The *FOXL2* Mutation (c.402C>G) in Adult-Type Ovarian Granulosa Cell Tumors of Three Japanese Patients: Clinical Report and Review of the Literature

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Adult-type granulosa cell tumor (AGCT) is a rare class of malignant ovarian tumor with unique features, characterized by slow growth, late recurrence, relatively good prognosis and unified cause in almost all patients. The *forkhead box L2 (FOXL2)* gene encodes an essential transcription factor in the ovary. FOXL2 is important in female sex determination, follicle recruitment, and granulosa cell development. About 70-97% of AGCTs were reported to carry a somatic mutation c.402C>G (C134W) in the *FOXL2* gene. However, it is unknown whether AGCTs of Japanese patients harbor the *FOXL2* c.402C>G mutation. Here, we report a mutational analysis of the *FOXL2* gene in four Japanese patients with AGCTs, and we review the literature to determine the precise incidence of *FOXL2* mutations in AGCTs. All four patients were analyzed by immunohistochemistry for FOXL2. Genomic DNA was extracted from paraffin-embedded tissues, and was analyzed to detect the c.402C>G mutation in *FOXL2* by direct sequencing. All tumors were stained with FOXL2. Three of the four tumors harbor the c.402C>G mutation. Based on the literature review, FOXL2 immunostaining is a highly specific marker for sex cord-stromal tumors (SCSTs), but it is not specific for AGCTs, one subtype of SCSTs. We identified 340 patients with the *FOXL2* mutation (c.402C>G) and determined that the incidence of the mutation is 91.9% in AGCT patients. Therefore, this *FOXL2* mutation is specific to AGCTs in the ovary and is useful for diagnosis of this disease.

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Introduction

Granulosa cell tumors (GCTs) of the ovary are sex cord-stromal tumors (SCSTs) and comprise less than 5% of all malignant ovarian tumors. GCTs have been classified into adult or juvenile clinicopathologic type (Schumer and Cannistra 2003). Adult-type GCTs (AGCTs) account for 95% of GCTs and most commonly develop during the perimenopausal or early postmenopausal period. GCTs are characterized by slow and indolent growth and a tendency for late recurrence with intervals of 10-20 years. Although the prognosis is often favorable, tumors that recur or are advanced-stage indicate poor prognosis, with < 80% of these patients dying from their disease (Young and Scully 1992). AGCTs are known for their genomic stability (Koukourakis et al. 2008) as compared to other solid tumors (Fuller and Chu 2004). However, Shah et al. (2009) recently analyzed the whole genome of four AGCTs using whole-transcriptome paired-end RNA sequencing. They found high incidence (86/89; 97%) of recurrent somatic mutation in the forkhead box L2 (FOXL2) gene in AGCTs (Shah et al. 2009). This was the first reported association between a somatic mutation in *FOXL2* and cancer. The expression of FOXL2 in GCTs is suppressed in the majority of juvenile-type GCTs, particularly those with the most aggressive pattern of progression (Kalfa et al. 2007).

In the present study, we investigated the immunohistochemical expression of FOXL2 and the presence of the mutation FOXL2 c.402C>G (C134W) in 4 Japanese patients with AGCTs. In addition, the literature on FOXL2mutations in AGCTs was reviewed to determine the precise incidence of FOXL2 mutations in AGCTs.

Materials and Methods

Patient collection

This study was performed in patients at the Shiga University of Medical Science Hospital. The protocols and consent forms were approved by the Institutional Review Board at Shiga University of Medical Science, and written informed consent was obtained from each patient before participating in the study. There were no patients of juvenile-type GCTs (JGCTs) in our hospital.

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Immunohistochemistry

Paraffin blocks of AGCTs were cut into 4-µm slices. Slides were then dewaxed and dehydrated for immunohistochemical analysis. Antigen retrieval was performed with 0.01 M citric acid (pH 6.0) in a water bath at 98°C for 30 min. Immunohistochemical staining was performed using the VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA, USA). Endogenous peroxidase was blocked with 3% hydrogen peroxide for 20 min at room temperature. Tissue sections were incubated overnight at 4°C with rabbit polyclonal anti-human FOXL2 antibody (ab5068, Abcam, Cambridge, MA, UK) at a 1 : 200 dilution, followed by a 1-h incubation with secondary antibody. An avidin-biotin immunoperoxidase system and DAB (Vector Laboratories) were used to visualize the bound antibody, and sections were counterstained with Harris hematoxylin. A patient was considered negative when no nuclear immunoreaction was observed, whereas a positive patient showed nuclear immunostaining. A normal ovarian tissue sample was used as a positive control. Negative controls were not exposed to primary antibody.

DNA isolation and FOXL2 mutational analysis

Genomic DNA was isolated from paraffin-embedded tissue blocks using the NucleoSpin Tissue kit (Machrey Nagel, Düren, Germany), in accordance with the manufacturer's instructions. The FOXL2 gene was amplified using a semi-nested technique, as described previously (D'Angelo et al. 2011). The first pair of oligonucleotides consisted of a forward primer (F1), 5'-CCAGTACATCAT CGCGAAGTTCCCG-3', and reverse primer (R), 5'-CTCCGGCCC CGAAGAGCC-3'. The thermal cycling conditions included an initial denaturation step for 5 min at 94°C, followed by 35 cycles of 30 sec at 94°C for denaturing genomic and amplified DNA, 30 sec at 60°C for annealing primer to DNA and 30 sec at 72°C for extension, and 7 min at 72°C for final extension. All PCR products (269 bp) were subsequently used as templates for nested PCR, in which the same reverse primer (R) and nested forward primer (F2), 5'-CAGCCTCAA CGAGTGCTTCAT-3', were used. The 194-bp obtained fragment contained codon 134. PCR products were purified using NucleoSpin Gel and PCR Clean-up kit (Machrey Nagel), and were subjected to direct sequencing using the ABI PRISM Big Dye X terminator v1.1 cycle sequencing kit (Life Technologies, Carlsbad, CA, USA). Sequencing fragments were analyzed by capillary electrophoresis with an automated ABI PRISM 3130x Genetic Analyzer (Life Technologies).

RNA extractions and quantitative real-time PCR

The expression levels of FOXL2 mRNA were evaluated by quantitative reverse transcription PCR. Total RNA was extracted from paraffin-embedded AGCT tissues using the NucleoSpin FFPE RNA kit (Machrey Nagel) in accordance with the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 μ g of total RNA using the PrimeScript RT reagent kit (Takara Bio, Shiga, Japan). PCR samples were prepared up to a final volume of 20 μ l using 1 × SYBR Green Master Mix (Roche, Laval, Canada). Primer sequences used were 5'-TTTGTCCCCTCAGTTTATTCC-3' (sense) and 5'-TGAATTTGGGCAGGAGACG-3' (antisense) for FOXL2 (Jamieson et al. 2010), and 5'-AAATCCCATCACCAT CTTCCA-3' (sense) and 5'-AATGAGCCCCAGCCTTCTC-3' (antisense) for glyceradehyde-3-phosphate dehydrogenase (GAPDH). All treatment pair reactions were performed in triplicate. Quantitative PCR was performed on the Roche LC480 system (Roche) under the following conditions: preamplification, 95°C for 5 min; amplification, 45 cycles of 95°C for 10 sec, 60°C for 10 sec, 72°C for 10 sec, 80°C for 2 sec, followed by a single acquisition; melting curve, 95°C for 5 sec, 65°C for 1 min, ramped to 98°C at 5°C per second with continuous acquisition. Ct values were used to compute levels of mRNA expression from the standard curve. Analytical data were adjusted based on the mRNA expression of GAPDH as an internal control.

Results

Clinicopathologic features are summarized in Table 1. The age of the 4 patients with AGCT ranged from 41 to 63 years (average, 56.25 years). Only Patient 2 was pre-menopausal; the other 3 patients were post-menopausal. In postmenopausal women, pre-operative endocrinological abnormalities included elevation of serum estradiol and suppression of follicle-stimulating hormone (Table 1). The size of the tumors ranged from 9 to 21 cm (average, 10.5 cm). All patients underwent total hysterectomy and bilateral salpingo-oophorectomy with or without partial omentectomy. All patients had stage IA disease and experienced an uneventful clinical course after surgery during a followup period ranging from 47 to 121 months. No symptoms of blepharophimosis, ptosis, and epicanthus inversus syndrome (BPES) were observed in any patient.

The expression status of FOXL2 in AGCTs was evaluated (Fig. 1). FOXL2 was expressed in AGCTs of all patients. Cell nuclei were robustly stained.

Employing direct sequencing analysis, we examined our tumor panels for the presence of the previously reported *FOXL2* mutation using DNA from paraffin-embedded tissue blocks. Three of four AGCT specimens were found to harbor the mutation [c.402C>G (p.C134W)]. The *FOXL2* (c.402C>G) mutation was heterozygous in all of the mutation-positive samples (Fig. 2).

In order to investigate whether *FOXL2* mutation influences mRNA levels, FOXL2 mRNA levels were determined by quantitative real-time PCR in AGCTs with the mutation (C134W) and in AGCTs without the mutation.

Patient 1 expressed ten-fold higher mRNA levels than Patient 3, while the mRNA levels of Patient 2 and Patient 4 were between those of Patient 1 and Patient 3. Thus, the levels of FOXL2 mRNA were not correlated in AGCTs, irrespective of the presence of the mutation (Fig. 3).

Discussion

GCTs are rare SCSTs occurring in women of all ages. These tumors account for only 3-5% of all ovarian tumors and have been classified into adult and juvenile clinicopathologic types. The tumors may exhibit a variety of histological patterns, which makes accurate diagnosis difficult, and the pathogenesis of the tumors is also poorly understood due to a lack of pathological unity.

FOXL2 is a winged helix/forkhead transcription factor. In humans, FOXL2 is expressed almost exclusively in ovarian tissue, in which it is involved in ovarian development (Cocquet et al. 2002; Schmidt et al. 2004). It has been

	Patient 1	Patient 2	Patient 3	Patient 4
Age at diagnosis (years)	62	41	63	59
Menopausal status	Post	Pre	Post	Post
Initial manifestations	Lower abdominal pain	Lower abdominal pain	Vaginal bleeding	Vaginal bleeding
Side of tumor formation	Right	Left	Right	Right
Tumor size (cm)	$11 \times 9 \times 5$	$21 \times 15 \times 12$	$10\times9\times10$	$7\times9\times9$
FSH (mIU/ml)	1.29	Not done	2.0	0.5
Estradiol (pg/ml)	64.37	Not done	49.3	14
Operation procedure	TAH-BSO	TAH-BSO-pOM	TAH-BSO	TAH-BSO-pOM
Stage	IA	IA	IA	IA
Follow-up (months)	47	51	76	121
Outcome	NED	NED	NED	NED

Table 1. Clinicopathologic feature of adult-type granulosa cell tumors.

NED, no evidence of disease; TAH, total abdominal hysterectomy; BSO, bilateral salpingo-oophorectomy; pOM, partial omentectomy; FSH, follicle-stimulating hormone.

The age of the 4 patients with AGCT ranged from 41 to 63 years. Only Patient 2 was pre-menopausal; the other 3 patients were post-menopausal. Elevated serum estradiol (E2) and suppressed FSH were observed in all post-menopausal patients. Tumor size ranged from 9 to 21 cm. All patients underwent total hysterectomy and bilateral salpingo-oophorectomy with or without partial omentectomy.

There was no recurrence in the patients after surgery over a follow-up period ranging from 47 to 121 months. We identified the only mutation of FOXL2 c.402C>G in the 4 patients with AGCTs.

reported that the somatic mutation in FOXL2 gene (c.402C>G) is present in more than 95% of ovarian AGCTs, but not in several other neoplasms (Schrader et al. 2009; Shah et al. 2009; Benayoun et al. 2010; Jamieson et al. 2010; Kim et al. 2010a; Lee et al. 2010; Al-Agha et al. 2011; Alexiadis et al. 2011; D'Angelo et al. 2011; Gershon et al. 2011; Hes et al. 2011). Although the functional consequence of the missense mutation remain unclear, Benayoun et al. (2013) reported that FOXL2 mutation induced genes that are associated with faster cell cycling and the downregulation of genes associated with cell death. Over time, such defects may be responsible at least partly for the malignant transformation of healthy granulosa cells into AGCT (Benayoun et al. 2013). Shah et al. (2009) identified a single somatic, missense mutation in FOXL2 (c.402C>G) using whole-transcriptome paired-end RNA sequencing of four GCTs. Subsequent direct sequencing of DNA from additional GCTs revealed that the mutation was present in 86 of 89 (97%) morphologically diagnosed adult granulosa cell tumors (Shah et al. 2009).

Eleven articles have reported patients of the somatic mutation in the *FOXL2* gene (c.402C>G) among ovarian AGCTs (Schrader et al. 2009; Shah et al. 2009; Jamieson et al. 2010; Kim et al. 2010a, b; Lee et al. 2010; Al-Agha et al. 2011; Alexiadis et al. 2011; D'Angelo et al. 2011; Gershon et al. 2011; Hes et al. 2011). No article have identified the mutations other than the mutation of *FOXL2* c.402C>G. Table 2 shows all of the patients in these articles, including our four patients, and shows the authors, number of patients studied for each series, number/percentage of mutated tumors, number/percentage of primary tumors in each

study. The number of patients in each series ranged from 3 to 89. The detection methods also varied widely, and included direct sequencing, a matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) genotyping assay, and various types of PCR. The percentage of mutated tumors in the reviewed series was consistent with existing reports, ranging from 70.4% to 100% (mean, 91.9%). The FOXL2 mutation occurred in more than 90% of mutated tumors in all but two studies (Alexiadis et al. 2011; D'Angelo et al. 2011), in which FOXL2 mutation occurred in 70.4% and 85.7% of tumors. Excluding this study, the FOXL2 mutation occurred in 94.0% of AGCTs. D'Angelo et al. (2011) suggested that no correlation was encountered between the presence of a FOXL2 mutation and various clinicopathologic parameters, expect for the presence of different sex-cord components.

In addition to a single somatic FOXL2 mutation in most AGCTs, germline FOXL2 mutations associated with BPES have also been reported. BPES patients represent an autosomal-dominant condition characterized by defects in eyelid development with (type I) or without (type II) premature ovarian failure (Fraser et al. 1988; Crisponi et al. 2001; De Baere et al. 2005, 2009; Raile et al. 2005; Nallathambi et al. 2007; Beysen et al. 2009; Dipietromaria et al. 2009; Méduri et al. 2010). Within the ovary, FOXL2 has been shown to be essential for ovary development and function (Pisarska et al. 2011). In BPES, FOXL2 mutations can induce cytoplasmic mislocalization as well as cytoplasmic or nuclear aggregation (Beysen et al. 2008; Dipietromaria et al. 2009), which most likely contributes to altered function in some patients. Interestingly, although germline FOXL2 mutations are present in BPES patients,



Fig. 1. Immunohistochemical analysis of FOXL2 in AGCTs and normal antral follicles.

Histology and immunohistochemistry for *FOXL2* in the samples heterozygous (A-F) or negative for the *FOXL2* C134W mutation (G, H). Hematoxylin and eosin staining (A, C, E, and G), immunostaining of *FOXL2* (B, D, F, and H). Patient 1 (A, B), Patient 2 (C, D), Patient 3 (E, F) and Patient 4 (G, H) shown in Table 1. Expression of *FOXL2* was determined in a follicular cystic ovary as a positive control (I). *FOXL2* protein was expressed in all patients. Expression levels of *FOXL2* did not differ between wild-type (B, D, F) and mutant samples (H). Original magnification, × 200. Scale bar, 100 μ m.



Fig. 2. Mutations in *FOXL2* gene codon 134 in granulosa cell tumors. Chromatograms of *FOXL2* gene at position 402, obtained from *FOXL2* gene analysis. Arrowhead indicates position 402 on the patient's chromatogram.



Fig. 3. FOXL2 mRNA expression in AGCTs.

AGCTs with the *FOXL2* (c.402C>G) mutation (Patients 1, 2 and 3) tended to have slightly higher mRNA expression than tumors with wild-type *FOXL2* (Patient 4). MT, AGCT with mutated-type *FOXL2* (C134W); WT, AGCT with wild-type *FOXL2*. GCTs do not develop in these patients. In type I patients, the predicted loss of ovarian function caused by the presence of the mutation during ovary development seems not to be related to aberrant GC proliferation and tumor development (Kalfa et al. 2007). In type II BPES patients, as *FOXL2* mutations do not appear to disrupt ovarian function, they likely exhibit normal regulation of ovarian gene targets. Thus, taking AGCTs and BPES into consideration, the phenotype is completely different based on where the mutation occurs in the *FOXL2* gene. That is to say, FOXL2 plays a diverse range of roles in granulosa cells.

FOXL2 is expressed in the granulosa cells of small and medium follicles (Crisponi et al. 2001; Pisarska et al. 2004; Schmidt et al. 2004; Ottolenghi et al. 2005) and is likely to play a role in granulosa cell proliferation and differentiation.

FOXL2 transcriptionally suppresses cellular differentiation (Bourguignon et al. 1998; Sullivan et al. 2001). FOXL2 was initially found to transcriptionally suppress the

Table 2. FOXL2 mutation frequency among ovarian adult-type granulosa cell tumors.

Source	Studied patient number	Mutated patient number	% of mutated patients	Heterozygous patient number	% of heterozygous patients
Shah et al. 2009	89	86	96.6%	83	96.5%
Schrader et al. 2009	11	11	100%	Not shown	Not shown
Kim, M.S. et al. 2010	56	53	94.6%	52	98.1%
Kim, T. et al. 2010	20	18	90.0%	Not shown	Not shown
Jamieson et al. 2010	56	52	92.9%	49	94.2%
Lee et al. 2010	3	3	100%	0	0%
Hes et al. 2011	3	3	100%	3	100%
Al-Agha et al. 2011	42	39	92.9%	Not shown	Not shown
D'Angelo et al. 2011	27	19	70.4%	Not shown	Not shown
Gershon et al. 2011	19	17	89.4%	Not shown	Not shown
Alexiadis et al. 2011	14	12	85.7%	11	91.7%
Present study, 2013	4	3	75%	3	100%
Total	344	316	91.9% (316/344)	201	94.3% (201/213)

Studies varied in size from 3 to 89 patients. The percentage of mutated tumors in the reviewed series was consistent, ranging from 70.4% to 100% (mean, 91.9%). *FOXL2* mutation occurred in more than 90% of mutated tumors in all but two studies, in which *FOXL2* mutation was found in 70.4% and 85.7% of patients. Ninety-four percent of patients carried a heterozygous mutation of *FOXL2*.

steroidogenic acute regulatory (StAR) gene (Bourguignon et al. 1998). StAR is a key molecule in steroidogenesis that translocates cholesterol from the outer to the inner membrane of mitochondria. The expression of StAR depends on the follicle size, and it is expressed in granulosa cells of large preovulatory follicles, but not small and medium follicles. FOXL2 binds to the human StAR promoter and suppresses its activity (Bourguignon et al. 1998), suggesting that transcriptional suppression by FOXL2 inhibits StAR expression in immature follicles. FOXL2 also acts as a transcriptional suppressor of both Cyp19 (P450aromatase) and Cyp11a (P450scc) in immature mouse ovary follicles (Bentsi-Barnes et al. 2010). P450aromatase converts testosterone to estradiol, a key product of differentiated granulosa cells, and P450scc cleaves the side chain of cholesterol to produce pregnenolone, the first step of steroid hormone synthesis. These results suggest that FOXL2 affects sex steroidogenesis in granulosa cells, which is involved in granulosa cell proliferation. FOXL2 also suppresses the transcription of cyclin D2, which directly regulates the cell cycle in granulosa cells (Sicinski et al. 1996). These results indicate that FOXL2 acts as a suppressor of premature differentiation and/or proliferation of granulosa cells, thus preventing the premature depletion of ovarian follicles, which occurs when FOXL2 function is altered in patients of AGCT and BPES.

The KGN cell line (Pisarska et al. 2004) has been used as a model of granulosa cells of the postnatal ovary. These granulosa cells are derived from an AGCT with the c.402G>C mutation in *FOXL2*. Therefore, they may actually reflect changes that occur in AGCTs. In this cell line, a large number of genes were regulated by FOXL2 (Diaz et al. 1988). These genes include cytokines such as interleukin-12 subunit α (IL12A) (Wolf et al. 1994) and interleukin-29 (IL29) (Srinivas et al. 2008) and anti-apoptotic factors such as BCL2 (B-cell lymphoma 2)-related protein A1 (BCL2A1) (Son et al. 2006) and immediate early response 3 (IER3) (Ahrens et al. 2002). Thus, mutations in *FOXL2* are thought to modify cell proliferation and apoptosis through differential regulation of a diverse set of genes in granulosa cells.

Histopathologic diagnosis of AGCTs is often difficult, and it is important to correctly identify this type of tumor because of its relatively favorable prognosis. Positive immunohistochemical stains for inhibin- α , calretinin and CD99 are widely used to identify SCSTs. In all 4 of our patients, the tumors were positively stained for inhibin- α , calretinin and CD99 (data not shown). Recently, Al-Agha et al. (2011) suggested that FOXL2 staining is a relatively sensitive and highly specific marker for SCST that works in formalin-fixed, paraffin-embedded tissue. However, FOXL2 is strongly expressed in both FOXL2-mutant SCST and a subset of SCST without the (c.402C>G) mutation in FOXL2 (Al-Agha et al. 2011). Furthermore, localization of the mutated FOXL2 protein was normal (Shah et al. 2009; Benayoun et al. 2010). In present study, localization of FOXL2 protein was consistent in wild-type and mutant samples (Fig. 1).

FOXL2 immunostaining is a highly specific marker for SCST, but it is not specific for AGCT within SCST. From this point of view, immunohistochemical analysis of FOXL2 is not sufficient to diagnose AGCTs. Therefore, analysis of *FOXL2* mutation is a more reliable tool for AGCT diagnosis than immunohistochemical analysis of FOXL2 and other markers, including inhibin- α , calretinin and CD99, considering the specificity of *FOXL2* mutation in AGCTs.

We examined FOXL2 mRNA expression in AGCTs in

both mutant and wild-type samples. FOXL2 mRNA levels varied even in mutant AGCTs. Patient 1 expressed ten-fold higher mRNA levels than Patient 3, while mRNA levels of non-mutant Patient 4 was between those of Patient 1 and Patient 3. Based on our data, mutations did not affect FOXL2 mRNA levels, although the number of patients was small. Our findings are similar to the report of Jamieson et al. (2010), who found that FOXL2 mRNA levels in the mutation-positive AGCTs were similar across the mutationnegative AGCTs and normal ovary samples. However, D'Angelo et al. (2011) suggested that tumors harboring mutations tended to have higher FOXL2 mRNA expression than those with wild-type FOXL2, although the differences were not significant. In addition, they reported that FOXL2 mRNA expression has a prognostic impact, not only in AGCTs, but also in JGCTs. In AGCT patients, a high mRNA FOXL2 expression had a worse disease-free survival than those with lower expression.

Taken together, the present results suggest that the effects of the *FOXL2* mutation on FOXL2 mRNA levels are unclear and that it is impossible to determine wild-type or mutant status based on FOXL2 mRNA levels alone. We were also unable to identify pathologic type by expression levels of FOXL2 mRNA, but FOXL2 mRNA levels may be useful for predicting prognosis.

In conclusion, this is the first report to show the frequency of *FOXL2* mutations in Japanese AGCT patients. Our data support previous studies regarding the high incidence of the somatic mutations in the *FOXL2* gene (c.402C>G) in AGCTs. The literature review revealed that *FOXL2* mutations are present in 94.0% of AGCTs; thus, the mutational analysis of *FOXL2* is more useful for diagnosing AGCTs than the routinely used immunostaining for FOXL2 and other markers of SCSTs. Furthermore, expression levels of FOXL2 mRNA may be useful for predicting prognosis.

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Conflict of Interest

The authors declare no conflict of interest.

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