Y-chromosome microdeletions and the MTHFR C677T polymorphism in Egyptian men with nonobstructive azoospermia

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Purpose
The aim of the present study was to assess the frequency of Y-chromosome microdeletions and *C677T* polymorphisms in the *MTHFR* gene in a sample of Egyptian men with nonobstructive azoospermia.

Patients and methods
This was a case–control study carried out using a PCR-RELP assay in 107 patients with nonobstructive azoospermia and 107 healthy fertile individuals who served as controls.

Results
Y-chromosome microdeletions were detected in 11 (10.3%) patients. There was no significant difference between patients and controls in the *MTHFR C677T* genotype distribution and allele frequencies ($P=0.507$).

Conclusion
Y-chromosome microdeletions play an important role in the development of nonobstructive azoospermia and there is no evident relation between the *MTHFR C677T* polymorphism and development of nonobstructive azoospermia.

Keywords:
azoospermia factor, *C677T* polymorphism, nonobstructive infertility, Y-chromosome microdeletions

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Introduction

Infertility is considered a major life crisis that threatens the stability of individuals' relationship. It is defined as the inability of a sexually active, couple not using contraception to achieve pregnancy in 1 year [1]. Infertility affects both men and women; it affects 15% of couples in the reproductive age [2]. A disorder of the male reproductive function can be detected in about 50% of the male partners of infertile couples [3]. In developing countries, the situation is worse and it has been reported that 30% of couples are infertile in some areas of the developing world [4,5]. Male infertility is a heterogeneous disorder, with various genetic and environmental factors that contribute toward the impairment of spermatogenesis [6]. Despite considerable progress in the understanding of human reproductive physiology, the underlying cause of male infertility remains undefined in about 50% of cases, which are referred to as idiopathic infertility [7]. Most idiopathic cases are likely to be of genetic origin because a large number of genes are involved in the process of human spermatogenesis [8]. When the semen sample shows no sperm, it is considered to be azoospermic; however, it is important to centrifuge the ejaculate because occasional specimens will contain a minuscule number of sperm that can be used in assisted reproduction, namely, intracytoplasmic sperm injection. Azoospermia can be classified into two broad categories: patients with spermatogenic failure (nonobstructive azoospermia) and patients with an obstructed ductal system and active spermatogenesis (obstructive azoospermia) [9]. Approximately 29% of azoospermic men have underlying genetic abnormalities [10]. Common genetic abnormalities include chromosomal or gene defects (nuclear or mitochondrial), and epigenetic alterations [11]. Y-chromosome deletions that are possibly related to male infertility were first reported in 1954 using the chromosome banding technique, which showed deletions on the distal part of the long chromosome arm (Yq) located on Yq11 [12]. The Yq contains three 'azoospermia factor' (AZF) regions: AZFa, AZFb, and AZFc. Deletions of the complete AZFc region are most frequently detected (69%), followed by deletions of the AZFb region (14%) and deletions of the AZFa region (6%). Deletions encompassing the complete AZFa or AZFb region are always associated with the complete absence of mature spermatozoa upon testicular biopsies. At the testicular level, the majority of the patients with an AZFa deletion have a Sertoli cell-only syndrome, whereas the most common phenotype among patients with an AZFb deletion is a maturation arrest of spermatogenesis [13]. Complete absence of the AZFc region, in contrast, causes a more heterogeneous phenotype, ranging from azoospermia to severe oligozoospermia. Spermatozoa can be found in ~70% of patients with an AZFc deletion [14]. Consequently, for these patients, intracytoplasmic sperm injection may be possible. The majority of Y-chromosome deletions are believed to arise *de novo*. However, in some cases, the deletion in the AZF region has been transmitted from the fertile father to the

infertile patient; the transmission of these deletions to the male offspring is obligatory, whether transmitted naturally or more often by assisted reproduction [15].

Folate participates in amino acid metabolism, purine and pyrimidine synthesis, and methylation of nucleic acids, proteins, and lipids. Dietary or genetically determined folate deficiency may impair the function of these metabolic pathways and lead to homocysteine accumulation [16]. Methylenetetrahydrofolate reductase (*MTHFR*) is a key regulatory enzyme involved in folate metabolism [17]. The *MTHFR* gene, located on the short arm of chromosome 1 (1p36.3), presents three common polymorphisms involving nucleotides C677T, A1298C, and G1793A. The change of C for T at position 677 causes the substitution of alanine for valine in the *MTHFR* protein and the consequent reduction in enzyme activity. The specific activity of the MTHFR enzyme is reduced by 35% in the presence of heterozygosis, genotype C/T, compared with the normal genotype C/C, and by 70% in homozygosis, genotype T/T [18,19]. It has been shown that severe *MTHFR* deficiency in male mice results in abnormal spermatogenesis and infertility [20]. Some studies have shown a significant correlation between *MTHFR* polymorphisms and human male infertility [21,22], whereas others did not find such association [23,24].

The aim of the present study was to assess the frequency of Y-chromosome microdeletions and *C677T* polymorphisms in the *MTHFR* gene in a sample of Egyptian men with nonobstructive azoospermia.

Patients and methods

The present study included 107 patients with nonobstructive azoospermia recruited from the Andrology Outpatient Clinic of the Department of Dermatology, Venereology and Andrology, the Main University Hospital, Faculty of Medicine, Alexandria University. In addition, 107 healthy fertile individuals with normal semen parameters and who had fathered at least one child in the past 2 years served as a control group.

The research plan was approved by the ethical committee of the Faculty of Medicine (2013) and a written informed consent was obtained from all participants.

Patients' inclusion criteria were cases with nonobstructive azoospermia (azoospermia was verified by at least two semen analysis and pelleting), previous testicular biopsy and histopathological assessment and absence of any evident cause for the condition. The exclusion criteria were hypogonadotropic hypogonadism, evident varicocele, history of radiation or chemotherapy, genital infection, trauma or torsion, history of mumps orchitis, history of testicular maldescent, any structural or numerical chromosomal anomalies, and any evidence of obstructive etiology.

Patients were subjected to a detailed assessment of history including medical history, history of trauma or torsion or late descent, drugs, surgical, and family history. Physical examination was performed including general and genital (testis, epididymis, vas deferens, and per-rectal examination) assessment. A hormonal assay of follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone, and prolactin was performed for patients using the chemiluminescence technique (Immulite 1000; Siemens, Munich, Germany).

A genetic study was carried out on blood samples where genomic DNA was extracted from human leukocytes and analysis for AZF deletion in patients and *C677T* polymorphisms in the *MTHFR* gene in patients and controls was carried out using the PCR-restriction fragment length polymorphism (PCR-RELP) technique. All PCR reagents were supplied from Fermentas including the primers. Genomic DNA was extracted from EDTA whole blood samples by a column method using a DNA extraction kit (Gene JET Genomic DNA Purification; Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Assessment of Y-chromosome microdeletions in the patient group

Detection of Y-chromosome microdeletion was performed using PCR multiplex according to the European Academy of Andrology (EAA) and the European Molecular Genetics Quality Network (EMQN) guidelines [25]. Briefly, the PCR was performed in two sets of multiplex A and B. Multiplex A contained the primer for sY86, sY127, and sY254, whereas multiplex B contained primers for sY84, sY134, and sY255. The two sets had primers for SRY and ZFY genes as an internal control. The PCR was performed in a total volume of 25 µl and the PCR conditions started with an initial activation step of 15 min at 95°C, followed by 35 cycles of 30 s denaturation (94°C), 90 s annealing (57°C), and 60 s elongation (72°C), ending with a last elongation step of 10 min and cooling to 4°C. The PCR products were visualized by 2% gel agarose stained with ethidium bromide. The expected band size was as follows: multiplex A: ZFY: 495 bp, SRY: 472 bp, sY254: 400 bp, sY84: 326 bp (AZFa) and multiplex B: ZFY: 495 bp, SRY: 472 bp, sY84: 326 bp (AZFa), sY134: 301 bp (AZFb), sY255: 126 bp (AZFc). Deletion of STS is only considered if failure of amplification occurs twice in the presence of a positive internal control and after simplex PCR.

Assessment of C677T polymorphisms in the MTHFR gene in patients and controls

Detection of the *MTHFR C677T* mutation was performed by PCR-RELP analysis using the following sets of primers: forward: 5′-CAT CCC TAT TGG CAG GTT AC-3′ and reverse: 5′-GAC GGT GCG GTG AGA GTG-3′ for amplification of a fragment of 265 bp, and then the amplified fragments were digested with the *Hin*fI enzyme. The PCR profile was as follows: initial denaturation at 95°C for 5 min, denaturation at 94°C for 30 s, annealing at 59°C for 30 s, extension at 72°C for 30 s for 35 cycles, followed by 72°C for 10 min [26]. The PCR reactions were performed in a Biocycler TC-S (HVD Life Science, Vienna, Austeria) thermal cycler apparatus.

Polymorphic 677C and 677T alleles were identified by restriction enzyme cleavage of the PCR product using the restriction enzyme *Hin*fI. Digestion products were analyzed by electrophoresis on a 3% agarose gel, stained with ethidium bromide. In the position 677 of the *MTHFR* gene, transition of the 'C' base, which is the wild base, to the 'T' base, produces a cut site for the *Hin*fI enzyme, which cuts the amplicons into two fragments of 171 and 94 bp. Then, the CC genotype would be reflected by a single band of 265 bp (uncut), the CT genotype by three bands of 265, 171, and 94 bp, and TT genotypes by two bands of 171 and 94 bp.

Statistical analysis

The data were entered and analyzed using SPSS (SPSS version 18; SPSS Inc., Chicago, Illinois, USA) system files software. A P value <0.05 was considered statistically significant.

Results

One hundred and seven Egyptian male patients with nonobstructive azoospermia were recruited in the present study (group I) and 107 normal fertile men served as controls (group II). Group I was further subdivided into group Ia with positive Y-chromosome microdeletions and group Ib with negative Y-chromosome microdeletions. The age of the patients ranged from 25 to 57 years (mean 33.14 ± 7.06 years) and the age of the individuals in the control group ranged from 21 to 49 years (mean 33.12 ± 6.4 years); there was no significant difference between the two groups ($P = 0.976$). The duration of infertility ranged from 1 to 25 years (mean 6.58 ± 5.38 years).

Y-chromosome microdeletions were detected in 11 (10.3%) patients and Table 1 shows the type of microdeletion and associated histopathological pattern and *MTHFR C677T* genotype distribution in the affected patients.

Comparison of clinical and laboratory parameters between group Ia and group Ib showed no significant difference between the two groups, except in the testosterone level, which was significantly lower in group Ia ($P = 0.011$). Also, there was no significant difference between the two groups in the *MTHFR C677T* genotype distribution ($P = 0.2$) (Table 2).

Assessment of the *MTHFR C677T* genotype distribution and allele frequencies in patients (group I) and controls (group II) is shown in Table 3, and there was no significant difference between both groups ($P = 0.507$) (Table 3). Allele distribution in patients was in Hardy–Weinberg equilibrium ($P = 0.309$).

Table 1 Type of Y-chromosome microdeletion, associated histopathology, and pattern of the *MTHFR C677T* polymorphism in azoospermic patients with positive Y-chromosome microdeletion (group Ia)

Type of Y-chromosome microdeletion	Histopathological pattern	MTHFR C677T genotype
Complete (a, b, c)	Sertoli cell only	Heterogeneous
Partial (a)	Sertoli cell only	Wild
Complete (c)	Early maturation arrest	Wild
Complete (c) and partial (b)	Early maturation arrest	Heterogeneous
Complete (a, b, c)	Tubular sclerosis	Heterogeneous
Complete (a)	Sertoli cell only	Heterogeneous
Partial (a)	Sertoli cell only	Wild
Partial (c)	Late maturation arrest	Heterogeneous
Complete (a, c) and partial (b)	Tubular sclerosis	Homogeneous
Complete (c) and partial (b)	Early maturation arrest	Wild
Complete (c)	Sertoli cell only	Heterogeneous

MTHFR, methylenetetrahydrofolate reductase.

Discussion

The underlying causal factors remain unknown in a large percentage of men with idiopathic infertility. However, evidences suggest that a variety of genetic causes are likely to be associated with idiopathic infertility. With the sequencing of the human genome and the implementation of large-scale functional genomics programs, there is now an opportunity to identify genetic causes of male infertility and to understand more precisely their effects on spermatogenesis.

The present study attempted to explore the role of Y-chromosome microdeletions and *MTHFR C677T* polymorphisms in Egyptian men with idiopathic dysfunctional azoospermia. They were recruited after a detailed

Table 2 Comparison of some clinical and laboratory parameters between group Ia and group Ib

	Group Ia ($n=11$)	Group Ib ($n=96$)	Test of significance	P value
Size of right testis (ml)	13.5 ± 3.5	13.3 ± 2	$t=0.337$	0.737
Size of left testis (ml)	13.55 ± 3.5	12.5 ± 2.5	$t=1.275$	0.205
FSH (mIU/ml)	18.1 ± 9.4	18.4 ± 11.9	$t=-0.060$	0.953
LH (mIU/ml)	9.36 ± 2.72	9.17 ± 4.25	$t=0.144$	0.885
Testosterone (ng/dl)	297.4 ± 87.7	387.8 ± 184.65	$t=-2.786$	0.011*
MTHFR C677T genotype [n (%)]				
Wild (CC)	4 (36.3)	60 (62.5)	Pearson's $\chi^2=2.957$	0.2
Heterogeneous (CT)	6 (54.5)	29 (30)		
Homogeneous (TT)	1 (9.1)	7 (7.3)		

FSH, follicle-stimulating hormone; LH, luteinizing hormone; *MTHFR*, methylenetetrahydrofolate reductase.
*Significant at $P<0.05$.

Table 3 *MTHFR C677T* genotype distribution and allele frequencies in patients (group I) and controls (group II)

	Genotypes [*n* (%)]			Allele frequencies	
	CC	CT	TT	C	T
Group I (*n*=107) (100%)	64 (59.8)	35 (32.7)	8 (7.5)	0.76	0.24
Group II (*n*=107) (100%)	62 (57.9)	32 (29.9)	13 (12.2)	0.73	0.27
Pearson's χ^2 tests			χ^2=1.36 *P*=0.507		

MTHFR, methylenetetrahydrofolate reductase.

assessment of history, clinical assessment, laboratory assay, and karyotyping.

Assessment of Y-chromosome microdeletion was performed using PCR multiplex according to the EAA and the EMQN guidelines. The protocol suggested by these guidelines has been optimized so that each of the two multiplex reactions contains a marker for each AZF region. Thus, when a complete deletion occurs in a sample, both PCR reactions should show a lack of amplification for the marker specific for that region [25]. The present study found Y-chromosome microdeletions in 10.3% of the patients included and the highest frequency of microdeletions was in the AZFc locus, followed by the AZFb and the AZFa loci. Y-chromosome microdeletion frequencies were reported in a wide variation in the literature ranging from 0.1 to 25% on the basis of the sample size in the studies, ethnic variations, and the type and number of primers used [27,28]. Comprehensive reviews comprising thousands of analyzed individuals show that the rate of Yq microdeletions ranges from 4 and 18% [29,30] and the rate of the tested Egyptian population in the present study is well within this range. The percentage of Y-chromosome microdeletions in the present study is close to the that of a study carried out on Egyptian infertile men in 2004 as the authors reported microdeletions in four patients out of 33 (12%) [31].

However, a study carried out in 2010 in Egypt on 49 patients reported a much higher percentage of microdeletions (37%) and the highest frequency of microdeletions was in the AZFb locus, followed by the AZFa locus and then the AZFc locus [32]. This was not in agreement with the results of the present study and many other studies that reported the highest frequency of microdeletion in the AZFc locus [33,34].

The present study found no significant differences in the mean levels of LH and FSH between patients with deletions and those without them. Similarly, a study carried out in 2004 comparing FSH levels between patients with and without deletions found no significant differences [35]. However, the present study found a significantly lower testosterone level in patients with deletions. This is a novel and verification of this finding and the value of low testosterone as a possible risk factor for the presence of Y-chromosome microdeletions needs to be studied further in larger groups of patients from different ethnic groups.

In the present study, there was no significant difference between patients and controls in the *MTHFR C677T* genotype distribution and allele frequencies. In the literature, the relationship between the *MTHFR C677T* polymorphism and the risk of male infertility remains controversial. In agreement with the present study, a study carried out in 2012 in south India on 206 infertile showed that the MTHFR C677T polymorphism was not associated with male infertility [36]. Similarly, Ebisch *et al.* [37] from the Netherlands studied 77 subfertile men and found no significant role of the *MTHFR* gene polymorphism in male infertility. In contrast, a Brazilian study carried out in 2011 reported that the presence of allele T of the *MTHFR C677T* polymorphism seems to be associated with both nonobstructive azoospermia and severe oligozoospermia [38]. A meta-analysis was carried out in 2011 to explore the association between the *MTHFR C677T* polymorphism and the risk of male infertility. It included 2275 cases and 1958 controls. In the overall analysis, no significant association was observed between the polymorphism and the risk of male infertility. However, in the subgroup analysis of ethnicity, it was found that *MTHFR 677T* led to an increase in the risk of male infertility in Asians, whereas no evidence of an association between the *MTHFR C677T* polymorphism and male infertility was observed in Whites. In addition, the stratified analysis showed that *MTHFR 677T* was associated with a significant increase in the risk of azoospermia. Thus, it is apparent that different ethnic groups yield different results in terms of the relation between the *MTHFR C677T* polymorphism and the risk of male infertility [39].

Conclusion and recommendations

Y-chromosome microdeletions play an important role in the development of nonobstructive azoospermia in Egyptian patients and in the era of microassisted reproduction and preimplantation genetic assessment, it is advisable to screen patients with nonobstructive azoospermia for these deletions. There is no evident relation between the *MTHFR C677T* polymorphism and development of idiopathic dysfunctional azoospermia in the Egyptian population.

The present study has some limitations as it was carried out on a limited sample of the population and the results need to be verified on a larger sample or in multicenter trials, nonobstructive.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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