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Y-CHROMOSOME MICRODELETIONS IN MEN WITH SEVERE SPERMATOGENETIC DEFECTS

Stela Racovita*[1], Svetlana Capcelea[1], Veaceslav Mosin[2], Svetlana Hadjiu [3,5], Ninel Revenco [3,5], Stanislav Groppa [4] and Mariana Sprincean[1, 5]

[1]Department of Molecular Biology and Human Genetics, [2]Department of Obstetrics and Gynecology, [3]Department of Pediatrics, Department of Neurology [4] *Nicolae Testemitanu* State University of Medicine and Pharmacy, [5]nstitute of Mother and Child, Chisinau, the Republic of Moldova

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***Corresponding author:**
Stela Racovita

ABSTRACT

Introduction: Male infertility has a heterogeneous etiology, most commonly caused by spermatogenesis disorders. Genetic factors explain about 30% of cases of male infertility associated with azoospermia and severe oligozoospermia. Y microdeletion is the most frequent known molecular genetic cause of severe impairment of spermatogenesis. **The Purpose:** To evaluate the frequencies and types of Y chromosomal microdeletion of the AZF regions, before Assisted Reproductive Techniques (ART) and to compare our results with other reports. **Materials and methods**: Infertile men were investigated during genetic counseling among infertile couples referred for ART treatment. The study group consisted of 98 men with azoospermia. They were between 21 and 44 years old (median age - 33 years). Semen analysis in all infertile men was assessed according to WHO, 2010. G-banding of metaphase chromosomes karyotype analysis were performed in all azoospermic patients. Genomic DNA was isolated and used to analyze AZF microdeletions by PCR. The regions and sequence-tagged sites of AZFa (sY84, sY86, DBY1, sY620), AZFb (sY117, sY127, sY134, SY143), and AZFc (sY254, sY255, sY153, SY158) were sequenced by multiplex PCR. The detections of sY14 (SRY) and ZFX/ZFY were employed as internal controls. Ten non-obstructive azoospermic men had Y chromosomal microdeletions. Six Y-microdeleted men underwent microsurgical observation of testicular architecture and quantitative histology of spermatogenesis in a strip of testicular tissue. The results were compared with the different type of Y microdeletion. **Results:** Deletions of Y chromosome were revealed in 10 (9,8%) of 98 patients with azoospermia. Deletions of AZFc - sY153, sY158, sY254 and sY255 locus were observed in five of ten azoospermic patients 50%. In two patients of ten 20% were detected with deletion of AZFb region, deleted markers were sY117, sY127, sY134, sY143. Deletions affecting both AZFb and AZFc loci were found in two patients 20%, with non-obstructive azoospermia. In only one case (1/10) 10% were detected microdeletions in each region of AZF: AZFa-sY84, sY86, sY620, DBY1; AZFb- s Y117, sY127, sY134, sY143; AZFc- sY153, sY158, sY254, sY255; and presence of the SRY gene, in the patient with XX male syndrome. AZFa deletions have not been detected in either patient. In all men with AZF microdeletions of the Y chromosome we found severe spermatogenic defects: however, we also didn't found, in all of them, mature sperm sufficient for ICSI. The patients were advised to use sperm from the donor for ICSI and IVF. **Conclusions:** Y chromosome microdeletions screening is important to define the aetiology of men with severe spermatogentic defects. This is important to provide a firm diagnosis and more effective solutions to couples with longstanding infertility, before ART.

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INTRODUCTION

Infertility is a global health problem, due to its high global prevalence and especially due to the significant negative consequences for quality of life (Datta, 2016).

Infertility isdefined by the World Health Organization (WHO) as the inability to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (Cavallini, 2015).

In approximately 15% of couples have difficulty or are unable to conceive (Naasse, 2015). A male factor is estimated to be present from 30% to 50% of cases, with sole responsibility in 30% of cases and a co-contributing female factor in 20% of factors (Katz *et al.,* 2017). The background of male infertility being extremely heterogeneous, most frequently being caused by disorders of spermatogenesis, clinically manifested by azoospermia and severe oligozoospermia. Genetic factors explain about 30% of cases of male infertility associated with azoospermia and severe oligozoospermia (Hamada *et al.,* 2013). The high frequency is justified by the involvement of numerous genes in the control of sexualization and reproduction (Reinke *et al.,* 2000). Y microdeletion is considered the most frequent known molecular genetic cause of severe impairment of spermatogenesis (Peterlin *et al.,* 2004). Deletion of the Y chromosome region containing the azoospermia factor (AZF) is located on the long arm of the Y chromosome (Yq11). Three regions on Y chromosome long arm (AZFa, AZFb and AZFc) have been identified in men with severe spermatogenic failure (Bichile *et al.,* 2014).Genes in these regions encode for proteins involved in the regulation of spermatogenesis (9). The frequency of these microdeletions in the general population is rare (1 in 4,000), but they occur in 10% of patients with idiopathic non-obstructive azoospermia and in 3 - 5% of men with severe oligozoospermia (Krausz *et al.,* 2018). The identification of Yq microdeletions is not only relevant for the diagnosis but it may have prognostic value prior to testicular biopsy (TESE- Testicular Sperm Extraction) in azoospermic men. In this regard, in case of complete AZFa, AZFb,c deletions of the Y chromosome testicular biopsy is not recommended because the chance of finding spermatozoa is very poor (Committee *et al.,* 2008). AZFc deletion, which are compatible with the presence of spermatozoa in the testis or in the ejaculate are obligatorily transmitted to the male offspring. The diagnosis of Yq microdletions has an obvious clinical significance, as it could have implications for the reproductive health and his children, therefore genetic counselling is mandatory (Lee, 2011).

MATERIALS AND METHODS

Patients: We analyzed infertile men opting for assisted reproduction at infertility clinics of the National Center for Reproductive Health and Medical Genetics and the Repromed Center in Chisinau, Republic of Moldova. Both institutions specialize in Assisted Human Reproduction where the vast majority of infertile couples from all over the country are opting for assisted reproduction. All of them underwent an andrological work-up, which included medical history, physical examination, and hormonal estimation. They were enrolled in the study because of poor sperm quality. The study group consisted of 98 men with azoospermia. They were between 21 and 44 years old (median age, 33 years). The written informed consent was obtained from each suject. The study protocol was approved by the national medical ethics' committee on research of the Nicolae Testemitanu State University of Medicine and Pharmacy, Chisinau, the Republic of Moldova. Semen analysis: spermiogram in all infertile men, was assessed according to published WHO guidelines Laboratory Manual for the Examination and Processing of Human Semen, 5th edition, 2010. All semen samples were collected in laboratory conditions after a recommended period of sexual abstinence of three to five days. Each sample was incubated at 37ºC and analyzed within an hour.

The spermiogram analysis was performed by the computerized method on the automated analyzer SQA IIC-P (Medical Electronic Systems, USA). Cytogenetic methods: Cytogenetic analyses were performed on preparations from cultures of peripheral blood lymphocytes and assessed by G- banding. The GTG (G-bands by trypsin using Giemsa) banding techniques were applied and 30 metaphases were counted, with minimum resolution of 550 bands in each patient. Nomenclature according to 2016 ISCN (International System of Cytogenetic Nomenclature) was used for reporting the results.

Molecular–genetic methods: Genomic DNA was prepared from peripheral-blood lymphocytes commercially - available blood DNA extraction kits. The DNA was amplified by multiplex Polymerase Chain Reaction (PCR) by using 14 Y chromosome specific STS (sequence- tagged sites). The STS primers used were sY84, sY86, DBY1, sY620 (AZFa); sY117, sY127, sY134, SY143 (AZFb); sY254, sY255, sY153, SY158 (AZFc) (Table 1). The detections of sY14 (SRY) and ZFX/ZFY were employed as internal controls. For each set of primers the PCR was carried out in a 25,5-µL reaction volume containing: 2 µl genomic DNA, 2,5 µl Dream Taq Green buffer, MgCl2, 2,5 µl dNTP (deoxy-nucleotidetriphosphate), 0.5 µl of each primer pair,18 µ H2O and 0.4 µl Taq DNA polymerase. Thermocycling (Biometra) were used for multiplex PCR set. Multiplex PCR sets No. 1, No. 2, No. 3 and No. 4 were performed together under the same PCR conditions as follows: initial denaturation at 95°C for 3 min; followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 45 s, extension at 72°C for 50s and a final extension at 72°C for 5 min. The reaction products were then analysed by electrophoresis at 3% agarose gels containing ethidium bromide and visualized under UV light. Six Y-microdeleted men underwent microsurgical observation of testicular architecture and quantitative histology of spermatogenesis in a strip of testicular tissue. The results were compared with the different type of Y microdeletion.

Table 1. Sequence-Tagged Sites (STS) and gene-specific primer sequences for deletion analysis

Multiplex PCR set No	STS	Region	Primer sequence	Size (bp)
1	*sY254*	AZFc	Forward 5′ *GGGTGTTACCAGAAGGCAAA-3′* Reverse 5′ *GAACCGTATCTACCAAAGCAGC-3′*	400 bp
	sY86	AZFa	Forward 5′ *GTGACACACAGACTATGCTTC-3′* Reverse 5′ *ACACACAGAGGGACAACCCT-3′*	320 bp
	sY127	AZFb	Forward 5*GGCTCACAAACGAAAAGAAA-3′* Reverse 5′ *CTGCAGGCAGTAATAAGGGA-3′*	274 bp
2	*sY255*	AZFc	Forward 5′ *GTTACAGGATTCGGCGTGAT-3′* Reverse 5′ *CTCGTCATGTGGCAGCCAC-3′*	126 bp
	sY84	AZFa	Forward 5′ *AGAAGGGTCTGAAAGCAGGT-3′* Reverse 5′ *GCCTACTACCTGGAGGCTTC-3′*	326 bp
	sY134	AZFb	Forward 5′ *GTCTGCCTCACCATAAAACG-3′* Reverse 5′ *ACCACTGCCAAAACTTTCAA-3′*	301 bp
3	*DBY1*	AZFa	Forward 5 *TATTGGCAATCGTGAAAGAC-3′* Reverse 5′ *TGCCGGTTGCCTCTACTGGT-3′*	277 bp
	sY117	AZFb	Forward 5 *GTTGGTTCCATGCTCCATAC-3′* Reverse 5′ *CAGGGAGAGAGCCTTTTACC-3′*	262 bp
	sY153	AZFc	Forward 5′ *GCATCCTCATTTTATGTCCA-3′* Reverse 5′ *CAACCCAAAAGCACTGAGTA-3′*	139 bp
4	*sY620*	AZFa	Forward 5′ *GGCTGATATATTTAACC-3′* Reverse 5′ *ACTCAAAACAACACAGTC-3′*	249 bp
	sY143	AZFb	Forward 5′ *GCAGGATGAGAAGCAGGTAG-3′* Reverse 5′ *CCGTGTGCTGGAGACTAATC-3′*	311 bp
	sY158	AZFc	Forward 5′ *CTCAGAAGTCCTCCTAATAGTTCC-3′* Reverse 5′ *ACAGTGGTTTGTAGCGGGTA-3′*	231 bp
Internal controls for each set of primers	*SY14*	ZFY	Forward 5′ *ACCRCTGTACTGACTGTGATTACAC-3′* Reverse 5′*GCACYTCTTTGGTATCYGAGAAAGT-3′*	495 bp
		SRY	Forward 5′ *GAATATTCCCGCTCTCCGGA-3′* Reverse 5*GCTGGTGCTCCATTCTTGAG-3′*	472 bp

RESULTS

A total of 98 infertile azoospermic men were studied, the median age, of infertile males was 33 years (range 21–44 year). The average duration of infertility was 7.9 years (range 1–24 years). Analysis of the AZF loci revealed microdeletions in 10 (9,8%) of 98 patients with azoospermia (Figure 1, Figure 2). The most common are microdeletions of the AZFc locus, deleted markers were sY153, sY158, sY254 and sY255 (Figure 1, Figure 2). In our sample, isolated deletions of this locus were observed in five of ten azoospermic patients with microdeletions - 50% (Figure 2). In two patients of ten 20% were detected with deletion of AZFb region, deleted markers were sY117, sY127, sY134, sY143 (Figure 1, Figure 2). Deletions affecting both AZFb and AZFc loci were found in two patients- 20%, with non-obstructive azoospermia (Figure 2). In only one case, 10% (1/10) were detected microdeletions in each region of AZFa-sY84, sY86, sY620, DBY1; AZFb- s Y117, sY127, sY134, sY143; AZFc- sY153, sY158 sY254, sY255; and presence of the SRY (Sex determining region on Y) gene (Figure 1, Figure 2, Figure 3). Full isolated AZFa deletions have not been detected in either patient.

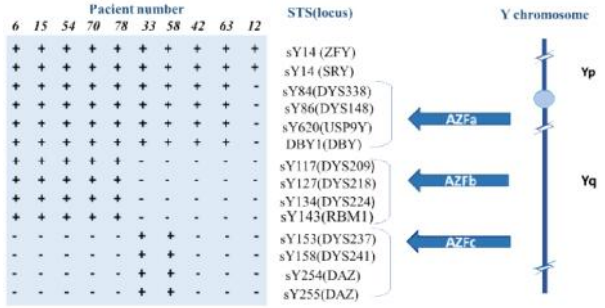

Figure 1. Schematic diagram illustrating different deletion patterns of the STS markers in the patients with deletions. +:PCR product was present;–:PCR product was not detected

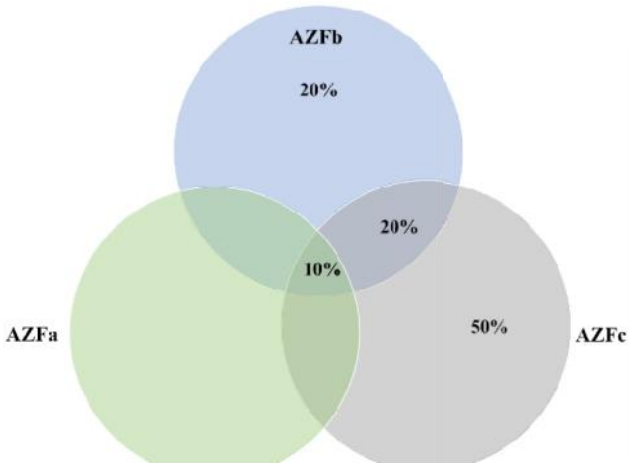

Figure 2. Venn diagram illustrating frequencies of different type of detected Y microdeletions in AZF regions. AZFc deletion were involved in 50% (5/10) of total deletions respectively. AZFb deletion were detected in 20% (2/10), combined deletions including AZFb and AZFc were also detected in 20% of all total deletions. Large deletion of all regions AZFa, AZFb and AZFc were found in 10% (1/10).

Clinical characteristic, hormonal analysis results, the cytogenetic evaluation of karyotype and testicular biopsy of azoospermic patients with AZF microdeletions are shown in Table 2.

The average age of 10 men with microdeletions of the Y chromosome was 32.7 years (range 28–38 year), with a history of infertility more than 3 years. Of the 10 patients with deletions, nine patients demonstrated no aetiological cause of infertility, whereas we found a deletion in one patient with varicocele. In two patients with AZFc deletion we detected a slight increase in follicle stimulating hormone (FSH) and serum testosterone concentration were redused. Significantly higher serum concentrations of FSH and relative normal levels of testosterone were in two cases with AZFb, and AZFb,c deletions. In nine cases with AZFc, AZFb, and AZFb,c microdeletions of the Y chromosome, were detected normal male karyotype (46,XY). In all men with AZFc deletions, the histological defects were variable, but no sperm were found. In men with AZFb microdeletions of the Y chromosome we found severe spermatogenic defects. However, we also didn't found, in all of them, mature sperm sufficient for ICSI (Intracytoplasmic sperm Injection). The patients were advised to use donor for ICSI and IVF (In vitro fertilization).

Microdeletions in each region of AZFa-sY84, sY86, sY620, DBY1; AZFb- sY117, sY127, sY134, sY143; AZFc- sY153, sY158 sY254, sY255; and presence of the SRY and ZFY (Zinc Finger protein, Y linked) gene, were detected in the patient with 46, XX karyotype, female characteristic (Figure 3, Figure 4, Table 2). The results of endocrine markers of the patient were as follows: slight increase in FSH of 10.5 mlU/ml (normal range of 2.0-10.0 mlU/ml); serum testosterone concentration 4.1 ng/ml (normal range 2.0-6.9 ng/ml); while luteinizing hormone (LH) was in the normal range of 7.7 mlU/ml (normal range 2.0-12.0 mlU/ml) (Table 2). Following clinical evaluation, the phenotype and psychological identity of the patient were male, height and weight being within the norm limits, genital organs characteristic of male sex with hypogonadism. The result of spermiogram presented azoospermia. The related results suggest the clinical diagnosis of sexual development disorder (XX syndrome, male phenotype). The presence of the SRY gene confirms and explains the male phenotype of the patient.

DISCUSSION

Our study shows that the microdeletion frequency (9.8%) found in 98 azoospermic men, from Republic of Moldova is comparable to the frequencies reported in the literature in a selected group of infertile men with severe spermatogenesis failure [Simoni *et al.*, 2004; Li, 2012; Nakashima, 2002; Rejeb, 2008; Stahl, 2010]. There is no correlation between total number of cases analyzed and Y microdeletion detected. According to a study conducted in Mexico, the frequency of AZF deletions is 12% of 50 with azoospermia [Shinka, 1996]. In the male population of China in 945 men with azoospermia, the frequency of microdeletions in the AZF region is identified in 11.5% [Li, 2012]. Similar data of 11.7% are reported by Japan on a sample of 60 azoospermic men and 11.8% by Tunisia on a sample of 76 men with azoospermia [Nakashima, 2002; Rejeb, 2008]. According to a study conducted in the USA on a group of 385 men with azoospermia, the incidence of AZF deletions being 10.4% [Stahl, 2008]. About 8.3% microdeletions in the AZF region were reported by Jordan, out of 34 azoospermic men and Norway 8.1% out of 37 men with azoospermia. In South Africa, the frequency of Y chromosome microdeletions in 50 men with azoospermia and severe

Table 2. Clinical features of azoospermic patients with Y chromosome microdeletions

No. patient	AZF deletion	Age	History of infertiity	Testosterone 2.0-6.9 ng/ml	FSH 2.0-10.0 mIU/ml	LH 3.0-12.0 mIU/ml	Sperm (mln/ml)	Karyotype	Testicular Biopsy
6	AZFc	32	5	2,2	11,5	4	0	46,XY	Sperm not found
15	AZFc	35	6	1,2	12,2	2,9	0	46,XY	Sperm not found
54	AZFc	31	3	5,9	9,9	6,7	0	46,XY	Sperm not found
70	AZFc	29	3	2	7,3	7,5	0	46,XY	Sperm not found
78	AZFc	28	3	3,2	5,2	5,1	0	46,XY	Sperm not found
33	AZFb	30	4	3,3	17,1	8,3	0	46,XY	Sperm not found
58	AZFb	34	3	5,4	5,7	12	0	46,XY	Not performed
24	AZFb,c	34	10	3,5	21,1	7,7	0	46,XY	Not performed
63	AZFb,c	36	8	3,0	6,9	8,4	0	46,XY	Not performed
12	AZFa,b,c	38	9	4,1	10,5	7,7	0	46,XX	Not performed

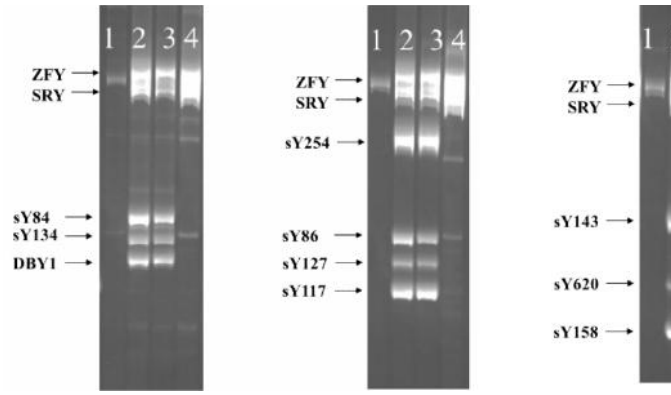

Figure 3. Results of electrophoresis for the detection of Y chromosome microdeletions: 1 – Female DNA; 2, 3 – Normal male sample; 4 – patient with large deletion of AZF (a, b, c) and presence of SRY and ZFY

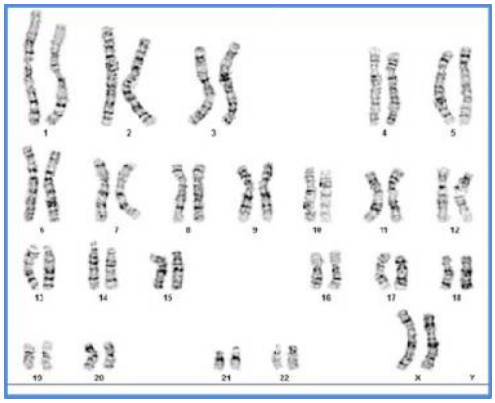

Figure 4: Patient age 38 years with 46,XX karyotype in male, and large deletion of Y chromosome (a, b, c) and presence of SRY gene

Also low frequencies are reported by Algeria - of 2%, Slovakia - of 4.6%, and Turkey - 1.3% in azoospermic men [Khabour *et al.*, 2014]. The frequencies of Y chromosome microdeletions are frequently diagnosed in a variable percentage from 2 to 20% of infertile men, in different geographical populations [Kerr *et al.*, 2000]. The heterogeneity of deletion results in the AZF region in different geographical regions may be due to the diagnostic method - the different numbers of STS primers used to detect mutation, a variation in the selective criteria used for recruited infertile patients or the fact that studies were performed on different ethnic populations [Stahl *et al.*, 2010]. According to data from the literature, the AZF region is absent in 1 of 8 azoospermic men with a normal karyotype [Stahl *et al.*, 2010]. In this study, we observed in nine cases AZFc, AZFb, and AZFb,c microdeletions of the Y chromosome, with normal male karyotype (46,XY). Deletion in AFZc (around 60 - 70%) is the most prevalent and frequent deletion in infertile men, followed by those in AZFb (1-5%), AZFa (0.5-4%), and AZFbc (1-3%) [22]. In our sample, we observed in 5(50%) of 10 (patients with microdeletions) deletionof AZFc region, AZFb was lost in 2/10 (20%) patients. In 2/10 (20%) cases were found AZFb and AZFc and AZFc, AZFb, AZFa were deleted in 1/10 (10%) case (Figure 1, Figure 2). This result is in agreement with the majority of the studies that indicate the high frequency of microdeletions in AZFc, followed by AZFb region [Balkan,2008; SãoPedro *et al.*, 2003; Omrani *et al.*, 2006]. Deleted markers in AZFc region were sY153, sY158, sY254 and sY255, which includes the DAZ (Deleted in Azoospermia) gene family (Figure 1). The DAZ family is reported to be the most common deletion gene in the AZFc region with a frequency of 13% in azoospermic males [Gary,2012; Vogt, 2003]. The DAZ gene cluster comprises several functional copies in the AZFc region, which encode proteins that bind to RNA (Ribonucleic Acid) being expressed exclusively in germ cells and control spermatogenesis. Deletions of the AZFc locus cause defects in spermatogenesis ranging in severity, from azoospermia due to Sertoli cells to oligozoospermia [Loginova, 2003]. The men with AZFc deletion have a favorable prognostic for sperm retrieval undergoing TESE. According to reports from many studies in patients with AZFc deletions mature sperm are obtained in approximately 50%, despite reduced fertilization rates and worse embryo scores after ICSI [Choi *et al.*, 2013]. In our study, testicular biopsy was performed in all five patients with AZFc deletion for sperm recovery, however, we didn't found, in all of them, mature sperm sufficient for ICSI.

Full AZFa deletions are rarely found among infertile males and are usually associated with complete absence of spermatogonia in the testicular tubule (Sertoli cell only syndrome type 1) [Mitchell *et al.*, 2017]. In our study this type that include full isolated AZFa deletions have not been detected in either patient (0%). The AZFα locus is located at the proximal part of the long arm of the Y chromosome and comprises approximately 1.1 Mb. This region includes the USP9Y (Ubiquitin specific peptidase 9, Y linked), DBY (DEAD box RNA helicases, Box 3, Y-linked), UTY (Ubiquitously transcribed tetratricopeptide repeat containing, Y linked) and TB4Y (Thymosin beta 4-isoform) genes. Foresta *et al*. reported well-defined spermatogenic alterations in individuals carrying deletions of both genes, USP9Y and DBY. In case of deletion of both USP9Y and DBY genes it causes severe defects in spermatogenesis, Sertoli Cell anly Syndrome thus this region being essential for fertility. The USP9Y gene is considered as a candidate gene for the AZFa phenotype [Selva, 2006]. In two cases we detected full AZFb, deleted markers were sY117, sY127, sY134, sY143 (Figure 1, Figure 2). This deletion is associated with azoospermia consequent to a meiotic

maturation arrest of germ cells [Mitchell, 2017]. Full AZFb deletions remove all copies of HSFY, EIF1AY, KDM5D, RPS4Y2, PRY and RBMY1. The RBMY (RNA-Binding Motif, Y linked) is one of the most important genes of the AZFb region with approximately six copies of this gene in the Y chromosome. Deletion of RBMY1 copies leads to decrease in sperm count, however, the presence of the two proximal RMBY1 copies is sufficient to avoid spermatogenic failure [Ergun-Longmire, 2005]. In one case we detected large AZF deletions involving AZFa, AZFb and AZFc loci and presence of the SRY and ZFY gene. The result of karyotype was 46,XX female characteristic (Figure 3, Figure 4, Table 2). Following the clinical evaluation of the patient, as well as the cytogenetic result, karyotype 46,XX, and molecular genetic that show the presence of the SRY gene and the lack specific markers of the Y chromosome, established a rare disorder of sex development (DSD) or de la Chapelle syndrome. Chapelle syndrome is a rare congenital condition with a frequency of 1:20000 newborns with a male phenotype. In majority of cases is due to an unequal crossing-over between the X and Y chromosomes during recombination of genetic material in prophase1 of meiosis and results in the translocation of the SRY gene from the Y chromosome to the X chromosome or autosomal chromosomes [Ergun-Longmire, 2005].

The presence of the SRY gene explains the male phenotype, in men with female karyotype. SRY is essential for initiating testis development and differentiation of the bi-potential gonad into Sertoli cells, which then support differentiation and development of the male germline (Colaco, 2018). In this case, extraction of testicular sperm is not recommended, residual spermatogenesis may not be present in the testicles. Men with deletions involving AZFa-b-c regions have the most severe spermatogenic damage. The presence of Sertoli cell syndrome and Leydig cell hyperplasia were found in other specialized studies after histological evaluation of testicular tissue in men (Ahmet Anık, 2003; Elena Vorona, 2007).

Conclusion

The spermatogenic loci AZFa, AZFb, and AZFc on the Yq11 chromosome control spermatogenesis in men and have an effect on fertility. Y chromosome microdeletions screening is important to define the aetiology of spermatogenesis defect. This is important to provide a firm diagnosis and more effective solutions to couples with longstanding infertility, before Assisted Reproductive Techniques.

Conflict of interest: The authors had no conflicts of interest to declare in relation to this article.

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