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The *MTHFR* C677T polymorphism is associated with mitral valve rheumatic heart disease

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Abstract. – OBJECTIVE: Rheumatic heart disease (RHD) is a serious complication of rheumatic fever (RF). Plasma homocysteine (Hcy) levels are increased in RHD patients. MTHFR catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate and plays a vital role in Hcy metabolism. We hypothesize that the *MTHFR* C677T polymorphism is associated with a risk of RHD.

PATIENTS AND METHODS: Eighty-six patients with RHD and 130 matched controls without a history of RHD were eligible for the study. The diagnosis of RHD was made according to modified Jones' criteria and echocardiography. Using echocardiography, RHD patients were further divided into mitral valve lesion (MVL) and combined valve lesion (CVL) groups. *MTHFR* C677T polymorphisms were genotyped by DNA sequencing. The chi-squared test was used to evaluate differences in genotypes.

RESULTS: Control genotypes were in Hardy-Weinberg equilibrium. The C677T homozygous genotype (OR = 4.09; 95% CIs 1.16-14.44; p = 0.020) and recessive model (TT *vs*. CC+CT; OR = 4.05; 95% CIs 1.17-14.04; p = 0.019) were significantly associated with MVL RHD.

CONCLUSIONS: This is the first study to investigate the association between the *MTHFR* C677T polymorphism and risk of RHD. The *MTHFR* C677T polymorphism is associated with RHD in patients with MVLs, perhaps via an Hcy-mediated cytokine effect.

Key Words:
Rheumatic heart disease, Mitral valve lesion, *MTHFR*, C677T polymorphism.

Introduction

Rheumatic heart disease (RHD) is a serious complication of rheumatic fever (RF) caused by an autoimmune reaction to Group A β-hemolytic streptococci. RHD can result in serious sequelae such as valve stenosis, arrhythmias, atrial dilation, and ventricular dysfunction[1]. Although the incidence and prevalence of RHD have decreased in developed nations since the early 1900s, RHD continues to be a major health hazard in many developing countries and in sub-Saharan Africa[2]**.** The prevalence of RHD is 24 per 10,000 children aged between six and 15 years in the Western district of Saudi Arabia, with the highest rates in rural areas and in females[3].

The mitral valve is affected in up to 50% of RHD cases[4,5], and mitral valve disease is a significant global public health concern[2]. In young patients, mitral regurgitation predominates, but mitral stenosis becomes progressively more common with age[6]. The exact pathogenesis of RHD remains poorly understood but is likely to involve a combination of environmental, genetic, and immunological factors. Recent studies have demonstrated that plasma homocysteine levels are higher in RHD patients than in controls[7,8].

Methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20) is one of three main homocysteine (Hcy) regulatory enzymes. MTHFR catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate and regulates the intracellular flow of folate via the conversion of Hcy to methionine[9]. *MTHFR* maps to chromosome 1p36.3[10], and two functional single nucleotide polymorphisms (SNPs) have been reported (C677T and A1298C) that alter MTHFR activity. C677T changes an alanine to valine at amino acid 222 (A222V; rs1801133) to produce thermolabile MTHFR[11,12]. 677TT homozygotes have a 50-60 percent reduction in MTHFR activity and raised plasma Hcy levels[13]. The C677T

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polymorphism has been associated with susceptibility to vascular disease[11], congenital heart disease[14-16], coronary artery disease[17,18], type 2 diabetes mellitus with diabetic retinopathy[19], common carotid atherosclerosis[20], Alzheimer's disease[21], oral clefts[22], and several cancers[23-25].

Therefore, Hcy levels are raised in patients with RHD, Hcy can produce free oxygen radicals and enhance inflammation[7,26], and there is strong evidence to support a relationship between *MTHFR* polymorphisms and Hcy expression. However, the role of *MTHFR* polymorphisms in RHD has not been evaluated to date. We therefore conducted a case-control study to test the hypothesis that *MTHFR* C677T polymorphisms are associated with the risk of developing RHD.

Patients and Methods

Patients

In total, 216 unrelated individuals of Saudi Arabian origin were recruited for this case-control study: 86 patients with RHD and 130 age, gender, and ethnicity-matched unrelated healthy volunteers without a history of rheumatic fever or autoimmune disease as controls. Subjects were recruited via The Centre for Genetics & Inherited Diseases (CGID), Taibah University and the Maternity & Children Hospital, Madina, Kingdom of Saudi Arabia. Institutional Ethics Committees of both institutions approved the study protocol. All adult study subjects gave informed consent and consent was provided by the parents or legal guardians of participants less than 18 years old.

Complete clinical and laboratory investigations were performed in all patients. The initial diagnosis of RHD was made based on modified Jones' criteria[27] and further confirmed by echocardiography. Based on the echocardiographic findings, the RHD was further divided into mitral valve lesion (MVL) or combined valve lesion (CVL) groups. Patients with suspected or definitive rheumatic fever but without valve involvement, heart complications, or other inflammatory conditions were excluded from the study.

Genotyping

Genomic DNA was extracted from 2 ml whole peripheral blood using the QIAamp DNA Mini Kit (Qiagen, Venlo, Netherlands). Extracted DNA was quantified by spectrophotometry (MaestroNano; MaestroGen, Las Vegas, NV, USA) followed by dilution to 10 ng/μl (working concentration) in standard Tris-EDTA buffer. The C677T region of the *MTHFR* gene was amplified using the primers and protocol described in[28]. Briefly, PCR reactions were carried out in a total reaction volume of 10 μl in thin-walled tubes containing 1.0 μl PCR buffer (10X), 1.0 μl $MgCl_2$ (25 mM), 1.0 μl dNTPs (10 mM), 2.0 pM each of the forward (5'-CAT CCC TAT TGG CAG GTT ACC C-3') and reverse (5'-GGG AAG AAC TCA GCG AAC TCA G-3') primers, 1.0 unit of Taq DNA polymerase (Applied Biosystems; Life Technologies, Carlsbad, CA, USA), and 40 ng genomic DNA. PCR amplifications were carried out using the ABI Veriti thermal cycler (Applied Biosystems). The amplified products were directly sequenced using BigDye™ chain termination chemistry on the ABI 3500 DNA analyzer (Applied Biosystems) as previously described[29]. Multiple alignment and sequence analyses were performed using Auto Assembler Software (Applied Biosystems).

Statistical Analysis

Allele frequencies were estimated using the gene count method. Genotype frequencies were assessed for Hardy-Weinberg equilibrium using the goodness-of-fit χ^2 test. Associations between genotypes and *MTHFR* C677T alleles and RHD were analyzed using the $\chi 2$ and Fisher's exact tests. Two-sided *p* values of less than 0.05 (95% level of confidence) were considered significant for statistical inference, and odds ratios and 95% confidence intervals (CIs) were calculated. All statistical analyses were performed using SPSS software (IBM® SPSS® Statistics 17, Chicago, IL, USA).

Results

The distributions of *MTHFR* C677T SNP genotypes and alleles in RHD and control groups are presented in Table I. The genotype proportions were 70.3% CC, 23.1% CT, and 6.6% TT in cases and 70.8% CC, 25.4% CT, and 3.8% TT in controls. The T allele frequency was 18.1% in cases and 16.5% in controls. The genotype distribution in the control group was in HardyWeinberg equilibrium (p=0.359). There were no statistical differences in genotype or allele frequencies between RHD cases and controls (Table II).

ORs and 95% CIs were calculated to assess the impact of the *MTHFR* C677T SNP on RHD

Table I. Genotype distribution and allele frequencies of the *MTHFR* 677C>T SNP in rheumatic heart disease.

	Control (%)	Overall RHD (%)	CVL (%)	MVL (%)
Genotype distribution				
CC	92 (70.8)	64 (70.3)	33 (76.7)	27 (62.8)
CT	33 (25.4)	21 (23.1)	10 (23.3)	10 (23.3)
TT	5 (3.8)	6 (6.6)	0 (0.0)	6 (14.0)
Allele frequency				
C allele	217 (83.5)	149 (81.9)	76 (88.4)	64 (74.4)
T allele	43 (16.5)	33 (18.1)	10 (11.6)	22 (25.6)
Test for HWE				
Chi-square	0.842	4.513	0.745	6.514
p-value	0.359	0.034	0.388	0.011

risk (Table I). An increased risk of RHD was observed in patients with the homozygous genotype (OR=1.73; 95% CIs 0.51-5.59; *p*=0.379) and also in the recessive 677T variant model (TT vs. CC+CT) (OR=1.76; 95% CIs 0.52-5.97; *p*=0.355); however, these were not statistically significant. In a subgroup analysis, the mutant homozygous genotype (OR=4.09; 95% CIs 1.16-14.44; *p*=0.02) and the recessive model (TT *vs.* CC+CT; OR=4.05; 95% CIs 1.17-14.04; *p*=0.019) were significantly associated with the MVL group (Table II). *MTHFR* C677T variants were not associated with CVL risk (Table II), and the distributions of *MTHFR* genotypes in different phenotypes were not significantly different (Table II).

Discussion

In this study, 86 RHD patients and 130 unrelated controls were genotyped to identify any association between the *MTHFR* C677T polymorphism and RHD in a Saudi Arabian population. There were no statistically significant differences in genotype distribution between RHD patients and controls or between phenotypes. However, the *MTHFR* C677T polymorphism was significantly associated with an increased risk of MVLs, but not CVLs, in RHD patients.

A number of studies have demonstrated that *MTHFR* polymorphisms are associated with various heart disease, autoimmune diseases, and cancer. *MTHFR* C677T is associated with pulmonary valve stenosis and aortic valve stenosis/subaortic stenosis in congenital heart disease[30], multiple sclerosis[31], rheumatoid arthri-

Table II. Results of association tests with *MTHFR* 677C>T SNP in rheumatic heart disease.

MTHFR 677C>T	OR (95% CI)	*p*-value
Overall RHD		
CC	Reference	
CT	0.92 (0.49-1.72)	0.783
TT	1.73 (0.51-5.89)	0.379
CT+TT vs. CC	1.02 (0.57-1.84)	0.944
CC+CT vs. TT	1.76 (0.52-5.97)	0.355
C allele	Reference	
A allele	1.12 (0.69-1.84)	0.662
CLV		
CC	Reference	
CT	0.85 (0.38-1.90)	0.682
TT	-	-
CT+TT vs. CC	0.73 (0.33-1.64)	0.448
CC+CT vs. TT	-	-
C allele	Reference	
A allele	0.66 (0.32-1.39)	0.273
MVL		
CC	Reference	
CT	1.03 (0.45-2.36)	0.940
TT	4.09 (1.16-14.44)	0.020
CT+TT vs. CC	1.44 (0.70-2.96)	0.328
CC+CT vs. TT	4.05 (1.17-14.04)	0.019
C allele	Reference	
A allele	1.73 (0.97-3.11)	0.063

Table III. *MTHFR* 677C>T SNP Genotype distribution in different phenotypes.

Phenotypes	status	CC	CT	TT	Chi square *p* value
Carditis	Yes	47	19	5	
	No	17	2	1	0.457
Arthritis	Yes	42	16	4	
	No	22	5	2	0.788
Subcuteneous nodules	Yes	1	1	0	
	No	63	20	6	0.766
Chorea	Yes	5	1	1	
	No	59	20	5	0.722
Erythema Marginatum	Yes	2	1	0	
	No	62	20	6	0.855
Acute phase reaction	Yes	52	16	4	
	No	12	5	2	0.744

tis[32], alopecia areata[33], and thyroid cancer[25]. We hypothesized that *MTHFR* would be a marker of susceptibility to RHD, since C677T allele carriers have been shown to have increased blood Hcy levels, Hcy levels are elevated in patients with acute rheumatic carditis and RHD[7], and plasma Hcy levels are strongly associated with the severity of coronary heart disease[34]. Hcy can undergo auto-oxidation in plasma and produce free oxygen radicals, thereby enhancing endothelial tissue damage and inflammation[12,26]; in this way, Hcy might contribute to the pathogenesis of RHD. Significantly higher Hcy levels have been detected in inflamed tissues or lesions than in the plasma itself, indicating that Hcy might be directly responsible for cellular and tissue damage and is pathogenetic in various immune disorders[35]. Chronic exposure of tissues and circulating cells to Hcy may also compound immune dysfunction and pathology[36].

Since increased serum Hcy levels are associated with active inflammation, one plausible mechanism for valve damage in RF may be via Hcy-mediated cytokine production. IL-8 and IL-6 are well established risk factors for the development of mitral valve RHD, and production of these cytokines is associated with elevated levels of Hcy in various cardiovascular diseases: hyperhomocysteinemia can upregulate plasma MCP-1 and IL-8 levels and promote the initiation and progression of atherosclerosis and venous thrombosis[37], while inflammation may be enhanced in homocysteine-related cardiovascular disease, possibly via IL-6-related mechanisms[38]. Angiotensin-converting enzyme (*ACE*) I/D polymorphisms are significantly associated with the development of valvular lesions in RHD patients[39], while patients with combined *MTHFR* TT+*ACE* ID alleles are three-times more susceptible to ischemic stroke than controls, suggesting that these two polymorphisms have epistatic effects[40]. Together with our study, these data support our hypothesis that the *MTHFR* C677T polymorphism might raise plasma Hcy levels and contribute to the pathogenesis of mitral RHD via a cytokine-mediated mechanism. However, our study was limited by having a relatively small sample size and plasma Hcy levels were not measured. Further work is needed to establish the underlying immunological mechanisms and a direct link with homocysteine expression.

Conclusions

To our knowledge, this is the first study to investigate the association between the *MTHFR* C677T polymorphism and susceptibility to RHD and its clinical features. The presence of a T allele increases the risk of developing MVL-pattern RHD; we postulate that this was via an Hcy-mediated cytokine effect. Larger studies are required in different ethnic backgrounds and with Hcy measurements to confirm the link between *MTHFR* C677T gene polymorphisms and risk of RHD.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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