original article

Prevalence of methylenetetrahydrofolate reductase gene polymorphisms (C677T, and A1298C) among Saudi children receiving dental treatment

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BACKGROUND: Methylenetetrahydrofolate reductase, the encoded by the *MTHFR* gene, plays a crucial role in converting the amino acid homocysteine to methionine. Two polymorphisms of the *MTHFR* gene, C677T and A1298C, reportedly reduce enzyme activity, resulting in hyperhomocysteinemia. Patients with C677T and A1298C polymorphisms may be at higher risk for developing abnormal hyperhomocysteinemia, which has been linked to catastrophic neurological including fatal outcomes.

OBJECTIVE: Determine the prevalence of the *MTHFR* gene variants C677T and A1298C among pediatric dental patients treated at King Abdulaziz University Hospital.

DESIGN: Cross-sectional.

SETTING: Clinics of pediatric dentistry department.

SUBJECTS AND METHODS: Healthy Saudi children 6–12 years old with no known allergies were screened for eligibility between May and December 2019. A single investigator collected saliva samples. The *MTHFR* C677T and A1298C polymorphisms were analyzed using polymerase chain reaction and restriction fragment length polymorphism.

MAIN OUTCOME MEASURE: The prevalence of *MTHFR* gene variants (C677T and A1298C) among the subjects.

SAMPLE SIZE: 138.

RESULTS: *MTHFR* C677T polymorphism was present in 36.2% of the sample and 90.0% of children carrying this allele were heterozygotes. *MTHFR* A1298C polymorphism was present in 91.3% of the sample and 77.0% of the children carrying this allele were heterozygotes. No linkage disequilibrium between *MTHFR* C677T and *MTHFR* A1298C was observed within this sample.

CONCLUSIONS: Our study found a high frequency of the *MTHFR* A1298C genotype, which was substantially more abundant than expected based on a Hardy-Weinberg distribution. Therefore, caution is advised in using N_2O in Saudi children as the increased prevalence of this *MTHFR* allele may increase the incidence of serious adverse effects among these children.

LIMITATIONS: Further studies are recommended with a larger sample size from randomly selected hospitals from different regions of Saudi Arabia.

CONFLICT OF INTEREST: None.

The *MTHFR* gene (OMIM: 607093) encodes methylenetetrahydrofolate reductase (*MTHFR*), which plays a crucial role in the endogenous conversion of homocysteine to methionine.[1] Reduced *MTHFR* activity can result in the accumulation of homocysteine in the blood plasma and cause hyperhomocysteinemia.[2,3] The most common causes of hyperhomocysteinemia are single nucleotide polymorphisms (SNPs) in the coding region of the *MTHFR* gene and the production of a variant enzyme that is less efficient than enzymes encoded by other alleles.[4-6] The two most common polymorphisms in the *MTHFR* gene are the C677T and A1298C polymorphisms. The alteration of cytosine (C) to thymine (T) at position 677 causes the replacement of the amino acid alanine for valine (A222V; UniProtKB: P42898) in the enzyme and subsequently reduces enzyme activity compared to enzymes with A677.[6] Specifically, the heterozygous CT genotype causes a 34% reduction in the *MTHFR* enzyme's activity, whereas the homozygous mutant TT genotype causes a 70% reduction in enzyme activity relative to that encoded by the CC genotype.[7] The mutation of adenine (A) to cytosine (C) at position 1298 results in the substitution of the amino acid glutamic acid with alanine (E429A). The A1298C polymorphism alone generally does not cause hyperhomocysteinemia.[8] However, the combined C677T and A1298C polymorphisms result in a 70% reduction in enzyme activity, indicating that the two polymorphisms have a synergistic effect on enzyme activity.[9] Several studies have documented the association of *MTHFR* C677T and A1298C polymorphisms with intellectual disability, psychosis, type II diabetes, venous thrombosis, and coronary heart disease.[10-15]

Based on the American Academy of Pediatric Dentistry (AAPD) nitrous oxide/oxygen (NO_2/O_2) inhalation is generally one of the safest and most effective techniques to reduce anxiety and produce analgesia in pediatric dental patients.[14,15] Nagele et al (2008) reported that patients with the *MTHFR* C677T and A1298C polymorphisms are at a higher risk for the development of abnormal hyperhomocysteinemia following exposure to N_2O anesthesia.[16,17] A later study conducted by Nagele et al reported that *MTHFR* C677T and A1298C had no influence on nitrous oxide-induced homocysteine increases.[18] However, severe catastrophic neurological outcomes, including fatalities, have been reported in children carrying *MTHFR* C677T and A1298C polymorphisms after treatment with N_2O.[14,18-20] Thus, patients who carry the *MTHFR* C677T and A1298C polymorphisms might be at increased risk for harmful side effects following N2O exposure.[19,20]

The prevalence of the C667T and A1298C genotypes of the *MTHFR* gene are known to vary among ethnic groups. More than 25% of Hispanics and 10% to 15% of North American Caucasians are estimated to be homozygous for the TT genotype.[21] However, the prevalence of the *MTHFR* C677T and A1298C polymorphisms among Saudi pediatric patients receiving dental treatments at King Abdulaziz University Hospital is unknown. N_2O is commonly used at the pediatric dental clinic at King Abdulaziz University Hospital. The goal of the current study was to determine the proportion of patients who are at an increased risk of negative side effects due to the *MTHFR* C677T and A1298C polymorphisms. This information will be useful for policy makers and medical practitioners with respect to N_2O use as a pharmacological agent in the management techniques used for pediatric dental patients.

SUBJECTS AND METHODS

Subject selection criteria and sample size calculation

The study was conducted in the Department of Pediatrics and Specialist Dental Clinic at King Abdulaziz University Faculty of Dentistry (KAUFD) in Jeddah, Saudi Arabia, between May and December of 2019. The study protocol was approved by the Human Research Ethics Committee of the School of Dentistry at King Abdulaziz University (024-03-20). All children who received dental treatment at the Pediatric Dentistry Department Clinic between May and December 2019 were screened for eligibility. The inclusion criteria included: 1) Saudi nationality, 2) healthy children 6–12 years of age receiving dental treatment at the Department of Pediatric and Specialist Dental Clinic at KAUFD, 3) no known allergy, and 4) no use of prescribed medications. The objectives and the nature of the study were introduced to the parents or guardians of eligible subjects. Those who agreed to participate were required to sign an informed consent written in Arabic before participation. Subjects who failed to provide the amount of saliva required for the analysis were excluded from the study. Subject data were recorded, including demographic data and family history of *MTHFR* enzyme polymorphism. All saliva samples were collected by a single investigator. When the results of the genetic analyses were obtained, the parents/guardians were informed directly.

Dental treatment under N_2O is considered an alternative behavioral management technique. At KAUFD's pediatric dentistry clinics, dental treatment is provided by trained pediatric dentist consultants and residents under the supervision of a pediatric dentistry consultant. Following the AAPD guidelines, each patient re-

ceived 100% oxygen for one to two minutes followed by titration of N_2O in 10% intervals without exceeding 50% N_2O. At the end of the treatment, once the N_2O was terminated, the patients were provided 100% oxygen for three to five minutes.[14]

Based on the results of Zappacosta et al,[22] the prevalence of the C667T and A1298C genotypes of the *MTHFR* gene was assumed to be 15%. Using OpenEpi software, we calculated that a random sample of 138 subjects would be sufficient to detect the true prevalence of these genes in the Saudi population with 95% confidence, assuming that the prevalence is in the range of 15% +/- 5%.[23]

Detection of MTHFR polymorphisms

1. Saliva samples were collected from patients using the Oragene-Discover (OG-500). Genomic DNAs from the saliva samples were extracted following the manufacturer's instructions (DNA Genotek, Kanata, ON). Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) were used to detect *MTHFR* polymorphisms using a previously described protocol.[24] Specifically, to genotype the C677T polymorphism, a 294-base pair (bp) fragment of the *MTHFR* gene was amplified using custom primers (forward: 5′-CTTTGGGGAGCTGAAGGACTACTA-3′; reverse: 5′-CACTTTGTGACCATTCCGGTTTG-3′; Macrogen, Korea). The PCR reaction was performed in 20 µL of a total volume containing 10 µL of 2X GoTaq Green Master Mix (Promega), 100 ng of genomic DNA, and 0.5 µM of each primer. Reactions were subjected to an initial denaturation at 95°C for 10 min, and then 35 cycles of denaturation (95°C, 1 min), primer annealing (65°C, 1 min), and extension (72°C, 1 min) with a final extension at 72°C for 10 min. PCR products were digested using the HinfI restriction enzyme and separated on a 3% agarose gel at 80 V for 120 min. The homozygous wild-type allele (C677C) was identified by a single band of 294 bp, the heterozygous (C677T) was identified by three bands of 294, 168, and 126 bp, and the homozygous (T677T) genotype was identified by two bands at 168 bp and 126 bp (**Figure 1A**).

To genotype the *MTHFR* A1298C polymorphism, a 163-bp fragment of the *MTHFR* gene,

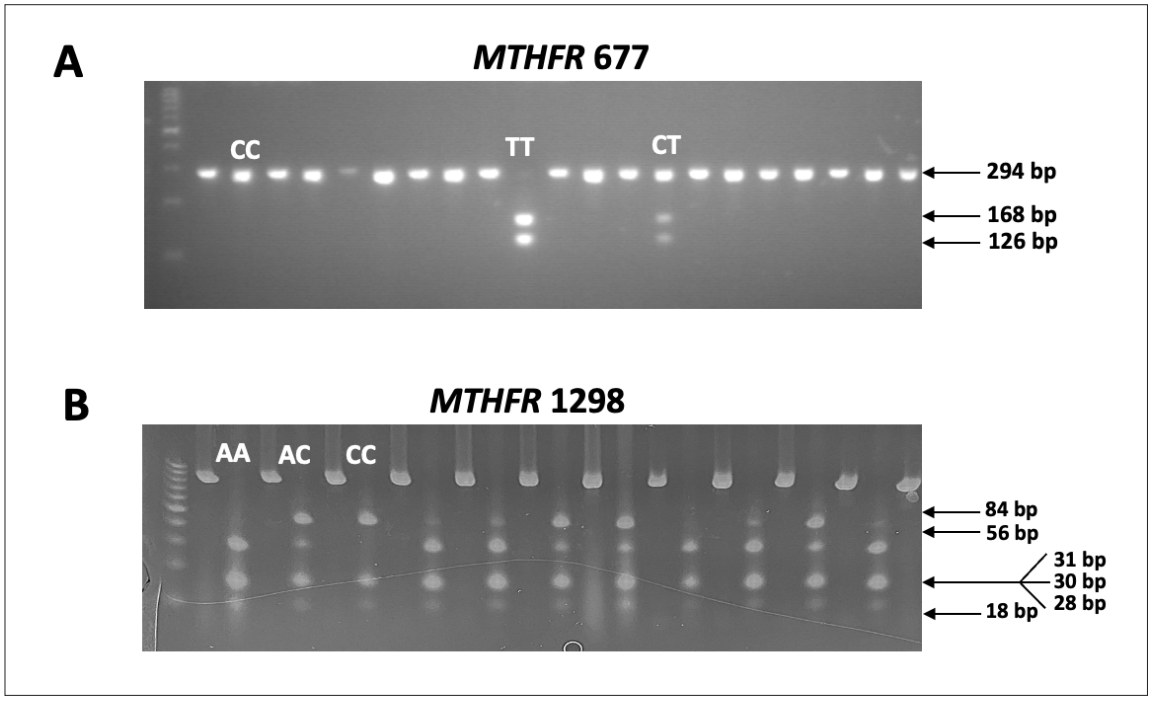

Figure 1. (A) PCR-RFLP results of the *MTHFR* C677T polymorphism. Agarose gel electrophoresis for *MTHFR* C677T after digestion with Hinf. Digestion of the homozygous wild-type CC genotype yields a single band of 294 bp, whereas the heterozygous CT genotype results in three bands of 294, 168, and 126 bp, and the homozygous TT genotype shows two bands at 168 bp and 126 bp. Lane 1: 100 bp DNA marker (B) PCR-based RFLP results of the *MTHFR* A1298A polymorphism. Agarose gel electrophoresis for A1298C polymorphism after digestion with MboII. Digestion of the homozygous AA genotype results in five bands of 56, 31, 30, 28, and 18 bp, whereas the heterozygous AC genotype results in three bands of 84, 56, and 30 bp, and the homozygous CC genotype leads by four bands of 84, 31, 30, and 18 bp. Since the molecular weights of 31, 30, and 28 bp are very similar, they could not be distinguished on the gel. The major visible bands were those of 84 bp and 56 bp. Lane 1:25 bp DNA marker.

Table 1. The *MTHFR* C677T and A1298C polymorphisms allele frequency.

Polymorphism	Allele	Percentage (95% CI)
MTHFR C677T	677C	80.1 (70.6-87.1)
	677T	19.9 (12.9-29.4)
MTHFR A1298C	1298A	43.9 (34.1-54.1)
	1298C	56.2 (45.9-65.9)

Table 2. Genotype frequencies of the *MTHFR* C677T and A1298C polymorphisms.

Polymorphism	Genotype	n	Percentage of subjects (95% CI)	Observed/ Expected[a]
MTHFR C677T	CC	88	63.8 (53.4-73.0)	0.99
	CT	45	32.6 (23.8-42.8)	1.00
	TT	5	3.6 (0.1-10.0)	0.97
MTHFR A1298C	AA	12	8.7 (4.3-16.5)	0.45
	AC	97	70.3 (60.2-78.8)	1.42
	CC	29	21.0 (13.8-30.5)	0.66

[a]Under Hardy-Weinberg equilibrium.

Table 3. Prevalence of the compound genotypes of the *MTHFR* C677T and A1298C polymorphisms.

Genotype	C677C	C677T	T677T
A1298A	6 (4.3%)	4 (2.9%)	1 (0.7%)
A1298C	59 (42.8%)	33 (23.9%)	3 (2.2%)
C1298C	27 (19.6%)	4 (2.9%)	1 (0.7%)

Data are number (%)

was amplified using custom primers (forward: 5′-CCTTGAACAGGTGGAGGCCAG-3′; reverse: 5′-GCGGTGAGAGTGGGGTGG -3′; Macrogen, Korea). The PCR reaction and thermal cycle were similar to the conditions used for C677T amplification, except for the annealing temperature, which was 62°C. These PCR products were then digested with MboII restriction enzyme and fragmented on a 4% agarose gel. The homozygous A1298A genotype was identified by the presence of five bands of 56, 31, 30, 28, and 18 bp. The heterozygous A1298C genotype was identified by three bands of 84, 56, and 30 bp (**Figure 1B**). The homozygous C1298C genotype was identified by four bands of 84, 31, 30, and 18 bp.

Statistical analysis

Allele and genotype frequencies of *MTHFR* polymorphisms, C677T and A1298C, were calculated by direct counting and compared using a chi-square test with the expected values calculated based on an assumption of Hardy-Weinberg equilibrium. The confidence intervals of allele and genotype frequencies were calculated using the normal approximation with continuity correction.[25] The linkage disequilibrium D′ and haplotype prevalence were carried out using a previously described method.[26]

RESULTS

Of 147 children initially eligible, 3 were excluded because of insufficient saliva, 6 refused to participate, leaving 138 included in the study. After all were genotyped, we first calculated the frequencies of the individual 677T and 1298C alleles. The frequency of the 677T allele was 19.9% while the frequency of the 1298C allele was 56.2%, indicating that the A1298C polymorphism was more abundant in the sample subjects (**Table 1**).

We examined the genotype distributions for the *MTHFR* C677T and A1298C polymorphisms to determine the abundance of the homozygous or heterozygous genotypes. The homozygous *MTHFR* T677T genotype was present in only 3.6% of the sample (**Table 2**), whereas the heterozygous *MTHFR* C677T genotype was present in 32.6% of the sample. Thus, the *MTHFR* C677C genotype comprised the remaining 63.8% of the sample. This genotype distribution for the *MTHFR* C677T polymorphism was consistent with Hardy-Weinberg equilibrium (χ^2=0.049, *P*=.97) (**Figure 2**). The homozygous *MTHFR* C1298C genotype was present in 21.0%, whereas the heterozygous *MTHFR* A1298C genotype was present in 70.3%. Thus, the 1298C polymorphism was present in 91.3% while the A1298A genotype was present in only 8.7%. This *MTHFR* A1298C genotype distribution was not consistent with the expected distribution based on a Hardy-Weinberg equilibrium (χ^2=18.25, *P*=.0001). Our data indicate that the *MTHFR* A1298C polymorphism is more abundant than expected among the sample of Saudis in the Western Province compared to the Central Province in another study (**Table 4**).[27,28]

Finally, we examined the distribution of the compound genotypes for the *MTHFR* C677T/A1298C polymorphisms. The frequency of the *MTHFR* C677C/A1298A compound genotype was only 4.3% of the sample population (**Table 3**). The *MTHFR* C677C/A1298C compound genotype was the most prevalent, at 42.8% of the sample population. The *MTHFR* T677T/C1298C compound genotype was the least prevalent, as it was present in only 0.7% of the sample population. The *MTHFR* T677T/A1298C and C677T/C1298C compound

genotypes also had a low abundance (2.2% and 2.9%, respectively). Furthermore, the *MTHFR* C677C/C1298C compound genotype was relatively common (19.6%), which indicated that subjects homozygous for the wild-type 677C were the primary carriers of the mutant 1298C allele. Linkage disequilibrium between C667T and A1298C polymorphisms (D'=−0.01, r^2= 0.0001) was not detected during analysis of the genotype combination. The four possible haplotypes of the 677 and 1298 loci were CA, CC, TA, and TC. As expected, the highest frequencies were found for the CC (44.9%) and CA (35.1%) haplotypes. the lowest frequencies were found for the TC (11.3%) and TA (8.7%) haplotypes.

DISCUSSION

The aim of this study was to determine the prevalence of *MTHFR* C677T and A1298C polymorphisms among pediatric patients who were treated at the King Abdulaziz University Hospital as a first step to limiting the adverse effects for children with reduced *MTHFR*-encoded enzyme activity receiving dental care and N_2O sedation. A sample of 138 Saudi pediatric patients was genotyped to determine the abundance of these polymorphisms. We determined that the *MTHFR* C677T polymorphism was present in 36.2% of the sample, compared to the global average of 33.6% reported in the dbSNP database (rs1801133).[29] Among those with the *MTHFR* C677T polymorphism, 90.0% were heterozygous. This distribution of the *MTHFR* C677T polymorphism also followed a Hardy-Weinberg distribution. The *MTHFR* A1298C polymorphism was present in 91.3% of the sample compared to the global average of 30.6% reported in the dbSNP database (rs1801131). Of those with the *MTHFR* A1298C polymorphism, 77.0% were heterozygous.

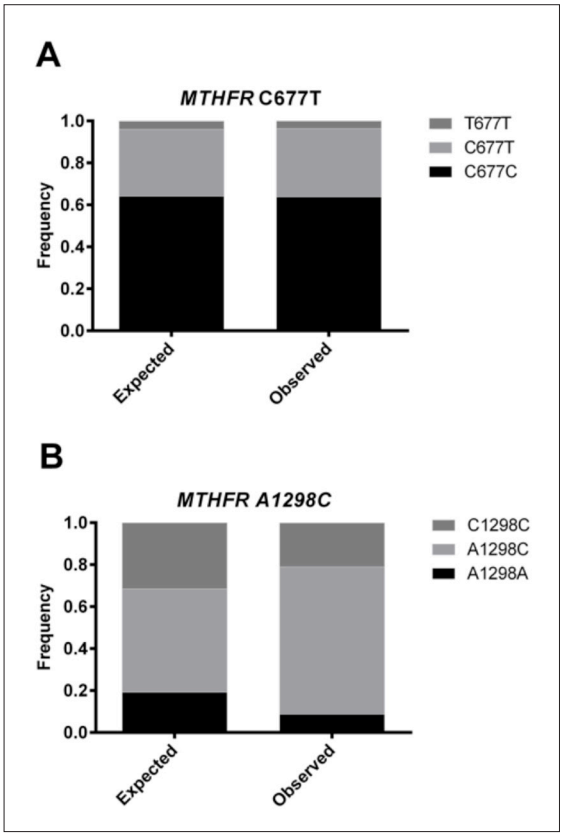

Figure 2. Expected and observed genotype frequencies for the *MTHFR* C677T and A1298C

This heterozygous *MTHFR* A1298C was substantially more abundant than expected based on a Hardy-Weinberg distribution.

Several studies have previously reported the prevalence of the *MTHFR* C677T and A1298C polymorphisms in other Saudi regions, but this is the first study

Table 4. Distribution of *MTHFR* C677T and A1298C polymorphisms among different ethnic populations in Saudi Arabia.

Reference	MTHFR A1298C			MTHFR C677T			n	Region
	CC (%)	AC (%)	AA (%)	TT (%)	CT (%)	CC (%)		
Current study	21.0	70.3	8.7	3.6	32.6	63.8	138	Western Province
(30)	---	---	---	4.0	21.0	75.0	105	Eastern Province
(31)	---	---	---	0	15.0	85.0	40	
(32)	---	---	---	2.4	24.6	73.0	884	Central Province
(27)	10.2	43.0	46.8	2.5	24.7	72.8	511	
(28)	---	42.5	57.6	3.0	23.0	74.0	250	
(33)	---	---	---	0	25.0	75.0	280	

to report them in the Western Province.[27,28,30-33] These data from the Western Province are largely consistent with previous work with respect to the *MTHFR* C677T polymorphism in the Eastern and Central provinces (**Table 4**). Only two previous studies have analyzed the presence of the *MTHFR* A1298C polymorphism.[27,28] Siraj et al[27] and Alghasham et al[28] identified that the heterozygous *MTHFR* A1298C genotype was present in 43.0% and 42.5% of patients in the central province, respectively. However, we found that this genotype was present in 70.3% of patients. This indicates that there is a notable difference in the prevalence of the *MTHFR* A1298C genotype between the Western and Central provinces. The observed abundance of the *MTHFR* A1298C genotype in the Western Province was also substantially higher than that reported for other populations including Italians at roughly 45%[22,34] and Chinese at roughly 29%.[25]

We observed that the most common compound genotypes were *MTHFR* C677C/A1298C, C677T/A1298C, and C677C/C1298C. Combined, these compound genotypes accounted for 86.3% of the sample population. Zappacosta et al reported a low abundance of the *MTHFR* C677C/A1298C compound genotype in an Italian sample that instead had a higher prevalence of *MTHFR* C677T/A1298A and T677T/A1298A compound genotypes.[22] Previous work has also indicated that the *MTHFR* C677C/A1298C compound genotype is much less abundant in American and Turkish populations, but equally abundant in an Indian population.[35] Thus, there is a somewhat large degree of variation in the distribution of compound genotypes among populations worldwide. The observed pattern of compound genotype combinations suggests that there is no linkage disequilibrium between the polymorphisms at nucleotide positions 677 and 1298.

The prevalence of minor adverse effects associated with N_2O sedation is low, ranging from 1.8% to 8.3%, with vomiting being one of the primary minor adverse effects.[36-39] Longer treatment duration and deeper N_2O sedation reportedly increased the incidence and severity of vomiting associated with N_2O sedation.[36,38] Significant adverse effects following N_2O sedation are extremely rare,[36-39] and a limited number of cases reported severe neurological complications in pediatric patients following N_2O sedation for over 80 minutes.[19,20] Although the exposure duration in the reported cases is longer than what pediatric patients are usually exposed to during dental treatment, the possible adverse effects should not be underestimated, especially for procedures requiring multiple and longer treatment durations.

Despite the AAPD contraindication on the use of N_2O in patients with *MTHFR* polymorphism,[14] no routine preoperative screening for *MTHFR* polymorphism has been recommended. In our study, a high frequency of the *MTHFR* A1298C genotype was detected among Saudi children, which emphasizes that detailed preoperative evaluation and investigation of the patients' previous medical and family history of *MTHFR* polymorphism can be suggested as a valuable tool to diagnosing *MTHFR* polymorphisms in patients who need treatment under N_2O.

Further studies to determine the association between *MTHFR* polymorphisms and the frequency and severity of adverse effects after dental treatment with N_2O are recommended. In addition, due to the limited sample size and the study's design, the direct cause and effect cannot be determined. Further studies with a larger sample size from randomly selected hospitals across different regions of Saudi Arabia are also recommended to accurately report the prevalence of *MTHFR* gene polymorphisms among Saudi children. Knowledge of the prevalence of *MTHFR* variants among our population will help us to better understand the likelihood that individual patients will experience N_2O-related adverse effects.

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