Characterization of collagens fibers (I, III, IV) and elastin in the extracellular matrix of normal and neoplastic canine prostatic tissues

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Abstract: Collagen (Coll) is the most common protein in the extracellular matrix, responsible for providing tissue structure and support. In some types of cancer, including prostate cancer (PC) Coll deregulation was described and related to tumor progression and metastasis. This study aimed to investigate Coll-I, III, IV and elastin in canine normal prostate and PC, using Picrosirius red (PSR) and Immunohistochemical (IHC) analysis. Eight normal prostates and 10 PC from formalin-fixed, paraffin-embedded samples were used. Collagen fibers area was analyzed with ImageJ software. The distribution of Coll-I and Coll-III was approximately 80% around prostatic ducts and acini, 15% among smooth muscle and 5% around in blood vessels, in both normal prostate and PC. Immunostaining for Coll-IV was observed in the basal membrane of prostate acini, smooth muscle, blood vessels, and never fibers of normal and PC samples. Elastic fibers were found in the septa dividing the lobules and around the prostatic acini of normal samples. A high amount of elastic fibers was observed around the ducts and the urethra in normal and PC. The distribution and area percentage of staining for collagen are similar in normal and neoplastic canine prostate when analyzed with PSR and IHC.

Keywords: dog; prostatic tissue; extracellular matrix; picrosirius; immunohistochemistry

1. Introduction

Cancer is the second leading cause of mortality worldwide. In men, prostate cancer (PC) is the third most common malignant neoplasia (after non-melanoma skin cancer and lung cancer) [1]. In advanced stage, human PC often shows metastasis to bones and resistance to anti-androgen treatment [2]. Similar to the men, dogs spontaneously develop PC [3, 4]. In dogs, PC it is a very aggressive and highly metastatic disease [5]. Usually, bone metastasis is diagnosed at a late stage of highly aggressive tumor subtypes [3]. Due to similarities in the clinical and pathologic aspects of PC in both species, some authors suggest that the dogs may be considered a good model for the study of human PC [4, 5, 6, 7].

Recently, has been demonstrated in the human PC an interaction between the tumor cells and the proteins of extracellular matrix (ECM). The ECM fibers play an important role in PC development
and progression [8, 9]. The ECM is a complex network of macromolecules [10]. The major constituents of ECMs are proteoglycans and fibrous proteins (collagen, elastin, fibronectin and laminin) [11]. Collagen (Coll) is the most common protein of the ECM [10]. The main collagen function is to provide structure, support and tensile strength, as well as, regulation of cell adhesion, chemotaxis, migration and direct tissue development [12]. In some human cancers, such as breast, colon and prostate, occurs a formation of abundant collagenous stroma (reactive stroma) in their tumor microenvironment (TME), responsible for the tumor metastatic process [8, 13, 14]. High density of type I collagen (Coll-I) and degradation of type IV collagen (Coll-IV) are frequently observed in solid cancer, associated with metastasis [9, 15-18]. In the veterinary medicine, few studies were conducted to understand the relationship between cancer and the collagens fibers, compared to human medicine [19]. Due the lack of information regarding tissue stroma in canine PC, this study aimed to characterize and compare the collagen fibers and elastin in the normal prostate and canine PC, using PSR and immunohistochemical test.

2. Materials and Methods

2.1. The Subjects

Eight canine normal prostates and 10 PC were retrieved from the archives of the Veterinary Pathology Service, FMVZ, UNESP, Botucatu, SP, Brazil. The prostates were collected from necropsies from animals that had an interval between death and necropsy less than 6 hours. Formalin-fixed paraffin-embedded (FFPE) samples from canine prostatic tissue were sectioned for histological diagnosis, which was performed by three pathologists (LGRC, CEFA, PEK), at the same time, in a multi-head microscopy. The histopathological classification was performed according to the human WHO from Tumors of the Urinary System and Male Genital Organs [20], which was recently adapted to canine PC [4]. (Supplementary Table 1). This study was approved by the institutional committee for the use of animals in research (#10.162/2016).

PC samples (10/10) were from intact male dogs, with age raging 8 up to 12 years. Six out of 10 samples were from mixed breed dogs, two cases (2/10) were from a German Shepherd dog, one case (1/10) from a Brazilian Mastiff dog and the other one from a Poodle (1/10). Regarding the clinical signs, five out of 10 patients showed tenesmus, three dogs had lameness (3/10) and two dogs (2/10) had loss of appetite. Five out of 10 patients had metastasis at the diagnosis and all dogs (10/10) died due to PC.

2.2. Picrosirius (PSR)

The slides were deparaffinized in xylene and rehydrated in alcohol. After, PSR staining was performed using a commercial kit (HistokitTM, Easypath, SP, Brazil), according to manufacturer’s instructions. The slides were examined in an optical microscopy with polarized light (Axio Imager A1, Zeiss®, Germany). The collagen fibers that presented red-orange birefringence were considered type I, while the collagen fibers with green birefringence were interpreted as type III (Coll-III) [21].

2.3. Immunohistochemistry (IHC)

The slides were subject to immunohistochemical using the peroxidase method. The antibodies, antigen retrieval, dilutions and incubation period are described in the Table 1. Endogenous peroxidase activity was inhibited with 4% hydrogen peroxide in methanol for 10 min at room temperature (RT). Then, the slides were treated with protein block serum-free for 15 min RT (Dako, Carpinteria, CA, USA). In each step, the slides were washed with Tris-buffered saline (pH 7.4). A LSAB system was used as secondary antibody; applied for 1 hour at RT, according to manufacturer’s instructions (Dako, Carpinteria, CA, USA). Peroxidase activity was reveled with 3',3'-Diaminobenzidinechromogen (DAB, Substrate System, CA, Dako). For the counterstained, Harris’s hematoxylin was used. As negative control, primary antibodies were replaced by Tris-buffered saline solution. A canine normal skin tissue was used as positive control tissue for all antibodies.
2.4. Interpretation of PSR and IHC staining

In the PC samples, areas with a higher percentage of neoplastic cells and minimal density of inflammatory cells were selected. In the normal samples, it was collected samples from the peripheral region of the prostate gland, avoiding areas close to the median septa, according to Ruetten et al. [22]. For normal and PC samples, it was captured five fields (20x magnification) with a digital camera (Axioncam MRc, Zeiss® Vision, Germany) for each protein and PSR. The stained areas were analyzed with Image J 1.49v software (National Institutes of Health, USA) and were assessed by setting a threshold, using the Image J threshold tool in accordance with the procedure described by Bauman et al., [23]. Briefly, the staining distribution and intensity of the collagens and elastic fibers were evaluated in both normal canine prostate and PC. For PSR staining, it was used a manual thresholding of hue (121-179), saturation (20-255), and brightness (10-255) values in Imagej [23]. For each marker in immunohistochemistry, we established a threshold values as follows: Coll-I: hue (0-170), saturation (69-255), and brightness (90-181), Coll-III: hue (111-176), saturation (10-98), and brightness (37-157), Coll-IV: hue (65-255), saturation (90-178), and brightness (101-255) and elastin: hue (0-146), saturation (0-175), and brightness (0-209).

2.5. Data analysis

Descriptive statistics was used to define the median and percentile of Coll-I, Coll-III and elastin in normal and canine PC. For statistical propose, we grouped samples with Gleason score 6 and 8 and compared with samples with Gleason score 10. Mann-Whitney U test was applied to compare the area percentage among normal and canine PC. Statistical significance was set at p< 0.05. All statistical analysis was done using GraphPad Prism 8 (GraphPad Software Inc. La Jolla, CA).

3. Results

Five out of 10 PC samples had Gleason 8 (5/10), four had Gleason score 10 (4/10) and one Gleason score 6 (1/10). The mean survival time was 152.1 days (±134.8). All prostatic samples (18/18) stained with PSR and the Coll-I was more abundant than Coll-III (Figure 1). The median expression of Coll-I by PSR in normal samples was 1.89 (1.196 - 3.839) and in PC samples was 2.24 (1.358 – 2.834). There was no statistical difference of Coll-I expression between normal and PC samples. Regarding the IHC for Coll-I, we also identified a higher proportion of Coll-I compared to Coll-III (Figure 1). The median expression of Coll-I in normal samples by IHC was 4.73 (1.367 – 8.414) and 6.18 (1.577 – 17.572) for PC samples. We identified a positive correlation of Coll-I expression between PSR and IHC techniques (R=0.6185; P=0.05). Thus, although the results are numerically different for both techniques, there is a correlation of the results. Besides that, we evaluated the distribution of the Coll-I thought the normal prostate. Approximately 80% of the Coll-I was located surrounding prostatic ducts and acini, 15% among smooth muscle and 5% around blood vessels (Figure 1). We also did not find statistical difference of Coll-I immunoexpression between normal and PC samples. Comparing Coll-I expression between samples with Gleason score 10 and 8/6, we did not find statistical difference (P=0.761)
Regarding Coll-III expression, we identified positive stain in all prostatic samples (18/18) for both techniques. PC samples showed a higher expression of Coll-III than normal samples by PSR (P=0.01). The median expression for Coll-III expression in normal samples was 1.64 (0.975 – 3.329) and 2.25 (1.067 – 3.605) for PC samples. We identified a higher Coll-III expression in PC samples compared to normal samples (P=0.05) by IHC. We identified a strong positive correlation (R=0.7805; P=0.007) of Coll-III expression between PSR and IHC techniques. Thus, samples with high Coll-III by PSR technique also showed a higher Coll-III expression by IHQ. We also qualitatively assessed the Coll-III distribution among the prostatic tissues. In both normal and PC samples, 80% of the Coll-III was located surrounding prostatic ducts and acini, 15% among smooth muscle and 5% around blood vessels (Figure 1). Table 2 shows the median, 25% percentile and 75% percentile values of Coll-I and Coll-III according to the diagnosis group and test applied. There was no statistical difference regarding the Coll-III expression and the Gleason score (P=0.0654).

Table 2. Median, 25% percentile and 75% percentile values of area percentage staining for Coll-I and Coll-III in the normal and canine PC, according to the method used.

<table>
<thead>
<tr>
<th></th>
<th>PSR test</th>
<th></th>
<th></th>
<th>IHC test</th>
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<tbody>
<tr>
<td></td>
<td>25%</td>
<td>Median</td>
<td>75%</td>
<td>p</td>
<td>25%</td>
<td>Median</td>
</tr>
<tr>
<td>Coll-I</td>
<td>N</td>
<td>1.25</td>
<td>1.89</td>
<td>2.27</td>
<td>2.85</td>
<td>4.73</td>
</tr>
<tr>
<td></td>
<td>PC</td>
<td>2.09</td>
<td>2.24</td>
<td>2.43</td>
<td>0.1298</td>
<td>3.31</td>
</tr>
<tr>
<td>Coll-III</td>
<td>N</td>
<td>1.33</td>
<td>1.64</td>
<td>2.06</td>
<td>1.81</td>
<td>3.22</td>
</tr>
<tr>
<td></td>
<td>PC</td>
<td>1.68</td>
<td>2.25</td>
<td>3.11</td>
<td>0.001</td>
<td>3.72</td>
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Immunostaining for Coll-IV was observed in the basal membrane (BM) of prostate acini, smooth muscle, blood vessels, and nerve fibers of normal and PC samples, although it was discontinuous (Figure 2). It was observed a Coll-IV immunostaining surrounding continuously the blood vessels in both normal and PC samples. The distribution of Coll-IV was approximately 70% in the BM, 15% in smooth muscle, 10% in blood vessels BM and 5% in nerve fibers in both groups. When we compared the normal prostate with PC samples, we did not find statistical difference, concerning Coll-IV expression (P=0.2722). However, it was identified absence of Coll-IV immunostaining in the tumors with Gleason score 10 compared to tumors with Gleason score 6 and 8 (P=0.0095) (Figure 2). The results of Coll-IV expression are described in Table 3.

Table 3. Median, 25% percentile and 75% percentile values of area percentage staining for Coll-IV and elastin in the normal prostate and canine PC samples.

<table>
<thead>
<tr>
<th>Group</th>
<th>Coll-IV</th>
<th>Elastin</th>
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<tr>
<td></td>
<td>Normal</td>
<td>PC</td>
</tr>
<tr>
<td>25%</td>
<td>1.11</td>
<td>0.28</td>
</tr>
<tr>
<td>Median</td>
<td>0.58</td>
<td>0.25</td>
</tr>
<tr>
<td>75%</td>
<td>1.72</td>
<td>0.42</td>
</tr>
<tr>
<td>p</td>
<td>0.2135</td>
<td>0.00229</td>
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Immunostaining for elastin was observed with similar intensity and distribution than Coll-IV, around blood vessels in normal tissues and PC. Elastic fibers were found in the septa dividing the lobules and around the prostatic acini of normal samples. A high amount of elastic fibers was observed around the ducts and the urethra in normal and canine PC (Figure 2). It was identified a higher expression of Elastin in PC samples compared to normal samples (P=0.0029). There was no statistical difference comparing Gleason score 10 with Gleason 6 and 8 samples (P=0.897).
Figure 1. The immunohistochemistry and PSR stain in normal tissue and canine PC. a: immunostaining of Coll-I in the stroma of normal prostate (case No. 3). b: immunostaining of Coll-III in the stroma of the normal prostatic tissue (case No. 3). c: PSR staining observed in an optical microscopy with polarized light, the collagens fibers present red-orange birefringence (Coll-I) and green birefringence (Coll-III) in a smaller amount (case No. 3). d: immunostaining of Coll-I in the stroma of prostatic neoplastic tissue (case No. 11). e: immunostaining of Coll-III in the stroma of canine PC (case No. 11). f: PSR staining in the canine PC with similar amounts of Coll-I and Coll-III (case No. 11).
Figure 2. Immunostaining for Coll-IV and elastin in canine prostatic tissue. a: Coll-IV immunostaining in the basal membrane of canine PC with cribiforme pattern (case No. 15). b: Absence of Coll-IV immunostaining in canine PC with solid pattern (case No. 12). c: Elastin fibers around the prostatic acini of normal samples (case No. 5). d. High amount of elastin fibers around the urethra (case No. 5).

4. Discussion

In cancer, the EMC is a network of macromolecules that allow the cellular evasion towards the defense of the organism, besides helping in their metastatic process [10]. Collagens fibers are important components of ECM remodeling in the TME, actually is known that their degradation and redeposition promote tumor infiltration, angiogenesis, invasion and migration [11]. In this study, we identified and characterized collagen and elastin fibers in the normal prostate and canine PC using PSR and IHC tests. This research also compared the correlation of both tests to identify Coll-I and III expression. Coll-I expression was not statistically different when compared normal prostates and canine PC samples using both techniques. Although PSR is less sensitive for staining of collagen fibers, the positive correlation between these two techniques, allows us to suggest that PSR is cheaper and routinely used in some histology laboratories, and can be a good choice. In humans, Bauman et al., [23] evaluated the Coll-I content in normal prostates and benign prostatic hyperplasia (PH) by PSR staining, with no statistical difference. We did not find studies evaluating collagens fibers with PSR and IHC simultaneously, in both human and canine PC. Regarding the immunostaining of Coll-I, statistical difference it was also not identified comparing normal and PC samples. However, in PC samples, we identified a higher median of Coll-I expression. The lack of statistical difference can be related to the low number of cases but also corroborates with Bauman et al., [23]

Wegner et al., [24] performed a study with PSR in the prostate gland of C57BL/6J mice. They found that fluorescent PSR imaging was more sensitive than polarized light for identify the collagen fibers. In addition, Fluorescent PSR imaging was compatible with the collagen expression by IHC test. The fluorescent PSR imaging method seems to be promising, but it must still be studied in the comparative oncology. Fewer studies with formalin-fixed paraffin-embedded (FFPE) samples of canine tumors were conducted to analyze the collagens fibers by PSR [19, 25, 26]. Bedoya et al., [27] used PSR staining in canine squamous cell carcinomas (SCCs), classified in well and poorly
differentiated. The percentage of Col-I was approximately 30% for low- and high-grade SCCs. Their results showed higher percentage of collagen fibers than observed in our study, comparing normal and canine PC, but these are different tumors, with different patterns and locations.

The interaction between the EMC components and metastatic progression is widely studied in human cancers. However, the literature lack information regarding the collagen fibers and elastin expression in human and canine PC tissues. The previous human literature is focused on the role of EMC components in prostate cancer cells [28, 29], instead human prostatic tissue [30, 31]. The Type I collagen degradation product (ICTP) was previously investigated in human PC, predicting bone metastasis type and activity [30]. However, the ICTP expression is evaluated in serum and these authors did not evaluated the collagen I expression in the prostatic samples. In this study, we evaluated the Coll-I immunoexpression in normal and neoplastic prostates, and did not study metastatic disease, which could be an interesting information.

Coll-I and III gene and protein expression were previously evaluated in human PC, using RT-qPCR and IHC [32]. These authors also correlated Coll-I expression with Gleason score. No correlation was found between protein and gene expression for both collagens. However, the IHC analysis showed that Coll-I and Coll-III was significantly reduced in PC, in all Gleason scores, when compared to benign areas. These authors suggested that collagen reduction in PC could be the result from high metalloproteinase (MMP) activity [32]. The MMPs have been correlated with the tissue invasion and tumor progression of different tumors [33]. In human PC, tissue inhibitors of matrix metalloproteinases (TIMPs) and MMPs dysregulation are caused by a significant gain of MMP-2 and 9 expression and TIMP-1 loss. The increased MMP-2 and 9 expression leads the degradation of collagen fibers being the evaluation of Coll-I expression trick. In canine PC, MMP-2 and MMP-9 were previously evaluated and authors found a protein overexpression, when compared PC to normal prostate. However, the authors did not compare the results with collagen expression [34]. Based on this previous description in canine PC, the MMP-2 and 9 overexpression can induce a collagen degradation interfering the collagen evaluation by IHC and PSR [34].

Comparing the Gleason score and the Coll-I and III immunostaining, we did not find statistical difference. Probably due the low number of cases. On the other hand, we identified a higher Coll-III expression in PC samples compared to normal sample by PSR and IHC. Duarte et al. [32] evaluated Coll-I, Coll-III, MMP2 and MMP9 expression in human prostate cancer by gene and protein expression. These authors found a Coll-III down expression compared to PH. Furthermore, high MMP 9 expression was found, indicating a degradation of Coll-III by MMP9. On the other hand, in human SCC, the Coll-III overexpression was associated with a worst prognosis sand higher tumor grade [35].

The pattern of Coll-IV expression in this study was similar to normal human prostate and PC [36]. An interesting finding was that solid canine PC had lower/absent Coll-IV expression than other PC patterns and normal tissues. Sinha et al., [37] found that Coll-IV immunostaining was less uniform or absent in the BM of poorly-differentiated human PC when compared to well-differentiated PC, HBP and normal human prostates. Similar results were also observed in feline and canine mammary tumors as well as in canine hemangiosarcoma [25, 26].

We found a normal pattern [37] of elastic fibers in canine prostate (in the septa dividing the lobules, around the alveoli, ducts and the urethra). The interesting result was that canine PC, had a statistical higher median of elastin fibers (p=0.00229) than normal tissue. Elastin fibers are involved in tumor invasion and metastasis [38], cell proliferation, adhesion, apoptosis and angiogenesis [39].

The collagen and elastin patterns in the prostate gland are dynamic and important differences can be found in different species. Beside that, the prostate gland anatomy, the animal breed, age and castration status can lead in different collagen and elastin fiber patterns [22]. In this study, we lack information regarding the region of the prostate gland (periurethral, peripheral or of the prostate biopsy) that the tissue specimens were collected. Thus, we strongly recommend the annotation of each region that the prostatic tissue was collected before evaluating the EMC components in canine prostate.
5. Conclusions

The distribution and percentage area of collagen are similar in normal and neoplastic canine prostate, when analyzed with PSR and IHC tests. In addition, the immunohistochemical localization of elastic system fibers is similar in both groups. Only, in canine PC with solid pattern was identified loss of Col-IV compared to others tumor patterns and normal prostate samples.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Histological type of 10 canine prostatic carcinomas, according to Eble et al., 2004 and Palmieri et al., 2014.


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References


