Evaluation of single nucleotide polymorphism C825T in the GNB3 gene with the development of type 2 diabetes of the South Indian population

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ABSTRACT

Diabetes mellitus, a widespread metabolic disorder, is caused by a deficiency of insulin or by a resistance to insulin and characterized by hyperglycemia. G-proteins, a class of signaling molecules, are involved in the signaling pathway of insulin. Any defect in the G-proteins leads to a defect in the GNB3 gene, coding for a G-protein that has been associated with the development of type 2 diabetes with related complications like hypertension and obesity. 70 subjects with type 2 diabetes and 70 control subjects were genotyped for the SNP using gene-specific PCR amplification and restriction digestion using the enzyme BsaJI, which cleaves the wide-type allele and not the mutant allele. The frequency of occurrence of the mutant and the wild-type alleles were statistically analyzed for significant relations. The statistical analysis with a p-value of 0.469 denotes the above, the 'acceptable' error rate. Therefore, the SNP C825T int eh GNB3 gene is not significantly associated with type 2 diabetes mellitus in the South Indian population.

Key words: BsaJI, G-protein, GNB3, PCR, RFLP, SNP, type 2 diabetes mellitus

INTRODUCTION

Type 2 diabetes mellitus results from defects in insulin secretion, almost always with a major contribution from insulin resistance (Albertii et al., 1999). Insulin Resistance (IR) is a condition where the body does not respond to insulin. Therefore, higher amounts of insulin are necessary for the desired effects. The high blood glucose levels continually stimulate the pancreas to synthesize more insulin. There is also a steady decline in the beta-cells of the pancreas, which results in decreased insulin production. It was previously known as 'Non-Insulin Dependent Diabetes Mellitus (NIDDM)' or 'Adult-Onset Diabetes Mellitus (AODM)'.

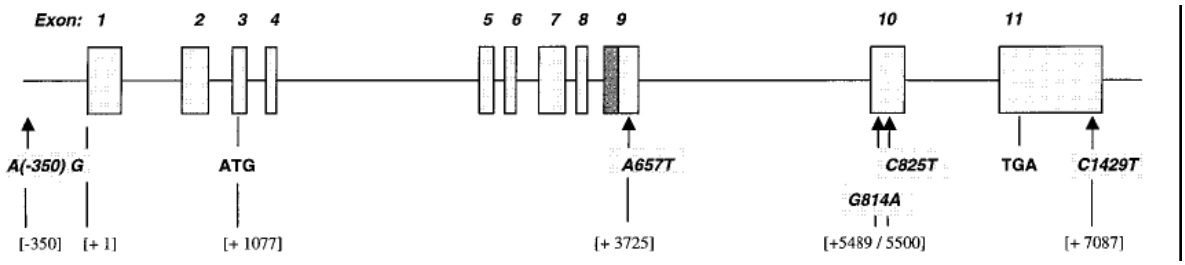
In type 2 diabetes mellitus, which was previously known as NIDDM or AODM, the pancreas produces insulin but the body does not respond to insulin. This is also known as 'insulin resistance'. As a result, the liver, muscles and fat tissues do not take up glucose from the blood. The glucose accumulates in the blood leading to hyperglycemia. The increased blood sugar levels also trigger the pancreas to produce more insulin, but it is not enough to keep up with the body's demand. Type 2 diabetes mellitus develops gradually. Lifestyle, family history and genetics play an important role in the development of type 2 diabetes.

Variations in the DNA sequences of human is vulnerable to develop diseases and respond to chemicals, drugs, vaccines and other agents. A single-nucleotide polymorphism is a DNA sequence variation occurring when a single nucleotide - A, T, C, or G - in the genome (or shared sequence) differs between members of a species (or between paired chromosomes in an individual). Single nucleotide polymorphisms (SNPs) have emerged as genetic markers of choice because of their high-density and relatively even distribution in the human genomes and have been used by many groups for fine mapping disease loci and for location of candidate genes (Chen, X., et al., 2003). SNPs are also thought to be key enablers in realizing the concept of personalized medicine.

G-proteins or Guanine Nucleotide Binding Proteins are a family of proteins involved in second messenger cascades during signal transductions. They alternate from 'inactive' guanosine diphosphate (GDP) to 'active' guanosine triphosphate (GTP), which is a binding state, and which proceeds to regulate downstream cell processes. Heterotrimeric G-proteins are also known as 'large G-proteins' and are made up of 3 subunits – alpha (α), beta (β) and gamma (γ). A large number of hormones, neurotransmitters, chemokines, local

mediators, and sensory stimuli exert their effects on cells and organisms by binding to G-protein-coupled receptors.

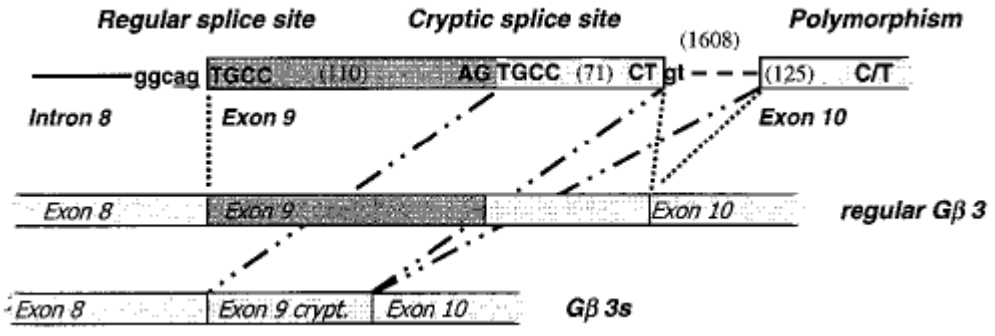
Guanine Nucleotide Binding Protein, subunit beta (β) 3, is the protein encoded by the gene GNB3 in human. It is a heterotrimeric G-protein, whose functions involve integrating signals between receptors and effectors, and modulating various signal transduction pathways. GNB3 is present on chromosome 12 at the location of 12p13 and has 340 amino acid residues. The gene has 7500 bp, with 11 exons and 10 introns. The start codon ATG being located in exon 3 and the stop codon TGA in exon 11 (Fig.1).



Adrenalin receptors, angiotensin II receptors, and glucagon receptors, among many others are G-protein-coupled. The lipolytic and glycogenolytic effects of adrenergic and glucagon receptor stimulation in hepatic, muscle, and fat tissues, mediated by G-proteins, reveal an antagonistic action to insulin.

The activation of G-proteins stimulates adenyl cyclase. This in turn induces hormone-sensitive lipase in adipose tissue, protein kinase A (PKA), and glycogen phosphorylase in muscle and fat cells, as well as in hepatocytes. Persistent stimulation may lead to insulin resistance and an increase in hepatic glucose output. G-proteins also regulate β-phospholipase C (PLC-β), which produces phosphatidylinositol (IP3), the calcium channel activator. The opening of calcium channels initiates insulin secretion. Therefore, G-proteins may contribute to the main pathophysiological mechanisms involved in type 2 diabetes, and the genes encoding its particular subunits are among the candidate genes for this disorder (Grzegorz Dzida et al., 2002).

The β3 subunit of the G-proteins is encoded by the GNB3 gene. The polymorphism resulting from cytosine-to-thymine substitution at position 825 (C825T), located in exon 10 of the gene. Cytosine substitution by thymine causes alternative splicing in exon 9, resulting in the loss of 41 amino acids from the polypeptide chain. The T825 variant of the gene is known to be associated with enhanced signal transduction via the G-protein system. Gβ proteins belong to the superfamily of propeller proteins, and all Gβ proteins identified so far consist of 7 WD repeats (referring to the conserved amino acids aspartate and tryptophan) that form a regular torus-like structure. Gβ3s results from alternative splicing of GNB3 and lacks the equivalent of 1 entire WD domain. In GNB3, the C825T polymorphism is located .1700 bp upstream of the alternative splice site (Fig. 2).



The 825T allele is associated with enhanced G-protein activation and, thus, increased cell proliferation, and it appears that the 825T allele exerts an additive effect on G-protein signaling in vitro as a result of the increased production of Gβ3s (Andersen, G., et al., 2006). The dimorphism (C825T) was found to be associated with increased cardiac potassium channel activity and increased α-adrenoceptor–mediated vasoconstriction in humans. Most importantly, the dimorphism has been shown to be associated with hypertension and obesity. In both the phenotypes, hypertension and obesity, are linked to the insulin resistance syndrome and atherosclerosis on one hand. Heterotrimeric G-proteins, on the other hand, are involved in the regulation of cellular

transmembrane sodium exchange that is found to be increased in insulin resistant subjects. Finally, worldwide distribution of the C825T dimorphism of GNB3 suggests that it might fulfill the criteria of a thrifty genotype (Wascher, C., et al., 2003).

MATERIALS AND METHODS

Samples Collection

The test samples (with type 2 diabetes) were chosen based on the WHO criteria (Albertii et. al., WHO 2006). 72 test samples were collected from the patients having T2DM, attending M.V. Hospital for Diabetes and Diabetes Research Centre (WHO collaboration), Royapuram, Chennai, South India. 68 control samples from healthy individual without the history of type 2 diabetes mellitus were also collected. 2.5 ml of venous blood was collected in a standard EDTA-tube (Vacutainer BD) from patients who have been fasting for 8 hours. Similarly, 2.5 ml of venous blood was collected under sterile conditions from the test patients and control individuals.

Genomic DNA Extraction

Genomic DNA was extracted from the collected blood (leucocytes) samples, using phenol- chloroform extraction method (Section of Cancer Genomics, Genetics Branch, NCI, National Institutes of Health, 2004). The extraction was qualitatively confirmed using 0.7% agarose gel electrophoresis and quantitatively analyzed using UV S-30 spectrophotometer (Boeco, Germany).

PCR Amplification

The polymorphism was detected using PCR amplification using specific primers. The primers were designed to amplify a fragment of length 268 bp, which contains the site of the SNP.
Forward: 5’ TGA CCC ACT TGC CAC CCG TGC 3’
Reverse: 5’ GCAGCA GCC AGG GCT GGC 3’
The reaction mixture was contained in 10 µl volume with the following composition (Table 1):

Table 1: PCR Components (10µl volume)

S. No.	Reagents	Volume (µl)
1	2X PCR Mix	5
2	Sample DNA	2
3	Forward Primer	0.1
4	Reverse Primer	0.1
5	Water (sterile)	2.8

The 2X PCR mix contains Prime Taq DNA Polymerase (1Unit/10 µl), 20 mM Tris HCl, 80mM KCl, 4mM $MgCl_2$, enzyme stabilizer, sediment, loading dye, pH 9, 0.5mM of each dATP, dCTP, dGTP, dTTP. The concentrations of both the forward and the reverse primers were 0.1µM. DNA amplification was carried out in a thermocycler (Eppendorf MasterCycler Gradient) (Table 2).

Table 2: PCR – Cycle Time and Temperature

S. No.	Reaction	Temperature (°C)	Time
1	Initial Denaturation	94	5 min
2	Denaturation	94	1 min
3	Annealing	60	30 seconds
4	Chain Elongation	72	30 seconds
5	Final Extension	72	5 min

Restriction Fragment Length Polymorphism (RFLP)

RFLP was used to distinguish between homologous DNA fragments based on the differing locations of a particular restriction site. The reaction was performed in a total volume of 10 µl consisting of 5 µl amplicon, Tris-HCl buffer, (100mM KCl, 1mM DTT, 1 mM EDTA, pH 7.4 at 25ºC) and 1 unit of BsaJI enzyme (Fermentas Life sciences, USA). The restriction site for the enzyme is 5'...C*CNNGG…3' and the source of the restriction enzyme is Bacillus stearothermophillus J695 (Z. Chen).

Samples were then digested overnight at 37°C and the digested restriction fragments were separated by electrophoresis on 1.5 % agarose gel (High EEO Pronastar, Cat. No. 8012) and visualized by ultraviolet transillumination after ethidium bromide staining.

In the wild type, i.e., presence of cytosine at position 825, the enzyme BsaJI has a restriction site and the fragment is cleaved into two restriction fragments of length 114 bp and 154 bp. In the presence of the SNP, the cytosine at position 825 is replaced with thymine, which removes the restriction site. However, the mutated type is not cleaved by the restriction enzyme, BsaJI.

Statistical Analysis

Clinical characteristics of all the subjects were expressed as mean ± SD. Mean of individuals with and without the SNP in following variables namely age, duration of diabetes, BMI, fasting blood glucose, postprandial blood glucose, HbA1c, T-cholesterol, triglyceride, HDL-cholesterol, LDL-cholesterol, VLDL cholesterol, urea and creatinine were calculated using the software SPSS ver. 15.0.

The significance of the observed RFLP results were calculated statistically using the software, Primer of Biostatistics. The p-value which shows the the probability of error and χ^2 was calculated.

RESULTS AND DISCUSSION

Blood samples collected from test (72) subjects were analysed for blood glucose levels. Analysis was done to confirm the presence of diabetes in the samples. Besides, the following physical and biochemical parameters were analysed for the data. The mean and standard deviations were calculated for the data using the software, SPSS for Windows ver. 15.0, and the values are tabulated.

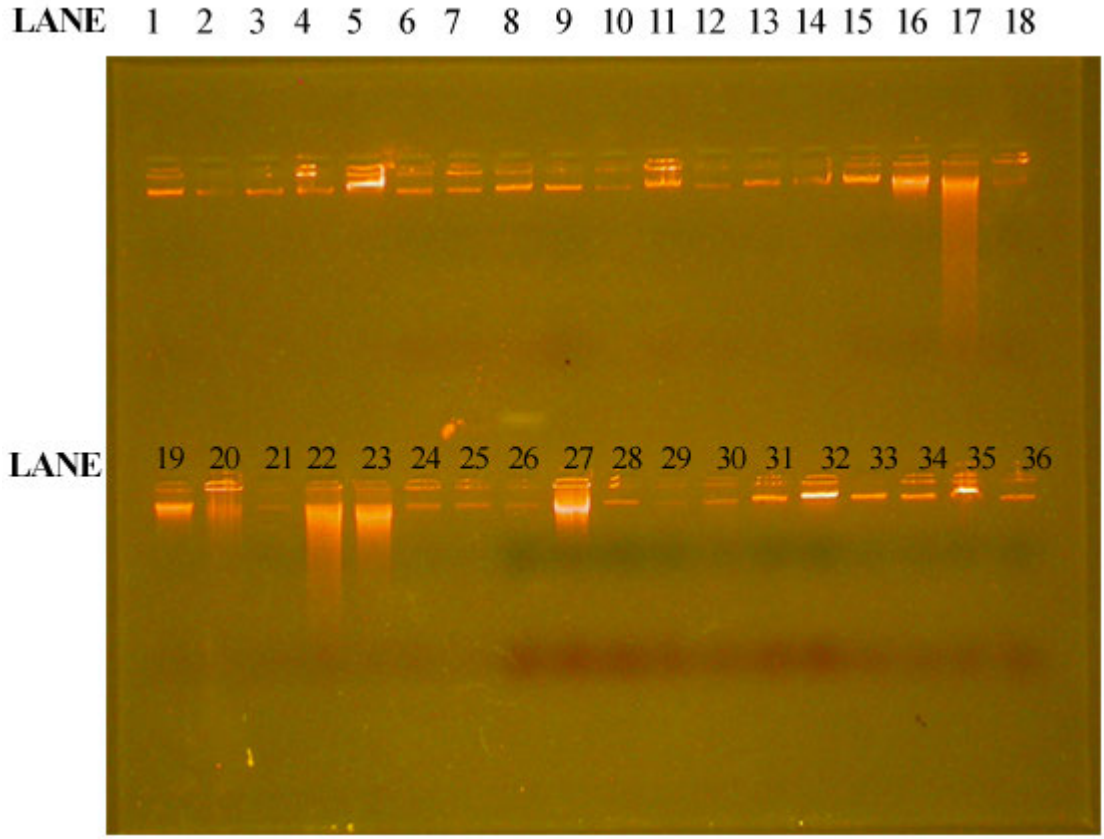
No. of subjects (n) = 72
No. of males = 42 (53.84%)
No. of females = 30 (41.6%)
(Table 3)

Table 3: Characteristics of the Test Study Group

S. No.	Criteria	Mean	Standard Deviation	Mean	Std. Deviation
1	Age	53.83	10.747	54.48	11.365
2	BMI (kg/m^2)	26.884	4.907	26.58	3.76
3	FBS (mg/dL)	165.48	57.893	159.09	52.002
4	PPBS (mg/dL)	263.16	90.393	245.29	80.692
5	Urea	22.28	6.806	22.97	7.917
6	Creatinine	0.760	0.174	0.803	0.1962
7	HbA1c	8.628	2.02	8.374	1.9314
8	TGL	155.75	118.649	150.96	113.324
9	Cholesterol	180.84	46.633	177.81	44.062
10	HDL	45.04	10.390	45.06	10.953
11	LDL	112.77	36.956	110.46	36.368
12	VLDL	23.32	18.898	22.24	16.462

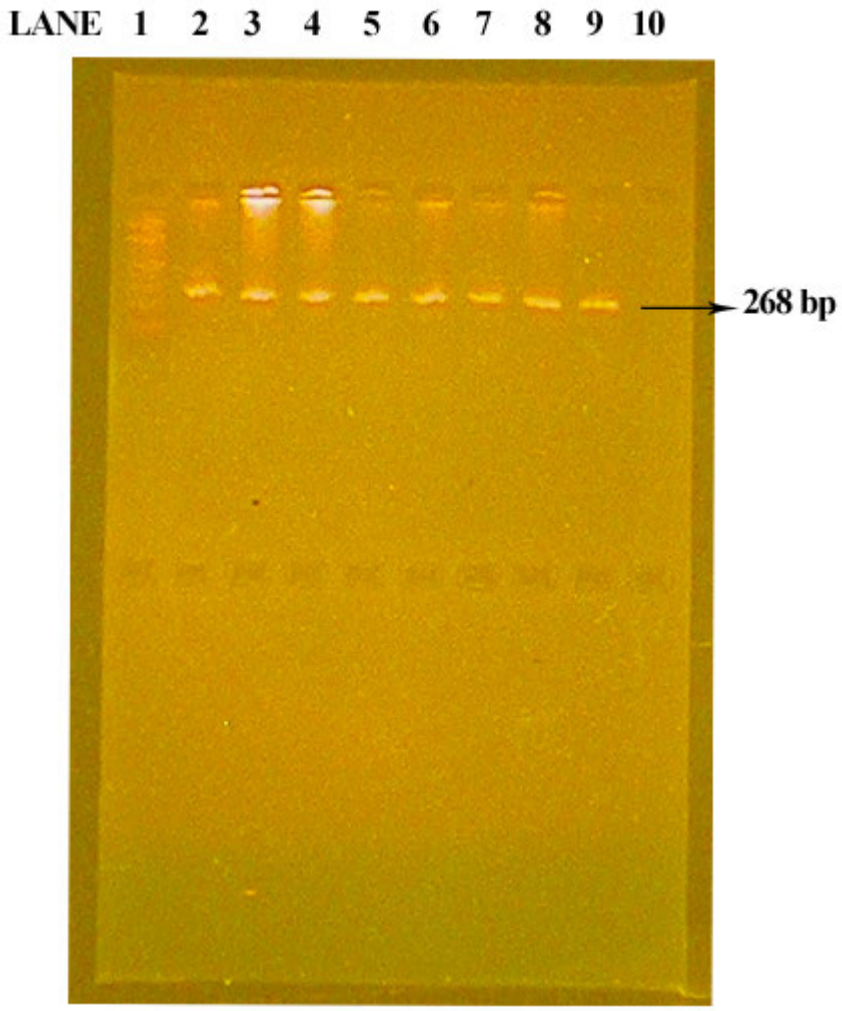
Genomic DNA Isolation

The genomic DNA was isolated from blood cells (leucocytes) using phenol-chloroform method from the collected blood samples and the isolation was confirmed qualitatively by gel electrophoresis using 0.7% agarose gel (Fig 3).



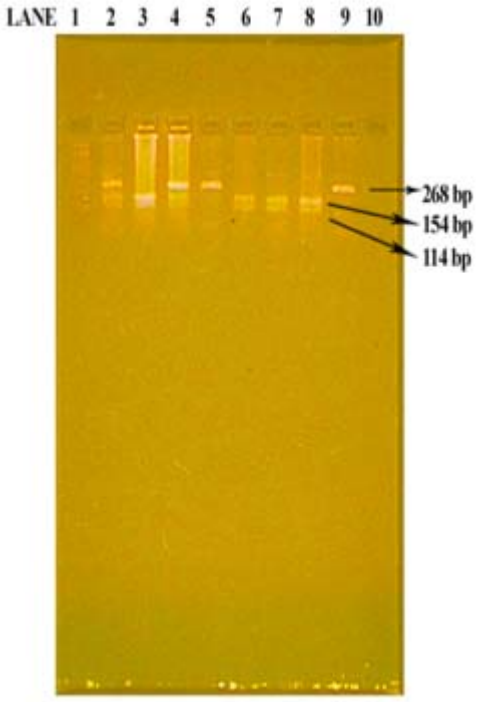
PCR Amplification

The isolated genomic DNA was amplified by PCR using gene-specific primers. The forward and reverse primers used are 5'- TCT GGA GAT TCT CCT ATT GGC - 3' and 5'- CTG GAA GAC AAC TAC AAG AG - 3' respectively. These primers amplify a 268 bp fragment of the GNB3 gene which contains the SNP C825T (rs5443). The PCR amplification was qualitatively confirmed by agarose gel electrophoresis on a 0.7% agarose gel (Fig 4).



Restriction Fragment Length Polymorphism (RFLP)

RFLP was performed on the PCR products, with the restriction enzyme, BsaJI (Bacillus stearothermophillus J695). This enzyme has a restriction site at C*CNNGG. The wild type of the GNB3 gene has CC at position 825 and so will have an intact restriction site for BsaJI. This will be cleaved into 2 fragments of lengths, 114bp and 154bp. The GNB3 gene with the polymorphism had TT or CT at position 825 and therefore has no restriction site for BsaJI. After digestion with BsaJI, the fragments were analysed using agarose gel electrophoresis on 2% (high EEO) agarose gel (Fig. 5).



The lanes with one DNA fragment of 268 bp are the samples which have the mutated allele and have not been cleaved by the restriction enzyme. The lanes with two DNA fragments contain the wild-type allele which have been cleaved by the restriction enzyme.

Statistical Analysis

The RFLP results were studied to determine the frequency of occurrence of the wild-type (CC) allele and the mutant (CT or TT) alleles. These values were analysed statistically for significance using the software, Primer of Biostatistics (McGraw-Hill) (Table 4).

Table 4: Allelic Frequencies of GNB3

	Control (n = 68)	Test (n=72)
Exposed	36	36
Unexposed	100	108

The ‘exposed’ stands for the number of mutant alleles and the ‘unexposed’ stands for the ‘wild-type’ alleles.

(Table 5) *Mutation in 1 allele: ** No Mutation: *** Mutation in 2 alleles: NS – Non-Significant (A level of < 0.05 is considered statistically significant).

Table 5: Genotypic Frequencies of wild-type and mutant alleles of GNB3

Subjects	Total (n)			CC** n (%)	CT* n (%)	TT*** n (%)	Chi square	p-value
	M	F						
Control	68	32	36	40(27.2)	20(13.6)	8(5.4)	0.525	0.469 (NS)
Type 2 DM	72	42	30	42(30.2)	24(17.2)	6(4.3)		

G-proteins are signaling molecules which are involved in the insulin signaling metabolic pathway. The GNB3 gene codes for the beta3 polypeptide of the G-proteins. A single nucleotide polymorphism of this gene at position 825 in exon 10, where cytosine is replaced with thymine, leads to a splice variant of the wild-type protein, which has enhanced activity. Cytosine substitution by thymine causes alternative splicing in exon 9, resulting in the loss of 41 amino acids from the polypeptide chain. Insulin uses a signaling mechanism involving G-proteins in maintaining the blood glucose levels. Also G-proteins are involved in many pathophysiological mechanisms which contribute to type 2 diabetes, like stimulation of adenylate cyclase, and the signaling pathways of adrenergenic and glucagon receptors in hepatic, muscle and fat-tissue cells. Therefore, polymorphism in this gene can be associated with the pathological mechanisms of type 2 diabetes.

Studies on this polymorphism have been made in various populations. Daimon et al., (2008) has shown significant association with the C825T polymorphism with type 2 diabetes with p = 0.002. Similarly, Danoviz ME et al., (2006) and Pollex et al., (2006) showed significant associations. Kiani et al., (2005) positively associated this polymorphism with type 2 diabetes in the Emirati population. In Asian Indian (US residing) populations, minor association between C825T and type 2 diabetes were shown by Pemberton TJ et al., (2008). This particular SNP has also been widely associated with hypertension which is both a risk factor for type 2 diabetes and a complication which occurs with type 2 diabetes.

Some studies have shown that though there is an association between hypertension and the GNB3 C825T polymorphism, there is no significant association with type 2 diabetes. Parra EJ et al., (2004) in an association study found no significant association with type 2 diabetes in the Hispanic American population. Andersen et al., (2006) showed only minor associations which were not statistically significant in the Danish population.

In this study, the associations between the C825T polymorphism (rs5443) and type 2 diabetes in the South Indian population were analyzed. No statistically significant associations were found between the SNP and the susceptibility of diabetes. Genotypes with the mutant allele (i.e. CT + TT) occurred more in the test samples (21.5%) as compared to the control samples (19%). But the frequencies of the T allele in the control and test (diabetic) samples were not significantly different. The allelic frequencies i.e., the number of mutant and wild-type alleles were calculated. The significance of this frequency in terms of p-value was found to be 0.469. The p-value shows the probability of error and 0.05 is treated as the 'acceptable' error rate in research experiments. Therefore, p = 0.469 indicates that there is no significant association between the GNB3 gene polymorphism, C825T, and type 2 diabetes in the South Indian population.

CONCLUSION

The genomic DNA isolated from the blood samples of type 2 diabetics of the South Indian population were analyzed for the association of the single nucleotide polymorphism C825T in the GNB3 gene with the development of type 2 diabetes. After RFLP and statistical analysis, a p-value of 0.469 was obtained, which is above the acceptable probability rate. Therefore, it is concluded that there is no significant association between the C825T polymorphism of the GNB3 gene and type 2 diabetes in the South Indian population.

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