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Is idiopathic male infertility really idiopathic ? Detection of DNA copy number variations and candidate chromosomal loci among azoospermic males by high resolution comparative genomic hybridization

Açıklanamayan erkek infertilitesi gerçekten idiopatik mi? Azoospermik erkeklerde karşilaştirmali genomik hibridizasyon yöntemi ile DNA kopya sayısı varyasyonları ve aday kromozomal lokusların belirlenmesi

Kanay Yararbas, Hatice Ilgın Ruhı, Kaan Aydos, Atilla Elhan, Ajlan Tukun

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Abstract

Purpose: Infertility is one of the most common health problems affecting about one of five couples, and male factor contributes to a considerable proportion of this condition. This study aimed to detect DNA copy number variations of azoospermic males by high resolution comparative genomic hybridization and suggest candidate chromosomal loci associated with male infertility.

Materials and methods: By using Comparative Genomic Hybridization (CGH), we aimed to detect previously unidentified genetic etiologic factors among infertile males. Thus it may be possible to explain some idiopathic cases and provide more accurate counselling to the affected couples. This technique may also allow predicting de novo infertility related loci.

Results: A total of 90 patients were analyzed by comparative genomic hybridization.

49 patients revealed at least one finding, whereas in 41 patients (46%) there was no copy number variations detected by our technique. A total of 21 spermatogenesis – related genes was present within the CNV loci.

Conclusion: The data obtained from this study show that infertile males may carry some DNA copy number variations hat may not be detected by conventional methods. With additional data, it may be possible to identify the etiologic significance of these variations.

Key Words: Male infertility, azoospermia, comparative genomic hybridization, cryptic chromosomal changes, copy number variations.

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Özet

Amaç: İnfertilite, beş çiftten birini etkileyen, en yaygın sağlık sorunlarından biridir. Erkek faktörü, bu durumun önemli bir kısmını oluşturur. Bu çalışmanın amacı, azoospermik erkeklerde karşilaştirmali genomik hibridizasyon yöntemi ile DNA kopya sayisi varyasyonlarının ve aday kromozomal lokuslarin belirlenmesidir.

Gereç ve yöntem: Bu çalışmada karşılaştırmalı Genomik Hibridizasyon (CGH) yöntemiyle infertil erkeklerde daha önce tanımlanamayan genetik etiyolojik faktörleri saptamayı amaçladık. Dolayısıyla bazı idiyopatik vakaları açıklamak ve etkilenen çiftlere daha doğru danışmanlık sağlamak mümkün olabilir. Bu teknik aynı zamanda de novo infertiliteye ilişkin loküslerin tahmin edilmesine izin verebilecektir.

Bulgular: Toplam 90 hasta karşılaştırmalı genomik hibridizasyon ile analiz edildi.

49 hasta en az bir bulgu ortaya çıkarırken, 41 hastada (% 46) tekniğimizle saptanan kopya sayısı değişimi yoktu. CNV lokuslarında toplam 21 spermatogenez ile ilgili gen mevcuttu.

Sonuç: Çalışmadan elde edilen veriler, infertil erkeklerin geleneksel yöntemlerle saptanamayacak bazı DNA kopya sayısı varyasyonları gösterebildiklerini göstermektedir. Ek verilerle, bu varyasyonların etyolojik önemini saptamak mümkün olabilir.

Anahtar sözcükler: Erkek infertilitesi, azoospermi, karşılaştırmalı genomik hibridizasyon, kriptik kromozomal değişiklikler, kopya sayısı varyasyonları.

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Kanay Yararbas, MD, Medical Geneticist; Current Institution: Acibadem Mehmet Ali Aydinlar University School of Medicine, Department of Medical Genetics, İstanbul kanayyararbas@gmail.com, Corresponding Author. (orcid.org/0000-0002-5314-3406)

Hatice Ilgın Ruhı, MD, Ass. Prof. Medical Genetics Ankara University Faculty of Medicine, Department of Medical Genetics, Ankara, hatice.i.ruhi@medicine.ankara.edu.tr (orcid.org/0000-0002-9438-5962)

Kaan Aydos, MD; Prof. Urology, Ankara University Center of Infertility, Ankara, doktoraydos@gmail.com (orcid.org/0000-0001-7691-5459)

Atilla Elhan, PhD; Ass. Prof. Biostatistics. Ankara University Faculty of Medicine, Department of Biostatistics, Ankara, elhan@medicine.ankara.edu.tr (orcid.org/0000-0003-3324-248X)

Ajlan Tukun, MD, PhD, Prof. Medical Genetics. Current Institution: Duzen Laboratories, Ankara, ajlantukun@gmail.com (orcid.org/0000-0001-7445-0918)

Introduction

Infertility is defined as the inability to conceive in one year period despite regular intercourse and using no contraceptive methods. Infertility is now defined as a disease.

Almost 20% of all couples are infertile. Many studies were conducted after Mathews Duncan first mentioned about the epidemiology of infertility [1]. Today it is known that at least half of the cases of infertility are due to the male factor. Assisted reproductive techniques made treatment possible to achieve successful pregnancies, while keeping the risk of passing the underlying genetic problem to the new generations.

Male factor infertility assessment usually involves three steps: history, physical examination and laboratory investigations. A careful historical examination can inform the practitioner about reversible factors of infertility such as medications, smoking, and endocrine abnormalities [2]. During the physical examination, attention should be paid to the presence of hypospadias, atrophic testes or varicocele. Laboratory analysis can begin with spermiogram [3]. Only by semen analysis the prognosis could be predicted as fertile, infertile and in between [4]. Male infertility is historically etiologically divided into 3 subgroups as pretesticular, testicular and posttesticular causes. Genetic factors such as Y chromosomal microdeletions, chromosome abnormalities, immotile cilia, congenital diseases such as cryptorchidism are grouped in post-testicular ones [5].

Determining the genetic defect helps in improving prognostic outcomes of assisted reproductive techniques [6]. It is thought that the genes that play a role in developing male reproductive system are also important in male infertility etiology [6]. The primary genes in sexual differentiation are *SRY* and *SOX9*. *INSL3* and *LGR8* are thought to be related with cryptorchidism [6]. The mutations of *TFM*, 5-alpha reductase, *DAX1* that have endocrine functions lead to anatomic abnormalities and defective testis formation [7]. New genes are also predicted to play a role in male infertility as research studies increase in number [8]. For example, it is now known that mitochondrial mutations are also causative in abnormal sperm morphology and motility [9, 10]. The differential diagnosis of azoospermia is important for finding the etiological factor, establishing the treatment opportunities and providing accurate counselling [11]. After all, about half of the cases remain idiopathic and are thought to be of genetic origin. The genetic factor may disturb the formation or the transportation of the sperm [11].

Chromosomal abnormalities account for approximately 5-10% of etiological factors of male infertility. There is a reverse relation between the sperm count and chromosomal abnormality chance but chromosomal changes may also occur in about 1% of normospermic males. Klinefelter syndrome is the leading genetic cause [12]. Other common chromosomal abnormalities can be listed as, the XYY male genotype, 45,X/46,XY mosaicism which may cause a streak gonad on one side due to mix gonadal dysgenesis resembling Turner phenotype, translocations involving Y chromosome which include Y – autosome translocations that may cause the spermatogenesis genes to be interrupted and particularly the ones between X and Y that may cause XX male status with no genes regulating spermatogenesis. Deletions of Yq, dicentric iso Yp and ring Y may also cause the same problem. Autosomal reciprocal or Robertsonian translocations, where t(13;14) is the most common one, may lead to inappropriate pairing in meiosis. Chromosomal inversions lead to loop formations in meiotic pairing, decrease in recombination frequency and formation of unbalanced outcomes. Finally, extra chromosome or marker chromosome may lead to meiotic arrest and instability. The main approach for these families involves accurate counselling. There are some checkpoints in male meiosis. If there is an unpaired chromosome in metaphase 1; arrest occurs, p53 free apoptotic pathways eliminate the cell and preserve the healthy cycle [12, 13].

Y chromosome is of importance in carrying sex determining spermatogenesis-related genes. Detection of losses in these genes is valuable for predicting the chance of passing the genetic defect to the following generations. The deletions and resulting loss of genes in Y chromosome are beyond the detection of microscopic analysis and brings the need of

molecular techniques. The frequency of Y microdeletions is thought to be at least 1%. It increases up to 10-15% in azoospermic and severely oligospermic males. The deletions occur mostly in Yq11, where the most involved regions are AZFc (60%), then AZFb, AZFb+c or AZFa+b+c (35%). Deletions are not known to be related with varicocele, cryptorchidism or hypogonadotropic hypogonadism. Complete loss of AZFa and AZFb is related to Sertoli cell-only syndrome (SCO) and in these patients, there is no appropriate spermatogenesis. The prognosis in Sertoli only patients is poor. Partial deletions in AZFa,b and deletions of AZFc may benefit from assisted reproductive techniques, but Y chromosome shows absolute paternal transmission, so the deletion also occurs in male offspring [14].

Molecular-based cytogenetic techniques has been used widely in the last decade because of their sensitivity and ease of automation. The most known are hybridization-based techniques such as comparative genomic hybridization (CGH) and array-based CGH [15].

CGH is known to be sensitive in detecting the mosaicism involving more than 40% of the sample. Archived material could also be used for CGH studies so that it may be useful for patient follow-up and research studies [16].

Materials and Methods

Patients

After the approval of ethics committee (IRB), patient selection was performed as follows:

- Infertile but otherwise healthy,
- Abnormal spermiogram: Non-obstructive azoospermia,
- Normal karyotype,
- No Y chromosomal microdeletions,

Informed consent was taken from all the patients. A total of 90 patients were analyzed.

High Resolution Comparative Genomic Hybridization (HR-CGH) Procedure

Genomic DNA was extracted from peripheral blood lymphocytes of the patients. 1µg of test and reference DNAs were labelled with Spectrum Green dUTP (Vysis, Illinois, USA) and Spectrum Red dUTP (Vysis, 30-803400) respectively, using a CGH nick translation kit (Vysis, Illinois, USA) according to the manufacturer's instructions with slight modifications. The fragment sizes of the nick translation products were confirmed by 1% agarose gel electrophoresis. After obtaining the correct fragment size (300-3000 bp range), hybridization was performed according to the Vysis CGH hybridization protocols as supplied with the CGH hybridization reagents kit (Vysis, Illinois, USA).

Briefly, 800 ng of patient DNA, 400 ng of male reference DNA, and human Cot1 DNA (Invitrogen, California, USA) were co-precipitated in the presence of x0.1 volumes of 3M sodium acetate and x2.5 volumes of 100% ethanol and redissolved in 3 µl H2O and 7 µl CGH hybridization buffer. Probe denaturation was performed by heating the probe mix for 5 min in 75°C water bath and pre-hybridization was achieved at 37°C for 20 min. Hybridization experiment was performed on commercially available metaphase spreads (46,XY) (Vysis, Illinois, USA). Denaturation of the slide was carried out in denaturation solution (70% formamide/2XSSC, pH 7-7.5) and the slide was dehydrated in ice-cold 70%, 85%, and 100% ethanol for 1 min each respectively. Hybridization was allowed to proceed for 96 hrs in a humidified chamber at 37°C. Post-hybridization washes were carried out in 0.4XSSC/0.3% NP40 at 75°C for 2 min and in 2XSSC/0.1% NP40 at room temperature for 30 seconds. The slide was counterstained with DAPI.

For each cell, DAPI, green and red components were captured using appropriate filters. The image processing was performed by use of Cytovision 3.1 software from Applied Imaging (Applied Imaging, Michigan, USA). Average green/red ratios were calculated for each chromosome in ideally 20 metaphases. For the identification of chromosomal imbalances, the ratios 0.50 and 1.50 were used as cut-off values for losses and gains, respectively. The centromeric and heterochromatic regions were not taken into account.

Statistical Analysis

After formatting the data as excel tables, the CGH findings were compared with clinical features by Chi-Square test and Fisher's exact test. <%5 *p* values were evaluated as significant values.

Results

A total of 90 patients were analyzed by comparative genomic hybridization. While all the patients had azoospermia, as additional findings, 14 patients (15.5%) had operation history for varicocele, 5 patients (5.5%) had a medical history of undescended testis. In 9 patients (10%) a history of failed assisted reproductive techniques (ART) was present.

We found at least one finding in 49 patients, whereas in 41 patients (46%) there was no copy number variations detected by our technique.

No copy number variations were detected on chromosomes 17, 20 and 21. Most involved chromosomes in copy number variations were the gonosomes. The most frequently involved chromosome arms were Xq (18 patients, 20%), 11q (13 patients, 14.4%), Xp (10 patients, 11%), Yq (9 patients, 10%) and 22q (9 patients, 10%).

Most frequently seen loss sites were within chromosomes 19p (7.8%), Xp (4.4%) and Xq (4.4%) while most frequently seen gain sites were within chromosomes Xq (15.6%), 22q (8.9%), Yq (7,8%), Xp (6.7%) and 11q (11.1%).

All the copy number variations detected by CGH are mapped to chromosomal bands in detail in Figure 1, showing losses in red and gains in green. The candidate autosomal and X chromosomal loci and the genes within those “gained” and “lost” copy number variation (CNV) regions as well as referred articles [17-37] are listed in Table 1.

Discussion

The data obtained from this study showed that more than half of the patients carry a detectable DNA copy number variation. Most of the findings were localized at a specific locus that contains a gene that might explain the clinical findings.

In order to increase the sensitivity and data volume, the changes detected in low threshold were recorded.

CGH is widely used since first described by Kallioniemi et al in 1992. Traditional molecular cytogenetic methods deal with limited loci whereas CGH takes advantage of identifying DNA copy number changes in the whole genome. But CGH does not detect balanced abnormalities that do not reveal DNA copy number changes. The heterochromatin sites and Y chromosome, centromeric and (sub) telomeric regions fail to give informative results. Also, chromosomes 1, 19 and 22 usually show aberrant hybridizations, so these chromosomes should be evaluated carefully. Our study revealed some aberrations on 19th and 22nd chromosomes. It would be accurate to take the patient’s clinical findings into account.

The CNV term refers to at least 1 kb of copy number alterations of DNA comparing to reference genome that can result in a deletion or a duplication. The evolution of genome-wide copy number screening methods had started with CGH and lately brought out single nucleotide polymorphism (SNP) arrays. The most important improvements are the increasing accuracy and resolution. CGH methodology is a qualitative method which does not show the size of the CNV. The findings are mapped to the chromosomal regions but there is no data about the accuracy of the results. The findings could be confirmed by another methodology.

Finally our data revealed no statistical significance due to lack of power. It’s obvious that increasing the patient number and selection of a more uniform patient profile may allow the collection of more predicting data.

As most of the classic research studies, such as Dohle et al’s [38] recommend, laboratory approach to azoospermic male involve chromosome analysis, Y chromosome microdeletion analysis and cystic fibrosis transmembrane regulatory gene (*CFTR*) analysis. The results from different centers are in line with Dohle’s who found 8 47,XXY males, 7 balanced translocations, 8 Y chromosome microdeletions and 14 *CFTR* mutations. These mutations and related anomalies, such as the bilateral absence of vas deferens, Young syndrome, are all obstructive causes. Some genetic causes diminish the hormonal balance as first observed and published by Thielemans et al [39]. Kallman, Prader Willi, Bardet Biedl syndromes cause hypogonadotropic hypogonadism. 17-alpha hydroxylase, 17-beta-hydroxysteroid dehydrogenase and 3-beta-hydroxysteroid dehydrogenase deficiency demolish androgen synthesis and 5-alpha reductase deficiency demolish testosterone metabolism. Irregular LH activity

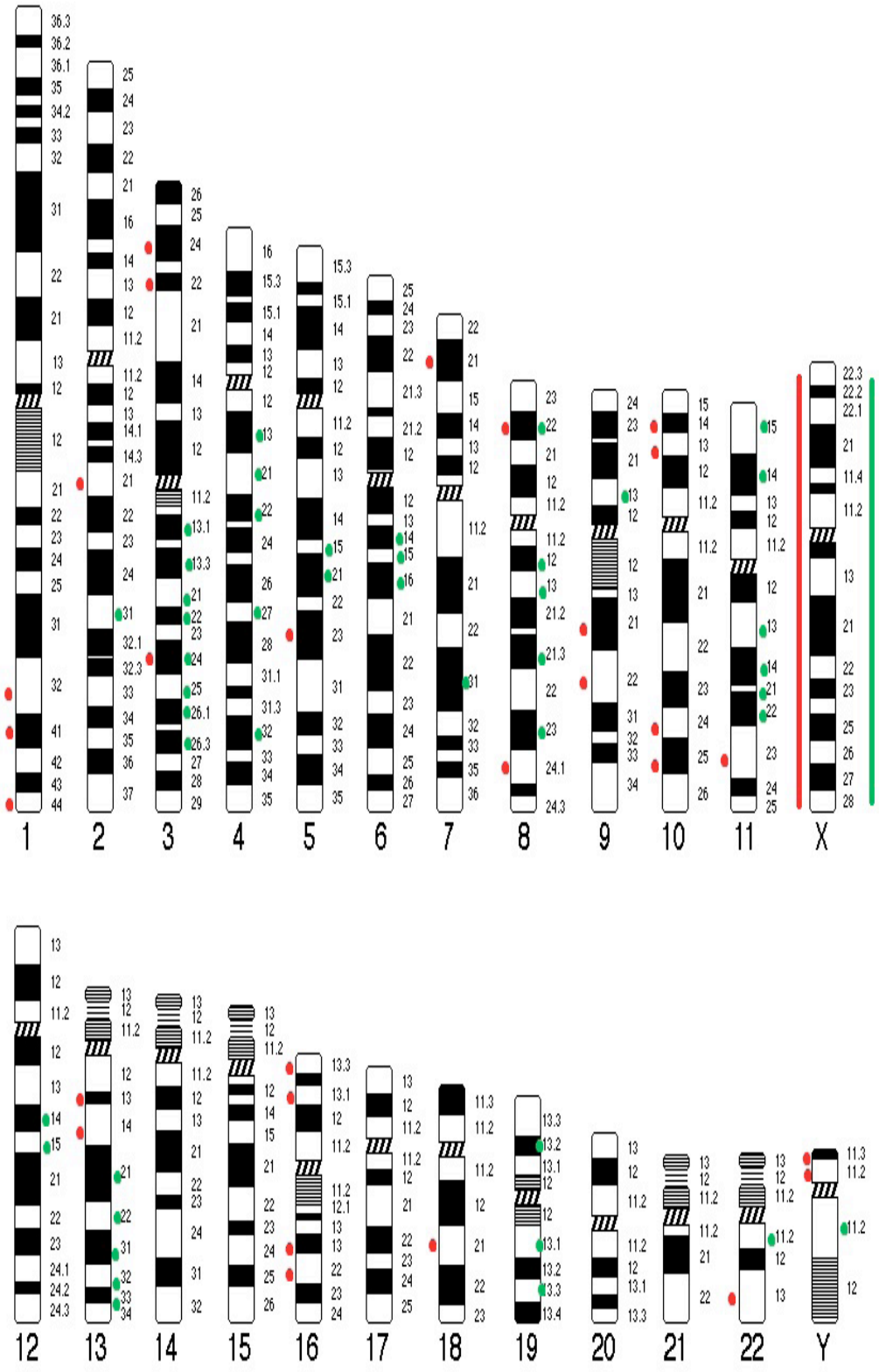

Figure 1. Detailed illustration of detected copy number variations, mapping to chromosomal bands. Please note that sex chromosomes are figured entirely because of high number of findings.

Table 1. The candidate autosomal and X chromosomal loci and the genes within those “gained” and “lost” CNV regions.

Chromosomal Locus (Sub)band	Type Gain (G) Loss (L)	phenotype	Clinically Relevant Gene affected within the aberrant region	Significance	Reference
1q32	L	azoospermia	ADORA1	Mammalian spermatozoa express ADORA1, where it is is functionally active. This gene was implicated to have role in fertilization process.	[17]
2q31	L	Varicocele azoospermia	NFE2L2 (nuclear factor (erythroid-derived 2)-like 2, NRF2):	This gene has been implicated in oxidative stress and it may be important in infertility related to a varicocele	[18]
3q26	G	Azoospermi	NYD-SP12 (SPATA16; spermatogenesis associated 16):	It codes a testis-specific protein Defective expression pattern is thought to be related to Sertoli only syndrome and defective spermatogenesis.	[19]
3q26	G	azoospermia	EIF5A2 (eukaryotic translation initiation factor 5A2):	Plays an important role in the continuity of spermatogenesis.	[20]
4q21+	G	Azoospermia Failed ART	CXCL10 (Chemokine (C-X-C motif) ligand 10):	. Chemokine synthesis is seen in Leydig cells during viral infections, so this gene could play a role in cases related to testicular inflammation.	[21]
5q15	G	Varicocele, azoospermia	NYD-SP16 (SPATA 9):	Responsible for production of one of the spermatogenesis-related proteins. The expression is lost in patients with Sertoli only syndrome.	[22]
6q15	G	Azoospermia Failed ART	SPACA1 (sperm acrosome associated 1; sperm acrosomal membrane-associated protein 32)	Functions in sperm acrosome morphogenesis and fusion with the ovum. The protein product is recognizable for the antisperm antibodies	[23]
7p21	L	azoospermia	DNAH11 (dynein, axonemal, heavy chain 11)	.Encodes a family member of dynein heavy chain. Responsible for cilia movement and functions as microtubule-dependent motor ATPase. Mutations result in primary ciliary dyskinesia, immotile cilia Kartagener and male sterility	[24]
8p22	L	Varicocele, azoospermia	MSR1	MSR1 was thought to be related to oxidative stress and prostate carcinoma. It could play an important role in the pathophysiology of varicocele.	[25]

Table 1. The candidate autosomal and X chromosomal loci and the genes within those "gained" and "lost" CNV regions (devamı)

9p13	G	azoospermia	TESK1 (testis-specific kinase 1):	Important in spermatogenesis.	[26]
10p13	L	azoospermia	VIM (vimentin):	Important in sperm morphology and related with constitutional sperm defects	[27]
11q13.1	G	azoospermia	TSGA10IP:	11q13.1. Related with TSGA10 that was introduced by Modarressi et al in 2001 Expressed in spermatocytes during spermatogenesis. (EntrezGene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=Pager&DB=gene).	[28]
11q22	G	azoospermia	TEX12 (testis expressed sequence 12):	It is similar to the gene expressed in mouse testis (EntrezGene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=Pager&DB=gene) and is important for synaptonemal complex assembly	[29]
16q13	L	azoospermia	TEPP (testis/prostate/placenta-expressed protein):	The selective expression is present in testis and some other tissues	[30]
19p13.2	G	azoospermia	INSL3	Related with descensus testis	[31]
19 p13.3	G	azoospermia	BGR-like gene	The expression is lost in azoospermic patients	[32]
19q13		azoospermia	LHB (luteinizing hormone beta polypeptide)	Important in stimulating spermatogenesis	[33]
19q13.3	G	azoospermia	DHX34	Member of a gene family functioning in spermatogenesis, embryogenesis, cell growth and cell division	[34]
Xp21	G, L	Azoospermia (both patients)	DAX1:	Known to be associated with congenital adrenal hyperplasia. Also important in testicular epithelization and maintenance of spermatogenesis	[35, 36]
Xq22.1	G	azoospermia	TAF7L	X chromosomal homolog of autosomal TAF7L. Spermatogenesis was shown to be impaired with improper functioning	[37]

decreases Leydig cell stimulation and causes testosterone deficiency. Testicular feminization and Reifenstein syndrome cause androgen insensitivity. Persistent Mullerian duct leads to male pseudohermaphroditism.

DNA copy number gains may lead to increase in gene dosage and may disturb its function. On the other hand, losses may result from submicroscopic deletions and in both situations, gene expression patterns may change. Occasionally unbalanced chromosomal rearrangements may not be shown in CGH profile as mentioned by Casas et al [40] so the sensitivity of CGH should not be predicted as 100% even though this happens rarely. Since our study population consists of patients with normal karyotype, this possibility did not bring any disadvantage. The sensitivity and specificity of the method were proved by using control DNA with known variations obtained from the tumour specimen (MPE 600 Control DNA, Vysis Inc.). Not to obtain any unbalanced changes should also be considered as a finding. Thus our normal results were considered as supportive for the conventional analysis. In order to the increase the specificity, different reference DNA could be used. Reference DNA of different gender could also be used as an internal control. The Same technique is useful for analyzing sex chromosome aberrations and XX males. In addition today array technologies bring more sensitive and specific design for detecting copy number variations.

Since the following generations are also considered to be at risk, counseling process is very important in male infertility [41]. So to clarify the underlying genetic etiology is obviously important. Despite the autosomal genes, defects on male chromosomes cannot be compensated. The abnormalities on sex chromosomal loci could be an etiological factor for male infertility since they play a role in spermatogenesis etc. In some of our patients, we found some gains or losses on Y chromosome. These loci might not be included in Y chromosome microdeletion kits but still could be involved in spermatogenesis [42].

Almost 1% of all males are infertile and approximately 15% of them are azoospermic. Our patient profile involved infertile azoospermic males, some of them with a medical history of varicocele, hormonal dysregulation, undescended testis. No special subtype was selected to be examined so results showed no statistical significance, but it is clear that increasing patient number with different subtypes might show remarkable findings.

Another remarkable point is that patients with CNV findings were not those who had additional findings such as varicocele. Similarly, only half of the patients with varicocele had CGH findings. These findings may still lead to generating a new hypothesis.

Assisted reproductive technique failure is also an important reason for patient referrals. Male factors are routinely examined, but especially submicroscopic changes could easily be missed. HR-CGH, particularly new techniques such as array CGH or SNP array may be chosen not as a second test or the test of choice especially when extra material, such as marker chromosomes, is detected but also for those cases still remaining as idiopathic after routine laboratory investigations [43].

X chromosomal changes were interestingly related with a whole chromosomal axis for enhancements but localized in losses. This supports the finding of mosaic X gains and supports the idea that spermatogenesis is diminished parallel to the severity of mosaicism in the testicular tissue. Since there is only one copy of X chromosome is present in men, and known that it is rich of testis specific genes, it is worth paying extra attention for X chromosomal studies in infertile males [44].

In conclusion the data obtained from this study show that infertile males may carry some DNA copy number variations that may not be detected by conventional methods. With additional data, it may be possible to identify the etiologic significance of these variations. There are many copy number variations in human genome. Although most of them are classified as benign, some may affect critical genes related with spermatogenesis within the deleted or duplicated area. Spermatogenesis is a complex process, which is estimated that, regulated by more than 2000 genes. So molecular karyotyping could be an alternative diagnostic method that can be used after standart chromosomal and molecular studies in azoospermic males.

Decleration of Interest

The authors report no conflicts of interest.

The authors declare that they all participated in the design, execution, and analyses of the study, and that they approved the final version of the manuscript.

The authors alone are responsible for the content and writing of this article.

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