

Analysis of partial AZFc (gr/gr, b1/b3, and b2/b3) deletions in Iranian oligozoospermia candidates for intracytoplasmic sperm injection (ICSI)

Mitra ATAEE¹, Fatemeh AKBARIAN², Mahba Ataei TALEBI³, Peyman DOLATI⁴,
Maryam MOBARRAKI¹, Abolfazl FARAJI¹, Massoud HOUSHMAND^{1*}

¹Department of Medical Genetics, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran

²Ale Taha Institute of Higher Education, Tehran, Iran

³Mendel Medical Genetic Laboratory, Tehran, Iran

⁴Department of Biotechnology, Payame Noor University, Tehran, Iran

Received: 11.10.2017 • Accepted/Published Online: 02.12.2017 • Final Version: 30.04.2018

Background/aim: Infertility is a main health issue. The human Y chromosome contains essential genes for spermatogenesis, especially those located on four major intervals defined as AZFa, AZFb, AZFc, and AZFd. A partial deletion of the AZFc region is reported as a significant risk factor for oligo-/azoospermia. The main purpose of this study was to investigate the prevalence of partial deletions in the AZFc region (gr/gr, b1/b3, and b2/b3) in Iranian oligozoospermic candidates for intracytoplasmic sperm injection.

Materials and methods: Multiplex PCR was used to assess the micro and partial deletions in 60 oligozoospermia infertile and 80 fertile men.

Results: Two cases (3.33%) showed AZFb deletion but no microdeletion was detected in the control samples. In the AZFc region, 20% of the patients showed deletions, in which 15% and 5% showed gr/gr and b2/b3 deletions, respectively. However, 10% of the healthy individuals also showed partial deletions, including gr/gr (7.5%) and b2/b3 (2.5%). No significant correlation was detected between the presence of gr/gr microdeletion in patients and controls ($P > 0.05$).

Conclusion: Our study showed that the partial AZFc deletions are not associated with male infertility in Iranian subjects.

Key words: Oligozoospermia, gr/gr, b1/b3, b2/b3, partial deletion, intracytoplasmic sperm injection

1. Introduction

Male infertility is characterized as a multifactorial syndrome with a wide variety of disorders. The etiology of the disease remains unknown in more than half of infertile men with a limited prognosis. In many cases, the patient expresses natural attributes of manhood, but the tests are unable to produce spermatozoa capable of inseminating oocytes. Today, couple infertility is identified as a health problem. According to the World Health Organization in 1987, infertility affects about 10%–15% of all couples, and approximately 50% of the cases involve the male factor. In men, infertility is often associated with oligospermia or azospermia (1). In general, a high proportion of male infertility is associated with systemic defects such as diabetes, obesity, varicoceles, and cystic fibrosis or infections such as mumps and herpes. Unbalanced levels of gonadotropin hormones such as testosterone, dihydrotestosterone, follicle stimulating hormone, and luteinizing hormone are amongst other causes of infertility.

Not only has the production of hormones, the structure of testis, ejaculation, and spermatozoa had reverse effects on the chance of pregnancy, but genetic abnormalities also have a major influence on infertility. Genetic abnormalities in infertile men are ten times more likely than in the general population (2). The Y chromosome carries different genes essential for spermatogenesis. This concept was first presented by Tiepolo and Zuffardi (3). Their molecular studies on Y chromosome deletions led to the diagnosis of azospermia factors (AZF) in the Yq11.2 region (3). The Y-chromosome euchromatin region embraces a distinct family of genes essential for spermatogenesis that are usually attributed to AZF. The deletions that occur in the three regions of Yq11, shown as AZFa, AZFb, and AZFc, seem to be associated with special defects in spermatogenesis (4).

AZFc is located on the distal region of the deletion interval 6 (6C-6E) of the Y chromosome. AZF deletion districts have a high prevalence, as seen in 2%–10% of

* Correspondence: massoudh@nigeb.ac.ir

men with severe oligozoospermia or azoospermia (3). The expansion of this area is around 5.3 Mb and includes 7 gene groups involved in spermatogenesis (5). Fractional analysis of the AZF regions using new molecular markers proved three types of microdeletion known as gr/gr, b1/b2, and b1/b3 (6). The correlation between relative AZF deletion and infertility is unknown (7).

Some of these deletions have minor effects on infertility, while others have high risks linked with spermatogenesis defects. As suggested by Yen (2001), the b1/b3 deletion takes place in the proximal region of AZFc, with a very low prevalence, where only a small number of spermatozoa in infertile men have been observed (8). The relationship between this deletion and infertility remains unknown.

The second relative deletion (gr/gr) removes a proximal 1.6 Mb segment from the AZFc region containing two copies of DAZ gene clusters (DAZ 1, 2) (7,9). Vogt et al. detected this deletion in 5 out of 63 oligospermic samples, compared to 107 normal subjects. Therefore, they suggested that gr/gr deletion may be responsible for the reduced sperm count (10).

At least two further AZFc partial microdeletions, such as b1/b3 and b2/b3 deletions, were responsible for spermatogenesis, in which the effect of b1/b3 deletion remains unidentified with a lower frequency than gr/gr deletion in the general population. Just like gr/gr deletions, b2/b3 deletions are also associated with specific Y chromosome deletion. The b2/b3 deletion was first described in 2004 by the Sjoerd Repping group. They screened 1563 men and in 25 of them the SY1191 marker was eliminated, thus indicating that b2/b3 deletions probably originated from a gr/gr inversion and later deletion between the amplicon b2 and b3 (7).

Our objectives were to determine the prevalence of partial microdeletions of the AZFc region in oligozoospermic candidates for intracytoplasmic sperm injection (ICSI) to evaluate their association with spermatogenic failure, and also to define the genetic association between partial AZFc deletions in both infertile patients and controls.

2. Materials and methods

2.1. Specimen collection

Sixty infertile men with sperm concentrations of $<20 \times 10^6$ sperm/mL were recruited, having at least 4 duration of infertility. Male patients with oligozoospermia were from Yazd Infertility Treatment Center and those with normal karyotype were selected for this study. Eighty subjects with proven fertility and normal sperm concentration ($>20 \times 10^6$ sperm/mL) were recruited as the controls.

2.2. DNA extraction

Total DNA was isolated from the peripheral blood of the patients using a QIAGEN DNA extraction kit.

2.3. Molecular screening

Polymerase chain reaction (PCR) was used to detect the eight different loci corresponding to different primarily investigated AZF loci, which included AZFa (sY86, sY84), AZFb (sY134, sY127), AZFc (sY255, sY254), and AZFd (sY145, sY153). Individual and multiplex-PCRs were set up preceding the following STSs: individual PCRs (sY86, sY84), multiplex 1 (sY127, sY254, ZFY), multiplex 2 (sY134, sY255, SRY), and multiplex 3 (sY145, SY153, SRY). Moreover, two ZFY and SRY primers were used as the control primers. Further details of the primer sequences are provided in Tables 1 and 2. The reaction included 50–100 ng of the genomic DNA in 25 μ L PCR reaction mix containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 2 mM dNTP, 10 pM of each primer, and 1 unit of Taq DNA polymerase. The initial denaturation step was at 94 °C for 5 min, followed by 28 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 45 s ending with a final extension for 5 min at 72 °C. The PCR products were electrophoresed on 2% agarose gel stained with ethidium bromide. Then samples without classical AZF deletion were investigated in order to find gr/gr, b1/b3, and b2/b3 polymorphisms. The samples lacking partial AZF microdeletions were examined to find the gr/gr, b1/b3,

Table 1. List of sequence tagged sites (STSs) primers used as control primers.

STSs	Size (bp)	Sequence
ZFY	495	F: 5' ACCRTCCTACTGACTGTGATTACAC3' R: 5' GCACYTCTTTGGTATCYGAGAAAGT3'
SRY	472	F: 5' GAATATTCCCGCTCTCCGGA3' R: 5' GCTGGTGCTCCATTCTTGAG3'
sY142	196	F: 5' AGCTTCTATTTCGAGGGCTTC3' R: 5' CTCTCTGCAATCCCTGACAT3'

Table 2. List of STSs primers used to screen micro and partial deletions of AZF regions.

STSs	AZF	Size (bp)	Sequence
sY84	AZFa	326	F: 5' AGA AGG GTC TGA AAG CAG GT3' R: 5' GCC TAC TAC CTG GAG GCT TC3'
sY86	AZFa	320	F: 5' GTG ACA CAC AGA CTA TGC TTC3' R: 5' ACA CAC AGA GGG ACA ACC CT3'
sY127	AZFb	274	F: 5' GGC TCA CAA ACG AAA AGA AA3' R: 5' CTG CAG GCA GTA ATA AGG GA3'
sY134	AZFb	301	F: 5' GTC TGC CTC ACC ATA AAA CG3' R: 5' ACC ACT GCC AAA ACT TTC AA3'
sY254	AZFc	400	F: 5' GGG TGT TAC CAG AAG GCA AA3' R: 5' GAA CCG TAT CTA CCA AAG CAG C3'
sY255	AZFc	126	F: 5' GTT ACA GGA TTC GGC GTG AT3' R: 5' CTC GTC ATG TGC AGC CAC3'
sY145	AZFd	125	F: 5' CAA CAC AAA AAC ACT CAT ATACTCG3' R: 5' GGG CAT TGT ATG TTA ATA AGA GTT3'
sY153	AZFd	135	F: 5' GCA TCC TCA TTT TAT GTC CA3' R: 5' ATG AGT CAC GAA AAC CCA AC3'
sY1191	AZFc	385	F: 5' CCAGACGTTCTACCCTTTCG3' R: 5' GAGCCGAGATCCAGTTACCA3'
sY1291	AZFc	527	F: 5' TAAAAGGCAGAACTGCCAGG3' R: 5' GGGAGAAAAGTTCTGCAACG3'

and b2/b3 polymorphisms. The presence of the mentioned deletions was evaluated by utilizing a single multiplex-PCR performed with two sY1191 and sY1291 STS markers (Table 2), and also sY142 marker used for detecting the controls (Table 1). The reaction conditions and PCR program were similar to the classical AZF as stated before. Then PCR products were separated on 2% agarose gel. The gr/gr and b2/b3 deletions were represented respectively by not being amplified in the PCR products with sY1291 and sY1191 primers, which results in the amplification of b1/b3 deletion.

2.4. Ethics statement

Sixty infertile male patients and 80 subjects with proven fertility were referred to the Yazd Infertility Treatment Center for diagnosis between January 2007 and November 2011. Documented, written consent was obtained from the patients as approved for the entire study protocol by the SMC governing ethics committee at the time.

2.5. Statistical analysis

Fisher's exact probability test was used with SPSS (version 13) to examine the association between the patient and control groups; P values < 0.05 were regarded as statistically significant.

3. Results

The multiplex-PCR analysis was used in order to determine AZFb and AZFc including gr/gr, b1/b3, and b2/b3 subdeletions in all subjects. In this study, two subjects (3.33%) were identified as having 1 AZFb deletion in using plus/minus STS analysis of the genomic DNA recommended by EAA/EMQN (10).

Nine subjects (15%) were found to have gr/gr subdeletion. However, six controls (7.5%) also showed gr/gr subdeletions. Fisher's exact probability test was used to examine the association of gr/gr deletions and male infertility. The test revealed $P > 0.05$. The null hypothesis was accepted, indicating that there was no significant association between gr/gr deletions and male infertility.

Three subjects (5%) indicated b2/b3 subdeletions, while two controls (2.5%) showed b2/b3 subdeletions. Fisher's exact probability test was used to examine the association of b2/b3 deletions and male infertility. The test revealed $P > 0.05$. The null hypothesis was accepted, indicating that there was no significant association between gr/gr deletions and male infertility. In addition, the b1/b3 deletion was absent from the both patients and controls (Table 3).

Table 3. List of subdeletions (gr/gr, b1/b3, and b2/b3) in the AZFc region.

Partial AZFc deletions	Patients	Controls	P-value
gr/gr	9 (15%)	6(7.5%)	P > 0.05
b1/b3	-*	-*	-
b2/b3	3 (5%)	2 (2.5%)	P > 0.05

*shows the absence of deletion in both patients and controls.

4. Discussion

It is commonly accepted that Y chromosome microdeletion is a determining factor in severe spermatogenic disorders. However, the relationship between AZFc partial deletions and male sterility is still discussed. In the present study, the absence of AZFc region deletions in oligozoospermic patients initiated the investigation for Y chromosome partial deletions in a number of patients who lacked classical deletions. As far as these partial deletions were observed in the general population of infertile patients, it was thought that they did not have any influence on male fertility. Many studies have been carried out to detect the relationship between subdeletions and sterility in recent years, but the results in different populations were not identical.

In the present study, gr/gr deletion showed the highest frequency among the patient and control samples. In the patient samples, 9 (15%) and 3 (5%) individuals showed gr/gr and b2/b3 deletion, respectively. However, in the control population, 6 (7.5%) and 2 (2.5%) individuals showed gr/gr and b2/b3 deletions, respectively, which indicated no significant difference between the patient and control samples ($P > 0.05$).

Based on the results from the Institute of Reproductive Medicine, the AZFc region has the highest level of microdeletions (79%), while the frequency of AZFb, AZFa, AZFb,c, and AZFa,b,c deletions are 9%, 3%, 6%, and 3%, respectively (11). However, other authors reported that the classical AZFb deletions were more common than the AZFc region in the patients (12,13).

The AZFb microdeletion halts spermatogenesis and sperm maturation, while AZFc deletion is followed by phenotypic variations from severe oligospermia to azoospermia. Partial deletions in AZFc can either lead to azoospermia or associate with the normal phenotypes. Therefore, one type of treatment for these individuals involves ICSI. However, ICSI is considered dangerous for those born from fathers with Y chromosome abnormalities, as only 2%–3% of ICSI candidates have microdeletions on their Y chromosome. It is estimated that if half of the oligospermia men undergo ICSI, the prevalence of male infertility will increase two-fold throughout 7 generations when the inherited deletions are passed to

the offspring (14). Another study showed no significant difference in the population of fertile men in comparison to the frequency of these subdeletions in Iranian male oligozoospermic and azoospermic patients (13). Results of the present study have shown that deletion frequencies in Iranian infertile men are different from those of men in European, Chinese, and Indian populations, which can be due either to the low number of specimens or the selection of patients. It was previously thought that partial deletions do not affect male fertility because these deletions are also observed in a population of fertile men. However, several groups evaluated these microdeletions in infertile men in order to illustrate their close relationship with infertility. Other studies reported significant correlations of gr/gr deletions (15–17) and insignificant associations of AZFc partial deletions with infertility in men (18–22). However, there are several factors that affect these results, such as demographic variations, differences in patient selection values, AZF in variable deletion regions, applying different STS markers, and environmental effects, and also the classification method used to define oligospermia with different amount of sperms varied, i.e. 5×10^6 , 2×10^6 , and 1×10^6 , where Vog considered $2 \times 10^6 >$ sperms per milliliter as an oligospermia standard (23) and in other studies performed by Carrara (24) and Hellani (25) this standard was considered $1 \times 10^6 >$ sperms per milliliter, which certainly had an important influence on the results of these studies (26). However, in the present study, the standard for selecting patients among the oligospermia men was a sperm count of less than 20×10^6 per milliliter.

The role of gr/gr deletion in infertility and its high rate of occurrence in the Iranian male population is probably related to a polymorphic trait of gr/gr deletion in infertility (18,19,27) or as a genetic factor that affects male fertility by interactions with other genetic and environmental factors (6). Visser et al. carried out metaanalysis experiments over 7 studies, observing a higher frequency of gr/gr deletion in oligozoospermia and azoospermia male patients compared to the control population. Furthermore, the amount of sperm in the semen was lower in males with gr/gr deletion, which is in agreement with our study (28).

A study by Van et al. led to the hypothesis that deletions can either impair sperm quality or cause devastating

effects on sperm functionality in fertilization processes, which will reduce the probability of having a son (29). Therefore, evaluating Y chromosome deletions is an important factor in ICSI precounselling sessions. Long-term studies on ICSI children are the most necessary in men with hypospermatogenesis, where ICSI may lead to 45, XO embryos (30).

In the majority of men with impaired spermatogenesis, no clear reason can be identified to be responsible for their infertility. Complete deletion of the Y chromosome has been well accepted as the major cause of severe spermatogenesis disorders. However, the relationship between partial AZFc deletions and male infertility is still controversial. In recent years, many studies demonstrated the relationship between these deletions and infertility but results in different populations varied.

References

1. Singh AR, Vrtel R, Vodicka R, Dhaifalah I, Konvalinka D, Santavy J. Genetic factors in male infertility and their implications. *Int J Hum Genet* 2006; 6: 163-169.
2. Griffin DK, Finch KA. The genetic and cytogenetic basis of male infertility. *Hum Fertil* 2005; 8: 19-26.
3. Tiepolo L, Zuffardi O. Localization of factors controlling spermatogenesis in the nonfluorescent portion of the human Y chromosome long arm. *Hum Genet* 1976; 34: 119-124.
4. Vogt PH. Human chromosome deletions in Yq11, AZF candidate genes and male infertility: history and update. *Mol Hum Reprod* 1998; 4: 739-744.
5. Poongothai J, Gopenath TS, Manonayaki S. Genetics of human male infertility. *Singapore Med J* 2009; 50: 336-347.
6. Repping S, Skaletsky H, Brown L, van Daalen SK, Korver CM, Pyntikova T, Kuroda-Kawaguchi T, de Vries JW, Oates RD, Silber S et al. Polymorphism for a 1.6-Mb deletion of the human Y chromosome persists through balance between recurrent mutation and haploid selection. *Nat Genet* 2003; 35: 247-251.
7. Repping S, van Daalen SK, Korver CM, Brown LG, Marszalek JD, Gianotten J, Oates RD, Silber S, van der Veen F, Page DC et al. A family of human Y chromosomes has dispersed throughout northern Eurasia despite a 1.8-Mb deletion in the azoospermia factor c region. *Genomics* 2004; 83: 1046-1052.
8. Yen P. The fragility of fertility. *Nat Genet* 2001; 29: 279-286.
9. Zhang F, Li Z, Wen B, Jiang J, Shao M, Zhao Y, He Y, Song X, Qian J, Lu D et al. A frequent partial AZFc deletion does not render an increased risk of spermatogenic impairment in East Asians. *Ann Hum Genet* 2006; 70: 304-313.
10. Fernandes S, Huellen K, Goncalves J, Dukal H, Zeisler J, Rajpert De Meyts E, Skakkebaek NE, Habermann B, Krause W, Sousa M et al. High frequency of DAZ1/DAZ2 gene deletions in patients with severe oligozoospermia. *Mol Hum Reprod* 2002; 8: 286-298.
11. Simoni M, Bakker E, Krausz C. EAA/EMQN best practice guidelines for molecular diagnosis of y-chromosomal microdeletions. State of the art 2004. *Int J Androl* 2004; 27: 240-249.
12. Mirfakhraie R, Mirzajani F, Kalantar SM, Montazeri M, Salsabili N, Pourmand GR, Houshmand M. High prevalence of AZFb microdeletion in Iranian patients with idiopathic non-obstructive azoospermia. *Indian J Med Res* 2010; 132: 265-270.
13. Salsabili N, Mirfakhraie R, Montazeri M, Ataei M, Yaghmaei P, Pourmand G. Gonadotropin and testosterone hormone's serum levels and partial deletions in the AZFc region in Iranian oligozoospermia infertile males. *Health J* 2011; 3: 566-570.
14. Faddy MJ, Silber SJ, Gosden RG. Intra-cytoplasmic sperm injection and infertility. *Nat Genet* 2001; 29: 131.
15. de Llanos M, Balleca JL, Gazquez C, Margarit E, Oliva R. High frequency of gr/gr chromosome Y deletions in consecutive oligospermic ICSI candidates. *Hum Reprod* 2005; 20: 216-220.
16. Ferlin A, Tessari A, Ganz F, Marchina E, Barlati S, Garolla A, Engl B, Foresta C. Association of partial AZFc region deletions with spermatogenic impairment and male infertility. *J Med Genet* 2005; 42: 209-213.
17. Lynch M, Cram DS, Reilly A, O'Bryan MK, Baker HW, de Kretser DM, McLachlan RI. The Y chromosome gr/gr subdeletion is associated with male infertility. *Mol Hum Reprod* 2005; 11: 507-512.
18. Hucklenbroich K, Gromoll J, Heinrich M, Hohoff C, Nieschlag E, Simoni M. Partial deletions in the AZFc region of the Y chromosome occur in men with impaired as well as normal spermatogenesis. *Hum Reprod* 2005; 20: 191-197.
19. Machev N, Saut N, Longepied G, Terriou P, Navarro A, Levy N, Guichaoua M, Metzler-Guillemain C, Collignon P, Frances AM et al. Sequence family variant loss from the AZFc interval of the human Y chromosome, but not gene copy loss, is strongly associated with male infertility. *J Med Genet* 2004; 41: 814-825.

In conclusion, our findings suggest the necessity of more extensive studies on Y chromosomal rearrangements, especially the difference in gene function between copies of AZFc genes, genomic copy number variation, and screening for AZFc deletions in larger cohorts of both case and control populations in different ethnic/geographic origins for better understanding of the contribution of AZFc deletions in spermatogenesis and its pathology in male infertility.

Acknowledgments

We would like to thank all Iranian oligozoospermia male patients from the Yazd Infertility Treatment Center, Yazd, Iran. This work was supported by a grant from Dr M Houshmand, to whom we give our greatest appreciation.

20. Stouffs K, Tournaye H, Elst JVD, Haentjens P, Liebaers I, Lissens W. Do we need to search for gr/gr deletions in infertile men in a clinical setting? *Hum Reprod* 2008; 23: 1193-1199.
21. Yang Y, Xiao CY, A ZC, Zhang SZ, Li X, Zhang SX. DAZ1/DAZ2 cluster deletion mediated by gr/gr recombination per se may not be sufficient for spermatogenesis impairment: a study of Chinese normozoospermic men. *Asian J Androl* 2006; 8: 183-187.
22. Zhang F, Lu C, Li Z, Xie P, Xia Y, Zhu X, Wu B, Cai X, Wang X, Qian J et al. Partial deletions are associated with an increased risk of complete deletion in AZFc: a new insight into the role of partial AZFc deletions in male infertility. *J Med Genet* 2007; 44: 437-444.
23. Vogt PH, Edelmann A, Kirsch S, Henegariu O, Hirschmann P, Kiesewetter F, Kohn FM, Schill WB, Farah S, Ramos C et al. Human Y chromosome azoospermia factors (AZF) mapped to different subregions in Yq11. *Hum Mol Genet* 1996; 5: 933-943.
24. Carrara RCV, Yamasaki R, Mazucatto LF, Veludo MAL, Sartorato EL, Pina-Neto JM. Somatic and germ cell cytogenetic studies and AZF microdeletion screening in infertile men. *Genet Mol Biol* 2004; 27: 477-482.
25. Hellani A, Al-Hassan S, Iqbal MA, Coskun S. Y chromosome microdeletions in infertile men with idiopathic oligo- or azoospermia. *J Exp Clin Assist Reprod* 2006; 3: 1.
26. Arruda JT, Bordin BM, Santos PR, Mesquita WE, Silva RC, Maia MC, Approbato MS, Florencio RS, Amaral WN, Rocha Filho MA et al. Y chromosome microdeletions in Brazilian fertility clinic patients. *Genet Mol Res* 2007; 6: 461-469.
27. Ravel C, Chantot-Bastarud S, El Houate B, Mandelbaum J, Siffroi JP, McElreavey K. GR/GR deletions within the azoospermia factor c region on the Y chromosome might not be associated with spermatogenic failure. *Fertil Steril* 2006; 85: 229-231.
28. Visser L, Westerveld GH, Korver CM, van Daalen SK, Hovingh SE, Rozen S, van der Veen F, Repping S. Y chromosome gr/gr deletions are a risk factor for low semen quality. *Hum Reprod* 2009; 24: 2667-2673.
29. van Golde RJ, Wetzels AM, de Graaf R, Tuerlings JH, Braat DD, Kremer JA. Decreased fertilization rate and embryo quality after ICSI in oligozoospermic men with microdeletions in the azoospermia factor c region of the Y chromosome. *Hum Reprod* 2001; 16: 289-292.
30. Siffroi JP, Le Bourhis C, Krausz C, Barbaux S, Quintana-Murci L, Kanafani S, Rouba H, Bujan L, Bourrouillou G, Seifer I et al. Sex chromosome mosaicism in males carrying Y chromosome long arm deletions. *Hum Reprod* 2000; 15: 2559-2562.