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Original Article *Özgün Araştırma*

ANALYSIS OF FACTOR V AND MTHFR GENES AS RISK FACTORS CONSTITUTING SUSCEPTIBILITY TO NEURAL TUBE DEFECTS: A CASE-CONTROL STUDY FROM TURKEY

Nöral Tüp Defektlerinde Duyarlılık Oluşturan Risk Faktörleri Olarak Faktör V ve MTHFR Genlerinin Analizi: Türkiye'den Bir Vaka-Kontrol Çalışması

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ABSTRACT

Objective: This study targeted to bring a molecular perspective to occult neural tube defects and thus develop future preventive personalized medicine strategies. The roles of three genetic variations namely Factor V Leiden (FVL) (rs6025), MTHFR A1298C (rs1801131), and MTHFR C677T (rs1801133) were investigated in a Turkish cohort including both the mothers and children to better analyze the potential inherited effects of these variations.

Material and Methods: Children affected with neural tube defects (NTDs) and control children without NTDs were included in the study together with their mothers. DNA extractions were performed from collected blood samples with standard salting-out procedure. Isolated DNAs were genotyped with Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (*PCR-RFLP*) method.

Results: In terms of Factor V Leiden (FVL) (rs6025) in NTD risk, there was no statistically important association between mothers with NTDs-affected children and control group mothers (p=0.639). However, a statistically significant association was observed when NTDs-affected children were compared with the ones not affected (p=0.0144). There was a statistically important association of MTHFR A1298C (rs1801131) both in mothers comparisons and comparison between NTDs-affected children and not-affected children (respectively, p=0.005; 0.008). MTHFR C677T (rs1801133) genotypes and/or alleles did not act as risk factors for NTD development neither in mothers nor in children in this study (p>0.05).

Conclusion: Our study indicates FVL mutation as an increased risk factor for NTD development, independently from the genotypes of mothers. MTHFR A1298C (rs1801131) homozygous AA genotype and A allele elevated the risk of NTD development both in mothers and children referring to the inherited role of this variation in Turkish population. However, MTHFR C677T (rs1801133) variation can not be considered as a risk factor in NTD development in our population.

***Keywords**: Neural tube defects (NTDs), genetic association, risk factors, Factor V Leiden,* 5-10-methylenetetrahydrofolate reductase *(MTHFR)*

ÖZ

Amaç: Bu çalışma konjenital olmayan nöral tüp defektlerine moleküler bir perspektif sunmayı ve geleceğe yönelik önleyici bireyselleştirilmiş tıp stratejileri geliştirmeyi hedeflemiştir. Üç genetik varyasyon; Factor V Leiden (FVL) (rs6025), MTHFR A1298C (rs1801131) ve MTHFR C677T (rs1801133) bu varyasyonların potansiyel kalıtımsal etkilerini daha iyi analiz etmek için hem anneleri hem de çocukları içeren bir Türk kohortunda araştırılmıştır.

Gereç ve Yöntemler: Nöral tüp defektli (NTD) çocuklar ve NTD'li olmayan kontrol grubu çocukları anneleriyle beraber çalışmaya dahil edilmiştir. Toplanılan kan örneklerinden standart tuzla çöktürme prosedürüyle DNA ekstraksiyonları gerçekleştirilmiştir. İzole edilen DNA'lar Polimeraz Zincir Reaksiyonu-Restriksiyon Fragment Uzunluk Polimorfizmi (PCR-RFLP) metoduyla genotiplendirilmiştir.

Bulgular: Factor V Leiden (FVL) (rs6025) mutasyonunun NTD riskini arttırması açısından NTD'li çocuğa sahip annelerle kontrol grubu anneleri arasında istatistiksel olarak önemli bir ilişki mevcut değildir (p=0.639). Ancak, NTD'li çocuklarla etkilenmemiş çocuklar karşılaştırıldığı zaman istatistiksel olarak önemli bir ilişki gözlenmiştir (p=0.0144). MTHFR A1298C (rs1801131) açısından ise hem annelerin kendi aralarındaki karşılaştırmada hem de NTD'li çocuklar ve etkilenmemiş çocuklar arasında istatistiksel olarak önemli bir ilişki mevcuttur (sırasıyla p=0.005; 0.008). MTHFR C677T (rs1801133) genotipleri ve/veya allelleri ise bu çalışmada ne annelerde ne de çocuklarda NTD gelişimi açısından risk faktörü olarak rol oynamadığı görülmüştür (p>0.05).

Sonuç: Çalışmamız, NTD gelişimi açısından FVL mutasyonunun annelerin genotiplerinden bağımsız olarak artmış bir risk faktörü olduğuna işaret etmektedir. MTHFR A1298C (rs1801131) homozigot AA genotipi ve A alleli bu varyasyonun Türk popülasyonunda kalıtımsal etkisine de vurgu yapacak şekilde hem annelerde hem de çocuklarda NTD gelişimi riskinde artışa yol açmaktadır. Ancak, MTHFR C677T (rs1801133) varyasyonu populasyonumuzda NTD gelişimi açısından bir risk faktörü olarak dikkate alınamamaktadır.

***Anahtar Kelimeler**: Nöral tüp defektleri, genetik assosiasyon, risk faktörleri, Factor V Leiden,* 5-10-methylenetetrahydrofolate reductase *(MTHFR)*

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INTRODUCTION

Neural tube defects (NTDs) are one of the severe congenital malformations with a worldwide prevalence of 1 per 1000 living birth (1,2). These defects occur by the failure of the neural tube to close nearly between the third and fourth weeks of human embryonic development and common types include anencephaly, spina bifida and encephalocele (3,4). Though the pathogenesis of NTDs seems to be quite complicated and surely involves environmental factors such as dietary conditions, the role of genetic factors can never be underestimated since a 40-fold increase in NTDs risk was shown in first-degree relatives (5). Dietary insufficiencies mainly focused on folic acid requirement since all women of childbearing age were recommended to take 400 µg folic acid per day from 4 weeks before impregnation through gestation week 12 to prevent a NTDs-affected pregnancy according to the Centers for Disease Control and Prevention, 1992 (6). Thus, in light of this early recommendation, it is not surprising that folate metabolism can act as a major genetic pathway together with some other signaling pathways such as planar cell polarity and glycometabolism (2). Besides the importance of folate metabolism pathway, another important factor in pregnancy can be blood clotting factors since the establishment and maintenance of a problem-free pregnancy is based upon a dynamic balance between coagulation and fibrinolysis (7).

5,10-methylenetetrahydrofolate reductase (MTHFR) gene is located at chromosome 1p36.3 and consists of 11 exons (8). MTHFR adjusts folate metabolism, DNA methylation, DNA synthesis, and repair and 677C>T mutation (rs1801133) of MTHFR gene leads to a defective enzyme activity of 50% less of the normal level and a common reason of increased homocysteine levels, previously identified as a venous thromboembolism (VTE) risk factor (4,9). Factor V is a blood protein forming fibrin in the clot and after enough fibrin has been made, it is inactivated by activated protein C (APC). Factor V Leiden is a mutated form of factor V which displays resistance to the action of APC and thus fibrin production can not be stopped and results with deep vein thrombosis (DVT) (10).

In light of the literature given above, the great importance of genes responsible in both folate metabolism and clotting system can never be ignored. Hence, this study targeted to bring a deep molecular perspective to offspring neural tube defects and develop preventive strategies in terms of personalized medicine in our population cohort. The roles of three genetic variations, namely Factor V Leiden (FVL) (rs6025), MTHFR A1298C (rs1801131), MTHFR C677T (rs1801133) were investigated in a Turkish cohort including both the mothers and children to better analyze the potential inherited effects of these variations.

MATERIALS AND METHODS

Study Population

The case group consisted of 65 women with NTDs-affected children and the same number of infants, investigated in the Department of Neurosurgery of Dicle University based on clinical manifestations. The control group consisted of 68 women and 68 children not affected by NTDs. The mean age of the mothers with NTDs-affected children was 36.7±5.3 and control mothers was 35.8±4.9. All samples were collected in accordance with the Declaration of Helsinki and the study was approved by the Medical Ethics Committee of Dicle University (Date: 18.03.2011; desicion no: 70).

Sample Preparation

Peripheral blood samples were collected from all participants in EDTA containing tubes and DNA isolations were performed according to the standard salting-out method of Miller et al (11). The extracted

DNA samples were stored at -20ºC for further genotyping experiments.

Genotyping

All genotyping experiments were performed with Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (*PCR-RFLP*) method as previously reported (12-14). Primer sequences were as follows: Factor V Leiden (FVL) (rs6025): F:5'-CATGAGAGACATCGCCTCTG-3'. R: 5'-GACCTAACATGTTCTAGCCAGAAG-3'; MTHFR A1298C (rs1801131): F: 5'-CTTTGG GGAGCTGAAGGACTACTAC-3'. R: 5'-CACTTTGTGACCATTCCGGTTTG-3'; MTHFR C677T (rs1801133): F: 5'-TGAAGGAGAAGGTGTCTGCGGGA-3'. R: 5'-AGGACGGTG CGGTGAGAGTG-3'. The resulting amplicons of Factor-V Leiden (FVL) (rs6025), MTHFR A1298C (rs1801131), and MTHFR C677T (rs1801133) were digested with restriction enzymes *Mnl1*, *MboII*, and *Hinf1*, respectively to enable genotypic discrimination between ancestral and mutant alleles. To control the success and consistency of repeat analysis, 20% of randomly selected samples were re-genotyped and the results yielded 100% concordance.

Detailed information of our genotyped data set is depicted in Table 1.

Statistical Analysis

GraphPad Prism was used for statistical comparisons. Differences of genotype and allele frequencies between cases and control groups were analyzed by χ^2 test with Yates' continuity correction. Statistical significance was set at $p<0.05$.

Table 1: Analyzed variants of genes

Gene symbol	Gene name	rs no	Consequence
FV	Factor V	rs6025	Missense variant
MTHFR	5,10-methylenetetrahydrofolate reductase	rs1801131	Missense variant
MTHFR	5,10-methylenetetrahydrofolate reductase	rs1801133	Missense variant

RESULTS

FVL (rs6025), MTHFR A1298C (rs1801131), MTHFR C677T (rs1801133) variations were analyzed in this study in order to evaluate the potential liability effects to occult neural tube defects in a Turkish cohort.

FVL (rs6025) mutations did not show a statistically significant association in terms of comparison between mothers with NTDs-affected children and control group mothers without NTDs-affected children while a significant association was present between NTDs-affected children and control group children, independent from the genotypes of the mothers (respectively p=0.639; 0.0144) (Table 2 and 3).

MTHFR A1298C (rs1801131) missense mutation pointed out to a more striking situation since a familial effect of this variation was also observed. The statistical analysis of mothers with and without NTDs-affected children showed a statistically important association with offering AA genotype, and A allele frequency as the risk factors for having a child with NTDs (respectively p=0.024; 0.005). The genotype comparisons of children of these case-control mothers revealed the hereditary importance of MTHFR A1298C (rs1801131) since AA genotype, and A allele were also statistically very meaningful in these NTDs-affected children (respectively p=0.013; 0.008) (Table 2 and 3).

MTHFR C677T (rs1801133) variation, on the other hand, showed no statistically important association neither in comparison between mothers nor between infants (respectively p=0.377; 0.846) (Table 2 and 3).

Table 2: The results of Factor V Leiden (FVL) (rs6025), MTHFR A1298C (rs1801131), and MTHFR C677T (rs1801133) variations in mothers with NTDs-affected children and control group mothers without NTDs-affected children

Genotypes		Mothers with NTDs-affected children N=65	Control mothers without NTDs-affected children N=68	χ^2	p	Allele frequencies	Cases	Controls	χ^2	p
MTHFR rs1801133	CC	48	54	0.054	0.812	C	111	122	0.779	0.377
	CT	15	14			T	19	14		
	TT	2	0							
MTHFR rs1801131	AA	37	54	5.111	0.024	A	99	122	7.748	0.005
	AC	25	14			C	31	14		
	CC	3	0							
Factor V Leiden rs6025	CC	61	66	0.225	0.635	C	126	134	0.220	0.639
	CT	4	2			T	4	2		
	TT	0	0							

Table 3: The results of Factor V Leiden (FVL) (rs6025), MTHFR A1298C (rs1801131), and MTHFR C677T (rs1801133) variations in children with NTDs-affected and control group children without NTDs-affected

Genotypes		NTDs-affected children N=65	Control children not affected with NTDs N=68	χ^2	p	Allele frequencies	Cases	Controls	χ^2	p
MTHFR rs1801133	CC	47	48	0.007	0.932	C	112	115	0.038	0.846
	CT	18	19			T	18	21		
	TT	0	1							
MTHFR rs1801131	AA	35	53	8.619	0.013	A	96	119	7.139	0.008
	AC	26	13			C	34	17		
	CC	4	2							
Faktör V Leiden rs6025	CC	56	64	1.038	0.308	C	120	132	2.132	0.014
	CT	8	4			T	10	4		
	TT	1	0							

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DISCUSSION

The association between FVL and the risk of venous thrombosis has already been established (15). This well-known fact was replicated in some other studies, among them a very recent one comprising a very large French cohort (3719 patients and 4086 controls) also documented this fact by indicating that Q534 allele was ~3-fold more frequent in cases than in controls (16). There are some studies in literature related to the potential effects of FVL (rs6025) in pregnancy including preeclampsia, spontaneous abortions, thrombophilia but except only one study, it has not been an investigation issue for neural tube defects. Yalcintepe et al. investigated thrombophilic gene polymorphisms in spontaneous abortions in Turkish population, but the effect of FVL was not significant in aborted materials (17). On the other hand, a previous study indicated the role of FVL genotypes in recurrent pregnancy loss (18). Very recently, a high prevalence of FVL variation in preeclamptic patients (9.6%) compared to controls (0.6%) in Sudanese women was shown by Ahmed et al (19). All the same, this value is still less according to the data of a meta-analysis which showed that FVL increased the risk of preeclampsia by almost 50% (20). FVL was not shown to be associated with pregnancy loss neither in Bosnian nor in Norwegian women (7,9). However, the meta-analysis results of a genetic association study comprising a very large mother/infant pairs (n=6755) indicated that though there was no association between FVL and fetal growth restriction, a possible association seemed to be possible with pre-eclampsia despite the values did not reach to statistical significance (20). There was no association of FVL with pre-eclampsia or recurrent pregnancy loss in Sinhalese women (21). It must be taken into account that the prevalence of thrombophilic polymorphisms can display ethnic differences and FVL allele was reported to be significantly more frequent in patients of Middle Eastern background compared to those of Northern European and Asian ethnicity (22). Heterozygous genotype of FVL was shown as a risk factor for the development of intraventricular hemorrhage (23). Besides the data given above that aim to analyze the potential importance of FVL in pregnancy-associated hazardous situations, to the best of our knowledge, there has been no study evaluating the effect of FVL in neural tube defects until to the study of Aydin et al. who reported a significantly high frequency of FVL in NTD group compared to control group (24). Their study also broadened the general agreement of the importance of folate supplementation to a deeper perspective by emphasizing that folate supplementation alone was not sufficient to prevent NTDs and the role of vitamin B12 levels was also important in terms of lowering NTD risk. In our study which is the second one in literature evaluating the role of FVL in NTD risk, there was no statistically important association between mothers with NTDs-affected children and control group mothers (p=0.639). However, a statistically important association was observed when NTDs-affected children were compared with the ones not affected (p=0.0144). Thus, our study also offers FVL mutation as an increased risk factor for NTD development, independently from the genotypes of mothers.

Important candidate genes in terms of constituting susceptibility to NTD development especially focus on MTHFR variations. Though some different kinds of MTHFR variations were also analyzed in literature, most refer to the values of the two types (MTHFR rs1801131 and MTHFRrs1801133) which were also targeted variations in this study. In our study, the association of MTHFR rs1801131 was statistically important both in mothers' comparisons, and comparison between NTDs-affected children and not-affected children referring to the inherited role of this variation. MTHFR rs1801131 homozygous AA genotype and A allele elevated the risk of NTD development. However, MTHFR rs1801133 genotypes and/or alleles did not constitute a risk factor for NTD

development neither in mothers nor in children in our study. In a Chinese population cohort, MTHFR C677T (rs1801133) homozygous TT was shown to be associated with an elevated risk of NTD, though a significant difference was not observed in terms of MTHFR rs1801131 variation. A gene-gene interaction effect was also emphasized in the same study which demonstrated that mothers carrying both TT genotype of MTHFR rs1801133 and CC genotype of COMT rs737865 displayed more than 2-fold increase in fetal NTD risk (3). MTHFR rs1801133 variation was shown as a potential risk factor previously in another Chinese cohort and again in a very recent study comprising of Chinese population in which mothers having TT and CT genotypes were shown to be more prone to have an NTD-affected offspring (2,25). The study of Fang et al. also suggested MTHFR rs1801133 variation is a risk factor with a dominant effect of TT genotype in the Han population of Northern China. MTHFR rs1801133 T allele was shown as a risk allele for NTD while a significant association for rs1801131 was not found in Eastern India population (1,4). On the other hand, in a study conducted in Northeast India, MTHFR rs1801131 CC genotype was reported to increase risk in relation to anterior encephalocele susceptibility (26). In a case-control study conducted on mothers and/or infants in USA, MTHFR rs1801131 and MTHFR rs1801133 heterozygous genotypes were found in the high folate intake group compared to homozygous mothers (27). In coherence with our results, MTHFR rs1801133 variation was not a major risk factor in terms of neural tube defects in USA population (28). Though our results in terms of FVL mutation evaluation was consistent with another Turkish population study, these researchers' data did not indicate the role of MTHFR rs1801131 and rs1801133 variations in NTD group in discordance with our results (24). Even this situation indicates the importance of not only conducting genome association studies in different populations but also performing replication studies.

In conclusion, we put forward the potential role of FVL (rs6025) in terms of NTD development in children and this is the second study in literature in this field. It is clear that the association of MTHFR genes with NTD susceptibility is controversial in different populations worldwide and more studies conducted in different populations are definitely required to draw a population-based risk factor analysis in terms of MTHFR genes. In our study based on Turkish population, neither MTHFR rs1801133 genotypes nor alleles were susceptibility factors for NTD development. However, MTHFR rs1801131 was a maternal risk factor; mothers having AA genotype and carrying A allele had a higher probability to have NTD-affected offspring. The comparison between NTDs-affected children and control children not affected with NTDs also highlighted the striking effect of AA genotype and A allele. We recommend further investigations with larger sample sizes in different populations to get a deeper insight into the etiology of NTDs. One other recommendation of our research team for future researchers could be extending the dimensions of this study by also adding paternal genotypes via conducting trio studies, and thus a more comprehensive evaluation based not only on mother's genes but also paternal risk factors can be elucidated.

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