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Determination of the association between the C677T and A1298C polymorphisms of the MTHFR gene and the development risk of azoospermia and oligozoospermia in Turkish infertile men

İnfertil Türk erkeklerinde MTHFR genindeki C677T ve A1298C polimorfizmlerinin azosperm ve oligosperm gelişim riski üzerindeki etkisinin araştırılması

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Summary

Aim: We aimed to investigate the relationship between unexplained male infertility, and the -677C/T (rs1801133) and -1298A/C (rs1801131) polymorphisms of the MTHFR gene in a group of Turkish infertile men with non-obstructive azoospermia and severe oligozoospermia in this study.

Materials and Methods: Study group includes 50 non-obstructive azoospermic patients, 50 severe oligospermic patients and 50 healthy controls with normal sperm parameters who had had more than one child. Genotyping was performed by generated amplicons from melting curve analysis after real time PCR.

Results: The distribution of the 677CC genotype was significantly higher in the control group than the infertile group (p= 0.046). There was a significant frequency of the polymorphic T allele in infertile patients higher than the control group (p=0.015). Neither the frequency, nor the allelic distribution of A1298C genotype was different between infertile groups compared with the control.

Conclusions: The MTHFR 677TT genotype is a genetic risk factor for unexplained male infertility, especially in the group with oligospermia and non-obstructive azoospermia.

Key Words: MTHFR, C677T, A1298C, polymorphism, azoospermia, oligospermia.

Özet

Amaç: *Bu çalışmada, non-obstrüktif azospermik ve şiddetli oligospermik infertil Türk erkeklerinde açıklanamayan infertilite ile MTHFR -677C/T (rs1801133) ve -1298A/C (rs1801131) gen polimorfizmleri arasındaki ilişkinin araştırılmasını amaçladık.*

Gereç ve Yöntem: *Çalışma grubu, non-obstrüktif azospermik 50 hasta, şiddetli azospermik 50 hasta ve sperm parametreleri normal olup birden fazla çocuğu olan sağlıklı 50 bireyden oluşmaktadır. Genotipleme, realtime PCR sonrası erime eğrisi analizi ile gerçekleştirilmiştir.*

Bulgular: *677CC genotipinin dağılımı kontrol grubunda infertil gruptan belirgin olarak daha yüksek saptanmıştır (p=0.046). İnfertil hasta grubunda polimorfik T alel frekansı kontrol grubundan daha yüksektir (p=0.015). A1298C genotipi için ise infertil grup ve kontrol grubu arasında frekans ve alelik dağılım açısından herhangi farklılık saptanmamıştır.*

Sonuç: *MTHFR 677TT genotipi özellikle oligospermli ve non-obstrüktif azospermli grupta açıklanamayan erkek infertilitesi için genetik risk faktörüdür.*

Anahtar Sözcükler: *MTHFR, C677T, A1298C, polimorfizm, azosperm, oligosperm.*

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Introduction

Infertility is a reproductive health problem that affects approximately 15% of all couples in the human population which defines as disability to conceive after 1 year of trying (1-3). Male factor has an important role in 50 % of the infertile couples (1). Numerous genes are necessary for normal sexual development, testis determination, testis descent, and spermatogenesis. Polymorphisms or mutations of these genes which consider as potential risk factors may cause spermatogenic failure (4). 10-15% of the male infertility due to severe oligozoospermia and azoospermia has been associated with a number of genetic risk factors such as chromosomal aberrations, gene mutations and polymorphisms (4-8).

Folate is necessary for DNA synthesis which is important for the development of spermatozoa and oocytes, methylation procedure and protein synthesis (2,7,9). Folate deficiency occurs frequently and known as a risk factor for various diseases, including cardiovascular diseases, neural tube defects and infertility (7,10-15). Homocysteine is a sulfur containing amino acid in methionine metabolism (7,16). Homocysteine concentration is influenced by deficiencies of essential co-factors as Vitamin B12, folic acid and polymorphisms or mutations in the enzyme coding sequence of the genes which is involved in homocysteine metabolism (16). Methylenetetrahyrofolate reductase (MTHFR) enzyme plays an important role in folate metabolism and catalyses the remethylation of homocysteine to methionine (7,17). MTHFR enzyme catalyzes the conversion of 5,10- methylenetetrahydrofolate to 5-methyltetrahydrofolate, a co-substrate for homocysteine re-methylation to methionine (18). It was reported that adult male mice testis have highest MTHFR levels than other major organs. That study indicates the importance of the MTHFR enzyme in spermatogenesis (19). To date severe mutations and polymorphisms have been identified in the MTHFR gene. -677C/T (rs1801133) and -1298A/C (rs1801131) are two well described, common polymorphisms in the MTHFR gene.

-677C/T (rs1801133) transition was first identified polymorphism in MTHFR gene which causes from cystidine to timin transversion at nucleotid 677. This genetic variant changes an alanine to a valine residue and encodes a thermobile form of MTHFR which decreases the enzymatic activity and increases the homocysteine levels by approximately 35% in heterozygote (CT) and 70% in homozygote polymorphic (TT) genotypes (14,18). -1298A/C (rs1801131) is another common variant of polymorphism in the MTHFR gene which converts adenine to cytosine substitution at nucleotide 1298 (20). This genetic variant changes a glutamine to an alanine and also encodes a thermobile form of MTHFR which decreases the enzymatic activity and increases the homocysteine levels.

The aim of this study was to investigate the frequencies of the -677C/T (rs1801133) and -1298A/C (rs1801131) polymorphisms of the MTHFR gene in patients with unexplained male infertility and was to evaluate their association with susceptibility to non-obstructive azoospermia or severe oligozoospermia.

Materials and Methods

One hundred infertile patients were recruited from the Ege University Urological Clinic and Family Planning and Infertility Investigation Centre and categorized into two groups according to the sperm analyses: 1st group was non-obstructive azoospermic patients (n=50); 2nd group was patients with isolated oligospermia with sperm density <10x10^{6}/ml (n=50). Control group was recruited from patients who had normal sperm parameters and more than one child (n=50). All participants gave their written informed consent. Semen analysis was performed strictly according to World Health Organization (WHO) 1999 guidelines. The diagnosis of azoospermia or severe oligozoospermia was made of the basis of two semen analyses performed according to the WHO recommended procedure. Non-obstructive azoospermia was determined after historical and physical examination, sperm analyses (including dip test), endocrine profile (FSH, LH, testosterone, Prl), ultrasound testicular volume and seminal vesicle valuation. Female causes of infertility were excluded from the initial study according to the information reported on the medical chart.

Blood specimens were collected in tubes containing EDTA. MTHFR -677C/T (rs1801133) and -1298A/C (rs1801131) gene polymorphisms were studied by real-time online PCR. Genomic DNA was extracted from peripheral leukocytes of the subjects using the High Pure PCR Template Preparation Kit (Roche Applied Science, Mannheim, Germany). Primers and probes were designed for detection of the MTHFR -677C/T (rs1801133) gene polymorphism. PCR master mix and conditions for MTHFR –677C/T detection were as follows; 5.4 µl H_2O, 0.6 µl 25 mM $MgCl_2$, 0.5 µl each primers (Forward Primer: 5’-CGAAGCAGGGAGCTTTGAGGCTG-3’, Reverse Primer: 5’-AGGACGGTGCGGTGA GAGAGTG-3’), 0.5 µl each probes (Fluorescein probe: 5'-TGACCTGAAGCACTTGAA GGAGAAGGTGTC–Fl-3', LCRed640 probe: 5'-LCRed640CGGGAGCCGATTTCAT CAT-P-3'), 1 µl LightCycler Master Hybridization Probes (Roche Applied Science), 1.5 µl genomic DNA and denaturation (94°C for 2 min), 45 cycles amplification (94°C for 0 s; 55°C for 10 s; 72°C for 15 s), melting analysis (94°C for 0 s, 40°C for 5 s, and 80°C 0 s, with a ramp rate of 0.6 in a step acquisition mode) and cooling step at 40°C for 30 s.

MTHFR A1298C LightMix® Kit (TIB MOLBIOL) was used to analyse the MTHFR -1298A/C (rs1801131) gene polymorphism. PCR master mix and conditions for MTHFR (-1298A/C) detection were as follows; 9.4 µl H_2O, 1.6 µl 25 mM $MgCl_2$, 2 µl reagent mix (parameter specific reagents containing primers and probes) (Tıb Molbiol), 2 µl LightCycler FastStart DNA Master Hybridization Probes (Roche Applied Science), 5 µl genomic DNA and denaturation (95°C for 10 min), 45 cycles amplification (95°C for 5 s; 60°C for 10 s; 72°C for 15 s), melting analysis (95°C for 20 s, 40°C for 20 s, and 85°C 0 s, with a ramp rate of 0.2 in a continuous acquisition mode) and cooling step at 40°C for 30 s. All experiments were carried out on the LightCycler™ Instrument (Roche Applied Science; Mannheim, Germany). Polymorphic alleles were identified by the specific melting temperatures (Tm) of the resulting amplicons.

For C677T polymorphisms, individuals with two copies of the C allele (C/C) show a single melting peak at 63.1°C, individuals with two copies of the T allele (T/T) show a single melting peak at 54.6°C, and individuals with both alleles (C/T) show two melting peaks at 54.6°C and 63.1°C in the melting curve analysis.

For A1298C polymorphisms, individuals with two copies of the A allele (A/A) show a single melting peak at 65°C, individuals with two copies of the C allele (C/C) show a single melting peak at 59°C, and individuals with both alleles (A/C) show two melting peaks at 59°C and 65°C in the melting curve analysis.

Statistical Analysis

All statistical analyses were conducted by SPSS statistical package, version 15.0. Distributions of continuous variables in groups were expressed as mean ± standard deviation. Categorical variables were compared between groups by use of χ^2 test. A value of $p < 0.05$ was considered significant.

Results

The distributions of genotype and allele frequencies were compared between unexplained infertile men and controls as well as infertile men with non-obstructive azoospermic vs. control group, oligozoospermic vs. control group as shown in Table-1 and Table-2.

Among the whole group of unexplained infertile men the frequencies of the 677C/C, 677C/T and 677T/T genotypes were 44%, 44% and 12% respectively. In the control group 677C/C genotype was present in 60% and 677C/T in 40% of the healthy controls. Homozygote polymorphic T/T genotype did not find in the control group. For A1298C polymorphism A/A, AC and C/C genotypes were present in 45%, 41% and 14% of the patients and 38%, 44% and 18% of the control group respectively. The distribution of 677CC genotype was significantly higher in control group than infertile group (*p*= 0.046; Table-1). There was a significant frequency of the polymorphic T allele in infertile patients higher than the control group (34% vs.20%, *p*=0.015, Table-1).

In the subgroup analyses; 677C/C, 677C/T and 677T/T genotypes were found in 46%, 44% and 10%; 1298A/A, 1298C/C and 1298A/C genotypes were found in 42%, 46% and 12% of the azoospermic patients respectively (Table-2). In the oligospermic patients; 677C/C, 677T/T and 677C/T genotypes were found in 42%, 44% and 14% of the cases; 1298A/A, 1298C/C and 1298A/C genotypes were found in 48%, 36% and 16% of the cases, respectively (Table-2). Neither the frequency of genotypes nor the allelic distribution was significantly different between infertile subgroups with oligospermia and non-obstructive azoospermia.

The combined haplotype frequencies were shown in Table 3 and our results displayed that patients carrying the MTHFR 677T/1298A haplotype is significantly higher in the infertile group (*p*=0.045, OR= 2.621) (Table-3).

Table-1. Genotype Distribution and Allele Frequency of the MTHFR C677T and A1298C Polymorphisms in the Control and Infertile Groups.

Polymorphisms	Genotypes	Control n=50	Infertil n=100	*p*	OR	95%CI
C677T	CC	30 (60%)	44 (44%)	0.046		R
	CT	20 (40%)	44 (44%)	0.727	0.523	0.262-1.044
	TT	0 (0 %)	12 (12%)	0.483	1.178	0.591–2.349
	CT+TT	20 (20%)	56 (56%)	0.046	1.909	0.955–3.806
	C	80 (80%)	132 (66%)	0.015	0.485	0.274– 0.858
	T	20 (20%)	68 (34%)		2.060	1.164–3.646
A1298C	AA	19 (38%)	45 (45%)	0.484		R
	AC	22 (44%)	41 (41%)	0.860	0.884	0.445–1.756
	CC	9 (18%)	14 (14%)	0.631	0.741	0.296–1.054
	AC+CC	31 (31%)	55 (55%)	0.037	0.749	0.374–1.499
	A	60 (60%)	131(65.5%)	0.374	1.265	0.771–2.076
	C	40 (40%)	69 (34.5%)		0.790	0.481–1.296

* OR: Odds ratio, CI: Confidence interval, R: Reference genotype

Table-2. Genotype Distribution of the MTHFR C677T and A1298C Polymorphisms in Infertile Subgroups.

	Genotype	Infertil sub-groups		Control vs. Azospermia			Control vs. Oligozoospermia		
		Azoospermia n=50	Oligozoospermia n=50	*p*	OR	95%CI	*p*	OR	95%CI
C677T	CC	23 (46%)	21 (42%)	0.229		R	0.109		R
	CT	22 (44%)	22 (44%)	0.839	1.178	0.532-2.609	0.839	1.178	0.532-2.609
	TT	5 (10%)	7 (14%)	0.765	0.567	0.256-1.255	0.614	0.482	0.217-1.071
A1298C	AA	21 (42%)	24 (48%)	0.838		R	0.419		R
	AC	23 (46%)	28 (36%)	0.999	1.084	0.493-2.384	0.317	1.619	0.735-3.568
	CC	6 (12%)	8 (16%)	0.576	0.621	0.203-1.898	0.999	0.867	0.305-2.467

* OR: Odds ratio, CI: Confidence interval, R: Reference genotype.

Table 3: Combined Haplotype Frequencies of C677T and A1298C Polymorphisms.

Haplotypes		EM Frequencies				
C677 T	**A1298C**	**Control**	**Infertile**	**P****	**OR**	**%95CI**
C	**A**	23	34	0.232		**R**
C	**C**	18	32	0.644	1.203	0.550–2.632
T	**A**	8	31	0.045	2.621	1.024–6.712
T	**C**	1	3	0.551	2.029	0.199–20.738

*Estimated frequencies calculated by Helix Tree Genetic Analyses V.0.9 Software.
**Logistic Regression Analyses, R: Reference genotype.

Discussion

Folate cycle is essential for various cellular functions which are involved in such processes as DNA synthesis, methylation procedure and protein synthesis (16). MTHFR is a regulatory enzyme in the homocysteine/folate pathway (15). The -677C/T (rs1801133) and -1298A/C (rs1801131) variants of the MTHFR gene has been reported to result in reduced enzymatic activity and impaired folate metabolism leading to increased homosisteine levels (11,12,21). There might be several possible mechanisms to explain the relation between the MTHFR C677T gene polymorphism and unexplained male infertility. DNA methylation is necessary for spermatogenesis. C677T polymorphism of the MTHFR gene increased the DNA hypomethylation and affects the differentiation of the germ cells. MTHFR C677T polymorphism reduced the enzymatic activity and increased the homocysteine levels. High level of homocysteine can lead to auto-oxidation which can damage the germ line DNA and sperm plasma membrane (4,14,15).

To date, there are several studies analyzed the association of the MTHFR C677T and A1298C polymorphisms in unexplained infertile males with non-obstructive azoospermia and severe oligozoospermia.

There are several studies performed in the populations and investigated the association between MTHFR gene polymorphisms and infertility (14,15,22). Their findings indicated an association of C677T polymorphism with unexplained infertility and they suggested that C677T gene polymorphism is a genetic risk factor for unexplained male infertility. It was reported that the frequency of the MTHFR TT homozygote and the CT heterozygote genotypes was significantly higher in the unexplained infertile patients, whereas MTHFR A1298C did not associated with infertility (23,24). However there are some contradictory results that reports MTHFR C677T gene polymorphism is not a risk factor in unexplained infertility (14,25). We thought that this discrepancy may depend on ethnic or geographic factors of the populations.

In our study, we investigated a possible association of C677T and A1298C polymorphisms in MTHFR gene on unexplained male infertility in a group of Turkish infertile men with non-obstructive azoospermia and severe oligozoospermia. We found that the frequencies of the T allele and the T allele carrier genotypes (CT+TT) were significantly higher in unexplained infertile patients (34%; 56%, respectively) than in control group (20%; 20%, respectively).

Conclusion

We conclude that the MTHFR 677TT genotype is a genetic risk factor for unexplained male infertility, especially with oligospermia and non-obstructive

azoospermia in unexplained infertile males.

MTHFR A1298C polymorphism was not associated with risk of the development of unexplained infertility in our group of oligospermic and non-obstructive azoospermic patients. Our findings supported by the other studies indicating that there is no relation (14,22-24). Our study is the first one in the Turkish population which shows that MTHFR C677T polymorphism is associated with the risk of the development of unexplained infertility.

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