Anti-Müllerian Hormone Profiles as a Novel Biomarker to Diagnose Granulosa-theca Cell Tumors in Cattle

Go KITAHARA1, Yasuo NAMBO2, Hosam EL-SHEIKH ALI1,3, Makoto KAJISA4, Mineto TAN15, Kazumi NIBE6 and Shunichi KAMIMURA1

1)Laboratory of Theriogenology, Faculty of Agriculture, University of Miyazaki, Miyazaki 889-2192, Japan
2)Hidaka Training and Research Center, Japan Racing Association, Hokkaido 057-0171, Japan
3)Theriogenology Department, Faculty of Veterinary Medicine, University of Mansoura, Mansoura 35516, Egypt
4)Sojo Agricultural Mutual Aide Association, Kagoshima 899-7601, Japan
5)Laboratory of Large Animal Theriogenology, Faculty of Agriculture, University of Tokai, Kumamoto 869-1404, Japan
6)Japan Animal Referral Medical Center, Kanagawa 213-0032, Japan

Abstract. This study was carried out to evaluate the blood profile and tissue expression of Anti-Müllerian hormone (AMH) as a biomarker for granulosa-theca cell tumors (GTCs) in cattle. Five cases with unilateral ovarian GTCs (GTC group) were investigated in comparison to other groups of Japanese Black cows, which had either cystic ovarian disease (COD group, n=5), a functional corpus luteum on Days 9 to 11 of the estrous cycle (Day 0=estrus; CL group, n=13) or received superovulation treatment (SOT group, n=13). We used transrectal ultrasonography and measured plasma AMH, estradiol-17β (E2), progesterone (P4) and testosterone (T) levels. Moreover, GTC tissues were collected and examined by immunohistochemical staining (IHC) for AMH. In the GTC group, ultrasound images of GTCs were variable and not definitive. However, the AMH level in the GTC group (n=3, 58.1 ± 66.3 ng/ml) was significantly higher than in the COD, CL and SOT groups (0.1 ± 0.1 ng/ml for GTC vs. COD, P<0.05; 0.2 ± 0.1 and 0.3 ± 0.2 ng/ml, respectively for GTC vs. CL and SOT, P<0.01). The other hormonal levels in the GTC group had no significant differences compared with the COD or SOT group. Neoplastic granulosa cells labeled with AMH antibody clearly demonstrated a variety of tissue patterns in all cases by IHC. To the best of our knowledge, this is the first study to investigate the blood profile and IHC of AMH in bovine GTCs. Our findings indicate that AMH may be a novel biomarker to diagnose GTCs in cattle.

Key words: Anti-Müllerian hormone (AMH), Cattle, Granulosa-theca cell tumors (GTCs), Immunohistochemical staining

Granulosa-theca cell tumors (GTCs) are the most common ovarian tumors in cattle [1], and the incidence may be less than 0.5% [2]. GTCs can affect various breeds [3] and occur at various ages [4] in cattle. GTCs produce a variety of steroid hormones, causing a subsequent elevation of plasma estradiol-17β (E2), progesterone (P4) and/or testosterone (T) levels [4]. Therefore, a variety of clinical signs, such as nymphomania [5], virilism [6] and mammary gland development [7], could be presented. In contrast, some in GTCs may not result in abnormal reproductive behavior at all [3]. Preliminary diagnosis of GTCs can be achieved by transrectal palpation and ultrasonography [1, 4]. GTCs should be suspected if a chronic cystic ovarian disease (COD) does not respond to standard treatment regimens or if the diameter of the ovary is more than 100 mm [1]. Thus, GTCs should be distinguished from other conditions, such as COD, oophoritis, ovarian abscesses and parovarian cysts, by these clinical signs [8]. However, the distinctions might be difficult depending on the clinical signs and other diagnostic methods, so a definitive diagnosis can only be made based on histopathological examination of the affected ovary [3]. In the treatment of unilateral bovine GTCs, unilateral ovariec-tomy is only indicated in cows that do not exhibit alterations in secondary sexual characteristics [9]. After removal, folliculogenesis in the contralateral ovary resumes, with subsequent recovery of fertility [4, 9, 10]. Therefore, the development of an accurate biomarker for the clinical diagnosis of bovine GTCs is necessary.

Anti-Müllerian hormone (AMH) is a member of the transforming growth factor β family that is expressed only in the gonads. In females, AMH is produced by the granulosa cells in the developing follicles of the ovary [11]. In humans, AMH has emerged as a possible marker for GTCs [12–14], polycystic ovary syndrome (PCOS) [15] and ovarian reserve [16]. In cattle, the AMH level has been found to not be significantly different during the estrous cycle in cattle and is used as a predictor for superovulatory response [17], but is not suitable as a biomarker for follicular cysts [18]. To the best of our knowledge, there is no report describing peripheral AMH levels or even its immunohistochemical localization in bovine GTCs. The objective of the present study was to evaluate the possibility of using AMH as a novel biomarker to diagnose bovine GTCs in comparison to COD, the luteal phase and postsuperovulation treatment.
Materials and Methods

Diagnosis of GTCT and COD

Five animals were suspected of having GTCTs. The breed, age and parity of each animal are shown in Table 1. These animals exhibited abnormal estrous cycles. Transrectal palpation and ultrasonography revealed a marked enlargement of one ovary (4 cases on the right side and a case on the left side) and atrophy of the contralateral ovary. Moreover, these ovaries were classified according to their ultrasound images into cystic and/or solid. The enlarged ovaries were extracted (3 cases by ovarioectomy and 2 cases after slaughter) and subjected to histopathological examinations, which confirmed our preliminary diagnosis.

Five Japanese Black cows beyond the voluntary waiting period (i.e., 60 days after parturition) were diagnosed with COD. Ultrasonographic examinations of the affected cows showed a unilateral follicle-like structure with a diameter ≥ 25 mm in the absence of a corpus luteum (CL), and their plasma P₄ levels in them were showed less than 1.0 ng/ml.

Superovulation treatment regime

CLs were identified by transrectal ultrasonography in 13 multiparous Japanese Black cows (9.2 ± 4.3 years old; average ± SD) at Days 9 to 11 of a normal estrous cycle (Day 0 = estrus). These cows were superovulated with a total of 20 mg FSH (Antrin 10, Kawasaki Seiyaku, Kanagawa, Japan) given twice daily intramuscularly over 3 days in a descending dose schedule (5, 5, 3, 3, 2 and 2 AU). At 48 and 60 h after the initial FSH treatment, luteolysis was induced by intramuscular injection of 30 mg progestinlan F₂₄ (PGF₂α, Pronalgon F, Pfizer, Tokyo, Japan). At 108 h after the initial FSH, the ovaries of these cows showed multiple preovulatory follicles.

Animal grouping

Four groups were enrolled in the present study. The first group included cattle with GTCTs (GTCT group, n=5), the second group contained cows affected with COD (COD group, n=5), the third group contained cows showing functional CLs at Days 9 to 11 of the estrous cycle (CL group, n=13) and the fourth group contained the same animals as the third group after subjecting them to superovulation treatment using FSH (SOT group, n=13).

Blood samples

Blood samples were collected via coccyeal venipuncture into heparinized vacutainer tubes (Venoject II, Terumo, Tokyo, Japan) and were immediately centrifuged (3000 rpm × 15 min at 4 °C). The plasma was harvested and stored at −30°C until the hormonal assay. Blood samples were individually collected once before any further interference in the GTCT group, when diagnosed in the COD group, at Days 9 to 11 in the CL group and at 108 h after the initial FSH injection in the SOT group.

Hormonal assay

The plasma samples of all animals in each group were assayed for AMH, E₂, P₄ and T levels, with the exception that AMH and E₂ could not be measured in 2 samples from the GTCT group (samples were not available at the assay time), so only P₄ and T profiles were available for these 2 cases. Plasma concentration of AMH was measured with an active MIS/AMH ELISA kit (DSL-10-14400; Beckman Coulter, Brea, CA, USA), which has been previously validated for cattle [19]. The sensitivity and intra- and interassay coefficients of variation for this assay were 0.006 ng/ml, <4.6% and <8.0%, respectively. Plasma E₂ concentration was measured with an Estradiol-17β ELISA kit (RE52041; IBL International GmbH, Hamburg, Germany). Plasma samples were extracted with diethyl ether, defatted with acetone/toluene and n-hexane and assayed [20, 21]. The sensitivity and the intra- and interassay coefficients of variation for E₂ were 9.7 pg/ml, <6.8% and <9.4%, respectively. Plasma P₄ and T concentrations were measured using an enzyme-linked fluorescent assay (VIDAS progesterone or testosterone, Japan bioMerieux, Tokyo, Japan). The sensitivity and intra- and interassay coefficients of variation for both the P₄ and T assay were 0.1 ng/ml and <10%, respectively. The data that did not meet the threshold of assay sensitivity were assumed to be the nadir in each assay.

Histopathology and immunohistochemistry

Histopathological examination of the affected ovaries was carried out after extraction. The size and weight of the extracted
large ovary were recorded. The representative tissue samples of suspected GTCTs were fixed in 10% neutral buffered formalin, embedded in paraffin wax by routine methods and sectioned at 5 μm for each sample. Sections were stained with haematoxylin and eosin (HE). The IHC procedure to detect AMH expression was carried out using the avidin-biotin-peroxidase complex method, as described previously [22]. After ABC staining, each slide was counterstained with hematoxylin. The primary antibody used was goat polyclonal anti-human AMH (1:500; sc-6886, Santa Cruz Biotechnology, Santa Cruz, CA, USA). To assess the specificity of the primary antibody, it was incubated with the blocking peptide (sc-6886P, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The AMH antibody was mixed with the peptide at 1:5 (w: w) overnight at 4 C and then used for IHC. The control sections were processed for IHC with omission of the primary antibody.

Data analysis

Plasma AMH, E₂, P₄ and T concentrations were compared between the CL group and SOT group using the Wilcoxon signed rank test and the Mann-Whitney U-test was used for all other intergroup comparisons. Statistical analyses were performed using the PASW® software (PASW®18, SPSS, Tokyo, Japan).

Results

Clinical findings

In the 5 cases of GTCTs, the age and parity at detection of GTCTs varied. The right ovary was affected more often by GTCTs than the left one, and the longest diameter was more than 100 mm in most cases (Table 1). In three cases, the ovary affected by GTCT was ovarioectomized, and COD was detected and treated in 2 of 3 ovarioectomy cases (#7980: 23 days after extraction; #0517: 34 days after extraction). These 3 ovarioectomy cases were conceived by artificial insemination (AI) 88.3 ± 77.1 (average ± SD) days after extraction. The ultrasound images of GTCTs showed various traits among the five cases (Fig. 1). The appearance was cystic in #5809, solid in #7980 and a mixture of cystic and solid in the other cases (#5085, 5017 and 4099) as demonstrated in Fig. 1.

Plasma hormonal levels

In 3 cases with GTCT (#0517, 4099 and 5809), the plasma AMH concentrations were 19.4, 134.6 and 20.3 ng/ml, respectively. These

Fig. 1. Ultrasound images of GTCTs. The images in A, B, C, D and E show cows #5085, #7980, #0517, #4099 and #5809, respectively. The bar is 10 mm.
AMH levels (58.1 ± 66.3 ng/ml) were significantly higher than in the COD, CL and SOT groups (0.1 ± 0.1 for GTCT vs. COD, P<0.05; 0.2 ± 0.1 and 0.3 ± 0.2 ng/ml, respectively GTCT vs. CL and SOT, P<0.01; Table 2). The plasma AMH concentration in the SOT group was significantly higher than in the CL group (P<0.01; SOT vs. CL), whereas there was no significant difference between the COD and CL or SOT groups (P = 0.70 for COD vs. CL; P = 0.12 for COD vs. SOT). Moreover, in the same 3 cases with GTCT, the plasma E2 concentration (15.2 ± 10.2 pg/ml) was significantly higher than in the CL group (4.9 ± 3.2 pg/ml; P<0.05); however, it was not significantly different compared with the COD or SOT group (4.6 ± 1.0 and 26.0 ± 11.0 pg/ml, respectively; P = 0.18 for GTCT vs. COD; P = 0.24 for GTCT vs. SOT). In all cases in the GTCT group, the plasma P4 concentration (2.8 ± 3.2 ng/ml) was significantly lower than in the CL group (9.1 ± 2.9 ng/ml; P<0.05); however, it was not significantly different compared with the COD and SOT groups (0.8 ± 0.0 and 1.3 ± 0.4 ng/ml, respectively; P = 1.00 for GTCT vs. OC; P = 0.63 for GTCT vs. SOT). There was no significant difference in plasma T concentration between all cases in the GTCT group and the other groups (0.3 ± 0.3, 0.4 ± 0.3, 0.5 ± 0.5 and 0.4 ± 0.4 ng/ml for GTCT, COD, CL and SOT, respectively; P = 0.55, 0.50 and 0.63 for GTCT vs. COD, CL and SOT, respectively).

### Discussion

GTCTs have been reported in cattle with various parities and reproductive statuses, such as heifers [4, 23] or cows [24] and gravid [25] or nongravid [6]. The weights of GTCTs also vary, from about 12 to 1,200 g [26]. In the present study, the cattle with GTCTs had different statuses, and there was a large difference in the weights of GTCTs, from 130 to 3000 g. Unilateral ovarioectomy is only indicated to treat valuable cows with unilateral GTCTs [9]. The contralateral ovary is usually inactive and atrophied by various hormones secreted by neoplastic granulosa cells, such as estrogen and inhibin [27]. Extracting GTCTs often results in COD in the contralateral ovary [7]; however, after treatment of this COD, they regain fertility. In the present study, the ovary affected by GTCT was extracted from 3 of the 5 cattle with GTCTs. 2 of these 3 cattle contracted COD and were treated. After that, all 3 cattle conceived within 176 days after extraction of GTCTs.

In mares, GTCTs may have a variety of ultrasonographic appearances. The most typical is a “honeycomb” appearance, which has many circular anechoic areas separated by echogenic trabeculae [28]. In humans, GTCT is usually diagnosed by ultrasonic and radiographic examination [12, 29]. Ultrasonography of GTCTs may reveal a large, echogenic, septated cystic mass arising from the ovary, or the mass may appear solid [12]. However, solid GTCTs are uncommon and have a heterogeneous appearance, with regions of hemorrhage, fibrosis, or both [2]. In cattle, GTCTs have regions of multiple follicular structures commingled with solid tissue densities, and an ultrasonographic pattern of the hypoechoic areas, ostensibly created by tissue compression, could improve the clinical diagnosis of bovine GTCTs [6]. In the present study, the ultrasound images of GTCTs showed three different appearances of tumors, cystic, solid or both, and were not definitive.

In humans, GTCTs are associated with excess estrogen production in 75% of cases [14], whereas the absence of estrogen secretion is observed in the other cases [13]. Also, GTCT cases rarely produce androgens. Women with progressive GTCTs have elevated AMH levels, but women in clinical remission have undetectable levels of

### Table 2. Comparison of blood hormonal levels in each group

<table>
<thead>
<tr>
<th>Group</th>
<th>Case No. or Numbers</th>
<th>AMH (ng/ml)</th>
<th>E2 (pg/ml)</th>
<th>P4 (ng/ml)</th>
<th>T (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTCT</td>
<td>#5085</td>
<td>-</td>
<td>-</td>
<td>7.0</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>#7980</td>
<td>-</td>
<td>-</td>
<td>0.5</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>#0517</td>
<td>19.4</td>
<td>16.1</td>
<td>5.6</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>#4099</td>
<td>134.6</td>
<td>4.5</td>
<td>0.8</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>#5809</td>
<td>20.3</td>
<td>24.9</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>58.1 ± 66.3a</td>
<td>15.2 ± 10.2bc</td>
<td>2.8 ± 3.2ac</td>
<td>0.3 ± 0.3b</td>
</tr>
<tr>
<td>COD</td>
<td>n=5</td>
<td>0.1 ± 0.1ac</td>
<td>4.6 ± 1.0ab</td>
<td>0.8 ± 0.0a</td>
<td>0.4 ± 0.3a</td>
</tr>
<tr>
<td>CL</td>
<td>n=13</td>
<td>0.2 ± 0.1b</td>
<td>4.9 ± 3.2b</td>
<td>9.1 ± 2.9b</td>
<td>0.5 ± 0.5b</td>
</tr>
<tr>
<td>SOT</td>
<td>n=13</td>
<td>0.3 ± 0.2a</td>
<td>26.0 ± 11.0c</td>
<td>1.3 ± 0.4c</td>
<td>0.4 ± 0.4b</td>
</tr>
</tbody>
</table>

The mean ± SD values that significantly differ are represented with different lowercase letters for each hormonal level. (P<0.05: a-bc in AMH and ac-b in E2, P<0.01: all others.)

### Histopathological findings

We observed multiple and variable-sized follicular structures and cell nests separated by fibroblasts and collagen in #5085, 0517 and 4099. Structures resembling follicular structures in #7980 and cell nests in #5809 were mainly seen in HE staining. The neoplastic tissues formed mono- or multilayers in the follicular structures or substantial in solid mass. In all cases except #5085, hemorrhages were noted in the neoplastic tissues and the cavities of the follicular structures. In #0517 and 5809, necrosis was noted in the neoplastic tissues. In neoplastic cells, such as granulosa cells, cosposincophil and a few mitotic cells were observed. In #5085 and 7980, neoplastic cells, such as theca cells, were clearly observed in the stroma. Based on these observations, a diagnosis of GTCT was made. Neoplastic granulosa cells labeled with AMH antibody clearly demonstrated a variety of tissue patterns with purplish red staining in all cases (Table 1, Fig. 2a, c). On the other hand, when tissues samples in all cases were incubated with the blocking peptide, more than a few were stained (Fig. 2b).
AMH [13, 14, 30]. When AMH remains elevated after extraction of GTCTs, residual disease is indicated. AMH levels are normal in 93% of other gynecologic and nongynecologic cancers [12]. In mares, E₂, testosterone and inhibin concentrations also fluctuate widely among mares with GTCTs and are not elevated in all mares with GTCTs [19, 31]. On the other hand, AMH concentrations were reportedly higher in all mares with GTCTs compared with cyclic and pregnant mares [32]. The relatively long half-life of AMH in calves compared with inhibin may account for the stability of circulating concentrations of AMH [22]. Cattle with GTCTs can have various hormonal levels, such as high levels of estrogen [30] and T [6], or the same hormonal levels [10] as normal cows. To our knowledge, there are no reports concerning the blood level of inhibin or AMH in cattle with GTCTs. In this study, the T levels in

Fig. 2. Histological findings in GTCTs by immunohistochemical (IHC) staining. The images presented are from cows in #0517 (1-a, 1-b and 1-c) and #5809 (2-a, 2-b, and 2-c). Neoplastic granulosa cells labeled with AMH antibody (a, c) and neoplastic granulosa cells that could not be labeled with AMH antibody after incubation with the blocking peptide are shown (b). Purplish red staining indicates granulosa cells in a GTCT labeled with AMH antibody (a, c).
all cows with GTCTs were less than 1 ng/ml, as in the CL group, whereas the E2 and P4 levels varied. The measured AMH levels of the cases were remarkably higher than those in the CL group.

GTCTs could be confused with COD, oophoritis, ovarian abscesses and parovarian cysts [8]. The honeycomb appearance shown by ultrasonography in GTCTs resembles the ultrasound images of ovaries with multiple follicles, such as in COD, in PCOS [33] or after superovulation treatment. Moreover, the honeycomb appearance is not necessarily indicative of GTCTs in cattle. E2, P4 and T levels vary among cattle with GTCTs, and the observed clinical signs are also different because of the various hormonal levels [6]. Therefore, GTCTs might not be easy to distinguish from other diseases based on the clinical signs, ultrasound images and gonadal steroid levels. In this study, peripheral AMH levels after superovulation treatment were significantly higher than before treatment, as in a previous report [17]. Moreover, AMH was significantly higher in all measured cases of GTCTs than in cases of COD or after superovulation treatment, but the other hormones, E2, P4 and T, were not significantly different.

IHC analysis for AMH has been reported in equine [22] and human [34] GTCTs. To the best of our knowledge, the present study is the first report characterizing bovine GTCT immunostaining for AMH. In GTCTs, immunoreactive AMH was expressed in the neoplastic granulosa cells in all three cases with GTCTs that had high blood AMH levels. Moreover, the other two cases for which samples were not available at the assay time presented similar AMH expression as the previous 3 cases. This observation is similar to findings in mares and women, and it shows that the neoplastic granulosa cells synthesized and secreted AMH. This study shows that GTCTs in cattle had various clinical statuses, ultrasound appearances and sex steroid hormone levels. It is the first study to show that blood AMH levels in all cattle with GTCTs were significantly higher than in cows that were normal, had COD or underwent superovulation treatment. Moreover, immunoreactive AMH was expressed in neoplastic granulosa cells in GTCTs. It might be considered that the granulosa cells uniquely secrete AMH, that their numbers in ovaries with GTCTs are increased more than in ovaries with normal structures and that therefore the plasma AMH levels in the cases with GTCT are higher than in normal cows. These findings may indicate that the blood AMH level could be a more useful biomarker to diagnose GTCTs than the traditional diagnostic aids.

Acknowledgments

This study was supported in part by a research grant (2011) from the Graduate School of Medicine and Veterinary Medicine, Miyazaki University.

References

15. Pellatt L, Rice S, Mason HD. Anti-müllerian hormone and polycystic ovary syndrome: a mountain too high? Reproduction 2010; 139: 825–833. [Medline] [CrossRef]


