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*Original Study*

# No Activating Mutations of FSH Receptor in Four Children with Ovarian Juvenile Granulosa Cell Tumors and the Association of These Tumors with Central Precocious Puberty

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**Abstract.** *Study Objective:* The stimulation of the follicle-stimulating hormone receptor (FSHR) by circulating FSH or some activating mutations of the FSHR may play a causal role in the development of granulosa cell tumors of ovaries.

*Study design:* We evaluated four patients with ovarian juvenile granulosa cell tumors (age range, 2.4 to 7.2; median, 2.9 years) and five healthy pubertal girls (age range, 16 to 18.5; median, 16.8 years) for activating mutations in exon 10 of the FSHR. The patients were followed and evaluated clinically. Genomic DNA was extracted from the peripheral blood. Exon10 of the FSHR was evaluated for mutations.

*Results:* All four patients presented with signs of precocious puberty. One patient, who had markedly accelerated growth velocity and advanced bone age, developed central precocious puberty after the removal of her tumor. Another patient was diagnosed to have a left ovarian cyst without tumor recurrence approximately 3.3 years after the removal of the tumor. Activating mutations were not found, but previously reported polymorphisms (Ser680Asn and Ala307Thr) of the FSHR were detected in three of four patients and in three of five controls. The follow-up period of these four patients ranged from 4.5 to 8.8 years, with a median value of 6.7 years.

*Conclusions:* We did not find any activating mutation in exon 10 of the FSHR in our patients, and one patient developed precocious puberty after removal of her tumor. The development of ovarian tumors in these patients may have been caused by mutations at other exons of the FSHR and G protein subunits, so the association noted between central precocious puberty and granulosa cell tumors might not be coincidental.

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## Introduction

Juvenile granulosa cell tumors (JGCTs) are usually encountered in prepubertal girls and young women. In 80% of reported cases, the patients were younger than 20 years of age.[1−5] JGCTs are rare, accounting for approximately 1% of all malignant tumors that occur in the first 2 decades of life.[1,6,7] Accelerated growth and bone development are important features of JGCTs.[1,4,7] The secretion of estrogens and androgens by these tumors leads to isosexual precocious pseudopuberty, including breast development, clitoral enlargement, and growth of pubic and axillary hair in approximately 80% of prepubertal girls with JGCTs. It has also been reported that all signs of pseudopuberty have disappeared after removal of the tumor.[4,7] Older patients with JGCTs commonly present with menstrual irregularities. Abdominal distention, pain, and palpable tumor mass are common findings in all age groups.[1,2,4,7] The prognosis for children with JGCTs is usually good if they are diagnosed in the early stages.[4,7,8] The tumor-related mortality rate was reported to be approximately 9% in children.[4]

Recently, central precocious puberty has been reported in some patients with ovarian granulosa cell tumors and in one patient with an ovarian lipid cell tumor. Signs of puberty developed concomitantly with the tumor or some years after its removal. In view of these occurrences, it has been speculated that gonadotropin stimulation might play an important role in

the development of the ovarian tumors.[9−11] Although this subject is still controversial, supraphysiological gonadotropins used in infertile women have been reported to induce JGCTs.[12,13] It has also been claimed that exposure to endogenously increased estrogen might result in premature activation of the hypothalamic-pituitary-gonadal axis.[1] The follicle-stimulating hormone (FSH) receptors that are necessary for the development and maturation of the gonads belong to the family of G protein−coupled receptors, which are complex transmembrane proteins characterized by seven transmembrane domains. Both luteinizing hormone (LH) and thyroid-stimulating hormone (TSH) receptors are members of this super-family of receptors. The FSH receptor (FSHR) gene, which has 10 exons and 9 introns, is mapped to chromosome 2p21 in the human. The human FSHR cDNA was cloned in 1991 by Minegish and colleagues. Its size is 54 kilobase pairs (kbp). The extracellular domain of the human receptor is encoded by nine exons, ranging from 69 to 251 base pairs (bp). The C terminal region of the extracellular domain along with the transmembrane and intracellular domains are encoded by exon 10 and contain more than 1234 bp. The human gene encodes 695 amino acids, including a signal peptide with17 amino acids. The nine introns vary greatly in their corresponding sizes from 108 bp for intron 7 to 15 kbp for intron.[14] Granulosa cell tumors also express FSHR, and FSHR stimulation by gonadotropic hormones may play a role in the growth and differentiation of these tumors. Some studies suggest that stimulation of the FSHR by circulating FSH or activating mutations of the FSHR may play a causal role in the development and growth of these tumors. Kotlar and colleagues found a heterozygous T → C mutation at nucleotide 1777 that converts codon 591 from phenylalanine to serine in 69% of the patients with ovarian sex cord tumors, which was an inactivating mutation. Based on their findings, we suggest that the mutation alters other signaling pathways, except for the cyclic adenosine monophosphate (cAMP) pathway.[15] In other limited studies, the presence of the mutations in the FSHR have not been shown.[16,17]

We evaluated our patients who had ovarian JGCTs in view of their clinical aspects and the activating mutations in exon 10 of the FSHR.

## Materials and Methods

### Subjects

Four patients who presented to the Pediatric Endocrinology Clinic of the Istanbul Faculty of Medicine with signs of precocious puberty, such as breast development and vaginal bleeding and who were diagnosed to have JGCTs, and five healthy pubertal girls without pubertal disorders were evaluated for mutation of the exon 10 of the FSHR gene. The ages of the patients ranged from 2.4 to 7.2 years, with a median value of 2.9 years at the time of diagnosis of the tumors. The age of the control group ranged between 16 and 18.5 years, with a median value of 16.8 years. The control group was selected from adolescents who were followed-up in the outpatient unit for adolescents in our clinics. They were healthy, and their puberty timing was in the normal ranges. Pubertal staging was graded according to Tanner staging in all patients.[18] The values of height, weight, and target height were expressed as standard deviation scores (SDS) according to the standards for Turkish children.[19] The molecular analyses were conducted at the Pediatric Endocrinology and Diabetology Section of the Department of Pediatrics of Indiana University School of Medicine. Informed consent was obtained from the control group as well as from the parents of the control group and those of the JGCT patients.

**Hormonal Studies and Bone Age**. The gonadotropin releasing hormone (GnRH) stimulation test was performed in two of these patients prior to diagnosis and after the removal of tumor in one patient. The GnRH test was not performed on one patient who presented to the Pediatric Endocrinology Clinic after removal of her tumor. After administration of 100 μg GnRH, LH and FSH levels were measured at 30-minute intervals for 2 hours. Basal and peak LH and FSH levels were determined. All serum hormone levels were measured by radioimmunoassay using kits provided by Diagnostic Products (Los Angeles, CA). Bone age was detected according to the method of Greulich and Pyle.

**Molecular Analyses**. Genomic DNA was extracted from the peripheral blood of the patients and the control group using a QIAamp Blood Maxi (Qiagen, Valencia, CA). Exon 10 of the FSH receptor gene was subdivided into three fragments because of its large size. These fragments were amplified by polymerase chain reaction (PCR) in PTC-100 TM Programmable Thermal Controller Peltier-Effect Cycling (MI Research, Watertown, MA) using three pairs (forward and reverse) of primers (Table 1). Amplification by the PCR occurred in 100-μL reaction mixtures containing 100 ng genomic DNA. The PCR mixture contained 20 mmol/L Tris-HCl (pH 8.4); 50 mmol/L KCl; 50 mmol/L MgCl2; 10 mmol/L each of deoxynucleotide triphosphate; 10 μM/L each of forward and reverse primers; and 2.5 U of Taq DNA polymerase (GibcoBRL, Life Technologies, Carlsbad, CA). PCR cycling parameters were adjusted as follows: an initial 95°C denaturation step for 2 minutes; annealing at 53° to 61°C for 30 seconds; extension at 72°C for 1 minute 30 seconds; followed

**Table 1.** Oligonucleotide Primers Used for PCR Amplification of the Exon 10 of the FSHR and PCR Products and Localization of the PCR Products in the FSHR Gene

| Fragment Number | Primers | Size (bp) | Localization |
|---|---|---|---|
| **1** | Forward: 5′ CTACCCTGCACAAAGACAGTG 3′<br>Reserve: 5′ AGTTTGCCAGTCAAATGGCAT 3′<br>(nucleotides 922−1360) | **570** | Part of inotron9, TM1 (1099−1161)<br>1st IC loop, TM2 (1190−1263)<br>EC loop, TM3 (1330−1395) |
| **2** | Forward: 5′ CATACCAAGAGCCAATATCAC 3′<br>Reserve: 5′ GTGAAACAGAACCAGCGAA 3′<br>(nucleotides 1270−1845) | **575** | 1st EC loop, TM3 (1330−1395)<br>2nd IC loop, TM4 (1456−1524)<br>2nd EC loop, TM5 (1585−1650)<br>3rd IC loop, TM6 (1722−1794) |
| **3** | Forward: 5′ ATCACTGTGTCCAAAGCAAAG 3′<br>Reserve: 5′ GATACATTTTCACATTGTGT 3′<br>(nucleotides 1866−2220) | **354** | TM7 (1825−1890)<br>Cytoplasmic tail (1891−2088) |

EC, extracellular loop; IC, intracellular loop; PCR, polymerase chain reaction; TM, transmembrane domain 1−7.

by 29 cycles of denaturation at 95°C (30 seconds); annealing at 53° to 61°C (30 seconds); extension at 72°C (1 min); and a final extension step of 7 minutes at 72°C. The annealing temperature was changed according to the fragments (53°C for first and second fragments, 61°C for third fragments). All PCR products were analyzed by electrophoresis in 1% agarose gels stained with ethidium bromide. The PCR products were purified using Micron PCR Centrifugal Devices (Amicon-Bioseparation-Millipore, Millipore, Bedford, MA). PCR products were directly sequenced by automated DNA sequencing at Davis Sequencing Center (Davis Sequencing Inc., Davis, CA).

## Results

**Clinical Findings**. All patients except for patient 3 presented with complaints of breast development, vaginal bleeding, and abdominal pain, which had started about 2 months prior to presentation. Patient 3, in addition to the above symptoms, had markedly accelerated growth and growth of pubic and axillary hair. Her parents had become aware of the breast development at age 6 months and the vaginal bleeding and pubic and axillary hair at age 18 months. She was admitted to a hospital, where a JGCT was diagnosed, followed immediately by a left salpingo-oophorectomy. With the reappearance of the findings, she was referred to the Pediatric Endocrinology Clinic of the Istanbul Faculty of Medicine.

Except for patient 3, there were no noteworthy features in the patients' family histories. The family history of patient 3, however, revealed that her mother had been followed for a diagnosis of myotonic dystrophy (adult form) since the age of 25. Neurological development was normal in all patients.

The patients' clinical and laboratory findings are summarized in Table 2. None of patients had dysmorphic features or café-au-lait spots. An increased growth velocity for age (15.2 cm/yr within the past 6 months) was noted in patient 3. Bone age was also markedly advanced in this patient. Although estrogen levels were very high in all patients, gonadotropin levels were not suppressed. Patient 3, especially, had markedly elevated FSH and estrogen levels. Pelvic ultrasonography showed enlarged uteri and ovaries for age in all patients. Bone scintigraphy and roentgenograms were normal.

All patients underwent unilateral salpingo-oophorectomy. All tumors were evaluated as stage1 according to the International Federation of Gynecology and Obstetrics classification of ovarian tumors,[1] and in all cases the diagnosis of JGCT was confirmed by histopathological examinations.

Because pubertal signs, including breast development, pubic and axillary hair, and vaginal bleeding did not regress in patient 3 after surgery, this patient was reevaluated. A pelvic ultrasonography at that time revealed a right multicystic ovary and an enlarged uterus for age. Cerebral magnetic resonance imaging was normal. Based on clinical and hormonal findings, a diagnosis of central precocious puberty was made in this patient. Leuprolide acetate, a GnRH analog, was started at age 3.1 years at a dosage of 3.75 mg/month. The patient showed a good response to the treatment and her hormone levels (basal LH: 1.6 mIU/mL; FSH: 4.4 mIU/mL; estradiol: 30 pg/mL) and growth velocity decreased to normal ranges for age. Pubertal development was arrested at stage 2 of breast and stage 1 of pubic and axillary hair. She is still on leuprolide acetate treatment. She was recently seen at age 9.8 years, and her weight was 38 kg (1.5 SD); her height was 145.6 cm (2.2 SD); and her bone age was 10.8 years. Her plasma levels for LH and FSH were 1.38 and 0.35 mIU/mL, respectively, and her estradiol level was 0.01 pg/mL.

All patients are still being followed at 3- to 6-month intervals. In patient 2, at age 7.8 years, physical examination and growth velocity were normal and she exhibited no pubertal signs; the pelvic ultrasound examination showed a big ovarian cyst (about 4 cm in

**Table 2.** Some Clinical and Laboratory Findings in Patients With JGCT at Presentation and Follow-up

| Patient Number* | Age (years) | Weight kg (SDS) | Height cm (SDS) | Bone Age (years) | Target Height cm (SDS) | Tanner Stage** | | | Hormonal Findings*** | | | | | Pelvic Ultrasonography | Follow-up (years) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | B | AH | PH | LH (mIU/mL) | | FSH | | $E_2$ (pg/mL) | | |
| | | | | | | | | | Basal | Peak | Basal | Peak | Basal | | |
| 1 | 2.4 | 15.2 (1.5) | 92.5 (0.6) | 2.5 | 158 (-0.3) | 2 | 1 | 1 | 1.5 | 4.4 | 1.5 | 2.5 | 130 | $13 \times 9$ cm, right multicystic ovarian mass | No problem (7.2) |
| 2* | 3.4 | 15 (0) | 101.2 (0.66) | 4.2 | 154 (-0.88) | 3 | 1 | 1 | 3.0 | 9.4 | 1.5 | 8 | 54 | $11 \times 9 \times 5$ cm, right multicystic ovarian mass | Left ovarian cyst at age 7.8 yrs (6.2) |
| 3* | 2.5 | | | | | 3 | 2 | 2 | 0.73 | | 8.24 | | 90 | $12 \times 10 \times 7$ cm, left solid ovarian mass | Central precocious puberty (8.8) |
| | 3.1 | 21 (4.66) | 110.1 (3.97) | 7 | 157 (-0.44) | 3 | 2 | 2 | 1.6 | 12.5 | 4.4 | 19 | 30 | Right ovary : multicystic appearance | |
| 4* | 7.2 | 23 (0) | 121.3 (0.01) | 6.8 | 162 (0.31) | 2 | 1 | 1 | 3.0 | | 1.0 | | 70 | $15 \times 4 \times 8$ cm, right ovarian solid mass | No problem (4.5) |

*Polymorphisms (A 307 $Thr^{P1}$/Ser 680 $Asn^{P2}$) were detected in the patient.
**AH, axillary hair; B, breast [18]; PH, pubic hair.[18]
***Normal ranges (prepubertal; basal): LH: 0.02–0.3 mIU/mL; FSH: 0.26–3 mIU/mL; Estradiol <15 pg/ml.

diameter) in the left ovary. Tumor markers (alpha-fetoprotein, CA 125, beta human chorionic gonadotropin, chorioembryonic antigen, etc.) and hormone levels were within normal limits. The ovarian cyst did not spontaneously regress so, considering the possibility of rupture of the ovarian cyst, cyproterone acetate was started at a dosage of 100 mg/$m^2$/day at age 8.5 years. Some regression in the size of the ovarian cyst was observed at age 9 years when pelvic ultrasonography was repeated. She is still on cyproterone acetate.

Patients 1 and 4 had no problems. In patient 4, physiological puberty started at about 10 years of age. The other patients have not developed normal physiological puberty findings yet. The range of the follow-up period in these patients ranged from 4.5 to 8.8 years, with a median value of 6.7 years (see Table 2).

**Molecular Analysis**. Direct sequencing of PCR products amplified from exon 10 of the FSH receptor of four patients and five controls did not reveal the presence of activating mutations in exon 10. But three patients (patients 2, 3, and 4) and three controls (1, 2, and 3) had a point mutation (G → A) at nucleotide 2105, resulting in the substitution of asparagine for serine at codon 680 (AGT → AAT); three patients (2, 3, and 4) and three controls (1, 3, and 4) had the point mutation (G → A) at nucleotide 985, resulting in the substitution of threonine for alanine at codon 307 (GCT → ACT). Both mutations were previously reported as polymorphisms in exon 10 of the FSH receptor gene.[14] The frequency of polymorphisms at codon 307 (three of four patients, three of five controls) and codon 680 (three of four patients, three of five controls) in the exon 10 of the FSH receptor were similar in the patients and in the control group.

## Discussion

The molecular pathogenesis of ovarian granulosa cell tumors is still unknown. Some studies suggest that gonadotropic hormones, particularly FSH, affect the development of ovarian tumors. These studies claim that granulosa cell tumors contain FSHRs and that tumorigenesis is affected by FSH and by activation of FSHR signaling pathways.[20–22]

Most mutations of the FSHR gene are found in exon 10,[14,23] so our subjects were evaluated only for exon 10 mutations. In our study, activating mutations were not detected. Kotlar and colleagues reported a study in which ovarian tumor and control DNA samples were evaluated for mutations in the exon 10 of the FSHR mutation (F591S) in 9 of 13 (69%) of the sex cord tumors and 2 of 3 ovarian small cell tumors. However, it was found that the mutant receptor did

not stimulate cAMP production in functional analyses. The tumor DNA was extracted from paraffin-embedded tissue samples. These authors suggest that the mutation plays a role in the development of the ovarian tumors, although it is surprising that this mutation was an inactivating mutation.[15] Fuller and colleagues evaluated a similar group of ovarian granulosa cell tumors for FSHR mutations. They could not find any FSHR mutations in 15 patients with granulosa cell tumors.[16] In addition, Ligtenberg and colleagues evaluated 23 ovarian granulosa cell tumors for mutations of whole FSHR gene, and they too could not detect any mutation.[17]

Two different polymorphisms, previously reported, were found in patients and in the control group. The frequency of occurrence of both polymorphisms was similar in both groups. Codon 307 was found in three of four patients; in controls, in three of five. Codon 680 was found in three of four patients and in three of five controls. The similarity of the frequency of polymorphisms in patients and controls led us to believe that they had no any clinical significance.

In some studies, the sequence of human ovarian FSHR[24] was reported to be different from the sequence of human testis receptor by seven nucleotides and five amino acids.[25] To date, two polymorphic sites in exon 10 of FSHR were reported. One of them was found in the extracellular domain at codon 307, which can be occupied by either alanine or threonine. The other was demonstrated at codon 680 in the intracellular domain, which can be occupied by either asparagine or serine.[14,26–30] Batista and colleagues showed allelic variants at codon 307 (in three of four patients) and at codon 680 (in one of four patients) in the girls with gonadotropin-independent precocious puberty resulting from autonomous cystic ovaries.[30] All studies suggest that these polymorphisms had no clinical significance in any of the subjects. However, it is still not known whether these polymorphisms have any physiological effects on FSH binding and signal transduction pathways. Although some studies suggest that a receptor variant or combination of variants may be related to a higher incidence of reproductive disorders,[29] valid evidence to prove this hypothesis does not exist.

Recently, some studies in patients with granulosa cell tumors have reported activating mutations of some parts of the G proteins, resulting in the contribution to tumorigenesis. Lyons and colleagues identified the presence of a mutation in the Gα i2 gene in 3 of 10 ovarian sex cord–stromal tumors. These 10 tumors consisted of 7 granulosa cell tumors.[31] On the other hand, Shen and colleagues[32] and Hannon and colleagues[33] recently examined 13 and 17 granulosa cell tumors, respectively, and activating mutations were not found. In another study, gsp oncogene mutation (R201C) was found in 4 of 14 sex cord–stromal tumors by Villares-Fragoso and colleagues. This study suggests that the putative oncogene gsp might play a significant role in the pathogenesis of these tumors.[22]

To our surprise, central precocious puberty was diagnosed in one of our patients after the removal of the tumor. Until now, only three cases of ovarian tumor–related central precocious puberty that developed after surgery have been reported.[9–11] One of these patients, who had an ovarian lipid-cell tumor, developed central precocious puberty 20 months after surgical cytoreduction, when she was 5.6 year old.[9] The other patient who developed central precocious puberty was a girl with a granulosa tumor. In this patient, puberty developed 4 years after the removal of tumor, when she was 4.25 year old.[10] Recently, a patient with a granulosa cell tumor was diagnosed with central precocious puberty within a short time after surgery. This patient also had a hypothalamic hamartoma at the tuber cinereum.[11] All of these patients had good responses to GnRH analog treatment. These reports suggest that previous exposure to endogenously increased estrogens, premature activation of the hypothalamic pituitary-gonadal axis, and gonadotropin stimulation from early childhood might have causative roles in the development of sex cord tumors.[9–11] In some animal studies, excess gonadotropin secretion and estrogenization seem to be related to the development of ovarian tumors. Taguchi and colleagues have suggested that neonatal treatment with estrogen (7,12 dimethylbenz (a) anthracene) in mice induces a high frequency of transformation in ovarian tissue and rapid growth of the ovarian tumors.[34] Murphy and colleagues also have thought that prolonged stimulation by gonadotropic hormones in mice leads to ovarian tumors.[35] Some human studies have demonstrated that both gonadotropic hormones given in supraphysiological doses and clomiphene citrate, which is used in infertile women for ovarian stimulation, induce granulosa cell tumors. Taking into consideration all these previous reports, we believe that the development of central precocious puberty in our patient with a granulosa cell tumor might not have been a coincidence. Persistent stimulation of the ovary by gonadotropins may have a direct carcinogenic effect, or the effect may be indirect, by inducing a high estrogen concentration.[12,13] Most reports concerning patients with estrogen-secreting tumors indicate that gonadotropin levels are suppressed as a result of the high estrogen levels.[36–38]

Our patients had high estrogen levels but, it is surprising to note, gonadotropin levels were not suppressed. Patient 3, especially, had markedly elevated FSH levels prior to GnRH stimulation. Central

precocious puberty was diagnosed in this patient after the removal of the tumor. The diagnosis of central precocious puberty was supported by a good response to GnRH analog treatment. Pescovitz and colleagues have reported that GnRH analog treatment is effective in patients with central precocious puberty as well as in patients with combined peripheral and central precocious puberty.[38] Overstimulation of the FSH or aberrant activation of its receptor may have contributed to the development of an ovarian tumor in our patient. On the other hand, elevated estrogen may have resulted in premature activation of the hypothalamic-pituitary-gonadal axis and onset of precocious puberty.

Activating mutations of FSHR were not seen in our study, but polymorphisms in FSHR were detected. One patient developed precocious puberty after removal of her tumor. The development of ovarian tumors in these patients may have been caused by mutations at other exons of the FSHR and G protein subunits, so the association noted between central precocious puberty and granulosa cell tumors might not be coincidental. New studies are needed to clarify the relationship, if any, between the development of ovarian tumors and the activation of FSHR-signal transduction pathways. In future studies, mutations that play roles in the development of tumors might be found at any level of the signal transduction pathways. In addition, these studies may show more concretely a relationship between central precocious puberty and the development of ovarian tumors. In view of the present data, we suggest that children with granulosa cell tumors be followed closely for the development of central precocious puberty, a complication that might adversely affect their final height and psychosocial well-being.

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