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Case report: The case of a 17 kg ovarian granulosa cell tumor in a Breton draft mare

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Granulosa cell tumor (GCT) is a benign tumor which affects the mare's ovaries. In this report, a case of unilateral GCT in an ovary, which weighed 17.04 kg, of a 9-year-old Breton draft mare is described. A transrectal ultrasonography exam revealed a unilateral multi-cystic enlarged ovary. Laparoscopic ovariectomy was difficult due to enlargement of blood vessels in the ovarian broad ligament. The mare was necropsied, and the pathological changes in the GCT-affected ovary and unaffected ovary were evaluated. The ovarian mass in the GCT-affected ovary had a cribriform pattern and was positive for anti-Müllerian hormone (AMH) and its receptor (AMHR2). The contralateral ovary showed no follicular development and was negative for AMH. AMHR2 was positively expressed in stromal cells. The AMH concentration in plasma was 4,210 ng/ml. This is the first report showing the presence of AMH (2,210 ng/ml) in ascites fluid, and it also shows that laparoscopic ovariectomy might not be suitable for larger ovaries affected by a GCT. Ultrasonographic, endocrine, and histopathological analyses were helpful for making a definitive diagnosis of GCT in this mare.

Key words: *anti-Müllerian hormone, contralateral ovary, granulosa cell tumor, mare*

J. Equine Sci.
Vol. 32, No. 2
pp. 67–72, 2021

Granulosa cell tumors (GCTs) are the most common tumors in the mare's ovaries, affecting granulosa cells and sometimes theca cells [7, 18]. They usually affect only one ovary, but they do affect both ovaries in rare cases [8, 21]. In most cases of GCTs, the mare fails to show an estrous cycle [18], except in non-classical GCT cases [21]. Ovarian ultrasonography, anti-Müllerian hormone (AMH) analysis of blood, and histopathology of ovarian tissue are considered reliable tools for GCT diagnosis [1, 3, 19, 20]. Surgical ablation of the affected ovary is the main treatment method. In mares, the largest ovarian mass diagnosed with GCTs was reported to be around 59.1 kg and was found in a Quarter Horse mare [18]. However, there is not enough detailed information available regarding hormone profiles and histopathology in equine GCT cases with such a giant ovarian mass. In addition, reports covering the histopathology of the contralateral ovary are limited. Therefore, the present report aimed to describe ultrasonographic appearances; laparoscopic approach for the affected ovary; hormone profiles in blood, cyst fluid, and ascites fluid; and morphologic abnormalities in both the affected and contralateral ovaries.

The mare presented here was a 9-year-old Breton draft horse weighing 886 kg. She had had 3 normal pregnancies before. In the previous breeding season, she was aborted due to placentitis and did not get pregnant. In April 2019, enlargement of the left ovary was first noticed (around 15 cm in diameter) with a multi-cystic appearance (Fig. 1A), and

Received: January 27, 2021
Accepted: April 14, 2021
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the right ovary had small follicles (Fig. 1B). The AMH level in blood circulation was 484 ng/m*l*. The mare did not show normal cycling. Four months later, the mare was referred to the Large Animal Clinic at the Obihiro University of Agriculture and Veterinary Medicine (OUAVM) because a veterinarian suspected that the mare might have a GCT. All procedures in this case study were approved by the Animal Experiment Committee of OUAVM, Japan.

Ultrasonographic examination using a convex probe revealed that the left ovary was around 30 cm in diameter (Fig. 1C). A GCT was suspected, and we attempted to perform a laparoscopic ovariectomy. The mare was intravenously sedated with 0.8 m*l* detomidine hydrochloride (0.01 mg/kg; Dozadine, Virbac Pty Ltd., New South Wales, Australia). The flank region was sterilely prepared and locally anesthetized with 280 ml lidocaine (Xylocaine injection 2%, Aspen Japan Co., Ltd., Tokyo, Japan). After making several small incisions, specialized trocars with camera were introduced into the abdominal cavity for visualization of the affected ovary. At the time of laparoscopic insertion, a significant portion of the abnormally giant ovary had sunk into the abdominal cavity, and the blood vessels of the ovarian broad ligament were enlarged (Fig. 1D), making it difficult to remove the ovary by laparoscopic surgery.

After the laparoscopic attempt to remove the affected ovary, the owner requested euthanasia of the mare due to the mare's body condition, owner's financial situation, and possibility of post-surgical complications in the case of performing a standard ventral midline approach under general anesthesia. Necropsy was performed to confirm the diagnosis and for educational and research purposes in the Pathology Laboratory, OUAVM, 7 days post-surgery.

Before euthanasia, a blood sample was taken for hormone analysis. After that, 4 m*l* medetomidine hydrochloride (5 μg/kg, IV; Dorbene Vet, Kyoritsu Seiyaku Corp., Tokyo, Japan), 18 m*l* ketamine hydrochloride (Ketalar for intramuscular injection 500 mg, Daiichi-Sankyo Co., Ltd., Tokyo, Japan), and 100 m*l* propofol (2% Propofol injection "Maruishi," Maruishi Pharmaceutical Co., Ltd., Osaka, Japan) were administered, and the mare was then euthanized by rapid intravenous injection of KCl. At necropsy, the size and weight of the left ovary were measured and found to be 34 × 20 × 25 cm and 17.04 kg, respectively (Fig. 1E). Multiple cysts filled with serous fluid were found on a cut surface of the left ovary (Fig. 1F), and the largest cyst was around 4 cm in diameter. The cyst fluid was dark red, and it was collected for hormone analysis. The right ovary was about 6 × 3 × 2 cm in size and had an atretic corpus albicans on the cut surface, but no follicles were found (Fig. 1G). Around the right fallopian tube, multiple cysts that were 0.5 to 3 cm in diameter were observed, and pale-yellow serous fluid was accumulated inside. In addition, the uterine mucosa was dark red, and focal hemorrhages of 0.2 to 3 cm in size were scattered. The mesovarium surrounding the GCT-affected ovary was exceptionally thickened (Fig. 1H). A large amount of light brown serous ascites was accumulated in the abdominal cavity (Fig. 1I). Ascites fluid was collected for hormone analysis, and both ovaries were collected and fixed in 10% neutral-buffered formalin for histological analysis.

Plasma, follicular, and ascites fluid samples were submitted to the laboratory of the Hidaka Training and Research Center, Japan Racing Association (JRA), for AMH measurement. Progesterone, estradiol, and testosterone hormone concentrations were measured using enzyme immunoassays (EIAs; ST AIA-Pack PROGIII, ST AIA-Pack iE2, and ST AIA-Pack Testosterone, Tosoh Bioscience, Inc., San Francisco, CA, U.S.A.) at the Laboratory of Equine Reproduction, OUAVM. Hormone profiles for plasma, follicular, and ascites fluid samples are shown in Table 1.

Formalin-fixed paraffin-embedded (FFPE) ovarian tissues were cut into 4 μm sections and stained with hematoxylin and eosin. Histopathologically, the left ovarian mass had a cribriform pattern characterized by sheets of granulosa cells with glandular perforation (Fig. 2A). The follicle-like structures were lined with a monolayer to multiple layers of granulosa cells (Fig. 2B) and contained serous fluid. Some granulosa cells formed Call-Exner bodies that grew around the eosinophilic substrate (Fig. 2C). Moreover, a Sertoli cell-like morphology with closely packed solid tubules lined by columnar and cuboidal granulosa cells was observed in the left ovary (Fig. 2D). The neoplastic cells had a polygonal shape, clear cytoplasm, and small round hyperchromatic nuclei. There was slight atypia, such as anisokaryosis of the nucleus. In addition, mitotic figures were observed. Based on these characteristics, the ovarian mass was diagnosed as a granulosa cell tumor. Granulosa tumor cells replaced most part of the left ovary. There was no luteal-like structure in the left ovary. The contralateral (right) ovary was atrophied, the parenchyma was replaced with fibrous tissue, and follicular development was not observed (Fig. 2E).

For immunohistochemistry (IHC) analysis, FFPE tissues were cut into serial sections with a thickness of 4 μm, and immunostaining was performed using methods described previously [19]. Areas stained with a brown color were considered to show positive staining. Immunostaining analysis showed that the granulosa cells in the GCT-affected ovarian tissue were immunopositive for AMH and AMHR2 (Fig. 2F and 2G). The contralateral ovary showed negative staining for AMH (Fig. 2H); however, a few stromal cells were positive for AMHR2 (Fig. 2I). Negative controls for each sample, for which primary antibody incubation was omitted, did not show positive staining (insert in Fig. 2F and 2H).

The ultrasonographic findings in the present case were in agreement with those in previous case reports which reported multi-cystic honeycomb appearances and unilaterally enlarged ovaries in equine GCTs [10, 17]. The structure of the contralateral ovary changed as time passed. The initial ultrasonographic exam revealed small follicles in the contralateral ovary; however, the ovary became atrophic and showed no follicular development at necropsy. This might have been related to inhibitory factors (inhibin or probably AMH) which were released by the GCT-affected ovary. Ovarian ultrasonography is not sufficient alone for the diagnosis of GCT, even though it is an important tool. For a definitive diagnosis, other laboratory analyses, such as histopathology and hormone measurements, are needed.

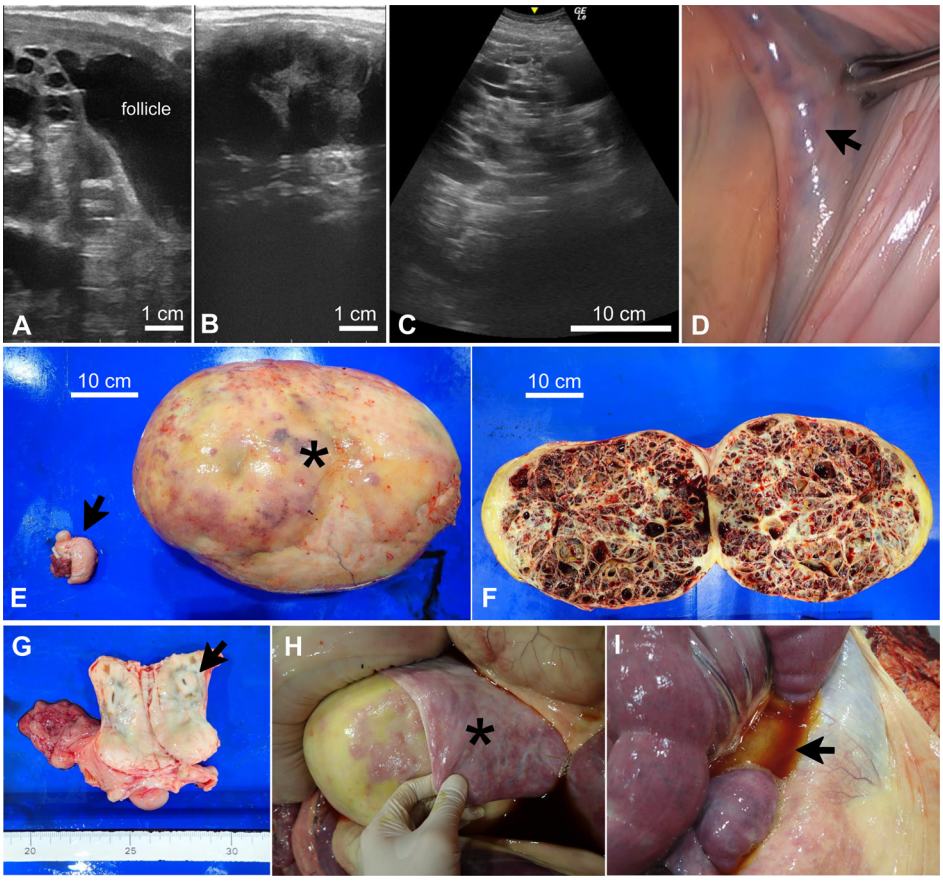

Fig. 1. Ultrasonographic, laparoscopic, macroscopic, and cross-sectional appearances of the enlarged and contralateral ovaries. (A) Ultrasonographic appearance of the enlarged ovary which had multiple small and large follicles (April). Bar=1 cm. (B) Ultrasonographic appearance of the contralateral ovary (April). Bar=1 cm. (C) Ultrasonographic 'honeycomb' appearance of the enlarged ovary, which is 30 cm in diameter (August). Bar=10 cm. (D) Laparoscopic visualization of the enlarged blood vessel (arrow) in the ovarian broad ligament. (E) Macroscopic structure of the enlarged (*) and contralateral (arrow) ovaries. Bar=10 cm. (F) Cross-sectional appearance of the enlarged ovary showing multiple cysts with a 'honeycomb' appearance. Bar=10 cm. (G) Cross-sectional appearance of the contralateral ovary showing a corpus albicans (arrow) but no follicular development. (H) Thickened mesovarium surrounding the granulosa cell tumor (GCT)-affected ovary (*). (I) Light-brown serous ascites (arrow) in the abdominal cavity.

Table 1. Concentrations of anti-Müllerian hormone (AMH), progesterone, estradiol, and testosterone hormones in plasma, cyst fluid, and ascites fluid samples

Samples	AMH (ng/m*l*)	Progesterone (ng/m*l*)	Estradiol (pg/m*l*)	Testosterone (ng/d*l*)
Plasma	4,210.00	0.22	66.50	4.76
Cyst fluid	6,640.00	1.35	15,200.00	324.10
Ascites fluid	2,210.00	0.26	21.80	3.38

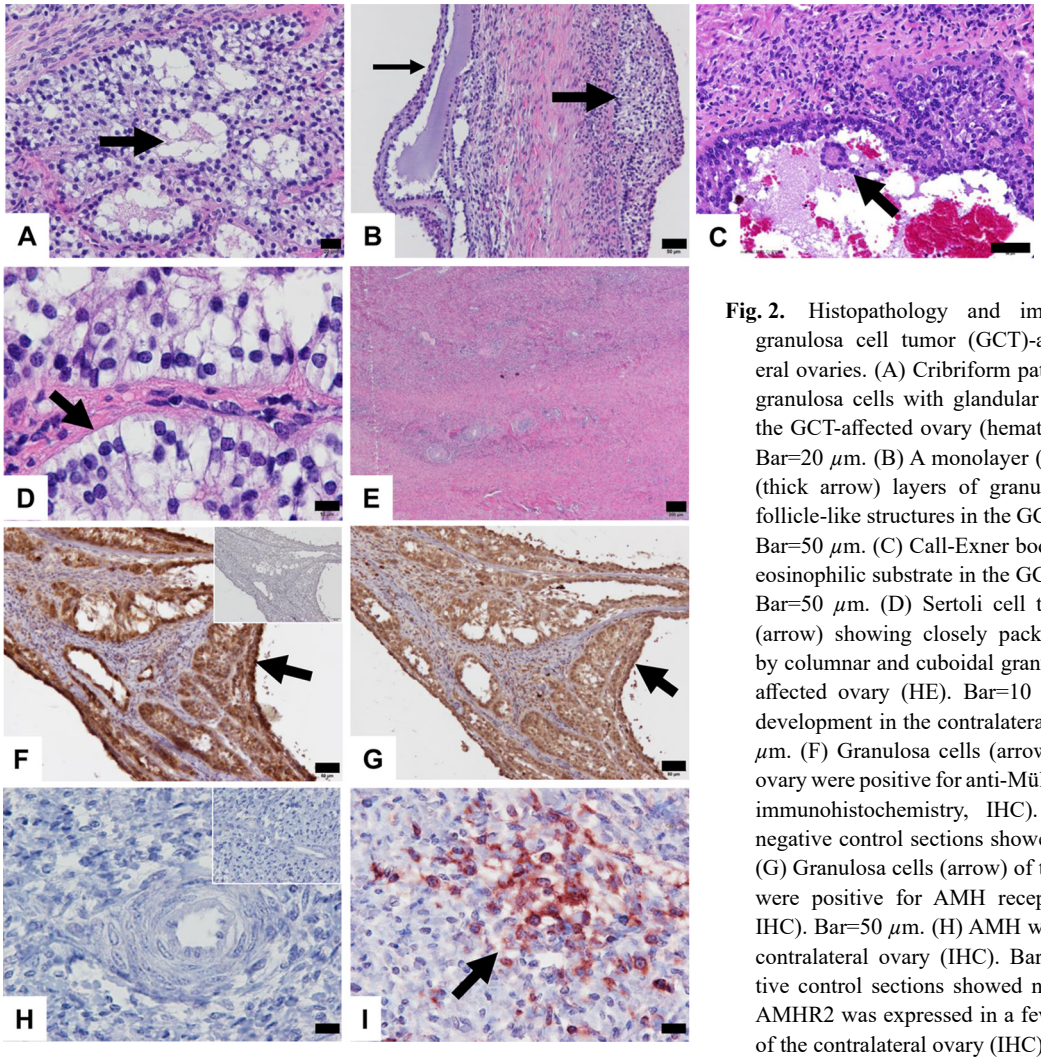

Fig. 2. Histopathology and immunostaining of the granulosa cell tumor (GCT)-affected and contralateral ovaries. (A) Cribriform pattern showing sheets of granulosa cells with glandular perforation (arrow) in the GCT-affected ovary (hematoxylin and eosin, HE). Bar=20 μm. (B) A monolayer (thin arrow) to multiple (thick arrow) layers of granulosa cells surrounding follicle-like structures in the GCT-affected ovary (HE). Bar=50 μm. (C) Call-Exner bodies (arrow) around the eosinophilic substrate in the GCT-affected ovary (HE). Bar=50 μm. (D) Sertoli cell tumor-like morphology (arrow) showing closely packed solid tubules lined by columnar and cuboidal granulosa cells in the GCT-affected ovary (HE). Bar=10 μm. (E) No follicular development in the contralateral ovary (HE). Bar=200 μm. (F) Granulosa cells (arrow) of the GCT-affected ovary were positive for anti-Müllerian hormone (AMH; immunohistochemistry, IHC). Bar=50 μm. Insert: negative control sections showed no positive staining. (G) Granulosa cells (arrow) of the GCT-affected ovary were positive for AMH receptor type 2 (AMHR2; IHC). Bar=50 μm. (H) AMH was not expressed in the contralateral ovary (IHC). Bar=10 μm. Insert: negative control sections showed no positive staining. (I) AMHR2 was expressed in a few stromal cells (arrow) of the contralateral ovary (IHC). Bar=10 μm.

Surgical ablation is the only treatment for a GCT-affected ovary [18]. In heavy draft horses, laparoscopic surgery is recommended because they have a higher risk of post-surgical complications and higher mortality rate after general anesthesia compared with light horse breeds [16]. Ultrasonic dissecting and coagulating devices used for laparoscopic surgery are designed for blood vessels up to 3 mm in diameter [13]. However, the case presented here had a giant ovary weighing around 17 kg, and thus the diameter of the blood vessel supplying blood to the affected ovary was too large for dissection. Consequently, a laparoscopic approach was impossible in this case. The mare might have had more chance of survival if laparoscopic surgery had been performed at early stage.

According to the histopathology, it was clear that the enlarged ovary of the current case contained a GCT, as there was an aggressive proliferation of granulosa cells occupying most of the ovarian mass. The immunoreactive AMH expression in the affected ovary and high concentration of AMH in plasma together implied that these neoplastic cells were actively secreting AMH into the blood circulation. On the other hand, the receptor for AMH was also positively expressed in the same neoplastic cells, suggesting autocrine and paracrine actions of AMH in the affected ovary [19]. If AMH has autocrine and paracrine actions in a GCT-affected ovary, what are these actions? There is a speculation that AMH might be an inhibitor of granulosa tumor cell growth [2], as the addition of AMH to a human GCT cell culture causes apoptosis and decreases the number of tumor cells. Considering this, it can be inferred that AMH secretion might be part of a feedback mechanism which inhibits neoplastic cell growth in the GCT-affected ovary in mares. However, in the current case, the amounts of AMH in even follicular cysts and blood were very high, and growth of the GCT mass was not inhibited. This could be explained by the fact that the tumor mass in the present case was too big to

show the effect of AMH, and this was supported by evidence showing that there is an inverse relationship between tumor size and the mRNA expression of AMH in human GCTs [2].

There are many case reports of equine GCTs that have focused on the affected ovary [10]; however, there is not enough information available about the contralateral ovarian structure and function. According to the histopathology in the present case, the contralateral ovary showed no follicular development. Immunostaining confirmed that the contralateral ovary did not secrete AMH. Interestingly, positive staining of AMHR2 in the contralateral ovary implied that AMH might have endocrine action there, for example, inhibition of follicular development. This idea was first hypothesized by Ball *et al*. [5], and it is also supported by the fact that AMH has an inhibitory effect on primordial follicle recruitment in mice [11]. However, there are no previous reports about direct or indirect effects of AMH on the contralateral ovary via its receptor (AMHR2) in equine GCTs. On the other hand, inactivity of the contralateral ovary is considered to be related to suppression of FSH due to the high amount of inhibin hormone secreted by the GCT-affected ovary [18]. Moreover, ovarian steroid hormones such as testosterone and estradiol may inhibit FSH secretion, although not all GCT-affected mares secrete high amounts of these steroid hormones [18].

Since the equine GCT is a hormonally active tumor [18], it is advisable to measure hormone concentrations in circulation. GCT-affected mares often have low levels of progesterone and higher levels of testosterone and inhibin in their blood [4, 18]. In this case, the plasma progesterone level was low (<1.0 ng/m*l*), indicating the absence of a functional corpus luteum. However, non-classical GCT cases sometimes have a functional corpus luteum and higher progesterone level [8, 14, 21]. Having high levels of testosterone (>10 ng/d*l*) in blood is related to stallion-like behavior and a high number of ovarian theca cells in GCT-affected mares [18]. The current case supported this, as it had a low amount of plasma testosterone (4.7 ng/d*l*), few theca cells, and no behavioral changes. In general, serum estradiol levels in equine GCT cases are not elevated; however, the case described here showed a slight increase in plasma estradiol (66.5 pg/m*l*) compared with the normal range (20–45 pg/m*l*) in cycling mares [18]. On the other hand, the estradiol level in cyst fluid (15,200 pg/m*l*) was lower compared with that in normal preovulatory follicular fluid (65,374 pg/m*l*) [6], suggesting lower aromatization action (production of estrogen from testosterone) in the affected ovary.

Recently, AMH has been reported to be the most reliable marker for diagnosing equine GCT, as compared with the inhibin and testosterone hormones [3, 9]. The cut-off value of serum AMH for equine GCT diagnosis is 4 ng/m*l* [3]. In this case, the plasma AMH level was almost 1,000 times higher than the cut-off value, which undoubtedly confirmed the diagnosis of GCT. According to a study in women with GCTs, positive correlation exists between serum AMH and the size of the GCT mass [12]. The current case supports the idea that the larger the tumor, the higher the AMH concentration.

To the best of our knowledge, this is the first report of the AMH level in ascites fluid in equine GCT (2,210 ng/m*l*); the level was lower than those in plasma (4,210 ng/m*l*) and ovarian cyst fluid (6,640 ng/m*l*), but it still seemed high. The reason why the AMH level in ovarian cyst fluid was higher than those in plasma and ascites fluid was probably related to the ovary itself being an original source of AMH production [9]. In women, the AMH levels in plasma and peritoneal fluid are well correlated [15]. However, it was unclear whether the AMH in the ascites fluid originated from the blood circulation or leaked directly from the GCT-affected ovary in the current case.

In conclusion, we described the findings of a case of unilateral GCT in a Breton draft mare which had a giant ovary weighing around 17 kg. The case described here clearly showed that the diameter of the blood vessel in the ovarian broad ligament is a critical factor when laparoscopic ovariectomy is attempted. The combination of histopathology and IHC analysis revealed that follicular development was inhibited in the contralateral ovary and that the contralateral ovary did not secrete AMH. However, the AMHR2 expression in the contralateral ovary, particularly in stromal cells, suggests that AMH might have an effect there. This is also the first report of AMH in the ascites fluid of a GCT-affected mare, suggesting the diagnostic potential of peritoneal fluid for equine GCT. All in all, ultrasonography, plasma AMH measurement, and histopathology analysis are important tools for equine GCT diagnosis.

Acknowledgments

This work was supported by the Racehorse Production and Training Research Grant Program of the Japan Racing Horse Association. The authors would like to thank A. Goto, A. Chiba, A. Tomikawa, T. Moriyama, and M. Nomura for technical assistance during laparoscopy and necropsy.

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