ORİJİNAL MAKALE / ORIGINAL ARTICLE

Balıkesir Sağlık Bilimleri Dergisi / BAUN Sağ Bil Derg
Balıkesir Health Sciences Journal / BAUN Health Sci J
ISSN: 2146-9601- e ISSN: 2147-2238
Doi: https://doi.org/10.53424/balikesirsbd.1382254

Immunohistochemical Characterisation of BMP-2, - 4, -7, TGF-β1 and Gremlin1 in Canine Osteosarcoma

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Geliş Tarihi / Received: *27.10.2023,* ***Kabul Tarihi / Accepted:*** *15.11.2023*

ABSTRACT

Objective: This study aims to evaluate the expression of Bone morphogenic proteins (BMP) -2, -4, -7, Transforming growth factor (TGF) -β1, and Gremlin1 in different subtypes of naturally occurring canine osteosarcoma (OS) by immunohistochemical method and contribute to a better understanding of the tumor microenvironment. **Materials and Methods:** Formalin-fixed, paraffin-embedded blocks of 16 naturally occurring canine OS were used. The tumors were classified according to the modified WHO's international histological classification of pet tumors. Compact bone tissues from five normal dogs were used as controls. **Results:** Immunohistochemically, BMP-2,-4, -7, TGF-β1, and Gremlin1 were not expressed in control tissues. BMP-2, -4, -7, TGF-β1, and Gremlin1 were expressed by undifferentiated mesenchymal cells and extracellular matrix in all OS subtypes. However, it was seen that there were differences in the expressions of these factors in different components of the tumor tissue. Although BMP-2, -4, -7, TGF-β1, and Gremlin1 have antagonistic effects in some pathways, they were co-expressed simultaneously in some regions in different OS subtypes. **Conclusion**: It was concluded that BMP-2, -4, -7, TGF-β1, and Gremlin1 could be expressed together in the same or different components of tumor tissues, and each can affect the behavior of tumor cells with their together or independent roles.

Keywords: BMP-2, BMP-4, BMP-7, Osteosarcoma, TGF-β1.

Köpek Ostesarkomunda BMP-2,- 4, -7, TGF-B1 ve Gremlin1'in İmmunohistokimyasal Karakterizasyonu

ÖZ

Amaç: Bu çalışmanın amacı, köpeklerde doğal yollarla oluşan osteosarkomun (OS) farklı alt tiplerinde Bone morphogenic proteins (BMP)-2, -4, -7, Transforming growth factor (TGF)-β1 ve Gremlin1 ekspresyonlarının immünohistokimyasal yöntemle değerlendirilmesi ve tümör mikroçevresinin daha iyi anlaşılmasına katkıda bulunmaktır. **Gereç ve Yöntem:** Doğal yollarla meydana gelen 16 köpek OS'u formalinde fikse edildi ve parafine gömüldü. Tümörler, WHO'nun modifiye uluslararası histolojik pet tümörleri sınıflandırmasına göre sınıflandırıldı. Kontrol için beş sağlıklı köpekten alınan kompakt kemik dokusu kullanıldı. **Bulgular:** İmmünohistokimyasal olarak, kontrol dokusunda BMP-2,-4, -7, TGF-β1 ve Gremlin1 ekspresyonu gözlenmedi. Tüm OS alt tiplerinde andiferensiye mezenşimal hücrelerde ve ekstraselüler matrikste BMP-2, -4, -7, TGF-β1 ve Gremlin1 ekspresyonu gözlendi. Bununla birlikte, tümör dokusunun farklı bileşenlerinde bu faktörlerin ekspresyonlarında farklılıklar olduğu görüldü. BMP-2, -4, -7, TGF-β1 ve Gremlin1'in bazı yolaklarda antagonistik etkileri olmasına rağmen, farklı OS alt tiplerinde bazı bölgelerde eş zamanlı olarak birlikte eksprese edildiği belirlendi. **Sonuç:** BMP-2, -4, -7, TGF-β1 ve Gremlin1'in tümör dokularının aynı veya farklı bileşenlerinde birlikte ifade edilebileceği ve her birinin birlikte veya bağımsız rolleriyle tümör hücrelerinin davranışını etkileyebileceği sonucuna varılmıştır.

Anahtar Kelimeler: BMP-2, BMP-4, BMP-7, Osteosarkom, TGF-β1.

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Bu makaleye atıf yapmak için / Cite this article: Kabak, Y. B., Inal, S., Sozmen M., & Gulbahar, M. Y. (2024). Immunohistochemical characterisation of BMP-2,- 4, -7, TGF- β1 and Gremlin1 in canine osteosarcoma. *BAUN Health Sci J, 13*(1), 75-82. https://doi.org/10.53424/balikesirsbd.1382254

INTRODUCTION

Osteosarcoma (OS) is a common primary malignant tumor of bone in dogs. About 85% of all canine bone tumors are malignant. It is frequent in large dog breeds. Although it is common among middle-aged dogs, it is seen in a broad age range. OS progresses rapidly and has a poor prognosis (Meuten, 2017).

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor (TGF) superfamily, except BMP-1, which is in the metalloproteinase family (Kessler et al., 1996). BMPs regulate bone and cartilage formation and repair, cell proliferation in the embryonic period, and regulation of bone homeostasis in the adult (Chen et al., 2004). BMP-2 stimulates the osteogenic differentiation of mesenchymal stem cells, initiates bone shaping and healing, and promotes the expression of other BMPs (Carreira et al., 2014; Park et al., 2009). BMP-4 is an osteochondrogenic factor and is essential for bone healing. BMP-7 stimulates cartilage and bone formation and plays a role in bone homeostasis and calcium regulation (Carreira et al., 2014).

Transforming growth factor-beta (TGF-β), belonging to the TGFβ superfamily, is involved in many essential physiological processes such as embryonic development, cell growth and differentiation, cell motility, extracellular matrix production, angiogenesis, apoptosis, and cellular immunity. The mammalian has three isoforms of TGF-β (β1, β2, and β3) (Haque & Morris, 2017). The role of TGF-β varies according to the stage of the tumor. It acts as a tumor suppressor by inhibiting cellular transformation in the early stage of tumorigenesis and preventing cancer progression. In the late period, it supports tumor development by facilitating epithelial-mesenchymal transformation, stimulating angiogenesis, immunosuppression, and metastasis (Haque & Morris, 20174; Wu et al., 2016).

Gremlin1 is a member of the DAN/Cerberus family, which is essential in regulating organogenesis and tissue differentiation. Gremlin1, a BMP-specific antagonist, exerts its antagonistic effect by preventing the binding of BMPs to extracellular BMPR-I and -II in the TGF-β signaling pathway (Gazzerro et al., 2005; Nguyen et al., 2014). Gremlin1, as a proangiogenic VEGFR2 agonist, has an angiogenic effect by binding to VEGFR2 in endothelial cells or by attaching directly to the surface of cancer cells (Kim et al., 2012; Mitola et al., 2010).

Canine OS is a good model for human OS, but some biological differences are likely (Kloen et al., 1997; Kubista et al., 2011). BMPs and TGF-β have been investigated in human and animal OS, but the results are highly controversial (Alfranca et al., 2015; Sulzbacher et al., 2002; Yoshikawa, Rettig, Lane et al., 1994; Yoshikawa, Rettig, Takaoka, et al., 1994). Moreover, there are few studies on Gremlin1 expression in human and canine osteosarcoma (Gu et al., 2019; Kim et al., 2012). Expressions of BMP-2, -4, and -7, TGF-β1, and Gremlin1 were not investigated together in canine OS. This study predicted that investigating the effects of BMP-2, -4, and -7, TGF-β1, and Gremlin1 on the development and behavior of OS in malignant bone tumors in dogs will contribute to the treatment protocols to be developed against OS.

MATERIALS AND METHODS

Tissue samples

Sixteen formalin-fixed paraffin-embedded canine OS tissues were used in this study. The samples used in the study are tissue blocks that came for diagnosis to Ondokuz Mayıs University, Faculty of Veterinary Medicine, Department of Pathology, between 2006 and 2017. Control tissues for each antibody and five normal bone tissues obtained from the dogs that had died of causes unrelated to tumor development were also used as healthy control tissues. Tumor and control tissues were fixed in 10% buffered formalin for 2 days and then decalcified in formic acid solution for approximately 4 weeks. After routine tissue follow-up, they were paraffin blocked. There is no information about whether it metastasizes to other tissues and what survival times are after OS diagnosis.

Microscopical examinations

Serial sections of 5 µ were taken from the blocks for H&E and immunohistochemical staining. The tumors were classified according to the modified WHO's international histological classification of pet tumors (Craig et al., 2016). Tumor tissues were graded according to the mitotic count, the degree of nuclear pleomorphism, and the percentage of necrosis, as described previously (Loukopoulos & Robinson, 2007). Two separate pathologists evaluated these OS.

Immunohistochemical examinations

Immunohistochemical staining was performed according to the manufacturer's protocol specified in the streptavidin-biotin-peroxidase kit (Ultravision Detection System, Thermo Scientific, Freemont, USA). After blocking the sections with proteinase K (Roche) and 3% hydrogen peroxide-methanol solution, protein blocking was performed. Sections were incubated in antibodies with BMP-2 (NBP1-1975, Novus Biologicals, 1:250), BMP-4 (LS-B3101, LSBio, 1:250), BMP-7 (bs-2242R, Bioss, 1:100), TGF-β1 (NB100-91995, Novus Biologicals, 1:100), and Gremlin1 (bs-1475R, Bioss, 1:250) at 4 °C overnight. As chromogen, 3-Amino-9-Ethylcarbazole (AEC substrate system, TA- 125-HA, Thermo Scientific, USA) was used. Counterstaining was performed with Mayer's hematoxylin.

The canine lung for BMP-2, the rat and canine kidney for BMP-4, and -7, the rat and canine spleen for TGF-β1, and the Gremlin1 canine small intestine tissues were used as positive controls. Sections were incubated with Phosphate-buffered saline (PBS) instead of the primary antibody as a negative control.

Evaluation of immunostaining

The immunostained area and staining intensity in tumor tissue in sections were evaluated at 200 final magnifications from a total of 10 different areas. Staining intensities were evaluated as 0, negative; 1, weak; 2, moderate; 3, strong. The immunostained area was determined by calculating the ratio of the stained areas to the total areas (0, negative; 1, < 25% low; 2, 26-75% moderate; 3, > 76% common). The immunoreactivity score (IRS) was obtained by multiplying the immunostaining intensity score by the immunostaining area score. IRS ranged from 0 to a maximum of 300.

Statistical analysis

All the data were expressed as median (minimum-maximum). Normal distribution analysis was not performed on the data as the number of samples within the groups was very small. It was assumed that all data did not show normal distribution. Multiple groups were compared using the Kruskal-Wallis test, while pairwise groups were evaluated by Mann-Whitney U tests with Bonferroni correction. Data were analyzed at the 95% confidence level, and a p-value < 0.05 was considered statistically significant. SPSS (Version 21.0) software was used for statistical analyses.

Ethical considerations

Since this study used tissues sent to our laboratory for diagnosis, ethics committee approval is not required.

RESULTS

Microscopic findings

Three of the OS included in the study were fibroblastic (FOS) (18.75%), four were chondroblastic (COS) (31.25%), five were productive osteoblastic (POOS) (25%), two were nonproductive osteoblastic OS (NPOOS) (12.5%), and two were determined giant cell-rich OS (GCROS) (%12.5). It was determined that 62.5% of the tumors were grade I, and 37.5% were grade II (Figure 1).

Immunohistochemical findings

In all OS subtypes examined in the study, undifferentiated mesenchymal cells were observed to express BMP-2, -4, and -7 cytoplasmically. The expression of BMP-2/ -4 in the nucleus of some of these cells was remarkable. BMP-2, -4, and -7 were also positive in the extracellular matrix surrounding these cells.

The osteoid matrix in POOS, similarly, the chondroid matrix and chondroblasts in COS gave an immunopositive reaction with BMP-2 (Figure 2A). In GCROS, giant cells, osteocytes in the trabeculae, and well-differentiated osteoblasts around the trabeculae did not express BMP-2. There was a statistically significant difference in BMP-2 antibody expression between the OS subtypes and the control group in the study ($P < 0.05$). Among the subtypes, only the difference between COS and NPOOS was significant ($P < 0.05$) (Table 1).

BMP-4 did not express the chondroid matrix in COS and osteoid matrix in POOS. However, some anaplastic osteoblasts and chondroblasts in these matrices had both cytoplasmic and nuclear expressions for BMP-4 (Figure 2B). In GCROS, giant cells were strongly immunopositive while some were not. Osteocytes in normal compact bone tissue were negative for BMP-4. Regarding BMP-4 antibody expression, the difference between OS subtypes and the control group and between POOS and GCROS were statistically significant ($P < 0.05$) (Table 1).

The osteoid matrix in osteoblastic OS was stained diffusely with BMP-7, the entire chondroid matrix and the peripheral regions of the bone trabecula were positive in COS. In addition, some anaplastic osteoblast, chondroblast, and giant cells exhibited cytoplasmic BMP-7 expression (Figure 2C). Osteocytes did not express BMP-7 in normal compact bone tissue. Regarding BMP-7 antibody expression, the difference between OS subtypes and the control group and between COS and GCROS were statistically significant ($P < 0.05$) (Table 1).

TGF-β1 expression was detected in all OS subtypes except GCROS. In general, TGF- β1 was found to be expressed cytoplasmically in anaplastic osteoblastic and chondroblastic cells as well as other undifferentiated malignant mesenchymal cells. It was also expressed in the extracellular matrix in these regions. In POOS, TGF-β1 expression was more intense in the peripheral regions of the osteoid matrix, especially in the regions where osteoblasts were localized. Similarly, in COS, the peripheral regions of the chondroid matrix were immunoreacted, while the centers of the osteoid and chondroid matrix did not express TGF-β1 (Figure 2D). However, TGF-β1 expression was negative in normal compact bone tissue used as a control. Except for GCROS, the difference in TGF-β1 antibody expression was statistically significant between the other subtypes and the control group ($P < 0.05$). The difference between COS, and POOS, NPOOS, GCROS ($P < 0.05$) and the difference between POS and COS and GCROS were statistically significant ($P < 0.05$) (Table 1).

All NPOOS, one POOS, and one GCROS sample of OS subtypes included in the study did not express Gremlin1. Gremlin1 was expressed in the cytoplasm of undifferentiated malignant mesenchymal cells, anaplastic osteoblastic and chondroblastic cells. It was also weakly expressed in the outer border of the osteoid matrix with some regions of the extracellular matrix in tumor tissue. Compact bone tissue used as control did not express Gremlin1 (Figure 3A-B). Statistics could not be made due to the small number of samples stained for the Gremlin1 antibody (Table 1).

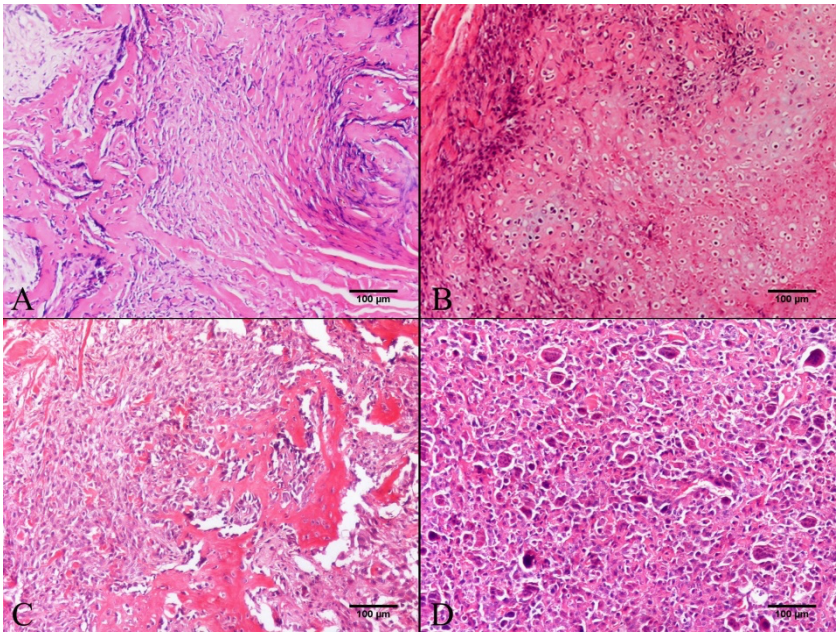

Figure 1. H&E histochemical staining results in primer canine OS. (A) FOS, (B) COS, (C) POOS, (D) GCROS. H&E, Bar=100 µm.

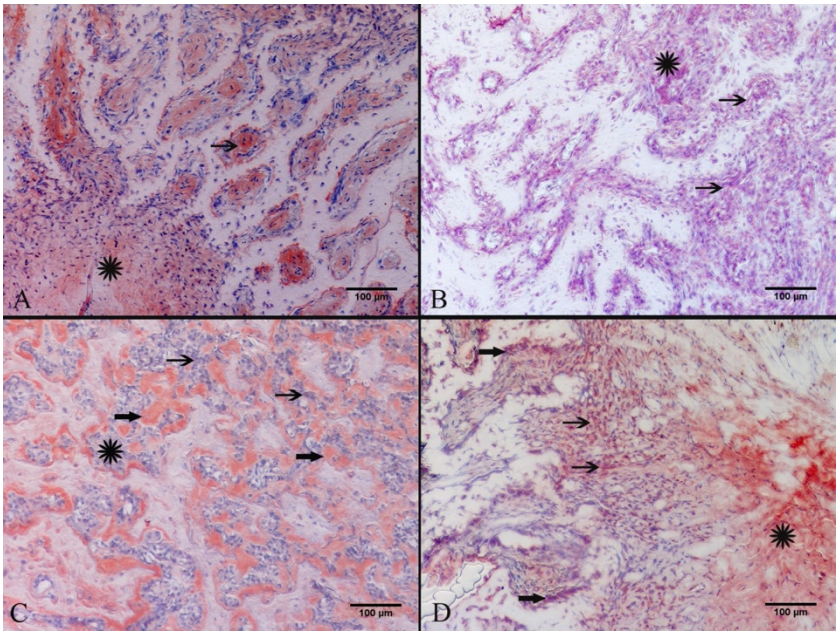

Figure 2. Immunohistochemical staining results for the BMP-2, BMP-4, BMP-7, and TGF-β1in primer canine OS. (A) BMP-2 expression in malignant mesenchymal cells (thin arrow) and the surrounding extracellular matrix (*) in FOS. (B) BMP4 expression in malignant mesenchymal cells and the surrounding extracellular matrix (*) and malignant mesenchymal cells (thin arrows) in FOS. (C) BMP-7 expression in malignant mesenchymal cells (thin arrows), the surrounding extracellular matrix (*), and the peripheral regions of the osteoid matrix (thick arrows) in COS. (D) TGF-β1 expression in malignant mesenchymal cells (thin arrows), the extracellular matrix (*), and the anaplastic osteoblastic cells (thick arrows) surrounding the bone trabecula in FOS. All microphotographs on the plate IHC and Bar=100 µm.

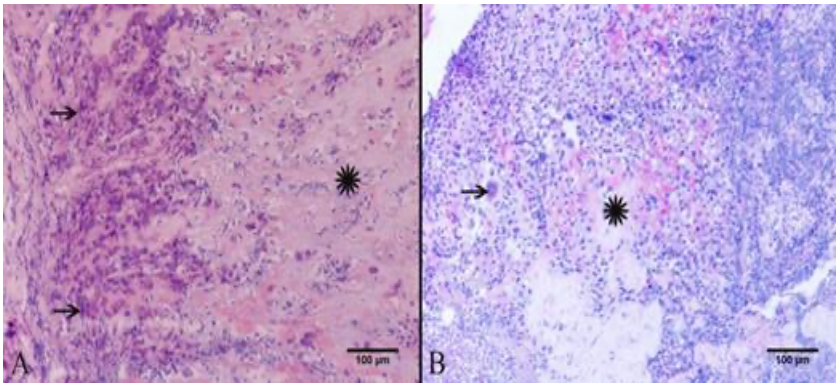

Figure 3. Immunohistochemical staining results for the Gremlin1 in primer canine OS. (A) Gremlin1 expression in chondroblastic cells (thin arrows) and the extracellular matrix (*) in COS. (B) Gremlin1 expression in extracellular matrix (*), and giant cell (thin arrow) in GCROS. All microphotographs on the plate IHC and Bar=100 µm.

Table 1. IRS for antibodies in subtypes of OS*.

	n	BMP-2	BMP-4	BMP-7	TGF-β1	Gremlin1
FOS	3	6 (6-9)[a]	5 (5-6)[a]	6 (4-6)[a]	2 (2-4)[a]	2
COS	4	6 (6-9)[c,e]	5.5 (5-7)[e]	7.5 (6-9)[b,e]	4 (4-6)[b,c,d,e]	3 (2-3)
POOS	5	6 (4-6)[g]	6 (5-7)[f,g]	4 (2-6)[b,g]	2 (1-2)[b,f,g]	2 (0-3)
NPOOS	2	4[c,h]	5[h]	3.5 (3-4)[h]	2[c,h]	0
GCROS	2	7.5 (6-9)[i]	4[f,i]	5 (4-6)[i]	0[d,f]	1 (0-2)
Control	5	0[a,e,g,h,i]	0[a,e,g,h,i]	0[a,e,g,h,i]	0[a,e,g,h]	0

*The data is presented as median (minimum-maximum). Mann-Whitney U test used. [a]The difference between FOS and Control is significant ($p < 0.05$). [b]The difference between COS and POOS is significant ($p < 0.05$). [c]The difference between COS and NPOOS is significant ($p < 0.05$). [d]The difference between COS and GCRO is significant ($p < 0.05$). [e]The difference between COS and Control is significant ($p < 0.05$). [f]The difference between POOS and GCROS is significant ($p < 0.05$). [g]The difference between POOS and Control is significant ($p < 0.05$). [h]The difference between NPOS and Control is significant ($p < 0.05$). [i]The difference between GCROS and Control is significant ($p < 0.05$)

DISCUSSION

Osteosarcoma has a complex cell heterogeneity and an abnormally produced immature osteoid matrix and arises as a result of poorly defined oncogenic events in this complex environment. Recent studies support that the bone microenvironment underlies OS initiation and progression (Corre et al., 2020). There are inconsistencies in the information regarding the expression of BMPs in OS. It has been reported in a human study that COS does not express BMP-2/4 (Yoshikawa, Rettig, Takaoka, et al., 1994). In one human study, all OS subtypes, including COS, were reported to express BMPs. This study reported that BMP-7 was expressed at the highest level in osteoblastic OS (Sulzbacher et al., 2002). Another human OS study using gene expression analysis reported that the BMP7 gene was expressed in osteoblastic OS at quite a different level than in non-osteoblastic OS (Kubista et al., 2011). We found that all OS subtypes examined in our study expressed BMP-2, -4, and -7 antibodies.

BMP-2/-4 expression is not at the same level at all stages of mesenchymal development, and there is no need for continued BMP-2/-4 expression after mesenchymal differentiation (Yoshikawa, Rettig, Takaoka, et al., 1994). Yoshiawa et al. showed that BMP-2/-4 are expressed in the cytoplasm of undifferentiated mesenchymal cells in most human OS (Yoshikawa, Rettig, Lane, et al., 1994; Yoshikawa, Rettig, Takaoka, et al., 1994). In our study, it was observed that BMP-2, -4, and -7 were expressed in the cytoplasm of undifferentiated mesenchymal cells, but BMP-2 and -4 were also expressed in the nuclei of some of these cells. A study on human OS reported that BMP-2/4 was not expressed in normal bone, osteoid and chondroid matrix and has little or no expression in osteoblastic and chondroblastic cells (Yoshikawa, Rettig, Lane, et al., 1994). However, osteoid and chondroid matrices in our study expressed BMP-2 and -7. Moreover, neoplastic osteoblasts and chondroblasts were also immunopositive for BMP-2, and -7. Although BMP-4 was not defined in the chondroid and osteoid matrix, some neoplastic chondroblasts and

osteoblasts at the periphery of these components showed immunoreaction for BMP-4. Sulzbacher et al. reported that BMP-2, -4, and -7 were expressed by neoplastic cells in human OS, similar to our findings (Sulzbacher et al., 2002). It is known that BMP-2 has a stimulating effect on the osteogenic differentiation of normal mesenchymal stem cells and also promotes the expression of other BMPs (Carreira et al., 2014; Park et al., 2009). BMP-4, and -7 is also an osteochondrogenic factor that promotes osteoblastic differentiation of mesenchymal stem cells (Carreira et al., 2014). The results we obtained in this study suggest that BMP-2, -4, and -7 expressions may contribute to the development of bone tumors.

In our study, BMP expression in the extracellular matrix, especially in the surrounding areas of undifferentiated mesenchymal cells, was noted in all OS subtypes. Complex events that occur during the development of bone tumors cause the release of some bone matrix growth factors such as BMPs, which promotes tumor cell proliferation and further bone resorption. This facilitates the movement and metastasis of tumor cells (Alfranca et al., 2015).

Aggressive clinical behaviors of the high-grade OS are associated with highly expressed TGF-β1 (Franchi et al., 1998). Nguyen et al. showed that TGF-β1 expression is higher in high-grade OS than in low-grade OS (Nguyen et al., 2014). Consistent with the literature, in our study, TGF-β1 expression was higher in grade II OS samples than in low-grade ones. In addition, COS expressed the highest TGF-β1 levels; these tumors were grade II, while two giant cell-rich OS samples that did not express TGF-β1 were grade I.

Franchi et al. reported that the chondroid matrix in the human OS does not express TGF-β1 (Franchi et al., 1998). In our study, peripheral regions of the osteoid, and chondroid matrix, neoplastic osteoblasts, and chondroblasts in tumor tissues expressed TGF-β1. In addition, undifferentiated mesenchymal cells, were also TGF-β1 immunopositive as noted in some other studies (Franchi et al., 1998; Kloen et al., 1997; Zhang et al., 2013). Zhang et al. reported that when the TGF-β1 signaling pathway was inhibited in human OS in vitro, OS cells could not form colonies and their differentiation properties were interrupted (Zhang et al., 2013). Cytokines and growth factors such as TGF-β1 produced by tumor cells facilitate tumor progression by disrupting the balance between bone resorption and bone formation (Lamora et al., 2016). Our results support Verrecchia and Reddini's thesis that this microenvironment expressing TGF-β1 in OS may indicate poor prognosis in primary bone tumors to promote angiogenesis, bone remodeling and cell migration (Verrecchia & Rédini, 2018).

Gremlin1 has been studied in certain human tumors (Karagiannis et al., 2015; Namkoong et al., 2006; Sato et al., 2016; Sneddon et al., 2006). In osteosarcoma, studies are few and results are inconsistent (Gu et al., 2019; Kim et al., 2012). A study investigating the tumor microenvironment showed that, unlike the stroma of normal tissues, Gremlin1 is expressed in the stroma of many carcinomas, thus providing a favorable microenvironment for the survival and spread of cancer cells (Sneddon et al., 2006). Sato et al. reported that high mRNA expression of Gremlin1 in human cervical cancer was significantly correlated with tumor size (Sato et al., 2016). Another human colon cancer study reported that Gremlin1 is secreted from cancer-associated fibroblasts and its expression is mainly localized at the invasion site (Karagiannis et al., 2015). A study with A549 cell culture suggested that Gremlin1 interacts directly with neoplastic cells independently of BMP and VEGFR2, triggering cell migration, invasion, and proliferation (Kim et al., 2012). Unlike other work, Gu et al. reported that the Gremlin1 gene and protein are significantly downregulated in different OS cell lines and their ability to proliferate and invade OS cells is reduced when they artificially upregulate Gremlin1 (Gu et al., 2019). Accordingly, they suggested that the metastasis ability of OS would be inhibited in the presence of Gremlin1 and that Gremlin1 is a marker showing a good prognosis (Gu et al., 2019). Gremlin1 was noted to be expressed in COS samples and tumor samples classified as grade II. Our results, Kim et al. (2012) confirmed the results. However, it should not be forgotten that tumor grade has no prognostic significance according to studies conducted in recent years (Schott et al., 2018).

CONCLUSION

In our study, BMP-2, -4, -7, TGF-β1, and Gremlin1 were expressed at the highest level in COS. FOS and osteoblastic osteosarcomas followed this. All of these proteins were expressed together in some components of the tumor tissue, especially in the undifferentiated mesenchymal cells and the extracellular matrix surrounding these cells. The fact that these proteins, which have opposing effects in some pathways, are expressed in the same regions and even by the same cells in different OS subtypes suggested that there may be other mechanisms other than those currently known.

Therefore, it was concluded that the tumor microenvironment is important in tumor development, invasion, and metastasis in different OS subtypes; further research on this subject is needed to predict tumor behavior and develop new treatment approaches.

Acknowledgement

The authors would like to extend their sincere thanks to anyone who contributed to this study.

A part of this study was previously presented as an oral presentation with the title "Immunohistochemical detection of gremlin, BMP-2, -7, and TGF-β1 in the naturally occurring canine osteosarcomas" at the 5th International Vet-Istanbul

Group Congress held in Ohrid, R. of Macedonia, 23-27 September 2018.

Conflict of Interest

The author declares no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

Author Contributions

Plan, design: YBK, MS; **Material, methods and data collection:** YBK, MS, SI; **Data analysis and comments:** YBK, MS, SI; **Writing and corrections:** YBK, MS, SI, MYG.

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