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Research Article

PREVALENCE OF GLUTAMATE CARBOXYPEPTIDASE II C1561T, REDUCED FOLATE CARRIER 1 A80G, AND METHIONINE SYNTHASE A2756G GENE POLYMORPHISMS IN PATIENTS WITH TYPE 2 DIABETES MELLITUS AMONG SOUTH INDIANS

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ABSTRACT

Objective: Glutamate carboxypeptidase II (GCPII), reduced folate carrier 1 (RFC1), and methionine synthase (MTR) genes involved in the folate metabolic pathway may play a key role in the pathogenesis of diabetes and its complications. The present study aimed to investigate the prevalence of genetic polymorphisms of GCPII C1561T, RFC1 A80G, and MTR A2756G in individuals with type 2 diabetes mellitus (T2DM) among South Indians.

Methods: The study subjects consisted of 100 healthy individuals and 200 patients with T2DM. Genetic polymorphisms (GCPII C1561T, RFCI A80G, and MTR A2756G) in the folate metabolic pathway were analyzed by polymerase chain reaction-restriction fragment length polymorphism method. Statistical analysis was performed to test the level of significance.

Results: With regard to GCPII C1561T and MTR A2756G gene polymorphisms, significant differences were not found when diabetic patients (with and without complications) and controls were compared according to different statistical models (dominant, recessive, and overdominant) $p>0.05$. A case–control genetic association analysis of RFC1 A80G gene polymorphism has shown that there was 3.7-fold increased risk for patients without complications and 4.9-fold increased risk for diabetic patients with complications.

Conclusions: Our findings suggest that the GCPII C1561T and MTR A2756G gene polymorphisms were not significantly associated with diabetes and its complications. Whereas, the RFCI A80G gene polymorphism involved in folate metabolism confers increased risk for diabetes and its complications in South Indian population.

Keywords: Polymorphism, Folate metabolism, Glutamate carboxypeptidase, Reduced folate carrier, Methionine synthase, Type 2 diabetes mellitus.

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INTRODUCTION

Folate, water-soluble B vitamin plays an important role in amino acid metabolism, purine and thymidylate synthesis, and DNA methylation. Many enzymes are involved in folate transport and uptake, the folate pathway, and homocysteine (Hcy) metabolism and various polymorphisms have been identified in these enzymes methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MTR), MTR reductase, reduced folate carrier (RFC), glutamate carboxypeptidase (GCP), etc., and are associated with various pathologies [1]. It has been noted that polymorphism in the folate pathway may alter enzyme activities, thereby interfere in DNA methylation, in the DNA synthesis, as well as in the genomic stability, which finally results in pathological conditions [2].

Dietary folates primarily exist in polyglutamate forms and are unable to cross the cell membrane when the glutamate tail is longer than three residues [3]. Before dietary folate is absorbed, polyglutamate folates are deconjugated to monoglutamates by folylpoly-γ-GCP (FGCP) in the small intestine. The 1561T allele of the GCPII gene, which codes for FGCP, may impair intestinal absorption of dietary folates [4]. The genomic sequence of GCPII comprises 19 exons and is localized to chromosome 11p11.2 [5,6]. The GCPII C1561T mutation (rs202676) is located in exon13 at the putative catalytic domain of the enzyme and is associated with 53% reduction of enzyme activity [7].

RFC1 is a cell surface transmembrane protein, which is involved in the bidirectional movement of folate across the membrane [8]. RFC has a much greater affinity for reduced folates such as 5- methyl THF which is the main form of circulating folate in the plasma [9]. The human RFC1 is located on chromosome 21q22 and the protein consists of 591 amino acid residues with a molecular mass of 65KD. A single-nucleotide polymorphism (SNP) (rs1051266) of the RFC1 gene is an A-to-G transition at nucleotide position 80, replacing a histidine (CAC) with an arginine (CGC) at codon 27 [10]. Loss of RFC expression or function results in profound physiological or developmental consequences. It has also been reported that synthesis of mutant RFC protein with impaired function results in antifolate resistance due to incomplete inhibition of cellular enzyme targets and low levels of substrate for polyglutamate synthesis [11].

Vital processes such as gene expression, Hcy metabolism, and neurotransmitter synthesis and degradation are governed by methylation [12]. MTR plays an important role in the conversion of Hcy to methionine which requires 5-methyl THF as a methyl donor group. The formation of this radical depends on the action of the enzyme MTHFR [13]. Furthermore, MTR is the only enzyme that can regenerate THF from 5-methyl THF [14]. SNP in MTR A2756G (rs1805087) leads to change from aspartic acid to glycine at codon 919 (D919G), resulting in a lower enzyme activity followed by Hcy elevation and DNA hypomethylation [15]. Moreover, several polymorphism studies are conducted in the folate pathway with respect to various diseases among the Indian population. Till date, very limited data are available regarding the GCPII C1561T, RFC1 A80G, and MTR A2756G gene polymorphisms and its association with type 2 diabetes among South Indians. Hence, the present study was designed to understand the role of GCPII C1561T, RFC1 A80G, and MTR A2756G gene polymorphisms in the development of diabetes and its complications among South Indians.

METHODS

Selection of subjects

A total of 300 subjects (n=100, with complications; n=100, without complications; and n=100, healthy individuals) aged 35–55 years were included in this study. The subjects represented in this study are the people who not only live in Tamil Nadu but also belong to an ethnic group from Tamil Nadu. They are a member of the Dravidian people of South India. Whole blood samples were collected after getting informed consent from the study subjects. The study was approved by the Institutional Ethical Committee of Asirvatham Hospital and the Biosafety Committee of Lady Doak College. This present study was conducted at the Lady Doak College from November 2015 to December 2017.

Genotyping of GCPII C1561T, RFC1 A80G, and MTR A2756G genes

Blood was collected in ethylenediaminetetraacetic acid-coated tubes and DNA isolation was carried out according to the phenol-chloroform method [16]. The quality of the DNA was checked in 0.7% agarose (HiMedia, Mumbai) gel electrophoresis and quantified using ultraviolet (UV) spectrophotometry (Hitachi, Japan). The genotyping of GCPII C1561T, RFC1 A80G, and MTR A2756G gene polymorphisms was made using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique as described by Devlin *et al*., 2000; Neagos *et al*., 2010; and Zara-Lopes *et al*., 2016, respectively [7,17,18]. The details of primer sequences, restriction enzymes, and band pattern of GCPII C1561T, RFCI A80G, and MTR A2756G gene polymorphisms are given in Table 1.

The amplified PCR products of GCPII C1561T, RFC1 A80G, and MTR A2756G gene polymorphisms were confirmed by 2% agarose (HiMedia, Mumbai) gel electrophoresis (Figs. 1-3), using the gel documentation system. The amplified products were digested with restriction enzymes (Fermentas Life Sciences, Germany), and the resulting fragments of the digested PCR products were visualized by performing agarose (3%) gel electrophoresis (Figs. 4-6) using a UV transilluminator.

Confirmation of PCR amplified products by sequencing

Randomly selected PCR amplified products were completely sequenced both strands in an automated ABI 3100 genetic analyzer (Chromous Biotech, Bengaluru, India) which showed 100% concordance with the results of PCR-based RFLP analysis. Basic local alignment search tool (BLASTN) analysis was performed to study the homology sequence of the amplified product.

Statistical analysis

Statistical analysis was performed using the SigmaStat 11.0 version software. The allele frequencies were calculated by the allele counting method. Genotype frequencies were investigated using the standard Chi-square analysis and checked for Hardy–Weinberg equilibrium (HWE). Odds ratio (OR) at 95% confidence intervals (95% CI) was calculated for different statistical models (dominant, recessive, and overdominant) using a 2×2 contingency table. $p \leq 0.05$ was set to be statistically significant.

RESULTS

The genotype and allele frequencies of GCPII C1561T, RFC1 A80G, and MTR A2756G gene polymorphisms in type 2 diabetes mellitus patients (with and without complications) and controls are shown in Table 2. OR was calculated to elucidate the prevalence of genetic polymorphisms involved in the folate metabolic pathway and the risk of diabetes and its complications in South Indian population.

Analysis of GCPII C1561T gene polymorphism

The PCR product of 244 bp fragment that resides in the GCPII C1561T gene was amplified and sequenced. Sequences of the PCR products of the GCPII gene obtained after DNA sequencing was subjected to BLASTN analysis revealed 99% similarity with those of other submitted GCPII C1561T gene sequences. In the present study, when the prevalence of GCPII C1561T gene polymorphism was investigated, the heterozygous genotype CT was found to be more prevalent among the study subjects. The mutant genotype TT was found to be completely absent among the studied population. The mutant "T" allele frequency was found to be 0.4 in patients with complications and 0.3 in both the patients without complications and the controls.

Chi-square analysis showed that the genotype distributions of GCPII C1561T gene polymorphism were deviated from the HWE in the studied population. Significant differences were not found when diabetic patients (with and without complications) and controls were compared according to different statistical models (dominant, recessive, and overdominant) $p>0.05$. The calculated OR showed that the GCPII C1561T gene polymorphism was not associated with type 2 diabetes among the South Indians.

Analysis of RFC1 A80G gene polymorphism

The PCR product of 230 bp fragment that resides in the RFC1 A80G gene was amplified and sequenced. When the sequences of the PCR products were confirmed by BLASTN analysis, the query sequences had a high degree of similarity (100%) with those of other RFC1 gene sequences in the database. The most prevalent genotype for the RFC1 A80G gene polymorphism was AG genotype in both patients with and without complications, whereas the occurrence of AA genotype was more prevalent in healthy individuals. The mutant G allele frequency was found to be 0.62 for diabetes patients with complications and 0.56 for patients without complications and 0.48 for controls.

The genotype distributions of RFC1 A80G gene polymorphism in patients with complications were in accordance with HWE (Chi-square/p=2.13/0.14). Whereas, deviation from HWE was observed in patients without complications (Chi-square/p=4.01/0.04) and in healthy individuals (Chi-square/p=17.52/2.9E-05). Significant differences were observed in the distributions of genotype frequencies of RFC1 A80G gene polymorphism between diabetes patients (with and without complications) and healthy controls with respect to AA versus AG+GG genotypes (dominant model) (patients without complications: p=0.0002, OR=3.76, 95% CI=1.88–7.53; patients with complications: $p \leq 0.0001$, OR=4.95, 95% CI=2.35–10.44). Nevertheless, no statistically significant difference was observed in recessive genetic model and lack of risk with diabetes was found for overdominant model. A case–control genetic association analysis of RFC1 A80G gene polymorphism has shown that there was 3.7-fold increased risk for patients without complications and 4.9-fold increased risk for diabetes patients with complications.

Analysis of MTR A2756G gene polymorphism

The PCR product of 498 bp fragment that resides in the MTR A2756G gene was amplified and sequenced. After direct DNA sequencing, the sequences of the PCR products were confirmed by BLASTN analysis. The query sequences had a high degree of similarity (99%) with other MTR gene sequences. In the present study, when the prevalence of MTR A2756G gene polymorphism was investigated, the mutant G allele

Table 1: Primer sequences, restriction enzymes, and band pattern of GCPII C1561T, RFCI A80G, and MTR A2756G gene polymorphisms

SNPs	Primer sequences	PCR products	Restriction enzymes	Band pattern
GCPII C1561T	F: 5'-CATTCTGGTAGGAATTTAGCA-3' R: 5'-AAACACCACCTATGTTTAACA-3'	244 bp	*AccI*	CC genotype-244 bp TT genotype-141,103 bp
RFC1 A80G	F: 5'-AG TGT CAC CTTCGT CCCCTC-3' R: 5'-CTCC CGC GTG AAG TTCTTG-3'	230 bp	*HhaI*	AA genotype-162,68 bp GG genotype-125,68,37 bp
MTR A2756G	F: 5'-CCA GGG TGC CAGGTA TAC AG-3' R: 5'-GCC TTT TAC ACT CCTCAA AAC-3'	498 bp	*HaeIII*	AA genotype-413,85 bp GG genotype-290,123,85 bp

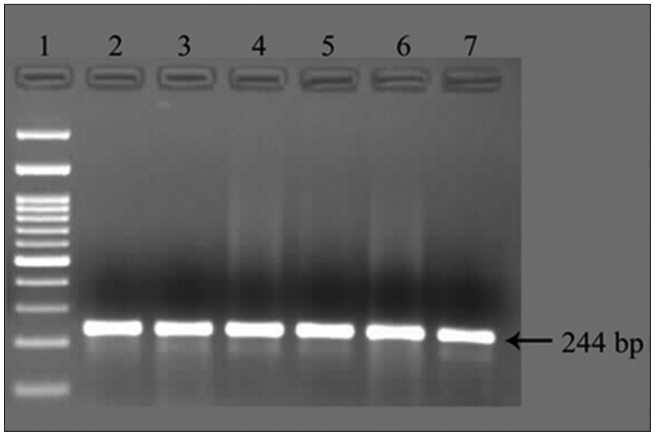

Fig. 1: Polymerase chain reaction (PCR) analysis of glutamate carboxypeptidase II C1561T gene. Lane 1: 100 bp DNA marker, lane 2–7: PCR product (244 bp)

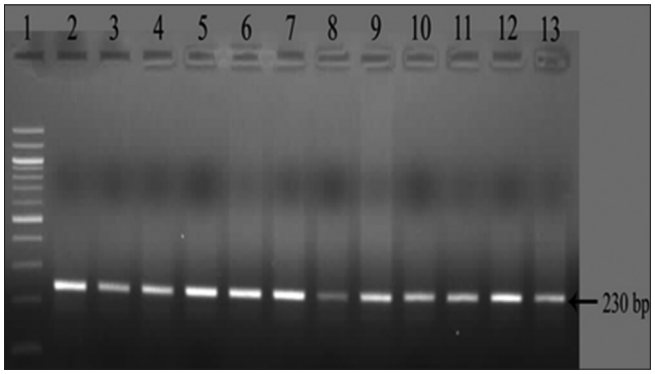

Fig. 2: Polymerase chain reaction (PCR) analysis of reduced folate carrier 1 A80G gene. Lane 1: 100 bp DNA marker, lane 2–13: PCR product (230 bp)

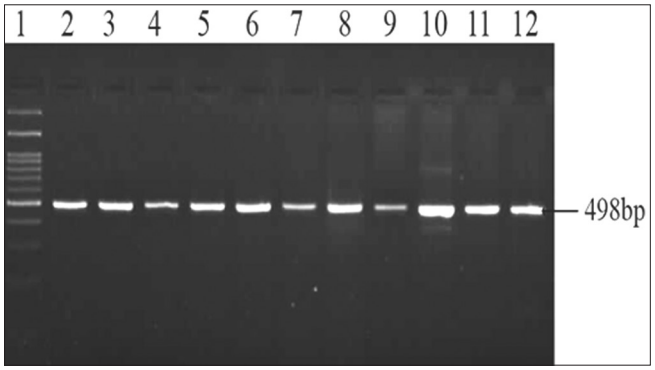

Fig. 3: Polymerase chain reaction (PCR) analysis of the methionine synthase A2756G gene. Lane 1: 100 bp DNA marker, lane 2–12: PCR product (498 bp)

frequency was found to be 0.3 in patients with complications, 0.29 in patients without complications, and 0.27 in the controls.

Chi-square analysis revealed that the genotype distributions in diabetic patients and controls were in accordance with HWE (patients with complications [Chi-square/p=3.61/0.05]; patients without complications [Chi-square/p=0.319/0.57]; and controls [Chi-square/p=1.34/0.24]). Significant differences were not found when diabetic patients (with and without complications) and controls were compared according to different statistical models (p>0.05). The results indicated that the MTR A2756G gene polymorphism was not associated with type 2 diabetes among the South Indians.

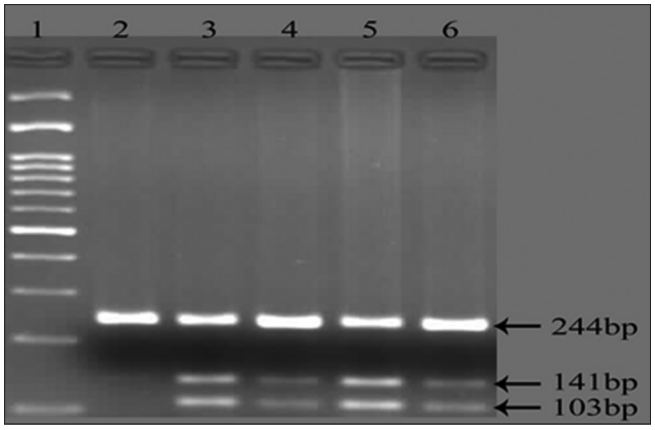

Fig. 4: Restriction digestion analysis of the glutamate carboxypeptidase II C1561T gene variant. Lane 1: 100 bp DNA marker, lane 2: wild genotype (CC) – 244 bp, lane 3–6: Heterozygous genotype (CT) – 141 and 103 bp

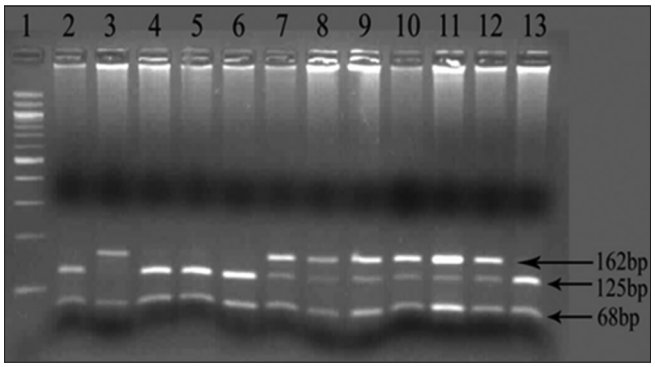

Fig. 5: Restriction digestion analysis of the reduced folate carrier 1 A80G gene variant. Lane 1: 100 bp DNA marker, lane 3: Wild genotype (AA) – 162 and 68 bp, lane 7–12: Heterozygous genotype (AG) – 162, 125, 68, and 37 bp, lane 2, 4–6, and 13: Mutant genotype (GG) – 125, 68, and 37 bp

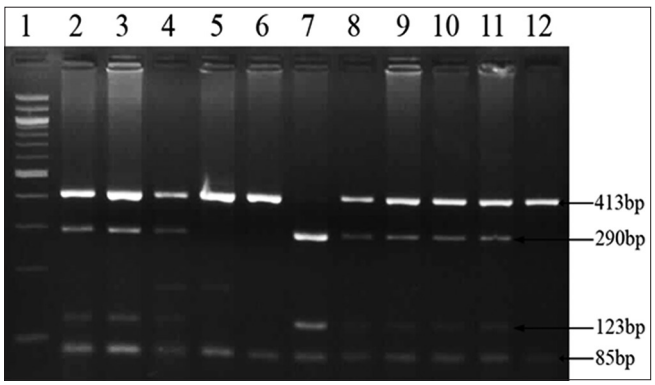

Fig. 6: Restriction digestion analysis of the methionine synthase A2756G gene variant. Lane 1: 100 bp DNA marker, lane 5, 6, and 12: Wild genotype (AA) – 413 and 85 bp, lane 2–4, 8–11: Heterozygous genotype (AG) – 413, 290, 123, and 85 bp, lane 7: Mutant genotype (GG) – 290, 123, and 85 bp

DISCUSSION

When the prevalence of the GCPII C1561T gene polymorphism among South Indian Tamil population was determined, no individuals with the homozygous mutant "TT" genotype were found among the studied population. Similar kind of result was also observed among the Netherlands and Indian population [4,19-21]. In our study, we

Table 2: GCPII C156IT, RFCI A80G, and MTR A2756G gene polymorphisms–genotype and allele frequencies in type 2 diabetic patients (with and without vascular complications) and controls

Genotype/Allele	Patients without complications (n = 100)	Patients with complications (n = 100)	Controls (n=100)
GCPII C156IT			
Genotypes			
CC	26	32	33
TT	74	68	67
CT	0	0	0
Alleles			
C	0.6	0.7	0.7
T	0.4	0.3	0.3
χ^2/p	34.4/<0.00001	26.5/<0.00001	25.3/<0.00001
RFCI A80G			
Genotypes			
AA	11	14	38
AG	54	59	29
GG	35	27	33
Alleles			
A	0.38	0.44	0.52
G	0.62	0.56	0.48
χ^2/p	2.13/0.14	4.01/0.04	17.52/2.9E-05
MTR A2756G			
Genotypes			
AA	45	50	51
AG	50	43	44
GG	5	7	5
Alleles			
A	0.7	0.71	0.73
G	0.3	0.29	0.27
χ^2/p	3.61/0.05	0.31/0.57	1.34/0.24
GCPII C156IT			
Patients with complications versus controls	Odds ratio	95% CI	p-value
CC versus CT+TT (dominant)	1.40	0.76–2.58	0.27
TT versus CT+CC (recessive)	-	-	-
CT versus CC+TT (overdominant)	0.71	0.38–1.31	0.27
Patients without complications versus controls			
CC versus CT+TT (dominant)	1.04	0.57–1.89	0.88
TT versus CT+CC (recessive)	-	-	-
CT versus CC+TT (overdominant)	0.95	0.52–1.72	0.88
RFCI A80G			
Patients with complications versus controls	Odds ratio	95% CI	p-value
AA versus AG+GG (dominant)	3.76	1.88–7.53	0.0002*
GG versus AG+AA (recessive)	0.75	0.40–1.37	0.35
AG versus AA+GG (overdominant)	0.28	0.15–0.51	<0.0001*
Patients without complications versus controls			
AA versus AG+GG (dominant)	4.95	2.35–10.44	<0.0001*
GG versus AG+AA (recessive)	1.09	0.60–1.96	0.76
AG versus AA+GG (overdominant)	0.34	0.19–0.62	0.0004*
MTR A2756G			
Patients with complications versus controls	Odds ratio	95% CI	p-value
AA versus AG+GG (dominant)	1.27	0.72–2.21	0.39
GG versus AG+AA (recessive)	1.0	0.28–3.56	1.0
AG versus AA+GG (overdominant)	0.78	0.45–1.37	0.39
Patients without complications versus controls			
AA versus AG+GG (dominant)	1.04	0.59–1.81	0.88
GG versus AG+AA (recessive)	1.43	0.43–4.66	0.55
AG versus AA+GG (overdominant)	1.04	0.59–1.82	0.88

GCP: Glutamate carboxypeptidase, RFC: Reduced folate carrier, MTR: Methionine synthase, n: Number of individuals, χ^2: Chi-square, CI: Confidence intervals, *p<0.05 statistically significant

have observed deviations from the Hardy–Weinberg law of population genetics for GCPII C1561T polymorphism among the study subjects. Furthermore, Hardy–Weinberg disequilibrium for RFC1 A80G gene polymorphism was observed in the diabetes patients without complications and the control groups. This is due to random selection samples, small sample size, and complexity of disease that involved both biological and genetic features [22].

The findings suggest that the GCPII C1561T and MTR A2756G gene polymorphisms were not associated with diabetes and its complications among South Indians. It has been revealed that the MTR A2756G polymorphism was not a risk factor for thrombosis among South Indians [23]. Furthermore, MTR A2756G polymorphism was not associated with coronary heart disease in Indian population [24]. Whereas, a study conducted in Indian population suggests that the presence of GCPII C1561T gene polymorphism increases the risk of coronary artery disease. It has been also reported that any perturbation in the GCPII activity might be associated with alterations in plasma folate and Hcy levels, thus playing a role in the pathophysiology of vascular diseases [25].

Several researchers have analyzed the influence of GCPII C1561T polymorphism on folate/Hcy concentrations and their association with the incidence of various disorders such as neural tube defects, gastric cancer, and cardiovascular disease [26-28]. However, few studies did

not find such an association which may be attributed to higher folate intakes due to mandatory food fortification with folic acid [29,30]. Folic acid derived from fortified foods is essentially in a monoglutamate form and does not require GCPII action for intestinal absorption, therefore bypasses the functional role of GCPII in the conversion of polyglutamate folates to monoglutamates [20]. The majority of the Indian population depends on diet as the primary source of folate and also lack of fortification program implies the functional role of GCPII C1561T gene polymorphism among the South Indian population.

In the present study, we found that the RFC1 A80G gene polymorphism may be at an increased risk for the development of diabetes and its complications among South Indians. The findings of Saxena have reported that the RFC1 A80G gene polymorphism might consider as an independent risk factor for the development of neural tube defects in the eastern region of Indian population [31]. Conversely, a study among Indians revealed no association between RFC1 A80G gene polymorphism and coronary artery risk [32]. However, such variations are observed due to the difference in the geographical regions and genetic background of the populations. It has been reported that the conflicting results are a commonplace in genetic association studies performed on different populations. Possible explanations for discrepant results include differences in the ethnicity (genetic background), the sample size (i.e., statistical power), the characteristics of the study subjects (e.g., undefined chronic illnesses), presence of nucleotide polymorphism(s) somewhere else in the examined genes, epigenetic alterations, linkage disequilibrium to other sequence variants in the vicinity of the studied locus, and prevailing environmental conditions [33]. Like any other study, this study has following limitations – small sample size, folate, and Hcy levels not analyzed.

CONCLUSIONS

In this study, the GCPII C1561T and MTR A2756G gene polymorphisms were not significantly associated with diabetes and its complications. Whereas, the RFCI A80G gene polymorphism involved in folate metabolism confers increased risk for diabetes and its complications in South Indian population. This finding has to be confirmed by increasing the sample size, which gives a better understanding of the role of SNPs in the pathogenesis of diabetes. Further, extensive studies on gene-nutrient and epigenetic interactions are needed for better understanding of the pathology of diabetes and its complications.

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AUTHORS' CONTRIBUTIONS

Conceived and designed the study: TA. Performed the experiment: KN. Analyzed the data: KN and TA. Interpretation of the results: KN, TA, AJP, and AJA. Wrote the paper: KN. Article editing: KN and TA. Coinvestigator of the project: AJP.

CONFLICTS OF INTEREST

The authors declare that there were no conflicts of interest for financial interests associated with this manuscript.

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