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Original Research

The c.677C>T polymorphism in the MTHFR gene associated with hypothyroidism

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Abstract

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Methyl tetrahydrofolate reductase (MTHFR) catalyzes the formation of tetrahydrofolate from folate. Mutation in the MTHFR gene may be the potential cause of hypothyroidism. We aimed to assess the genetic association of c.677C>T and c.1298A>C polymorphisms in the MTHFR gene with hypothyroid and hyperthyroid conditions. We registered 100 participants; 50 were clinically diagnosed with thyroid dysfunction and a control group of 50 individuals. All the samples were analyzed for serum TSH, T3, and T4 values. DNA was extracted and PCR was carried out to amplify exon 4 and 7. Restriction fragment length polymorphism (RFLP) was performed for genotype C677T and A1298C alleles and confirmed by sequencing. Serum values confirmed that 32/50 patients were with hypothyroidism and 18/50 with hyperthyroidism. Genotyping data showed no deviation from Hardy-Weinberg equilibrium in the three groups. The c.677C>T polymorphism was found significantly associated (p-value=0.021) with hypothyroidism while no significant association (p-value=0.365) with hyperthyroidism. The genotype data of allele c.1298A>C showed no significant association with hypothyroidism (p-value=0.324) and hyperthyroidism (p-value=0.303) patients. This study concludes that c.677C>T polymorphism in the MTHFR gene is possibly associated with hypothyroidism in Pakistani thyroid disease patients and can be a potential genetic marker for early risk assessment.

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Introduction: Hypothyroidism (under-active thyroid state) and hyperthyroidism (hyper-active thyroid state) are the most common endocrine abnormalities, influencing almost every metabolic process in the human body. The worldwide prevalence of both hypothyroidism and hyperthyroidism varies from country to country. In Pakistan, the prevalence of the former is 4.1% and that of the latter is 1.2% (1). Moreover, these conditions are more common among women than men. People above the age of 55 or 60 more commonly suffer from hypothyroidism. The usual onset of hyperthyroidism is between 20 to 50 years of age (2). There are plenty of causes of these two clinical conditions but the leading cause of hypothyroidism is Hashimoto's Thyroiditis and that of hyperthyroidism is Graves' disease. Hashimoto Thyroiditis and Graves' disease are again counted among common autoimmune human diseases, with a prevalence of 2% in countries where iodine intake is sufficient (3). The subclinical disease condition characterized by the production of anti-thyroid antibodies with the lack of clinical features has an even higher prevalence (4, 5). Several genes are associated with the thyroid gland. The MTHFR or 5,10-methylenetetrahydrofolate reductase is a protein-coding gene that encodes for 70 kDa (6), NADPH-dependent enzyme known as MTHFR. The MTHFR enzyme controls the catalysis of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, an active form of folate. This 5-methyltetrahydrofolate then acts as a co-substrate for re-methylation of homocysteine to methionine (7). The MTHFR gene performs a variety of molecular functions and controls numerous biological processes in the human body. The important functions include MTHFR enzyme activity and binding of FAD, NADP, protein complex, and, modified amino acids. There are two most common polymorphisms including c.677C>T and c.1298A>C. The c.677C>T polymorphism, also known as rs1801133, C677T, or Ala222Val, is the most common and extensively studied mutation of the MTHFR gene. The Ala222Val polymorphism causes major conformational changes in the MTHFR protein and a significant reduction in the FAD-binding affinity resulting in the alteration of the secondary, tertiary, and quaternary structure (8). This lead to the thermoliable of the MTHFR enzyme. The other common polymorphism is c.1298A>C, also known as rs1801131, A1298C or E429A. This change in the MTHFR gene also reduces the enzyme activity but, this reduction is not as dramatic as caused by c.677C>T (9). But c.1298A>C does not cause the thermolabile enzyme. These mutations are associated with multiple disorders and clinical conditions. The two polymorphisms may co-exist and enhance the effect of each other and the condition is known as compound heterozygous state. The decreased activity of MTHFR protein due to Ala222Val and Glu429Ala is mainly of serine phosphorylation sites (10). This provides insights into the structural-functional relationship of the most common polymorphism of the MTHFR gene. Mutation in the MTHFR gene may be the potential cause of hypothyroidism, so screening for this mutation is recommended (11). We designed this study to screen the prevalence of c.677C>T and c.1298A>C polymorphisms in the MTHFR gene and their association with thyroid disorder.

Methodology: This study was conducted after the approval from the Departmental Ethical Committee, Virtual University of Pakistan. The current study included two groups; the diseased and the controls. We registered 100 participants at random; 50 clinically diagnosed with thyroid dysfunction having no other associated disease, and a control group of 50 individuals without family history after informed consent. Blood samples (2-3 mL) were collected from the registered participants for serum thyroid function tests. About 1mL of blood from the sample was used for thyroid function test while the remaining was collected in the vacutainers containing EDTA as an anticoagulant. Thyroid profile testing was done using highly sensitive RIA/IRMA (radioimmunoassay/immuno-radiometric assay) procedures for serum TSH, T3 and, T4 levels (12). DNA extraction from leukocytes was performed using the standard organic method with some modifications (13). Some of the serum vacutainers were also used for genomic DNA extraction from clotted blood. The clot was separated, sheared, and homogenized for DNA extraction from cryopreserved clotted human blood (14). DNA quantification was done using NanoDrop and agarose gel electrophoresis. Primers were designed using primer3 software (accessed online from https://bioinfo.ut.ee/primer3-0.4.0/ for amplification of c.677C>T and c.1298A>C MTHFR Polymorphisms (15). Isolated DNA samples were subjected to PCR for c.677C>T and c.1298A>C MTHFR Polymorphisms. Primers, template DNA, and other reagents shown in Table 2 were added for amplification of the two alleles. Touch down PCR technique was used and amplification conditions were set in the following manner: initial denaturation at 95°C for 5 min (1 cycle); denaturation at 95°C for 30 s, annealing at 53.3°C for 30 s, extension at 72°C for 30 s (30 cycles) and final extension at 72°C for 10 min, hold at 4°C (1cycle).

The PCR product of 300 bp of c.677C>T allele and 329bp of c.1298A>C sized were obtained. The amplified product was digested for restriction fragment length polymorphism (RFLP) analysis overnight at 37°C with 10 U of TaqαI and MboII restriction endonucleases, respectively. Sanger's sequencing was performed to validate the results of RFLP analysis. Allele frequency differences in the three groups were compared with the help of Fisher's exact test. The X2 test was used to compare the genotype frequency differences between Hypothyroidism or Hyperthyroidism and the controls. The statistical significance criterion was set at $p<0.05$.

Result: The disease group constituted hypothyroidism and hyperthyroidism patients, with mean age 61.4±5.5years and 59.4±5.7 respectively. The control group included healthy individuals with normal thyroid function and having mean age of 39.07±9.342. 6.3% and 4.8% of the total study population were hyperthyroid and hypothyroid males, respectively. Whereas 11.1% and 12.7% of the study population were hyperthyroid and hypothyroid females, respectively while 19% were normal males and 27% were normal females.

We found significant differences in serum levels of TSH and T4 for control and thyroid disorder patients.

PCR products having C allele at c.677 position were not cleaved by the TaqαI enzyme, and a single fragment of 300 bp was obtained. Whereas, Samples with the T allele were cleaved by the TaqαI enzyme, generating 158 bp and 142 bp sized fragments. With heterozygous genotype or haplotype CT three fragments were obtained, i.e. 300 bp, 158 bp and 142 bp (Figure 1).

The observed MTHFR genotypic and MTHFR allelic frequencies did not show any deviation from Hardy Weinberg equilibrium, in both the controls and the hypothyroid and hyperthyroid patients. The allelic frequencies of both MTHFR c.677C>T and c.1298A>C variants are shown in table 4.

RFLP analysis shown in Table 4, compares the frequency of the MTHFR c.677 C>T polymorphism between controls, hypothyroid, and hyperthyroid patients. In controls, the C allele is present in 78% and the T allele in 22%. In hypothyroid patients, the C allele frequency is 60.93% (P = 0.021), indicating a significant difference from controls, while the T allele frequency is 39.06%. In hyperthyroid patients, the C allele is found in 69.44% (P = 0.365), and the T allele in 30.55%, showing no significant difference from controls as indicated in Figure 2.

The genotypic frequencies of the MTHFR c.677C>T and c.1298A>C polymorphisms in the control, hypothyroid, and hyperthyroid patients are shown in table 5.

Discussion: We have conducted this study to identify the association of the two most common MTHFR gene polymorphisms, c.677C>T and c.1298A>C with hypothyroidism/hyperthyroidism. The available published data suggests that there is no study conducted on an association between thyroid disorder (hypothyroidism/hyperthyroidism) and MTHFR polymorphisms in the Pakistani population. The c.677C>T is reported in multiple conditions including those associated with ischemic stroke (16), protective role for renal function (17), grave's disease (18) risk for development of lung and thyroid cancer (19, 20). Our finding of a statistically significant association between c.677C>T polymorphism and hypothyroidism supports the premise that individuals with MTHFR c.677C>T polymorphism are more susceptible to developing hypothyroidism. The association between hypothyroidism and MTHFR polymorphism, especially c.677C>T polymorphism is still quite controversial and needs more elucidation. Whereas, relating hyperthyroidism with these two common variations in the MTHFR gene, has been rarely done. The majority of earlier studies about this issue, ''an association between MTHFR polymorphisms and thyroid disorder'' do not directly involve either of these clinical states, i.e. hypothyroidism or hyperthyroidism; they rather report an association between MTHFR polymorphisms and auto-immune thyroid disorders (21), particularly the Graves' disease. (21) tried to associate autoimmune thyroid disorders, Graves' disease, and Hashimoto thyroiditis with the polymorphisms in MTHFR (c.677C>T and c.1298A>C), MTRR, and three other genes. They did not find any association between any of the MTHFR polymorphisms and thyroid autoimmunity causing hypo- or hyperthyroidism.

However present study has a finding that little contrast to their result, that c.677C>T polymorphism is associated with hypothyroidism, so it can be considered as a genetic risk linked with hypothyroidism.

Another study by (22) has reported an association between Hashimoto thyroiditis-induced hypothyroidism and two common MTHFR variants. With this finding, it became easy to support the fact that hyperhomocysteinemia and hypomethioninemia in an underactive thyroid state is a product of the alliance between reduced FAD synthesis and MTHFR c.677C>T and c.1298A>C polymorphisms. In the individuals, having c.677C>T polymorphism there is a loss of enzyme activity (with CT genotype: 40% and with TT genotype: 70%). The main function of the MTHFR enzyme is to support the synthesis of methyl donor (5 MTHF) for the re-methylation of homocysteine to methionine. Since the enzyme has lost its partial/complete functional activity due to MTHFR c.677C>T polymorphism, there will be more homocysteine and methionine, than it should normally be. This happened particularly in the case of hypothyroidism because, in a hypothyroid state there is less thyroxine, which is needed for the conversion of riboflavin into FAD; a co-factor of the MTHFR enzyme. Therefore, just like previous studies (23, 24), this study reports that thyroid status has an important role in allowing c.677C>T MTHFR polymorphism to express phenotypically; as it controls the accessibility to FAD co-factor.

On the other side, the current study also accords well with the novel idea that MTHFR variations happening within women prevent them from the development of Greaves' disease (25). No association was found between hyperthyroid patients (the majority members of the group were females, i.e. 78%) and MTHFR polymorphisms; since Graves' disease is a leading cause of hyperthyroidism, it can be assumed that c.677C>T and c.1298 A>C variants make women less susceptible to develop hypothyroidism. The root cause for the differential distribution of MTHFR genotypes and allelic frequencies for c.677C>T and c.1298 A>C polymorphisms is regional/ethnic variation (26-28). The A1298C MTHFR shows as a protective factor for the hypothyroidism disorder whereas the MTHFR C677T appears to be a risk factor (29).

A significant difference between the distribution of C and T alleles for c.677C>T MTHFR variation is identified between controls and the hypothyroidism (p= 0.02); indicating that this mutation is affiliated with the underactive thyroid state. It is concluded that hypothyroidism and c.677C>T MTHFR polymorphism are correlated; the hypothyroid state controlling the phenotypic expression of this polymorphism and polymorphism is involved in the conversion of thyroxine to triiodothyronine. An interplay between hypothyroidism and c.677C>T MTHFR polymorphism thereby, generates hyperhomocysteinemia and hypomethioninemia. There is no significant association between c.677C>T MTHFR

polymorphism and hyperthyroidism in our study. Moreover, there is possibly no significant association between c.1298A>C MTHFR polymorphism and either hypothyroidism or hyperthyroidism. But, these conclusions do require further confirmation with larger cohort sizes. We can conclude that c.677C>T polymorphism in the MTHFR gene can be considered a genetic risk factor or biomarker for predisposition to hypothyroidism.

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Table 1. Primer set 1, for c.677C>T Polymorphism, flanking a region of 261 nucleotides within the *MTHFR gene*.

ID	Sequence 5'-----3'	Tm	GC%	Length	Product Size
MTHFR-1F	GTCTCTTCATCCCTCGCCTT	59.17	55.00	20	

MTHFR-1R	TGGGGTGGAGGGAGCTTAT	59.60	57	19	300
MTHFR-2FP	CACTGCCCTCTGTCAGGAGT	60.46	60.00	20	329
MTHFR-2RP	ACAGGATGGGGAAGTCACAG	59.96	55.00	20	

Table 2. PCR reaction mixture for *MTHFR* polymorphisms

Reagent	Quantity
Sterile deionized water	13.1 µL
10x *PCR buffer (Thermo Scientific, Ltd)	2.5µL
2.5mM dNTP mix (Thermo Scientific, Ltd)	2.5µL
$MgCl_2$ (Thermo Scientific, Ltd)	3 µL
Primer 1 (10pmols/µL)	1µL
Primer 2 (10pmols/µL)	1µL
Taq DNA Polymerase, 5U/µL (Thermo Scientific, Ltd)	0.4µL
Template DNA (30-50ng/ µL)	2µL
Total	**25µL**

Table 3. One Way ANOVA for age, TSH, T3, and T4 among normal, hypothyroid, and hypothyroid study subjects

Variable	*Normal*			*Hypothyroidism*			*Hyperthyroidism*		*F(2,48)*	*P*
	M	*SD*		*M*	*SD*		*M*	*SD*		
TSH	2.08	0.9		27.57	6.9		7.39	1.4	174.62	0.000
T3	1.64	1.2		0.75	0.3		2.29	2.9	2.56	0.088
T4	97.65	22.2		70.51	26.2		135.0	52.0	11.65	0.000

Note:M=Mean, *S.D*=standard deviation, *p=significance*, *F*=a ratio of the variance

Table 4. Allelic frequencies of the *MTHFR* c.677 C>T and c.1298 A>C polymorphisms among controls and patients with thyroid disorder

Genetic Polymorphism	Controls	Hypothyroid Patients	P values	Hyperthyroid Patients	P values
***MTHFR* c.677 C>T, n (%)**					
C (%)	78 (78)	39 (60.93)	0.021	25 (69.44)	0.365
T (%)	22 (22)	25 (39.06)		11 (30.55)	
***MTHFR* c.1298 A>C, n (%)**					
A (%)	73 (73)	47 (73.43)	0.324	32 (88.88)	0.303
C (%)	17 (17)	17 (26.56)		4 (11.11)	

Table 5. Genotypic frequencies of the *MTHFR* c.677 C>T and c.1298 A>C polymorphisms among controls and patients with thyroid disorder.

Genetic Polymorphism	Controls	Hypothyroid Patients	P values	Hyperthyroid Patients	P values
***MTHFR* c.677 C>T, n (%)**					
CC	32 (64)	11 (34.37)	0.031	8 (44.44)	0.239
CT	14 (28)	17 (53.12)		9 (50)	
TT	4 (8)	4 (12.5)		1 (5.55)	
***MTHFR* c.1298 A>C, n (%)**					
AA	35 (70)	17 (53.12)	0.301	15 (83.33)	0.422
AC	13 (26)	13 (40.62)		2 (11.11)	
CC	2 (4)	2 (6.25)		1 (5.55)	

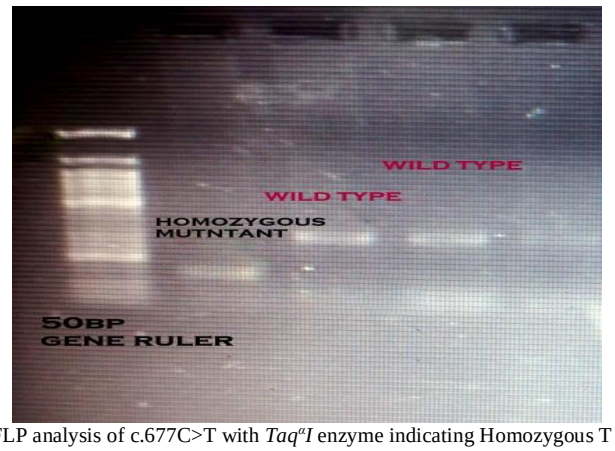

Figure 1. RFLP analysis of c.677C>T with *Taq*$^{\alpha}$*I* enzyme indicating Homozygous T and C allele.

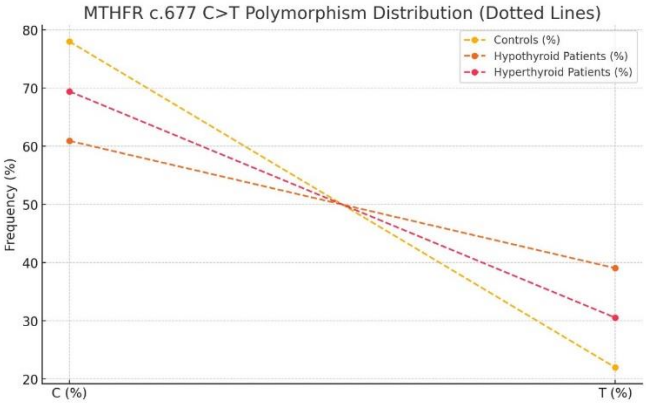

Figure 2. *MTHFR* c.677 C>T polymorphism across controls, hypothyroid patients, and hyperthyroid patients for the C and T alleles