

Deletion and Testicular Expression of *DAZ* (Deleted in Azoospermia) Gene in Patients with Non-Obstructive Azoospermia

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Abstract

Background: Deletions of the *DAZ* (deleted in azoospermia) genes within the human Y chromosome's *AZFc* region are the most common cause of spermatogenesis failure. These deletions are usually assessed by analyses of genomic DNA extracted from peripheral leukocytes. *DAZ* genes are expressed in male germ cells. In this prospective study, we investigated *DAZ* expression and deletion in 102 consecutive infertile men presenting with non-obstructive azoospermia in Avesina Research Institute, Tehran, Iran during 2005-6.

Methods: In this prospective study, we extracted genomic DNA from peripheral blood leukocytes for detection of *DAZ* deletions and testicular biopsies for histopathological assessment and analyses of *DAZ* expression level by reverse transcription polymerase chain reaction. *DAZ* levels were normalized to expression of the housekeeping *Phosphoglucomutase 1* gene.

Results: In four out of 102 patients (3.9%), we found *DAZ* deletion. *DAZ* expression was observed in 60 (61.2%) of 98 other patients. Expression was not detected in patients with Sertoli cell-only syndrome, but observed in 37 of 40 (92.5%) patients with maturation arrest and 20 of 26 (76.9%) with hypospermatogenesis.

Conclusion: The absence of *DAZ* expression could result in quantitative reduction of germ cells and might be observed despite of normal genomic DNA constitution. We recommend to check *DAZ* testicular expression and genomic DNA deletion, in non-obstructive azoospermia. This is more recommended to avoid transmission of genetic abnormalities which might lead to infertility in male offspring, when assisted reproductive techniques (ART) are performed.

Keywords: *Infertility, Deleted in Azoospermia Gene, Y Chromosome, Azoospermia, Iran*

Introduction

Deletions of Y-chromosomal regions are known as an important cause of spermatogenesis defects in human (1, 2). Three distinct *AZF* (azoospermia factor) loci have been identified on the Y chromosome (*AZF*a, b and c) which are required for normal fertility (3).

*AZF*c deletions are shown to be related with severe oligozoospermia and azoospermia, in testes with different histopathological findings varying from hypospermatogenesis to degrees of meiotic arrest or Sertoli cell-only (SCO) syndrome. Other studies suggest that the entire *AZF*b and/or *AZF*b-c deletions may influence the ability of Y chromosome to pair and consequently cause azoospermia (4).

Among different genes on *AZF* regions, *DAZ* (deleted in azoospermia) within *AZF*c region is the gene with the highest frequency of deletion. Deletions of *DAZ* are observed in approximately 10% of men with spermatogenesis defects (1, 2).

DAZ gene family has an autosomal member in human 3p24 chromosome, known as *DAZL1* (*DAZ*-Like). *DAZL1* is a single copy gene which is expressed in human testis and fetal gonocytes and encodes a 3.3kb transcript (5-8). The structure of *DAZ* arises from transposition, repeat amplification and pruning of the autosomal gene *DAZL1* during primate evolution (9, 10).

Multiple copies of *DAZ* are present on the Y chromosome. *DAZ* genes are arranged in two clus-

ters, containing two genes in a head-to-head orientation. These four gene copies are 99.9% identical in introns, exons and flanking sequences (9, 10) and at least three of them are transcribed (9). DAZ genes encode proteins with one or two RRM (RNA recognition motif) and eight to 18 copies of DAZ repeat (9).

The multicopy nature of DAZ has limited the understanding of its actual role in human spermatogenesis (11). The expression of DAZ is observed in spermatogonia and early primary spermatocytes. These findings propose that DAZ functions in the first phases of spermatogenesis differentiation or earlier, in the maintenance of the spermatogonial stem cell populations (12, 13).

Deletions of DAZ are usually assessed by polymerase chain reaction on genomic DNA extracted from peripheral leukocytes. This method is not sufficient for investigation of DAZ because it does not allow the distinction of intragenic deletions, point mutations or deletions which are not able to remove the whole cluster (11, 14,15).

In order to determine the importance of DAZ genes in spermatogenesis, we investigated the peripheral blood leukocytes DAZ DNA deletion as well as testicular expression in men with non-obstructive azoospermia using RT-PCR. We also evaluated the histopathological findings of the samples to find the possible effect of DAZ expression on spermatogenesis progression.

Materials and Methods

Patients and Testicular Samples

We obtained 5 ml of blood and testicular samples from 102 unrelated infertile men with non-obstructive azoospermia who undergone testicular sperm extraction (TESE) in Avesina Infertility Clinic, Tehran, Iran during 2005-2006. We had ten testicular samples from fertile patients who undergone orchidectomy as well as patients with obstructive azoospermia as controls. Multiple testicular biopsies were performed to obtain the samples, unless the patient did not agree with multiple biopsies. Idiopathic non-obstructive azoospermia was defined as the absence of spermatozoa

in at least two semen analyses. Patients with obstructive azoospermia were excluded after clinical examination additional tests by andrologists. Cytogenetic analysis for all the patients showed a normal 46,XY karyotype. Patients' information including age, family history of male infertility, history of any testis developmental and pathological problems, clinical examinations (testis site, consistency and size), plasma level of follicle stimulating hormone (FSH), leutinizing hormone (LH), testosterone and semen analysis characteristics were obtained from database of Avesina Infertility Clinic laboratories. The exclusion criteria were possible known causes of testicular damage, such as cryptorchidism, varicocele, seminal tract infections, drug use, endocrinopathies, post-mumps orchitis, testicular trauma or torsion.

Approval from the Avesina Research Institute's ethics and human rights committee was obtained for the use of specimen and study protocols. Before biopsy, all participants were signed the informed consent form.

Histopathological Assessment

All patients specimen were sent to Pathology Laboratory of Avesina Infertility Clinic in Bouin's fixative and after processing, slides were stained by hematoxylin and eosin. According to histopathological findings, we classified the patients into three groups: (i) Sertoli cell only-syndrome (absence of germ cells in the seminiferous tubules); (ii) maturation arrest (germ cells arrest at different stages of spermatogenesis) and (iii) hypospermatogenesis (reduction of normal spermatogenesis).

DNA Extraction from Blood Cells

Genomic DNA was extracted from peripheral blood leukocytes with use of salting out technique. DNA was stored in -20 °C for further use.

DNA and RNA Extraction from Testicular Cells

The approximate size of testicular samples was 2×2×2 mm and all samples were stored in -70 °C until RNA extraction. Total RNA was extracted by the RNA-Bee™ (Tel-Test, Protech Technology Enterprises Co., Ltd, Friendswood, TX, USA). Briefly, tissue samples (50-100 mg) were homo-

genized and lysed in the presence of 1ml RNA-Bee reagent. Addition of chloroform followed by centrifugation separated the solution into an aqueous phase (containing RNA) and an organic phase. After transferring the aqueous phase, RNA was recovered by precipitation with isopropyl alcohol. Total RNA was eluted in 20 μ L DEPC-treated water. Quantification of RNA was performed by absorbance at 260 nm using spectrophotometer (Biophotometer, Eppendorf, Berlin, Germany). The mean RNA concentration was 410.2 μ g/ μ L and OD 260/280 ratio was about 1.8.

cDNA Synthesis

For the synthesis of complementary DNA, 10 μ L of total RNA was heated in 80 °C for 10 min followed by cooling on ice. Master mixture included 4 μ L of 5x reverse transcriptase (RT) buffer containing Tris-HCl (pH =8.3) (Fermentase, Burlington, Canada), 10 mM of each dNTP, 20 pmol/ μ L random primer, 20 U RNase inhibitor (Roche, Mannheim, Germany), 200 U of Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase (RevertAid™, Fermentas, Burlington, Canada), and 1.5 μ L of DEPC-treated water. RT temperature profile was 42 °C for 60 min, 70 °C for 10 min, and final cooling to 4 °C. The cDNA was stored at -20 °C until further use.

Polymerase chain reaction

cDNA was checked for the integrity using the house keeping gene phosphoglucomutase 1 (PGM1) primers which amplify region 1718-2104 (from exon 10 to exon 11). The product size of PGM1 primers is 386 bp, and can not be amplified through the genomic DNA. In addition, we checked the expression of Synaptonemal Complex Protein 3 (SYCP3) which is expressed in germ cells, as a marker of germ cell existence in all cDNAs (16). PCR for DAZ genes were performed using sY254 primers for samples with similar cDNA integrity (Table 1). sY254 primers amplify the exons 2 and 3 and expected RT-PCR product size was 106 bp, in contrast with genomic DNA PCR product which was 380 bp.

PCR was carried out in 20 μ L of reaction volume containing: 1 μ L of either testicular cDNA or genomic DNA extracted from peripheral blood leukocytes, 1 U Taq polymerase (Roche, Mannheim, Germany), 10mM of each dNTP, 10 pmol of each primer, 100 mM Tris-HCl (pH=8.3), 500 mM KCl, 25 mM MgCl₂ (MBI, Fermentas, Burlington, Canada). Amplification was performed for 34 sequential cycles, including 30 S of denaturation at 94 °C, 30 S of primer annealing at 60 °C, and 30 S of extension at 72 °C followed by 7 min final extension at 72 °C. Before the first cycle, all samples were incubated for 3 min at 94 °C. PCR reaction products were separated on 1.5% agarose gel by electrophoresis in TAE (Tris -acetic acid-EDTA) buffer, then stained with ethidium bromide and visualized under UV light. The results were considered as positive or negative.

Statistical Analysis

The Statistical Package for Social Sciences (SPSS, Chicago, IL, USA) version 11.2 and Microsoft Excel (Seattle, WA, USA) spreadsheet were used for data entry and analysis.

Comparisons between groups were performed using χ^2 -tests (Pearson and Fisher's exact test, two-sided) and student *t*-test at alpha level of 0.05 was used to indicate statistical significance.

Results

Non-obstructive azoospermia was diagnosed in 102 patients, and their histopathological findings were hypospermatogenesis (n= 27), maturation arrest (n= 43), Sertoli cell-only syndrome (n= 32) (Fig. 1).

DAZ Deletion

Patients were considered normal for DAZ genes when PCR on testicular genomic DNA and DNA extracted from peripheral leukocytes amplified sY254 fragment. We observed four out of 102 (3.9%) patients with deletion of DAZ genes in both testicular and peripheral leukocytes DNA. In order to confirm DAZ deletion in these patients, we amplified DYS448 Y chromosome short tandem

repeat (Y-STR) which is almost five Mbp far from DAZ region in Y chromosome and observed the deletion again (data not shown). Two patients with DAZ deletion had arrest of spermatogenesis at the level of primary spermatocytes, as well as one at the level of spermatid and one with hypospermatogenesis. Two of above four patients had family history of male infertility in one of their brothers. However, we did not have access to the patients family in order to check the deletion of DAZ. None of patients were mosaic for DAZ genes.

DAZ Expression

One hundred and two testicular samples with normal PGM1 amplification were investigated for DAZ expression using RT-PCR (Fig. 2). In four patients with DAZ deletion no DAZ mRNA was seen, as mentioned above.

DAZ mRNA was seen in three out of 32 patients with Sertoli cell-only syndrome (9.3%). Analysis of samples with maturation arrest revealed normal amplification in 37 (92.5%) out of 40 patient samples, in contrast with expression in 20 (76.9%) patients with hypospermatogenesis. All of these patients had apparently normal DAZ genes constitution. Absence of DAZ mRNA expression in hypospermatogenesis patients was not significantly higher than maturation arrest. Table 2 shows the findings of testicular histopathology, genomic PCR and RT-PCR analysis.

FSH (*t*-test: $P < 0.0001$) and LH (*t*-test: $P = 0.002$) plasma levels were significantly higher and testicular volume (*t*-Test: $P < 0.0001$) were significantly lower in patients without DAZ expression. Testosterone plasma level showed no significant difference between these two groups of patients.

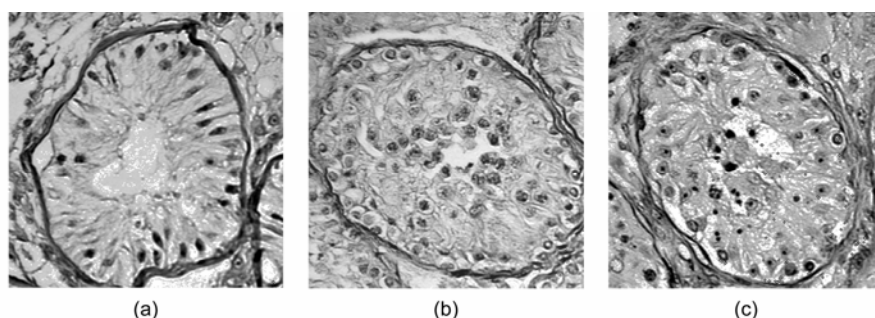


Fig. 1: Histopathology of testicular samples with H & E staining (X200). (a) Sertoli cell-only syndrome; (b) Maturation arrest; (c) Hypospermatogenesis.

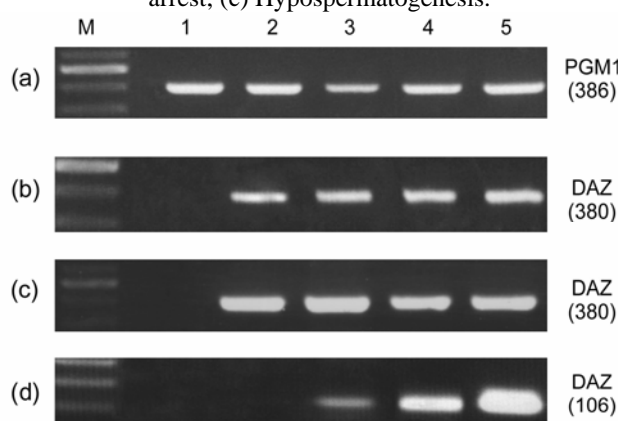


Fig. 2: RT-PCR and PCR products of PGM1 and DAZ genes in testicular and blood samples on 1.5% agarose gel stained with Ethidium Bromide. (a) RT-PCR products of PGM1 gene in testicular cDNA; (b) PCR products of DAZ genes in peripheral blood leukocyte DNA; (c) RT-PCR products of DAZ genes in testicular DNA; (d) RT-PCR products of DAZ genes in testicular cDNA. 1: Patient with maturation arrest and DAZ deletion; 2: Patient with hypospermatogenesis and absence of DAZ expression; 3: Patient with Sertoli cell-only syndrome and DAZ expression; 4: Patient with hypospermatogenesis and DAZ expression; 5: Positive Control.

Table 1. Sequences of oligonucleotides used in DAZ PCR

Primers	Sequence	DNA (bp)	RNA (bp)
PGM1	5'-TCCGACTGAGCGGCACTGGGAGTGC-3' 5'-GCCCGCAGGTCTCTTTCCCTCACA-3'	-	386
sY254	5'-GGGTGTTACCAGAAGGCAAA-3' 5'-GAACCGTATCTACCAAAGCAGC-3'	380	106

Table 2: RT-PCR products of DAZ gene in testicular samples of men with non-obstructive azoospermia.

Testicular Histopathology	Cause of Azoospermia	DAZ PCR	DAZ RT-PCR
Sertoli cell-only syndrome	Idiopathic	Normal	3/32 ^a
		Deletion	0/0
Maturation Arrest	Idiopathic	Normal	37/40
		Deletion	0/3
Hypospermatogenesis	Idiopathic	Normal	20/26
		Deletion	0/1
All	Idiopathic	Normal	60/98
		Deletion	0/4

^a Number of positive results/Number of patients.

Discussion

Y chromosome microdeletions represent one of the most frequent causes of azoospermia, among potential factors influencing male fertility (10). Therefore, analysis of Yq microdeletions is an important test in diagnosis of infertile patients, specially for candidates of assisted reproductive techniques (15).

It is observed that frequency of Y chromosome deletion increases in patients with spermatogenesis defect (17). On the other hand, the testicular pathology of men with deletion of AZFc region could not be associated with a specific interruption phase of spermatogenesis (3). However, most of problems in understanding the biological function of DAZ and the genotype-phenotype relation arise from the multicopy nature of the gene. There is still no definitive proof for requirement of DAZ in spermatogenesis (18).

DAZ deletions are generally screened by PCR on genomic DNA extracted from peripheral leukocytes in infertile patients (19). In this study, we analysed DAZ deletion using above method and DAZ expression using RT-PCR of testicular samples from infertile men with Sertoli cell-only syndrome, maturation arrest and hypospermatogenesis.

We did not observe DAZ mRNA expression in patients with Sertoli cell-only syndrome, as a probable result of lack of germ cells, which is in contrast with some of previous studies (18). We only had three patients with Sertoli cell-only syndrome who expressed DAZ in testis. This might be explained by the nonhomogeneous nature of the testis and the fact that the biopsies were not taken from the same location for histology and gene expression in each testis (18). On the other hand, DAZ deletion was observed in patients with hypospermatogenesis and maturation arrest, but in none of patients with Sertoli cell-only syndrome. This confirms the previous studies in which the deletion of this region was not sufficient to cause complete loss of spermatogenetic line (20). DAZ mRNA expression was detected in patients with maturation arrest and hypospermatogenesis, but we had some patients in this groups with apparently normal DAZ genes constitution and no expression. In these patients, absence of DAZ expression could be a possible cause of disease. In addition, based on genomic and cDNA sequence analysis, at least three Y-chromosomal DAZ genes (DAZ 2, 3, and 4) are transcribed, but there is still doubt about DAZ1 transcription

(9). sY254 primers are amplified in all four copies of DAZ genes. Therefore, negative PCR results of genomic DNA or testicular cDNA indicates loss of all members of gene family or absence of all DAZ mRNA, respectively. However, a normal positive result is less informative and merely indicates the presence of at least one gene copy. Therefore, if we assume DAZ1 as a pseudogene, the above negative results are expected when the active genes are deleted and consequently not transcribed. This finding can also be described if abnormalities of DAZ promoter, transcription or post-transcription phases exist in these patients. However, nothing is confirmed about regulation of DAZ expression (15) and further studies are required to confirm the association between DAZ expression and pathophysiology of spermatogenesis arrests.

The prevalence of expression in patients with maturation arrest was higher than hypospermatogenesis. This finding could show that DAZ is transcribed in a germ cell-specific manner. It is observed that problems in DAZ expression are more correlated with quantitative reduction of germ cells instead of impairment of the maturation progression (12, 13, 15).

This study suggests that lack of DAZ expression might be observed despite of apparent no genomic DNA deletion. Therefore, we assume that checking blood genomic DNA for DAZ deletion in men with non-obstructive azoospermia is not sufficient and is recommended to be accompanied by DAZ testicular expression and testicular genomic DNA analysis. This is more important when there is a possibility of assisted reproductive techniques. If DAZ deletion is detected, sex selection using preimplantation genetic diagnosis (PGD) is suggested, to avoid male infertile offspring.

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