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Association of c.1298A>C Variation in Methylenetetrahydrofolate reductase Gene with Neural Tube Defects: Case-Control study in Syrian population

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Abstract: Neural tube defects (NTDs) are the most common multifactorial congenital disorders worldwide. Some genetic variations in *Methylenetetrahydrofolate reductase* (*MTHFR)* gene were shown to be associated with NTDs. In this case-control study, we aimed to investigate the effect of *c.1298A>C* variation on NTDs risk in a cohort of Syrian population. The study consisted of 262 individuals distributed into two groups; the first group involved 72 isolated NTDs-cases and 86 healthy controls, the second group included 30 mothers of NTDs-offspring as cases and 74 mothers with no previous family history of NTDs or any other birth defects, as controls. No significant association was found between the *c.1298A>C* variant and the risk of NTDs. However, a significant association was found in both over-dominant and heterozygous co-dominant genetic models, in maternal *c.1298A>C* variation suggesting a risk factor for having NTDs by more than 2-fold. Furthermore, our data point out to a possible interaction between maternal-fetal genotypes and nutritional factors in inducing NTDs. Our findings strongly emphasize the leading role of maternal genotype in determining the pregnancy outcomes. Awareness is needed in our society to the importance of adequate diet and taking nutritional supplements before and during pregnancy. This is the first study on NTDs and their etiology in Syria, further studies are needed.

Key words: Folate, Genetic Variations, Hardy-Weinberg equilibrium (*HWE*), *Methylenetetrahydrofolate reductase* gene, Multifactorial disorders, Neural tube defects (NTDs).

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Introduction:

Neural tube defects (NTDs) are a group of most common congenital malformations (1) that affect the central nervous system and axial skeleton (2). NTDs are complex multifactorial disorders, both environmental and genetic factors contribute to their occurrence (3). Suitable consumption of periconceptional folic acid can reduce 50-70% of NTDs risk (4). The *Methylenetetrahydrofolate reductase* (*MTHFR*) gene is one of the most considered candidate genes that play in NTDs etiology due to its vital role in folate metabolism (5). Several case-control studies have found an association between some polymorphic variants such as *c.677 C>T* (p.Ala222Val) and *c.1298 A>C* (p.Glu429Ala) in the *MTHFR* gene and NTDs (6, 7). In Syria, no previous studies were carried out on NTDs and their causes, therefore our aims was to investigate the possible association of the *c.1298 A>C* variation at *MTHFR* gene with NTDs risk in a cohort of Syrian population.

Material and Methods:

The sample study was divided into two groups, First group involved: 72

isolated NTD-cases staying at Children's University Hospital in Damascus city for treatment. NTDs-cases with chromosomal aberrations or any other birth defects were considered as exclusion criteria. In addition, 86 randomly selected healthy individuals with no family history, participated as control group. Detailed information about each participant was filled in a separate file, included: type of NTDs, with/without family history, and clinical symptoms. The Second group included: 30 mothers of NTDs-affected children served as cases. The controls consisted of 74 mothers, who have previously given birth for at least two healthy children, and have no family history of NTDs or other birth defects in their families. Simple questionnaires were accomplished for each participant including parental consanguinity, maternal diet type (vegetarian / non-vegetarian / both), intake of vitamin supplements before or during pregnancy, maternal diseases and drugs intake if any. All cases and controls were informed about the study, and a written consent was taken from all patients' families and participants. All participants recruited were Syrians, and from different governorates. This study was conducted from August 2015 until August 2016, and has been approved by the ethical committee of Damascus University-Syria.

Serum Folate concentration analysis:

Peripheral blood samples from mothers of the second group were collected in clot activator vacutainer tubes after at least 4 hours of fasting. Folate concentration analysis was done by *folateIII, ROCHE* kit using *Hitachi Elecsys 2010, Roche* analyzer according to the manufacturer's protocol.

Genomic DNA isolation:

Blood samples were collected in EDTA vacutainer tubes. Genomic DNA was isolated from peripheral blood using *Vivantis Technologies, GF-1, Blood DNA Extraction Kit*, as instructed by the manufacturer.

***PCR-RFLP* analysis:**

Amplification of a segment around the *c.1298A>C* variant was performed for all cases and controls, using two pairs of primers (8) to amplify a 163bp fragment (Table 1). All PCR reactions (25 µl final volume) contained 12.5 µl of Dream Taq PCR Master Mix (2X) (Thermo Scientific), 10µM of each of primer, 6.5 µl of nuclease-free water, and 3 µl of genomic DNA. Each PCR experiment contained a negative control (nuclease-free water) for contamination detection. PCR reactions were done using Eppendorf Master Cycler. The amplified products were electrophoresed in 2% agarose gel containing ethidium bromide, visualized and photographed using a UV transilluminator (Olympus).

Table (1): PCR conditions for amplification of *c.1298A>C* variant in the *MTHFR* gene

Initial denaturation	95° C for 3 minutes
35 cycles	95° C for 60 sec
	63 °C for 85 sec
	72 °C for 30 sec
Elongation cycle	72 °C for 7 minutes
Final hold	4 °C

PCR-RFLP method: The amplified PCR fragments of 163 bp were digested with *MboII* restriction enzyme (Thermo Scientific, 1500U) according to manufacturer's instructions. Fragments were analyzed by 4 % ultra-agarose gel electrophoresis (UltraPure™ Agarous, Invitrogen, California, United States), using 50 bp DNA Ladder (Thermo Scientific, GeneRuler).

Statistical analysis:

All statistical analyses were performed using MedCalc® (Version 14.8.1) and SPSS 16.0 softwares. Chi-square (χ^2) and Independent Samples *t-test* were used as main tests. The standard *P*-value ≤ 0.05 was considered statistically significant.

Results:

The first group aged (2 days -7 years), comprised of 72 isolated NTDs-cases; 68 Spina bifida aperta cases and only 4 cases with Encephalocele. The control group consisted of 86 healthy individuals aged (1 day to 33 years). The Second group contained 30 mothers of NTDs-cases, aged (16 to 55 years), and 74 control mothers ages from 16 to 46 years old (Table 2).

Table (2): Characteristics of study groups.

First group							
NTDs-Cases	**Type of NTDs n (%)**		Count	**Female: male ratio**	**Controls**	Count	**Female: male ratio**
	Spina Bifida	**Encephalocele**					
Females	37(51.38%)	4 (5.55%)	41	1.3	Females	55	1.77
Males	31(43.05%)	-	31	0.7	Males	31	0.56
Total	68(94.44%)	4 (5.55%)	**72**		*Total*	**86**	
Second group							
Mothers of NTDs-Cases	Type of NTDs in offspring n (%)		Count **30**		**Control Mothers**	Count **74**	
	Spina Bifida	**Anencephaly**					
	28 (93.33 %)	2 (6.66 %)			-		
§Mean age ± SD	27.73 ± 8.3				28.85± 6.97		

§SD: Standard Deviation

PCR amplification of a fragment around the *c.1298A>C* variant yielded the expected size 163 bp (Figure 1). Enzyme digestion showed different restriction patterns compatible with different genotypes.

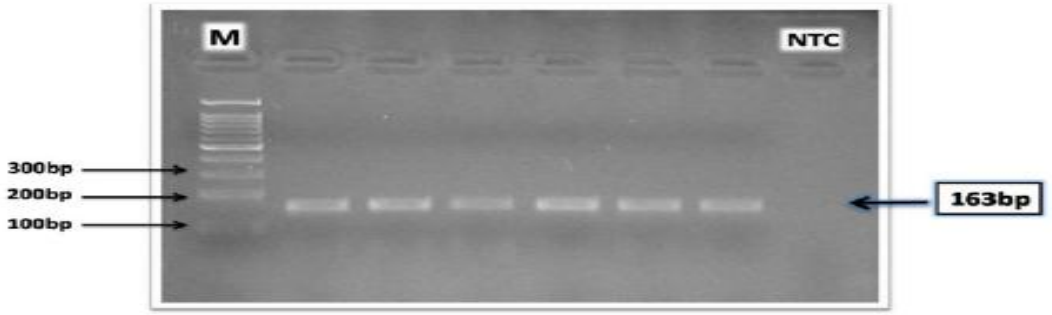

Figure (1): Agarose gel electrophoresis of the PCR products showing a single PCR fragment of 163 bp. M, molecular marker (100 bp). NTC: negative control for contamination detection.

Genotype frequencies at *MTHFR c.1298A>C* in NTDs-patients and in controls deviated from *HWE* as (*P*-values=0.007, 0.004) respectively, but no significant differences were found in the *C* allele frequencies (*P*=0.4178) (OR: 0.8182, 95% CI: 0.5035-1.3295). On the other hand, distribution of genotypes in the second group has revealed deviation from *HWE* (*P*=0.017) in mothers of NTDs-cases, while no deviation was found in control mothers (*P*=0.935). No significant differences were found in the *C* allele frequency (*P*=0.4371) (OR: 1.2838, 95% CI: 0.6837-2.4104) (Table 3 and 4). No significant association was found in any genetic models amongst cases and controls of the first group for *MTHFR c.1298A>C*, While it was significant at over-dominant and heterozygous co-dominant genetic models amongst cases and controls of the second group (Table 4).

Maternal serum Folate concentration analysis was only possible for 20 mothers of NTDs affected offspring and 58 of the controls. The average concentration was approximate for mothers of NTDs and controls (11.97 ± 4.64 ng/ml vs. 12.01 ± 3.95 ng/ml) respectively. However, no significant relation was found between maternal serum folate concentration and the risk of NTDs (*P-value* = 0.971). A significance difference in dietary supplementation intake between individuals of the second group was found (*P* <0.0001); as 91% of the controls had taken dietary supplementation sometime during their pregnancies, while 45% only of NTDs'mothers had received any of it.

Table (3): *MTHFR* c.1298A>C Genotypes and alleles frequencies in sample study groups

Study Groups		Genotypes frequency (%)			HWE	2n	Alleles (%)	
		AA	AC	CC	*P*-value		A	C
First Group	NTDs-Patients	33(45.83)	38(52.77)	1(1.38)	***0.007**	144	104(72.22)	40(27.77)
	Controls	34(39.53)	49(56.97)	3(3.48)	***0.004**	172	117(68.02)	55(31.97)
Second Group	NTDs'Mothers	9(30)	20(66.66)	1(3.33)	***0.017**	60	38(63.33)	22(36.66)
	Control Mothers	35(47.29)	32(43.24)	7(9.45)	0.935	148	102(68.91)	46(31.08)

HWE: Hardy-Weinberg Equilibrium
***P*-values < 0.05 –not consistent with HWE concept.**

Table (4): Association analysis of genetic models for *MTHFR* c.1298A>C in study groups.

Study groups	Dominant (AA vs AC+CC)		Recessive (AA+AC vs CC)		Over-dominant (AC vs AA+CC)		Homozygous co-dominant (AA vs CC)		Heterozygous co-dominant (AA vs AC)		Allele contrast (A vs C)	
	§OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
First group	0.7727 (0.41-1.4565)	0.4253	0.3897 (0.03965-3.8299)	0.4189	0.8439 (0.4497-1.5838)	0.5973	0.3434 (0.03398-3.4715)	0.3652	0.7990 (0.4215-1.5145)	0.4916	0.8182 (0.5035-1.3295)	0.4178
Second group	2.0940 (0.8476-5.1735)	0.1092	0.3300 (0.03883-2.8056)	0.3100	2.6250 (1.0806-6.3766)	***0.0331**	0.5556 (0.06036-5.1136)	0.6037	2.4306 (0.9674-6.1067)	***0.0588**	1.2838 (0.6837-2.4104)	0.4371

***Statistically significant associations (*P* ≤ 0.05)**
§ OR: Odd Ratio

Discussion:

Several studies suggest a range of genetic and environmental factors that involved in NTDs events, as genetic features play a crucial role in responding to environmental factors (3, 9). Most statistical genetic studies confirm that disease penetrance may be associated with a certain genotype that can pose a risk to individuals, and increases the frequency of the variant allele amongst case group, leading to deviate from *HWE* (10).

Our study revealed that the distribution of genotypes for *c.1298A>C* variant was not consistent with *HWE* neither in NTDs-cases nor in healthy controls. In addition, association analysis results were not significant at any genetic models, amongst cases and controls of the first group. Our results suggest no significant association between *c.1298A>C* variant and NTDs risk. These inferences agree with many other studies (11, 12), however, oppose others (8, 13). Furthermore, the *c.1298A>C* variant diminishes the enzyme activity, suggesting a secondary involvement of *c.1298A>C* in NTDs occurrence, and highlights the environmental role of fetal development and the occurrence of congenital abnormalities (8, 14).

No significant differences were found in the frequency of *C* allele for c.*1298A>C* variant in the second group, however, genotype frequencies of NTDs-cases‘mothers deviated from *HWE* (*P*= 0.017). Moreover, a significant association was found in both over-dominant, as well as heterozygous co-dominant genetic models, with OR>1, suggesting that the heterozygous genotype *AC* in mothers may be considered as a risk factor for having NTDs by more than 2-fold. These data agree with few previous studies which found an association between *c.1298A>C* variant in mothers and having NTDs-affected offspring (12, 14). Furthermore, a previous study proposed that the *c.1298A>C* variant could become clinically important during pregnancy, when the requirements of folate are high due to the growth of the fetus and placenta (15). On the contrary, other researchers observed no association between c.1298A>C in mothers and NTDs outcomes (16, 17). These discrepancies may be due to ethnic diversity, the difference in geographical regions and environmental conditions (18). In general, our data emphasize the importance of maternal genetic make-up in determining the micro-environment nature at early stages of embryonic development, which agrees with previous studies (19). No significant differences were observed in means of maternal serum folate concentrations between cases and controls (*P*=0.971). These results are in agreement with previous studies conducted in Iran (20), and Turkey (21). Our questionnaire results revealed that the percentage of control mothers who took dietary supplements during their pregnancies was higher than cases, and showed a strong significant difference (*P*<0.0001). These results are consistent with other several studies which consider folate deficiency during pregnancy is one of the most serious risk factors for NTDs (21).

Unfortunately, we were not able to recruit pregnant women diagnosed with NTDs fetuses for better assessment of the effect of folate concentration on the NTDs. Therefore, the effect of folate concentration on NTDs during

pregnancy should be considered in future studies.

In conclusion, Genetic variations are poorly studied in Syria. Further studies are needed to investigate the prevalence of NTDs and their possible causes, as well as studying the effect of other biomarkers and genetic variations that may play in the etiology of NTDs.

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Ethics approval and consent to participate:

This study was considered by the Ethics Committee of faculty of sciences on 6-July-2015, and has obtained the necessary ethics requirements of the Ethical Committee of Damascus University- Syria and approved by the Committee on 20- July -2015.

Consent for publication:

All authors approved the submission of the manuscript for publication.

Competing interests:

The authors declare that they have no competing interests.

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