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Screening of Y Chromosome Microdeletions and Cytogenetic Analysis in Infertile Men

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Abstract
In our study we aimed to demonstrate Y microdeletions with using sY254 sequence tagged sites (STS) marker in primary infertile male patients by using Polymerase Chain Reaction (PCR) method and determine the correlation between cytogenetic evaluation of patients. Twenty five male patients referred from Manisa and it's regions, who were diagnosed with primary infertility in the urology department of Manisa Celal Bayar University were included in our study. Thirteen individuals had azoospermia, twelve individuals had oligozoospermia. Ethics committee approval of our patients were recieved from Manisa Celal Bayar University. Blood material was taken under sterile conditions from individuals. Total DNA was isolated from peripheral blood. DNA purity was checked. Target gene regions of total DNA were amplified with PCR method by using oligonucleotide primers. sY14 STS marker used as internal control and pure water used as negative control. Cytogenetic study was made with lymphocyte cultures, human metaphase chromosomes were analysed with light microscopy. According to our analysis results no microdeletion was determined and all of the patients had normal karyotype (46, XY).

Keywords: Y microdeletion, sY254, STS, Infertility, Azoospermia, Oligozoospermia

INTRODUCTION

Infertility, which is defined as the inability to conceive after a year of unprotected sexual intercourse, affects almost 15% of couples. Male factors are responsible in about 50% of the cases [1,2,3,4]. Male infertility is a multifactorial syndrome. The major factors who contribute to infertility are; erectile dysfunction, infections of the reproductive system, gonadal endocrine disorders [Follicle Stimulating Hormone (FSH), Leutinizing Hormone (LH), testosterone], antisperm antibodies, exposure to chemical agents and radiations, testicular cancer, varicocele, anatomic abnormalities (hypogonadism, anorchia), genetic aberrations and others [4, 5, 6, 7, 8]. However, in many cases (in around 30%-50%) the causes of male infertility is still unknown, this condition is defined as idiopathic infertility [1, 4, 7]. Both genetic and environmental factors are responsible for reducing male infertility [1,9].

Genetic factors decrease reproductivity in males around 10-15% by affecting physiological processes of hormonal homeostasis, spermatogenesis, and sperm quality [3,10]. Some genetic causes of male infertility are chromosomal abnormalities, Y chromosome microdeletions, X-linked and autosomal gene mutations. After Klinefelter syndrome, Y chromosome microdeletions are the most frequent genetic cause of male infertility [1, 2, 10].

Between all human chromosome, Y chromosome is the shortest chromosome and it has the least number of genes. Y chromosome lenght is 60 Mb, 3 Mb associated with pseudoautosomal regions (PAR1 on short arm of the Y chromosome (Yp) and PAR2 on long arm of the Y chromosome (Yq)) and 57 Mb associated with nonrecombining regions (NRY) also known male specific Y (MSY). On the NRY region, heterochromatic and euchromatic regions are found. Known Y chromosome genes are in the euchromatic region. Euchromatic regions has nearly 23 Mb length; 8 Mb is located on the Yp and 14,5 Mb is located on the Yq [1, 6, 10, 11]. Y chromosome is essential for human sex determination, male germ cell improvement and continuation for normal spermatogenesis [1, 6, 9, 11, 12]. In 1976 Tiepolo and Zuffardi described the correlation among Y chromosome deletions and male infertility for the first time [10, 11, 12]. Y chromosome microdeletions are one of the most important genetic etiology of male infertility in the world. Generally male infertility is a de novo event of genetic origin and come to exist during spermatogenesis which involved more than 4000 genes. The long arm of the Y chromosome includes genes and gene families comprised in spermatogenesis [3, 9, 10]. Y chromosome microdeletions are responsible for not only to spermatogenesis defect, but also in recurrent pregnancy loss [14]. On the euchromatic portion of the Y chromosome long arm, microdeletions happen in 10%-15% of idiopathic primary testiculopathies (azoospermia and severe oligozoospermia) [10, 11, 12, 13]. These microdeletions present in the pathogenic mechanisms for infertile males [15].

Genes located on the euchromatic region of the long arm of the Y chromosome, interval 5 and 6, locus 11 (Yq11), called azoospermia factor (AZF) region and was genetically mapped in 1996 by Vogt et al., plays an essential role in spermatogenesis [1, 2, 6, 9, 11, 12]. Genes of AZF region are organized in three different nonoverlapping subregions; AZFa, AZFb and AZFc, these are necessary for normal spermatogenesis [4, 5, 9]. In recent years, the result of the sequence of the MSY and the molecular mechanism, a new AZF region which called AZFd was discovered. AZFd is located inthe place which AZFb and AZFc regions are overlapping [1, 5, 10]. Microdeletions of AZF regions result in several spermatogenetic changes and these microdeletions are associated with

azoospermia, oligozoospermia, diverse testis histology ranging from sertoli cell-only (SCO) syndrome, hypospermatogenesis (HSG) and maturation arrest [4, 9, 15].

AZFa region include ubiquitin-specific protease 9 (USP9Y) and dead box on the Y chromosome (DBY) [6]. This region is located on proximal Yq11 (Yq11.21) [6, 9]. Complete AZFa deletions are relevant to severe testicular phenotype, SCO syndrome, azoospermia. Partial AZFa deletions are extremely rare [1, 4, 14].

AZFb region include RNA binding motif on the Y chromosome (RBMY) [6]. This region is located on distal Yq11 (Yq11.23) [6, 9]. Complete AZFb deletions are associated with maturation arrest at meiosis I, and azoospermia and oligozoospermia. Partial AZFb deletions are changeable phenotypes from HSG to SCO syndrome extremely rare [1, 4, 6, 14].

AZFc region has the deleted in azoospermia (DAZ) gene cluster [6]. This region is located on distal Yq11 (Yq11.23) [6, 9]. Complete AZFc deletions are variable clinical and histological phenotypes which may range from severe azoospermia to mild oligozoospermia and SCO syndrome. Partial AZFc deletions are changeable phenotypes from HSG to SCO syndrome [1, 4, 6, 11]. There are many STS markers which are related with AZF regions for screening Y microdeletions. sY254 is one of the STS marker is mainly associated with DAZ gene of the AZFc region (Figure 1.) The absence of sY254 demonstrate complete deletion of AZFc region [15, 16].

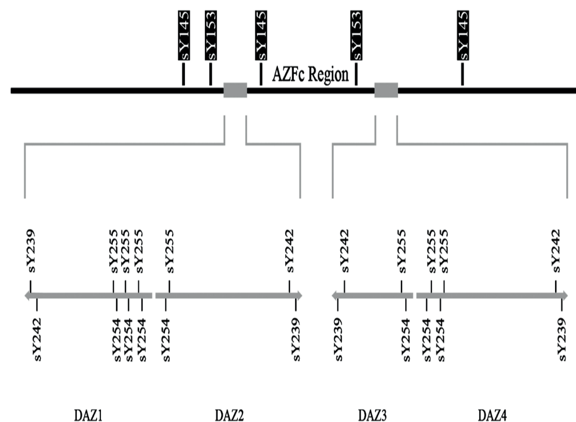

Figure 1. AZFc and putative AZFd region's relative STSs positions (STSs marked in bold belong to the putative AZFd region) [16]

In present, assisted reproductive technologies (ART) like testicular sperm extraction (TESE), intracytoplasmic sperm injection (ICSI), in vitro fertilization (IVF) help infertile men with azoospermia and oligozoospermia for succesful fertilizations and pregnancies. So, before performing ART procedure, determine Y microdeletions, especially AZFc region deletions, is very important diagnostic tool for obtain favourable results [2, 4, 5].

In this study, aimed to determine Y chromosome microdeletions with using sY254 STS marker and f method in 25 infertile men who were diagnosed with primary infertility in the urology department of Manisa Celal Bayar University and also ethics committee approval were recieved from Manisa Celal Bayar University.

MATERIALS AND METHODS

Material Collection

Semen analysis, reproductive hormone estimation (FSH and LH), karyotype and Y chromosome microdeletion analysis were examined for all patients.

Molecular Analysis

Peripheral blood materials was taken under sterile conditions from 25 patients in EDTA vacutainer tubes (BD Vacutainer) and were stored at -20 ^{O}C until DNA extraction. Total DNA was isolated from peripheral blood using DNA isolation kit (Macherey Nagel) according to manufacturer instructions. DNA samples were stored at -20 ^{O}C until PCR application. DNA samples purity were measured with spectrofotometre (Perkin Elmer). DNAs with an OD260 / OD280 ratio between 1.8-2.0 were taken into the study. Target gene regions were amplified with PCR method by using sY254 STS marker oligonucleotide primers (MWG Eurofins) (Table 1), reaction buffers and appropriate PCR programme. sY14 STS marker oligonucleotide primers (MWG Eurofins) used as internal control and pure water used as negative control. PCR products were run on 2 % agarose gel (Serva) and then imaged with a gel imaging system (Syngene).

Table 1. STS marker, primer sequences and PCR product size [5]

STS Marker	Primer sequence	AZF region	PCR product size (bp)
sY 254	F-GGGTGTTACCAGAAGGCAAA R-GAACCGTATCTACCAAAGCAGC	AZFc	370
sY 14	F-GAATATTCCCGCTCTCCGGA R-GCTGCTGCTCCATTCTTGAG	Internal control SRY	472

Cytogenetic Analysis

Peripheral blood materials was taken under sterile conditions from 25 patients in sodium heparin vacutainer tubes (BD Vacutainer) and were stored at -20 ^{O}C. Lymphocyte cultures were made from peripheral blood. GTG banding process was applied to preparates. Human metaphase chromosomes obtained by cytogenetic analysis were observed with light microscopy. For each patient 20 metaphases area were analyzed.

Semen and Hormone Analysis

Semen samples were collected after 3-5 days period of sexual abstinence into a sterile container (Falcon). Samples were analyzed for semen volume and sperm concentration, measured according to the reference values.

Blood materials was taken under sterile conditions from 25 patients in vacutainer serum separator tubes (BD Vacutainer) and were stored at -20 ^{O}C. Levels of serum FSH and LH were detected with biochemicals methods and measured according to the reference values. All semen and hormone analysis were done in the same laboratory.

RESULTS

Our patient group consisted of twenty five infertile men. Thirteen individuals were diagnosed with azoospermia and twelve individuals were diagnosed with oligozoospermia.

Clinical diagnosis, age, semen volume, sperm concentration, FSH and LH values of the patient group were given in Table 2.

Table 2. Clinical diagnosis, age, semen volume, sperm concentration, FSH, LH values of the patient group

Patient no	Clinical diagnosis	Age	Semen volume (ml)	Sperm concentration (x 10^6/ml)	FSH (mIU/ml)	LH (mIU/ml)
1	Azoospermia	28	3	0	24.47	13.47
2	Azoospermia	32	5	0	26.00	14.54
3	Azoospermia	35	4	0	26.66	15.44
4	Azoospermia	40	2	0	23.45	13.78
5	Azoospermia	21	4	0	25.45	14.66
6	Azoospermia	26	6	0	27.66	15.44
7	Azoospermia	23	5	0	25.00	17.54
8	Azoospermia	24	5	0	26.55	14.44
9	Azoospermia	33	6	0	24.44	13.77
10	Azoospermia	35	3	0	20.46	14.76
11	Azoospermia	36	4	0	23.55	13.54
12	Azoospermia	40	5	0	20.13	13.76
13	Azoospermia	23	6	0	26.99	16.55
14	Oligozoospermia	26	3	13	26.33	17.43
15	Oligozoospermia	29	4	10	-	-
16	Oligozoospermia	32	5	6	-	-
17	Oligozoospermia	37	5	4	-	-
18	Oligozoospermia	40	5	7	-	-
19	Oligozoospermia	32	5	15	-	-
20	Oligozoospermia	23	6	12	-	-
21	Oligozoospermia	20	4	15	-	-
22	Oligozoospermia	40	6	6	27.13	15.64
23	Oligozoospermia	23	5	4	24.13	14.34
24	Oligozoospermia	28	5	10	25.78	15.23
25	Oligozoospermia	33	4	16	19.54	15.00

The average age of patient group was calculated as 30,36. The average age of patient group who had azoospermia was calculated as 30,46. The average age of patient group who had oligozoospermia was calculated as 30,25.

Semen volume average of patients group was 4,6 ml. Semen volume average of patients who had azoospermia was calculated as 4,46 ml but sperm was not found. Semen volume average of patients who had oligozoospermia was calculated as 4,75 ml and sperm concentration was between 4 to 16 (x 10^6/ml).

In the cytogenetic analysis, 20 metaphase areas of each patient preparate were examined and they were observed to have normal karyotype (46, XY) (Table 3).

Table 3. Cytogenetic analysis results

Clinical diagnosis	Analyzed field count	Karyotype
Azoospermia	20	46, XY
Azoospermia	20	46, XY
Azoospermia	20	46, XY
Azoospermia	20	46, XY
Azoospermia	20	46, XY
Azoospermia	20	46, XY
Azoospermia	20	46, XY
Azoospermia	20	46, XY
Azoospermia	20	46, XY
Azoospermia	20	46, XY
Azoospermia	20	46, XY
Azoospermia	20	46, XY
Azoospermia	20	46, XY
Oligozoospermia	20	46, XY
Oligozoospermia	20	46, XY
Oligozoospermia	20	46, XY
Oligozoospermia	20	46, XY
Oligozoospermia	20	46, XY
Oligozoospermia	20	46, XY
Oligozoospermia	20	46, XY
Oligozoospermia	20	46, XY
Oligozoospermia	20	46, XY
Oligozoospermia	20	46, XY
Oligozoospermia	20	46, XY
Oligozoospermia	20	46, XY

As the results of our PCR analysis using sY254 STS marker oligonucleotide primers, on Y chromosome no microdeletion was determined (Figure 2).

Figure 2. I; 100 bp marker, II-XI, infertile patient AZFc sY2454 region and sY14 region

DISCUSSION

Y chromosome microdeletions and chromosomal abberrations are the most important genetic cause in infertile men affected with azoospermia and severe oligozoospermia [3]. The most common genetic reason of male infertility due to spermatogenesis deficiency is Y chromosome microdeletions, the incidence is 2.7-55.5% in infertile men [15].

The AZF region microdeletions in some cases of male infertility who have azoospermia or oligozoospermia are thought to be pathogenetically involved [1,3]. Deletions of Yq11.23 in AZF region lead to idiopathic azoospermia and oligozoospermia, the rate of incidence is about 8-12% in worldwide [6].According to the studies, AZF region deletions affect approximately 13% of men with non-obstructive azoospermia and in approximately 7 to 10% of men with oligozoospermia. Testicular tissue sections

taken from infertile men who has azoospermia with Yq11 aberrations were analyzed and intense spermatogenesis disruption were determined. Thus AZF region functions are absolutely necessary for differentiation and proliferation of human male germ cells [1]. In our study, we analyzed infertile men patients group who had 13 (52%) azoospermic patients and 12 (48%) oligozoospermic patients.

Even though chromosomal abnormalities in infertile men may lead to recurrent pregnancy loss, microdeletions in the AZFc region of the Y chromosome may have an important function in embryo efficiency or in protection of pregnancy [1]. The other important genetic factors which can result with male infertility are numerical and structural chromosomal abnormalities. This abnormalites can be detected with cytogenetic analysis. If a men have non-obstructive azoospermia and their total motile sperm count <5 million, American Urological Association and European Academy of Andrology Guidelines recommend cytogenetic analysis [13]. Male infertility which come to exist with Y chromosome abnormalities is characterized by azoospermia (absence of sperm), severe oligozoospermia ($<5\times10^6$ sperm/ml semen) and moderate oligozoospermia ($5–20\times10^6$ sperm/ml semen) [5]. In our patients group, thirteen patients had azoospermia and when we analyzed their semen samples, semen volume average was calculated as 4,46 ml but sperm was not found. Twelve patients had oligozoospermia and when we analyzed their semen samples, semen volume average was calculated as 4,75 ml and sperm concentration was between 4 to 16 (x 10^6/ml).

Since 1970, researches have shown that deletion of the long arm of the Y chromosome is associated with spermatogenic failure and leads to partial or complete spermatogenic arrest. The loci involved in sperm production and differentiation using molecular methods have been defined [9]. Small deletions, which are located on the Yq, are considerably smaller than other structural chromosomal abnormalities, can not be seen with microscope, can not be detected by karyotyping so these are called microdeletions. They can only be detected by using PCR method [2, 10]. Y chromosome microdeletions can not be detected with cytogenetic analysis, but rarely Y chromosome abnormalities may refer Y chromosome microdeletions presence [13]. The incidence of Y chromosome microdeletions in patients who affected with azoospermia (they have no sperm count) or affected with oligozoospermia (they have sperm count less than 5 million per milliliter) is 2-10% or more. Frequency of Y chromosome microdeletions may changeable because of the patient selection criteria and population composition. Sometimes it can be encountered with many false diagnosis protocols. The frequency of Y chromosome microdeletions observed in various studies may vary with heterogeneity in the type and number of PCR markers which were applied [14]. In this study, our patients group consisted of infertile men who had oligozoospermia and azoospermia. We used the PCR method to investigate microdeletions of Y chromosome. In PCR analysis using sY254 STS marker oligonucleotide primers, Y chromosome microdeletion was not detected in their related gene region. Also we used cytogenetic analysis method to investigate chromosomal abnormality on the Y chromosome. Altough we analyzed 20 metaphase area for each patient, chromosomal abnormality was not found. All of the patients had normal karyotype (46, XY).

According to our study results, possible microdeletions that may cause infertility may be found in other gene regions. It is thought that the probability of microdeletion detection may increase with increasing the number of patients and the number of genes regions which were screened.

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