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RESEARCH ARTICLE

Na^+/K^+-ATPase and bone morphogenetic protein-2 expressions in parenchymal and microenvironmental cells of canine mammary tumours

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ABSTRACT

The most common canine tumour is mammary tumour, which resembles breast cancer in humans. Microenvironment is a crucial factor in the formation of breast cancers. In order to distinguish between benign and malignant canine mammary tumours, this study looked at the immunohistochemical expression of Na^+/K^+-ATPase and bone morphogenetic protein-2 (BMP-2) in tumour and microenvironmental cells. The aim of this study was to evaluate the expression of Na^+/K^+-ATPase and BMP-2 in canine mammary tumours and their relationship with malignancy. In this investigation, 10 normal breast tissues were used as controls, and 28 benign and 46 malignant mammary tumours were taken from the archives of the Department of Pathology. The findings showed that malignant tumours expressed more Na^+/K^+-ATPase and BMP-2 than did normal breast tissue. Both markers had a negative or slight expression in benign tumours, whereas they considerably increased in malignant tumours. Both tumour parenchymal and microenvironmental cells in malignancies expressed Na^+/K^+-ATPase and BMP-2. Na^+/K^+-ATPase expression was observed to be more prominent in cells when compared to BMP-2. These findings also suggest that Na^+/K^+-ATPase and BMP-2 could be employed in the future to help diagnose canine and possibly human breast cancers earlier or as possible targets for treatment.

KEYWORDS

Na^+/K^+-ATPase, BMP-2, dog, immunohistochemistry, mammary tumour

INTRODUCTION

Dogs are more likely to develop cancer due to their extended longevity (Withrow and Vail, 2007). One of the top causes of demise in both humans and animals is breast cancer. The focus of this research is the detection of biomarkers that can be used to diagnose and treat cancer. New methods are being developed to measure tissue and plasma levels of these biomarkers (Baker, 2000; Pepe et al., 2001; Diamandis and Yousef, 2002). The altered expression or dysfunction of Na^+/K^+-ATPase may be linked to the development of many diseases, including malignancies, in addition to the myriad physiological activities it performs in a range of biological systems (Blok et al., 1999; Rajasekaran et al., 1999, 2003a, 2003b; Espineda et al., 2003). Numerous cancers have rising and falling intracellular sodium and potassium levels, respectively. This shows that the processes controlling sodium and potassium homeostasis inside cells are no longer strictly regulated in cancer cells. These modifications might be connected to changes in the activity of the Na^+/K^+-ATPase pump. Na^+/K^+-ATPase may be crucial in the aetiology of several diseases including cancer, due to its significant role in numerous physiological activities of diverse biological systems (Rajasekaran et al., 2003b; Espineda et al., 2003).

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The TGF-β superfamily includes multifunctional growth factors called bone morphogenetic proteins (BMPs). BMP-2 facilitates the epithelial-to-mesenchymal transition (EMT), which increases the invasiveness and motility of malignant breast tumour cells both *in vivo* and *in vitro* (Katsuno et al., 2008; Kang et al., 2009; Jin et al., 2012). Breast cancer development and stem cell transition may be influenced by BMP-2 (Clement et al., 2016). However, the reasons for the elevated levels of BMP-2 and Na+/K+-ATPase in breast tumours are mainly unknown. In addition to a cluster of cancer cells, a tumour mass may also contain a variety of extracellular matrix, secreted substances, invading inflammatory cells, and resident host cells. Although the make-up of the tumour microenvironment differs depending on the type of tumour, the primary elements are blood vessels, stromal cells, immune cells, and extracellular matrix. To encourage their growth and progression, tumour cells alter the functioning of host cells, alter important chemical events, and activate physical and biological reactions inside the tissues from which they originated. The tumour microenvironment is a complicated and dynamic entity that is frequently ignored. It is believed that the tumour microenvironment actively promotes the development of cancer rather than being a passive component (Truffi et al., 2020). An essential, necessary, dynamic, and reciprocal relationship between cancer cells and tumour microenvironment elements emerges in the early stages of tumour growth, ensuring cancer cell survival, local invasion, and metastatic spread (Alkasalias et al., 2018; Cruz-Bermúdez et al., 2019; Anderson and Simon, 2020).

The tumour microenvironment has drawn attention to fibroblasts, endothelial cells, myofibroblasts, pericytes, dendritic cells, macrophages, and other immune cells (Bussard et al., 2016; Hossen et al., 2019). Supporting tumour cells are drawn from the host's local stroma and perform a number of crucial functions, such as promoting neoangiogenesis, extracellular matrix remodelling, drug resistance, cellular invasion, migration, and evading immune surveillance through the production of various growth factors, chemokines, and cytokines (Hanahan and Coussens, 2012). Important elements in the development and growth of tumours are interactions between tumoural cells and the host stroma (Dvorak, 1986).

Only a few studies have examined BMP-2 and Na^+/K^+-ATPase in canine mammary cancers (Wensman et al., 2009; Freeman et al., 2010; Klopfleisch et al., 2010). All of the investigations, however, exclusively covered tumour parenchymal cell responses. According to reports, Na^+K^+-ATPase, and BMP-2 play a critical role in particular in osteogenic proliferations, boost osteoblastic cell proliferation and have a synergistic effect (Tang et al., 2021). However, it is unknown how these indicators interact with tumoural cells in canine mammary cancers. This study aims to determine whether there is a relationship between Na^+/K^+-ATPase and BMP-2 expression and malignancy criteria in tumoural and microenvironmental cells of benign and malignant canine mammary tumours.

MATERIALS AND METHODS

Tissue samples

Ten normal breast tissues and a total of 74 canine mammary tumour tissues, including 46 malignant and 28 benign tumours from the archives of the Department of Pathology were used in this investigation.

Tissue processing and histopathological method

Using a rotary microtome (Leica RM2155, Leica Microsystems, Wetzlar, Germany), paraffin blocks were cut at a thickness of 5 µm for microscopic evaluations. For histopathological examination, haematoxylin and eosin (HE) staining was performed on each slide (Luna, 1968).

Immunohistochemistry

After histopathological analysis, two serial sections were immunohistochemically stained to show the expression of BMP-2 and Na^+/K^+-ATPase. The secondary kit and the primary antibodies were acquired from Abcam (Cambridge, UK). Commercial kits were used in accordance with the manufacturer's instructions for the immunohistochemical investigation 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]. The secondary antibody was Mouse and Rabbit Specific HRP/DAB Detection Kit-Micropolymer (ab236466). Slides were deparaffinised and rehydrated in descending concentrations of alcohol and water for immunohistochemical detection. Furthermore, neither antibody needed any pretreatment. Hydrogen peroxide (3%) was used to inhibit endogenous peroxidase activity and for protein block to prevent non-specific signalling. The sections were then treated with primary antibodies at 4 °C overnight. Antibody dilution solution, rather than the primary antibody, was used for the negative controls. After 10 min of complement application, goat anti-rabbit IgG antibody HRP-conjugate was used. Sections were rinsed three times in phosphate-buffered saline (PBS) in all steps of procedures except the protein block and primary antibody. The slides were incubated with DAB chromogen, counterstained with haematoxylin, and analysed with a light microscope (Topsakal et al., 2019).

The immunopositivity of each slide was examined, and a semiquantitative analysis was performed, as will be discussed in more detail below. Using an arbitrary visual scale with grading scores ranging from (−) to (+++), semiquantitative analysis was conducted to evaluate the strength of the immunohistochemical reactivity of tumour cells with markers as follows: When viewed under a microscope, (−) stands for a negative stain, (+) for a focal weak stain, (++) for a diffuse weak stain, and (+++) for a diffuse strong stain (Ozturk et al., 2018). A pathologist with specific training completed the scoring, and all sections were double-checked. For morphometric studies and microphotography, the Database Manual cellSens Life Science Imaging Software System (Olympus Co., Tokyo, Japan) was utilised.

ImageJ 1.48 was used to calculate and analyse the immunohistochemical scores of positive cells (National Institutes of Health, Bethesda MD).

Statistical analysis

SPSS (Statistical Package for Social Sciences) 22.0 (SPSS Inc., Chicago, Illinois, USA) was used for the statistical analysis. The data are displayed as mean ± SD. The ANOVA test was employed in the statistical analysis to evaluate whether there were any differences between the immunoscores of benign and malignant tumours and the control group. Duncan tests were additionally employed to compare continuous variables between groups. Statistics were considered significant at P values <0.05.

RESULTS

Based on the necropsy records it was noted that malignant tumours were bigger and irregularly formed, while benign tumours had lower diameters and were round to oval in shape. Malignant tumours usually exhibited cystic and necrotic areas.

Severe arterial hyperaemia was the most prevalent histological finding in all tumour cases. In benign tumours, haemorrhagic foci were infrequent. Although most benign tumours retained the morphology of acini and duct, some cases of benign mammary adenoma showed growth throughout the lumen. In these cases, the expanding mammary glands was found to be surrounded by thick fibrous connective tissue. Pleomorphic cells and mitotic figures were incredibly rare in the aggregate (Fig. 1). A dog was found to have ductal adenoma with papillary growth restricted to the ducts. The histology of benign mixed mammary tumours showed anaplasia and metaplasia of osseous and cartilaginous tissue, but no mitoses. Table 1 presents the Na^+/K^+-ATPase and BMP-2 expression scores as well as the histological categorisation of these tumours.

According to the histopathology of malignant mammary tumours, glandular epithelial cells often range in shape from flat to cubic. These cases frequently had abnormal cells, papillary expansions, and cystic formations. Although this varied from case to case, mitotic activity was generally fairly high. Furthermore, the majority of malignant mammary tumours displayed growth of fibrous tissue. Necrotic areas, particularly in the centres of the masses, were the most frequent microscopic finding in cancer cases. Neutrophil leukocytes and mononuclear cells are the main types of inflammatory cell infiltrates that have been discovered, especially in necrotic areas. Bone and cartilage metaplasia was prevalent in carcinosarcomas, and malignancy criteria were also present in these tissues. The typical histological features of all malignant cases were high mitotic activity, pleomorphic and anaplastic cells, and necrotic areas. Furthermore, the existence of metastases was the key indicator of malignancy.

This study examined the connections between Na^+/K^+-ATPase and BMP-2 expression and tumour type. The findings showed that Na^+/K^+-ATPase and BMP-2 were considerably expressed in malignant tumours but only slightly or moderately expressed in benign tumours. In benign cases, BMP-2 expression was either absent or minimal, whereas it greatly increased in malignant breast tumours. Additionally, distinct responses to the expressions of BMP-2 and Na^+/K^+-ATPase were observed in various

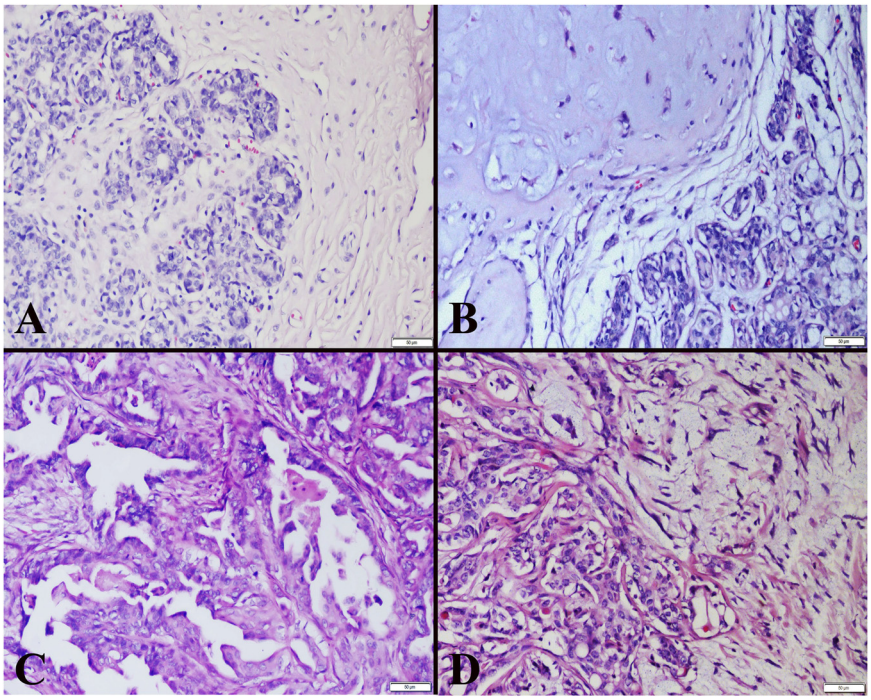

Fig. 1. Histopathological appearance of the tumours. (A) Mammary adenoma, (B) benign mixed tumour, (C) tubopapillary carcinoma, and (D) carcinosarcoma. Haematoxylin and eosin (HE), scale bars = 50 µm

Table 1. Na^+/K^+-ATPase and bone morphogenetic protein-2 (BMP-2) expression scores of the mammary tissue and of benign and malignant mammary tumours with pathological classification

No.	Tissue or diagnosis of the tumours	Na^+/K^+-ATPase	BMP-2
1–10	Normal mammary tissue	8 (−) and 2 (+)	7 (−) and 3 (+)
11–25	Benign mixed tumour	12 (+) and 3 (++)	3 (−), 11 (+) and 1 (++)
26	Intraductal papillary adenoma	1 (+)	1 (+)
27–34	Simple adenoma	7 (+) and 1 (++)	2 (−) and 6 (+)
35–37	Fibroadenoma	2 (+) and 1 (++)	3 (+)
38	Ductal papilloma	1 (+)	1 (+)
39–48	Solid carcinoma	4 (++) and 6 (+++)	1 (+), 6 (++) and 3 (+++)
49–60	Anaplastic carcinoma	3 (++) and 9 (+++)	4 (++) and 8 (+++)
61–72	Tubopapillary carcinoma	8 (++) and 4 (+++)	5 (++) and 7 (+++)
73–76	Anaplastic carcinoma	4 (+++)	2 (++) and 2 (+++)
77–80	Simple carcinoma	2 (++) and 2 (+++)	1 (++) and 3 (+++)
81–83	Tubular adenocarcinoma	3 (+++)	2 (++) and 1 (+++)
84	Lipid-rich carcinoma	1 (+++)	1 (++)

Data are given as number of cases and (scores).

regions of the same tumour mass. Na^+/K^+-ATPase and BMP-2 were discovered to be significantly expressed in areas where malignancy was more prevalent. Both parenchymal and microenvironmental cells had BMP-2 and Na^+/K^+-ATPase, and the combination was associated with malignancy. Expression in metastatic tumours was noticeable as compared to non-metastatic cases. The cells that expressed the highest levels of BMP-2 and Na^+/K^+-ATPase also exhibited very high mitotic activity.

Despite the presence of Na^+/K^+-ATPase expression in benign tumours, it was considerably upregulated in all malignant tumour types. Expressions were discovered in myoepithelial, stromal, and mammary gland cells (Fig. 2). In benign tumours, BMP-2 expression was found to be absent or very low, whereas it was found to be highly expressed in malignant tumours (Fig. 3). Both markers had very low or negative expression in the control mammary gland. The immunohistochemistry scores for the benign and malignant cases are statistically compared in Table 2. Graphs of the statistical scores for each type of tumour are displayed in Fig. 4. The tumour progression and pathogenetic mechanism are shown in Fig. 5.

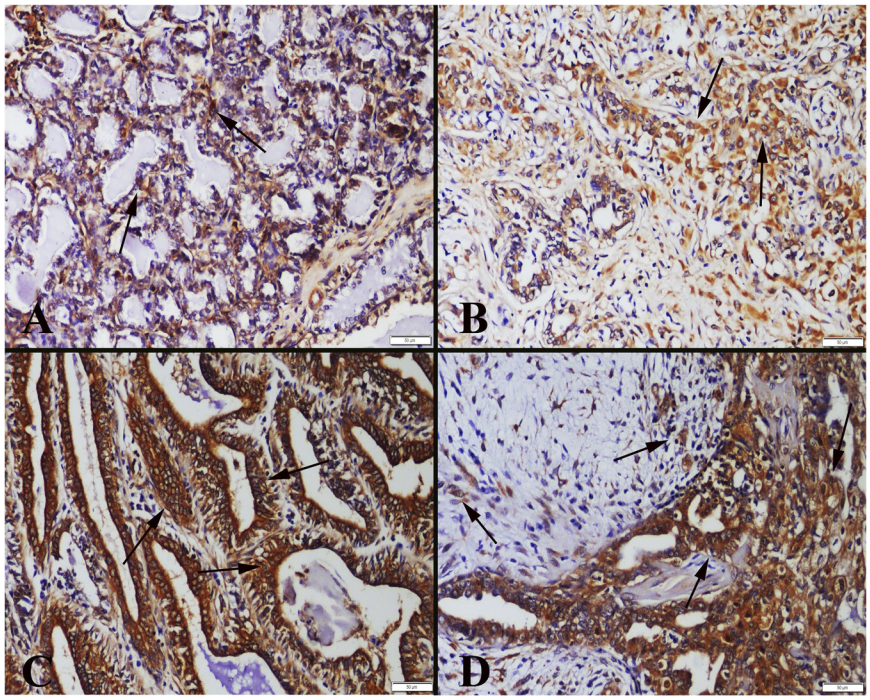

Fig. 2. Na^+/K^+-ATPase expressions of the tumour and microenvironmental cells. (A) Slight expression (arrows) in mammary adenoma, (B) slight expressions (arrows) in benign mixed tumour, (C) marked expressions (arrows) in tubopapillary carcinoma, and (D) increased expressions (arrows) in carcinosarcoma. Streptavidin–biotin peroxidase method, scale bars = 50 µm

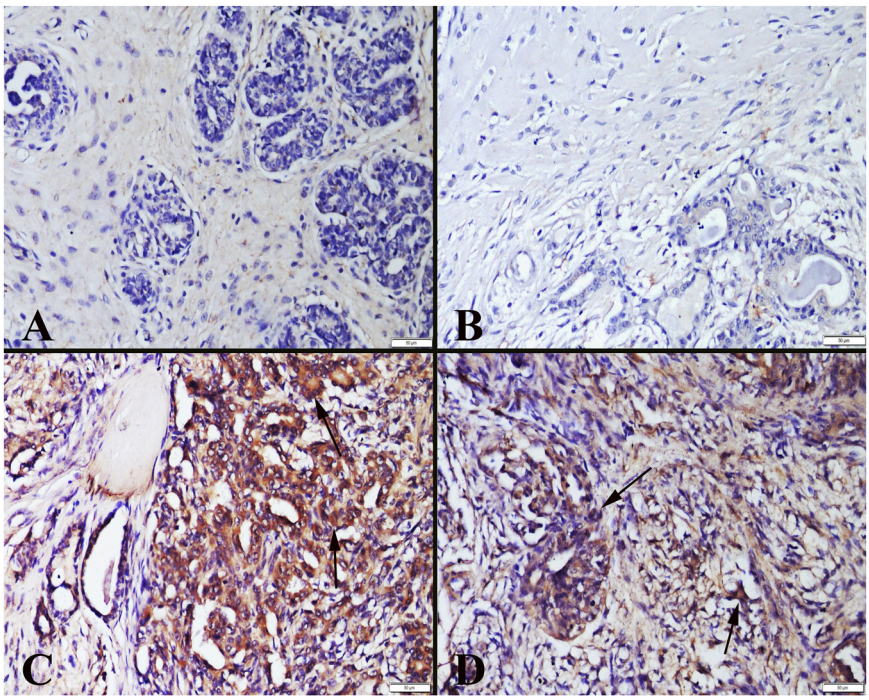

Fig. 3. Bone morphogenetic protein-2 (BMP-2) expressions of the tumour and microenvironmental cells. (A) Negative expression in mammary adenoma, (B) negative expressions in benign mixed tumour, (C) marked expressions (arrows) in simple carcinoma, and (D) increased expressions (arrows) in carcinosarcoma. Streptavidin–biotin peroxidase method, scale bars = 50 µm

Table 2. Statistical analysis results of Na^+/K^+-ATPase and BMP-2 expressions in benign and malignant mammary tumours

	Control	Benign	Malignant	*P* value
Na^+/K^+-ATPase	0.20 ± 0.13^a	1.17 ± 0.39^b	2.62 ± 0.48^c	<0.001
BMP-2	0.30 ± 0.15^a	0.85 ± 0.44^b	2.50 ± 0.54^c	<0.001

Values are presented as mean ± standard deviation. Data with different superscripts indicate significant differences from each other.

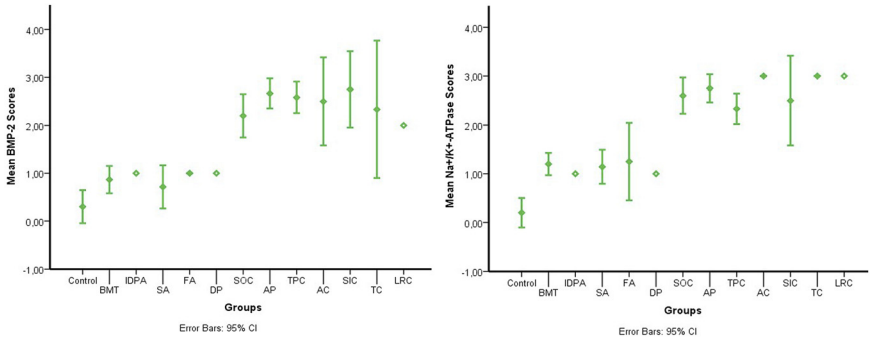

Fig. 4. Graphical representation of the statistical scores of the different tumour types. Normal mammary tissue (control), benign mixed tumour (BMT), intraductal papillary adenoma (IDPA), simple adenoma (SA), fibroadenoma (FA), ductal papilloma (DP), solid carcinoma (SOC), anaplastic carcinoma (AP), tubopapillary carcinoma (TPC), anaplastic carcinoma (AC), simple carcinoma (SIC), tubular adenocarcinoma (TC), lipid-rich carcinoma (LRC)

DISCUSSION

Since breast cancer has increased in prevalence in both humans and animals in recent years, it is now one of the main causes of death (Johnston et al., 2001; Ferlay et al., 2015). The development of biomarkers for the early diagnosis or treatment of breast cancer has thus been the subject of extensive investigation. New methods are being developed quickly to measure tissue and plasma levels for the detection of these biomarkers (Nerurkar et al., 1989; Baker, 2000; Diamandis and Yousef, 2002). Identifying the expression of Na^+/K^+-ATPase and BMP-2 in parenchymal

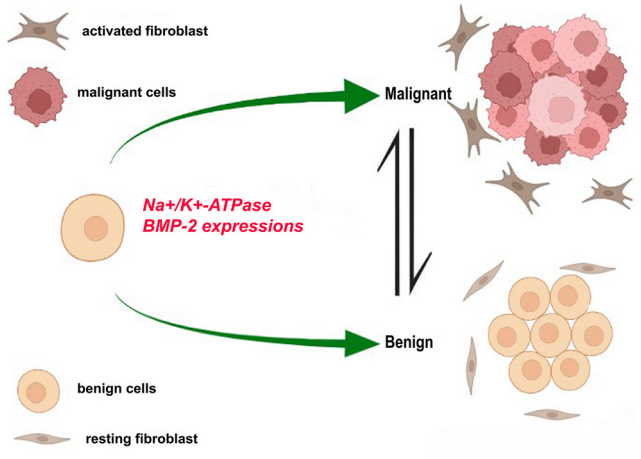

Fig. 5. Illustration of the pathogenetic mechanism of the expression and tumour progression

and microenvironmental cells in benign and malignant canine mammary tumours was the aim of this study, which also aimed to evaluate any prospective diagnostic candidates for these molecules.

A significant plasma membrane ion transporter with three different subunit types is called Na^+/K^+-ATPase (Geering, 1990). It is not surprising that the dysfunction or changed expression of Na^+/K^+-ATPase could be related to the pathophysiology of many diseases given the variety of physiological roles of Na^+/K^+-ATPase in different biological systems. As a result, Na^+/K^+-ATPase expression and activity have been reported to be changed in a variety of human malignancies. Na^+ rose intracellularly while K^+ decreased in several tumour cases. There may be some modifications to the intracellular Na^+ and K^+ balance in tumoural cells. Changes in the activity of the Na^+/K^+-ATPase pump may be the source of these modifications (Blok et al., 1999; Rajasekaran et al., 1999; Espineda et al., 2003). In recent studies, it has been reported that Na^+/K^+-ATPase, one of the main intracellular enzymes, may be a valid target in cancer treatment, and inhibitors of Na^+/K^+-ATPase can be a treatment option for cancer cells in non-toxic low nanomolar concentrations (Banerjee et al., 2018; Pucci et al., 2019; Bejček et al., 2021). Furthermore, only little information is available about how Na^+/K^+-ATPase activity is related to canine mammary cancers. Freeman et al. (2010) reported that Na^+/K^+-ATPase is more highly expressed in neoplastic cells compared to normal mammary epithelium in the dog. However, they did not examine the microenvironmental cells in their study (Freeman et al., 2010). Similar to that, our study found that malignancy was strongly associated with an increase in Na^+/K^+-ATPase expression in tumoural cells. Additionally, Na^+/K^+-ATPase was expressed by microenvironmental cells, and this expression was stronger in cases of cancer.

Growth factors are important for the development of tumours. Members of the transforming growth factor (TGF) superfamily, BMPs play a crucial role in embryonic development and tissue homeostasis by controlling apoptosis, motility, cellular differentiation, and proliferation (Nohe et al., 2004; Ye et al., 2009; Davis et al., 2016). According to Huang et al. (2017), BMP-2 induces epithelial-to-mesenchymal transition (EMT) and invasion of breast cancer cells in women's breast tumours. However, there are not many reports linking BMP-2 to canine mammary cancers (Wensman et al., 2009; Klopfleisch et al., 2010). Both of these earlier investigations solely looked at the parenchymal cells of tumours and no data were provided on the microenvironmental cells. The current study demonstrated a strong correlation between BMP-2 and canine mammary tumour malignancy. In this study, the parenchymal and microenvironmental cells of mammary tumours also showed very significant expression, with expression being substantially higher in cases of malignant tumours.

The significance of the tumour microenvironment has recently been emphasised in tumour studies. It is composed of a heterogeneous mixture of inflammatory cells surrounding the tumour cells and endogenous host stroma, and it plays a significant role in tumour progression (Bussard et al., 2016). In general, immunohistochemical studies of tumoural tissues solely looked at parenchymal expressions of the markers. However, stromal cells emit a range of pro-tumorigenic substances, and the microenvironment is crucial to the development of tumours. This study demonstrated that BMP-2 and Na^+/K^+-ATPase were also expressed by microenvironmental cells in canine mammary tumours. The expressions were substantially associated with malignancy, and BMP-2 and Na^+/K^+-ATPase were similarly expressed in both parenchymal and microenvironmental cells.

Studies on human breast tumours often involve either cell cultures or experimental animal models. Breast tumours in humans and dogs have been discovered to be closely related. As a result, scientists have started to analyse human breast tumours against canine mammary tumours. Numerous researches have revealed that various parameters can be adapted to women's breast and canine mammary tumours (Sultan and Ganaie, 2018). In terms of spontaneous tumour growth, homologous genome sequencing, genetic differences, coexistence and many other factors, human and canine malignancies are closely linked. Additionally, multiple studies have found many similarities, including the use of similar markers in evaluations and the presence of inflammatory responses in the tumour microenvironment (Queiroga et al., 2011; Carvalho et al., 2016). Recent years have seen a sharp rise in comparisons between human and canine breast cancers (Nerurkar et al., 1989; Sorenmo et al., 2009; Cassali, 2013; Ozmen, 2020). As a result, research on canine breast tumours is frequently interpreted in association with human tumours. The findings of this study also suggested that BMP-2 and Na+/K+-ATPase expressions may contribute to the aggressiveness of human breast tumours. In this study, we employed 10 normal canine mammary tissues, 46 malignant and 28 benign mammary tumours from the archives of the Department of Pathology. To begin with, all tumoural masses were re-evaluated for any possible misdiagnosis. After that, sections of each tumour

were immunohistochemically stained and examined for the expression of BMP-2 and Na^+/K^+-ATPase. The sections were also examined to see if there was a connection between malignancy and the release of BMP-2 and Na^+/K^+-ATPase from parenchymal and microenvironmental cells. Despite the low number of tumours evaluated in the study, it was one of the most significant investigations on the elevated expression of markers from parenchymal and tumoural microenvironmental cells.

The synergistic effects of BMP-2 and Na^+/K^+-ATPase, particularly on osteogenesis, have been described in recent years; however, it is unknown how these two markers work against malignancies. Tang et al. (2021) demonstrated the synergistic effects these two chemicals have on tumour growth. The results of the current study showed that the expression of Na^+/K^+-ATPase and BMP-2 differed considerably between benign and malignant tumours and significantly increased with malignancy in canine mammary tumours. These outcomes agreed with the findings of earlier research (Freeman et al., 2010; Kaszak et al., 2018). Microenvironmental cells also expressed these markers, although they were primarily expressed by tumoural parenchymal cells. It was not feasible to ascertain whether there was a correlation between Na^+/K^+-ATPase and BMP-2 expressions and survival time because the study was an archival investigation. However, a decreased survival rate in dogs may be related to higher Na^+/K^+-ATPase and BMP-2 expression in malignant tumours. On-going research is anticipated to clarify this issue. Further research should look at and correlate the plasma levels of these Na^+/K^+-ATPase and BMP-2 expressions with tissue levels.

In conclusion, despite extensive research on the biomarkers of mammary tumours in both humans and animals, it has not yet been possible to identify the optimal biomarker for early diagnosis or determining malignancy (Kaszak et al., 2018). It is becoming more and more obvious that the tumour microenvironment, which comprises a diverse array of tumoural cells and host stroma that supports the tumour cells, is essential for the development of the tumours (Bussard et al., 2016). The response of the tumoural stroma may be connected to the development and spread of the tumour. Canine and human breast tumours remarkably resemble each other (Hawai et al., 2013).

It is well recognised that the tumour microenvironment plays a crucial role in the growth and survival of the tumour cells. Numerous studies have focused on the expression of markers in tumour cells while ignoring the expression of markers in cells from the microenvironment. The notion that the microenvironment is significant in the therapy of tumours has been advanced in recent years (Chen et al., 2018; Chen and Song, 2019).

According to the results of the current investigation, tumour stromal reactions need to be checked for malignancy. According to this study, the microenvironment has a role in tumour malignancy and expresses markers that are linked to malignancy. Additionally, this study showed that increased Na^+/K^+-ATPase and BMP-2 expressions in canine mammary tumours are linked to malignancy and can be utilised as a screening tool for cancer. Additionally, the findings of this study suggest that BMP-2 and Na^+/K^+-ATPase may be used for the early diagnosis or prognosis of canine mammary tumours. Further studies are needed for determining the serum levels of these markers. Additionally, assessing the microenvironment may help with tumour diagnosis and effectiveness of the therapy.

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