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진성반음양과 혼합 생식샘 발생장애를 가진 환자 생식샘의 조직학적 및 분자유전학적 분석

Histopathological and Molecular Genetic Analysis of the Gonads in Patients with True Hermaphroditism and Mixed Gonadal Dysgenesis

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Histopathological and Molecular Genetic Analysis of the Gonads in Patients with True Hermaphroditism and Mixed Gonadal Dysgenesis

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이 논문을 의학박사학위 논문으로 제출함

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ABSTRACT

True hermaphroditism (ovotesticular DSD) and mixed gonadal dysgenesis (MGD) have an overlapping clinical feature including ambiguous genitalia, endocrinologic data, and karyotypes, however, differential diagnosis between the two conditions is clinically important because the risk of malignant germ cell tumor, fertility potential, and surgical treatment methods are quite different. Although the pathogenesis of the two conditions remains unclear, the occurrence of both conditions in the same family or in the twins have been reported, which led us to hypothesize that these two conditions could be genetically related. To clarify the genetic relationship between the two condition, we analyzed the clinicopathologic features and molecular genetic profiles of gonadal tissue in patients with ovotestis (n=13) and MGD (n=12) by whole exome sequencing. Cytogenetic results of the 13 patients with ovotestis showed 46, XX (n=9) or 46, XX/46, XY chimerism (n=4), whereas 12 patients with MGD showed 45, X/46, XY (n= 5) most commonly and other mosaic cell lines, which could be derived from 46, XY through mitotic or meiotic errors, suggesting that the two conditions are not genetically related. Histologically, the most significant difference was the presence or absence of well-developed ovarian tissue in ovotestis and MGD, respectively, however, there were previously undescribed minor component of additional dysgenetic gonadal tissue at the peripheral portion of the ovotestis. In whole exome sequencing, various types of somatic and germline mutations were identified in both groups, but clinical significance or gene function have not been clarified yet. Four patients (30.8%) with ovotestis in our study had 46, XX/ 46, XY chimerism, which could be the cause of ovotesticular development. One of eight patients with 46, XX/SRY-negative ovotestis revealed WNT4 and WNT7A mutation, which might have downregulated β-catenin pathway, and subsequent testicular differentiation, but remaining seven patients with 46, XX/SRY-negative, the cause of ovotestis development could not be explained. Based on the gene ontology analysis, the mutated genes in ovotestis, including FASN, ACADVL, and ME1 gene were significantly enriched in pathway associated with lipid metabolism, whereas those in patients with MGD were associated with cytokine production, small molecule metabolism, endocrine process, steroid biosynthesis. In conclusion, ovotestis and MGD appear to have

different pathogenetic mechanisms, although some of the clinical and histopathologic features are overlapping. Functional relationship between the mutated genes and the abnormal sexual development need to be further clarified.

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INTRODUCTION

Disorders of sexual development (DSD) have been reclassified into 46, XX DSD, 46, XY DSD, and sex chromosome DSD according to the 2006 Chicago classification [1]. However, risk of germ cell malignancy and the patient management need to be judged by individual diagnostic categories based on the histopathologic features of the gonads correlated with clinicalfeatures, which include complete and partial androgen insensitivity, congenital adrenal hyperplasia, complete gonadal dysgenesis, mixed gonadal dysgenesis, true hermaphrodite and so forth, rather than chromosomal composition used in the 2006 Chicago classification.

Ovotestis or true hermaphroditism is a rarest disorders of sexual development (DSD) accounting for 1: 100,000 live births, and is defined as the condition having a well-developed ovarian and a testicular tissue in the same individual. Both ovarian and testicular tissue may coexist as ovary on one side and testis on the other (lateral type), ovotestis on one side and normal testicular or ovarian tissue on the other (unilateral type) or bilateral ovotestes, in which ovary and testis are positioned side by side or with vertical connection. Histologically, the gonadal tissue in ovotestis show appropriate histological development for the age of the patients; the testicular tissue is characterized by well-developed tunica albuginea on the surface, well developed seminiferous tubules containing germ cells and Sertoli cell components, while the ovarian tissue contains adequate number of primordial follicles within ovarian-type stroma with or without developing follicles.

In contrast, mixed gonadal dysgenesis (MGD) is a syndrome of broad clinical, cytogenetic and histopathologic spectrum, characterized in most cases by the presence of a normal appearing testis on one side and a contralateral dysgenetic gonad, which may be rudimentary not having differentiated into an ovary or a testis or in other cases it may be completely absent. Histologically, the dysgenetic gonad forms neither well-developed ovarian primordial follicles nor prepubertal or mature seminiferous tubules, instead, it shows wide ranges of histologically abnormal gonadal tissue ranging from completely atrophic gonadal stromal tissue only (streak gonad), to disorganized primitive sex cord-like structure without germ cell component, and to scattered germ cells admixed with disorganized

primitive sex cord-like structure in the ovarian type stroma or mixture thereof.

Patients with these two conditions, ovotesticular DSD and MGD, may have an overlapping clinical phenotype including ambiguous genitalia, endocrinologic data, and karyotypes. However, it is important to differentiate one from the other based on the histopathologic features of the gonad because of discrepant risk of malignant germ cell tumor and different surgical treatment method [1, 2]. In the past, early gonadectomy was recommended for all cases of DSD with Y chromosome component, however, according to current approaches, gonadal tumor risk is predicted based on the histopathological and molecular diagnosis, on which the timing of the gonadectomy depends on. Risk of germ cell malignancy has been reported up to 35% in cases of gonadal dysgenesis (mixed, partial or complete) in contrast to less than 3% in cases of ovotesticular DSD [1]. Gender assignment is also a difficult problem. It should be based on various factors including potential for normal function of the external genitalia, genital appearance, potential for fertility, surgical options, need for lifelong replacement therapy, views of the family, and, sometimes cultural practices and the parent's agreement.

Ovotesticular DSD and gonadal dysgenesis occurred in the same family or in the twins have been reported [3-5], which raised the question whether the two conditions are different manifestations of the same disorder of gonadal development. Since the development of male or female gonad is governed by a balance between two antagonistic pathways, it is conceivable that mutations in the genes responsible for ovarian development might have induced a testicular differentiation as well as an ovarian differentiation in a susceptible 46, XX/SRY-negative subject. Subsequently, more vulnerable ovarian germ cells/sex cord structure in the microenvironment during early embryonic period could have induced early degeneration of ovarian follicles, which formed MGD.

We hypothesized that true hermaphroditism and MGD might be genetically related or at least some overlapping pathogenetic mechanism in the abnormal gonadal development. To clarify the relationship between the two conditions, we investigated the clinicopathological and molecular genetic features of the patients with ovotesticular DSD and MGD.

MATERIALS AND METHODS

1. Case Collection

To study the clinicopathological findings of the patients with ovotesticular DSD and MGD, 13 cases of ovotesticular DSD (True hermaphroditism) and 12 cases of MGD were retrieved from the surgical pathology files of the Department of Pathology, Asan Medical Center, Seoul, Korea, over a 20-year period (2000-2020). The diagnosis was determined based on the gross and microscopic features of the resected or biopsied gonadal tissue combined with intraoperative findings of contralateral gonad, if the latter was not resected. In 14 cases, tissue from normal appearing gonad was not taken for histopathologic confirmation, but presence or absence of normal gonad was judged by the surgeon, which was adopted for the diagnosis of ovotestis or MGD.

Clinical information was obtained from medical records, which included age, presenting symptoms, karyotypes, cytogenetic result for Y-chromosome specific sequence (SRY), phenotypes of the external genitalia, hormonal levels, reared gender, type of surgical corrections, follow-up period, and the occurrence of germ cell malignancy if it occurred.

Review of hematoxylin and eosin-stained slides of all 25 cases was performed by two pathologists (S-RL, K-RK). Dysgenetic change was defined as abnormal histopathological findings, which was described in Table 1, regardless of size of the lesion. The presence of any abnormal gonadal features in the absence of well developed gonad, even a single microscopic focus, was interpreted as dysgenetic change of the gonad.

2. DNA Extraction and Whole Exome Sequencing

Hematoxylin and eosin-stained slides were reviewed and encircled by an experienced pathologist to identify gonadal and non-gonadal normal tissues. A total of 24 formalin-fixed and paraffin-embedded tissue samples obtained from 17 patients were available for whole exome sequencing, which included 11 ovotesticular DSD (9 gonad only samples and 2 paired gonad and normal tissue samples) and 6 MGD (5 paired gonad and normal tissue samples and one normal tissue sample).

Genomic DNA was isolated from the manually microdissected area of each tissue. The

samples were prepared using an Agilent SureSelectXT Target Enrichment kit (Agilent Technologies, Santa Clara, CA, USA) after DNA quality assurance. Whole exome sequencing was performed using the Illumina Hiseq platform (San Diego, CA, USA).

3. Bioinformatics Analysis

The sequenced reads were aligned to the GRCh37 using BWA-MEM (v0.7.12) under the default settings. The aligned reads were processed with a GATK workflow that included marking and removing for PCR duplicates using Picard (v2.17.3), indel realignment and base quality score recalibration using GATK (v3.8.0).

Somatic variant calling was conducted using two modes (paired mode and tumor only mode) of Mutect2 from GATK (v4.1.2.0). To remove potential germline variants from candidates of somatic variants, germline-resource (af-only-gnomad.raw.sites.b37.vcf.gz) and a panel of normal made from all DSD normal samples were used.

Germline variant calling was conducted using HaplotypeCaller from GATK (v4.1.2.0). The somatic and germline variants were subsequently annotated using vcf2maf (v2.0) with Clinvar. Variants with a total depth of less than 20, SNVs with a depth of less than 3, and indels with a depth of less than 5 were filtered out. We excluded the 3' flank, 3' UTR, 5' flank, 5' UTR, IGR, introns and silent variants.

For filtering of candidate disease-causing variants, variants with minor allele frequencies of <=0.01 in the general and East Asian population of the Genome Aggregation Database (gnomAD) and the Exome Aggregation Consortium (ExAC) were selected. Variants classified in the Clinvar database as pathogenic, likely pathogenic, or variants of uncertain significance (VUS) were selected. To predict the effect of amino acid change on protein function, we used Sorting Intolerance From Tolerant (SIFT) and Polymorphism Phenotyping version 2 (PolyPhen2). Variants that are predicted to be functionally deleterious and probably damaging were selected. Common germline variants from 1772 samples of Korean Reference Genome Database (KRGDB) (http://coda.nih.go.kr/coda/KRGDB/index.jsp) were removed and germline variants detected in more than 50% of 16 gonadal samples were also removed. The biological significance and functional interpretation of gene sets were evaluated using the Gene Ontology Enrichment Analysis of ENSENBL gene IDs with

clusterProfiler package in R (v3.16.1). A GO category was considered significantly enriched in genes if the FDR-adjusted p value was <0.05. For comparison with public database, the list of 1141 genes known to be associated with DSD from the comparative toxicogenomics database (CTD) was used. The Catalogue of Somatic Mutations in Cancer (COSMIC) mutational signatures were assessed using MuSiCa (http://bioinfo.ciberehd.org:3838/MuSiCa/).

RESULTS

1. Clinical Features

The clinical features of all 25 patients are summarized in Table 2 and Table 3. The age of 25 patients at the time of diagnosis ranged from 3 days to 31 years (median: 2 months). Of 25 patients with DSD, only two patients with ovotestis were detected after 3 years old, who presented with vaginal bleeding and palpable pelvic mass at the age of 31, and with bilateral cryptorchidism at the age of 9.

Ambiguous external genitalia including unilateral or bilateral cryptorchidism (11 of 25, 44%), hypospadias (8 of 25, 32%), clitomegaly (5 of 25, 20%), micropenis (3 of 25, 12%), and bifid scrotum (2 of 25, 8%) was observed in all patients except one with ovotestis who presented with vaginal bleeding.

On cytogenetic analysis, eight of 13 patients with ovotestis had a 46, XX karyotype and five showed sex chromosome abnormalities including 46, XX/46, XY chimerism and a 46, X, del(X)(q25) karyotype. Of a total of 12 patients with MGD, 45, X/46, XY was the most common feature (n=5) with various sex chromosome abnormalities and one with a 46, XY karyotype (Table 2).

Eight (61.5%) of 13 patients with ovotesticular DSD were raised as females, whereas seven (58%) of 12 patients with MGD were raised as male (Table 3).

At the time of diagnosis, one patient with ovotesticular DSD, whose age was 31, had mixed germ cell tumors and two patients with MGD had gonadoblastoma.

The mean postoperative follow-up period ranged from 1 to 248 months (mean 90 months)

in patients with ovotestis and from 32 to 238 months (mean 121 months) in those with MGD. During the follow-up period, newly detected malignant germ cell tumor was not observed.

2. Histopathological Features

A total of 13 cases with ovotesticular DSD were divided into the three subgroups based on the location of the ovotestis: lateral type in three cases (23%), unilateral in nine (69%), and bilateral in one (8%). Among nine cases with unilateral type, opposite gonad of the ovotestis was ovary in eight case and testis in one. Of 10 cases with unilateral or bilateral types, five cases showed a side-by-side connection between ovary and testis and five cases were vertically connected. Eight cases (61.5%) of all ovotesticular DSD had various types of additional dysgenetic gonad. Dysgenetic features included thin or absent tunica albuginea (6 cases, 46.1%), tubular structures resembling primitive sex cord (6 cases, 46.1%), and anastomosing seminiferous tubules with edematous change of interstitial tissue (7 cases, 53.8%).

In 12 cases with MGD, the dysgenetic gonad was composed of streak gonadal tissue without germ cells or sex-cord structure (one case, 8.3%), primitive sex cord-like structure with or without germ cells (9 cases, 75%), and primitive sex cord-germ cell structure resembling miniature of the gonadoblastoma-like lesion (two cases, 16.7%). Normal appearing gonad of MGD also showed some abnormal features including edematous change of interstitial tissue (6 cases, 50%), thin or absent tunica albuginea (4 cases, 33%), and anastomosing seminiferous tubules (3 cases, 25%). Out of 12 cases with MGD, five cases (42%) contained a minor portion showing dysgenetic features at the periphery of normal appearing gonad. The minor dysgenetic areas consisted of primitive sex cord-like structure with or without germ cells (3 cases, 25%), microscopic foci of gonadoblastoma-like lesion (one case, 8.3%), and streak gonadal tissue (one case, 8.3%).

3. Analysis of Exome Sequencing

The average of 95,883,336 reads were generated per each sample. The mean duplication rate was 21.8%. The mean mapped read depth was 50.9X and 85.5% of target regions was

covered at 10X or greater.

Fourteen cases (87.5%) out of 16 cases had a total of 338 pathogenic somatic mutations including 270 missense mutations, 35 nonsense mutations, 12 splice site mutations, 2 translation start site mutations and 19 small indels. The median numbers of somatic mutations per sample in 11 ovotesticular DSD and 5 MGD were 22.0 (range: 4-37, mean: 21.64) and 10.0 (range: 8-37, mean: 20), respectively.

When genes recurrently mutated in at least two patients were selected, MUC4, PIGO, SCN5A and SMG6 had the highest mutation frequencies (3/16 cases, 19%). PIGO, SCN5A and SMG6 were found in both conditions, whereas MUC4 was found only in ovotesticular DSD (Fig.3A). The three somatic mutations in MUC4 included one frameshift deletion, one in-frame deletion and one in-frame insertion. Somatic mutations involving MUC4, KRD36C, ATAD3B, BAI1, and CRIPAK genes were frequently detected in ovotestis, while mutations involving SCN5A, OBSCN, SGSM3, SRRM2, and ACSF3 genes were frequently found in MGD.

We compared somatic mutations of 16 cases with 1141 genes known to be associated with DSD from the comparative toxicogenomics database (CTD). Somatic mutations involving 27 genes were detected in 12 cases (75%) including 8 ovotesticular DSD (73%) and 4 MGD (80%). The ovotesticular DSD and MGD had genetic alteration in one gene, the fatty acid synthase (FASN), in common. However, there was no gene that have a significant difference in both groups among other genes known to be associated with DSD (Fig.3B).

There was a total of 10 genes differently mutated in both groups. The MUC4, KRD36C, ATAD3B, BAI1, and CRIPAK genes were mutated only in ovotestis cases, whereas mutations of the SCN5A, OBSCN, SGSM3, SRRM2, and ACSF3 genes were frequently detected in MGD (Fig.3C).

Among the pathogenic somatic mutations, we compiled the list of genes known to be associated with DSD and conducted an enrichment analysis of the genes. The top 10 GO terms significantly enriched in ovotestis and MGD are shown in Figure 5. The top-ranked GO terms in ovotestis were “regulation of lipid metabolic process”, “ossification”, and “gland development” (Fig.4A). The GO terms included the genes FASN, ACADVL, ME1, THRA, SPP1, WNT4, FBN2, ARG1, and PSEN1. In contrast, the top-ranked GO terms in

MGD were “negative regulation of cytokine production” and “regulation of small molecule metabolic process” (Fig.4B). The GO terms included the genes NFKB1, INHBB, POMC, and FASN.

A total of 1601 germline mutations including 1332 missense mutations, 8 nonsense mutations, 2 mutations in gene encoding RNA-binding protein, 60 splice region mutations, 5 splice site mutations, 2 translation start site mutations, and 192 small indels were identified in all 16 cases (Fig.5A). The median numbers of germline mutations per sample in 11 ovotesticular DSD and 5 MGD were 99.0 (range: 78-121, mean: 99.55) and 106.0 (range: 88-110, mean: 102.2), respectively.

Comparing to 1141 genes known to be associated with DSD, our cases had germline mutations involving 74 DSD-related genes in 15 cases (93.8%). In common, the ovotesticular DSD and MGD had germline mutations in the genes GHR, AKAP13, CYP1B1, and NCL (Fig.5B).

A total of eight genes including four pseudogenes were differently mutated in both groups. ATN1 and FADS6 genes were mutated only in ovotestis, while COL17A1 was mutated only in MGD (Fig.5C).

After compiling the list of genes showing pathogenic germline mutations, an enrichment analysis of the genes was performed. The top 10 GO terms significantly enriched in ovotestis are shown in Figure 6A. The top-ranked GO term was “collagen-containing extracellular matrix” and included the 44 genes such as LAMB3, NTN3, and COL7A1. The GO terms significantly enriched in MGD are shown in Figure 6B. The top-ranked GO term was “actin filament binding” and included the genes MYBPC2, KLHL17, UTRN, XIRP1, FSCN2, SHROOM3, NEB, SYNE3, OBSL1, VIL1, AVIL, and MYH4.

We analyzed the COSMIC mutational signatures to identify the mutational pattern. In both conditions, the detected mutations were associated with COSMIC signature 1 (Fig.7).

Table 1. Histopathologic analysis of gonadal histology

	Gonad	Histopathological findings
Normal	Testis	Thick tunica albuginea
		Compact, round to ovoid regular seminiferous tubules
	Ovary	Ovarian type stroma and numerous primordial follicles containing oocytes
Abnormal	Testis	Thin or absent tunica albuginea
		Anastomosing seminiferous tubules
		Primitive sex cord-like structure with or without germ cells
		Edematous change in interstitial tissue
	Ovary	Ovarian type stroma without adequate number of primordial follicles
		Primitive sex cord-like structure with or without germ cells

Table 2. Clinical features in patients with ovotestis and MGD

		Ovotestis (n=13)	MGD (n=12)
Age at presentation (mean)		3 days ~ 31 years (3.2 years)	8 days ~ 3 years (0.3 years)
Presenting symptoms			
	Ambiguous external genitalia	12 (92.3%)	12 (100%)
	Vaginal bleeding*	1 (7.7%)	0
Age at surgery	Prepubertal	12 (92.3%)	12 (100%)
	Postpubertal	1 (7.7%)	0
Reared gender	Male	5 (38.5%)	7 (58%)
	Female	8 (61.5%)	5 (42%)
Karyotypes	46, XX	8 (61.5%)	0
	46, XX/46, XY chimerism	4 (30.8%)	0
	46, X, del(X)(q25)	1 (7.7%)	0
	46, XY	0	1 (8.3%)
	45, X/ 46, XY	0	5 (41.7%)
	45, X/ 46, XX, +mar	0	1 (8.3%)
	45, X/ 46, XX or 46, XY	0	1 (8.3%)
	45, X/ 46, XY, +abnormal Y chromosome	0	2 (16.7%)
	45, X/ 47, XYY, +abnormal Y chromosome	0	1 (8.3%)
	45, X/ 46, XY/ 47, XYY, +abnormal Y chromosome	0	1 (8.3%)

*One patient with ovotestis presented with vaginal bleeding at the age of 31 and the ovotestis was incidentally discovered; MGD, mixed gonadal dysgenesis

Table 3. Follow-up results for gonadal tumors

		Ovotestis (n=13)	MGD (n=12)
Postoperative follow-up period (mean, months)		1 ~ 248 mo (90 mo)	32 ~ 238 mo (121 mo)
Malignant tumor at diagnosis		1 (MGMT)*	2 (Gonadoblastoma)
Type of gonadectomy	Bilateral gonadectomy	5 (38.5%)	6 (50%)
	Unilateral gonadectomy	8 (61.5%)**	6 (50%)
Newly detected tumor during follow-up		none	none

*, MGCT, mixed germ cell tumors; **, partial gonadectomy was performed in 4 out of 8 patients with ovotestis; mo, months

Table 4. Histopathological analysis of ovotestis

		Ovotestis (n=13)
Gonadal types	Unilateral	9 (69%)
	- Ovotestis-ovary	8 (89%)
	- Ovotestis-testis	1 (11%)
	Bilateral	1 (8%)
	Lateral	3 (23%)
Connection of ovary and testis	Side by side	5 (50%)
	Vertical	5 (50%)
Additional dysgenetic gonad	Present	8 (61.5%)
	Absent	5 (38.5%)
Dysgenetic features	Thin or absent tunica albuginea	6 (46.1%)
	Anastomosing seminiferous tubules	7 (53.8%)
	Primitive sex cord-like structure	6 (46.1%)

Table 5. Histopathological analysis of MGD

		MGD (n=12)
Dysgenetic features in normal appearing gonad	Edematous change of interstitial tissue	6 (50%)
	Thin or absent tunica albuginea	4 (33%)
	Anastomosing seminiferous tubules	3 (25%)
	Dysgenetic gonad in addition to well-formed gonad	5 (42%)
	- Primitive sex cord structure	3 (60%)
	- Ovarian type stroma	1 (20%)
	- Gonadoblastoma-like lesion	1 (20%)
Dysgenetic gonad	Primitive sex cord-like structure with/without germ cells	9 (75%)
	Streak gonad	1 (8.3%)
	Gonadoblastoma-like lesion	2 (16.7%)

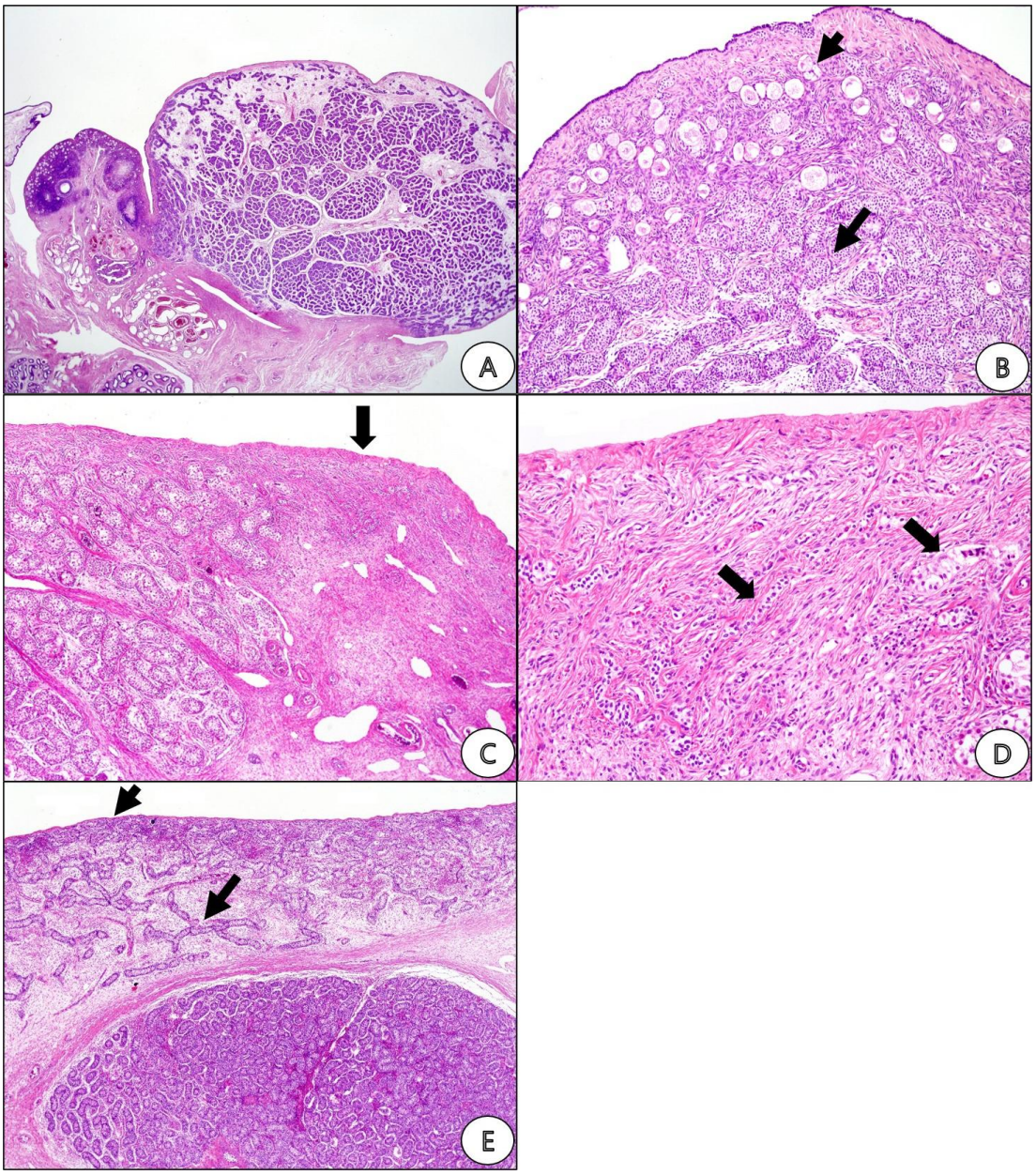

Figure 1. Histopathological features of ovotestis. (A) The ovary and testis were connected side by side in the same gonad. (B) The ovarian tissue in the outer portion (short arrow) and testicular tissue in the inner portion (long arrow) were vertically connected. (C) 61.5% of ovotestis had dysgenetic gonad (arrow) in addition to normal gonad. (D) The dysgenetic gonads were composed of primitive sex cord-like structures (arrows), (E) thin or absent tunica albuginea (short arrow), and anastomosing seminiferous tubules (long arrow).

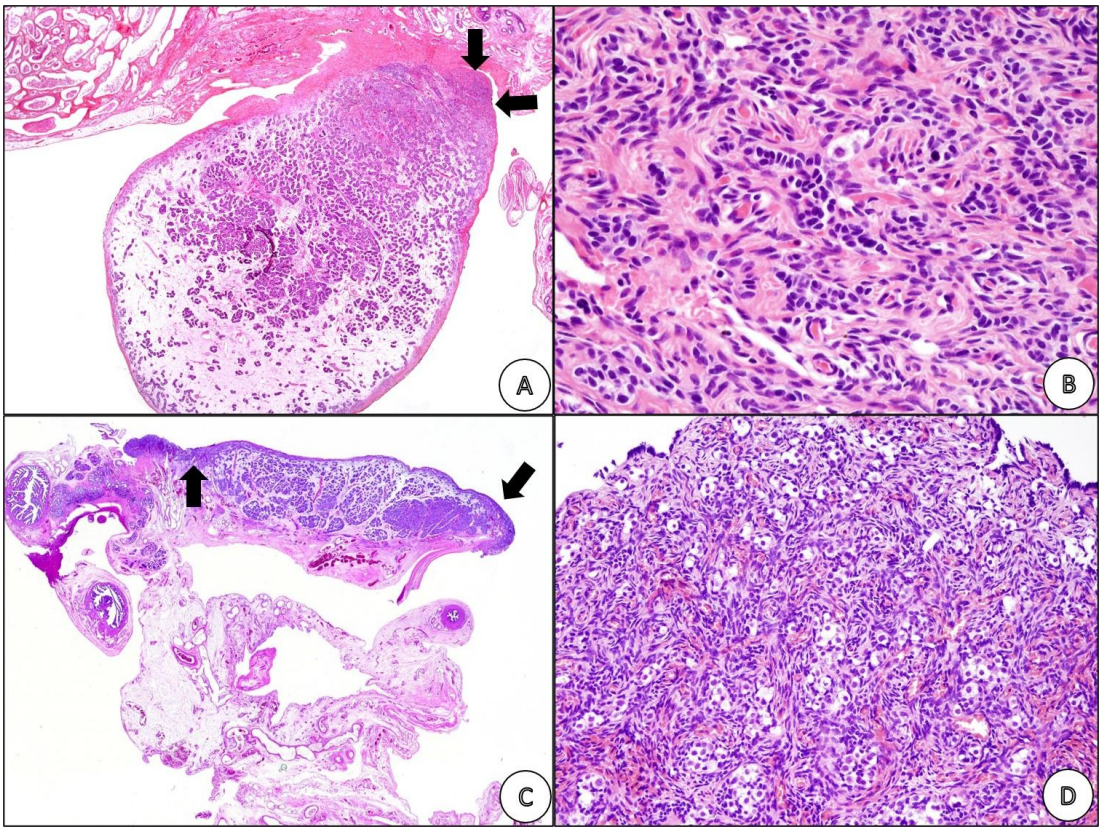

Figure 2. Histopathological features of mixed gonadal dysgenesis (MGD). 42% of the MGD had dysgenetic gonadal tissue at the periphery of well-formed normal appearing testis. (A, arrows) One patient with right testis and left dysgenetic gonad. The left dysgenetic gonad consisted of a nearly normal appearance of testis and a minor portion of dysgenetic change (arrow). (B) The dysgenetic gonadal tissue was composed of primitive sex cord-like structures with a few germ cells. (C) One patient with right streak gonad and left testis. The normal appearing testis showed prepubertal well-formed seminiferous tubules in the center and with two dysgenetic foci at the periphery covered with thin tunica albuginea (arrows). (D) Gonadoblastoma-like component was identified in the dysgenetic portion.

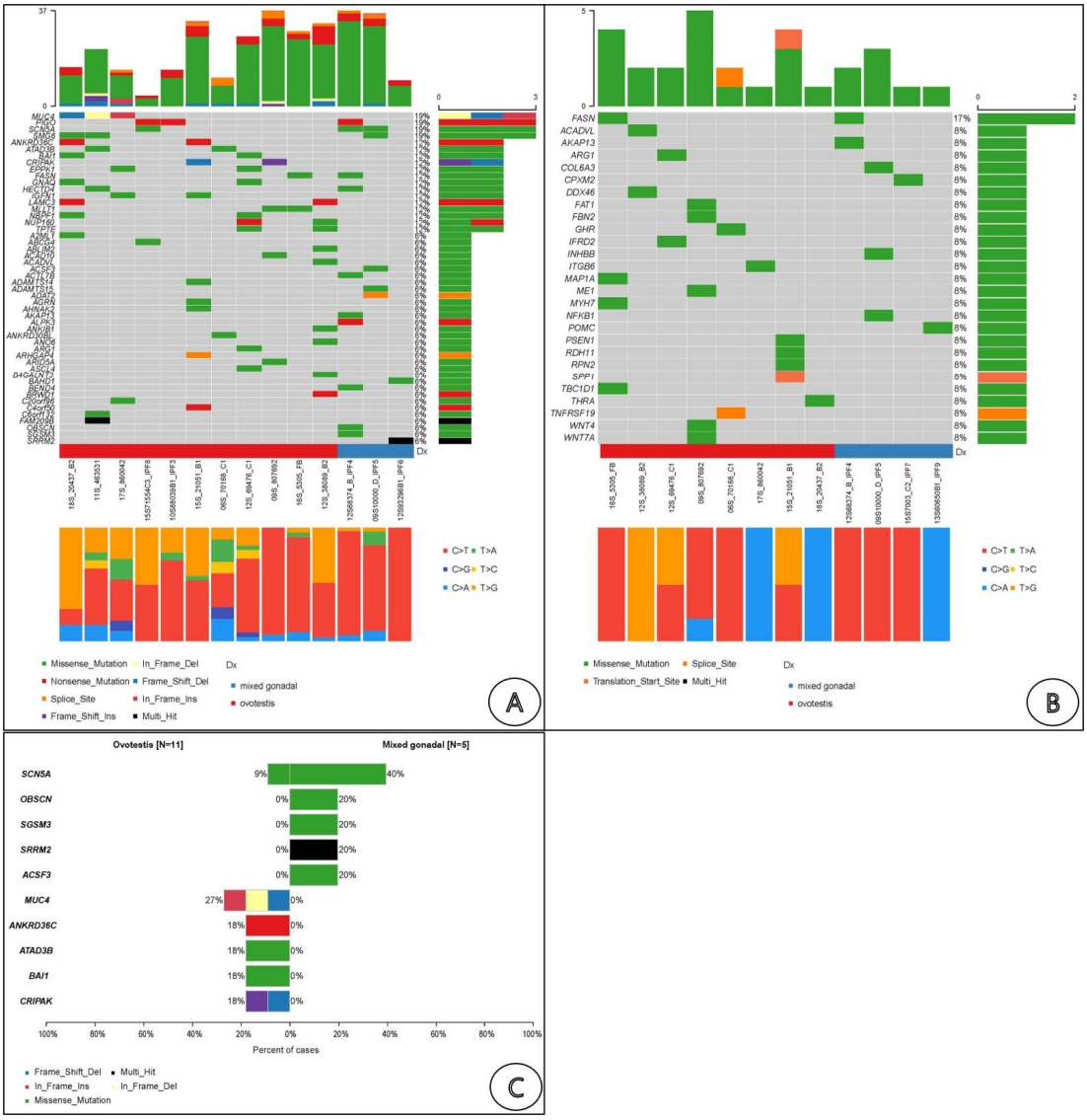

Figure 3. Pathogenic somatic mutations of the patients with ovotestis and MGD. (A) Various types of somatic mutations involving 312 genes were detected in 14 (87.5%) of 16 cases. (B) Somatic mutations involving 27 DSD-related genes were found in 12 cases (75%). There was no gene that have a significant difference in both groups except FASN gene. (C) Ten genes differentially mutated between ovotestis and MGD were identified.

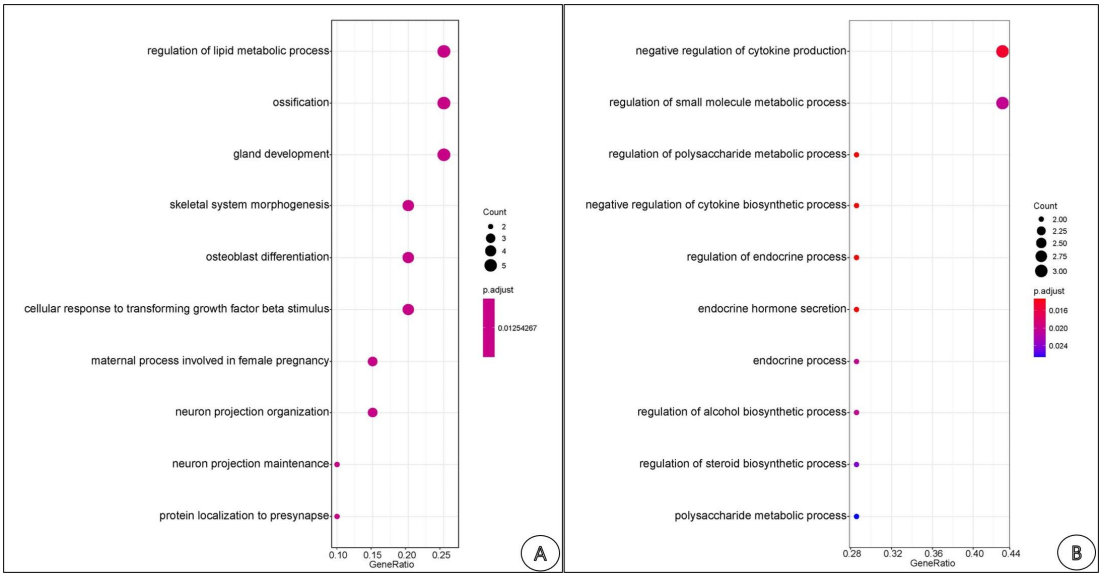

Figure 4. Gene ontology analysis of genes with somatic mutations. (A) In ovotestis, the top-ranked GO terms were “regulation of lipid metabolic process”, “ossification”, and “gland development”. (B) In MGD, the top-ranked GO terms were “negative regulation of cytokine production” and “regulation of small molecule metabolic process”.

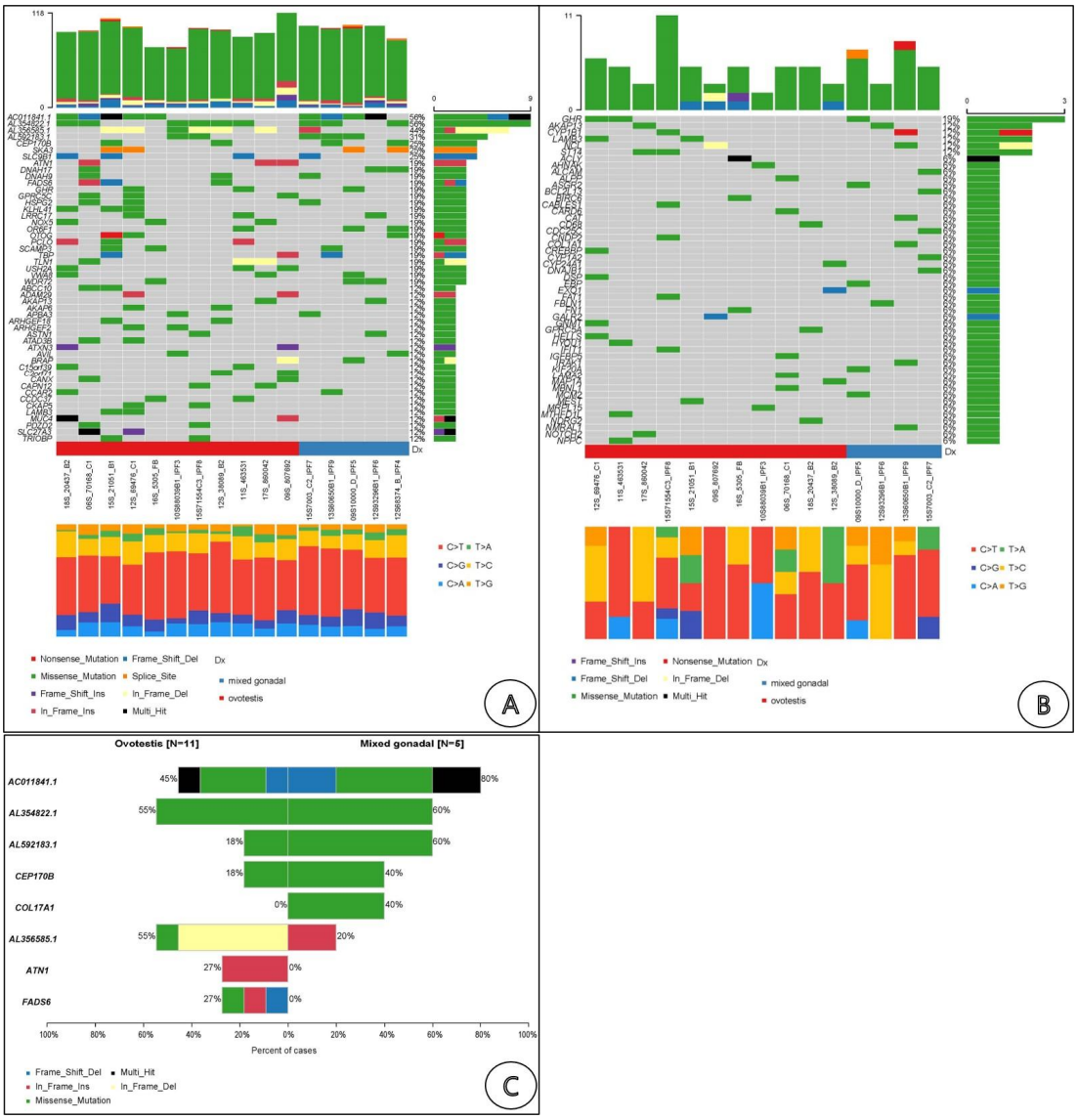

Figure 5. Pathogenic germline mutations of the patients with ovotestis and MGD. (A) Various types of germline mutations involving 1343 genes were detected in all 16 cases (100%). (B) Germline mutations involving 74 DSD-related genes were found in 15 cases (93.8%) of 16 cases. (C) Eight genes including four pseudogenes were differentially mutated between ovotestis and MGD.

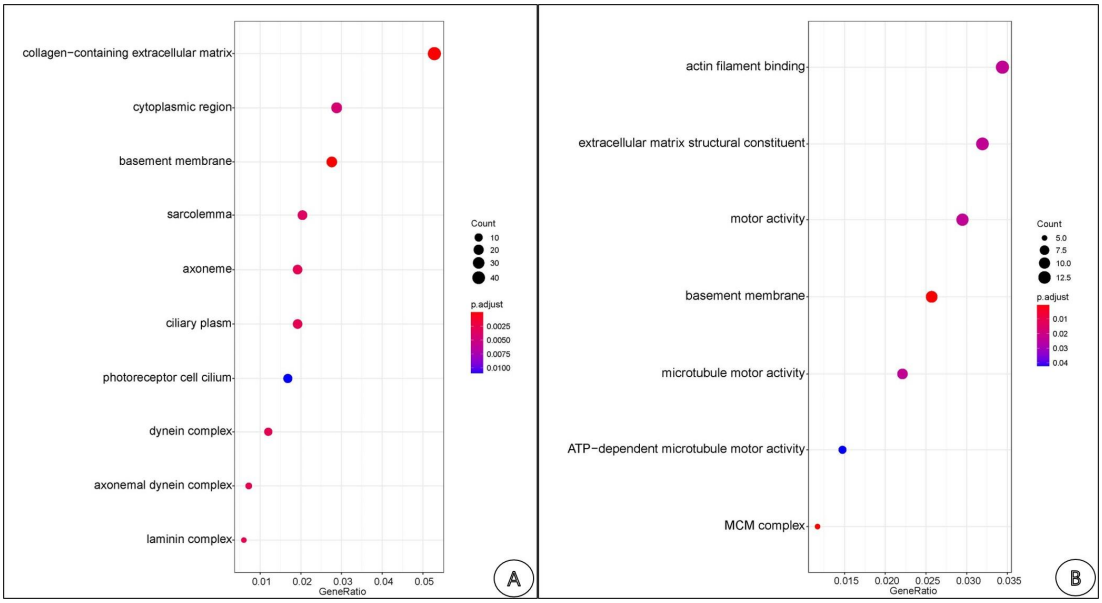

Figure 6. Gene ontology analysis of genes with germline mutations. (A) In ovotestis, ten GO terms were significantly enriched. The top-ranked GO term was “collagen-containing extracellular matrix” (B) In MGD, the top-ranked GO term was “actin filament binding” among GO terms significantly enriched.

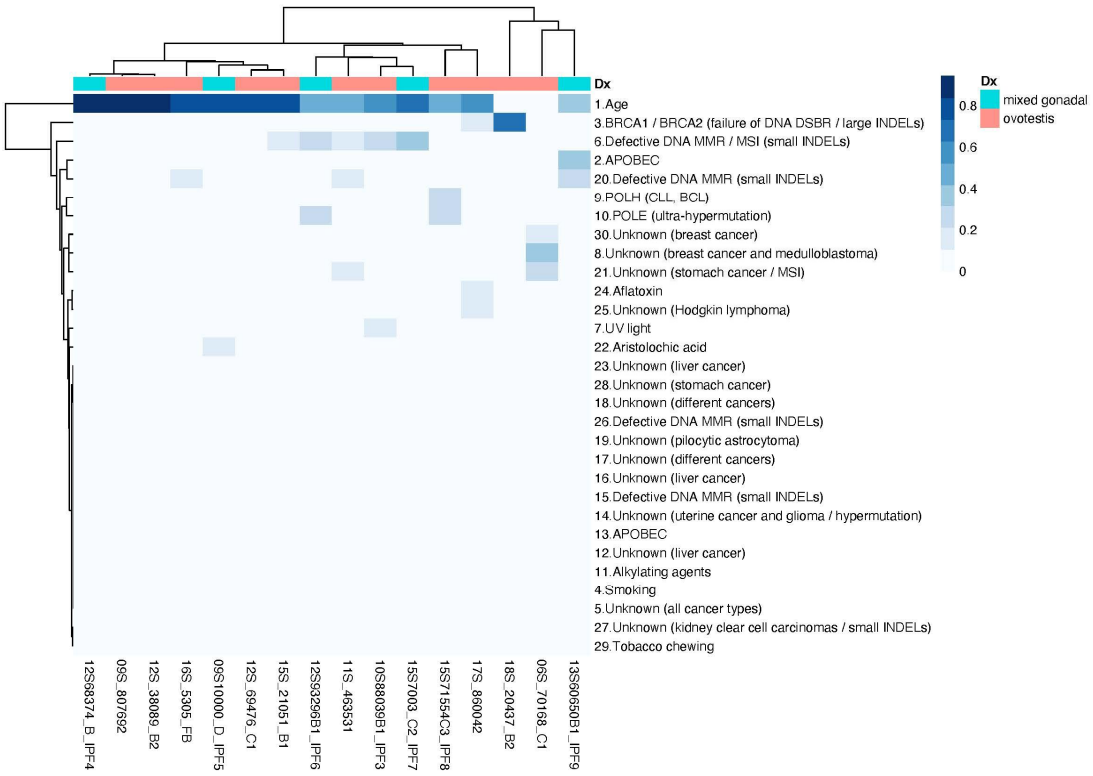

Figure 7. Analysis of COSMIC mutational signatures. In both ovotestis and MGD, the detected somatic mutations were associated with COSMIC signature 1 that are correlated with age at diagnosis.

DISCUSSION

It has long been assumed that the ovarian development was the default state during sexual development. Therefore, majority of researches were focused to uncover the molecular clue for testicular development, while the study of ovarian development received very little attention. However, more recently, disruption of a single gene was shown to induce complete testicular development and female-to-male sex reversal in the absence of the testis-determining gene, SRY.[6, 7] Subsequently, male-to-female sex reversal, or development of ovotestis, was identified in human 46, XX/SRY-negative patients carrying mutations in the R-spondin1 (RSPO1) gene, which is a WNT signaling pathway activator. [6, 8] Currently, it is clear that genetic induction of WNT/β-catenin signaling can promote ovarian development from a gonad programed otherwise to become a testis,[9] and the RSPO1/WNT/β-catenin pathway acts at the top of the ovarian differentiation cascade. The development of male or female gonad is governed by a balance between two antagonistic pathways; for ovarian development female genes need to be expressed and male genes actively repressed at the same time, and is reversed for testicular development.[10]

In male, SRY upregulates SOX9, which triggers testis differentiation [11, 12]. In addition to SRY and SOX9, there are some other genes, which can be considered testis-promoting, differentiation and/or maintenance genes, which include FGF9, PTGDS, SOX8, NR5A1, SOX3, NR0B1, PDGFRA, DMRT1, AMH, NGF, NTF3 and NGFR as the most important examples [13]. On the other hand, in the female, the activated RSPO1/WNT4/β–catenin signaling pathway induces ovarian development [14, 15].

Ovotesticular DSD is caused by gain-of-function variants in genes important for testis differentiation or loss-of-function variants in ovarian differentiation genes [16]. Development of dual gonads may be easily explained in the presence of mosaicism, gene mutations and chimerism, however, it is difficult to clarify the cause of ovotesticular development in 46, XX or 46, XY, particularly if SRY is negative or no SRY mutation is found. Molecular studies have demonstrated that around 90% of 46, XX individuals with ovotesticular DSD are SRY-negative [17], and 10% of 46, XX can be caused by translocation of SRY gene onto the X chromosome (SRY-positive 46, XX DSD) [18],

duplication of SOX9 [19, 20], ectopic gonadal expression of SOX9 [21], duplications in the SOX9 upstream enhancer [22], the presence of an extradose or to an overexpression of an SOX9 enhancer region [22]. NR2F2 loss-of-function mutation [23], mutations in the SRY, SOX9 and DMRT1 genes [24], excess DAX1, and activation of the testis enhancer of SOX9 [25].

Eight of 13 (61.5%) patients with ovotesticular DSD had 46, XX in our study, all of whom were SRY negative. The genetic mechanism for testis development in 46, XX/SRY negative ovotestis patient remains unexplained. One patient with 46, XX/SRY-negative ovotesticular DSD in our study had a novel somatic mutation (p.T219M) in WNT4 and a known somatic mutation (p.D134N) in WNT7A, without mutation of genes to cause testicular differentiation. According to the 1000 Genomes project, it is known that allele frequency of the WNT7A (p.D134N) mutation is 0.1% and 0.02% in East Asian and global population, respectively. The mutation is a rare variant that has a minor allele frequency of less than 1%, however, the clinical significance of this variant has not been reported. WNT4 is a growth factor involved in multiple developmental processes including the formation of the kidney, adrenal gland, breast, and the female reproductive system. The specific molecular mechanism of WNT4 action during gonadal development remains unidentified, however, WNT4 is expressed in the gonads of both sexes before sex determination and is subsequently down-regulated in the male gonad. Wnt4 is implicated in Müllerian duct regression, the formation of sex-specific vasculature, the inhibition of steroidogenesis and in sex-specific cell migration events.[26] WNT7A is known to activate canonical β-catenin signaling pathway and WNT7A depletion in 5637 HMI and T24 cells decreased levels of active β-catenin and its downstream target genes involved in the epithelial-to-mesenchymal transition (EMT) and extracellular matrix (ECM) degradation. [27] In the patient with WNT4 and WNT7A mutations, disrupted balance between two antagonistic pathways, namely defective repression for female genes for ovarian development and defective expression for male genes for testicular differentiation could be responsible for ovotesticular development. Another one with 46, XX/SRY-negative ovotestis had a novel germline mutation (p.D96Y) in CYP11B1, the gene encoding 11β–hydroxylase deficiency. The CYP11B1 mutation is the second most common cause of congenital adrenal hyperplasia, resulting in accumulation of 11-deoxycortisol,

hyperaldosteronism and hyperandrogenism [28]. In our case, hormonal analysis revealed normal testosterone (2ng/dl), cortisol (3.9ng/dL), aldosterone (30.3ng/dL), and ACTH (45.9pg/ml) levels with high level of estradiol (26pg/ml). There was no clear hormonal evidence of 11β–hydroxylase deficiency such as the results of ACTH stimulation test or marked elevation of 11-deoxycortisol. To predict possible effect of the detected mutation on the function of 11β–hydroxylase and ovotesticular developmental mechanism, further study is needed.

Four patients (30.8%) with ovotestis in our study had 46, XX/ 46, XY chimerism, which can be the cause of ovotesticular development. By definition, chimerism is produced by fusion of two separate zygotes which are fertilized by two different sperms or resulting from the fertilization of a second polar body, while mosaicism results from a mitotic error in a single zygote. In practice, when two cell lines of 46, XY and 46, XX cell lines are identified in an individual, chimerism should be considered and it should be distinguished from mosaicism by using autosomal polymorphic markers.

Cytogenetically, the most common karyotype of ovotesticular DSD is a 46, XX, but 46, XX/46, XY chimerism or a variety of other chromosomal mosaicisms are observed [29, 30]. On the other hand, the most common karyotype of MGD is a 45, X/46, XY and various mosaic karyotypes with XY cell line are observed [31, 32]. In our study, 61.5% of the patients with ovotestis had 46, XX and the remaining had 46, XX/46, XY chimerism, whereas many of the patients with MGD had 45, X/46, XY and the remaining had diverse mosaicism, which could be produced through mitotic error from 46, XY. Therefore, the basic cytogenetic composition was thought to be different between the two conditions, although the difference between the two conditions could have been caused by the potential selection bias due to a small number of cases.

During the surgical pathology practice, we often encountered ambiguous histologic features, in which additional dysgenetic gonadal tissue was identified at the periphery of well-formed ovotestis. The additional dysgenetic gonadal tissue at the periphery of ovotestis were histologically indistinguishable from those of dysgenetic gonad of the MGD, and was quite frequently seen in 8 of 13 patients (61.5%) with ovotestis, which raise the question if it is a variant feature of MGD. These overlapping histopathologic features between the two

conditions would make the differential diagnosis difficult, especially in small biopsied samples. The ovarian tissue of ovotesticular DSD had relatively normal histologic features with adequate number of primordial follicles, whereas the testicular component was frequently abnormal, which included thin or absent tunica albuginea, anastomosing seminiferous tubules, and primitive sex-cord like structures (Figure 1D, long arrows). These additionally abnormal gonadal features in the ovotestis have never been described in the literature, and the clinical significance cannot be determined

Of the eight ovotesticular DSD patients with a minor portion of additional abnormal gonadal component, six patients had 46, XX or its derivate karyotypes, whereas two patients had 46, XX/46, XY chimerism, suggesting that clear relationship between the presence of additional abnormal gonadal tissue component and the karyotype.

In our study, partial gonadectomy was performed in four of 13 patients with ovotestis. However, germ cell tumors were not developed from residual gonads during the follow-up period, suggesting that the presence of additional dysgenetic tissue may not be source of malignant germ cell tumor, although follow up period was not long enough (range: 1~248 months, mean: 90 months).

The incidence of malignant germ cell tumor in patients with ovotesticular DSD is significantly lower than in patients with MGD. Although it does occur with an incidence less than 5% of patients with ovotestis [1, 33], mostly in patients with the 46, XX/46, XY karyotype [34], in contrast to the highest tumor risk up to 35% in TSPY (testis specific protein Y encoded) positive gonadal dysgenesis [1]. The distinction is also important for the sake of preserving their fertility.

The patients with ovotesticular DSD are generally infertile, however, individuals with ovotestis reared as female, if the patient has a uterus, may have fertility potential since they have frequently functional ovarian tissue as has been evidenced by menstruation. Successful pregnancy and delivery have been described in many cases.[2] However, correct distinction is not possible through any of the external genital phenotype, karyotype or endocrine data.

In our study, various somatic and germline mutations were identified in both ovotestis and MGD. The detected mutations were associated with COSMIC signature 1, which is characterized predominantly by C>T substitution at NpCpG trinucleotides, and results from

an endogenous mutational process initiated by spontaneous deamination of 5-methylcytosine [35]. This finding suggests that numerous mutations that occurred in both conditions might be caused by endogenous mutational process instead of exogenous mutagenic exposure.

Although various somatic and germline mutations were identified in both groups, there were only a few common mutations in both conditions. Those individual mutations have neither been previously reported in patients with DSD, nor have their functions been clarified. However, based on the gene ontology analysis there are certain differences in biological significance of the mutated genes between the two groups. In ovotesticular DSD, pathway associated with lipid metabolism was significantly enriched. Three cases with ovotestis revealed missense mutations in FASN, ACADVL, and ME1 genes. ACADVL gene encodes protein that catalyzes mitochondrial fatty acid β–oxidation pathway, ME1 gene encodes a cytosolic NADP-dependent enzyme that generates NADPH for fatty acid biosynthesis, both of which have common roles in the lipid metabolic pathway. The FASN encodes enzyme that catalyzes the biosynthesis of palmitate from acetyl-CoA and malonyl-CoA in the presence of NADPH. Previous studies have shown that the FASN modulates estradiol-stimulated estrogen receptor (ER)-α transcriptional activation in breast and endometrial cancer cells [36, 37]. Another study has shown that reduction of ER-β2 is associated with male-biased gene transcription, suppressed female-responsive gene expression and primordial germ cell migration in fish gonad. Indeed, reduction of ER-β2 induces germ cell degradation and loss, which in some cases ultimately affected the XX female sexual development [38]. Although the exact mechanisms and roles of lipid metabolism and steroid hormone pathways in the development of ovotesticular DSD must be delineated further, our study indicates that lipid metabolic pathway is associated with ovotesticular DSD.

CONCLUSION

Ovotestis and MGD appear to have different pathogenetic mechanisms, although some of the clinical and histopathologic features are overlapping. Functions of the mutated genes in both conditions related to the sexual development need to be further clarified in the future.

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