

MTHFR and IL-4 Gene Polymorphisms Are Not Associated with Primary Dysmenorrhea in Young Adults

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ABSTRACT Primary dysmenorrhea is one of the most common conditions among young adult females. The purpose of the present study was to investigate possible associations between the functional MTHFR gene C677T and IL-4 gene intron 3 VNTR polymorphisms with primary dysmenorrhea susceptibility in a Turkish population. One hundred and fifty-nine unrelated young women with primary dysmenorrhea and 135 unrelated healthy age-matched controls. Genomic DNA were isolated and MTHFR gene C677T polymorphism genotyped using polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) assay and IL-4 gene intron 3 VNTR polymorphism genotyped by using PCR with specific primers. The distribution of genotype and allele frequencies was not statistically different between the primary dysmenorrhea patients and healthy controls ($p>0.05$). According to the findings of first study of intron 3 VNTR polymorphism in the IL-4 gene and C677T polymorphism in MTHFR gene, these polymorphisms do not lead to increased susceptibility to primary dysmenorrhea.

INTRODUCTION

Primary dysmenorrhea known as painful menses in women without pelvic pathology usually begins during adolescence and continues during adulthood. It is presented as crampy pelvic pain beginning shortly before or at the beginning of menses and continuing up to three days. In the presence of pelvic organ pathology, dysmenorrhea is defined as secondary. Dysmenorrhea is one of the most common gynecologic complaints among adolescent and young adult females. The prevalence of dysmenorrhea is highest in adolescent women, with estimates ranging from 20 to 90 percent, depending on the measurement method used (Banikarim et al. 2000; Davis and Westhoff 2001; French 2005; Strini   et al. 2003; Davood 2006; Iacovides et al. 2013).

The pathophysiology of primary dysmenorrhea is not precisely understood. There are continuing research from several points of view such as endometrial and myometrial physiology, hormonal physiology and polymorphism

studies. Gene polymorphisms can alter the function of the related protein. The inheritance of a polymorphism or their combinations may predispose an individual to specific disorders because of changed role of affected gene product in the related physiologic process. There are several polymorphism studies found genetic susceptibility to primary dysmenorrhea. Among these, cytochrome P450 2D6 (CYP2D6) and glutathione S-transferase Mu (GSTM1) polymorphisms (Wu et al., 2000), cytochrome P450 1A1 (CYP1A1) MspI and CYP1A1HincII polymorphisms (Li et al. 2007, 2008), familial Mediterranean fever gene (MEFV) polymorphisms (Erten et al. 2013; Ocak et al. 2013), glutathione S-transferase theta 1 (GSTT1), glutathione S-transferase pi 1 (GSTP1), and estrogen receptor 1 (ESR1) (Woo et al. 2010) have been investigated.

5, 10-Methylenetetrahydrofolate reductase (MTHFR) plays important role in DNA integrity via folate metabolism. Interleukin-4 (IL-4), secreted by Th2 lymphocytes, eosinophils and mast cells, is a key cytokine having cytotoxic, anti-tumor and numerous anti-inflammatory effects. As genetic factors have been implicated in the pathogenesis of primary dysmenorrhea, the purpose of the present study was to investigate possible associations between the functional MTHFR gene C677T and IL-4 gene intron 3 VNTR polymorphisms with primary dysmen-

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orrhea susceptibility in a Turkish population. According to our knowledge, there is no study assessing the association of intron 3 VNTR polymorphism in the interleukin-4 (IL-4) gene and C677T polymorphism in methylenetetrahydrofolate reductase (MTHFR) gene with the susceptibility of primary dysmenorrhea. As genetic factors have been implicated in the pathogenesis of primary dysmenorrhea, the purpose of the present study was to investigate possible associations between the functional MTHFR gene C677T and IL-4 gene intron 3 VNTR polymorphisms with primary dysmenorrhea susceptibility in our population.

MATERIAL AND METHODS

Study Population

A total of 159 unrelated young women who had not responded well to non-steroidal anti-inflammatory drugs in the menstrual period, and who had presented to the outpatient gynecology service with the complaint of recurrent pain episodes related to menstruation were included in the study as primary dysmenorrhea cases. A complete gynecological evaluation was done for all the patients. Patients were included if their menstrual cycles lasted 21 to 35 days, with menstruation lasting 3 to 7 days and they experienced painful menses in the past 5 years with the pain starting one day before or on the day of onset of bleeding. They had no gastrointestinal, gynecologic, or autoimmune disorders, and pelvic surgery. Women with secondary dysmenorrhea and polycystic ovary syndrome were excluded. A total of 135 unrelated healthy age-matched controls were chosen with no previous history of dysmenorrhea without any identifiable gynecological pathology after annual gynecologic check-up examination and if they used no medications including regular non-steroidal anti-inflammatory drugs and oral contraceptives. After the approval of Human Research Ethics Committee of university, informed consent was obtained of all the participants. The experimental procedures were carried out in accordance with the Declaration of Helsinki and related institutional regulations. A clinical data collection form were designed to record age, age at menarche, parity, quality of life score, and family history of dysmenorrhea, marital status, medi-

cation usage as oral and intramuscular for relief of pain, and smoking. The visual analog rating scale of health-related quality of life was used. The respondent is asked to place a line perpendicular to the visual analog scale line at the point that represents their health quality and with a ruler, the score is recorded by measuring the distance on the 10-cm line from the "0 as none" anchor to the mark of patient, obtaining a range of scores from 0 to 10 cm.

Genotype Determination

Genomic DNA was extracted from 2-mL whole venous blood samples according to the manual of a commercial DNA isolation kit (Sigma-Aldrich, Taufkirchen, Germany).

The MTHFR C677T polymorphism (rs1801133) was analyzed by PCR based RFLP methods as described previously (Frost et al. 1995). In brief, the PCR protocol was consisted of an initial melting step of 5 min at 94°C; followed by 35 cycles of 30 s at 94°C, 30 s at 61°C, and 30 s at 72°C; and a final elongation step of 5 min at 72°C. PCR primers (5'-TGAAGGAGAAGG TGTCTGCGGGA-3' and 5'-AGGACGGTGCGG TGA GAG TG-3') were used to amplify a portion of the MTHFR gene from 100 ng of genomic DNA in a 25 µL reaction containing 2.5 µL of 10xPCR buffer, 200 µM dNTP, 10 pM each primers, and one unit of Taq DNA polymerase. After amplification, the 198 bp PCR product was digested with Hinf I in a 15 µL reaction solution containing 10 µL of PCR product, 1.5 µL of 10x buffer, and two units of Hinf I at 37°C overnight. The digestion products were separated on 3% agarose gels and fragments stained with the ethidium bromide were photographed on an ultraviolet transilluminator. Wild type (CC) individuals were identified by only a 198 bp fragment, heterozygotes (CT) by both the 175/23 bp and 198 bp, and homozygote variants (TT) by the 175/23 bp.

To detect 70 bp VNTR polymorphism of IL-4 gene PCR assay as described by Mout et al. (Mout et al. 1991) was used. In brief, PCR was performed with a 25 µL reaction mixture containing 50 ng DNA, 0.8 L M of each primer, 200 L M of each dNTP, 2.5 mM MgCl₂, 0.5 U Taq polymerase, 10x KCl buffer (MBI, Fermentas). Amplification was carried out using primers F 5' AG-GCTGAAAGGGGAAAGC-3', R 5'-CTG TTCACCTCAACTGCTCC-3' with initial dena-

turation at 95°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 45 s, extension at 72°C for 1 min and final extension at 72°C for 10 min. The PCR products were visualized on 3% agarose gel stained with ethidium bromide. PCR product was of 183 bp for P1 allele and 253 bp for P2 allele.

In order to validate the accuracy and reproducibility of this method, each PCR reaction included internal controls for each genotype. Second PCR was performed to confirm samples which results are not clear. Also, to confirm the accuracy of the genotyping, repeated analysis was performed on randomly selected samples to detect discrepancies.

Statistical Analysis

Statistical analysis was performed using and the OpenEpi Info software package version 2.3.1 (www.openepi.com). Descriptive statistics as mean±SD percentage values were presented. ANOVA, t, and chi-square test were used to ana-

lyze clinical parameters and polymorphism data as appropriate. Odds ratios (ORs) for polymorphisms were calculated as appropriate. A *p* value of less than 0.05 was considered as significant.

RESULTS

The study was completed with 159 women with dysmenorrhea and with 135 healthy controls. Table 1 presents the clinical parameters according to MTHFR gene C677T and IL-4 gene intron 3 VNTR polymorphisms in 159 study patients with primary dysmenorrhea. There were no association between the MTHFR gene C677T and IL-4 gene intron 3 VNTR polymorphisms and the age, parity, and quality of life score of primary dysmenorrhea patients (*p*>0.05). The age at menarche was found significantly higher in primary dysmenorrhea patients with TT genotype of MTHFR gene C677T polymorphism than those with CC and CT genotypes (*p*<0.05). The ages at menarche of primary dysmenorrhea patients with CC and CT genotypes of MTHFR

Table 1: Clinical parameters according to MTHFR gene C677T and IL-4 gene intron 3 VNTR polymorphisms in 159 study patients with primary dysmenorrhea

Characteristic	Total (n=159)	MTHFR gene C677T polymorphism				Signifi- cance	IL-4 gene intron 3 VNTR polymorphism		
		CC (n=79)	CT (n=64)	TT (n=16)			P2P2 (n=124)	plus P1P2 P1P1 (n=35)	Signifi- cance
Age (years)	25.5 ±4.0	24.9 ±3.7	25.9 ±4.2	26.3 ±4.3	0.215		25.5 ±4.03	25.5 ±3.9	0.997
Age at menarche (y)	12.3 ±0.6	12.3 ±0.6	12.2 ±0.5	12.6 ±0.9	0.021		12.2 ±0.6	12.5 ±0.6	0.041
Parity (n)	1.2 ±1.014	1.2 ±1.1	1.1 ±0.9	1.7 ±1.03	0.436		1.3 ±1.07	0.9 ±0.8	0.242
Quality of life score (1-10)	6.9 ±1.0	6.8 ±1.0	6.9 ±1.0	7.0 ±1.0	0.701		6.9 ±1.0	6.8 ±1.0	0.705
Family history of dysmenorrhea, n (%)	67 (42.1)	35	29	3(4.5)	0.135		52 (77.6)	15	1.000
Yes	92 (57.9)	(52.2)	(43.3)	13 (14.1)			72 (78.3)	(22.4)	
No		(47.8)	(38.0)					(21.7)	
Marital status, n (%)	67 (42.1)	26	35	6(9.0)	0.030		48(71.6)	19	0.122
Married	92 (57.9)	(38.8)	(52.2)	10 (10.9)			76(82.6)	(28.4)	
Other		(57.6)	(31.5)					(17.4)	
Medications, n (%)	144	69	60	15	0.385		111	33	0.525
Oral	(0.6)	(41.7)	(41.7)	(10.4)			(77.1)	(22.9)	
Intramuscular	15 (9.4)	10	4 (26.7)	1 (6.7)			13(86.7)	2(13.3)	
Smoking, n (%)	15 (9.4)	9 (60.0)	5 (33.3)	1 (6.7)	0.690		12(80.0)	3(20.0)	0.986
Yes	44	70	59	15			112	32	
No	(90.6)	(48.6)	(41.0)	(10.4)			(77.8)	(22.2)	

Data were presented as mean±SD or percentage and analyzed by ANOVA, t, and chi-square tests as appropriate. MTHFR, methylene tetrahydrofolate reductase; IL-4, interleukin-4.

gene C677T polymorphism were found comparable ($p>0.05$). The age at menarche was found significantly higher in primary dysmenorrhea patients with P1P2 plus P1P1 genotype of IL-4 gene intron 3 VNTR polymorphism than that with P2P2 genotype ($p<0.05$). The ratios of married women with CC and CT genotypes of MTHFR gene C677T polymorphism were found significantly different compared to other genotypes ($p<0.05$). The ratio of married women with TT genotype of MTHFR gene C677T were not different compared to other genotypes ($p>0.05$). The ratios of women with family history of dysmenorrhea, oral medication use, and smoking were found similar with regard to the genotypes of MTHFR gene C677T polymorphism ($p>0.05$). In primary dysmenorrhea patients, the distributions of P2P2 and P1P2 plus P1P1 genotypes of IL-4 gene intron 3 VNTR polymorphism were found similar among the clinical parameter of age, parity, quality of life score, and ratios of family history of dysmenorrhea, to be married, oral medication use, and smoking ($p>0.05$).

Table 2 shows the genotype and allele frequencies of MTHFR gene C677T and IL-4 gene intron 3 VNTR polymorphisms in primary dysmenorrhea patients and healthy controls. With regard to the to the genotypes and alleles of MTHFR gene C677T and IL-4 gene intron 3 VNTR

polymorphisms, the primary dysmenorrhea patients and healthy controls were found similar ($p>0.05$). With regard to the CC plus CT vs. TT and CC vs. CT plus TT and alleles C and T, there was no significant odds ratios ($p>0.05$). With regard to the P2P2 plus P1P2 vs. P1P1 and P2P2 vs. P1P2 plus P1P1 and alleles P2 and P1, there was no significant odds ratios ($p>0.05$).

DISCUSSION

In this case-control study including 159 young women with primary dysmenorrhea and 135 healthy controls, we assessed the MTHFR gene C677T and IL-4 gene intron 3 VNTR polymorphisms. Overall, selected clinical parameters including age, age at menarche, parity, quality of life score, family history of dysmenorrhea, marital status, drug use for pain relief, smoking were found comparable with regard to the genotypes of both MTHFR gene C677T and IL-4 gene intron 3 VNTR polymorphisms in young women with primary dysmenorrhea. Overall, in patients with primary dysmenorrhea, pain reduced quality of life in all genotypes of studied polymorphisms. In patients with primary dysmenorrhea, we found no meaningful increase in the genotype and allele frequencies of intron 3 VNTR

Table 2: Genotype and allele frequencies of MTHFR gene C677T and IL-4 gene intron 3 VNTR polymorphisms in primary dysmenorrhea patients and healthy controls

	Primary dysmenorrhea (n=159)	Controls (n=135)	Significance	OR (CI 95%)
<i>MTHFR Gene C677T</i>				
Genotypes				
CC	79 (49.7)	78 (57.8)	0.191	
CT	64 (40.3)	50 (37.0)		
TT	16 (10.1)	7 (5.2)		
CC plus CT: TT	143 (89.9): 16 (10.1)	128 (94.8): 7 (5.2)	0.126	2.0 (0.8-5.5)
CC: CT plus TT	79 (49.7): 80 (50.3)	78 (57.8): 57 (42.2)	0.168	1.4 (0.9-2.2)
Alleles				
C	222(69.8)	206 (76.3)	0.079	1.4 (0.9-2.0)
T	96 (30.2)	64 (23.7)		
<i>IL-4 Gene Intron 3 VNTR</i>				
Genotypes				
P2P2	124 (78.0)	99 (73.3)	0.590	
P1P2	31 (19.5)	33 (24.4)		
P1P1	4 (2.5)	3 (2.2)		
P2P2 plus P1P2: P1P1	155 (97.5): 4 (2.5)	132 (97.8): 3 (2.2)	0.886	1.1 (0.2-6.2)
P2P2: P1P2 plus P1P1	124 (78.0): 35 (22.0)	99 (73.3): 36 (26.7)	0.357	0.8 (0.4-1.3)
Alleles				
P2	279 (87.7)	231 (85.6)	0.440	0.8 (0.5-1.3)
P1	39 (12.3)	39 (14.4)		

Data were presented as number and percentage and analyzed by Chi-square and odds ratio (OR) tests.

MTHFR, methylene tetrahydrofolate reductase; IL-4, interleukin-4; OR, odds ratio; CI, confidence interval.

polymorphism in the IL-4 gene and C677T polymorphism in MTHFR gene compared to those of healthy controls. We think that these results need to be confirmed because the limitations of this study including small number of cases with primary dysmenorrhea.

Primary dysmenorrhea is the most common gynecological condition among women of reproductive age (Iacovides et al. 2013). A strong effect of family history of dysmenorrhea and risk of dysmenorrhea was shown in previous studies, which is in line with some previous studies reporting a similar association, suggesting genetic susceptibility to dysmenorrhea among women with variant genotypes in a number of metabolic gene polymorphisms (Ju et al. 2014). Several risk factors are associated with more severe episodes of dysmenorrhea such as earlier age at menarche, long menstrual periods, obesity, lower socioeconomic status, smoking, and alcohol consumption. Primary dysmenorrhea usually begins in adolescence after the establishment of ovulatory cycles. In primary dysmenorrhea, pain is caused by myometrial activity resulting from uterine ischemia modulated by endometrial prostaglandins inducing smooth muscle contraction of underlying myometrium and multiple other factors altering perception and the severity of the pain. In a recent study, Tu et al. (2013) investigated structural changes in patients with magnetic resonance imaging in women with dysmenorrhea. They suggested that with the early onset of dysmenorrhea, structural alterations in brain may lead to a cumulative maladaptive response that predisposes to other clinical pain conditions.

About one third of patients describe their pain as severe; however, there is a discrepancy between the burden of primary dysmenorrhea and seeking medical treatment for this condition. There is a linear association between the severity of pain and disability caused by dysmenorrhea. In accordance with the severity of dysmenorrhea, frequently accompanying symptoms include diarrhea, nausea and vomiting, fatigue, light-headedness, headache, and dizziness. Dysmenorrhea has been reported to affect the ability of women to carry out daily activities. In addition, Iacovides et al. (2013) demonstrated that women with dysmenorrhea had a significant reduction in quality of life when they had severe menstrual pain compared with their own pain-free follicular phase and compared with

controls during menstruation. There is no laboratory and imaging studies providing specific information for the establishment of diagnosis of primary dysmenorrhea (www.sogc.org).

MTHFR is a central enzyme regulating the metabolic function of folate that plays a pivotal role in DNA synthesis, repair, and methylation. A common C677T polymorphism in MTHFR results in reduced enzymatic activity and increases the risk for the development of cardiovascular disease, Alzheimer's disease, and depression in adults, and of neural tube defects in the fetus. The mutation also confers protection for certain types of cancers (Trimmer 2013). This common polymorphism is also associated with hyperhomocysteinemia that has been reported to be an increased risk factor for neural tube defects and cardiovascular disease (Thomas and Fenech 2008). Gene-nutrient/environmental and gene-racial/ethnic interactions have been shown to affect the impact of MTHFR genetic variants (Toffoli 2008). The role of MTHFR C677T polymorphism has been investigated in several gynecologic and obstetric disorders such as cervical cancer (Wu 2013), ovarian cancer (Ma 2013), menopause (Li et al. 2010), polycystic ovarian syndrome (Radaković and Goldstajn 2007), preeclampsia (Wang et al. 2013), and recurrent pregnancy loss (Makino et al. 2004).

Previous studies have established that local immune-inflammation in the endometrium plays a key role in the regulation of menstruation (Ponnampalam et al. 2004; Ponnampalam 2006; Talbi et al. 2006). The protein encoded by IL-4 gene is a pleiotropic cytokine produced by activated T cells (www.ncbi.nlm.nih.gov/gene/3565). Actions of IL-4, a typical Th2 cytokine, on several non-immune cells have been reported (Chomarat and Banchereau 1997). In addition, actions of IL-4 have been reported in endometrial tissues as stimulating the proliferation of endometrial stromal cells (Ou Yang 2008). Urata et al. (2013) demonstrated that IL-4 in combination with prostaglandin E2 may enhance estrogen production in endometrial tissues, implying an elaborate mechanism that Th2 immune response augments inflammation-dependent progression of the disease. Ma et al. (2013) demonstrated that genes encoding pro-inflammatory cytokines (IL1B, TNF, IL6, and IL8) were up-regulated and genes encoding TGF- β superfamily members (BMP4, BMP6, GDF5, GDF11, LEFTY2, NODAL, and MSTN) were down-regulated in the peripheral

blood mononuclear cells during menstruation. They noted that the expression pattern of cytokine genes revealed dysregulated inflammation responses related to the extensive down-regulation of TGF- β superfamily members related to anti-inflammatory responses but the up-regulation of genes coding for pro-inflammatory cytokines in young women with primary dysmenorrhea.

A growing body of in vitro and clinical evidence suggests that gene polymorphisms may be an important factor in the physiological derangement in several disorders including obstetric and gynecologic conditions. In this study, the researchers found that the distributions of genotype and allele frequencies of MTHFR gene C677T and IL-4 gene intron 3 VNTR polymorphisms were similar in the primary dysmenorrhea patients and healthy controls. These results suggest that intron 3 VNTR polymorphism in the IL-4 gene and C677T polymorphism in MTHFR gene are not significant risk factors increasing susceptibility to primary dysmenorrhea.

CONCLUSION

In conclusion, there is no relation between primary dysmenorrhea and MTHFR, IL-4 genes but further randomized controlled studies about this possible relation are surely needed.

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