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CXC Chemokine Ligand 12 and G Protein-Coupled Receptor 30 Expressions in Canine Mammary Tumors of Mixed Origin

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ABSTRACT

Canine mammary tumors (CMT) included complex interactions in the etiopathogenesis. It is the most common problem of neoplasia in female dogs. The aim of this study was to reveal the roles of chemokine CXC Ligand 12 (CXCL12) and G protein coupled receptor 30 (GPCR30) expressions in the inflammatory process and neoplastic development in canine mammary glands. Therefore, after clinical and pathomorphological evaluation, 18 cases of mixed origin malignant forms (mixed-type carcinoma-n=6, complex-type carcinoma-n=3, carcinosarcoma-n=7, carcinoma and malignant myoepithelioma-n=2)] were examined in the study. Inflammatory cells accompanying neoplastic changes, were determined to consist of predominantly neutrophils and leukocytes, followed by lymphocytes, plasma cells and macrophages. The CXCL12 and GPCR30 expressions were scored. immunohistochemically. Most of the expressions for both markers were moderate in the mammary gland and duct epithelial cells, myoepithelial cells and inflammatory cells. Fibrocytes and fibroblasts gave a mild reaction in general, and no reaction was found in the myxoid, chondroid and osteoid matrix. There was considered to be a close relationship between mixed composition CMT and subacute inflammation, and thus it was concluded that inflammtory cells may trigger or initiate neoplastic transformation in the cellular environment including differentiated cells of the mammary gland.
Key words: Chemoattractive cytokine, G protein, clinicopathology, mammary tumor, dog.

Mikst Orjinli Köpek Meme Tümörlerinde CXC Chemokine Ligand 12 ve G Protein Coupled Receptor 30 Ekspresyonları

ÖZ

Köpek meme tümörleri etiyopatogenezinde karmaşık bir seri etkileşimleri içerir. Dişi köpeklerde en yaygın karşılaşılan problemdir. Bu çalışmada, köpek meme tümörlerinde yangısal süreç ve tümör gelişimde bir çeşit chemokin olan chemokine CXC Ligand 12 (CXCL12) ile G protein coupled receptor 30 (GPCR30) ekspresyonlarının rollerini ortaya koymak amaçlandı. Bu amaçla, klinik ve patomorfolojik değerlendirmeden sonra, malignant formda mikst orjinli 18 olgu (mixed-type carcinoma-n=6, complex-type carcinoma-n=3, carcinosarcoma-n=7, carcinoma and malignant myoepithelioma-n=2) çalışmaya dahil edildi. Neoplazik değişikliklere eşlik eden yangısal hücreler ağırlıklı olarak nötrofil lökosit ve sırasıyla lenfosit, plazma hücresi ile makrofajlardan oluşuyordu. İmmunohistokimyasal olarak CXCL12 ve GPCR30 ekpsresyonları skorlandı. Her iki belirteçte ekspresyonların çoğu meme bezleri, duktal epitel hücreler, miyoepitel hücreleri ile yangısal hücrelerde orta şiddetteydi. Ancak, fibrosit ve fibroblastlarda genelde hafif şiddetteydi. Mikst orjinli köpek meme tümörleri ile subakut yangı arasında yakın bir ilişki olduğu düşünüldü. Ayrıca, yangısal hücrelerin meme bezlerinin farklılaşmasını içeren hücresel çevrenin bu mikst neoplazik dönüşümünü tetikleyebileceği veya başlatabileceği sonucuna varıldı.
Anahtar kelimeler: Şemoatraktif sitokin, G protein, klinikopatoloji, meme tümörü, köpek.

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INTRODUCTION

Canine mammary tumors (CMTs) are the most frequent neoplasms in intact female dogs (Egenvall et al., 2005). The tumors have an increasing incidence at approximately 6-7 years of age, although 10-11 years is also a potential risk for bitches (Priester, 1979; Boldizsar et al., 1992; Misdrop, 1996; Moe, 2001; Egenvall et al., 2005). It has been reported that increased age, progestagen treatment or intact status may be related to an increased risk of mammary neoplasia in bitches (Perez-Alenza et al., 2000). Nearly half of CMTs are diagnosed as malignant (Misdorp et al., 1999) and tumors with malignant transformation constitute almost half of all mammary tumors in dogs (Perez-Alenza et al., 2000).

The prediction of clinical behavior in malignant mammary tumors is difficult because of the heterogeneous form in pathological aspects of clinical behavior (Perez-Alenza et al., 2000). These tumours exhibit a complex histological pattern because they comprise elements from the epithelium and the mesenchyma and have the capacity to undergo malignant transformation, thereby resulting in mainly carcinomas and less frequently carcinosarcomas and sarcomas in mixed tumours (Misdrop et al., 1999; Cassali et al., 2011).
Chemokines are a superfamily of small molecule chemoattractive cytokines that regulate many cellular functions (Dewan et al., 2006). The CXC chemokine ligand (CXCL12) is expressed in a variety of cells, including stromal cells (fibroblasts and endothelial cells) (Muller et al., 2001; Salvucci et al., 2002). CXCL12 correlates with common sites of metastatic breast cancer (Crump et al., 1997). CXCL12 has linkage to CXCR4, which is known as a specific receptor for chemokines, and it activates several signal transductions in the cells. As a result of this mechanism, multiple effector molecules regulate cell survival, proliferation, chemotaxis, chemoinvasion, migration, and adhesion. In this regard, decreased stimulation in CXCR4 may create the development of neoplastic transformation and uncontrolled proliferation in the cells (Luker and Luker, 2006).

The biological effects of the chemokines are mediated by seven-transmembrane-domain receptors that constitute a subset of the G protein–coupled cell surface receptor (GPCR) superfamily (Zlotnik and Yoshie, 2000). Similar to the CXCL12-CXCR4 interaction, GPCRs, the largest family of cell-surface receptors, regulate cellular motility, growth and differentiation, all of which are understood to play an important role in the biology of cancer (Spiegelberg and Hamm, 2007). This regulation mechanism may trigger malignant transformation of mammary or breast cells (Li et al., 2005; Dorsam and Gutkind, 2007). In addition, GPCRs, which are thought to be one of the main mediators in the inflammation process, have a potential correlation between subacute and chronic inflammations and malignant transformation in the cells (Dorsam and Gutkind, 2007).

The aim of the present study was to investigate interactions between CXCL-12 and GRCR30 expressions in mixed origin CMTs. It is hoped that this research will contribute to a better understanding of the biology of mixed origin CMTs.

MATERIALS and METHOD

Animals

The study was performed according to the principles outlined in decision no. 2014/8 of the Ethics Committee of Animal Research of Turkey.

The study included a total of 48 female dogs with the complaints of mammary masses that were submitted to the Obstetrics and Gynaecology Clinics of the Veterinary Faculties of the University of Ankara and Ondokuz Mayis University. The ages of dogs ranged from 4 to 14 years. The dogs were of different breeds including mostly Terriers, German Shepherd, Pekingese, Cocker Spaniel, Kangal and mixed breeds. Clinical diagnosis was made of all the masses localizated on the left or right mammary lines. Permission was obtained from the owner of each dog and the affected mammary lobes or affected mammary lines including masses were removed by regional mastectomy, unilateral or bilateral complete mastectomy depending on the clinical stage and the number of tumours in the mammary lobes.

After the operation, the mammary tissues with suspected neoplasia were sent to the Department of Veterinary Pathology, Ankara University for diagnosis. In accordance with the aim of the present study, only 18 cases with suspected mixed neoplastic composition were included for evaluation, unlike a previous study (Anadol et al., 2017).

General Physical and Clinical Examinations

General physical examinations were performed routinly for all animals. In each case, all mammary glands and regional lymph nodes were examined and evaluated clinically. In addition, the number of tumours per animal, location, adherence to skin, adherence to underlying tissues, and tumour ulceration were recorded.

Macroscopic and Histopathological Examinations

The tissues were observed and evaluated on the basis of general macroscopic criteria. The tissues were fixed in 10% buffered formalin solution, and were then passed through degraded alcohol and xylol series in autotechnicon (Leica) and embedded in paraffin wax. Sections 5 μm in thickness were cut from the paraffin block and routinely stained with haematoxylin and eosin (H&E). After the histopathological analysis, the neoplastic changes in the mammary glands were evaluated according to the Goldschimidt et al. classification (2011).

Immunohistochemical Analysis

The strept-avidin-biotin complex peroxidase (Strept ABC-P) method was used to show CXCL12 and GPCR30 expressed cells. The sections on positive charged glass slides were deparaffinized and rehydrated by passing through xylene and degraded alchol series. Antigenic retrieval was performed using citrate buffer EDTA solution (pH=6.0) (Bioptica). The sections were kept for 20 minutes at 750 W. For retrieval of endogeneous peroxidase activity, the sections were treated with 3% H2O2-methanol solution for 5 minutes at room temperature. Non-specific protein activities in tissue were retrieved using blocking serum (Novocastra, Leica, RE7120-K). Primary antibodies (CXCL12 at 1/200 dilution, ABIN1585802, Antibodiesonline, , GPCR30 at 1/200 dilution, ab188607, Abcam) were droppered onto the sections and incubated at +4ºC overnight. Then biotinylated and Horse Radish Peroxidase marked sera were consecutively added and the sections were incubated for 45 minutes at 37°C. As chromogen, Diaminobenzidine (DAB) was added and left for 5 minutes. For counterstaining, Gill's haematoxylin was used. After passing through degraded alcohol and xylol series, the sections were mounted with a non-aqueous medium (Entellan). Until the DAB stage, the sections were washed in TBS with Tween 20 (TBST) for 1 minute twice after the end of each step except the protein blocking step. The findings were evaluated under light microscope (Leica DM4000) and visualised on the digitalized camera attachment.

Scoring of mitotic index and immunohistochemical expressions in canine mammary tissues

The mitotic index was calculated according to the Nottingham grading system in the Elston-Ellis modification of the original Scarff-Bloom-Richardson (SBR) grading system, which is popularly known as the contemporary European Breast Cancer Grading System (Simpson et al., 2000). In this scoring system, mitotic figures were counted histomorphologically by screening 10 microscopic areas at the periphery of the tumour at x400 magnification (10 High Power Fields-HPFs). Up to 9 mitoses per 10 fields were scored as 1 point, 10-19 mitoses 2 points, and more than 20 or more mitoses, 3 points. Immunohistochemical expressions were evaluated by counting the expressed cells at x400 magnification in 10 HPFs. The immunoexpressions were scored semiquantitatively as 0-10% (negative), 10-30% (mild positive), 30-70% (moderate positive), and 70-100% (strong positive).

RESULTS

General physical and clinical findings

In the physical examination, no problems were determined apart from the excessive masses in the mammary lobes. In the clinical examination, the unilateral / bilateral masses in the mammary lobes were generally not painful, but some showed painful characteristics due to ulceration and/or necrosis. During palpation of the masses, it was noticed that the affected lobes were warmer to the touch compared to the other mammary lobes. Some were fluctuant in consistency. On the radiographic examinations, there were no cases showing metastatic foci in any location (mainly regional lymph nodes and lungs).

Macroscopic and histopathological findings

Macroscopically, the masses were generally swollen with an elastic and sometimes fluctuant consistency. The dimensions and localizations are shown in Table-1. Cut sections were generally multilobular in appearance. In 5 samples, there was pus or mucoid substance discharge from cystic or cavernous areas in the cut sections. In general, the masses were yellowish-grayish white in color.

Histopathologically, complex-type carcinoma (n=3) included a malignant epithelial component and a benign myoepithelial component. The neoplasm was characterized by irregular tubules, sometimes necrosis and irregular bundles within a myxoid matrix consisting of myoepithelial cells within the interstitium. Mixed-type carcinoma (n=6) included a malignant epithelial component and benign mesenchymal component. The neoplasm was characterized by irregular tubules, myoepithelial cells and foci of cartilage and/or bone. Carcinosarcoma (n=7) included malignant epithelial cells and cells morphologically resembling connective tissue elements. Carcinoma and malignant myoepithelioma (n=2) included malignant epithelial cells and myoepithelial cells. There were varying degrees of mitotic activities in the cases: low mitotic index (10 malignant cases), moderate mitotic index (4 malignant cases) and finally high mitotic index (2 malignant cases). Inflammatory cells were found in 11 cases and no inflammatory cell infiltration into neoplasic areas

was determined in 7 cases. The composition of inflammatory cells was predominantly neutrophils, leukocytes, lymphocytes, plasma cells and macrophages, respectively (Figures 1-3).

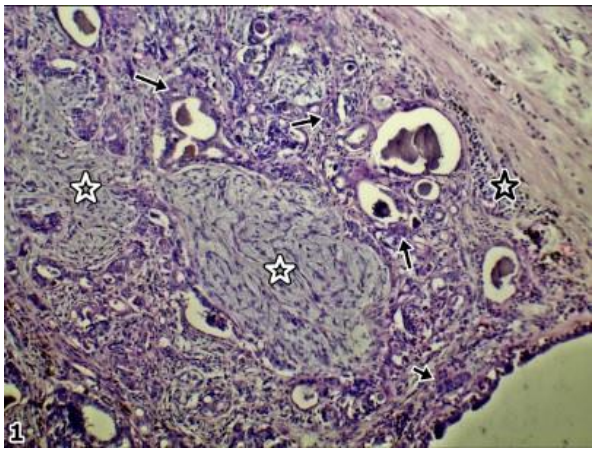

Figure-1. Anaplasic mammary gland cells (arrow), myoepithelial cells (black stars) and inflammatory cell infiltration (white stars), mixed type carcinoma, x100, H&E.

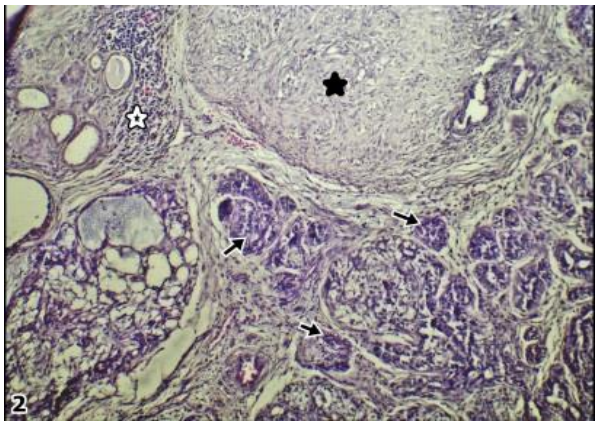

Figure-2. Anaplasic mammary gland cells (arrows), myxoid matrix (white stars) and inflammatory cell infiltration (black stars), complex type carcinoma, x100, H&E.

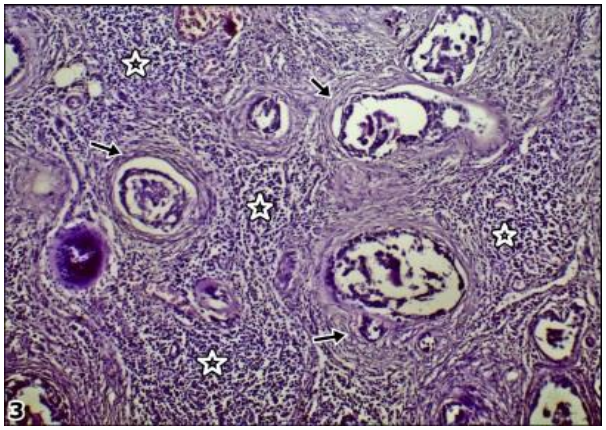

Figure-3. Anaplastic mammary gland cells and peripheral myoepithelial cell proliferation (arrows), inflammatory cell infiltration (white stars), carcinoma and malignant myoepithelioma, x100, H&E.

C-X-C motif chemokine 12 protein (CXCL12) and G Protein Coupled Receptor 30 (GPCR30) expressions

CXCL12

Epithelial cells were found positive mildly (n=7) and moderately (n=11), myoepithelial cells were expressed mildly (n=1) and moderately (n=15) and no reaction was seen in 2 cases. Fibrocytes and fibroblast expression were found at mild and moderate levels in 7 cases for each. The remaining 4 cases were negative. Inflammatory cells were found positive mildly (1 of cases), moderately (n=8) and strongly (n=2) and no expressions were determined in 7 cases (Figures 4-5).

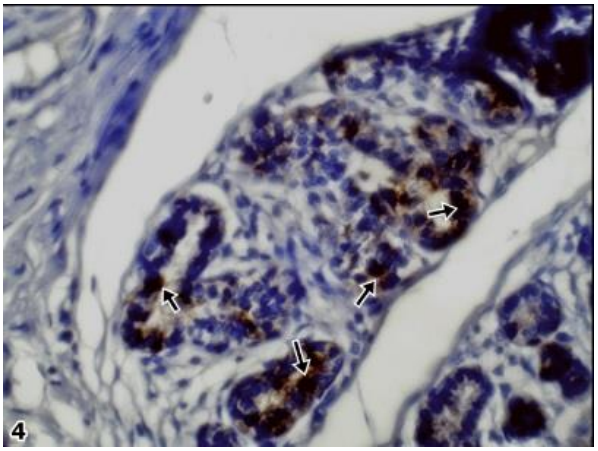

Figure-4. CXCL12 expressions in cytoplasm of anaplastic mammary gland cells (arrows), complex type carcinoma, x400, immunoperoxidase staining.

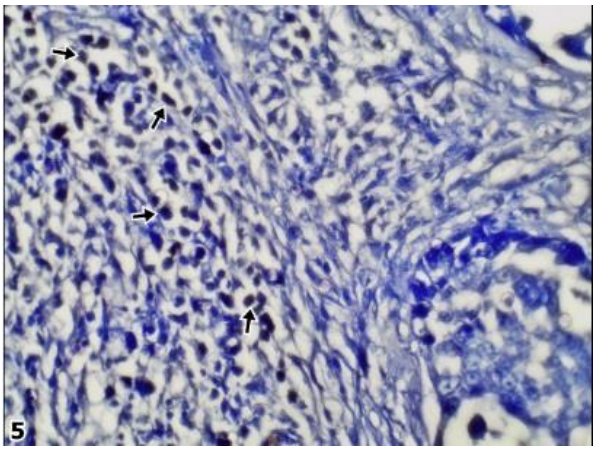

Figure-5. CXCL12 expressions in cytoplasm of mononuclear cells (arrows), complex type carcinoma, x400, immunoperoxidase staining.

GPCR30

Epithelial cells were found positive mildly (n=5), moderately (n=13), myoepithelial cells were expressed mildly (n=2) and moderately (n=16) and there was no reaction in 2 cases. Fibrocytes and fibroblast expression were found mildly (n=9) and moderately (n=3) with no reaction determined in 6 cases. Inflammatory cells were found positive moderately (n=11) and no expression in 7 cases

(Figures 6-8). The scored expressions are shown in Table-1.

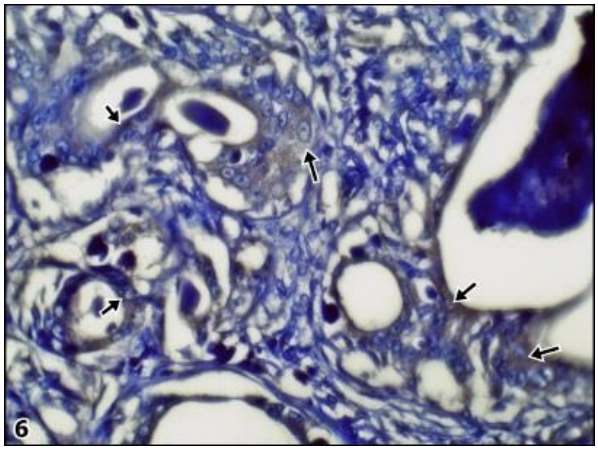

Figure-6. GPCR30 expressions in cytoplasm of anaplastic mammary gland cells (arrows), complex type carcinoma, x400, immunoperoxidase staining.

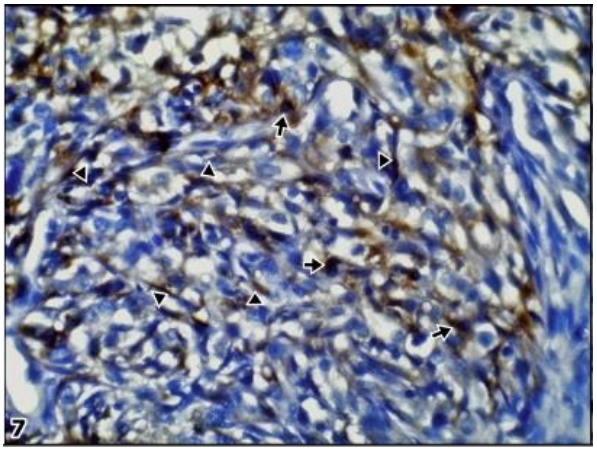

Figure-7. GPCR30 expressions in cytoplasm of malignant mesenchymal and myoepithelial cells (arrows), carcinosarcoma, x400, immunoperoxidase staining.

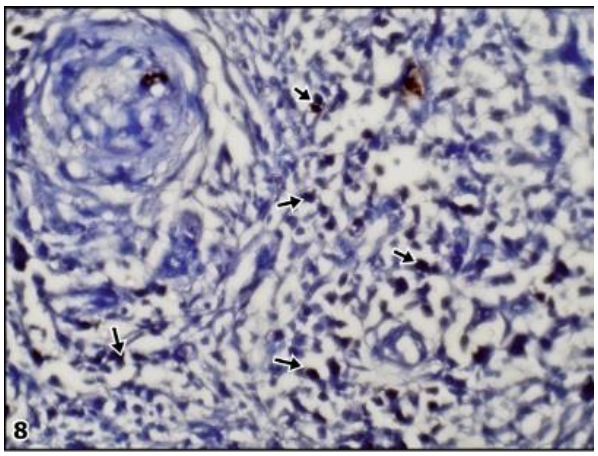

Figure-8. GPCR30 expressions in inflammatory cells (arrows), carcinosarcoma, x400, immunoperoxidase staining.

DISCUSSION

Previous studies on the etiopathogenesis of canine mammary tumours (CMT) have focussed on factors determining the malignant transformation of benign mixed tumours (Ramalho et al., 2006). In this malignant transformation, certain components of the extracellular matrix of the cellular environment participate in the process of malignant transformation (Cassali et al., 2011).

For many years, there have been different opinions regarding tumours of mixed composition, and they are not yet fully understood (Cassali et al., 2011). Some researchers have stated that mixed tumors originate from mesenchymal tissues including cartilage, bone and stromal connective tissue, while others have stated that the mesenchymal components originate from myoepithelial cells (Hurley et al., 1964; Pulley et al., 1973; Tateyama and Cothcin,1977). In both this study and a previous study by the current authors, evaluation was made of both mammary carcinoma and mixed tumors classified using the Goldschmidt et al. system (2011). According to the results obtained, malignant tumors of mixed composition exhibit malignancy in the epithelial cells compared to the preponderance of the mitotic index. Thus, the current study results showed great parallelism with previous studies.

In this study, a possible interaction was described between neoplastic mammary gland epithelia - mesenchymal cells in their micro-environment and inflammatory cells. In this context, increased expressions were observed of C-X-C motif chemokine 12 protein (CXCL12) and GPCR 30 or G-protein-coupled estrogen receptor-1 (GPER1), which are two members of the inflammatory mediator family, found in mixed composition benign and malignant tumors.

Table-1. Identities and mass localization in cases with Canine Mammary Tumor, histopathological diagnosis and immunohistochemical expression.

Case Number	Identification Age-breeds	Localization of masses and dimension	Histopathological Diagnosis	Mitotic index	CXCL12 expressions	G30 Protein expressions
1	Terrier 14 years old	Right abdominal cranial 2x2x4 cm	Carcinoma mixed type	1	E + My + Fib ++	E + My ++ Fib +
2	German Shephard dog 13 years old	Right abdominal cranial 1x0.5x1 cm, Left abdominal caudal 2x2x1 cm	Carcinosarcoma Lymphocyte and neutrophil leucocyte infiltration	2	E+ IC++ My++ Fib+	E+ IC++ My++ Fib+
3	Terrier 8 years old	Left abdominal cranial 3x2x1.5 cm	Carcinosarcoma Lymphocyte, plasma cell and neutrophil leucocyte infiltration	2	E + IC+++ My- Fib-	E ++ IC++ My++ Fib-
4	Mixed breed age not known	Left abdominal caudal 3x3x4 cm	Carcinosarcoma Lymphocyte and neutrophil leucocyte infiltration	2	E+ IC++ My++ Fib-	E++ IC++ My++ Fib++
5	Terrier 8 years old	Left abdominal caudal 2x2x2 cm	Carcinoma mixed type Lymphocyte infiltration	3	E ++ IC+++ My++ Fib-	E + IC++ My+ Fib-
6	Boxer 13 years old	Left abdominal cranial 3x2x4 cm	Carcinosarcoma Lymphocyte and neutrophil leucocyte infiltration	2	E++ IC++ My++ Fib-	E++ IC++ My++ Fib-
7	Spaniel Cooker 13 years old	Right abdominal cranial 3x4x3 cm	Carcinosarcoma	1	E+ My- Fib-	E+ My+ Fib-
8	Pekingese 14 years old	Left abdominal cranial 1x0.5x2 cm	Carcinoma mixed type	3	E ++ IC++ My++ Fib+	E ++ IC++ My++ Fib++
9	Mixed breed 10 years old	Right abdominal cranial 3x2x5 cm, Left abdominal caudal 2x2x3 cm	Carcinoma mixed type	2	E++ My++ Fib++	E++ My++ Fib++
10	Kangal 4 years old	Left inguinal 4x5.x1.3 cm	Carcinosarcoma Lymphocyte and neutrophil leucocyte infiltration	1	E++ IC++ My++ Fib++	E+ IC++ My++ Fib++
11	Terrier 12 years old	Right inguinal 2.5x1.7x1.5 cm	Carcinoma complex type Lymphocyte, plasma cell and neutrophil leucocyte infiltration	1	E+ IC++ My++ Flb++	E+ IC++ My++ Fib-
12	Mixed breed Age not known	Left abdominal caudal 3.5x1.8x1.9 cm	Carcinoma mixed type	1	E++ My++ Fib+	E++ My++ Fib+
13	Mixed breed 13 years old	Left inguinal 6x3x0.5 cm	Carcinoma complex type Plasma cell infiltration	2	E ++ IC+ My++ Fib-	E++ IC++ My++ Fib-
14	Mixed breed 12 years old	Left inguinal 8x6x4 cm	Carcinoma and malignant myoepithelioma	1	E++ My++ Fib+	E++ My++ Fib-
15	Mixed breed Age not known	Right abdominal caudal 7x5x3 cm	Carcinoma complex type	1	E++ My++ Fib-	E+ My++ Fib-
16	Mixed breed 12 years old	Right thoracal cranial 5x4x4.5 cm	Carcinosarcoma Neutrophil leucocyte infiltration	1	E+ IC++ My++ Fib++	E+ My++ Fib-
17	Mixed breed 6 years old	Left abdominal caudal 5x5.5x3.5 cm	Carcinoma and malignant myoepitelioma Lymphocyte infiltration	1	E++ IC++ My++ Fib++	E++ IC++ My++ Fib++
18	Mixed breed 8 years old	Right abdominal cranial 4x3.5x3.2 cm	Carcinoma mixed type Lymphocyte and neutrophil leucocyte infiltration	1	E++ My++ Fib++	E++ IC++ My++ Fib++

Mitotic index: 0 mitotic figure (o point), 1-9 mitotic figures (1 point), 10-19 mitotic figures (2 points), higher than 20 mitotic figures (3 points), E: Glandular and ductal epithelial cells, My: Myoepithelial cells, Fib: Fibrocytes and fibroblasts, IC: Inflammation cells, Scores of cells: + (mild positive), ++(moderate positive), +++ (strong positive)., Mitotik indeks: 0 mitotik figür (0 puan), 1-9 mitotik figür (1 puan), 10-19 mitotik figür (2 puan), 20'den fazla mitotik figür (3 puan)„ E: Bez ve akıtıcı kanal epitel hücreleri, My: miyoepitel hücreleri, Fib: Fibrosit ve fibroblastlar, IC: Yangı hücreleri, Hücrelerin skorlanması: + (hafif pozitif), ++ (orta şiddette pozitif), +++ (güçlü pozitif).

An important activator of neoplastic change is CXCL12, which is produced by inflammatory cells in the tumor micro-environment and may recruit other cancer cell types (Luker and Luker, 2006). CXCL12 induced-CXCR4 has been shown to be linked to components of the extracellular matrix (ECM). (Hartman et al., 2005). Therefore, migration of cells and interactions with extracellular matrix molecules such as matrix metalloproteinases (MMP) 2 and 9 make the formation of a mixed tumor much easier after the molecules have overexpressed CXCL12 (Kang et al., 2005). It has been reported that interaction in the cellular micro-environment can increase invasion and motility in the cells and enhance tumor malignancy (Boimel et al., 2012). In the present study, this increased cellular micro-environment activity was observed in several cases in which malignant tumors had mixed composition. It was thought that the CXCL12 produced by numerous inflammatory cells created a signal not only for the mammary gland and duct epithelium, but also for the extracellular matrix in the tumor micro-environment.

CXCR4 and its chemokine ligand CXCL12 are known to be members of the G protein coupled receptors family (GPCRs) (Muller et al., 2001). The GPCRs are the largest family of cell-surface molecules involved in signal transmission. They play a role in tumour growth and metastasis in particular. GPCRs fulfil these roles through expression in proliferating malignant cells. Conseuently, they make an important contribution to tissue remodelling, inflammation, angiogenesis, and cancer. In this context, there is a possible relationship between chronic inflammation and cancer similar to CXCL12 produced by inflammatory cells. In the signalling mechanism, G-protein-linked Prostoglandin E2 receptors trigger an alternative stimulation to cycloxygenase 2 (COX2). In this neoplastic progression, the pro-inflammatory function of COX is initiated (Dorsam and Gutkind, 2007; Feigin et al., 2014). A relationship between increased COX2 levels and high tumor malignancy was also reported in our previous study (Anadol et al., 2017). In that study, it was thought that CXCL12 might have a key role in triggering COX2 and subsequent neoplastic progression in the canine mammary gland after subacute and chronic inflammation.

On the other hand, GPCR30 or G-protein-coupled estrogen receptor-1 (GPER), which is another key factor in the GPCRs family, activates some signals for 17 beta-estradiol (Filardo et al., 2000). In this cascade, after 17beta-estradiol is bound to GPCR30, it causes a dissociation in the heterotrimeric G-protein complex. (Luttrel et al., 1999). Epidermal Growth Factors (EPGF) are activated and subsequently this situation enhances cellular proliferation (Maggiolini et al., 2004; Girgert et al., 2012). The relationship between GPCR30 and breast cancer have been previously reported (Girgert et al., 2012). In the light of the aforementioned findings, it was thought that GPCRs triggered neoplastic activity in both epithelial and mesenchymal cells through a dual mechanism after affecting prostaglandin and estradiol signals during the inflammation process.

In conclusion, both CXCL12 and GPCR30 can be considered to have a central role within the inflammation process of canine mammary glands. After expression in inflammatory cells, both may trigger a cascade of neoplastic transformation in the epithelium and the extracellular matrix as the micro-environment. As a result of this activation, the condition may result in malignant tumors in dogs. This study can be considered to shed light on the etiopathogenesis of mixed type tumors.

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