(Original Research)

Localization of anti-Müllerian hormone and its receptor in granulosa cell tumors of thoroughbred mares

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Abstract

Granulosa cell tumor (GCT) is a sex cord stromal tumor in mares and causes infertility. The objective of this study was to localize anti-Müllerian hormone (AMH) and its receptor, anti-Müllerian hormone receptor type 2 (AMHR2), in ovarian tissue samples obtained from eleven Thoroughbred mares diagnosed with GCT. Immunohistochemistry (IHC) revealed positive immunostaining for both AMH and AMHR2 in the granulosa-like cells of GCT. Furthermore, double immunofluorescence staining revealed the presence of co-localization of AMH and AMHR2 in granulosa-like cells of GCT in mares. These findings suggest that granulosa-like cells of GCT are a target of AMH, indicating AMH may have paracrine and autocrine function on granulosa-like cells of GCT in mares. Moreover, this study is the first to show the co-localization of AMH and its receptor, AMHR2, in GCT-affected ovaries of Thoroughbred mares by using double immunofluorescence staining.

Key word: anti-Müllerian hormone; anti-Müllerian hormone receptor type 2; granulosa cell tumor; mare; immunohistochemistry

1. Introduction

Granulosa cell tumors (GCT) are the most common tumor which affect ovarian tissue in mares [1, 2]. Mares with GCT show abnormal clinical signs including unilateral ovarian enlargement, stallion-like behavior, anestrus or persistent estrus behavior [3]. In addition, mares with GCT may become infertile due to the suppressed activity of the contralateral normal ovary, and if GCT are ablated by surgery, the normal ovary will become active, with recovery from infertility [3, 4].

In most cases, veterinary practitioners diagnose equine GCT based on history and rectal examination with ultrasonography which often reveals a polycystic or honeycomb appearance in GCT affected ovaries of mares [3]. However, the gross appearance and therefore the ultrasonographic appearance of GCT varies and may not fit the classically described honeycomb appearance on ultrasonography. Therefore, endocrine diagnostic aids such as inhibin, testosterone or anti-Mullerian hormone determination may also be used.

Currently, plasma anti-Müllerian hormone (AMH) concentrations can be used as a potential biomarker for diagnosing GCT in mares [5-7]. It was previously reported that median AMH concentrations were 72.6 ng/mL in mares with GCT versus 0.70 ng/mL in mares with other ovarian abnormalities [8].

AMH, a homodimeric glycoprotein, is a member of transforming growth factor-ß family. During organogenesis, secretion of AMH from male gonadal tissue is important for blocking Müllerian duct growth, thereby preventing development of the female tubular tract in the male fetus [9].

In mares, AMH is secreted from granulosa cells of GCT and growing and antral follicles in normal ovaries [7]. In female animals, AMH is responsible for inhibition of follicular recruitment and sensitivity of growing follicles to FSH [10]. However, the

potential role of AMH in equine GCT is still unclear. Almeida et al (2011) revealed the AMH and its receptor – AMHR2 in granulosa components of GCT ovary in mares by using immunohistochemistry (IHC) [5].

In order to check if there is paracrine and autocrine activity of AMH in equine GCT, we aimed to investigate the co-localization of AMH and AMHR2 in equine GCT by using IHC and double immunofluorescence staining in the current study.

2. Materials and Methods

2.1. Sampling

Ovarian samples were taken from 12 Thoroughbred mares which were clinically diagnosed with GCT in the Hidaka region, Japan. Mares were between 4 and 26 years old. Before taking the affected ovaries, blood samples were taken and centrifuged in order to measure plasma AMH concentration (AMH Gen II ELISA kit; # A73818, Beckman Coulter, Inc., CA, USA). Ovarian tissues were fixed in 4% paraformaldehyde phosphate buffer solution, and embedded in paraffin. The paraffin block was sectioned at four microns with a REM-710/SB gliding microtome (AS ONE Corp., Osaka, Japan). For histopathological evaluation, tissue sections were stained with hematoxylin and eosin.

All procedures in this study were performed in according to the guidelines of the Institutional Animal Welfare and Experiment Management Committee of Japan Racing Association (JRA), Hidaka Training and Research Center.

2.2. Immunohistochemistry (ABC method)

Tissue sections were deparaffinized and hydrated (xylene, graded alcohol series),

and endogenous peroxidase was inactivated (0.3% H2O2 in methanol, 30 min). Antigen retrieval was performed in an autoclave (20 min at 121°C) by using antigen unmasking solution (# H-3300, Vector Laboratories Inc., CA, USA). After that, sections were blocked with normal serum (Vectastain ABC Kit, # PK-6101, 6105, Vector Laboratories Inc., CA, USA). Then, sections were individually incubated with anti-AMH-Goat antibody (sc-6886, Santa Cruz Biotechnology Inc., CA, USA, 1:500) and anti-AMHR2-Rabbit antibody (# AP-7111b, ABGENT, CA, USA, 1:100) in humidified chambers overnight at 4°C [5]. [5]. After that, sections were incubated with biotinylated secondary antibody (30 min) followed by the incubation of streptavidin-HRP complex (30 min). After the color development with DAB solution, slides were counterstained with hematoxylin solution.

As a positive control, a male fetal gonad of 131 days old was used. As a negative control, parallel slide which was stained in the same procedure using PBS as a substitute for the primary antibody was used.

Slides were examined with a BX53 system microscope (BX53-33, Olympus Corp., Tokyo, Japan) and cellSens Standard software (Olympus Corp., Tokyo, Japan).

2.3. Double immunofluorescence staining

Deparaffinization and antigen retrieval steps were the same as the ABC method. After antigen retrieval, a blocking reaction (30 min) was performed by using 1% skim milk (dissolved in PBS). Primary antibodies which were used for IHC were also used for primary antibody incubation (overnight at 4°C). After incubation of primary antibody, slides were incubated with DyLight 594-labeled anti-Goat IgG-Donkey antibody (# 24-145-073013, ImmunoReagents Inc., NC, USA) and FITC-labeled anti-Rabbit IgG-Donkey antibody (# A120-108 F-10, Bethyl Laboratories, TX, USA) as secondary

antibodies for 1 hour at room temperature. After that, slides were mounted in VECTASHIELD Hard Set Mounting Medium containing DAPI (# H - 1500, Vector Laboratories Inc., CA, USA).

Slides were examined with All-in-One fluorescence microscope (BZ-X 710, Keyence Corp., Osaka, Japan) and BZ-X Analyzer (Keyence Corp., Osaka, Japan).

3. Results

In the current study, plasma AMH concentrations in GCT-affected mares were 583±205 ng/mL (Table 1).

Histopathological analysis of GCT-affected ovarian tissues revealed both cystic and solid forms of GCTs which were composed of multi-layered granulosa-like cells around the follicular and solid areas, and proliferated, polyhedral shaped, eosinophilic theca-like cells which surrounded the granulosa cell layer in some GCT samples (Fig 1. a, e, i, m). Also, we observed macro-, micro-follicular cystic areas and insular patterns in GCT-affected ovaries.

Immunohistochemistry analysis of the present study revealed that immunoreactive AMH and AMHR2 were expressed in granulosa-like cells (Fig 1. b, c, f, g, j, k, n, o) in GCT samples, although AMH staining intensity was variable in each tissue. However, theca-like cells in some tissue samples showed positive staining of AMH and AMHR2 (Fig 1. b, c), while other stromal cells were not positive for AMH and AMHR2.

Double immunofluorescence staining using anti-AMH and anti-AMHR2 antibody demonstrated that both immuneoreactive AMH and AMHR2 were mainly localized in granulosa-like cells of GCT-affected ovaries (Fig 2.).

4. Discussion

Equine GCTs are an important cause of infertility with secondary anestrus in most mares with GCT. It is well known that AMH is secreted into the blood from the GCT-affected ovary, thereby, measuring the circulating AMH provides an accurate diagnosis of GCT [5-8].

In the current study, plasma AMH concentrations in GCT affected mares (583±205 ng/mL) were measured as much higher than plasma AMH concentrations in normal cyclic mares (0.96±0.08 ng/mL) [5]. It confirms that plasma AMH concentration could be an important biomarker to be evaluated simultaneously with ultrasonography and histopathology analysis for the definitive diagnosis of equine GCT. Interestingly, range between the lowest (2013 ng/mL) and the highest (7.6 ng/mL) concentration of plasma AMH in GCT cases was extremely broad. However, it was difficult to determine any correlation between the size of GCT-affected ovaries and plasma AMH concentration in GCT-affected mares because of the small number of GCT samples and variability of the GCT cases in this study.

The present study showed that the most of GCT cases in mares were benign, and histopathological findings in ovarian tissue affected with GCTs were consistent with study on equine GCT [11]. We observed more proliferation of granulosa cells in the follicular and solid parts of GCT affected ovarian tissue as compared with normal ovaries.

In order to localize AMH and AMHR2 in GCT-affected ovarian tissue of mares, we performed immunohistochemistry and double immunofluorescence staining. This is the first report localizing the AMH and AMHR2 in GCT-affected ovaries of Thoroughbred mares by using double immunofluorescence staining. All GCT cases showed positive

staining of AMH and AMHR2 mainly in granulosa-like cells. These findings were consistent with the observation which was conducted in GCT-affected ovarian tissues by using immunohistochemistry analysis in equine [5, 7], bovine [12] and human GCT cases [13]. Our study also showed the importance of AMH as an immunohistochemical biomarker for confirming the equine GCT diagnosis using biopsy and tissue samples of GCT-affected ovary before or after the surgical ablation.

In women, Anttonen et al [14] has suggested that AMH may downregulate and suppress GCT growth through interaction with the AMHR2. According to the present study, the expression of AMHR2 in granulosa-like cells implies that there could be paracrine or autocrine action of AMH in the GCT-affected ovary. Some researchers assume that elevated AMH in circulation may suppress the activity of the contralateral ovary [7], nevertheless, the biological action of AMH to the contralateral normal ovary in GCT-affected mares is still unknown.

In the further, association between plasma AMH concentration, histopathological changes of equine GCT and intensity of mRNA expression of GCT-affected ovarian tissue should be clarified. Moreover, localization of AMH, AMHR2 proteins and mRNA expression of AMH, AMHR2 genes in contralateral normal ovary of GCT-affected mares should be investigated.

5. Conclusion

In conclusion, this is the first study using double immunofluorescence staining for co-localizing AMH and AMHR2 in GCT-affected ovarian tissue of mares. The results suggest that AMH secreted from the granulosa-like cells of equine GCT may act as autocrine or paracrine action on tumor cells (granulosa-like cells). As well as, we

consider that AMH is a good biomarker of immunohistochemistry analysis for diagnosing equine GCT.

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Conflict of interest statement

The authors declare that they do not have any competing interests.

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Fig. 1. Histopathological structure and localization of immunoreactive AMH and AMHR2 in the GCT-affected ovarian tissues of mares. Granulosa-like cells (arrow head) and theca-like cells (arrow) were positive for AMH and AMHR2. GCT 1 (a-d): Granulosa cell layer and underlying theca-like cells of macro-follicular pattern (400x). GCT 2 (e-h): Granulosa-like cell showed Sertoli cell-like morphology and Insular structure (400x). GCT 3 (i-l): Part of macro-follicular patterns (40x). GCT 4 (m-p): Multiple micro-follicular and Insular patterns (40x).

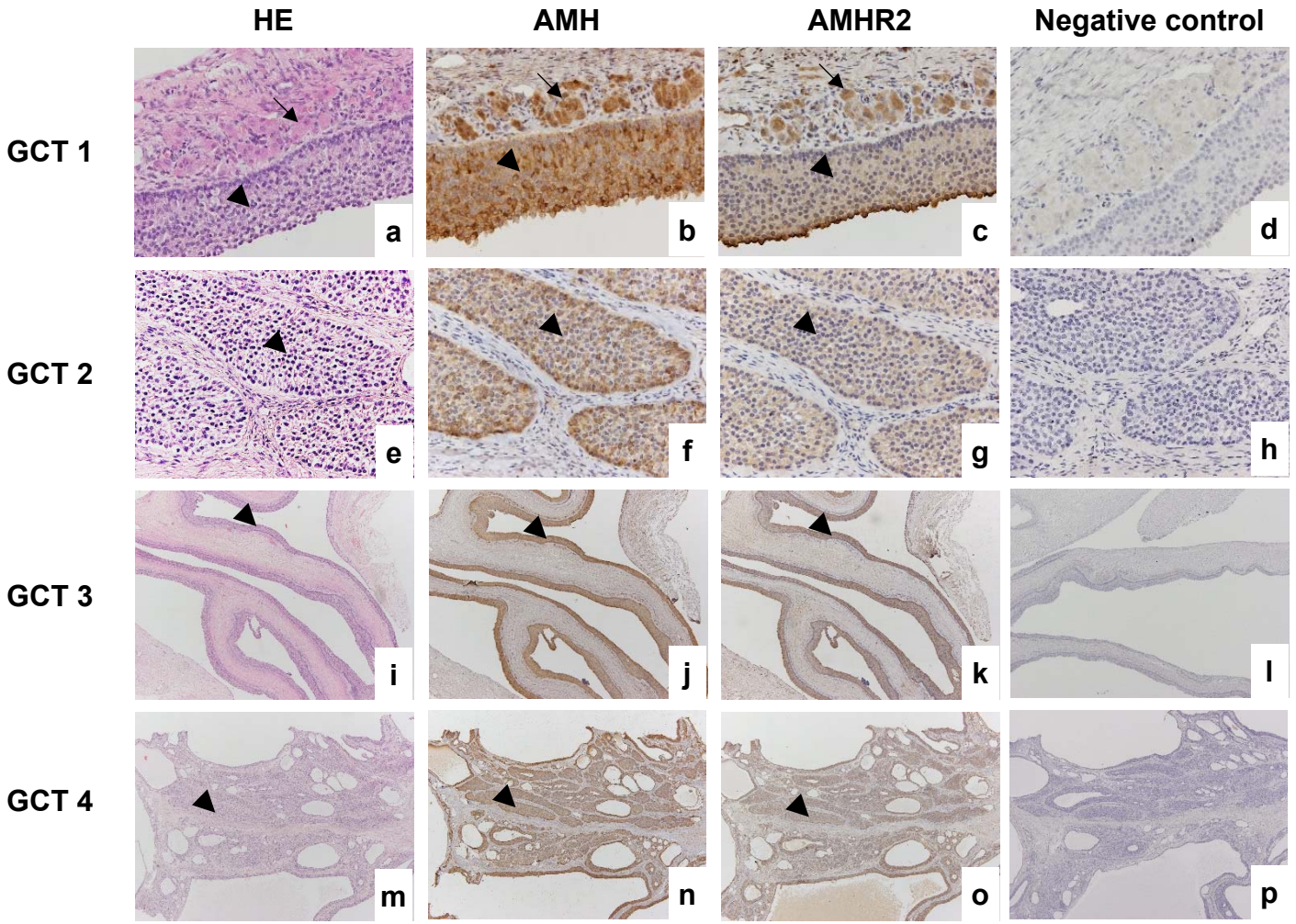
Fig. 2. Co-localization of immunoreactive AMH and AMHR2 in the ovarian tissue of equine GCT (double immunofluorescence staining, 400x, 20μm scale bar). (a) H&E. (b) Nucleus (DAPI). (c) Granulosa-like cells were AMH positive (Dylight 594-red). (d) Granuloso-like cells were AMHR2 positive (FITC-green). (e) Merge of AMH and AMHR2 in Granulosa-like cell.

Table 1. Diameter of the enlarged ovaries, presence of ultra-sonographic honeycomb appearance and plasma AMH concentration in GCT affected mares.

Sample no	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Diameter of the ovary (cm)	13.3	10	9	11.7	20	10.3	9.3	8	10	8.1	8
Honeycomb appearance	+	+	+	+	cyst	+	+	-	+	-	solid
Plasma AMH (ng/mL)	1347	2013	1200	106.7	81.6	94.6	50.2	7.6	22.2	295.7	1190.5
Mean (AMH ng/mL)	**583±205**										

Highlights

- AMH and AMHR2 are localized in granulosa-like cells of both solid and follicular type of GCT-affected ovary in mares.
- There could be paracrine or autocrine action of AMH in granulosa-like cells of GCT-affected ovary in mares.
- Plasma AMH measurement and Immunohistochemistry analysis are crucial tools for definitive diagnosis of equine GCT.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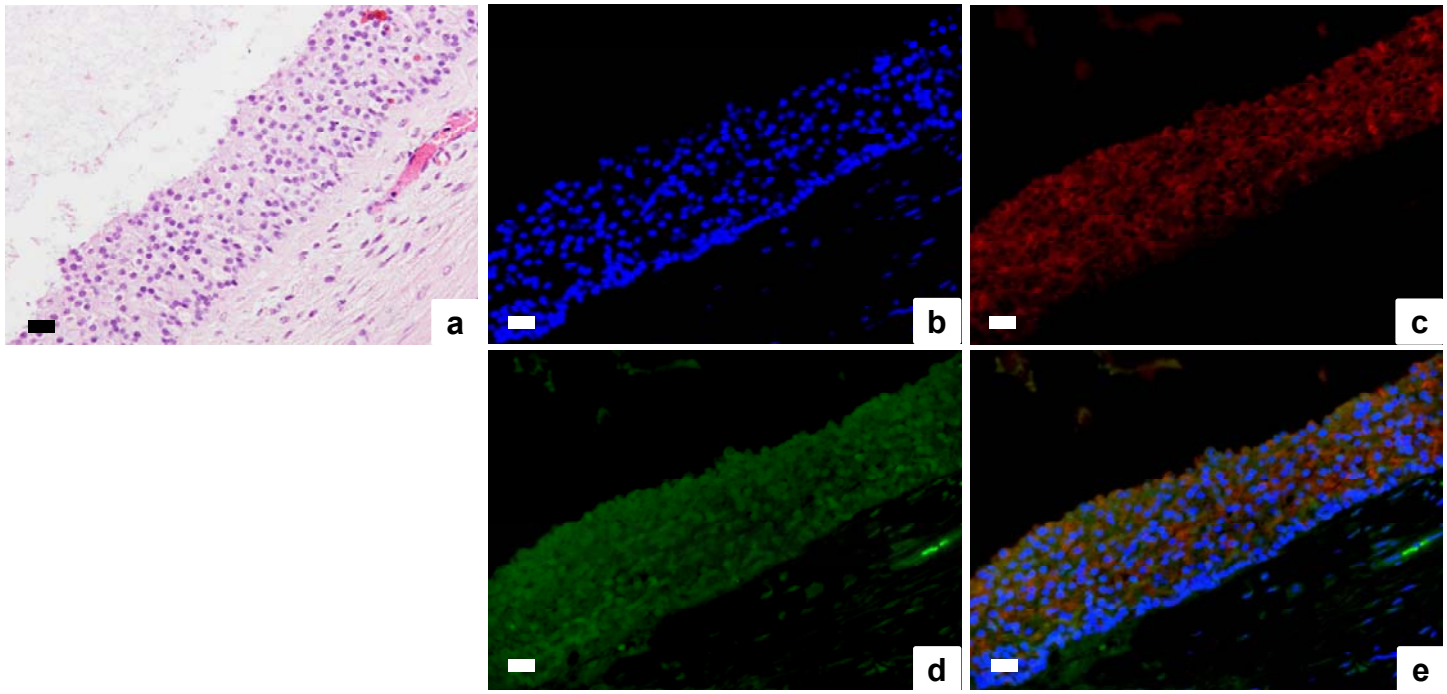
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