PREVALENCE OF Y CHROMOSOME MICRODELETIONS IN IRANIAN INFERTILE MEN

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Abstract- This study was designed to determine the frequency of Y chromosome AZF (Azoospermia Factor) subregions' microdeletions in patients with idiopathic nonobstructive azoospermia and severe oligozoospermia. Subjects included 40 men who had been referred to infertility clinics for assisted reproduction, 37 were azoospermic and 3 had severe oligospermia. Medical history and physical exam revealed no evidence of infection, obstruction of seminal tract, endocrine failure or chromosomal anomalies. Hormonal study was performed for all patients. Twenty six men had biopsies of the testes including 11 patients with hypospermatogenesis, 9 patients with maturation arrest, 4 patients with sertoli cell only syndrome and 2 patients with tubular sclerosis. In 14 men who did not have a testicular biopsy multiple, epididymal and testicular sperm aspirations under anesthesia failed and testicular sperm extraction was subsequently performed for ICSI. DNA was isolated from blood samples. Polymerase chain reaction (PCR) amplification of 11 loci spanning the AZFa, AZFb and AZFc subregions of the Y chromosome using sY81, sY83, sY127, sY130, sY131, sY147, sY149, sY157, sY158, sY254 and sY276 was performed. Microdeletions of the Y chromosome were found in two of the patients (5%), who had azoospermia. Deletions were restricted to DAZ (deleted in azoospermia) locus in AZFc subregion. One of the patients had a history of cryptorchidism and the second had undergone a left side varicocelectomy. Testicular pathology showed sertoli cell only syndrome in both of them. Our experience adds to the current logic that men with azoospermia or severe oligospermia should be evaluated for Yq11 microdeletions before deciding to operate varicoceles or else scheduling them for assisted reproductive techniques.

Acta Medica Iranica, 41(3): 164-170; 2003

Key Words: Nonobstructive azoospermia, AZF microdeletions

INTRODUCTION

Failure of spermatogenesis is the result of a multitude of causes such as systemic diseases, malnutrition, endocrine disorders, environmental hazards and genetic factors. Genetic defects such as mutations and chromosomal abnormalities acount for at least 30% of male infertility (1). The fact that there are deletions in the long arm of Y chromosome that are associated with azoospermia has been known for over two decades, and an azoospermia factor (AZF) in men with Yq chromosome microdeletions has been proposed (2,3). AZFs localized in interval 6 may play an important role in regulation of spermatogenesis (4). Further studies have led to the proposal of the existence of at least 3 AZF subregions termed AZFa, AZFb, and AZFc.

Received: 27 May 2002, accepted: 16 April 2003

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Deletion of AZFa is associated with lack of germ cells or Sertoli cell only syndrome. Deletion of AZFb is associated with spermatogenesis arrest and finally deletion of AZFc gene products is associated with failure of maturation process of post-miotic germ cells. Although this hypothesis remains controversial, it is accepted that the completion of spermatogenesis requires multiple genes, not only on the Y chromosome but elsewhere as well. Recently another AZF subregion named AZFd, localized between AZFb, and AZFc has been described, which has complicated the issue (1). With the advent of polymerase chain reaction (PCR) and construction of a Y-chromosome sequence-tagged site (STS) map, microdeletions were detected at a frequency of 0.4-55.5%.This varying frequency is probably related to the criteria on which the patients are selected (5). In this study we investigated Yq11 microdeletions with the use of PCR analysis in a group of men with idiopathic nonobstructive azoospermia.

MATERIALS AND METHODS

Fourty infertile men were studied from the infertility clinics of two Tehran University of Medical Sciences hospitals. All patients had already multiple semen analyses. A complete medical history was obtained and a physical examination was performed. Inclusion criteria were azoospermia or severe oligospermia (<1000000/ml) on multiple samples. Among the patients, those with defective spermatogenesis secondary to infection, obstruction of the seminal tract, endocrine failure and chromosomal anomalies shown on history or clinical examination were excluded. Hormonal study was performed and FSH, LH and testosterone plasma concentrations were measured by Radioimmunoassay (RIA). Most patients had a long history of primary infertility and were candidates for Intra-Cytoplasmic Sperm Injection (ICSI). Twenty six patients had testicular biopsies showing different degrees of spermatogenesis arrest. Reports of biopsies consisted of 11 patients with hypospermato-genesis, 9 patients with some degrees of maturation arrest, 4 patients with sertoli cell only syndrome and 2 patients with tubular sclerosis.14 patients did not have testicular biopsy; in this group multiple epididymal and testicular sperm aspirations under anesthesia failed and testicular sperm extraction was subsequently performed for ICSI. Blood samples were taken from the patients after obtaining informed consent.

DNA extraction method

DNA was isolated from leukocytes according to the standard proteinase K/ phenol-chloroform method. Four mililiters of distilled water was added to 2 ml of whole blood, and mixed throughly to lyse the erythrocytes. The mixture was centrifuged at 4000 rpm for 15 minutes, to pellet the leukocytes. Two mililiters of lysis buffer (100 mM Nacl, 25 mM EDTA, 1% SDS and 300 mcg proteinase K) was added to the pellet, mixed thoroughly, and incubated overnight at 37°C. The lysed cells were extracted by equal volume of a mixture of phenol (equilibrated to neutral PH by saturation with Tris-HCl at PH: 8) and chloroform twice. This procedure was repeated twice again with chloroform only. DNA was precipitated by addition of 1/5 volume of 9.5 M ammonium acetate and 2 times volume of cold ethanol. DNA concentration was reached to 1-2 mcg /mclit for use in PCR.

STSs and PCR

11 STSs used to map Yq interval 5 and 6, were selected from the STS map of Vollrath et al. and Rejio et al. (6,7). These STSs were Y specific and did not have homologs on the X chromosome or autosomes.

Each patient's sample was typed for all STSs using PCR. For each PCR 2 microlitres (100-200 ng) of patient's DNA extracted from peripheral blood cells were used. A PCR buffer containing 50 mM KCL, 1.5 mM $MgCl_2$, 10 M Tris-Hcl, PH: 8.3 and Taq polymerase (2u), dNTP(0.2 mM each of dTTP, dCTP, dGTP, dATP), oligonucleotide primers (10 pM each of the forward and reverse primers) were added to the patients` DNA. Thermocycling consisted of an initial denaturation of 2 minutes at 94°C and of 30 cycles of 1 minute at 94°C(melting), 1 minute at 60°C (annealing) and 2 minutes at 72°C (extension). STSs included sY 81, sY 83, sY 127, sY 130, sY 131, sY 147, sY 149, sY 157, sY 158, sY 254, sY 276. Two microlitres of tracking dye (Ethidium bromide) were added to each sample before running it on a 3% agarose gel. Each set of PCR reactions included a normal male control (positive control) and a normal female control (negative control). A patient's sample was considered positive for a STS if it produced the PCR product of the expected size under the same condition and negative if a product of the expected size was not obtained after three PCR attempts.

RESULTS

The study group consisted of 40 patients, 37 with azoospermia and 3 with severe oligospermia (<1000000/ml). The subjects ranged in age from 25-65 years (mean age 34.4 years). Clinical data of patients are summarized in table 1. There was no overlap in positive historical findings.

Eight patients had normal size testes measured by an orchiometer and 32 had atrophic testes.Nine patients had a elevated FSH (>10 mIU/ml) and one patient had history of hyperthyroidism.

Table 1. Positive findings in medical history of our patients

History of cryptorchidism	5
History of varicocelectomy	15
History of inguinal hemiorrhaphy (unilateral)	5
Family history of infertility	3

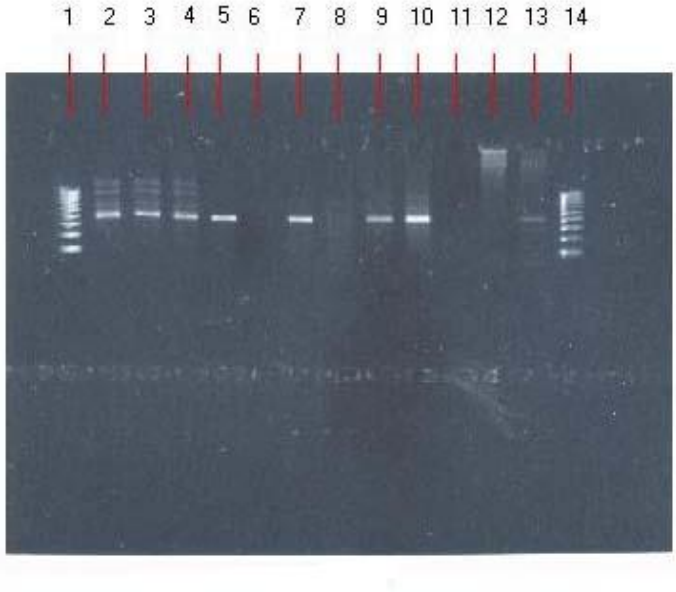

Fig. 1. Ethidium bromide- stained PCR products (amplification of sY254) of 9 patients with azoospermia shown in lanes 2 to 10, including 2 patients with microdeletion in sY254 shown in lanes 6 and 8. See the text for details.

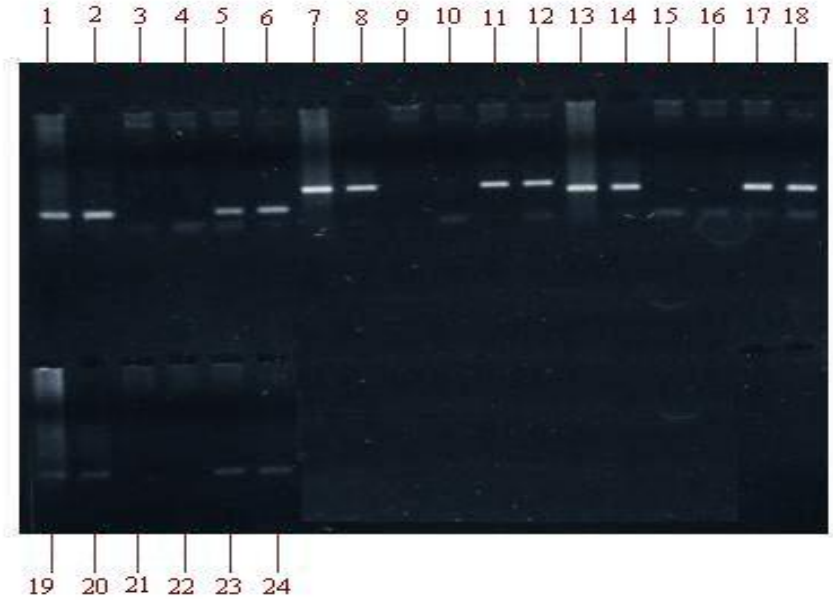

Fig. 2. Example of PCR amplification, of 2 patients with microdeletion shown in Fig. 1, using STSs sY149, sY157, sY158, and sY147 to prove lack of deletion in other areas

The results of testicular pathology and semen analyses are shown in table 2.

Table 2. Results of testicular histology reports and sperm counts

Pathology report	No. of patients	Sperm count
Hypospermatogensis	11	All azoospermic
Maturation arrest	9	6 azoospermic. 3 severe oligospermic
Sertoli cell only syndrome	4	All azoospermic
Tubular sclerosis	2	All azoospermic
Pathology unknown	14	All azoospermic

Our study showed two cases in whom no amplification was detected for the STS sY254 specific for the DAZ (deleted in azoospermia) locus. One of these patients was a 33-years old azoospermic man with a history of bilateral cryptorchidism whose testicular pathology was sertoli cell only syndrome. The second patient was a 34-years old azoospermic man who had undergone a left side varicocelectomy and his testicular biopsy had shown sertoli cell only syndrome. Agarose gel electrophoresis containing the PCR amplification products of these two patients is shown in Fig. 1. In this figure lanes 1 and 14 correspond to 100 base pairs DNA markers, lanes 2, 3, 4, 5, 7, 9 and 10 correspond to azoospermic patients without deletions. Lanes 6 and 8 are related to azoospermic patients with deletion in sY254 STS. Lane 11 is water control, Lane 12 is female control, and lane 13 is male control.

These 2 patients had no deletions in other areas of Y chromosome as depicted in Fig. 2. In this figure STS sY149 has been used in lanes 1 to 6, sY157 in lanes 7 to 12, sY158 in lanes 13 to 18, and sY147 in lanes 19 to 24. Lanes 1, 7, 13, 19, correspond to the first patient, lanes 2, 8, 14, 20, correspond to the second patient, lanes 3, 4, 9, 10, 15, 16, 21, 22, correspond to female control, and lanes 5, 6, 11, 12, 17, 18, 23, and 24 correspond to male control.

DISCUSSION

There is no doubt that molecular biology and molecular genetics are going to play a pivotal role in helping to understand the basis of human reproduction failures. Nowadays men with extremely low sperm counts or even men with azoospermia in whom only testicular biopsy can provide a handful of sperm can be subjected to assisted reproduction via ICSI, however it is important to establish the causes of the defect. The connection between infertility, genetics and elucidation of regulatory elements governing normal spermatogenesis is going to have a great impact in clinical practice. First, it may provide an explanation for the problem and this alone can relieve some of the anxiety or guilt feelings associated with the spontaneous reproductive failure. Second, it may provide genetic evidence that some forms of male infertility are genetic in origin and potentially transmissible to male offspring. Third, it may direct the clinicians in avoiding surgeries which probably have poor results (i.e. varicocele operation in one of our patients with microdeletion) or in avoiding prescribing prolonged hormonal and nonhormonal therapies, which in these instances, would not be able to improve the fertility status. Finally, the information on the genetic origin of infertility may ultimately help the couple to decide on whether or not to begin the entire cycle of assisted reproduction (8,9). Spermatogenesis is a long and complex process and our knowledge of the genetic control of spermatogenesis remains very limited. It is reasonable to assume that the process of spermatogenesis is regulated by the accurately coordinated expression of many genes (1). It has been postulated that factors controlling human spermatogenesis are located on the distal portion of the euchromatin segment of the long arm of the Y chromosome, Yq11 (10). This spermatogenesis locus lying in Yq11.23 as demonstrated with high resolution banding techniques has since come to be known as azoospermia factor (AZF) (11). The AZF locus has been localized to interval 6 of the Y chromosome (1). Further studies have led to the proposal of the existence of three AZF subregions termed AZFa, AZFb, and AZFc. Deletion of AZFa is assosiated with lack of germ cells or sertoli cell only syndrome, deletion of AZFb is assosiated with spermatogensis arrest and AZFc gene products are involved in the maturation process of postmiotics germ cells (12). It is generally accepted that the completion of spermatogenesis requires multiple genes not only on the Y chromosome but elsewhere as well. The issue has been further complicated by description of another AZF subregion named AZFd localized between AZFb and AZFc. Patients with microdeletions restricted to AZFd may have mild oligospermia or even normal sperm counts associated with abnormal morphology (13). Four candidate gene families for AZF have been identified; they are RNA-binding motif (RBM), deleted in azoospermia (DAZ) Drosophila fat

facet related Y (DFFRY) and chromodomain Y (CDY) (14-17) (Fig. 3).

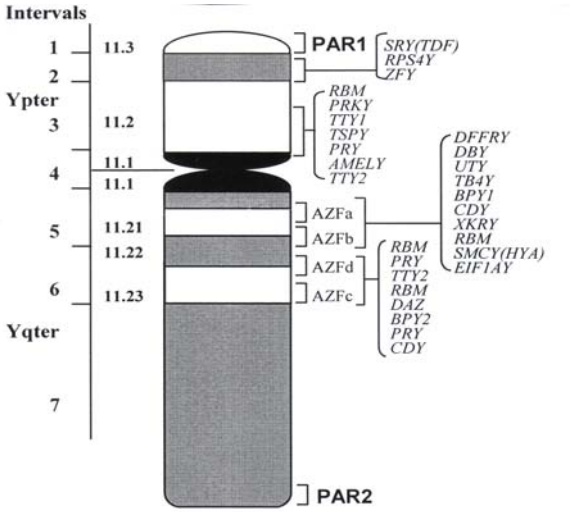

Fig. 3. Gene map of the human Y chromosome. The Y chromosome is divided into seven intervals. The Azoospermia Factor (AZF) is divided into AZFa, AZFb, AZFc, and AZFd regions and mapped into intervals 5 and 6. PAR, pseudoausomal region

If we assume that about 1 in 1000 males is azoospermic and that about 12-15% of them will have Y-chromosome microdeletions, then the frequency of this genetic defect is about 1 in 8000-10000 births, but infact Y-chromosome microdeletions have been reported of various prevalences (0.4-55.5%) and this is probably related to the criteria by which the patients are selected and it has been recommended that Y microdeletion analysis should be limited to azospermic or severely oligospermic men and candidates for ICSI (5,8). In our study Y microdeletions were detected in 2 patients (5%). Both of them had deletion in DAZ gene in AZFc region.The frequency of AZF deletion events in proximal and middle Yq11 (AZFa and AZFb) is the same, ranging below 1% in men with idiopathic azoospermia. On the other hand the frequency of deletions in distal Yq11 (AZFc) seems to be generally higher and is described between 5-20% in different laboratories (18). Our results are compatible with the minimum of reported range. Deletions in the AZFc region involving the DAZ gene have been reported to be more prevalent in severe hypospermatogenesis than in sertoli cell only syndrome, suggesting that deletions of this region are not sufficient to cause complete loss of the spermatogenic line (19). Deletions in the AZFc subregion involving DAZ were observed in two of our patients and this gene represents the best candidate for AZFs (4). A functional DAZ homologue (DAZLA1) on human chromosome 3p (which is considered a fertility factor in women) and presence of multiple copies of DAZ in the AZFc subregion may prevent complete loss of spermatogenesis. Our results were not in agreement with most previous literature reporting that sertoli cell only syndrome is associted with microdeletions of the AZFa subregion. It may be possible that deletions in more than one subregion are common features in patients with sertoli cell only syndrome (20,21). Because we did not study extensively deletions within AZFa subregion, we cannot exclude the possibility that deletions within this subregion may be present. It is also probable that if more testicular tissue specimens of these patients were examined, some foci of spermatogenesis could have been found. Both of our patients with microdeletions had interesting points in their history. One of them was an excryptorchid man and it has been reported that Y microdeletions are probably responsible for a bilateral testicular damage, a consequence of which is cryptorchidism (22). The other patient had a history of unsuccessful varicocelectomy. It has been reported that men with varicocele and genetic lesions have a poorer response to varicocele repair than men without coexisting genetic lesions (9). Most of our patients were candidates for ICSI and it has already been documented that microdeletions in the male parent are transmitted to male offspring who are conceived with ICSI. Knowledge of the prevalence of microdeletions in a series of ICSI candidates is important to predict the potential epidemiological consequences of this type of treatment (2). Also one study showed that ICSI in oligozoospermic men with microdeletions in the AZFc region of the Y chromosome leads to a lower fertilization rate and poorer embryo quality and the couple should know this probability (23). In conclusion our data support an important role of deletions of Yq in the pathogenesis of male infertility, which have an important implication in the clinical evaluation of infertile men and in planning assisted human conception.

Acknowledgement

We wish to thank vice dean for research of Tehran University of Medical Sciences for financial support.

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