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RESEARCH ARTICLE

Detection of *MTHFR* C677T Genotyping for Spina Bifida Defects using ARMS-PCR Technique

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ABSTRACT

Neural tube defects (NTDs) are complex structural deformities in newborn infants. These defects are caused by a blend of genetic and environmental factors in the abnormal phenotype. The current study aims to determine the genetic variation polymorphism for methylenetetrahydrofolate reductase (MTHFR) C677T rs180133 genotyping and to detect single nucleotide polymorphisms (SNPs) in a DNA sample of Spinal Bifida (SB) and compare it with control newborn children. Blood samples were collected from newborns (50 children under one year old) (25 cases of spinal bifida (SB) and 25 controls). The DNA was extracted and amplified by the tetra ARMS-PCR technique (Amplification Refractory Mutation System Polymerase Chain Reaction). ARMS-PCR technique consists of four primers (two outer and two inner) to amplify target DNA sequences for different genotypes for the distribution of the genetic variation of MTHFR genotyping of different genotypes (wild-type (CC) and mutant-type (TT) alleles). The results indicate that the genetic mutation of the enzyme tetrahydrofolate reductase was associated with spina bifida in newborns and could serve as a biomarker to detect the development of spina bifida in newborns.

Keywords: Genotyping, Methylenetetrahydrofolatereductase, Neural tube defect, Spine bifida, Tetra primer ARMS PCR technique

Introduction

Neural tube defect is a general term encompassing congenital malformations of the central nervous system (CNS). Neural tube defects (NTDs) are a type of birth defect that occurs when the neural tube fails to close during early gestation. It is the result of the incomplete closure of the neural tube and incidence worldwide ranges from 1.0 to 10.0 per 1,000 births.[1,2] NTDs can be categorized as “open” or “closed” based on whether the neural tissue is covered. This condition can lead to anencephaly, spina bifida, and other congenital abnormalities.[3] Spina bifida (SB), a type of NTD, occurs when the development of the fetus’s spine is incomplete during the first month of pregnancy.[4] The mortality rate for spina bifida is 7%, whereas it is 46% for encephalocele and 100% for anencephaly.[5] The severity of the condition can vary, ranging from mild with no noticeable symptoms to severe with nerve damage. Spina bifida is usually evident at birth and often occurs within the first 28 days of pregnancy before a woman even realizes she is pregnant.[6,7] Infants with anencephaly typically survive only a few days after birth.[8] Folate metabolism genes have a function in the folate metabolism cycle recent research has revealed that the incidence rate of SB is approximately 3.63 per 10,000 live births in the United States (U.S.) and 18.6 per 10,000 worldwide. Although the mortality rate for liveborn infants with SB has decreased to around 8%, it remains more than 10 times higher than the national average for all U.S. births. Survivors often experience motor impairment, bowel and bladder dysfunction, and neurological complications.

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Notably, the presence of Arnold-Chiari II malformations, characterized by the downward displacement of the cerebellar vermis and tonsils, is associated with SB, and can significantly impact motor, cranial nerve, and cognitive functions, affecting the quality of life for SB patients and their families. Both genetic and environmental factors play a role in the development of spina bifida. While the genetic component is estimated to contribute to 60–70% of cases, non-genetic risk factors like decreased folate consumption, maternal anticonvulsant therapy, diabetes mellitus, and obesity also play a role.[9] Genes of the methionine cycle involved in the folate metabolism cycle have been studied as potential risk factors for SB. The candidate genes of the methionine cycle to evaluate the association with NTD risk (i.e., DHFR, MTHFR, MTHFD1, MTR, MTRR, and TYMS and methyltransferases.[10] The methionine cycle, which is crucial for translating genes and involves the conversion of 5-MTHF and Homocysteine (Hcy) to methionine, S-adenosyl-methionine (SAM), and S-adenosyl Hcy, is particularly relevant.[11,12] One specific candidate gene in this pathway is the methylenetetrahydrofolate reductase MTHFR gene. This enzyme plays a role in the processing of amino acids and it is important for a chemical reaction involving forms of folic acid vitamin (vitamin B9), which undergoes a mutation called Single nucleotide polymorphisms (SNP) resulting in a C to T transition at nucleotide 677.[13] This mutation has been associated with an increased risk of developing NTDs in certain populations. The MTHFR gene is located on chromosome 1 p 36.3.[14] The MTHFR enzyme plays a significant role in processing amino acids and it is essential for the conversion of folic acid into 5-methyltetrahydrofolate.[15,16] This conversion is necessary for the multi-step process that transforms the amino acid homocysteine into methionine, a vital component for protein production and other essential compounds in the body.[17] Variations in the MTHFR gene can impact the processing capacity of methylenetetrahydrofolate reductase folate and potentially increase the risk of NTDs.[18,19] First-degree relatives, such as siblings and children, have an increased risk of developing spina bifida compared to the general population.[20] Therefore, the current study aimed to investigate the role of C677T MTHFR gene polymorphism, in children with spina bifida within the Iraqi population and serve as a biomarker for the detection of the MTHFR genotype risk factor and development of SBs in the Iraqi population.

Materials and methods

Sampling

The study was designed as a case-control prospective study dependent on this research on 50 Iraqi infants (25 with cases of SBs and 25 healthy control) in the age range 0–2 years both sexes in the period from August 2022 to October 2022 from hospitals in Iraq. Two mL of venous blood samples in an EDTA tube were collected for the genotyping study. All the participants were evaluated by a qualified physician neurosurgical in the department of hospitals in Hillah and Baghdad cities after obtaining the Institutional Ethical Committee approval and written informed consent from study subjects after clinical examination for everyone.

Table 1. Specific Tetra Primer sequences for C677T *MTHFR* gene.

SNP Primer *MTHFR* C677T	Primer Sequence	Length (bp)
IFP (T allele)	5′ AAGGTGTCTGCGGGCGT3′	165
IRP (C allele)	5′AAAGCTGCGTGATGATGAAATA GG3′	181
OFP	5′CCCAGTCCCTGTGGTCTCTTC3′	305
ORP	5′AGGGAGCTTATGGGCTCTCCT3′	305

IFP: Inner forward primer, ORP: Outer reverse primer, OFP: Outer forward primer, IRP: Inner reverse primer.

Genomic DNA extraction

The Genomic DNA samples were isolated from 2ml whole blood using a DNA Extraction and Purification Kit (Bioneer, Korea) following the instructions of the manufacturer protocol. The concentration of DNA and purity were measured using a nano-drop and quality was checked by electrophoresis using a 1% agarose gel.

Genetic analysis of MTHFR C677T

In the current study, primers were designed based on an online tool in a National Center for Biotechnology Information (NCBI) database repository which is available for free to the public use, following the optimal standard criteria. Then synthesis was performed by Bioneer, Korea. The primer sequences are as follows:

Inner forward primer IFP (T allele), Inner reverse primer IRP (C allele), Outer forward primer OFP and Outer reverse primer ORP. Genotyping for the *MTHFR* (rs180133, C677T) variant was carried out through ARMS-PCR. The ARMS-PCR product reaction volume was 25 μl containing 1 μl IFP, 1 μl IRP, 1 μl OFP, 1 μl ORP, 12.5 μl of Green Master Mix, and 3 μl of gDNA, and 5.5 μl of Nuclease free water. The conditions of the ARMS-PCR were performed on a thermal cycler (Bioneer, Korea) and included an initial pre-denature at 95 ˚C for 10 minutes (min.) followed by 35 cycles under the following conditions: denaturation at 94 ˚c for 2 min. annealing at 57 ˚C

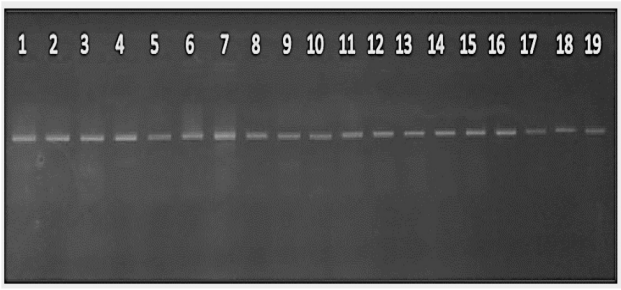

Fig. 1. Genomic DNA electrophoresed on 1% agarose, 5 Volt/1cm. Lane 1–13 refers to cases and Lane 14–19 refers to controlling children.

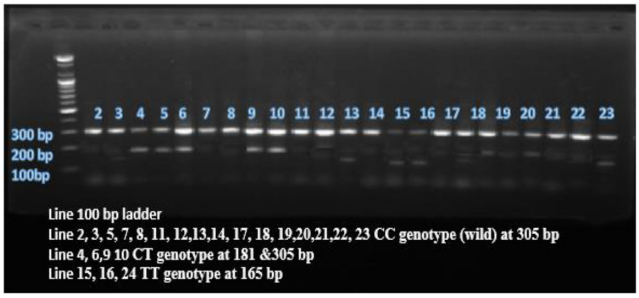

Fig. 2. Electrophoresis of ARMS-PCR products for *MTHFR* C677T polymorphism cases' group.

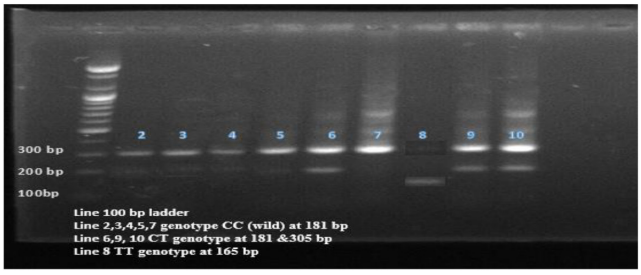

Fig. 3. Electrophoresis of ARMS-PCR products for *MTHFR* C677T polymorphism of control group.

for 1mint, extension at 72 ˚C for 1mint, and a final extension cycle of 72 ˚C was for 5 mints. The ARMS-PCR products were visible on 2% agarose gel, stained with ethidium bromide, and visualized by UV light using a DNA ladder (100 bp). The distribution of the *MTHFR* genotype in the control mother was found to be in Hardy-Weinberg equilibrium (HWE) as shown below: HWE: $(p2 + 2pq + q2 = 1)$ Where p is defined as the frequency of the dominant allele and q as the frequency of the recessive allele for a trait controlled by a pair of alleles (A and a).

Statistical analysis

The Statistical Analysis System[21] program was utilized to assess the impact of various factors on study parameters. Hardy-Weinberg equilibrium (HWE) was examined for a genetic epidemiology study, with a significance level set at $P < 0.05$. Logistic regression analysis was used to calculate all odds ratios (ORs) and 95% confidence intervals (CIs) to demonstrate the degree of association.

Results and discussion

The results of the current study show that the distribution of sex was 12 (48%) males, 13 (52%) females for the case group 14 (56%) males, and 11 (44%) females for the control group. These results show there were no significant differences between both study groups for the sexes. A similar result there were no significant differences between both study groups for the sexes was reported by Behere *et al.*[22] The gDNA produced a sharp intense band product with high integrity and purity when electrophoresed on a 1% agarose as shown in Fig. 1. The concentration of gDNA was counted by nano-drop spectrophotometer ranging from 80–120 ng/μl and the 260/A280 ratio provided ranged between 1.8 and 1.9 in purity.

The result of the *MTHFR* C677T genotyping product used for ARMS-PCR was electrophoresed in agarose gel, Figs. 2 and 3 for both studied groups giving three polymorphisms, including the wild-type homozygous CC genotype having a single band with length of 305 bp, the mutant homozygous TT genotype having a single band with length of 165 bp, while the heterozygous CT genotype having 2 bands with length of 181 bp and 305 bp.

The results of MTHFR gene polymorphism as shown in Table 2 provided the genotype of the wild-type homogenous CC 18 (72%) and 22 (88%) for the cases and controls, respectively, with a P-value of 0.527 and a reference Ref[1] On one hand, the results of the mutant homogenous TT were 2 (16%) and 0 (0%) for the cases and controls, respectively, with a P-value of 0.289 and an OR of 0.052 (0.02–0.27). These results revealed there were no significant differences between homogenous CC and TT genotypes for the MTHFR C677T. On the other hand, the results of the MTHFR gene polymorphism for the heterogeneous CT genotype were 4 (16%) for the cases and 3 (12%) for the controls, with a P-value of 0.705 and an OR of 0.613 (0.32–1.150). These results of the CT genotype were not significantly different between the two studied groups of children. Moreover, the results of MTHFR genotyping of C allele frequency were 0.8 and 0.94 for cases and controls, respectively, with an OR of 0.219 (0.10–0.74) In addition, the results of MTHFR genotyping of T allele frequency were 0.2 and 0.6 for cases and controls, respectively, with an OR

Table 2. *MTHFR* C677T Genotype distribution and allele frequency in both study groups.

MTHFR C677T / Genotyping N = 50	Cases N = 25	Controls N = 25	P- value	OR (95%) of SBs
CC	18 (72%)	22(88%)	0.527NS*	Ref.** = 1
CT	4(16%)	3(12%)	0.705NS*	0.613(0.32–1.150)
TT	3(12%)	0(0%)	0.289NS*	0.052(0.02–0.27)
Allele Frequency C	0.8	0.94	0.453NS*	0.219(0.10–0.74)
Allele Frequency T	0.2	0.06	0.052NS*	0.307(0.14–0.91)

NS*: Non-Significant, Ref.**: Reference = 1.

of 0.307 (0.14–0.91). These results explain that there were no risk factors for the SB case. The results indicate the distribution of MTHFR gene polymorphisms (CC, TT, and CT genotypes) and allele frequencies in cases and control for no significant differences between the two groups for the CC genotype, and for the TT genotype, the OR suggests a potential protective effect, as do the results for the CT genotype.[23] The C allele frequency analysis shows no significance in cases compared to controls, with an OR indicating a potential protective effect. In addition, the T allele frequency shows no significant difference in cases compared to controls, indicating a potential protective effect. was associated with neural tube defect risk in patients and it might be counted as a molecular marker for evaluating the susceptibility of NTDs. A similar result showed that the presence of a mutant allele in homozygous or heterozygous conditions for both SNPs had increased the risk associated with NTDs.[24]

Based on the current results, it seems that the observed and expected allele frequencies for the CC, TT, and CT genotypes in both cases and controls were compared to assess deviations from HWE as shown in Table 3. The results, according to HWE, produced each of the observed and expected results as follows: In these cases, the CC allele frequency produced 18 and 16 (64%) as observed and expected, respectively. On the one hand, the TT allele frequency produced 3 and 1 (4%) as observed and expected, respectively. On the other hand, the results of CT allele frequency were 4 and 8 (32%) for observed and expected, respectively. These results were significant between the CT allele frequency observed and expected, with a p-value of 0.0001. Moreover, in controls, the CC allele frequency produced 22 and 22.09 (88.36%) as observed and expected, respectively. On the one hand, the TT allele frequency produced 0 and 0.09 (0.36%) as observed and expected, respectively. On the other hand, the results of CT allele frequency were 4 and 2.82 (11.28%) for observed and expected, respectively. These results were significant between CT allele frequency observed and expected, with a p-value of 0.0001. In the case and control groups, the observed and expected frequencies for the CC, TT, and CT genotypes were compared. The significant difference between the observed and expected CT allele frequency suggests a deviation from HWE for this genotype in the case and control groups. These deviations from HWE could be indicative of various factors such as non-random mating, genetic drift, mutation, selection, or population substructure within the studied population. The Hardy-Weinberg equilibrium is used in genetic populations to describe the expected and predicted frequencies of genotypes under certain conditions under various factors such as non-random mating, genetic drift, mutation, selection, or population substructure. Several causes, including non-random mating, genetic drift, mutation, selection, and population substructure, can be indicated by deviations from HWE.[25] Similar results reported that there is an association between the CT heterozygote variant of the MTHFR C677T gene and SB risk factors that is *significantly* correlated with the *genotype* (*CT*/TT) of the *MTHFR C677T gene* polymorphism.[26]

Table 3. *MTHFR* C677T Genotype distribution according to Hardy–Weinberg equilibrium in both study groups.

MTHFR Genotyping N = 50	Observed No	Expected No (%)	P-value of Exp.
Cases			
CC	18	16 (64%)	
CT	4	8 (32%)	0.0001 **
TT	3	1 (4%)	
Controls			
CC	22	22.09 (88.36%)	
CT	3	2.82 (11.28%)	0.0001 **
TT	0	0.09 (0.36%)	

** ($P \leq 0.01$).

The amino acid alanine is converted to valine via the MTHFR SNP mutation, which occurs at position 677 within exon 4 and includes a transition from cytosine (C) to thymine (T). DNA, RNA, and proteins need one carbon (a methyl group) for methylation and re-methylation, usually catalyzed by MTHFR. MTHFR

also catalyzes the conversion of homocysteine into methionine.[27] These associations may be influenced by various factors such as nutrition, environment, and genetics.[28,29] Exposure to environmental factors such as physical and chemical mutagenic agents. The mutation and the risk of developing SB differences in the findings could be linked to ethnicity, country of origin, or demographic factors, as reported by.[30] Others have hypothesized that folate has a role in DNA methylation, which results in the overexpression of some genes involved in autoimmunity associated with the progression of NTDs.[31,32] Previous studies have reported an association between maternal MTHFR C677T genetic genotyping and adverse birth outcomes, particularly in pregnant women with insufficient folate intake.[33] The TT genotype of the MTHFR C677T allele has been associated with an increased risk of preterm birth and low birth weight under various genetic models.[34,35] The pore supplementation of folic acid during early pregnancy is necessary to prevent potential pathological conditions caused by folate deficiency.[36,37] Inadequate folate intake may be due to poor dietary intake of green vegetables, fruits, legumes, and animal-origin food products or a decreased bioavailability of folate influenced by genetic factors, such as carriers of the MTHFR 677CT/TT polymorphism.[38,39] Many studies showed the closely related genetic factors (SNPs) interactions with the risk of congenital heart disease (CHD). They indicated genetic polymorphisms of the maternal MTHFR gene were significantly associated with the risk of fetal CHD in the Chinese population.[40] While the current study has several strengths, including examining the relationship between MTHFR gene genotyping and SBs, there are also a few limitations. These include a small sample size, a semi-quantitative assessment of maternal dietary folate intake derived from questionnaires, and a lack of information regarding the fetal MTHFR genotype, which makes it impossible to determine the relative contribution of the fetal genotype to the observed effects. Studies in different ethnic populations and with a larger sample are required to confirm these findings.

Conclusion

The MTHFR C677T genetic mutation has been associated with certain health in newborns, but its contribution to the development of most SB is not considered significant. Although the presence of the MTHFR mutation can be a biomarker and risk factor for SBs in certain populations, such as the Iraqi population, more research is necessary to fully comprehend its influence on neural tube development.

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Authors' declaration

- Conflicts of Interest: None to declare.
- Ethical Clearance: We confirm that this manuscript's data, tables, and figures are ours. The authors signed ethical consideration approval.
- Ethical clearance: The local ethical committee at the University of Baghdad approved the project.
- No animal studies are present in the manuscript.
- No potentially identified images or data are present in the manuscript.
- Ethical Clearance: The local ethical committee at the University of Baghdad approved the project.

Authors' contribution statement

This work was a collaborative effort among all authors H.H.H was responsible for sample collection, while H.K.A and Z.F.A authors contributed to the research experiment and writing of the manuscript. The final manuscript was revised and approved by all authors.

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الكشف عن التنميط الجيني لانزيم الميثيلين تتراهيدروفولات المختزل C677T لعيوب السنسنة المشقوقة باستخدام تقنية سلسلة البلمرة بنظام تضخيم الطفرة الحرارية رباعي البوادئ

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الخلاصة

عيوب الأنبوب العصبي هي تشوهات هيكلية معقدة عند الأطفال حديثي الولادة. تنجم هذه العيوب عن مزيج من العوامل الوراثية والبيئية في النمط الظاهري غير الطبيعي. تهدف الدراسة الحالية إلى تحديد تعدد أشكال التباين الوراثي في التنميط الجيني لإنزيم ميثيلين تتراهيدروفولات المختزل والكشف عن تعدد أشكال النوكليوتيدات المفردة في عينة الحمض النووي لمرضى السنسنة المشقوقة ومقارنتها مع الأطفال الاصحاء حديثي الولادة. تم جمع عينات الدم من الأطفال حديثي الولادة (50 طفلاً أقل من سنة واحدة) (25 حالة من حالات انشقاق العمود الفقري و25 حالة من الاصحاء). تم استخراج الحمض النووي وتضخيمه بواسطة تقنية تفاعل البلمرة المتسلسل لنظام تضخيم الطفرة الحرارية (تفاعل البلمرة المتسلسل لنظام الطفرة المقاومة للحرارة). تتكون تقنية تفاعل البلمرة الرباعي المضخم من أربعة بادئات (اثنان خارجيان واثنان داخليان) لتضخيم تسلسل الحمض النووي المستهدف للأنماط الجينية المختلفة (أليلات النوع البري والنوع الطافر). تشير النتائج إلى أن الطفرة الجينية للانزيم تتراهيدروفولات المختزل كانت مرتبطة بـالسنسنة المشقوقة عند الأطفال حديثي الولادة ويمكن ان يكون كمؤشر حيوي للكشف عن تطورأمراض السنسنة المشقوقة للاطفال حديثي الولادة.

الكلمات المفتاحية: عيب الأنبوب العصبي، انشقاق العمود الفقري، إنزيم ميثيلين رباعي هيدروفولات اختزال، التنميط الجيني، سلسلة البلمرة بنظام تضخيم الطفرة الحرارية رباعي البوادئ