Partial Deletions of Y-Chromosome in Infertile Men with Non-obstructive Azoospermia and Oligoasthenoteratozoospermia in a Turkish Population

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Abstract. Many genetic alterations have been identified to aid in understanding the genetic basis of male infertility, however, the cause of 30% of male infertility remains unknown. Some studies indicated that subdeletions of Y chromosome may be a reason for male infertility caused by testicular failure. In this regard, we aimed to investigate frequency of AZFc region subdeletions and their clinical effects in patients with idiopathic infertility. A total of 333 male infertile patients with non-obstructive azoospermia (NOA) or oligoasthenoteratozoospermia (OAT), and 87 normozoospermic controls were screened to detect gr/gr, b1/b3 and b2/b3 subdeletions. We recorded higher gr/gr deletion frequency in normozoospermic controls compared NAO and OAT groups (p=0.026). There were no significant differences in b2/b3 subdeletion rates among groups (p=0.437). In the OAT group, follicle-stimulating hormone levels of cases with b2/b3 deletion were statistically lower than cases without b2/b3 deletion (p=0.047). No statistical correlations were indicated among subdeletions, sperm count and assisted reproductive technology (ART) outcomes. These data demonstrate that gr/gr and b2/b3 subdeletions may not play a significant role in the etiopathogenesis of male infertility and ART outcomes in the studied population.

Infertility is the inability for a couple to achieve pregnancy after at least 1 year of unprotected sexual intercourse. Being multifactorial in nature, infertility attributes its roots to an array of causes. The most common causes of infertility include: undescended testis, varicocele, antisperm antibodies, hypogonadism and a whole range of genetic disorders, such as numerical and structural chromosomal aberrations (1).

Chromosomal abnormalities, Y-chromosome microdeletions, and mutations of cystic fibrosis transmembrane conductance regulator (CFTR) gene are well-established causes of infertility in azoospermic or severe oligozoospermic men. In addition, a number of copy number variations have been described to be associated with azoospermia and severe oligozoospermia (2).

The long arm of Y-chromosome (Yq) contains highly repetitive homologous DNA sequences arranged in an inverted orientation. Of these, approximately eight palindromic sequences are susceptible to non-allelic homologous recombination and are known to carry many genes critical to the development of male germ cells. These DNA sequences are arranged in detached intervals of AZFa, P5/proximal P1 (AZFb), b2/b4 (AZFc), and partially overlapped P5/distal P1and p4/distal P1 (AZFbc) regions. Deletions of the genes in these intervening sequences can be the result of faulty recombination and therefore, known to cause azoospermia and spermatogenic failure (1-5). All these three AZF regions are constitutional domains for proper spermogenesis (6). Common deletions occur in AZFc region (~80%) causing non-obstructive azoospermia (NOA) and severe oligozoospermia (OAT) (<5 million sperm/ml) (7), then deletions in AZFa and AZFb regions with frequency of 0.5-4.0% and 1-5%, respectively.

The two regions AZFa and AZFb have a deletion frequency of 1-3% (8-11). Repping and colleagues established that AZFc subdeletions (partial deletions) (12). b1/b3, b2/b3 and gr/gr are three main groups of Y-chromosome AZFc partial deletions (Figure 1). However recent studies from various ethnic populations have produced conflicting results (12-15).

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Key Words: Non-obstructive azoospermia, oligoasthenoteratozoospermia, male infertility, Y chromosome deletion, gr/gr, b1/b3, b2/b3.
This study aimed to evaluate AZFc b1/b3, b2/b3 and gr/gr partial deletion frequency in infertile men with a sperm count of less than 5 million sperm/ml and normozoospermic controls among the studied population were used so as to assess possible effects on outcomes of assisted reproductive technology (ART).

Materials and Methods

The total number of NOA and OAT infertile men involved in this study was 404. Eighty-seven normozoospermic men attending Andrology Clinics at the Department of Urology and the In Vitro Fertilization Centre at Ondokuz Mayis University (OMU) were also enrolled in the present study. The protocol of this study together with consent form was approved by the OMU Clinical Research Ethics Committee (OMU TAEK 2011/484). All participants signed a consent form stating their full consent and their own free will to participate in the study after receiving detailed information about the study. Demographic data, medical histories, physical examination findings and laboratory results were collected retrospectively. Firstly, patients underwent a routine physical examination. Testicular volumes were measured with Prader Orchidometer (Andromed). All blood samples were taken between 07:00-11:00 for hormonal examination. Total testosterone (TT), follicle-stimulating hormone (FSH), prolactin (PRL), luteinizing hormone (LH) and estradiol (E2) levels were quantified with radioimmunoassay at the OMU Biochemistry Laboratory (16).

Semenz analysis micro-testicular sperm extraction (TESE) was performed on 119 (54.5%) cases of the NOA group for sperm retrieval to use in ART and histopathological examination of testes.

The exclusion criteria for patients was obstructive azoospermia (cases with low ejaculate volume, normal FSH level, unilateral/bilateral vas deferens agenesis, epididymal obstruction due to previous surgical procedures, acidic semen, and previous vasectomy), secondary infertility, testicular malignancy, orchidectomy, radiation exposure or prescribed drug usage, chromosomal aberrations and Y chromosome microdeletions. Consequently, 22 cases with Klinefelter syndrome, 12 cases with hypogonadotropic hypogonadism, another 12 cases with Y-chromosome microdeletions, two cases with 46,XX testicular disorder of sex development and 23 cases with obstructive azoospermia were not included in the study. After repeated semen analysis in accordance with the World Health Organization 2010 criteria (17), the study group was divided into two groups based on sperm count: 115 patients with OAT and 218 patients with NOA. All patients and controls were from the Central Black Sea Region of Turkey.

Power analysis was performed to determine the actual sample size needed. The AZFc subdeletion frequency was considered to be 5% in fertile men. The size of the control group was estimated to be 55 with 95% confidence interval (CI) and 80% power at the level of significance 0.05. Therefore, 87 normozoospermic male were enrolled in the study as a control group. Fifty-five out of these 87 controls were volunteers each of which had fathered at least one healthy child.

Y-Chromosomal subdeletion analysis. Whole blood samples were collected for isolation of genomic DNA using a conventional ‘salting out’ procedure (18). The quality of DNA was assessed using a spectrophotometer (Multi-scan Go; Thermo Scientific, Turku, Finland). We then tested the presence or absence of partial AZFc deletions using polymerase chain reaction (PCR). Sex determining region Y (SRY) gene was used as a positive control. sY1161, sY1191, sY1201, sY1206 and sY1291 sequence tagged site (STS) markers were used to detect Yq AZFc subdeletions. PCR conditions and primer sequences were used as given elsewhere (19). Amplicons were distinguished on 2% agarose gel and then visualized using gel documentation system after staining with ethidium bromide. The deletions of b2/b3 and gr/gr were represented by the absence of markers Y1191 and Y1291, respectively, and presence of the other four STSs (2). The b1/b3 subdeletions were qualified by absence of sY161, sY1191 and sY1291 STSs and existence of sY1201 and sY1206. We considered STS deletion as occurring only after there were at least three unsuccessful amplifications in the presence of amplification of positive control. We performed at least three additional PCRs to eliminate false deletions and maximize accuracy of results.
We assessed AZFc subdeletion frequency in infertile man with OAT and NOA in a Turkish population. Our results indicate that there is no association between the frequency of AZFc subdeletions and male infertility.
Deletions of gr/gr have roles in various spermato genesis statuses from normozoospermia to azoospermia by reducing the number of copies in AZFc regions (14, 22-24).

Repping and colleagues showed an association between gr/gr AZFc subdeletions (12) and also showed a relation between b2/b3 partial deletion and male infertility 1 year later (25). Interestingly, these subdeletions were demonstrated in oligozoospermic and azoospermic men, as well as in normozoospermic men (23). A study with 337 infertile and 263 normozoospermic fertile men from Italy demonstrated one (0.29%) b2/b3 subdeletion and statistically higher gr/gr deletion 17 (5.3%) in the infertile group, whereas only one (0.4%) gr/gr deletion was found in the fertile group and consequently, a statistically higher frequency of gr/gr deletions were reported in infertile patients (14). Several other studies demonstrated a possible association between male infertility and gr/gr subdeletion (26-28). However, our observation show higher frequency of gr/gr partial deletions in the control group (12.6%) compared to NOA (4.1%) and OAT (7%) groups (p=0.026). Several studies also did not observe any significant relation between gr/gr subdeletions and male infertility. A study with 96 NOA and 87 normozoospermic males from Sri Lanka detected four gr/gr deletions in both NOA and normozoospermic groups and concluded that gr/gr deletion was not enough for spermatogenic failure alone (29). Zhang and colleagues reported a relationship between gr/gr deletion and infertility in cases from Holland, Spain and Italy, however the same authors reported no relationship between case studies from eight ethnic groups in East Asia (30). Likewise there was no relationship between gr/gr deletions and infertility in studies from Israel China, Malaysia, Egypt and Chile (13, 31-36). In a meta-analysis (6,388 cases and 6,011 controls), Stouffs and colleagues reported the frequency of gr/gr deletions as 4.69% in normozoospermic controls and 6.86% in cases with spermatogenic impairment. Their results demonstrated gr/gr deletions were significantly higher in oligozoospermic (not azoospermic) cases (p<0.001) (14).

Ye and colleagues performed a study to search for AZFc subdeletions in the Yi ethnic minority in China (37). Similarly, they detected gr/gr subdeletion in 7.6% of cases and 8.5% of controls and b2/b3 deletions in 6.3% of cases and 8.5% of controls, consistent with East Asian studies (30). The results of gr/gr subdeletion association studies suggested ethnic and geographical variability (1). Therefore, a high frequency of gr/gr subdeletion in normozoospermic controls can be explained by ethnic and geographical variability. Three meta-analyses studies have shown that gr/gr partial deletions increase the risk of infertility in Caucasian populations and suggested homogeneous ethnicity grouping for further studies (13, 24, 38).

| Subdeletion | Embryo development, n | Pregnancy, n | p-Value | p-Value |
|-------------|-----------------------|--------------|---------|---------|
|             | −         | +          | −       | +       |
| b1/b3       |            |            |         |         |
| With deletion | 1        | 0          | 0.118   | 1       | 0       | 0.253   |
| Without deletion | 63      | 84         |         | 49      | 57      |         |
| b2/b3       |            |            |         |         |
| With deletion | 4        | 9          | 0.258   | 4       | 7       | 0.344   |
| Without deletion | 60      | 75         |         | 46      | 50      |         |
| gr/gr       |            |            |         |         |
| With deletion | 3        | 1          | 0.215   | 2       | 0       | 0.216   |
| Without deletion | 61      | 83         |         | 48      | 57      |         |

| Histopathology, n | SCO | Maturation arrest | HSG | p-Value |
|-------------------|-----|-------------------|-----|---------|
| b1/b3             |     |                   |     |         |
| With deletion     | 0   | 0                 | 0   | 0.958   |
| Without deletion  | 56  | 9                 | 9   |         |
| b2/b3             |     |                   |     |         |
| With deletion     | 3   | 0                 | 0   | 0.589   |
| Without deletion  | 53  | 9                 | 10  |         |
| gr/gr             |     |                   |     |         |
| With deletion     | 1   | 1                 | 0   | 0.233   |
| Without deletion  | 55  | 8                 | 10  |         |

SCO: Sertoli cell only, HSG: hypospermatogenesis.
The studies on b1/b3 and b2/b3 partial deletions are rare compared to gr/gr subdeletions and their results remain controversial (39-42). Wu and colleagues screened gr/gr and b2/b3 partial deletions in a Han-Chinese population of 699 subjects, including 451 idiopathic infertile patients and 248 fertile controls. They found b2/b3 deletion frequency was significantly higher in idiopathic infertile patients than control group (odds ratio (OR)=2.93; 95% confidence interval (CI)=1.34-6.39) (40). Similarly, two independent studies from China and Egypt reported a higher frequency of b2/b3 deletions than gr/gr deletions in cases with spermatogenic failure in the Han population (41, 42). On the other hand, several trials showed no relationship between b2/b3 subdeletions and male infertility. Huckenbroich and colleagues studied the relation of b2/b3 partial deletions in 170 normozoospermic, 348 NOA and oligozoospermic males in a German population and reported higher frequency of b2/b3 deletions in normozoospermic males. In addition, the frequency of gr/gr deletions was similar among groups in their study. They concluded that these subdeletions could not be a reason for spermatogenic failure or infertility alone in the German population (23). A recent meta-analysis indicated that b2/b3 subdeletions elevate infertility risk in the Mongolian populations and the East Asian region, however, not in Europeans, Caucasians, Dravidians and South Asians (43).

We compared sperm counts between OAT and controls, but no significant difference was indicated in cases with or without gr/gr deletion ($p=0.398$ and $p=0.961$) and nor for b2/b3 deletions ($p=0.346$ and $p=0.158$). On the other hand, a meta-analysis study showed a statistically significant difference between gr/gr deletion and azoo-/oligozoospermic and suggested gr/gr deletions significantly reduce sperm counts and are thus associated with low semen quality (20).

We did not find any relationship between testicular histopathology and subdeletions. Similarly, in a study from Japan, the authors observed gr/gr deletions in eight cases with Sertoli cell only syndrome, in two cases with maturation arrest and in one case with hypospermatogenesis (44).

High FSH levels are closely related to testicular deficiency and impaired spermatogenesis (45). Schoor and colleagues found that the FSH level was higher than 7.6 mIU/ml in 89% of NOA cases (46). In the present study, FSH levels of cases with b2/b3 deletion were significantly lower than in cases without deletion in the OAT group ($p<0.005$). Moreover, sperm counts of cases with b2/b3 deletion was higher than concentration of cases without deletion in the OAT group. Consequently, b2/b3 subdeletions might not affect spermatogenesis.

Sin and colleagues found gr/gr deletions more frequent (86.2%) in haplogroup D (19). In most studies, haplogroup analysis was not performed, therefore it is not possible to determine the frequencies of certain haplogroups in men with or without gr/gr deletions (13). We did not investigate Y-chromosome haplogroups in our study, therefore, we cannot explain the frequency of Y-chromosome partial deletions in certain haplogroups in men living in the Central Black Sea Region of Turkey. Subdeletion rate can be affected by ethnicity depending on haplogroup distribution. In the present study, we do not know whether the high frequency of gr/gr subdeletion in normozoospermic controls is related to haplogroup.

The number of studies investigating the relationship between subdeletions and micro-TESE/ICSI outcomes are limited. Stahl and colleagues performed micro-TESE in azoospermic men with gr/gr deletions. Their sperm retrieval rate was 64% (14/22) and it was similar to the sperm retrieval rate of the idiopathic azoospermic cases in the same clinic (47). Similarly, in another study from Spain, the authors observed that the ICSI outcome was same in the four absinth gr/gr deletion compared to cases without deletion (26). We did not find statistical correlations between subdeletions and ART outcomes (sperm retrieval, pregnancy and testicular histology, embryo development).

The frequency of Yq chromosome gr/gr phenotypes are heterogeneous and depend on environmental factors, epigenetic modifications, lifestyle, penetrance of the gene, additional microdeletions in AZF regions, germinal mosaicism, gene–gene interactions and concurrence with other male infertility disorders (8). Geographic and ethnic origins of population may influence the frequency of deletion genotypes (3).

**Conclusion**

To our knowledge, this is the first study aiming at investigating b1/b3, gr/gr and b2/b3 subdeletions of the AZFc region in a Turkish population. Our results showed that AZFc partial deletions may not play a significant role in infertility, sperm counts, and ART outcomes in Turkish men.

**Declaration of Interest**

The Authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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