Cukurova Medical Journal
ÇUKUROVA ÜNİVERSİTESİ TIP FAKÜLTESİ

Cukurova Med J 2023;48(1):117-126
DOI: 10.17826/cumj.1193382

RESEARCH

Association of infertility and methylenetetrahydrofolate reductase genotypes in Turkish couples

Türk çiftlerde infertilite ve metilentetrahidrofolat redüktaz genotipleri arasındaki ilişki

Halil İbrahim Pazarbaşı[1,2], M. Bertan Yılmaz[3], İbrahim Ferhat Ürünsak[4], Nurşen Keser[5], Hatice-Korkmaz Güvenmez[1]

1Cukurova University, Faculty of Art and Science, Department of Biology, Adana. Turkey
[2]Izmir Biomedicine and Genome Center, Department of Genomic Sciences and Moleculer Biotechnology, İzmir, Turkey
[3]Cukurova University, Faculty of Medicine, Department of Medical Biology, [4]Department of Obstetrics and Gynecology, Adana, Turkey
[5]Republic of Turkiye Ministry of Agriculture and Forestry Adana Food Control Laboratory Directorate, Adana, Turkey

Abstract

Purpose: Infertility is described as unexplained when all of the tests of a basic infertility evaluation return within normal limits and present in 15% of infertile couples. Some studies indicate that there is an association between methylenetetrahydrofolate reductase (*MTHFR* C677T and A1298C) mutations and unexplained infertility in male or female grown adults. The objective of this study was to analyze the distributions of *MTHFR*'s C677T and A1298C genotypes in couples with unexplained fertility problems (UFP) and healthy controls.

Materials and Methods: Two common variants C677T and A1298C of the *MTHFR* gene were screened in infertile couples (n =60 for C677T polymorphism; n=62 for A1298C polymorphism) and controls from the Cukurova region of Turkey. C677T and A1298C mutations in the *MTHFR* gene were detected by the SNP analysis (Fragment analysis) kit of the multiplex PCR amplification/ligation products. Homocysteine levels (in serum) were determined by the human hcy ELISA kit and folate values were determined by the Beckman coulter Unicel DxI 800 chemiluminescence test kit at the Central Laboratory of Balcali Hospital in Cukurova University.

Results: In this study, an association between unexplained infertility and *MTHFR* C677T polymorphism was not found. However, we found an association between *MTHFR* A1298C polymorphism and males with UFP (%7) and controls (%19). A statistically significant difference was observed between the infertile and control groups regarding i) the folate and homocysteine values of *MTHFR* C677T heterozygous individuals; ii) the

Öz

Amaç: Açıklanamayan infertilite, infertil çiftlerin %15'inde görülen ve konvansiyonel tetkiklerle sebebi tespit edilemeyen bir durumdur. Literatürde, erkek veya kadınlarda methylentetrahydrofolate reductase gen polimorfizmleri (*MTHFR* C677T ve A1298C) ve açıklanamayan kısırlık arasında ilişki bulunduğunu gösteren çalışmalar mevcuttur. Çalışmamızda *MTHFR* genindeki C677T ve A1298C polimorfizmleri ile açıklanamayan infertilite arasındaki ilişkinin araştırılması amaçlanmıştır.

Gereç ve Yöntem: Açıklanamayan infertilite tanısı alan çiftler ve sağlıklı kontrollerde *MTHFR* geninin iki yaygın polimorfizmi (C677T polimorfizmi için n=57 birey; A1298C polimorfizmi için n=62 birey) multiplex PCR amplifikasyon/ligasyon ürünlerinin SNP (Tek nükleotid polimorfizmi) analizi yöntemi ile çalışılmıştır. Çukurova Üniversitesi, Balcalı Hastanesi Merkez Laboratuvarında homosistein düzeyleri (serumda) human hcy ELISA kiti ile, folat değerleri ise Beckman coulter Unicel DxI 800 kemilüminesans test kiti ile belirlendi.

Bulgular: Çalışmamızda, açıklanamayan infertilite ve kontrol gruplarında kadın ve erkek cinsiyetleri arasında *MTHFR* C677T polimorfizminin genotip ve allel sıklığı açısından istatistiksel olarak anlamlı bir fark bulunmamıştır. A1298C polimorfizmi açısından CC genotip oranlarının açıklanamayan infertilite tanısı almış erkeklerde (%7) kontrol (%19) grubuna göre daha düşük ve istatistiki olarak anlamlı olduğu bulunmuştur. İnfertil ve kontrol grupları arasında serum folik asit ve homosistein düzeyleri karşılaştırıldığında; *MTHFR* C677T heterozigot

Address for Correspondence: Halil İbrahim Pazarbaşı, Cukurova University, Faculty of Art and Science, Department of Biology, Adana, Turkey E-mail: halilpazarbasi89@gmail.com
Received: 23.10.2022 Accepted: 11.01.2022

homocysteine values of the *MTHFR* C677T normal individuals; iii) the homocysteine values of *MTHFR* A1298C heterozygous, normal and homozygous individuals; iv) the *MTHFR* C677T heterozygous and normal individuals; v) the homocysteine values of *MTHFR* C677T normal individuals; vi) the folate values of the *MTHFR* A1298C heterozygous and normal individuals.

Conclusion: The etiopathogenesis of unexplained infertility remains largely unexplored. However, the relationship of the folate/homocysteine findings with the *MTHFR* polymorphisms under study is not clear. The results of our study support a relationship between the *MTHFR* A1298C polymorphism and male fertility problems.

Keywords: Unexplained infertility, *MTHFR* polymorphisms, A1298C, C677T, folate metabolism

bireylerin folat ve homosistein değerleri arasında; *MTHFR* C677T normal bireylerin homosistein değerleri arasında; *MTHFR* A1298C heterozigot, normal ve homozigot bireylerin homosistein değerleri arasında; kadın infertil ve kontrol gruplarında *MTHFR* C677T heterozigot ve normal bireylerin folat değerleri arasında; *MTHFR* C677T normal bireylerin homosistein değerleri arasında; *MTHFR* A1298C heterozigot ve normal bireylerin folat değerleri arasında istatistiki olarak önemli fark bulunmuştur.

Sonuç: Açıklanamayan infertilitenin etyopatogenezinde hala bilinmeyen noktalar bulunmaktadır. Folat/homosistein bulgularının çalışılan polimorfizmler ile ilişkisi net değildir. Çalışmamızın sonuçları, *MTHFR* A1298C polimorfizmi ve erkek fertilite problemleri arasındaki ilişkiyi desteklemektedir.

Anahtar kelimeler: Açıklanamayan infertilite, *MTHFR* polimorfizmleri, A1298C, C677T, folat metabolizması

INTRODUCTION

Infertility is described as the inability to conceive for one year, despite the regular and unprotected sexual life of the couples. Infertility is a common reproductive health issue affecting approximately 15% of married couples. Half of these cases include men, and 60-75% of male infertility cases are idiopathic because the underlying molecular mechanisms are largely unknown[1]. If pregnancy has not been achieved within one year in a couple with physically unobstructed coital activity 2-3 times per week, then basic infertility investigation should be conducted[2], including semen analysis according to the latest criteria of the World Health Organization in men and in women, basal body temperature monitoring for ovulation detection, ultrasonographic follicular monitoring, monitoring of serum progesterone levels within the 20-22 days of the menstrual cycle, biopsy from the endometrium two days prior to the expected menstruation, as well as hysterosalpingography, hysterosonography, laparoscopy, hydrolaparoscopy and selective salpingography for evaluating the tubal opening and uterus. Peritoneal factors in women are evaluated only by laparoscopy and hydrolaparoscopy. If these basic test results are not abnormal, other infertility causes are excluded and unexplained infertility is diagnosed[3].

Unexplained infertility is a condition that cannot be detected by conventional tests and is observed in 15% of infertile couples[3]. Since the cause of infertility cannot be determined, the methods used in treatment are aimed to increase the fertility rates rather than abolishing them. For this purpose, treatment methods such as waiting therapy, clomiphenecitrate, ovarian hyperstimulation with gonadotropin, intracervical insemination, intrauterine insemination and in vitro fertilization are applied either alone or in combination.

Previous studies have shown that as male infertility can occur later in their life, a significant fraction of cases of this type of infertility may be of genetic origin as well[4]. Infertility in men is assessed by physical examination complemented by appropriate laboratory tests. In these evaluations, the etiology of male infertility is defined and the appropriate treatment methods are determined.

After the general physical examination, biochemical analyses, such as semen and hormone (luteinizing hormone (LH), follicle stimulating hormone (FSH), testosterone, prolactin) analysis, as well as thyroid function tests are performed, together with assessement of testicular dimensions, secondary sex characteristics and vas deferens. Then, chromosome and molecular analyses such as Y chromosome microdeletion and cystic fibrosis gene mutation (CFTR) tests, are conducted [5]. After these evaluations, a person-specific treatment method can be applied.

Apart from the applied treatments and previous pregnancies, family history is also considered very important contributor to infertility. In addition, the sexual development of couples, especially of men in adolescence and childhood, must be evaluated. It has been reported that some comorbid diseases in infertile individuals (e.g. diabetes) can trigger infertility[5].

Whether patients have undergone surgery, as well as the exposure to gonadotoxins, are also very important in assessing infertility. In addition, family history for cystic fibrosis and diseases that cause sexual differentiation should be investigated. Factors associated with the women are not reported adequately in the literature. Moreover, the genetic etiologies of 2 out of 10 pregnancies which differ from aneuploidies and were terminated as spontaneous abortion are not known.

Chronic folate and methyl deficiency result in abnormal DNA methylation, DNA single- of double-strand breaks, and defects in chromosome segregation. Clinical and experimental studies indicate that genomic DNA hypomethylation is associated with chromosomal structural changes and abnormal segregation. Methylentetrahydrofolate reductase (*MTHFR*) plays a significant role in RNA, DNA and protein metabolisms and is closely associated to spermatogenesis. The enzyme MTHFR is responsible for converting 5,10-methylene tetrahydrofolate (5,10-methylene THF) to 5-methyl THF. 5,10-methylene tetrahydrofolate (5,10-methylene THF) is the basic folate form in circulation and serves as a methyl group donor for methylation of homocysteine (Hcy) to methionine. The methionine synthase (MTR) enzyme catalyzes the next reaction. Methionine synthase enzyme requires vitamin B12 or cobalamin (Cbl) as cofactors and mediates the formation of S-adenosyl methionine (SAM), a methyl donor required for DNA methylation reactions. Therefore, plays an significant role in the regulation of DNA methylation. It is known that the *MTHFR* C → T polymorphism at position 677 decreases enzyme activity by reducing the affinity of the enzyme for the flavin-adenine-dinucleotide (FAD) cofactor[6,7,8]. It has been reported that the CT genotype of the *MTHFR* 677 polymorphism decreased the enzyme activity by 35% and the homozygote TT genotype by about 70%[8,9]. Regarding the A1298C polymorphism, the homozygous CC genotype is related to a more distinctive decrease in enzyme activity relative to the heterozygous genotype[8,10]. As a result of the decreased *MTHFR* activity, the level of 5-methyl tetrahydrofolate is reduced, and the 5,10-methylene tetrahydrofolate and plasma homocysteine levels are increased. High homocysteine levels indicate defective folate metabolism.

The scope of this study is to determine the allele frequencies of polymorphisms (C677T, A1298C) occurring in the *MTHFR* gene involved in folate metabolism and to evaluate the results in terms of infertility risk and genetic diversity. Moreover, it is aimed to determine the relationship between blood homocysteine-folate values and polymorphisms, and whether they have common effects for infertility, to investigate whether the diversity in these gene regions represents a risk factor for infertility and to calculate the incidence of polymorphisms in the relevant genes in the Turkish society.

MATERIALS AND METHODS

Two different polymorphisms of the *MTHFR* gene were investigated in this study. Blood samples from couples who applied to Çukurova University, Faculty of Medicine, Department of Obstetrics and Gynecology and Infertility Policlinic were used. The study group was composed of infertile couples and the control group consisted of fertile couples. The Clinical and Local Ethics Committee of Çukurova University approved the study procedure used in this investigation, which was given the number 2014/30. The participation of all the patients and the control group in the study was provided by filling the informed consent forms for DNA study prepared in accordance with the rules specified in the information and commitment form of the Research Project of Çukurova University Medical Faculty Ethical Committees.

Couples whose infertility could not be explained despite evaluations of basic infertility tests, such as the presence of ovulation, adequate sperm production and patency of the fallopian tubes were included in the study with the diagnosis of unexplained infertility. Those couples who provided reasoning to explain infertility in the baseline infertility test evaluations and couples with a history of abortion were excluded from the study.

DNA isolation and determination of homocysteine and folate values of patients

In order to isolate the DNA of each individual and to determine the homocysteine and folate values, blood from each patient and control individuals samples were collected into three different tubes. Two of the tubes were purple-capped with EDTA and one of them was stored at -20 ° C for use in DNA isolation after blood collection. The second tubes with EDTA were centrifuged at 1000 rpm, the plasma samples were transfered into sterile Eppendorf tubes and

stored at -20 ° C. Anticoagulant was not present into the third red tubes containing blood. After blood collection, the tubes were centrifuged at 1000 rpm, the serum samples were transfered into sterile Eppendorf tubes and stored at -20 °C so as to measure homocysteine levels. Serum homocysteine levels were determined by the human hcy ELISA kit and the folate values were determined by the Beckman coulter Unicel DxI 800 chemiluminescence test kit at the Central Laboratory of Balcali Hospital in Cukurova University. The study was conducted in accordance with the procedure provided by the Beckman firm.

DNA was isolated from leukocytes from the peripheral blood collected in EDTA tubes from the patient and control groups using the Invitrogen Genomic DNA Mini Kit (CA, USA). Since it is important to assess the amount and purity of the isolated DNA samples for polymerase chain reaction (PCR) and endonuclease cleavage reactions, DNA concentration and purity were measured spectrophotometrically at 260 and 280 nm. Absorption values measured at these wavelengths were used to determine quantities of isolated nucleic acids as ng/µl or µg/ml in a fairly pure manner. The isolated DNA was stored at -20 ° C after its concentration was measured on the spectrophotometer. DNA isolation was performed again from low-concentration samples.

Genotyping studies of two polymorphisms of the *MTHFR* gene (C677T and A1298C)

With the ABI Genetic Analyzer, C677T and A1298C mutations in methylenetetrahydrofolate reductase gene were detected by the SNP analysis (Fragment analysis) kit of multiplex PCR amplification/ligation products. Thus, the genotypes of patients and control groups were determined by this method. The probes contained in the kit were FAM for *MTHFR* C677T and JOE for A1298C as Fluorochrome. The steps of the method can be summarized as follows: isolation of DNA, measurement of concentration of DNA, multiplex PCR, probe ligation, electrophoresis and analysis phase.

The preparation of multiplex PCR involved: primer mixture 2 µl, PCR mixture 5 µl, Taq polymerase 0.1 µl, buffer 2 µl and DNA 1 µl (total volume= 10 µl). PCR was performed as follows: denaturation at 96° C for 5 minutes, amplification at 95°C/60°C/72°C for 45/60/45 seconds (as 35 cycles), respectively, extension at 72°C for 5 minutes, enzyme inactivation at 99,9°C for 30 minutes, and amplification product storage at 4°C. Components and volumes of probe ligation used in genotyping studies are probe mixture 2 µl, DNA ligase 0.5 µl, 10X ligase buffer 2 µl, distilled water 5.5 µl (Total volume=10 µl). The probe ligation reaction protocol used in genotyping studies is as follows: ligation at 95°C for 30 seconds, at 60°C for 2 minutes (35 cycles), enzyme inactivation at 99° C for 10 minutes and storage at 4°C for later use. Capillary electrophoresis was performed on the ABI 3130 Genetic Analyzer and the genotypes were read from the analysis screen (Figure 1).

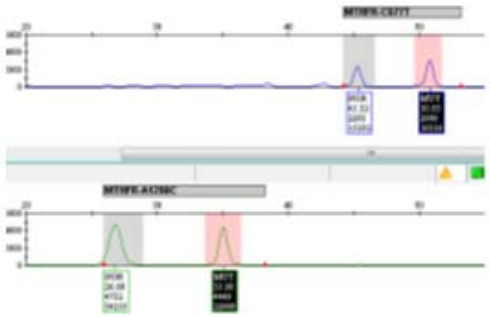

Figure 1. Genotyping analysis screenshot for *MTHFR* C677T and A1298C polymorphisms. The sample with normal and mutant peaks has a heterozygous genotype; if it has only a normal peak, it is genotyped as a homozygous normal; if it has only mutant peak, it is genotyped as a homozygous mutant.

Statistical analysis

Student t-test was used for the statistical evaluation of the clinical data. Statistical analyses were performed using the SPSS 11.5 software and Pearson Chi-square (x^2) test for determining differences in allele and genotype distributions of patient-control groups. The threshold p-value for statistical significance level was set at 0.05 ($p < 0.05$).

RESULTS

In this study, in the group with unexplained infertility, the distributions of *MTHFR* C677T polymorphism for CC, CT and TT genotypes were 52%, 42% and 6%, respectively. In the control group the results were 48%, 42% and 10%, respectively (Table 1). In the group with unexplained infertility, the allele frequency for the C allele was 72.5%, and 27.5% for the T allele; in the control group, the allele frequency

for the C allele was 69%, and 31% for the T allele (Table 2). In our study, there was no statistically significant difference in the genotype and allele frequency of *MTHFR* C677T polymorphism between the unexplained infertility and control group (p> 0.05). In the group of women with unexplained infertility, the distribution of the *MTHFR* C677T polymorphism for CC, CT and TT genotypes were 37%, 50% and 13%, respectively, whereas in the women control group, these values were 52%, 35% and 13%, respectively. In the male group with unexplained infertility, the distribution of *MTHFR* C677T polymorphism for CC, CT and TT genotypes were 67%, 33% and 0, respectively, whilst in the male control group, they were 45%, 48% and 7%, respectively (Table1). In our study, no statistically significant difference between the male and female genders of unexplained infertility and control groups in terms of genotype and allele frequency of *MTHFR* C677T polymorphism was observed (p=0.896> 0.05 for genotypes; p=0.886> 0.05 for allele frequency).

In our study, the distribution of the *MTHFR* A1298C polymorphism for AA, AC and CC genotypes were found to be 38%, 50% and 12% in the group with unexplained infertility, and 47%, 37% and 16% in the control group, respectively. The allele frequency in the group with unexplained infertility was 63% for A allele and 37% for C allele; in the control group, it was 65% for A allele and 35% for C allele (Table2). In our study, there was no statistically significant difference in the genotype and allele frequency of *MTHFR* A1298C polymorphism between the unexplained infertility and control group (p=0.298> 0.05 for genotypes; p=0.891> 0.05 for allele frequency).

Table1.Genotypes of infertile and control groups regarding the *MTHFR* C677T and A1298C polymorphisms

Infertile Group (1-60)							**Total**	**P-Value**
MTHFR C677t	Normal		Heterozygote		Homozygote			
	Cc	%	Ct	%	Tt	%		
	31	(52)	25	(42)	4	(6)	60	
Female	11	(37)	15	(50)	4	(13)	30	
Male	20	(67)	10	(33)	0	(0)	30	
Control Group (61-122)							Total	0.896> 0.05
MTHFR C677t	Normal		Heterozygote		Homozygote			
	Cc	%	Ct	%	Tt	%		
	30	(48)	26	(42)	6	(10)	62	
Female	16	(52)	11	(35)	4	(13)	31	
Male	14	(45)	15	(48)	2	(7)	31	
Infertile Group (1-60)							Total	P-Value
MTHFR A1298c	Normal		Heterozygote		Homozygote			
	Aa	%	Ac	%	Cc	%		
	23	(38)	30	(50)	7	(12)	60	
Female	13	(43)	12	(40)	5	(17)	30	
Male	10	(33)	18	(60)	2	(7)	30	
Control Group (61-122)							Total	
MTHFR A1298c	Normal		Heterozygote		Homozygote			0.298> 0.05
	Aa	%	Ac	%	Cc	%		
	29	(47)	23	(37)	10	(16)	62	
Female	15	(48)	12	(39)	4	(13)	31	
Male	14	(45)	11	(36)	6	(19)	31	

In Table 1, those individuals with CC genotype regarding the *MTHFR* C677T polymorphism are considered as normal, the individuals with CT genotype are considered as heterozygous, and individuals with TT genotype are considered as homozygous. The individuals with AA genotype regarding the *MTHFR* A1298C polymorphism are considered as normal, individuals with the AC genotype are considered as heterozygous, and individuals with the CC genotype were considered as homozygous.

Table 2. Allele frequencies of infertile and control groups regarding the *MTHFR* C677T and *MTHFR* A1298C polymorphisms.

	MTHFR C677T Allele Frequencies		MTHFR A1298C Allele Frequencies	
	C	T	A	C
	n (%)	n (%)	n (%)	n (%)
Infertile	87 (72.5)	33 (27.5)	76 (63)	44 (37)
Control	86 (69)	38 (31)	81 (65)	43 (35)
p value	0.796 > 0.05		0.827 > 0.05	

In our study, the distribution of *MTHFR* A1298C polymorphisms for AA, AC and CC genotypes was 43%, 40% and 17% in the female group with unexplained infertility, respectively, while in the female control group, these values were 48%, 39% and 13%, respectively. In the male group with unexplained infertility, the distribution of the *MTHFR* A1298C polymorphism for AA, AC and CC genotypes were 33%, 60% and 7%, respectively, whereas in the male control group, they were 45%, 36% and 19% (Table1). In our study, the frequency of the AA and AC genotypes of *MTHFR* A1298C polymorphism between male and female genders for unexplained infertility and control groups were not statistically significant in terms of A and C allele frequencies (p> 0.05) (Table 2).

Regarding the A1298C polymorphism, CC genotype ratios were found to be lower in men with unexplained infertility compared to the control group; this difference was statistically significant (p <0.05).

A statistically significant (p <0.05) difference was found in both groups when the folate values of infertile and control group *MTHFR* C677T heterozygous (CT) individuals were compared. However, no statistically significant difference was found between the folate values of *MTHFR* C677T normal (CC) and homozygous mutant (TT) individuals of the infertile and control groups (p> 0.05).

There is a statistically significant difference (p <0.05) between the homocysteine values of *MTHFR* C677T heterozygous (CT) and normal (CC) individuals for the infertile and control groups. Nonetheless, there was no statistically significant difference between the homocysteine values of *MTHFR* C677T homozygous mutant (TT) individuals of infertile and control groups (p> 0.05).

There was no statistically significant difference between the folate values of *MTHFR* A1298C heterozygous (AC), normal (AA) and homozygous mutant (CC) individuals for infertile and control groups (p> 0.05). When the homocysteine values of *MTHFR* A1298C heterozygous (AC), normal (AA) and homozygous mutant (CC) individuals for infertile and control groups were compared, a statistically significant difference (p <0.05) was found.

In this study, we found statistically significant differences between i) the folate and homocysteine values of *MTHFR* C677T heterozygous individuals in the infertile and control groups, ii) the homocysteine values of *MTHFR* C677T normal individuals (CC), iii) the homocysteine values of *MTHFR* A1298C heterozygous (AC), normal (AA) and homozygous (CC) individuals, iv) the folate values of *MTHFR* C677T heterozygous and normal individuals in the infertile and control groups, v) the homocysteine values of *MTHFR* C677T normal individuals, and vi) the folate values of *MTHFR* A1298C heterozygous and normal individuals.

DISCUSSION

The cases in which the cause of infertility cannot be traced with the standard tests are defined as unexplained infertility. It is generally thought that oocyte quality, fertilization or implantation disorders contribute to unexplained infertility and these disorders are not diagnosed by Standard tests[11]. In addition to the known Standard tests, *MTHFR* C677T and A1298C polymorphisms are also examined as part of the etiology of unexplained infertility[6,7,8,9,10]. Gene polymorphisms may vary according to ethnic origin and race and play a role in biodiversity. Since the MTHFR enzyme affects the folic acid cycle and homocysteine level, polymorphisms in the *MTHFR* gene affect the corresponding gene product.

MTHFR has a significant role in the regulation of DNA methylation. The 677 C → T polymorphism is

known to reduce the enzyme's activity by reducing the affinity of the MTHFR enzyme for the flavin-adenine-dinucleotide (FAD) cofactor[6,7,8]. It has been reported that *MTHFR* 677 CT genotype reduces the enzyme activity by 35% and the homozygous TT genotype by about 70%. In terms of the *MTHFR* A1298C polymorphism, the homozygous CC genotype has been reported to be associated with a more distinct decrease in enzyme activity as compared to the heterozygous genotype[8,9,10]. As a result of decreased *MTHFR* activity, the 5-methyl tetrahydrofolate level decreases, and, conversely, the levels of plasma homocysteine and 5,10-methylene tetrahydrofolate are increased. High levels of homocysteine indicated effective folate metabolism.

Reduction in MTHFR enzyme activity due to variations in the corresponding gene results in DNA hypomethylation. Abnormal folate levels and methylation metabolism may lead to abnormal chromosome distribution[12]. Folate level has an important role in homocysteine metabolism. Folate deficiency is often observed and associated with hyperhomocysteinemia, which is considered as a risk factor for many diseases, including infertility. According to a study by Wong *et al.,* the combined treatment of zinc sulphate and folic acid was reported to increase the total normal sperm count in subfertile and fertile men[13].

Increased levels of fibrinogen, factor II, VII, X, XII, and plasminogen activator inhibitor 1 (PAI – 1), as well as decreased protein S amounts, lead to increased clotting tendency in normal pregnancies. Thrombophilia (hypercoagulability or prothrombotic state) involves a group of coagulation disorders in which the tendency of thrombosis (blood clots in blood vessels) increases[14]. The increased tendency to coagulation can be acquired or inherited. The importance of hereditary thrombophilia in recurrent pregnancy loss and infertility has been emphasized[15]. The most common cause of hereditary thrombosis is point mutation in the Factor V Leiden (FVL). The risk of thrombosis in these mutation carriers is 5–10 times higher in heterozygotes and 80- 100 times in homozygotes. The risk of thrombosis is increased 2-fold in heterozygous prothrombin mutation carriers (Factor II G20210A) and 2-fold in homozygous hyperhomocysteinemia (*MTHFR* C677T). It is speculated that changes in the coagulation factors during pregnancy may cause abortion via congestion in placental bed veins due to hemostatic errors. An effective uteroplacental circulation is essential for the successful course of pregnancy and may be affected by hemostatic disorders. Maternal thrombophilias (*MTHFR* C677T and A1298C mutations, FVL, Prothrombin G20210A, D-Dimer) are very important in both the occurrence and maintanance of pregnancy, and obstetric aspects as well. Uteroplacental circulatory disorder is an important factor in fetal losses. Most studies reveal a strong association between FVL mutations and vascular placental insufficiency in patients with recurrent fetal losses[11,16,17].

Since exogenous hormone usage may influence the coagulation and fibrinolytic system and stimulate the prothrombotic phenotype, inherited thrombophilias representing the risk associated vascular disease and thrombophilia may increase the risk in women who applied assisted reproductive techniques[14].

Homocysteine is an amino acid that is implicated in methionine metabolism. It is catabolized (cystationine B synthase) during the transsulfuration pathway or converted to methionine by remethylation (5,10 methylenetetrahydrofolate reductase). Defects in both pathways lead to an increase in homocysteine. Homocysteine represents an independent risk factor for atherosclerosis and venous thromboembolism (VTE).

On the basis of the findings of our study for unexplained infertility and control groups, no statistically significant difference between the female and male genders with regards to allele and genotype frequency of *MTHFR* C677T polymorphism was found ($p > 0.05$). As far as the A1298C polymorphism is concerned, CC genotype ratios were found to be statistically significantly lower in men with unexplained infertility compared to the control group. In a study of Adıgüzel *et al.*, there was no significant difference between the women patients and the control group regarding the *MTHFR* C677T mutation. Thus, it could not be inferred that *MTHFR* C677T mutations might cause unexplained infertility[11]. In a study conducted by Casadei *et al.* (2010), 100 women with unexplained infertility and 200 healthy fertile women were included in the study. The two groups of women were examined for the presence of *MTHFR*C677T mutation and no statistically significant difference was found[18]. In a study by Singh and colleagues performed in Indian society, it was shown that homozygosity (TT genotype) in terms of C677T mutation was a risk factor for idiopathic male infertility. In the second study of the same group, the CC genotype was

reported as a genetic risk factor for idiopathic male infertility in the Indian population in terms of *MTHFR* A1298C polymorphism[19]. Moreover, Wu *et al.*, in a study of spermatozoa in China, demonstrated that hypermethylation of the *MTHFR* gene promoter was associated with idiopathic male infertility[20]. Herodež *et al.* found a significant relationship between *MTHFR* C677T and A1298C polymorphisms and unexplained infertility in men in a study conducted with Slovak couples[1].

In our study, we found statistically significant differences between a) folate and homocysteine values of *MTHFR* C677T heterozygous individuals in the infertile and control groups, b) homocysteine values of *MTHFR* C677T normal individuals, c) homocysteine values of *MTHFR* A1298C heterozygous , normal and homozygous individuals, d) folate values of *MTHFR* C677T heterozygous and normal individuals in the infertile and control groups, e) homocysteine values of *MTHFR* C677T normal individuals and f) folate values of *MTHFR* A1298C heterozygous and normal individuals.

The abnormalities in folate metabolism which lead to reproductive problems are not fully elucidated[21]. Inadequate methylation of critical metabolites has been proposed as a possible regulator of cell division problems[21]. The homocysteine levels of homozygous individuals in terms of *MTHFR* C677T or A1298C alleles were found to be normal unless there was a deficiency in folate intake[21].The findings of this study, which was published by the researchers Coulam and Jeyendran in 2009, support the hypothesis that proteins which are thought to be involved in fibrinolysis and coagulation do not have hemostatic function as mentioned in previous studies. The genes encoding thrombogenic proteins are not only involved in coagulation and fibrinolysis, but also in fertilization, embryonic development, tissue regeneration[22], recurrent implantation failure[23], recurrent pregnancy losses[23,24,25,26] and congenital anomalies[27]. Thrombophilic gene mutations are thought to have an effect on recurrent abortion frequency and may be related to clotting in placental vessels[28]. It has been reported that in the case of recurrent implantation failure, hypofibrinolysis affects trophoblast migration[29,30], and the effect on unexplained infertility necessitates folic acid metabolism[21].

In a study published in 2009 by the researchers Coulam and Jeyendran, *MTHFR* C677T polymorphism was associated with unexplained infertility. In other studies, *MTHFR* C677T polymorphism TT homozygosity was also associated with recurrent pregnancy losses[24] and congenital anomalies such as neural tube anomalies[27].

Regarding the *MTHFR* C677T polymorphism, homozygous women with recurrent unexplained abortion exhibited an increase in folate level and a decrease in homocysteine level after folic acid administration[31]. In addition, it was reported that folic acid treatment in TT homozygous women with the *MTHFR* C677T polymorphism led to an increase in the rate of fraternal twins[32] and decrease in neural tube anomalies.

The etiopathogenesis of unexplained infertility remains largely unknown. Based on the findings of our study, there is no significant relationship between the *MTHFR* C677T polymorphism and unexplained infertility. This could be partially explained in terms of the genetic differences of the populations. On the other hand, a limitation of our study is that the data observed were obtained from a population restricted to Turkish couples from the Cukurova region. However, in men, a significant relationship between the A1298C polymorphism and unexplained infertility was noteworthy. In order to further support this, a higher number of cases should be investigated in addition to the studies in the literature related to both polymorphisms of the *MTHFR* gene. Thrombophilia plays an important role in the etiopathogenesis of unexplained infertility. In those cases with unexplained infertility and thrombosis history, investigation of mutations in the *MTHFR* gene would be useful for the diagnosis and treatment of unexplained fertility.

Yazar Katkıları: Çalışma konsepti/Tasarımı: HİP, HKG; Veri toplama: HİP, NK; Veri analizi ve yorumlama: HİP, NK; Yazı taslağı: HİP, MBY; İçeriğin eleştirel incelenmesi: HİP, MBY, İFÜ, HKG; Son onay ve sorumluluk: HİP, MBY, İFÜ, NK, HKG; Teknik ve malzeme desteği: HİP, İFÜ; Süpervizyon: HİP, HKG; Fon sağlama (mevcut ise): yok.
Etik Onay: Bu çalışma için Çukurova Üniversitesi Tıp Fakültesi Girişimsel Olmayan Klinik Araştıramalar Etik Kurulundan 04.04.2014 tarih ve 30/14 sayılı kararı ile etik onay alınmıştır.
Hakem Değerlendirmesi: Dış bağımsız.
Çıkar Çatışması: Yazarlar çıkar çatışması beyan etmemişlerdir.
Finansal Destek: Bu çalışma "Çukurova Üniversitesi Araştırma Projeleri Finansman Birimi" tarafından FYL-2014-2830 proje numarası ile desteklenmiştir.
Yazarın Notu: Çalışmamızda çiftlerin homosistein ve folat seviyelerinin ölçülmesinde sağladıkları destek için Prof. Dr. Salih Çetiner ve Prof. Dr. Özlem G. Öztürk'e teşekkürlerimizi sunuyoruz.

Author Contributions: Concept/Design : HİP, HKG; Data acquisition: HİP, NK; Data analysis and interpretation: HİP, NK; Drafting manuscript: HİP, MBY; Critical revision of manuscript: HİP, MBY, İFÜ, HKG; Final approval and accountability: HİP, MBY, İFÜ, NK, HKG; Technical or material support: HİP, İFÜ; Supervision: HİP, HKG; Securing funding (if available): n/a.
Ethical Approval: Ethical approval was obtained for this study from the Ethics Committee of Non-Interventional Clinical Research of the

Faculty of Medicine of Çukurova University with the decision dated 04.04.2014 and numbered 30/14.
Peer-review: Externally peer-reviewed.
Conflict of Interest: Authors declared no conflict of interest.
Financial Disclosure: This work was supported by "Cukurova University Research Projects Funding Unit" with project number FYL-2014-2830.
Acknowledgement: We would like to thank to Prof.Dr. Salih Çetiner and Prof. Dr. Özlem G.Öztürk for their support they provided for the analyses of homocysteine and folate levels of the couples.

REFERENCES

1. Herodež ŠS, Zagradišnik B, ErjavecŠkerget A, Zagorac A, Takač I, Vlaisavljevıč V et al. MTHFR C677T and A1298C genotypes and haplotypes in Slovenian couples with unexplained infertility problems and in embryonıc tissues from spontaneous abortions. Balkan J Med Genet. 2013;16:31-40.
2. Smith S, Pfiefer SM, Collins J. Diagnosis and management of female infertility. JAMA. 2003;290:1767-70.
3. Mutlu MF, Bastu E, Oktem M. Unexplained infertility: A current overwiew. Gazi Medical Journal. 2013;24(1).
4. Ozpak L, Pazarbasi A. Cytogenetics of male infertility. Archives Medical Review Journal. 2011;20:230-45.
5. Mark HFL, Sigman M. Cytogenetics of male infertility. Medical Cytogenetics (Ed. HFL Mark):247-73. New York, Marcel Dekker, 2000.
6. Guenther BDF, Sheppard CA, Tran P, Rozen R, Matthews RG, Ludwig ML. The structure and properties of methylenetetrahydrofolate reductase from escherichia coli suggest how folate ameliorates human hyperhomocysteinemia. Nat Struct Mol Biol. 1999;6:359-65
7. Yamada K, Chen Z, Rozen R, Matthews RG. Effects of common polymorphisms on the properties of recombinant human methylenetetrahydrofolate reductase. Proc Natl Acad Sci U S A. 2001;98:14853-8.
8. Eggermann T, Schwanitz G. Abnormal folate metabolism and maternal risk for down syndrome. In Genetics and Etiology of Down Syndrome (Ed S Dey):97-107. Rijeka, Croatia, InTech, 2011.
9. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet. 1995;10;1:111-13.
10. Van Der Put NM, Gabreëls F, Stevens EM, Smeitink JA, TrijbelsFJ, Eskes TK. et al. A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? Am J Hum Genet. 1998;5:1044-51.
11. Adıguzel FI, Adıguzel C, Seyfettinoğlu S, Urunsak IF. Açıklanamayan infertilite etiyolojisinde olası risk faktörü olarak herediter trombofilinin yeri. Bakırköy Tıp Dergisi. 2015;11:162-66.
12. Park JH, Lee HCh, Jeong YM, Chung TG, Kim HJ, Kim NK et al. MTHFR C677T polymorphism associates with unexplained infertile male factors. J Assist Reprod Genet. 2005;22:361-68.
13. Wong WY, Merkus HM, Thomas CM, Menkveld R, Zielhuis GA, Steegers-Theunissen RP. Effects of folic acid and zincsulfate on male factor subfertility: A double-blind, randomized, placebo-controlled trial. Fertil Steril. 2002;77:491-98.
14. Fatini C, Conti L, Turillazzi V, Sticchi E, Romagnuolo I, Milanini MN. et al. Unexplained infertility: Association with inherited thrombophilia. Thromb Res. 2012;129:e185-e188.
15. Letsky EA, Swiet M. Maternal hemostasis coagulation problems of pregnancy in; Thrombosis and Hemorrhagie. (Edts: Loscalzo J, Schafer AI) Blackwell Scientific Publications. 1994:965-98.
16. Brigden ML. The hypercoagulable state. Who, how, and when to test and treat. Postgrad Med. 1997;101:249-62.
17. Ridker PM, Miletich JP, Buring JE, Ariyo AA, Price DT, Manson JE et al. Factor V leiden mutation as a risk factor for recurrent pregnancy loss. Ann Intern Med. 1998; 128:1000-3.
18. Casadei L, Puca F, Privitera L, Zamaro V, Emidi E. Inherited thrombophilia in infertile women: implication in unexplained infertility. Fertil Steril. 2010;94:755-7.
19. Singh K, Singh SK, Raman R. MTHFR A1298C polymorphism and idiopathic male infertility. J Postgrad Med. 2010;56:267-9.
20. Wu W, Shen O, Qin Y, Niu X, Lu C, XiaY et al. Idiopathic male infertility is strongly associated with aberrant promoter methylation of methylenetetrahydrofolate reductase (MTHFR). PloS One. 2010;5:e13884.
21. Coulam CB, Jeyendran RS. Thrombophilic gene polymorphisms are risk factors for unexplained infertility. Fertil Steril. 2009;91:1516–7.
22. Rawlins ND, Barrett AJ. Evolutionary families of peptidases. Biochem J. 1993;290:205–18.
23. Coulam CB, Jeyendran RS, Fishel LA, Roussev RG. Multiple thrombophilic gene mutations are risk factors for implantation failure. Reprod Biomed Online. 2006;12:322–7.
24. Goodman CS, Coulam CB, Jeyendran RS, Acosta VA, Roussev R. Which thrombophilic gene mutations are risk factors for recurrent pregnancy loss? Am J Reprod Immunol. 2006;56:230–6.
25. Coulam CB, Wallis D, Weinstein J, Dasgupta DS, Jeyendran, RS. Thrombophilic gene mutations among patients experiencing recurrent miscarriage and deep vein thrombosis. Am J Reprod Immunol. 2008;60:426-431.
26. Bianca S, Barrano B, Cutuli N, Indaco L, Cataliotti A, Milana G. et al. Unexplained infertility and inherited thrombophilia. Fertil Steril. 2009;92;1:e4.

27. Botto LD, Yang Q. 5,10-methylenetetrahydrofolate reductase gene variants and congenital anomalies: a HuGE review. Am J Epidemiol. 2000;151:862–77.
28. Many A, Schrieber L, Rosner S, Lessing JB, Eldor A, Kupferminc MJ. Pathologic features of the placenta in women with severe pregnancy complications and thrombophilia. Obstet Gynecol. 2001;98:1041–4.
29. Axelrod HR. Altered trophoblast functions in implantation-defective mouse embryos. Dev Biol. 1985;108:185–90.
30. Aflalo ED, Sod-Moriah UA, Potashnik G, Har-Vardi I. Differences in the implantation rates of rat embryos developed in vivo and in vitro: possible role for plasminogen activators. Fertil Steril. 2004; 81:780–5.
31. Nelen Wl, Blom HJ, Thomas CM, Steegers EA, Boers CH, Eskes, TK. Methylenetetrahydrofolate reductase polymorphism affects the change in homocysteine and folate concentrations resulting from low dose folic acid supplementation in women with unexplained recurrent miscarriages. J Nutr. 1998;128:1336–41.
32. Kallen B. Use of folic acid supplemantation and risk for dizygotic twinning. Obstet Gynecol Surv. 2005;60:212–4.