Immunohistochemical Analysis of Na⁺/K⁺-ATPase and Bone Morphogenetic Protein-2 Expressions in Both Parenchymal and Stromal Cells from Benign and Malignant Canine Mammary Tumors

ozlem ozmen (✉ ozlemozmen@mehmetakif.edu.tr)

University of Mehmet Akif Ersoy, Faculty of Veterinary Medicine <https://orcid.org/0000-0002-1835-1082>

Research Article

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Abstract

Canine mammary tumors are the most common type of dog tumor, and they are similar to human breast tumors. Na^+/K^+ -ATPase is a common plasma membrane ion pump with important physiological and pathophysiological functions. In mammary tumors, the tumor microenvironment was composed of a heterogeneous population of tumor cells and nearby endogenous stromal cells. Bone morphogenetic proteins (BMPs) regulate fetal development, tissue homeostasis and differentiation, and a variety of cellular functions. The purpose of this study is to examine the immunohistochemical expression of Na^+/K^+ -ATPase and BMP-2 in tumoral and stromal cells from benign and malignant canine mammary tumors. In this study, ten benign and ten malignant mammary tumors from the archives of the Department of Pathology were used, with five normal breast tissues serving as controls. The results of the revealed that tumors had higher levels of Na^+/K^+ -ATPase and BMP-2 expressions than normal mammary tissue. While both markers were expressed negatively or mildly in benign tumors, they increased significantly in malignant tumors. Both Na^+/K^+ -ATPase and BMP-2 are expressed by tumoral and stromal cells in canine mammary tumors. When compared to compared to BMP-2, Na^+/K^+ -ATPase expression was found to be more severe. This study found that Na^+/K^+ -ATPase and BMP-2 can be used as markers of malignancy in canine mammary tumors and that stromal cells also play an important role in tumor progression. These findings also indicated that Na^+/K^+ -ATPase and BMP-2 may be used for early diagnosis or as a potential target for treatment of canine and human breast tumors in the future.

Introduction

Cancer incidence in dogs has increased due to their longer lifespan (Whithrow et al., 2007). Breast cancer has recently been identified as one of the leading causes of death in both humans and animals. As a result, studies on the detection or treatment of biomarkers that can be used in cancer diagnosis and treatment are at the forefront. For the detection of these biomarkers, new techniques for measuring both tissue and plasma levels are being developed (Baker et al., 2000, Pepe et al., 2001, Diamandis and Yousef, 2002).

Aside from the numerous physiological functions of Na^+/K^+ -ATPase in a variety of biological systems, its altered expression or malfunction may be associated with the pathogenesis of many diseases, particularly cancers (Blok et al., 1999, Rajasekaran et al., 1999, Rajasekaran et al., 2003a, Rajasekaran et al., 2003b, Espineda et al., 2003). In many tumors, intracellular potassium and sodium levels fall and rise, respectively. This situation suggests that the mechanisms governing intracellular homeostasis for potassium and sodium are no longer tightly regulated in cancer cells. These alterations could be related to the changes in Na^+/K^+ -ATPase pump activity. Because of its important roles in numerous physiological functions of various biological systems, Na^+/K^+ -ATPase may play a pivotal role in the pathogenesis of various diseases such as cancer (Rajasekaran et al., 2003b, Espineda et al., 2003).

Bone morphogenetic proteins (BMPs) are multifunctional growth factors that belong to the TGF- β superfamily. In vivo and in vitro, BMP-2 promotes invasiveness and motility of malignant mammary tumor cells by facilitating epithelial-to-mesenchymal transition (EMT) (Katsuno et al., 2008, Kang et al., 2009, Jin et al., 2012). BMP-2 may play a role in stem cell transformation and breast cancer initiation (Clement et al., 2016). However, the mechanisms underlying the increased levels of Na⁺/K⁺-ATPase and BMP-2 in breast cancers are largely unknown.

Endothelial cells, fibroblasts, pericytes, myofibroblasts, dendritic cells, macrophages, and other immune cells in the tumor microenvironment have all received attention (Bussard et al., 2016). Supporting cells of the tumors are recruited from the local stroma of the host and play several important roles, including promoting extracellular matrix remodeling, neoangiogenesis, drug resistance, cellular migration, invasion, and evasion of immunosurveillance through production of various growth factors, cytokines, and chemokines (Hanahan and Coussens, 2012). Interactions between tumor cells and host stroma are important factors in tumor progression and growth (Dvorak, 1986).

There are only a few reports on Na⁺/K⁺-ATPase and BMP-2 in canine mammary tumors (Wensman et al., 2009, Freeman et al., 2010, Klopfleisch et al., 2010). However, all of the studies only reported on tumoral parenchymal cell reactions. The purpose of this study is to investigate the immunohistochemical expression of Na⁺/K⁺-ATPase and BMP-2 in both parenchymal and stromal cells of benign and malignant canine mammary tumors and to see if there is a link between the expression and the malignancy criteria.

Materials And Methods

Tissue samples:

In this study, a total of 20 canine mammary tumors (10 malignant and 10 benign) and 5 normal breast tissues from the Department of Pathology archive were used.

Tissue processing and histopathological method:

For histopathology and immunohistochemistry, paraffin blocks were cut at a thickness of 5 μ m using a Leica RM2155 rotary microtome (Leica Microsystems, Wetzlar, Germany). Moreover, all slides were stained with hematoxylin and eosin (HE) on a regular basis (Luna, 1968).

Immunohistochemistry:

Following histopathological evaluation, selected sections were immunohistochemically stained to demonstrate the expression of Na⁺/K⁺-ATPase and BMP-2. All primary antibodies and secondary kit are purchased from Abcam (Cambridge, UK, 1/100). Using a routine streptavidin-biotin peroxidase technique, commercial kits were used for immunohistochemical examination of Na⁺/K⁺-ATPase [Anti-Sodium Potassium ATPase Antibody-Plasma Membrane Marker (ab58475), 1/50 dilution] and BMP-2 [Anti-BMP2 Antibody (ab14933), 1/100 dilution], according to the manufacturer's instructions. The Mouse and Rabbit

Specific HRP/DAB Detection Kit-Micropolymer (ab236466) was used as the secondary antibody. For immunohistochemical detection, slides were deparaffinized and rehydrated in descending concentrations of alcohol and water. Furthermore, both antibodies did not require any pretreatment. Endogenous peroxidases were inhibited with 3% hydrogen peroxide, and nonspecific signaling was inhibited with protein block. The section was then incubated 4°C overnight with primary antibodies. There were both positive and negative controls used. For negative controls, phosphate buffer solution (PBS) was used instead of the primary antibody. Complement was applied for 10 min, followed by Goat Anti-Rabbit IgG Antibody HRP-conjugate. Except for the protein block and primary antibody steps, sections were washed three times with PBS. DAB was used as the chromogen, and the slides were then counterstained with hematoxylin and examined under a light microscope (Topsakal et al., 2019). All of the slides were analyzed for immunopositivity, and a semiquantitative analysis was carried out, as described further below.

To assess the severity of the immunohistochemical reaction of tumor cells with markers, semiquantitative analysis was performed using an arbitrary visual scale with a grading score ranging from (-) to (+++) as follows: (-) denotes a negative stain, (+) a focal weak stain, (++) a diffuse weak stain, and (+++) a diffuse strong stain when examined under a microscope (Ozturk et al., 2018). The scoring was done by a specialized pathologist, and all sections were evaluated twice by the same person. The Database Manual cellSens Life Science Imaging Software System (Olympus Co., Tokyo, Japan) was used for morphometric analyses and microphotography.

Statistical analysis:

For statistical analysis, SPSS (Statistical Package for Social Sciences) 15.0 software (SPSS Inc., Chicago, Ill, USA) was used. The data are presented as mean $\pm$ SD. In the statistical analyses, the ANOVA test was used to see if there were any differences between the immunoscores of malignant and benign tumors and the control group. Furthermore, Duncan tests were used to compare continuous variables between groups. P values < 0.05 were considered statistically significant.

Results

The most common histopathological finding in all mammary tumor cases was severe hyperemia in vessels. Hemorrhagic foci were uncommon in benign tumors. Although the appearance of the acini and duct was preserved in benign tumors, proliferation throughout the lumen was frequently observed in benign mammary adenoma cases. In these cases, thick fibrous connective tissue was found around the proliferating mammary glands. Mitotic figures and pleomorphic cells were extremely uncommon in the mass (Fig. 1). In a dog, ductal adenoma with papillary growth and limited in ducts was diagnosed. Histology of benign mixed mammary tumors revealed osseous and cartilage metaplasia and anaplasia, but no mitoses. Table 1 shows the histopathological diagnosis as well as the Na⁺/K⁺-ATPase and BMP-2 expression scores.

Table 1
Na⁺, K⁺-ATPase and BMP-2 expression scores of the mammary tissue
and benign and malignant mammary tumors with pathological
classification

No	Tissue or diagnosis of the tumors	Na ⁺ , K ⁺ -ATPase	BMP-2
1	<i>Normal mammary tissue</i>	-	-
2	<i>Normal mammary tissue</i>	-	-
3	<i>Normal mammary tissue</i>	-	-
4	<i>Normal mammary tissue</i>	-	-
5	<i>Normal mammary tissue</i>	-	-
6	<i>Benign mixed tumor</i>	+	+
7	<i>Benign mixed tumor</i>	+	+
8	<i>Benign mixed tumor</i>	++	-
9	<i>Benign mixed tumor</i>	+	+
10	<i>Benign mixed tumor</i>	+	+
11	<i>Mammary adenoma</i>	+	+
12	<i>Mammary adenoma</i>	+	+
13	<i>Mammary adenoma</i>	++	+
14	<i>Mammary adenoma</i>	+	+
15	<i>Mammary adenoma</i>	+	+
16	<i>Mammary adenocarcinoma</i>	++	++
17	<i>Mammary adenocarcinoma</i>	+++	+++
18	<i>Mammary adenocarcinoma</i>	++	+++
19	<i>Mammary adenocarcinoma</i>	+++	+++
20	<i>Mammary adenocarcinoma</i>	++	++
21	<i>Malign mix tumor</i>	++	+++
22	<i>Malign mix tumor</i>	+++	++
23	<i>Malign mix tumor</i>	+++	++
24	<i>Malign mix tumor</i>	+++	+++
25	<i>Malign mix tumor</i>	++	+++

The histopathology of malignant mammary tumors revealed that the shapes of glandular epithelial cells commonly range from cubic to flat. In these cases, cystic structures, papillary extensions, and atypical cells were common. Mitotic activity varied from case to case but was consistently very high. Furthermore, fibrous tissue proliferation was seen in the majority of malignant mammary tumors. The most common microscopic finding in malignant cases was necrotic areas, particularly in the centers of the glandular masses. Inflammatory cell infiltrates have been found primarily in neutrophil leukocytes and mononuclear cells, particularly in necrotic areas. In malignant mixed tumors, cartilage and bone metaplasia were common, and malignancy criteria were also found in these tissues. In all malignant cases, mitotic activity, anaplastic and pleomorphic cells, and necrotic areas were typical histological structures. Moreover, the most important criterion for malignancy was the presence of metastases.

In this study, the relationship between Na⁺/K⁺-ATPase, BMP-2, and malignancy, which are the basis of our study, are investigated. According to the results of the study, they were significantly expressed in malignant tumors and only slightly to moderately expressed in benign tumors. BMP-2 expression was either negative or slight in benign cases, but it increased significantly in malignant mammary tumors. In addition, different reactions to Na⁺/K⁺-ATPase and BMP-2 expressions were noted in different areas of the same tumor mass. In areas where malignancy was more prevalent, significant expression of Na⁺/K⁺-ATPase and BMP-2 was found. Na⁺/K⁺-ATPase and BMP-2 were also found in both parenchymal and stromal cells, and the reaction was linked to cancer. By comparing the non-metastatic cases, expression was marked in metastatic tumors. It was also discovered that the cells with the most Na⁺/K⁺-ATPase and BMP-2 expression had intense mitotic activity.

Despite the fact that Na⁺/K⁺-ATPase was expressed in benign tumors, it was significantly increased in all malignant tumor types. Expressions were found in mammary gland cells, stromal cells, and myoepithelial cells (Fig. 2). BMP-2 expression was observed to be negative or very mild in benign tumors and severe in malignant tumors (Fig. 3). Table 2 displays the statistical analysis results of the immunohistochemical scores. Pathogenetic mechanism of the expression and tumor progressions illustrated in Fig. 4.

Table 2
Statistical analysis results of Na⁺, K⁺-ATPase and BMP-2 expressions in benign and malignant mammary tumors.

	Control	Benign	Malignant	P value
Na ⁺ , K ⁺ -ATPase	0.00 ± 0.00 ^a	1.20 ± 0.42 ^b	2.50 ± 0.52 ^c	< 0.001
BMP-2	0.00 ± 0.00 ^a	0.90 ± 0.56 ^b	2.20 ± 0.78 ^c	< 0.001
*: Values are presented as mean-standard deviation.				
**: Data with different superscripts indicate significant differences from each other.				

Discussion

Mammary cancers have become more common in both humans and animals in recent years, and they are now regarded as one of the leading causes of death (Ferlay et al., 2015, Johnston et al., 2001). As a result, there is a lot of research on biomarkers that can be used for early detection or treatment of breast cancer. For the detection of these biomarkers, new techniques for measuring both tissue and plasma levels are rapidly developing (Baker et al., 2000, Diamandis and Yousef, 2002, Nerurkar et al., 1989). The goal of this study is to evaluate any potential candidate of Na^+/K^+ -ATPase and BMP-2, as well as to determine the expression of these markers in both parenchymal and stromal cells in benign and malignant canine mammary tumors.

Na^+/K^+ -ATPase is a major plasma membrane ion transporter with three different types of subunits (Geering, 1990). Due to the numerous physiological functions of Na^+/K^+ -ATPase in various biological systems, it is unsurprising that dysfunction or altered expression of Na^+/K^+ -ATPase could be linked to the pathogenesis of many diseases. As a result, there have been some reports of altered expression and activity of Na^+/K^+ -ATPase in various human cancers. In many tumors, intracellular Na^+ increased, while K^+ decreased. In malignant cells, there may be some changes in intracellular homeostasis for Na^+ and K^+ . These alterations could be caused by changes in Na^+/K^+ -ATPase pump activity (Blok et al., 1999, Rajasekaran et al., 1999, Espineda et al., 2003). Furthermore, canine mammary tumors and Na^+/K^+ -ATPase activity are poorly understood (Freeman et al., 2010). They discovered that neither normal nor neoplastic stromal cells express Na^+/K^+ -ATPase. This study showed an increase in Na^+/K^+ -ATPase in parenchymal cells of canine mammary tumors, which was closely related to malignancy. In contrast to Freeman et al., there was an increased expression in stromal cells.

Growth factors play critical roles in tumor progression. BMPs are members of the transforming growth factor- β (TGF- β) superfamily, which regulates apoptosis and motility, cellular differentiation, and proliferation and is particularly important in tissue homeostasis and embryonic development (Davis et al., 2016, Nohe et al., 2004, Ye et al., 2009). Huang et al. reported that BMP-2 promotes EMT and invasion of the breast cancer cells in women (Huang et al., 2017). However, there are very few reports for BMP-2 and canine mammary tumors (Wensman et al., 2009, Klopffleisch et al., 2010). Both previous studies only examined parenchymal cells of the tumors, no information of stromal cells was found. This study showed that BMP-2 is closely related to malignancy of the canine mammary tumors. In this study, intense marked expression was also observed in the parenchymal and stromal cells of mammary tumors, with expression being much stronger in malignant cases.

Recently in tumor studies, the importance of the tumor microenvironment has been highlighted. It is made up of a heterogeneous mixture of endogenous host stroma and tumor cells, and it serves an important function during the disease progression period (Bussard et al., 2016). In general, authors examined only parenchymal expressions of the markers in immunohistochemical studies of tumoral tissues. However, stroma plays an important role in tumoral progression, and stromal cells secrete a variety of pro-tumorigenic factors. This study showed that stromal cells in canine mammary tumors also expressed

Na⁺/K⁺-ATPase and BMP-2. The expressions were similar to those found in parenchymal and stromal cells and were strongly linked to cancer.

Generally, either experimental animal models or cell cultures have been used for studies on human breast tumors. Human and canine breast tumors have been found to have a close relationship. Therefore, researchers have begun to evaluate human breast tumors using canine breast tumors. Numerous studies have shown that a variety of factors can be adapted to human and canine tumors (Sultan and Ganaie, 2018). Human and canine tumors are closely related in terms of spontaneous tumor development, homologous genome sequencing, genetic differences, coexistence, and many other factors. In addition, numerous studies have revealed many similarities, such as the presence of inflammatory reactions with the tumor microenvironment and the use of similar markers in evaluations (Carvalho et al., 2016, Queiroga et al., 2011). Recently, the comparisons between human and canine mammary tumors have been increasing rapidly (Nerurkar et al., 1989, Cassali, 2013, Sorenmo

et al., 2009). Therefore, studies on canine breast tumors are also interpreted in human tumors. The results of this study also showed that Na⁺/K⁺-ATPase and BMP-2 expressions may play a role in human breast tumor malignancy.

In this study, we used ten malignant and ten benign mammary tumors, as well as five normal breast tissues from our department archives. To begin, tumoral masses were reassessed for potential misdiagnoses. Following that, sections of all tumors were immunohistochemically stained and tested for Na⁺/K⁺-ATPase and BMP-2 expressions. In addition, the sections were investigated to determine whether there is a relationship between malignancy and the release of Na⁺/K⁺-ATPase and BMP-2 from parenchymal and stromal cells. Despite the small number of tumors used in our study, it was one of the most important studies on the increased expressions of markers from tumoral stromal cells as well as parenchymal cells.

According to the findings of this study, the expression of Na⁺/K⁺-ATPase and BMP-2 in canine mammary tumors differed significantly between benign and malignant tumors and increased significantly with malignancy. These results were consistent with previous studies (Freeman et al., 2010, Kaszak et al., 2018). Although these markers were strongly expressed by tumoral parenchymal cells, they were also expressed by stromal cells. Since the study was an archive study, it was not possible to determine whether there was a relationship between Na⁺/K⁺-ATPase and BMP-2 expressions and survival time. However, increased expression of Na⁺/K⁺-ATPase and BMP-2 in malignant tumors has been linked to a lower survival rate in dogs. More in-depth studies are expected to shed light on the subject. In addition, in new studies, plasma levels of these Na⁺/K⁺-ATPase and BMP-2 expressions should be examined and correlated with tissue levels.

Conclusions

Despite numerous studies on the biomarkers of mammary tumors in both humans and animals, an ideal biomarker to predict early diagnosis or estimate malignancy has yet to be discovered (Kaszak et al., 2018). It is becoming increasingly clear that the tumor microenvironment, which consists of a heterogeneous group of tumoral cells and host stroma that provides support to tumor cells, plays a critical role in disease progression (Bussard et al., 2016). The reaction of tumoral stroma may be related to tumor progression and metastasis. Canine mammary tumors are strikingly similar to human mammary tumors (Hawai et al., 2013). The current study found that stromal reactions of the tumors should be evaluated for malignancy. This study also indicated that higher levels of Na^+/K^+ -ATPase and BMP-2 expressions in canine mammary tumors are associated with malignancy and can be used to screen for cancer. In addition, the results of this study showed that inhibiting Na^+/K^+ -ATPase and BMP-2 in both parenchymal and stromal cells could be used in the treatment of canine or human breast cancers in the future. Further studies are needed to assess serum concentrations of these markers and their inhibition in treatment. Moreover, evaluation of the stroma may be more effective in determining tumor malignancy and treatment.

Declarations

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Code availability: Not applicable

Authors' contributions: **Ozlem Ozmen**, Designed the study, evaluate gross and immunohistochemical findings and wrote the draft of the manuscript.

Additional declarations for articles in life science journals that report the results of studies involving humans and/or animals: Not applicable

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Figures

Figure 1

Histopathological appearance of the tumors. (A) Mammary adenoma, (B) benign mixed tumor, (C) mammary adenocarcinoma, and (D) malignant mixed tumor, HE

Figure 2

Na⁺/K⁺-ATPase expressions of the tumoral parenchymal and stromal cells. (A) Slight expression (arrows) in mammary adenoma, (B) slight expressions (arrows) in benign mixed tumor, (C) marked expressions (arrows) in mammary adenocarcinoma, and (D) increased expressions (arrows) in malignant mixed tumor, streptavidin-biotin peroxidase method

Figure 3

BMP-2 expressions of the tumoral parenchymal and stromal cells. (A) Negative expression in mammary adenoma, (B) negative expressions in benign mixed tumor, (C) marked expressions (arrows) in mammary adenocarcinoma, and (D) increased expressions (arrows) in malignant mixed tumor, streptavidin-biotin peroxidase method

Figure 4

Illustration of the pathogenetic mechanism of the expression and tumor progressions