INTERNATIONAL JOURNAL OF UROLOGY
International Journal of Urology (2018) doi: 10.1111/iju.13863

Original Article

Male infertility in Sertoli cell-only syndrome: An investigation of autosomal gene defects

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Abbreviations & Acronyms
Amp = amplification
CFTR = cystic fibrosis transmembrane conductance regulator gene
CGH = comparative genomic hybridization
CNV = copy number variation
COL1A1 = collagen, type I, alpha 1
Del = deletion
ESM = extracellular matrix
gDNA = genomic DNA
H19 = imprinted maternally expressed transcript
HOXD9 = homeobox gene
KCNQ1 = potassium voltage-gated channel subfamily Q member 1
LOH = loss of heterozygosity
SC = synaptonemal complex
SCOS = Sertoli cell-only syndrome
SNP = single-nucleotide polymorphisms
SYCP3 = synaptonemal complex protein 3

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Received 7 May 2018; accepted 11 October 2018.

Objectives: To detect autosomal genetic defects and to determine candidate genes in Sertoli cell-only syndrome infertile men.
Methods: Single-nucleotide polymorphism + comparative genomic hybridization microarray technology was carried out on 39 Sertoli cell-only syndrome infertile patients in the present study. Array comparative genomic hybridization compares the patient's genome against a reference genome, and identifies uncover deletions, amplifications and loss of heterozygosity.
Results: A link between defective spermatogenesis genes and infertility was examined, and amplifications and deletions in several genes were detected, including homeobox gene; synaptonemal complex element protein 1; collagen, type I, alpha 1; imprinted maternally expressed transcript; and potassium voltage-gated channel subfamily Q member 1.
Conclusions: The present data suggest that several genes can play an important role in spermatogenesis and progression of Sertoli cell-only syndrome.

Key words: array comparative genomic hybridization, infertility, Sertoli cell-only syndrome.

Introduction

Infertility is defined as the inability to have a child despite not using any contraception and having regular sexual intercourse for at least a year.[1,2] There are approximately 15% infertile couples in the world, of whom 30–40% are related to active male infertility. At least 30% of male infertility is idiopathic.[3,4] Male infertility is usually caused by deterioration of the sperm parameters. Semen quality is conventionally assessed by examining the number, motility and morphology of sperms in ejaculate. The absence of any sperm in ejaculate is called azoospermia.[5]

One of the causes of azoospermia is germ cell aplasia; that is, SCOS, which occurs in the testis tissue in seminiferous tubules in the complete absence of germ cells.[6] Sertoli cells are large, prismatic cells located in seminiferous tubules and specialized in secretion; but, they do not proliferate after puberty. In spermatogenesis, Sertoli cells provide the necessary nutrients and physical support for germ cells, and protect them from the autoimmunity of the neighboring defense cells by maintaining spermatogenic cells within their own cells through close contact.[7,8]

SCOS is a histological diagnosis, for which two different forms have been defined: congenital primitive SCOS and secondary SCOS (acquired or mixed) due to pathology.[9] Although having spermatozoa is not possible in primitive SCOS, it is difficult but possible in secondary SCOS that develops due to severe pathogenesis after testicular injury. The size of testicular and azoospermic semen samples of patients ranges from small to normal. Phenotypically, these samples have a normal male structure. As there are no germ cells, levels of follicle-stimulating hormone are usually increased. In contrast, plasma testosterone and luteinizing hormone levels are normal.[10]

A number of factors can cause SCOS; for example, microdeletions of the Y chromosome, numeric and structural disorders of the chromosome, undescended testis, radiation, cytotoxic drugs, and viral infections.[11,12] However, the etiology of SCOS, which is known to be multifactorial, has not been fully elucidated. In male patients with SCOS, it is thought that autosomal gene defects might also cause this disease.[13]

In recent years, DNA microarray technologies have been used to define mutations responsible for infertile phenotypes in men.[14] DNA microarray platforms, such as CGH + SNP, allow for reliable detection of chromosomal anomalies that develop as a result of changes in the CNVs in DNA and comparison of multiple genomes. It is thought that CNVs that differ from one individual to another have an effect on the expression of genes through direct gene dosage or by changing the control of gene expression.[15]

The present study investigated the autosomal gene defects causing SCOS in male volunteers with a SCOS diagnosis to identify responsible candidate genes using CGH + SNP microarray analysis.

Methods

Study participants

The study group consisted of patients who were referred to Marmara University Faculty of Medicine Department of Urology and Andrology Polyclinic, and Zeynep Kamil Women and Children's Diseases Training and Research Hospital Urology Polyclinic with the complaints of infertility, and were diagnosed with SCOS by microdissection testicular sperm extraction procedure, which was carried out twice. In the present retrospective study, SCOS patients with pathologically definite diagnosis were selected. According to the pathology reports, all patients participating in the study had congenital primitive SCOS. There is no sperm in primitive SCOS cases, and the patients are irreversibly infertile. According to the histopathological diagnosis of testicular biopsies, sclerotic appearance in the seminiferous tubules, thickening of the peritubular basement membrane and only Sertoli cells were observed in the intratubular areas. In addition, some reports of the patients indicated that there was enlargement in interstitial areas, atrophy in Leydig cells and Leydig cell hyperplasia. The histopathological findings of all patients were diagnosed as SCOS (Fig. 1a,b). Karyotyping, Y microdeletion and CFTR mutation screening were carried out for all patients with a SCOS diagnosis. Patients with Y chromosome microdeletion and CFTR mutation; those with numerical and structural chromosomal defects according to the chromosome analysis; systemic or endocrine diseases that could cause infertility, cryptorchidism, genital system obstruction, varicocele-associated testicular atrophy or a history of bilateral mumps orchitis; and those who had undergone chemotherapy or radiotherapy, or cytotoxic and/or radiological therapy were excluded from the study. After applying the exclusion criteria, 39 infertile male patients with primitive SCOS of unknown origin aged 25–45 years were included in the study. The average age of the patients was 30.2. On CGH microarray platforms, the reference (control) DNAs specified by the manufacturer according to the nature of the study are used. In the present study, DNA of a healthy man with fertile, normal XY and who had no disease was used as a control, as recommended for the Agilent 4 × 180K study protocol (NA12891-European Male). Before the study, written approval was obtained from the Non-Interventional Clinical Research Ethics Committee of Marmara University Faculty of Medicine (approval number: 23.09.2011-2).

DNA isolation

A Roche High Pure PCR Template Preparation Kit (Roche, Basel, Switzerland) was used to obtain DNA from peripheral blood samples. The protocol recommended by the manufacturer for DNA isolation was applied exactly. A NanoDrop ND-1000 UV-VIS spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to assess the gDNA concentration and purity. Only samples with a A260/A280 value of 1.8–2.0 in spectrophotometric measurement of gDNA were included in the further analysis. Each sample that passed this control step was fixed at a concentration of 50 ng/μL and an amount of 0.5 ng.

Array CGH

For all the samples, an Agilent SurePrint G3 Human CGH + SNP Microarray Kit 4 × 180K was used. In the 4 × 180K microarray chip, the probe length is 60 nucleotides and the resolution is 5 kb on average. The chip consists of 180 000 probes, 110 712 CGH and 59 647 SNP, and allows simultaneous analysis of four samples. On CGH microarray platforms, the reference DNAs specified by the manufacturer according to the nature of the study are used. In the current study, we used a control DNA as recommended for the Agilent 4 × 180K study protocol. For all the samples, enzymatic cleavage and fluorescent marking of gDNA, and hybridization and microarray analyses were undertaken in accordance with the standard protocol. After microarray scanning, feature extraction 10.5.1.1 and Agilent CytoGenomics 2.0.6.0

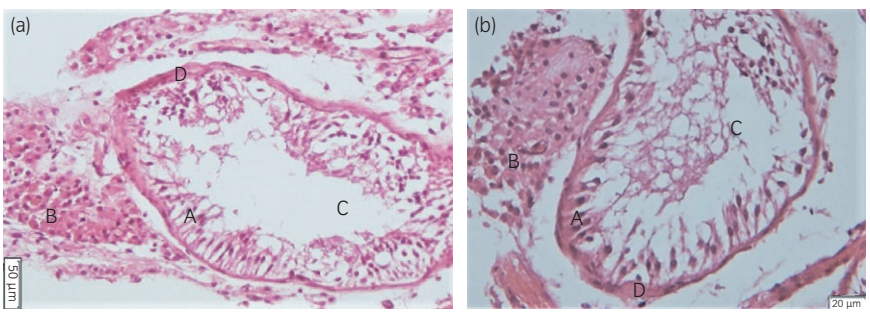

Fig. 1 (a,b) Pathological photographs of two cases with SCOS. SCOS: seminiferous tubules lined by Sertoli cells only and germ cells are completely absence. (A) Only Sertoli cells in the intracellular area, (B) hypertrophic Leydig cells, (C) seminiferous tubules and (D) basal membrane.

software (Agilent Technologies, Santa Clara, CA, USA) packages were used to extract the data from the image file (tiff) and convert it to log rates to analyze aberrations. The quality control reports of all samples were examined and determined to be appropriate for an array analysis.

Results

Clinical data

The clinical parameters caused by SCOS are presented in Table 1. The follicle-stimulating hormone level of patients increased compared with the normal value. Patients with SCOS also had lower testosterone serum levels. Sperm concentration and amount were not present in patients (Table 1). The clinical profile of the SCOS patients is appropriate for the clinical diagnosis parameters. A significant finding that could relate to candidate genes could not be detected in terms of hormone level.

Array CGH

Agilent Cytogenomics Feature Extraction 10.5.1.1 software was used to analyze Amp, Del changes and LOH in 39 infertile male patients with SCOS who underwent SNP + CGH microarray scanning. For each patient, the Amp/Del and LOH diagrams were created based on the changes observed throughout the genome. Figures 2 and 3 present the Amp/Del and LOH diagrams of patient 11 as an example.

The a-CGH analysis revealed Del, Amp and LOH in some genes previously associated with spermatogenesis defects and male infertility. In the current study, CNVs were identified in the following genes: *HOXD9*, *SYCE1*, *COL1A1*, *H19* and *KCNQ1*. The location, size and dosage changes (Amp/Del) of these genes in the chromosome are shown in Table 2, and their CNVs are shown in Table 3.

Table 1 Comparison of clinical parameters in the study group

	Sertoli cell-only syndrome (n = 39)
Testicular volume (mL)	28 ± 12
Luteinizing hormone (1.0–9.0 IU/L)	8.1 ± 3.5
Follicle-stimulating hormone (1.0–13.0 IU/L)	15.1 ± 4.6
Testosterone, free (9.0–30.0 ng/dL)	14.7 ± 6.2
Sperm concentration (10^6/mL)	0
Total sperm count (10^6)	0
a + b motility (%)	–
Normal morphology (%)	–

Data presented as mean ± standard deviation.

Discussion

The majority of male infertility problems are caused by spermatogenic defects. Spermatogenesis is a highly complex process in which a large number of genes and proteins function together, and very few genes have been identified that are known to cause SCOS in this process.[16] Del of a genomic segment can result in hemizygosity for the deleted segment and cause loss of function or mutation in the normal allele, thus leading to failure in prevention of the disease by this allele. An increased number of copies, such as Amp, can cause imbalances due to the overexpression of duplicated genes, or, when intragenic can alter product structure and function.[17]

In the present study, gene-dosage changes and LOH were investigated in 39 cases of well-characterized SCOS that caused infertile phenotype using the array CGH method to identify candidate genes that might be responsible for infertility. The array analysis revealed CNVs in the *HOXD9*, *SYCE1*, *COL1A1*, *H19* and *KCNQ1* genes.

Each homeotic gene is expressed at different times and in different tissues during embryogenesis. Proteins encoded by homotypic genes control the expression of other genes. Homeotic genes also have a role in regulating the cell division rate, cell site and final differentiation products, and determining the shape, size and surface properties of cells. All homeotic genes are formed by duplication, and contain a common region of 180 nucleotides (60 amino acids) called homeobox. Homeobox proteins provide a series of coordinated expressions when linked to DNA promoter and

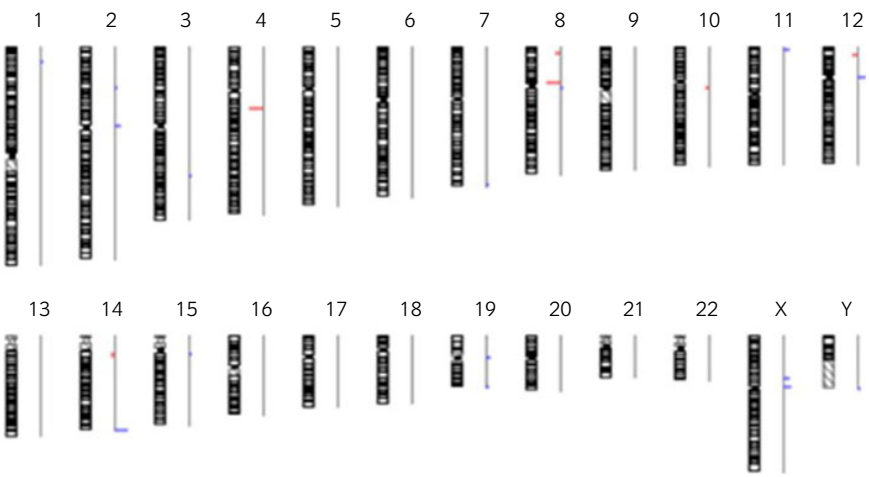

Fig. 2 Amp/Del diagram of all chromosomes of patient 11 (blue: Amp; red: Del).

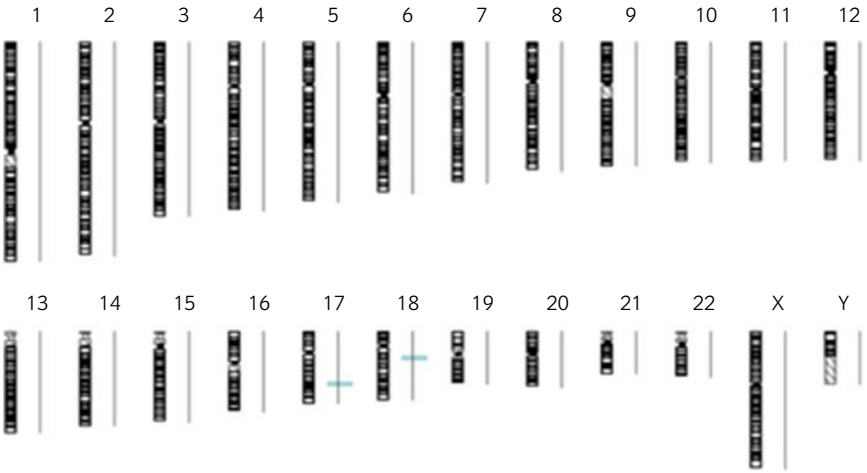

Fig. 3 LOH diagram of all chromosomes of patient 11 (blue: LOH).

Table 2 Locations, sizes and gene dosage changes of chromosomes in CNV defective genes (Amp and/or Del)

Location	Start	Stop	Size (bp)	Gene	Amp and/or Del
2q31.1	176 987 545	176 988 288	744	*HOXD9*	Amp and Del
10q26.3	135 254 039	135 377 532	123 494	*SYCE1*	Amp and Del
17q21.33	48 275 860	48 277 213	1354	*COL1A1*	Amp
11p15.5	2 016 675	2 016 774	100	*H19*	Amp and Del
11p15.5	2 721 354	2 722 525	1172	*KCNQ1*	Amp and Del

Table 3 CNV changes of genes according to patient

Patient no.	*HOXD9*	*SYCE1*	*COL1A1*	*H19*	*KCNQ1*
SCOS 1				Del	
SCOS 2				Del	
SCOS 3	Amp	Del			
SCOS 4					
SCOS 5		Amp			
SCOS 6				Del	
SCOS 7					
SCOS 8					Del
SCOS 9					
SCOS 10					
SCOS 11					
SCOS 12					
SCOS 13	Amp			Amp	Amp
SCOS 14					
SCOS 15	Amp				
SCOS 16					Del
SCOS 17				Del	
SCOS 18					Del
SCOS 19		Amp	Amp	Del	
SCOS 20			Amp		
SCOS 21	Amp				
SCOS 22					
SCOS 23					
SCOS 24	Del	Amp		Del	Del
SCOS 25					Del
SCOS 26			Amp	Del	
SCOS 27		Amp	Amp		Del
SCOS 28			Amp		
SCOS 29					
SCOS 30					
SCOS 31					
SCOS 32		Amp			Del
SCOS 33			Amp		
SCOS 34	Amp		Amp		
SCOS 35			Amp		
SCOS 36					
SCOS 37	Amp		Amp		
SCOS 38	Amp		Amp		
SCOS 39		Amp	Amp		

enhancer (control) sequences. Hox genes are a subgroup of homeobox genes. In vertebrate animals, these genes are found in gene clusters. In mammals, there are four clusters called Hox classes (HOXA, HOXB, HOXC and HOXD).[18] In a study, frameshift mutations were observed in the HOXA9, HOXA10 and HOXA11 genes in mice; and dramatic changes were occurred in the reproductive systems of both male and female mice. It was determined that these mutations led to developmental problems in the uterus and oviduct of female mice, and the vas deferens and epididymis of male mice.[19] In the current study, the identification of Amp in patients 3, 13, 15, 21, 34, 37 and 38, and Del in patient 24 in the HOXD9 gene indicates that the Amp/Del changes in this gene might have a direct effect on the development of the male reproductive system. However, the data in the literature are insufficient to reach a definite conclusion on this issue.

The SC, a structure formed by the assembly of a group of proteins, provides a link between homologous chromosomes in the prophase step of meiosis I. SC plays a crucial role for homologous chromosomes in terms of the formation of synapses, and proper segregation and distribution of chromosomes. The SC proteins are found in male and female gametogenesis at the pachytene phase of meiosis I. After the pachytene phase, SC usually breaks down and is no longer seen. Mutations in genes that form SC proteins are known to cause meiotic defects.[20,21] One of the proteins that constitute SC is SYCP3. In a study evaluating patients with spermatogenesis anomalies (spermatogonial arrest, testicular atrophy and SCOS) based on the mRNA expression levels of the *SYCP3* gene, these levels were found to be lower in the patient group compared with healthy individuals.[22] In the current study, although there was no change in the *SYCP3* gene, another associated SC protein, the *SYCE1* gene, was observed

to change. Del in one patient and Amp in six patients were detected in the *SYCE1* gene. It is believed that the SYCE1 protein is involved in stabilization and recombination of SC, and defects of this gene cause anomalies during spermatogenesis.

In the present study, Amp was identified in 11 patients in the "*COL1A1*" gene. Collagen fibrils, the major component of connective tissue, are mainly composed of three a-chains. Collagen monomers are synthesized in cells, secreted into the ESM and forms collagen fibrils.[23] In their study in 2004, Chen *et al.* found increased levels of *COL1A1*, *COL1A2* and pro-collagen gene expression in immature mouse testes.[24] Another study evaluating the testis tissue of 6- and 60-day-old male mice reported expression of *COL1A1*, *COL1A2* and pro-collagen in spermatogonium type A cells, but did not observe expression in Sertoli cells.[25] In addition, *COL1A1*, *COL1A2* and pro-collagen expression in 6-day-old mice was at a much higher level than in 60-day-old mice. As is known, the proteins of *COL1A1*, *COL1A2* and pro-collagen gene expression are incorporated into the ESM structure to form collagen fibrils. ESM located between Sertoli cells and germ cells regulates the movement, development and differentiation of cells; nutrient and matter exchange; migration of cells; and the relationships between the cells. Disorders of the ESM structure might stop Sertoli cells from providing germ cells with the necessary nutrients and metabolites, thus leading to the differentiation of germ cells and prevention of cell migration within ESM. In the current study, it is considered that the Amp of the *COL1A1* gene identified in 11 patients led to the development of SCOS by impairing the molecular structure of ESM and preventing the support of Sertoli cells for germ cells.

The mutation of the *COL1A1* gene is generally reported to be associated with osteogenesis imperfecta. Osteogenesis imperfecta patients show signs of defects in tissues where large type I collagen is present. These characteristics are: recurrent fractures, blue sclera, hearing loss, development disorders and dentinogenesis imperfect. In the present study, Amp was identified in 11 patients in the *COL1A1* gene, but no patient was diagnosed with osteogenesis imperfecta. An increase in the number of copies in the *COL1A1* gene might result in a tissue-specific imbalance in the gene product. Increased CNV can cause overexpression of the *COL1A1* gene in the testis tissue, but might not affect other tissues. An increased amount of collagen in testicular tissue might disrupt ESM. Disorders of the ESM structure might stop Sertoli cells from providing germ cells with the necessary nutrients and metabolites, thus leading to the differentiation of germ cells and prevention of cell migration within ESM.

The DNA methylation process in male germ cells continues after birth and is completed in the pachytene phase of meiosis I. Studies investigating spermatogenesis-specific genes suggest that the successful completion of DNA methylation in the sperm genome might represent a critical aspect of sperm maturation. Methylation is unique in the male germ cell line compared with somatic tissues. It begins in the prenatal period and is completed during postnatal spermatogenesis. DNA methylation in the sperm genome is an extremely important reaction in the maturation of spermatozoa. During transition of spermatogonia to spermatocytes, both loss and recovery of methylation are observed.[26,27]

In the present study, Amp and Del changes were detected in the *H19* and *KCNQ1* genes known as imprinting genes. The *H19* gene is specifically methylated during the development of large germ cells in men. In a study investigating whether imprinting defects led to defective spermatogenesis, it was found that the H19 gene was not properly methylated in patients with moderate and severe oligozoospermia.[28] Under normal conditions, the *H19* gene is imprinted during the premeiotic phase of spermatogenesis through the methylation of the paternal allele. If the paternal allele is not sufficiently imprinted, spermatogenesis does not take place in a normal manner and oligozoospermia eventually develops. According to the results of the present study, hypospermatogenesis and abnormal genomic imprinting are associated, and sperms of oligozoospermic men carry an increased risk of transmission of imprinting mistakes.[28,29] Among the infertile male patients with SCOS, Del of the *H19* gene was observed in seven patients, and Amp of this gene was detected in one patient. Although the Del of the *H19* gene in the paternal allele is not expected to be reflected on the phenotype, in the case of a maternal allele, it might negatively affect germ cell development through its effect on the expression in the *Igf2* gene. This indicates direct correlation with male infertility.

It was shown that in infertile men with protamine deficiency, significant hypermethylation occurred in the imprinting region of the KCNQ1 region in the sperm structure, and in the same patients, the H19 locus is hypomethylated. It was emphasized that the hypermethylation of the imprinting region of the *KNCQ1* gene resulted in weak sperm parameters.[30] The *KCNQ1* gene is active in the maternal allele and inactivated in the paternal allele. The allele in which Del takes place has a critical role, as in all imprinting genes. In the current study, Del of the *KCNQ1* gene was found in seven patients, and Amp of this gene was seen in one patient. Patient 13 had Amp of the *H19* and *KCNQ1* gene, and patient 24 had Del of the same genes. We believe that Del that occurs in an active gene as a result of methylation does not affect the phenotype, whereas Del of an active gene might cause infertility.

Future studies should determine the parental origin (maternal/paternal) of the changes in the imprinting genes of *H19* and *KCNQ1*, and include a methylation analysis to compare the results.

The results of the current study revealed noteworthy Amp/Del changes in the imprinting genes of *H19* and *KCNQ1*, as well as the *HOXD9*, *SYCE1* and *COL1A1* genes that were previously reported to be effective in the spermatogenesis process.

CNV as a DNA segment of ≥1 kb that is present at a variable copy number in comparison with a reference genome. CNVs have been shown to affect expression of genes and gene dosage. CNVs can contain a whole genome, gene fragments, a large number of large, regulatory elements or can be found in extrinsic regions. The content of the fragment undergoing Del or duplication is important for phenotypic results. In the present study, the combination of Amp and/or Del of

various candidate genes for SCOS was defined in the same patient.

The CNVs in these genes might act together, and cause the disease phenotype and/or severity. CNVs that are too long in the genome could contain a large number of genes. These might be the underlying causes of "neighbor gene syndromes" or genomic disorders.

CNV in one of these genes might cause disease by acting on the regulator region (enhancer) of the other gene, and can be moved beyond the limits of the regulatory effects. Thus, the disease phenotype might be emerging.

HOXD9, *SYCE1*, *COL2A1*, *H19* and *KCNQ1* genes are involved in the development of the male reproductive system and spermatogenesis process. Amp and Del in these genes lead to a change in gene dosage. Changes in the number of copies CNV in these genes can cause an increase or decrease in the amount of protein that is effective in the spermatogenesis process. Del of a genomic segment creates hemizygosity for the cleaved region, and failure to prevent normal allelic disease due to loss of function in the allele might result in a genetic disorder. An increase in the number of copies can lead to imbalances as a result of overexpression of the duplex genes, or when they are intragenic, they can change the structure of the product and hence the function. All genes (*HOXD9*, *SYCE1*, *COL2A1*, *H19* and *KCNQ1*) play a role in the reproductive system. Protein products of these genes might be affecting common signaling pathways in the spermatogenesis process. Dosage changes in these genes could cause germ cell destruction by affecting various protein signaling pathways and might result in the SCOS phenotype.

In the present study, Del/Amp combinations were determined in candidate genes in the same patient. Although array CGH analysis can identify Del or Amp on a genome-wide scale with high resolution, it did not explain Del/Amp combination. In the same patient, detection of a Del/Amp combination in different genes might cause SCOS to change the gene dosage profile. Here, we have shown the investigations of dosage imbalances for candidate genes. However, as the severity of the disease does not show a significant difference between the cases, it cannot be associated with the Del/Amp combination. Increased understanding of the molecular basis will arise from a greater understanding of the genes that might be implicated in the development of SCOS, and identifying the molecular pathways. Advanced analysis techniques and a larger number of patients are required to establish the relationship between these changes and the histopathology of SCOS.

Acknowledgments

This study was supported by a grant from the Research Foundation of Marmara University, Turkey (Project No: BSAGC-YLP-040112-0002).

Conflict of interest

None declared.

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