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Common Methylenetetrahydrofolate Reductase Polymorphisms (A1298C & C677T) in Ectopic Trophoblasts and Methotrexate Treatment Failure in Tubal Pregnancies

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ABSTRACT

Objectives: The success rate of methotrexate (MTX) therapy varies among tubal ectopic pregnancies. Common methylenetetrahydrofolate reductase (MTHFR) polymorphisms (C677T&A1298C) have been suggested to alter MTX effect. This study aimed to assess and compare MTX treatment failure rates with respect to MTHFR polymorphisms in trophoblasts of ectopic tubal pregnancies.

Material and methods: A retrospective chart review of tubal ectopic pregnancies was conducted and 34 eligible cases were found. Paraffinized blocks of ectopic trophoblastic tissues were retrieved from the archives of pathology department. Common MTHFR polymorphisms were studied on microdissected trophoblastic tissues. Sixteen cases with history of failed MTX therapy (study group) and 18 control cases were compared for their pertinent clinical characteristics and common MTHFR polymorphisms (C677T&A1298) data.

Results: In the study group, there were 8 (50%) C677T single nucleotide polymorphisms (SNP) and 9 (56.7%) A1298C SNP. Polymorphism rates were not found to be different between two groups for neither polymorphism ($p > 0.05$ for both). Number of compound heterozygotes was 3 (18.7%) in study group and 5 (27.7%) in controls ($p = 0.693$). In addition, MTHFR polymorphism presence seemed to have no effect on interval serum β-hCG concentration change in MTX-fail group ($p=0.693$).

Conclusions: Our data implied that common MTHFR polymorphisms of ectopic trophoblastic tissue are not associated with MTX failure in patients with tubal pregnancies. Additionally, serum β-hCG concentration changes caused by MTX treatment and studied MTHFR polymorphisms are likely independent.

Key words: tubal pregnancy, methotrexate, methylenetetrahydrofolate reductase, polymorphism, trophoblast

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INTRODUCTION

Ectopic pregnancy is a significant health problem. Although it is difficult to estimate, its incidence is about 1.5% in all pregnancies and approximately 0.3 maternal deaths occur per 100.000 live births [1, 2]. Increased risk for other ectopic pregnancies and fallopian tube damage are common morbidities of this condition. Early diagnosis reduces its morbidity and mortality.

Medical treatment with methotrexate (MTX) is a suitable alternative to surgery in many cases. MTX acts as a folate antagonist and shows its anti-mitotic activity primarily in tissues with high proliferative capacity such as chorionic villi. It binds to dihydrofolate reductase enzyme irreversibly and inhibits the formation of reduced folate. Methylene-tetrahydrofolate reductase (MTHFR) is a well-studied enzyme with a specific function of converting

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5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate in homocysteine and methionine metabolism [3]. Since MTHFR involves in folate metabolism and MTX acts as a folate antagonist, efficacy of MTX in patients with MTHFR gene polymorphisms has been an area of interest [4, 5]. In this regard, certain polymorphisms in this gene have been shown to alter MTHFR enzyme's catalytic activity. Namely, A1298C and C677T polymorphisms are common single nucleotide changes in the MTHFR gene at position 1298 from adenine to cytosine and at position 677 from cytosine to thymine, respectively. These changes, especially homozygous alterations at C677T (TT genotype) have been shown to decrease its functional capacity [6]. Thus, there have been many studies on MTHFR gene polymorphisms and their association with several health conditions ranging from rheumatoid arthritis to cancers [5, 7-10].

Although it is widely used in the treatment of ectopic pregnancy, clinical response to MTX may vary even in carefully selected cases. Therefore, we aimed to investigate the possible association between common MTHFR polymorphisms (A1298 and C677T) and MTX treatment failure in patients with tubal pregnancies. Recently, this relation was not found on the blood samples of tubal ectopic pregnancies that have been treated with MTX [11]. In the present study, we focused on trophoblasts, rather than maternal blood samples in order to delineate possible fetal polymorphism effect.

MATERIAL AND METHODS

Patients

This was a retrospective study conducted at Adnan Menderes University Hospital, Aydin- Turkey. Local ethics committee approval was obtained for the study. Medical records of patients who underwent surgery for tubal ectopic pregnancy in the last three years (from March 2014 to March 2017) have been screened. Charts of 40 cases were retrieved. These patients were divided into two groups based on their history of MTX treatment. Patients who had received MTX treatment but underwent surgery later for incomplete response were included into "study group". Since it was not possible to obtain tubal samples from patients who had been successfully treated with MTX, surgically treated tubal ectopic pregnancies without prior MTX exposure were chosen as control group. Inclusion criteria for the study group were failed MTX treatment (either less than 15% drop of serum β-hCG levels between the 4^{th} and 7^{th} day of MTX protocol or clinical/laboratory signs of tubal rupture) and having surgical treatment afterwards. Control group comprised of cases that have not matched the following MTX treatment criteria, therefore underwent surgery for treatment without MTX trial: serum β-hCG level < 5.000 mIU/mL, adnexal mass diameter less than 35 mm measured by transvaginal ultrasound, hemodynamic stability, no history of hypersensitivity to MTX, no history of renal, liver, pulmonary or gastrointestinal disorders, normal laboratory values (no leucopenia or pancytopenia, normal transaminase, blood urea nitrogen and creatinine levels) [12]. Exclusion criteria for both groups were cesarean scar pregnancy, interstitial ectopic pregnancy and cervical pregnancy. All patients in the study group had single dose MTX protocol (50 mg/m^2, intramuscular).

Microdissection of Tubal Samples

Hematoxylin and eosin (H&E) stained slides of tubal ectopic pregnancies were reviewed and areas of interest with chorionic villi were marked via fine-point permanent marker under light microscope (Olympus BX53, Olympus Co., Tokyo, Japan). Formalin fixed-paraffin embedded (FFPE) tissue blocks those belong to the marked slides were retrieved from the archives of pathology department. They were held in -20^0 C for 20 minutes and 6 µm thin sections were obtained via microtome (Thermo Shandon HM 430 Sliding Microtome, Thermo Fisher Scientific Inc., MA, USA). Wrinkling of the sections during microtome cutting were straightened in 45^0C water bath for 5 minutes and placed on separate slides. After processing in xylene and ethanol series for dewaxination and dehydration, slides were air dried. By matching these deparaffinized sections with previously marked H&E stained sections, areas of interest having chorionic villi were re-located. These regions were then carefully dissected under the light microscope at X40 magnification by means of pointed surgical blade [13]. Micro-dissected tissue samples were transferred and into separate microcentrifuge tubes (Eppendorf 1.5 mL microcentrifuge tubes, Merck KGaA, Darmstadt, Germany).

DNA Extraction and SNP Genotyping

DNA extraction from FFPE samples was achieved with a commercially available kit according to the manufacturer's protocol user instructions (NucleoSpin DNA FFPE XS, MACHEREY-NAGEL GmbH&Co KG, Germany). Each sample was diluted in 100 µl distilled water. Following this procedure, single point mutation analyses for MTHFR C677T and A1298C were performed via SNP biotech Real-time PCR kit (SNP Biotechnology Research, Development and Production Ltd., Co., Turkey). Briefly, 20 µl wild-type (normal) and 20 µl mutant master-mix placed into individual tubes. Then 5µl of extracted DNA was added to each tube and Real-time PCR (QPCR) process was started. Conditions were; 95^0C for 10 min, at 95^0C for the Hot Start for 15 seconds, 60^0C for 1 minute and 30 cycles (Thermal Cycler CFX96 Real-Time PCR equipment, BioRad, USA). HEX dye was used as controller. Assessments were then done by using carboxyfluorescein (FAM) dye labeled MTHFR 677 and 1298 polymorphism probes.

Statistics

Statistical analysis was performed using SPSS (Statistical Package for the Social sciences) version 18 for Windows (SPSS Inc., Chicago, IL). The Kolmogorov-Smirnov test was used to assess the normality of numeric variables. For the numeric variables that were normally distributed, comparison between two groups was made by independent sample t test and descriptive statistics are presented as mean ± standard deviation. For the numeric variables that were not normally distributed, comparison between two groups was made by Mann–Whitney U test and descriptive statistics are presented as median (interquartile range). To analyze the categorical data, a chi-square test was used, and descriptive statistics are presented as frequency (%). The p values below 0.05 were considered statistically significant.

RESULTS

Out of 40 cases that were retrieved from the medical records, two of them were excluded for cesarean scar pregnancy and one for interstitial pregnancy. Three cases were excluded for missing pertinent medical information. A total of 34 cases were left for final analysis. Among them, 16 (47%) cases had surgical intervention due to failed MTX treatment (study group). Remaining 18 (53%) cases had surgery without prior MTX treatment (control group).

Table 1. Clinic and demographic data comparison

	Study Group (MTX-Fail) (n = 16)	Controls (n = 18)	p
Age [years]	30.5 ± 5.2	32.7 ± 5.8	0.266
Parity*	1 (0-3)	1 (2)	0.495
BMI	25.7 ± 5.9	29.1 ± 8.4	0.320
Previous ectopic pregnancy, n [%]	4 (25)	2 (13.3)	0.654
Previous cesarean, n [%]	7 (56.2)	4 (43.8)	1.0
Previous endometrial curettage, n [%]	2 (12.5)	5 (33.3)	0.220
Gestational Age [days]	43.3 ± 9.1	49.2 ± 7.0	0.092
MTX to surgery interval [days]	10.0 ± 5.1	N/A	N/A
Change in β-hCG concentration after MTX, [mIU/mL]**			
C677T, wild type	603.5 (111.7–1281.0)	N/A	0.645
C677T, mutant	180.0 (-151.2–2491.2)		1.0
A1298C, wild type	679.0 (1.0–827.0)		
A1298C, mutant	299.0 (37.0–1615.5)		
Hemoglobin level at hospitalization [g/dl]	12.1 ± 1.2	12.0 ±1.8	0.847

BMI — body mass index; MTX — methotrexate; N/A — not available; * data is given as median and interquartile range; **data is given as median and 25th –75th percentile

Clinical characteristics and demographic data of study and control group were presented in Table 1.

MTHFR genotypes C677T and A1298C were identified in all DNA samples extracted from FFPE sections of micro-dissected tubal ectopic chorionic villi. All results including genotype and allele frequencies were shown in tables with their corresponding p values (Tab. 2 and 3).

When polymorphisms were compared between study and control groups, no significant differences were found ($p > 0.05$). In this comparison, both homozygous and heterozygous genotypes were taken into account. There were 2 homozygote A1298C polymorphism (CC) carriers in the study group and 4 in the control group. C677T homozygosity (TT) was seen in three cases in study group, and in two cases in control group. As for compound heterozygosity (having both polymorphisms), there were three (18.7%) cases in the study group and 5 (27.7%) cases among controls. Number of patients who were compound heterozygotes were not different between groups either ($p = 0.693$).

Table 2. Comparison of allele frequencies between groups

MTHFR C677T	Study Group n [%]	Controls n [%]	Total	p
C	21 (65.62)	24 (66.66)	45 (66.17)	
T	11 (34.37)	12 (33.33)	23 (33.82)	1.000
Total	32 (100)	36 (100)	68 (100)	
MTHFR A1298C				**p**
A	21 (65.62)	21 (58.33)	42 (61.76)	
C	11 (34.37)	15 (41.66)	26 (38.23)	0.961
Total	32 (100)	36 (100)	68 (100)	

MTHFR — Methylenetetrahydrofolate reductase

Table 3. MTHFR genotype frequency comparison between groups

MTHFR C677T	Study Group n [%]	Control Group n [%]	p
Wild Type (CC)	8 (50)	8 (44.44)	
Heterozygote (CT)	5 (31.25)	8 (44.44)	0.538
Homozygote (TT)	3 (18.75)	2 (11.11)	
Total	16 (100)	18 (100)	
MTHFR A1298C			
Wild type (AA)	7 (43.75)	7 (38.88)	
Heterozygote (AC)	7(43.75)	7 (38.88)	0.731
Homozygote (CC)	2 (12.5)	4 (22.22)	
Total	16 (100)	18 (100)	
COMPOUND HETEROZYGOTES			
C677T /A1298C	3 (18.75)	5 (27.77)	0.693

MTHFR — Methylenetetrahydrofolate reductase

The differences between two β-hCG levels (on the day of MTX was administered and on the operation day) with respect to polymorphism statuses were measured in the study group to further evaluate MTX treatment and polymorphism relations. No significant differences were observed in β-hCG concentration change between patients with and without polymorphisms (p = 1.0 for A1298C and p = 0.645 for C677T, Tab. 1).

DISCUSSION

Ectopic pregnancy is still a common health problem with limited treatment options. MTX is the main therapeutic agent that is currently used when medical treatment is an option. It has an acceptable rate of success, especially in the early phases of trophoblast invasion. However, failures with MTX treatment are not rare [14]. The established MTX treatment indication criteria in tubal ectopic pregnancies are well defined to maximize its effectiveness and safety [12]. Also, according to a recent meta-analysis, there is no difference between single dose and multiple dose regimens in terms of success rates [15]. Therefore, it is reasonable to look for other factors such as individual genotypic differences that can influence the MTX treatment success in tubal ectopic pregnancies.

Certain single nucleotide changes in MTHFR gene have been suggested to affect clinical response to MTX in variety of clinical situations such as in rheumatoid arthritis and childhood acute lymphoblastic lymphoma [4, 5]. Limited number of studies have investigated this effect in ectopic and molar pregnancies. By systemic administration, MTX reaches trophoblastic tissue via maternal circulation and shows its antimetabolite/antiproliferative action on rapidly proliferating trophoblasts. For that reason, we worked on fetal trophoblastic tissues rather than maternal blood samples to investigate the relation between common MTHFR polymorphisms and MTX treatment failure.

The results of this study indicated that MTHFR A1298C and C677T polymorphisms are not associated with MTX treatment failure in tubal ectopic pregnancies. In a similar study by Kutuk et. al., no significant differences in MTHFR polymorphism frequencies were reported between 21 MTX-unresponsive and 88 responsive patients [11]. They also obtained paternal blood samples to infer fetal genotype and did not found any association either.

Qu et al. investigated the similar hypothesis on 62 patients with low-risk gestational trophoblastic neoplasia. They analyzed molar tissues for same MTHFR polymorphisms and concluded that C677T homozygote mutation (TT) was associated with ineffective MTX treatment in this group of patients [16]. However, another study found no association between common MTHFR polymorphisms and clinical response to MTX therapy, when they genotyped the blood and molar tissue samples of gestational trophoblastic neoplasia cases [17]. Our results in ectopic trophoblastic tissue supported this finding even though study population and dose of MTX differed from theirs.

We also compared the interval change in serum β-hCG levels (from the day of MTX administration to day of surgery) between patients with MTHFR polymorphisms and those without. There was no difference in interval β-hCG levels between groups. Naturally, this parameter was observed only in the study group, since control group had no MTX exposure. This finding also implies that MTHFR A1298C and C677T polymorphisms of fetal trophoblastic tissue have no effect on MTX treatment efficacy. However, there are studies reporting increased efficacy of MTX in the presence of these polymorphisms in rheumatoid arthritis patients [18, 19].

In terms of population's background MTHFR polymorphism frequencies, a previous large Turkish study reported the frequencies for C677T, C677C, T677T as 42%, 47%, 9%, respectively and for A1298C, A1298A, C1298C as 43%, 46%, 10%, respectively [20]. Only the frequency of homozygous C1298C SNP in our control group was found higher than the expected background frequency (22% vs. 10%).

One of the possible drawbacks of our study might be the use of manual technique rather than laser microdissection for sampling from FFPE tissues to minimize tissue contamination. Nevertheless, manual microdissection technique is a universally accepted method among pathologists for retrieving the area of interest for further molecular testing [13]. Furthermore, a previous study has genotyped MTHFR gene from maternal and paternal blood samples to infer ectopic trophoblasts' genotype and their results were similar to ours. Other disadvantage of our study was the technical and ethical impossibility of trophoblastic tissue sampling from the patients whom had successful MTX treatment. Thus, for control group, we collected the records of patients with ectopic pregnancy without MTX exposure. Nevertheless, it could be assumed that approximately 90% of the control group patients would have been successfully treated by MTX, since majority of studies have reported such high success rates in eligible cases [21–24].

For similar future MTHFR polymorphism studies in ectopic pregnancies, paternal screening along with maternal screening would be valuable. Moreover, serum folate, homocysteine and vitamin B12 level measurements in a much larger population treated and not treated with MTX might add significant implications [11, 25–27]

CONCLUSIONS

In conclusion, our results suggest that common MTHFR polymorphisms in trophoblasts and MTX treatment failure in tubal ectopic pregnancies are probably not related.

Furthermore, these polymorphisms may not affect MTX related serum β-hCG concentrations.

Conflicts of interest

The authors report no conflict of interest.

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