Kafkas Univ Vet Fak Derg
19 (Suppl-A): A237-A242, 2013
DOI: 10.9775/kvfd.2013.8653

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

CASE REPORT

Granulosa Theca Cell Tumor in An Arabian Mare: Are Immunohistochemically Loss of GDF-9 and BMP-6 Proteins Associated with High GATA-4, Inhibin-α, AMH Expressions?

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Makale Kodu (Article Code): KVFD-2013-8653

Summary

Granulosa-theca cell tumor (GTCT) in an Arabian mare was diagnosed by clinical, pathomorphological and immunohistochemical (IHC) examinations. Immunohistochemically, it was tried to clarify the possible roles of Transforming Growth Factor–β superfamily members [Growth Differentiation Factor-9 (GDF-9), Bone Morphogenetic Protein-6 (BMP-6), anti-Müllerian Hormone (AMH) and inhibin-α], GATA family members (GATA-4 and GATA-6) and Insulin-like Growth Factor family (IGF-1 and IGF-2) on GTCT and results was compared with two negative control ovarian tissues. Moderate positivities with AMH, inhibin-α, IGF-2, GATA-4, and mild positivities with IGF-1, GATA-6 were obtained whereas no positivity could be shown with GDF-9 and BMP-6 in neoplastic ovarian tissue. Additionally, mild positivities were obtained with all markers in control stainings. Further molecular studies for transcription factors regulating activation of genes in response to mitogenic and stress signals in equine GTCT tumorigenesis are needed to elucidate whether the high GATA-4, AMH, and inhibin-α immunopositivities play a significant role on GDF-9 and BMP-6 deficiency.

Keywords: *Mare, Granulosa-Theca Cell Tumor, GATA-4, GATA-6, AMH, Inhibin, GDF-9, BMP-6, Immunohistochemistry*

Arap Kısrakta Granuloza-Teka Hücre Tümörü: İmmunohistokimyasal Olarak GDF-9 ve BMP-6 Protein Kayıpları Yüksek GATA-4, İnhibin-α ve AMH Ekspresyonları İle İlişkili mi?

Özet

Bir Arap kısrağında granuloza-teka hücre tümörü (GTHT); klinik, patomorfolojik ve immunohistokimyasal (İHK) incelemelerle teşhis edildi. İmmunohistokimyasal olarak GTHT'de; Transforming Growth factor-β ailesi [GDF-9 Growth Differentiation Factor-9 (GDF-9), Bone Morphogenetic Protein-6 (BMP-6), anti-Müllerian Hormone (AMH) ve inhibin-α], GATA ailesi (GATA-4 ve GATA-6) ve Insulin-like Growth Factor ailesi (IGF-1 and IGF-2) üyelerinin olası rolleri ortaya konmaya çalışıldı ve bulgular iki negatif kontrol ovaryum dokusuyla karşılaştırıldı. Neoplastik ovaryum dokusunda AMH, inhibin-α, IGF-2, GATA-4 orta derecede, IGF-1, GATA-6 ise hafif derecede pozitif sonuç verirken GDF-9 ve BMP-6 pozitiflik göstermedi. Ayrıca kontrol grubu dokuların boyamalarında tüm belirteçler ile orta derecede pozitif sonuçlar elde edildi. Kısraklarda GTHT tümörogenezisinde yüksek GATA-4, AMH ve İnhibin-α düzeylerinin, GDF-9 ve BMP-6 eksikliğindeki önemli rolünü belirlemede mitojenik ve stres sinyallerine yanıttaki genlerin aktivasyonundan sorumlu transkripsiyon faktörlerinin de yeni moleküler çalışmalar ile incelenmesi gerekliliğini ortaya koymuştur.

Anahtar sözcükler: *Kısrak, Granuloza-Teka Hücre Tümörü, GATA-4, GATA-6, AMH, İnhibin, GDF-9, BMP-6, İmmunohistokimya*

INTRODUCTION

Granulosa-theca cell tumor (GTCT) is one of the most common ovarian neoplasm found in the mare [1-4] and represents 2.5% [5,6] to 5.6% [3,7] of equine neoplasms. GTCT is a steroidogenic tumor in domestic animals [1,3,4,8,9] which

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originates from the sex cord or specialized ovarian stroma [6]. Diagnosis is based upon history, findings on clinical examinations, macroscopical and microscopical evaluations of excised tissue [4,7].

Transforming growth factor-β (TGF-β) superfamily members are extracellular signaling molecules which regulates biological processes as cellular growth, differentiation, motility, and apoptosis. TGF-βs are subdivided into TGF-βs/Activins/Nodals, and Bone Morphogenetic Proteins (BMPs)/Growth Differentiation Factors (GDFs)/Anti-Müllerian Hormone (AMH) or Müllerian Inhibitory Substance (MIS) [10]. TGF-β superfamily members have important efficacy in intra-ovarian molecular interactions. These proteins play active roles in primordial follicle development, granulosa and theca cell proliferation, and follicular atresia, development of gonadotropin receptors in somatic cells, oocyte maturation, ovulation, luteinization, and corpus luteum formation [11,12].

Inhibins are proteins produced by gonads which act in an endocrine manner to inhibit follicle stimulating hormone (FSH) synthesis and secretion from pituitary [13]. Subtype inhibin-α is expressed from most of GTCTs and is used as a marker as AMH [14].

GATA family (GATA-1 to-6) is a member of zinc finger transcription factors, and GATA-1,-2,-4,-5, and -6 have been implicated to reproductive development or function in mammals [15,16]. Expression of some essential gonadal genes is regulated by transcription factors GATA-4 and GATA-6 [14].

Insulin-like growth factors (IGFs) are peptides which are related to proinsulin. IGFs regulate mitosis, differentiation, and growth as well as survival of cells when FSH concentrations are declining or low. IGFs regulate follicular growth and also promote the actions of gonadotropins on ovary. IGF-I plays important role in folliculogenesis. IGF-II stimulates thecal cell steroidogenesis, thereby determines the ability of androgen synthesis of thecal cells [17].

Although the molecular pathogenesis of GTCT in mares have not been fully understood, defects in ovarian and intrafollicular paracrine/autocrine signaling pathways have been considered to be important. However, the influence of AMH and inhibin-α on equine GTCT have been studied recently [18] as in human medicine, the effects of other growth and differentiation factors on tumoral pathobiology are not clear. Therefore, the aim of this study was to search the availability of immunolabeling some TGF-β superfamily, IGF, and GATA family members (GDF-9, BMP-6, AMH, inhibin-α, GATA-4, GATA-6, IGF-1, IGF-2) and to discuss the possible role of these proteins in pathogenesis of GTCT in mares as a first report.

CASE HISTORY

An 8-year-old, non-parity Arabian mare was referred to Equine Hospital with complaints of anestrus, lameness, and aggressive behaviour. On rectal examination, the right ovary was identified as a rough mass; approximately 15 cm in diameter, the left ovary was detected to be firm and inactive. Transrectal ultrasonographic examination revealed irregularly shaped multicystic areas with a honeycomb appearance. The left ovary confirmed to be small and inactive. A presumptive diagnosis of GTCT was made. Ovariectomy via a right ventral, diagonal paramedian approach was performed under general anaesthesia. The gross and histopathological examinations of the tumor were performed after removal of affected ovary.

Two healthy ovaries were used as negative controls in immunohistochemical evaluations. Control ovaries were obtained from normally cycling but humanely euthanized mares due to incurable emergency conditions in accordance with the decision of authorized veterinarian.

Materials were evaluated macroscopically and tissue samples taken from ovary were fixed in 10% neutral buffered formalin. Tissue samples were evaluated with routine tissue process and embedded in paraffin. Tissue sections were stained with Hematoxylin-Eosin (H&E) and IHC.

For immunohistochemistry, Avidin-Biotin Complex Peroxidase (ABC-P) was applicated to tissue sections. The deparaffinized and dehydrated sections were kept for 3 min in microwave 700 W with 0.1 M citrate buffer as antigen retrieval solution, and hold in 3% hydrogen peroxide-methanol solution for 20 min. After blocking with non-specific blocking sera (DAKO, X1010) at 4°C overnight, the sections were incubated with polyclonal primary sera [GDF-9, BMP-6, AMH, inhibin-α, IGF-I, IGF-II, GATA-4, GATA-6, *(Table 1)*] at the same conditions and time. After washing with PBS, the sections were incubated for 45 min with biotinylated goat anti-rabbit antibodies at room temperature. After washing with PBS again, the immune complexes were detected by secondary antibodies marked with horseradish peroxidase (HRP) (DAKO LSAB+System HRP- kit, cat. no: K0679). DAB chromogen for revealing the reaction (Cat no. RE7105 and RE7106, Novocastra Lab.), and Mayer's haematoxylin for counter staining was used. Negative control sections were treated as described above by being exempted from primary antibodies. All results were evaluated under light microscope (Leica, DM 4000B) and imagined by camera attachment (Leica, DFC-420). Positivities which obtained from all primary sera were counted on 10 different microscopic fields at x100 magnification. The immunopositivities were scored as: 0 (no reaction), 1+ (mild), 2+ (moderate) and 3+ (strong).

The diagnosis of GTCT was confirmed by macroscopical and histopathological examinations of the excised tissue. Macroscopically, the ovary was weighed of 827 g. and measured 18x11x8.5 cm in diameter. Its consistency was generally firmness but some areas was fluctuated. Its surface was roughly, well vascularizated and tan yellow coloured. On cut surface, there were come across multiple

Table 1. *Antibody panel of primary sera*
Tablo 1. *Temel serumların antikor tablosu*

Primary sera	Optimal Dilution	Catolog Codes
Polyclonal goat anti mouse GDF-9	1:100	Santa Cruz Biotechnology, C-20, sc-7407
Polyclonal goat anti mouse BMP-6	1:100	Santa Cruz Biotechnology, S-20, sc-27408
Polyclonal goat anti mouse AMH	1:100	Santa Cruz Biotechnology, C-20, sc-6886
Polyclonal goat human inhibin-α	1:100	Santa Cruz Biotechnology, T-17, sc-22048
Polyclonal goat anti mouse IGF-I	1:100	Santa Cruz Biotechnology, G-17, sc-1422
Polyclonal goat anti mouse IGF-II	1:100	Santa Cruz Biotechnology, N-20, sc-1415
Polyclonal goat anti mouse GATA-4	1:100	Santa Cruz Biotechnology, G-4, sc-1237
Polyclonal goat anti mouse GATA-6	1:100	Santa Cruz Biotechnology, G-6, sc-7244

fluctuated cyst, seized from 0.3 mm to 2 cm in diameter and filled with yellowish fluid. Histopathologically, sections were generally composed of neoplastic granulosa cells with hyperchromatic nuclei and polygonal shape. These neoplastic cells were surrounded by compact fibrous stroma, contained also theca cells.

Immunohistochemically, AMH and inhibin-α were more dense in cytoplasms of neoplastic granulosa cells *(Fig. 1-a,-b)*. Furthermore, IGF-2 and GATA-4 with moderate positiveness (> 50%/10 microscope areas) were also observed in both granulosa and theca cells *(Fig. 1-d,-e)*. However, it was not attended to any positive reaction with GDF-9 and BMP-6 in neoplastic cells, mild positivities (20-50%/10 microscope areas) with IGF-I and GATA-6 markers were detected in neoplastic granulosa and theca cells, respectively *(Fig. 1-c,-f)*.

In negative control sections, mild positivities (20-50% /10 microscope areas) were obtained from whole antibodies which mentioned above in follicular epithelial cells, thecal cells and stromal components in few areas.

DISCUSSION

The oocyte plays an important role in growth and differentiation of the follicle and in directing its own fate [19]. Gene expression profiles of GTCTs in women found to be similar to that of granulosa cells of preantral and small/medium antral follicles [14]. This situation may be similar in another monovular species, in mares too.

Among BMPs, BMP-6 is demonstrated to be strongly expressed in granulosa cells of tertiary follicles and oocytes in human. BMP-6 is found to stimulate gene expression of inhibin/activin βA and βB subunits but not inhibin-α subunit in cultured human granulosa cells. BMP-6 is also found to stimulate mRNA expression of FSH receptor and AMH. Follicles with high BMP-6 expression may be more likely to survive FSH decrease in serum and to reach dominant follicle stage [20]. GDF-9 is an oocyte-derived factor which plays important roles in primordial follicle recruitment, granulosa and theca cell proliferation/atresia, steroidogenesis, oocyte maturation, ovulation, luteinization, and corpus luteum formation [19]. GDF-9 promotes follicular growth and synergistically inhibits FSH-induced granulosa cell differentiation in primates, ruminants and rodents. It is also demonstrated that follicular development was improved when GDF-9 function was upgraded, whereas ovarian dysfunctional follicular abnormalities leading to infertility were detected when GDF-9 expression was reduced [21].

In the case, expressions of BMP-6 and GDF-9 in GTCT cell lines were not observed in contrast to positivities in controls. It was thought that the ovary quietly progressed in the way of neoplastic changes, and tumor cells were not under effects of growth and differentiation stages of follicles. As BMP-6 is present in healthy tertiary follicles and GTCTs are mostly consisted of small and multicystic follicles, it may be expected to show low immunopositivity. Deficiency in GDF-9 may play a role in tumoral development in this case because of emerging abnormalities on granulosa and theca cells in its insufficiency.

In recent studies, immunolabeling for AMH was not detected in small, primordial follicles with a single layer of flattened granulosa cells, but was detected in follicles consisted of granulosa cells with more than one layer in normal equine ovaries. AMH immunolabeling increased in antral follicles with multiple layers of granulosa cells. The intensity of AMH immunolabeling was decreased in antral follicles with diameter >30 mm compared to small antral follicles. Follicles undergoing atresia had a slight AMH immunolabel, while atretic follicles without granulosa layer did not. Corpora lutea also did not show AMH immunolabeling [18,22].

In this case, moderate positivities with AMH were detected in neoplastic granulosa cells. The result obtained from GTCT with small and multiple cystic structures were compatible with the abundant expressions of AMH in healthy preantral and small antral follicles. This similarity is thought to be related with size and number of follicles. Ball et al.[18] reported that AMH immunostaining may be a useful tool for detection of GTCT in mares because of high AMH expression characteristics of such tumors. Elevation

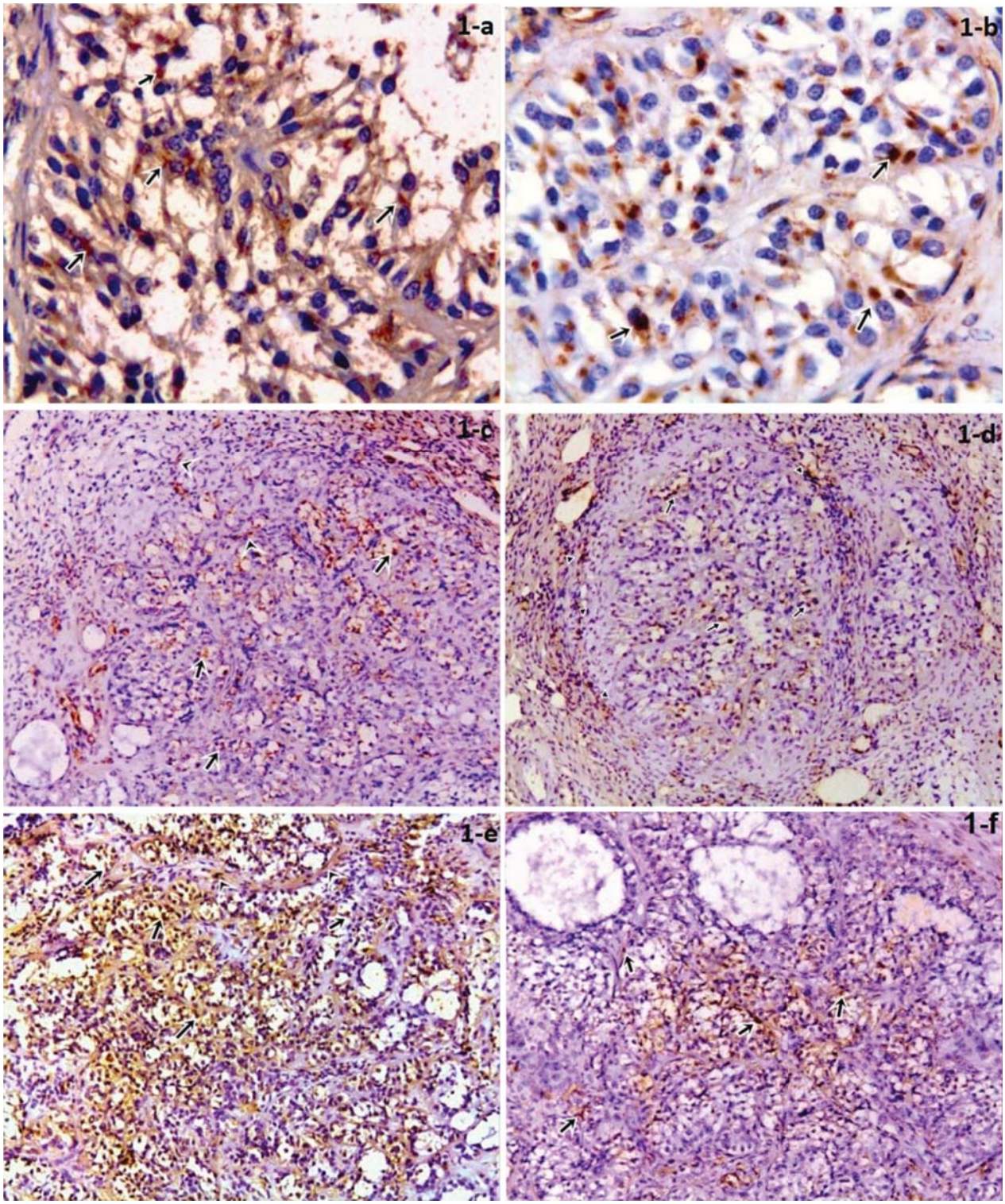

Fig 1. Immunohistochemical stainings with AMH, Inhibin-α, IGF-1, IGF-2, GATA-4 and GATA-6, respectively, **a-** Moderate positiveness with AMH in cytoplasm of neoplastic granulosa cells (arrows), ABC-P method, x400, **b-** Moderate positiveness with Inhibin-α in cytoplasms of neoplastic granulosa cells *(arrows)*, ABC-P method, x400, **c-** Mild positiveness with IGF-1 in cytoplasms of neoplastic granulosa cells *(arrows)* and spindle shaped cells *(arrowheads)*, ABC-P method, x40, **d-** Moderate positiveness with IGF-2 in cytoplasms of neoplastic granulosa cells *(arrows)* and theca interna and externa cells (arrowheads), ABC-P method, x40, **e-** Moderate positiveness with GATA-4 in both cytoplasms of neoplastic cells *(arrows)* and some thecal cell *(arrowheads)*, ABC-P method x40, **f-** Mild positiveness with GATA-6 in cytoplasms of neoplastic thecal cells *(arrows)*, ABC-P method x40

Şekil 1. AMH, Inhibin-α, IGF-1, IGF-2, GATA-4 ve GATA-6 ile immunohistokimyasal boyamalar, **a-** Neoplastik granuloza hücre sitoplazmasında AMH ile orta derecede pozitiflik (oklar), ABC-P method, x400, **b-** Neoplastik granuloza hücre sitoplazmasında inhibin-α ile orta derecede pozitiflik *(oklar)*, ABC-P method, x400, **c-** Neoplastik granuloza hücre sitoplazmasında IGF-1 ile hafif derecede pozitiflik *(oklar)* ve iğ şekilli hücreler *(ok başları)* ABC-P method, x40, **d-** Neoplastik granuloza hücre sitoplazmasında IGF-2 ile orta derecede pozitiflik *(oklar)*, teka interna ve teka eksterna hücrelerinde *(ok başları)*, ABC-P method, x40, **e-** GATA-4 ile neoplastic hücre sitoplazmasında *(oklar)* ve bazı teka hücrelerinde orta derecede pozitiflik *(ok başları)*, ABC-P method x40, **f-** Neoplastic teka hücre sitoplazmasında GATA-6 ile hafif derecede pozitiflik *(oklar)*, ABC-P method x40

of serum AMH concentrations are also specific for GTCT, therefore serum AMH analysis may be used to monitor progression of tumor and to assess postoperative recurrence in mares as well as in women.

Inhibins play important roles in folliculogenesis by influencing granulosa cell maturation and proliferation, oocyte maturation and steroid hormone production. Lack of inhibin production is associated with several ovarian diseases, furthermore increased inhibin-α levels are detected in ovarian cancers [23].

The moderate positivity with inhibin-α versus undetermined positivity with GDF-9 and BMP-6 may be conceived as the attenuating effect of inhibin-α on GDF-9 and BMP-6 in this case of GTCT. Inhibins are capable of binding type-2 activin and BMP receptors through their β-subunits acted via type-3 TGF-β receptor. Therefore, high inhibin levels can antagonize the actions of activins, BMPs and potentially other TGF-β superfamily members [13].

Inhibin-α is a tumor suppressing gene in mice and deleting of this gene results in aggressive granulosa/sertoli cell tumors, but in contrast with mice, granulosa cell tumors in women are associated with high levels of inhibin in circulation which is thought to occur due to a loss in responsiveness to inhibins by tumor cells [13]. Inhibin-α is also present with high concentrations in both serum and ovarian tissue samples of mares with GTCT. Thus, inhibin-α is used as a marker for GTCT in mares as well as in women. Additionally, Ball et al.[18] found AMH expression was correlated with serum inhibin concentration in a study of equine GTCT. This knowledge supports the immunohistochemical findings of intensive positivity on neoplastic granulosa cells with AMH and inhibin-α markers in our case.

GATA-4 is associated with gonadal sex differentiation in embryo, and both GATA-4 and -6 play role in gonadal development. In adult gonads, GATA-4 and GATA-6 get involved in steroidogenesis [15]. Overexpressed GATA-4 may activate gene promoter of AMH [15] and inhibin-α [24]. In adult mice ovary, GATA-4 is expressed by healthy granulosa cells, some thecal cells, and interstitial cells. GATA-6 is detected in granulosa cells, and strongly expressed in corpora lutea [15]. GATA-4 expression is found to be high in human GTCTs [14]. Therefore, this characteristic expression conceives GATA-4 to be a potential prognostic tool for GTCTs. It is suggested that GATA-4 plays a role in granulosa cell differentiation via Cyclin D2 promoter [14]. In our case, the obtained reactions were also in this manner. Especially, GATA-4 was expressed moderately in granulosa and theca cells while mild reaction with GATA-6 was encountered. Accordingly, GATA-4 is found to have more prognostic value for GTCT diagnosis in mares when compared to GATA-6.

As well as intracellular GATA-4 phosphorylation is achieved by protein kinase A (PKA) pathway under effects of gonadotropins, it can occur by mitogen activated protein kinase (MAPK) signaling pathway under influences of stress and mitogenic effects [25]. Additionally, adrenal gland tumors were observed in mice due to high gonadotropic effects following gonadectomy [26]. High simultaneous expressions of LH receptors, GATA-4, and inhibin-α were detected in these tumors [24,26].

IGF-1 has significant tasks in antral follicles such as granulosa cell proliferation, estradiol, progesteron production and inhibin-A, activin-A, follistatin secretion by granulosa cells, oocyte viability, oocyte maturation, follicle dominance, multiple ovulation, and also increase in follicle sensitivity to gonadotrophin and to LH-R in granulosa and theca cells in mammals [27]. Mild positivity with IGF-1 in neoplastic ovarian tissue in our case may be a sign of IGF-1 inhibition that results in follicular development failure which is followed by formation of small cystic structures.

IGF-2 plays important roles in initiating primordial follicle growth, granulosa cell proliferation in secondary and antral follicles, progesteron and estradiol production by granulosa cells, and stimulating steroidogenesis by thecal cells in antral follicles [27]. Predictably, IGF-2 concentrations may remain constant or be increased when androgen synthesis from thecal cells, and aggressive and/or stallion-like behaviours in mares with GTCT are taken into account. The obtained moderate positivity with IGF-2 may be expressed in this context.

In the case, it was thought that there is a close relationship between inhibin-α and IGF-2 (due to their moderate staining) when evaluated as regards to initiation of follicle growth and granulosa cell proliferation. In this context, these two factors may trigger possible primary effect in the way of neoplastic changes as to be understood from literature knowledge.

In conclusion, AMH and inhibin-α showed the highest immunopositivity in neoplastic granulosa cells among other markers and proved their characteristic expression in equine GTCT. GATA-4 also showed high positivity characteristically in both neoplastic granulosa and thecal cells whereas any positivities could not be observed with BMP-6 and GDF-9. According to our results AMH, inhibin-α, and GATA-4 are all have a role as diagnostic tools for equine GTCT. When considering all these findings, the markers play a role in granulosa theca cell tumorigenesis in the case. It may be usefull for diagnosis and evaluating prognosis if any positivities were obtained from these markers in this type of GTCT cases. Then, these markers can properly use as diagnostic tool. Further study on these factors will have important implications for our understanding the tumorigenesis of the most common ovarian neoplasia in mares.

Acknowledgement

The authors are grateful to Turkish Equestrian Club for supplying the study materials.

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