaplasia).

7. We consider that these outbreaks of intestinal metaplasia found in the bladder occur as regenerative aspects associated with chronic injuries, without malignant potential.

8. Using molecular techniques we have demonstrated the presence of bovine papilloma virus in urothelial samples collected from cattle. Although the literature provides data on the presence of viral subtypes 1 and 4, they were not found in this study.

9. BPV-2 viral strain was found in 42% of the studied samples. The viral DNA was present in almost all tumor cases (the only exception being a benign tumor - papilloma), and in no non-neoplastic lesions.

10. The presence of virus in the normal mucosa demonstrates that, in some cases, the infection with BPV-2 may be latent, and in case of a weakened immune
system, or overlapping the action of environmental and nutrition carcinogens, (bracken fern - *Pteridium spp.*) it can be activated causing urothelial lesions.

11. Our study first used nuclear fractal dimension analysis in urothelial lesions in cattle. From the biophysics point of view, the results confirm that the response of cells to carcinogenic factors is to change its geometrical complexity and reduce the fractal dimension.

12. This paper demonstrated that malignant urothelial cells lose their nuclear characteristics, aspect which can be quantified using fractal dimension analysis (FD).

13. There is an undisputed merit to the use of animal models in studying the pathogenesis of bladder lesions, even if, for now, an association can not be made between histological grade of the neoplasms and symptoms; given the fact that the samples are obtained from the slaughterhouse, currently, there is no question of treatment or observing the changes.

**RECOMMENDATIONS**

A future extension of the present work is to identify different ways of development of each specific type of cancer and a better understanding of the molecular mechanisms that play a role in carcinogenesis.

For this we recommend extraction of viral DNA of different strains of BPV and tracking the frequency with which each is found in various geographical areas of Romania.

Evaluation of intestinal metaplasia in different epithelial neoplasms, especially urothelial, remains a challenging topic and open to researchers in conducting future studies in this field.