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MTHFR C677T and A1298C Gene Polymorphisms, Hyperhomocysteinemia, and Intimal Medial Thickness as Risk Factors of End-Stage Renal Disease in Children on Hemodialysis

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Background: The methylenetetrahydrofolate reductase (MTHFR) C677T gene polymorphism has been shown to be associated with cardiovascular disease and in patients with end-stage renal disease (ESRD). However, the relationship between MTHFR polymorphisms and cardiovascular disease (CVD) in patients on hemodialysis has not been examined. The aim of this study was to assess the association of polymorphisms of MTHFR gene with homocysteine (Hcy) and intimal medial thickness (IMT) in children on hemodialysis. Methods: We performed a case-control study comprising 55 pediatric patients with ESRD and 55 healthy children as controls. Plasma Hcy was measured in all the subjects and these subjects were genotyped for 2 MTHFR polymorphisms (C677T and A1298C). Results: We observed significantly higher Hcy levels in patients compared to controls. The frequency of MTHFR 1298CC genotype was significantly higher in ESRD children than in controls (21.82 % vs 5.45 %) and the frequency of the MTHFR 677TT genotype differs significantly between groups (18.18 % vs 0.00 %). The frequency of co-occurrence of MTHFR 677CT/1298AC and 677TT/1298CC was significantly higher in patients than controls and was associated with an increased risk of disease ($p < 0.05$). MTHFR 1298AC+CC genotypes were associated with higher Hcy levels. IMT was also significantly higher in patients with the 1298AC+CC genotypes ($p < 0.05$). Conclusion: A1298C polymorphism of MTHFR gene appears to be associated with the severity of carotid atherosclerosis and co-occurrence of MTHFR polymorphisms has a synergistic effect on increased risk of disease susceptibility. ***J Clin Basic Cardiol 2012; 15 (online): 7–12.***

Key words: *A1298C, C677T, MTHFR, end-stage renal disease, children, single nucleotide polymorphism*

Introduction

Hemodialysis (HD) patients have a much higher overall mortality rate than the general population [1, 2]. Cardiovascular disease (CVD), a frequent complication in these patients, is a major cause of the mortality [3]. In this setting, important risk factors for cardiovascular disease include hypertension, diabetes mellitus, and increased oxidant stress [4]. Hyperhomocysteinemia has recently been identified as a predictor of atherosclerotic complications in the general population [5–8]. It is also frequent among patients with renal failure [9, 10]. Several recent studies showed that in dialysis patients, hyperhomocysteinemia was a risk factor for cardiovascular complications [10–12].

Serum and intracellular levels of homocysteine (Hcy) are regulated by remethylation to methionine or transsulfuration to cysteine [13]. The methyl donor in the vitamin B_{12}-dependent remethylation is 5-methyltetrahydrofolate generated from the reduction of 5,10-methylenetetrahydrofolate by the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR). Elevated plasma concentrations of total Hcy (tHcy) result chiefly from genetic defects in the enzymes involved in homocysteine metabolism or from nutritional deficiency of the vitamin cofactors [14]. Because renal uptake and metabolism normally account for about 70 % of Hcy elimination from plasma [10, 15], impaired renal function also results in hyperhomocysteinemia [9, 10]. Hcy causes endothelial cell dysfunction and injury via production of potent reactive oxygen species during its autooxidation [14, 16]. This metabolite is thrombogenic, as it increases thromboxane formation, antagonizes nitric oxide, enhances platelet aggregation, and inhibits protein C and thrombomodulin [14, 16]. Hcy also is a potent mitogen for vascular smooth muscle cells [17, 18]. Therefore, in end-stage renal disease, elevated plasma Hcy concentrations could contribute to the high prevalence of cardiovascular disease and the increased mortality rate [10–12, 19]. Hyperhomocysteinemia at an earlier stage could also accelerate progression of chronic renal disease [19].

Two polymorphisms, MTHFR C677T and MTHFR A1298C, decrease MTHFR activity and tend to increase Hcy levels [20]. A common C-to-T mutation at nucleotide position 677 (C677T) has been identified in the gene coding for MTHFR, which is involved in the remethylation of Hcy [20]. The C677T mutation causes a valine for alanine substitution, which decreases MTHFR activity and tends to elevate plasma concentrations of tHcy in individuals who are homozygous for the mutation (TT genotype) [20]. This genotype also has a reported association with coronary heart disease [21, 22], but this remains controversial [23]. In subjects with normal renal function, the TT genotype causes only a 25-% increase in plasma tHcy compared to subjects with other genotypes [23], but in patients with end-stage renal disease undergoing maintenance dialysis, the TT genotype causes a 40–100-% increase in plasma tHcy [19, 24, 25] compared to other genotypes that already have 2–3 times higher concentrations of tHcy than normal subjects. The compound heterozygosity of MTHFR A1298C polymorphism with other MTHFR polymorphisms has been associated with either increased or decreased Hcy levels [20].

In this study, we examined the association of these 2 polymorphisms of the MTHFR gene, ie, C677T and A1298C, with ESRD in children with hyperhomocysteinemia and intimal medial thickness TMT).

Methods

55 Egyptian pediatric patients with end-stage renal disease on hemodialysis were included in the study. HD children were selected from the hemodialysis unit of the Center of Pediatric Nephrology and Transplantation (CPNT), Children's Hospital, Cairo University. The study was performed from Janu-

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Table 1. Demographic profile of the study population

	Maintenance hemodialysis	Controls
n	55	55
Age (years)	10.62 ± 3.49	10.7 ± 4.51
Gender		
Male	29 (52.73 %)	30 (54.55 %)
Female	26 (47.27 %)	25 (45.45 %)
BMI (kg/m^2)	18.89 ± 3.00	20.60 ± 1.44
SBP (mmHg)	126.13 ± 17.36*	94.54 ± 9.70
Indexed SBP	1.03 ± 0.14**	0.73 ± 0.05
DBP (mmHg)	85.13 ± 13.76*	62.55 ± 9.10
Indexed DBP	1.00 ± 0.10**	0.75 ± 0.05
eGFR (ml/min/1/1.73m^2)	11.20 ± 3.35**	87 ± 8.8
Dialysis (years)	2.74 ± 1.58	–
Kt/V	1.68 ± 0.45	–
Creatinine (mmol/l)	567.5 ± 128.2*	63.65 ± 28.28
Predialysis urea (mmol/l)	25.19 ± 7.00*	2.77 ± 0.90
Total cholesterol (mg/dl)	193.04 ± 50.37*	160.31 ± 17.75
Albumin (g/dl)	3.68.35*	4.93.39
Calcium (mmol/l)	2.48 ± 0.10*	2.57 ± 0.42
Serum phosphate (mmol/l)	1.26 ± 0.15**	1.63 ± 1.10
Hemoglobin (g/dl)	10.53 ± 1.83*	14.20 ± 1.50
Homocysteine (μmol/l)	35.21 ± 37.24*	16.11 ± 14.50
CIT (mm)	0.49 ± 0.09**	0.40 ± 0.03

BMI: Body Mass Index; SBP: systolic blood pressure; DBP: diastolic blood pressure; eGFR: estimated glomerular filtration rate; Kt/V: adequacy of hemodialysis; CIT: carotid intimal thickness.
*p < 0.05 or **p < 0.01 vs controls and HD

ary 2011 to June 2011. The causes of renal failure were: hereditary nephropathies (n = 21), obstructive uropathies (n = 6), neurogenic bladder (n = 4), glomerulopathy (n = 3), renal hypoplasia or dysplasia (n = 2), and unknown causes (n = 19). Inclusion criteria for HD patients included a constantly elevated serum creatinine level above the normal range (ranging from 3.4–15.8 mg/dl) and dialysis for at least 6 months. They were treated with hemodialysis for 3–4 h 3 times weekly with a polysulfone membrane using bicarbonate-buffered dialysate. Duration of hemodialysis was 2.82 ± 1.37 years. 31 HD patients were taking anti-hypertensive treatment. The following classes of drugs were employed: α-adrenoceptor antagonists in one HD patient, betablockers in 9, ACE inhibitors in 17, and calcium channel blockers in 4.

To control for differences in age and body size, blood pressures were indexed to the age-, gender-, and height-specific 95th percentile for each subject (measured systolic [SBP] or diastolic blood pressure [DBP] was divided by the age-, gender-, and height-specific 95th percentile). Hypertension was defined as indexed SBP or DBP ≥ 1.0. None of HD patients had cardiovascular events on the basis of examination and detailed clinical history.

All control subjects (n = 55) were healthy with no clinical signs of vascular or renal disease and no family history of renal disease as assessed by medical history and clinical examination, as well as a lack of medications taken at the time of the study. Healthy control subjects were selected to be matched for age and gender to the patient groups, as well as within the same BMI limits. They were collected from the pediatric clinic (apart from the medical services unit) of the National Research Centre (NRC) which is one of the biggest research centers in Egypt. Informed consent for genetic studies was obtained from the parents of all participants. The protocol of the study was read and approved by the ethics committee of the NRC in Egypt.

Biochemical Markers

Venous blood samples were collected in the morning after an overnight fast on a midweek dialysis day before the dialysis session. Three ml of venous blood was collected in EDTA vials for the extraction of genomic DNA. Pre- and post-dialysis kidney function tests were determined by standard laboratory methods. Hemoglobin, albumin, serum calcium, serum phosphorous, blood urea nitrogen, and creatinine were measured for all patients and controls using an automatic biochemistry analyzer (Olympus America Inc, Center Valley, PA, USA).

Quantitative Determination of Homocysteine Levels in Serum

The Diazyme homocysteine enzymatic assay is based on a novel assay principle that assesses the co-substrate conversion product instead of assessing the co-substrate itself. The concentration of homocysteine in the sample is indirectly proportional to the amount of NADH converted to NAD+.

Table 2. MTHFR polymorphisms: genotype and allele frequency in ESRD children and controls

	HD	Controls	OR	95-% CI	p
n	55	55	–	–	–
Genotypes					
677CC	28 (50.91 %)	35 (63.64 %)	0.97	0.72–1.32	0.88
677CT	17 (30.91 %)	20 (36.36 %)	1.04	0.62–1.73	0.87
677TT	10 (18.18 %)*	0 (00.00 %)	0.46	0.32–0.65	0.002
1298AA	15 (27.27 %)	44 (80.00 %)	0.32	0.20–0.52	0.52
1298AC	28 (50.91 %)	8 (14.55 %)	0.87	0.19–3.88	0.86
1298CC	12 (21.82 %)**	3 (5.45 %)	0.09	0.03–0.26	0.0001
Alleles					
677C	73 (66.36 %)	90 (81.82 %)	0.70	0.54–0.92	0.16
677T	37 (33.64 %)	20 (18.18 %)	1.52	1.04–2.21	0.17
1298A	58 (52.73 %)	96 (87.27 %)	2.92	1.81–4.75	0.35
1298C	52 (47.27 %)**	14 (12.73 %)	0.16	0.08–0.32	0.0001

OR: odds ratio: CI: confidence interval. *P < 0.05 or **p < 0.001.

The kit was supplied by Diazyme laboratories (USA), catalog # D Z568A [26].

Intima-Media Thickness Measurement
Carotid artery ultrasound was carried out to measure (IMT) using the Vivid 3 (Norway) ultrasonography machine with 3- and 7-MHz linear array transducers. Imaging protocols were followed as described by Sidhu et al [27].

Genotyping of MTHFR Gene Variants
DNA was extracted using the spin column QIAamp blood kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) amplification and enzymatic digestion of the products were done. For the methylene tetrahydrofolate reductase C677T polymorphism genomic DNA of 0.5–2 mg was amplified each with a forward primer '50 TGAAGGAGAAGGTGTCTGC GGGA-3' and a reverse primer '50 AGGACGGTGCGGT GAGAGTG-3' (supplied by Biosynthesis). PCR was performed with a Biometra thermoblock under standard conditions, for 40 cycles and with 72 °C as annealing temperature. After amplification, the PCR product was digested with restriction endonuclease HinfI and electrophoresed in a 4–% agarose gel. With the enzyme HinfI (Fermentas, Lithuania), the respective genotypes were defined as:
1. Wild type (677CC; indicating the absence of the restriction site)
2. Heterozygote (677CT) produced 198, 175, and 23 base pair fragments
3. Homozygous mutants (677TT) produced 175 and 23 base pair fragments

For the methylene tetrahydrofolate reductase A1298 C polymorphism genomic DNA of 0.5–2 mg was amplified each with a forward primer '50 CTTTGGGGAGCTGAAGGAC TACTAC-3' and a reverse primer '50 CACTTTGT GACCATTCCGGTTTG 3' (supplied by Biosynthesis). PCR was performed with a Biometra thermoblock for 35 cycles and with 72 °C as annealing temperature. After amplification, the PCR product was digested with restriction endonuclease MboII and electrophoresed in a 3–% agarose gel with the enzyme MboII (Fermentas, Lithuania). The respective genotypes were defined as:
1. Wild type (1298AA) if they produced 5 fragments of 56, 31, 30, 28, and 18 base pair
2. Heterozygote (1298AC) if they produced 6 fragments of 84, 56, 31, 30, 28, and 18 base pair
3. Homozygous mutants (1298CC) if they produced 4 fragments of 84, 31, 30, and 18 base pair.

The major visible bands were those at 84 and 56 base pair [28].

Statistical Analysis
The Statistical Package for Social Science (SPSS) program, version 16.0, was used for data analysis. Data were summarized as mean ± SD, range, or percentage. Histograms and normality plots were used for evaluating the normality of data. For those data with skewed distribution, log transformation was performed before a t-test.

A sample size of 20 will give us approximately 80 % power (alpha = 0.05, 2-tail) to reject the null hypothesis of zero correlation. We used power calculations performed by the Power and Precision program (Biostat) to determine the number of chromosomes required to detect a significant difference between the polymorphism frequency in the reference population and the expected frequency. Data were evaluated between the experimental groups by independent samples t-test. Allele and genotypic frequencies for MTHER C677T and MTHFR A1298C alleles were calculated with the gene-counting method. Hardy-Weinberg equilibrium was tested by using the Pearson Chi-square (χ^2) test. A 2 × 2 contingency table was used for test of the differences of allele frequencies between cases and controls. Odds ratios (OR) with 95-% confidence intervals (CI) were estimated for the effects of high-risk alleles. Clinical characteristics of ESRD children with different MTHER C677T and MTHFR A1298C genotypes were compared using an independent t-test. $P < 0.05$ was considered statistically significant.

Results

Anthropometric, clinical, and biochemical parameters in controls and ESRD children are shown in Table 1. Hcy levels were significantly higher in the patient group compared to controls ($p < 0.05$).

Prevalence of MTHFR Genotypes
The genotype and allele frequencies for the MTHFR C677T and A1298C polymorphisms are shown in Table 2. The population was in Hardy-Weinberg equilibrium (HWE) for MTHFR C677T and MTHFRA1298C genotypes. The homozygous mutant of MTHFR677 TT genotype was significantly higher in our study population ($p < 0.05$). The fre-

Table 3. Association of various parameters with different MTHFR genotypes in ESRD children.

	677CC	677CT+TT	1298AA	1298AC+CC
n	28	27	15	40
BMI (kg/m^2)	18.97 ± 2.77	18.75 ± 3.52	18.53 ± 3.01	17.16 ± 3.06
SBP (mmHg)	116.32 ± 18.62	124.23 ± 19.01	119.68 ± 18.88	123.57 ± 19.85
DBP (mmHg)	76.94 ± 13.59	85.00 ± 15.02*	79.57 ± 13.49	85.32 ± 14.25*
Creatinine (mmol/l)	442.00 ± 174.15	571.95 ± 140.56*	476.48 ± 165.31	581.67 ± 148.51
Urea (mmol/l)	24.88 ± 7.40	24.34 ± 8.08	25.17 ± 7.28	25.39 ± 4.37
Albumin (g/dl)	3.67 ± 0.28	3.44 ± 0.27	3.73 ± 0.27	3.55 ± 0.29
Calcium (mmol/l)	2.32 ± 0.42	2.36 ± 0.31	2.41 ± 0.44	2.38 ± 0.31
Phosphate (mmol/l)	1.63 ± 1.11	1.51 ± 1.22	1.02 ± 1.08	1.54 ± 1.25
Cholesterol (mg/dl)	188.78 ± 55.03	198.11 ± 43.07	181.55 ± 51.09	187.77 ± 53.25
CIT (mm)	0.49 ± 0.10	0.49 ± 0.09	0.53 ± 0.15	0.48 ± 0.07*
Homocysteine (μmol/l)	38.25 ± 36.56	42.31 ± 43.02	34.66 ± 24.85	42.34 ± 30.73*

All values in mean ± SD (Standard deviation), *p < 0.05 was considered significant.

quencies of MTHFR 1298CC genotype and mutant allele C were significantly higher in patients compared to controls ($p < 0.001$) and were associated with an increased risk of ESRD.

The association of various parameters (biochemical and clinical) with MTHFR genotypes is shown in Table 3. Increased serum creatinine levels were observed in MTHFR 677CT+TT as compared to 677CC genotype ($p < 0.05$). Mean IMT in patients with 1298AC+CC genotypes was significantly higher compared to 1298AA ($p < 0.05$). No significant change was observed in mean IMT between MTHFR 677CT+TT and 677CC genotypes. No significant association of mean plasma Hcy levels was seen between the MTHFR C677T genotype. However, patients with 1298AC+CC genotype had significantly higher levels of Hcy ($p < 0.05$) compared to the 1298AA genotype.

MTHFR C677T and A1298C Polymorphisms

The cross-tabulation of genotype combinations of MTHFR C677T and A1298C are given in Table 4. The frequency of co-occurrence of MTHFR 677CT/1298AC and 677TT/1298CC was significantly higher in patients than controls and was associated with an increased disease risk ($p < 0.05$).

Discussion

Hyperhomocysteinemia has been commonly observed in patients with chronic renal failure, owing to both altered metabolism and reduced renal excretion [29]. Significantly increased Hcy levels in ESRD patients were also observed. An association between mild hyperhomocysteinemia and occlusive vascular disease has been reported although the findings are inconsistent [30–32]. Many studies have shown that folic acid can reduce elevated homocysteine levels [33, 34] but has not yet been shown to improve the progression of atheroma or cardiovascular outcome in chronic renal failure (CRF). A recent study on the effect of high-dose folic acid on the progression of atherosclerosis and cardiovascular events in patients with CRF has shown that high-dose folic acid does not slow atheroma progression or improve either cardiovascular morbidity or mortality in patients with CRF [35]. Further interventional studies are needed to see if folate/vitamin supplementation could have beneficial effects in patients with chronic renal failure.

Significantly higher serum PO4 and calcium levels were observed in ESRD patients in our study. Similar observations have been made by others in patients with chronic renal failure [36]. The increased levels of Ca/PO4 have been suggested to contribute to arterial calcium deposition in these patients. We observed increased carotid IMT in ESRD children, which further supports a possible association of high serum Ca/PO4 with cardiovascular disease in these patients. In an independent sample t analysis, serum calcium phosphate levels were similar in all MTHFR genotypes, however, IMT showed association with MTHFR 1298AC+CC genotypes. Thus these observations do not suggest a possible association between MTHFR genotypes and arterial calcifications, but further studies are needed with larger sample size to confirm these findings.

Polymorphisms in MTHFR gene have been shown to be associated with increased homocysteine levels and possibly an effect on cardiovascular mortality. Patients with chronic kidney diseases who are on dialysis are known to have increased plasma Hcy concentrations. However, there is inconsistent evidence of any association between MTHFR genotypes and ESRD. We studied the respective frequencies and co-occurrence of 2 functional MTHFR polymorphisms (C677T and A1298C), associated with plasma Hcy concentrations in ESRD children. We found that the homozygous mutant of MTHFR677 TT genotype was significantly higher in our study population and is associated with an increased disease risk; however, the highest disease risk was observed with the 1298CC genotype. The MTHFR C677T polymorphism has been inconsistently shown to be associated with chronic kidney diseases and renal failure in different ethnic populations. Previous studies in many ethnic groups including Chinese [37], Polish [38, 39], and Japanese [40] have demonstrated that MTHFR C677T may be a risk factor for renal diseases [41]. However, others failed to find such an association [42].

The MTHFR A1298C polymorphism is associated with decreased enzyme activity and could result in increased Hcy levels, suggesting a possible role in the pathogenesis of chronic renal failure [43]. The homozygous wild genotype (AA1298) has been reported to be protective in patients with diabetic nephropathy in the presence of low folate levels [44], however, others have not found any association of A1298C genotypes alone or in combination with plasma Hcy levels in hemodialysis patients [45].

The frequency of the variant C allele of 1298 gene was significantly higher in our pediatric population with ESRD. Fung et al [46] reported that the MTHFR-coding polymorphism at A1298C is associated with renal decline in African-Americans with hypertensive nephrosclerosis. We also observed significantly higher Hcy levels in patients with AC+CC genotypes. Thus, a higher frequency of CC genotype present in our population makes it extremely relevant in terms of its potential impact on ESRD in our pediatric population.

Intimal thickening is an adaptive process of the arterial wall, presumably related to atherogenesis and increased IMT of the carotid arteries has been considered a useful marker of atherosclerosis and cardiovascular complications [27, 47, 48]. We found that patients with 1298AC+CC genotypes had significantly higher IMT as compared to 1298AA genotype, which suggests that these genotypes may be predisposing these patients to higher risk of CVD.

Co-occurrence of 1298CC with 677CC genotype has also been shown to be associated with an increased cardiovascular disease risk in hemodialysis patients [44]. Koupepidou et al

Table 4. Combined genotype frequencies of MTHFR C677T and A1298C in pediatric patients and controls, n (%).

	Patients			Controls		
n_{tot}	55			55		
	MTHFR677CC	MTHFR677CT	MTHFR677TT	MTHFR677CC	MTHFR677 CT	MTHFR677TT
MTHFR1298AA	10 (7.27)	2 (20)	3 (3.64)	30 (54.55)	14 (21.82)	0 (0.00)
MTHFR1298AC	8 (21.82)	14 (27.27)*	6 (5.45)*	5 (18.18)	3 (3.63)	0 (0.00)
MTHFR1298CC	10 (7.27)	1 (3.64)	1 (3.64)*	0 (0.00)	3 (1.82)	0 (0.00)

*$p < 0.05$

observed that essential hypertensive patients with 677CT/1298AC genotypes were predisposed to develop hypertensive nephrosclerosis and chronic renal failure [49]. Födinger et al demonstrated that combined the MTHFR677TT/1298AA genotype was a predictor of total Hcy plasma levels in dialysis patients [43]. Thus, the increased risk with these genotypes may be due to their influence on Hcy levels [50]. We found that co-occurrence of MTHFR 677CT/1298AC and 677TT/1298CC was significantly higher in patients than in controls and was associated with an increased disease risk.

The present study has certain limitations. The source pediatric population of the study was an Egyptian population only, which renders generalizability to the other ethnic groups uncertain. Future studies are required to analyze the various MTHFR genotypes with disease prevalence in different ethnic pediatric populations.

Conclusion

Our results suggest that the presence of mutant alleles 1298C and 677T confers an increased risk for ESRD in children and a co-occurrence of these polymorphisms has a synergistic effect on increased risk of disease susceptibility.

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Conflict of Interest

The authors declare that they have no competing interests.

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