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Analysis CTLA-4 gene Polymorphism in CTLA- 4 gene polymorphism in type 1 and 2 diabetes mellitus among South Indian Population

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Abstract

CTLA-4 in found to be involved in the pathogenesis of type 1 and 2 diabetes such as polymorphisms which affects the protein function that is relevant to the disease process. According to the previous research reports, CTLA-4 gene also been linked to a wide range of autoimmune disorders, such as multiple sclerosis, rheumatoid arthritis, celiac disease, and primary biliary cirrhosis. Jaya et al., (2018) Type 1 diabetes mellitus (T1DM) is an organ-specific autoimmune disease characterized by T cell-mediated destruction of pancreatic islets. The genetic factors involved consist of at least five vulnerability genes: Hence the objective of the study was to investigate for associations of CTLA-4 +49A/G polymorphisms with T1DM and T2DM using polymerase chain reaction and gene sequence analysis. The results found that the frequency distribution of CTLA4+49A/G genotype and alleles between T1DM, T2DM patients and normal controls were statistically significant. (OR 2.286; 95%CI 0.804 to 6.945; P=0.118), and the CTLA-4 +49A/G polymorphism was recognized as a risk susceptibility factor for T1DM and T2DM. The study concluded that significant association between CTLA-4 +49A/G polymorphism and T1DM and T2DM. So, the study suggested that the CTLA4 gene polymorphism is closely linked to the pathogenesis of diabetes. Further studies are necessary to investigate how genetic and environmental factors affect the pathogenesis of DM to provide a basis for early diagnosis, genotyping and treatment of DM.

Introduction

Diabetes mellitus (DM) is a highly prevalent disease characteristic of elevated glucose level as a result of dysregulated metabolism. There are currently 285 million people with diabetes worldwide, and nearly 500 million people is expected to develop diabetes by 2030 (Shaw *et al.*, 2010). The chronic vascular complications of diabetes pose a great threat to patients' lives and impose a heavy economic burden. The World Health Organization estimated that the economic loss due to diabetes in China has amounted to $557.7 billion between 2005 and 2015 (Cheng *et al.*, 2011). Diabetes mellitus, often known simply as diabetes, is a group of common endocrine diseases characterized by sustained high blood sugar levels (WHO, 2023; MSD,2022) Diabetes is due to either the pancreas not producing enough insulin, or the cells of the body becoming unresponsive to the hormone's effects (Shoback and Gardner, 2011). Classic symptoms include thirst, polyuria, weight loss, and blurred vision. If left untreated, the disease can lead to various health complications, including disorders of the cardiovascular system, eye, kidney, and nerves (Kitabchi, 2009). Untreated or poorly treated diabetes accounts for approximately 1.5 million deaths every year (WHO, 2023).

The etiology of diabetes is yet to be fully understood. It is now recognized that diabetes is not a disease of a single cause, but a syndrome of multiple causes, among which are genetic, autoimmune and environmental factors (Ozougwu *et al*., 2013; Xie *et al.*, 2020). There are two major subtypes of diabetes mellitus: type 1 and type 2 diabetes. Type 1 diabetes results from cell-mediated autoimmune destruction of β-cells, causing decreased insulin production (Eizirik *et al.*, 2020). Type 2 diabetes is characteristic of progressive loss of islet function and insulin resistance in peripheral tissues, eventually requiring insulin therapy. Therefore, immunological factors are extremely important in the pathogenesis of both type 1 and 2 diabetes (Aylward *et al.*, 2018).

The capability of some pancreatic β-cells to secrete insulin is lost by the time diabetes develops symptoms, therefore prediction and prevention of diabetes are very important.[5] It is helpful to know the genetic profile of diabetes to prevent it. Understanding the genetic underpinnings of DM is critical to develop effective treatments of the disease (Dajani *et al.*, 2017). A recent study suggested that CTLA-4, a costimulatory molecule that activates T cells, might be one of the candidate biomarkers related to DM susceptibility (Borysewicz-Sańczyk*et al.*, 2020). *CTLA-4* consists of four exons and three introns and is located on the long arm of

chromosome 2q33. Similar to CD28, CTLA-4 also belongs to the immunoglobulin superfamily, but has a higher affinity to B7 molecule expressed on the antigen-presenting cells (APCs) than CD28, making CTLA-4 a potent suppressor of T-cells (Chikuma, 2017). CTLA-4 has multiple polymorphic loci. The exon 1+49 A/G polymorphism is the sole polymorphism that alters the major amino acid sequence of CTLA4 (Hosseini *et al.*, 2020; Ren *et al*., 2022). The cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) gene is located on the long arm of chromosome 2q33. It consists of four exons and encodes a costimulatory molecule that is expressed on the surface of activated T cells. CTLA-4 and CD28 (also located on 2q33) are members of the immunoglobulin superfamily and bind to the B7 molecule on antigen-presenting cells. This completes the activation initiated when the antigen-specific cell-surface T-cell receptor (CD3 complex) engages the antigen bound to a major histocompatibility complex class II molecule on the surface of an antigen-presenting cell (Vaidya *et al.,* 2004).

CTLA-4 has a greater affinity for the B7 molecule than does CD28, and it downregulates T-cell function. Therefore, it may play a crucial role in T-cell-mediated autoimmunity and thus in susceptibility to autoimmune diseases, including type 1 diabetes mellitus. CTLA-4-deficient mice rapidly develop lymphoproliferative disease with multiorgan lymphocytic infiltration and tissue destruction, with particularly severe myocarditis and pancreatitis, and die 3–4 weeks postpartum. CTLA-4 has also been thought to be potentially associated with a wide range of autoimmune disorders, such as autoimmune endocrinopathies, multiple sclerosis, rheumatoid arthritis, celiac disease, and primary biliary cirrhosis (Vaidya *et al.,* 2002). It is considered the most likely candidate gene for type 1 diabetes susceptibility for the IDDM12 locus on chromosome 2q33 (Fotini *et al.,* 2005). Hence according to above said consequences, the study lead to following objectives: (i) to study the prevalence of Type 1 and 2 diabetes mellitus (DM) (T1DM and T2DM) (ii) to analyze the blood glucose concentration in DM patients and control group (iii) investigate the association of CLTA-4 gene polymorphism with Type 1 and 2 diabetes mellitus (DM) (T1DM and T2DM).

Methodology

Study Design

A total of 60 clinically proven DM patients and age and sex matched 60 non-diabetic healthy subjects as control included in this study. The sample and related clinical data was collected

from Dr.Kannan's Endocrinology Clinic, Madurai. The collected blood samples were used for the genetics study.

I. Genomic DNA Preparation: (Sambrook *et al* 2001; Veeramuthumari *et al.,* 2011)

The blood samples were thawed at room temperature and 300µl of blood was transferred to centrifuge tubes. Equal volume of PBS was added to it and incubated for 20minutes and centrifuged at 3000 rpm for 5minutes. The supernatant was removed and the pellet was resuspended in 900µl of RBC lysis buffer and mixed thoroughly. This was centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded. To the pellet 600µl of ice-cold cell lysis buffer was added and mixed well, then 200µl of ammonium acetate was added to the mixture to precipitate the proteins and centrifuged at 3000rpm for 7minutes.The Supernatant was separated and 1000µl of Isoproponal was added and the tube was inverted till the DNA was precipitated and centrifuged at 7000rpm for 2minutes.The precipitated genomic DNA was washed with 600µl of 70% ethanol and allowed to air dry. The DNA was resuspended in TE buffer and stored at -20^{o}C. The isolated DNA was confirmed by using 0.7% Agarose gel electrophoresis.

II. Analysis Single-strand conformational polymorphism of CTLA4 polymorphisms:

CTLA-4 exon 1 position 49 (A/G: Codon 17 Thr/Ala) polymorphism was defined employing polymerase chain reaction. A 25µl polymerase chain reactions (PCR) recipe was prepared using 2µl of 5µM Forward primer (5'-GCTCTACTTCCTGAAGACCT-3') and Reverse primer (5'-AGTCTCACTCACCTTTGCAG- 3') (Fermentas Life Sciences, Germany),~200 ng Genomic DNA, 1 U of Taq polymerase (Bangalore Genei, India) dNTP's 200µM (New England Biolabs, Beverly, MA), 10XPCR buffer with $MgCl_2$ and the volume was made up with nuclease free water. Thermocycling conditions were as follows: Initial denaturation - 94°C for 4 minutes, annealing - 58°C for 45 seconds, extension - 72°C for 45 seconds, denaturation - 94°C for 45 seconds, final extension - 72°C for 4 minutes. The polymerase chain reaction was carried out using a thermocycler (Eppendorf, Germany). The amplicons were elctrophoretically confirmed (2% agarose). (Rau *et al.,* 2001; Veeramuthumari *et al.,* 2011; 2013 and Philip and Isabel, 2011). The amplified PCR product was used for the sequence analysis.

III. Statistical Analysis

The comparison of alleles and genotypes was performed by chi-square and Student's *t*-tests. The genetic equilibrium coincidence was analyzed using the Hardy–Weinberg equilibrium method. Differences with $P<0.05$ were determined to be statistically significant.

Results and Discussion

The present study included that a total of 60 clinically proven diabetes mellitus patients and 60 non-diabetic healthy subjects as control group. The differences in terms of age and gender between the diabetic group and the control group were statistically significant and were comparable (Table:3). The Hardy–Weinberg equilibrium test was performed on the genotype frequencies of the two loci in the control group and diabetic subjects (P>0.05).

Table 3: Clinical Characteristics of Diabetes population

	Gender		**Age (Years)**	**Fasting Blood Glucose (mM)**
	Male	**Female**		
DM	36(60%)	24(40%)	56.8±13.1	
T1DM	12(20%)	7(12%)	43.8±15.3	14.76±5.33
T2DM	24(40%)	17(28%)	57.5±12.5	10.38±4.35
Control group	27(45%)	33(55%)	47.6±18.3	4.87±2.35

T1DM-Type 1 diabetesT2DM- Type 2 diabetes

The current study data showed that 86% of the people were type 2 diabetes mellitus. 19% were type 1 diabetes mellitus.

Figure 5: Isolated DNA confirmed using Agarose gel electrophoresis

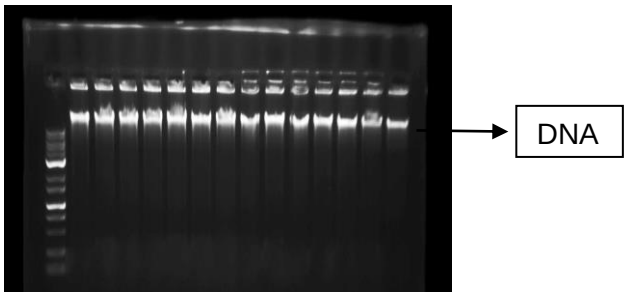


Figure 6: Amplified PCR product using Agarose gel electrophoresis

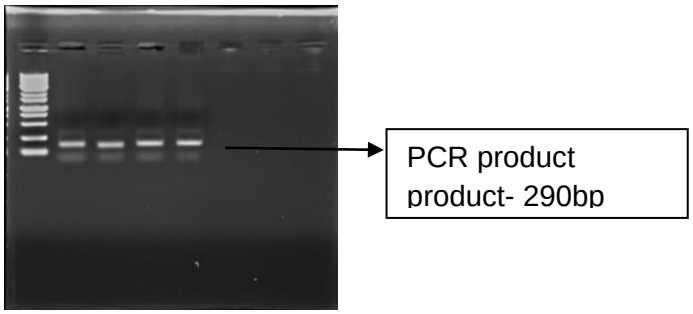


Figure 7: Sequence analysis of A/G at position 49 CTLA-4 Polymorphisms in DM Patients:

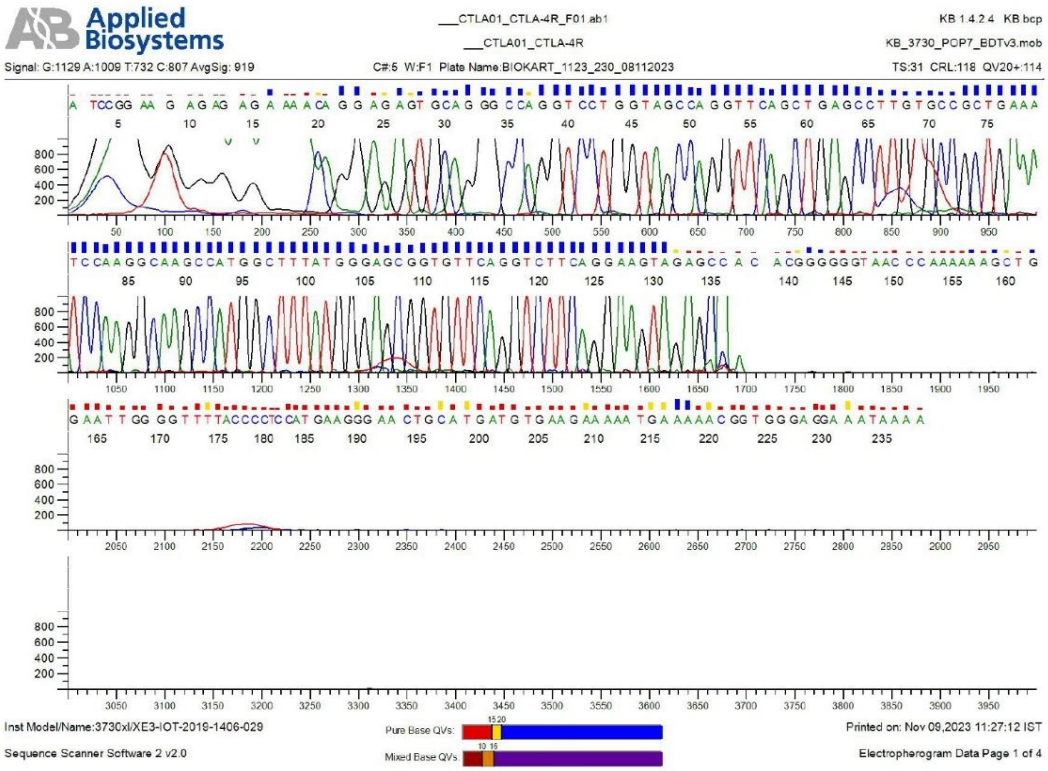
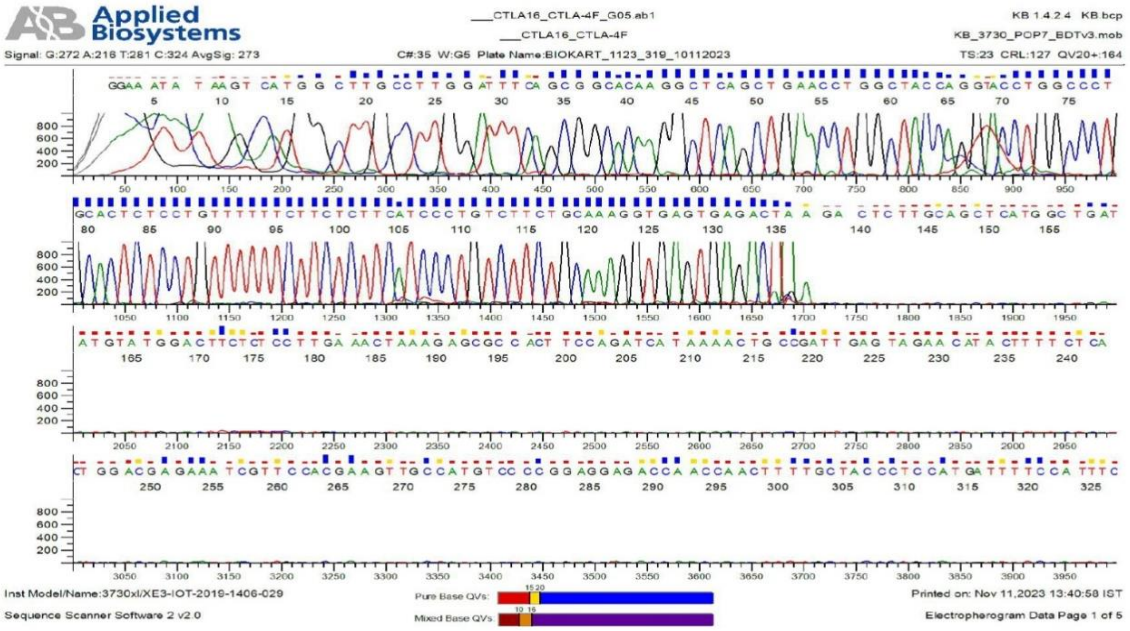
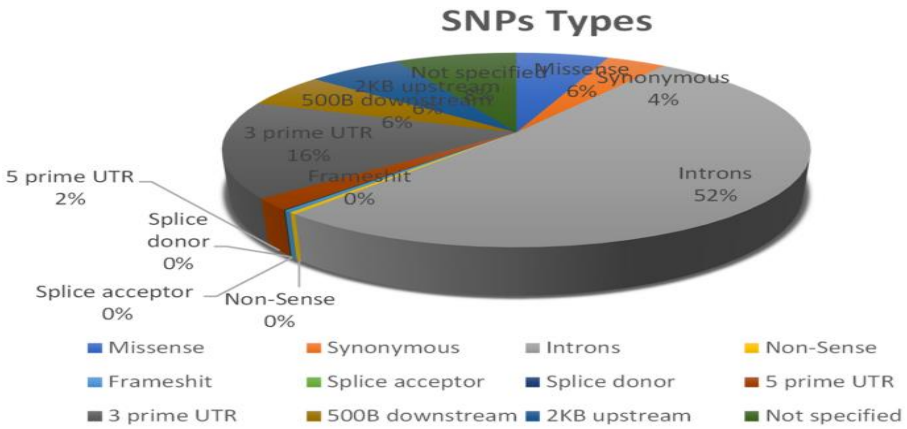


Figure 8: Sequence analysis of A/G at position 49 CTLA-4 Polymorphisms in Control group:



The Sequence study results found that CTLA-4A/G polymorphism among the diabetes mellitus population. The findings also demounted that the CTLA-4 gene might be a susceptibility gene for type 1 and 2 diabetes, and CTLA-4 polymorphisms are important predictors of diabetes. According to the various scientist reports, the current genetic study also showed that A/G polymorphism at 49 position in exon 1 of the CTLA-4 gene is also associated with the risk of latent autoimmune diabetes (LADA) and type 1(T1DM) and 2 diabetes mellitus (T2DM).

Figure 9: Percentage of all the SNPs in human CTLA4 gene – NCBI (Irfan *et al.*, 2022)



The NCBI database result showed that the 48% of the SNPs observed in Exon regions. Hence the study found that CTLA-4 gene plays a crucial role in immunological functions of various internal organs like thyroid, pancreas, liver, heart, muscles ect.

Recent studies have found that there are many important risk loci that are closely associated with the development of diabetes, among which CTLA-4 has been identified as a susceptibility gene for T1DM (-Borysewicz Sańczyk *et al.,* 2020). Studies correlating CTLA-4 polymorphisms with T1DM have been carried out among different populations in several countries. It has been shown that the occurrence of T1DM in Korean, Portuguese, Chilean, and Azerbaijani people is not significantly associated with CTLA-4+49 A/G gene polymorphism (Angel *et al.,* 2009; Almasi *et al.,* 2025) whereas in European, Chinese, African, Iranian, Croatian, Polish, Finnish, Belgian, Estonian, Tunisian, and Egyptian people there is a significant association, where the GG genotype of the CTLA-4+49 locus is a risk factor for T1DM (Mosaad *et al.,* 2012). In contrast, there are contradictory studies in Japan, with some studies showing that CTLA-4 exon 1+49 GG genotype is closely associated with type 1 diabetes and others showing no association between the two (Mochizuki *et al.,* 2002). The present study showed that the AA genotype of the CTLA-4 exon 1+49 locus was strongly associated with the development of T2DM, but not with T1DM, which is inconsistent with the previous findings. This discrepancy may stem from the relatively small patient number and hence suboptimal representation of T1DM patient population. However, it was found that DM patients with the G allele at the CTLA-4+49 locus were more likely to develop T1DM (Mochizuki *et al.,* 2002), while those with the A allele were more likely to develop T2DM (Haller *et al.,* 2004).

Approximately 10% of the patients with T2DM have autoantibodies in their body fluids at the time of diagnosis and these patients are insulin resistant, with gradually decreasing islet function as the disease progresses and eventually requiring insulin therapy. The presence of islet-specific antibodies in latent autoimmune diabetes in adults (LADA) is similar to that of T1DM, but its symptoms are similar to those of T2DM (Mollo *et al.,* 2013). Dong *et al.,* (2014) study showed that the GG or AG type of CTLA-4+49 is a susceptibility gene for LADA and can be used to

diagnose and differentiate LADA from T2DM. Hence the present study report also coincide with the Dong *et al.,* findings. Our study included a relatively small number of patients with T1DM, which could limit the rigor of comparison among patients with different genotypes. Further, more comprehensive analysis of the CTLA-4 polymorphism in patients with different diseases using Hardy–Weinberg equilibrium test and multivariate logistic regression analysis could likely enhance our understanding of the pathogenetic role of the CTLA-4 polymorphism.

Ren *et al.,* (2022) data suggested that the gene polymorphisms of CTLA-4, including CTLA-4+49A/G and CTLA4-318C/T, are important predictors of DM. CTLA-4 might be a susceptibility gene for T2DM. Patients with T2DM carrying the T allele at the CTLA4-318 C/T locus are more predisposed to diabetic ketosis. Irfan *et al.,* (2022) identifed three damaging missense SNPs rs1553657429, rs1559591863 and rs778534474 in coding region of CTLA4 gene. Among these SNPs the rs1553657429 showed a loss of potential phosphorylation site and was found to be highly conserved. The prediction of gene– gene interaction showed the interaction of CTlA4 with other genes and its importance in different pathways. This investigation of damaging nsSNPs could be considered in future while studying CTLA4 related diseases and can be of great importance in precision medicine.

So, based these available studies there has been no more number of studies on the frequencies of the polymorphisms among Indian population, therefore the representation of the study sample pool to the general population should also be verified in the future once relevant data become available. The present study would promote effective drug development through DNA-based drug design in the near future.

Conclusion: The results found that the frequency distribution of CTLA4+49A/G genotype and alleles between T1DM, T2DM patients and normal controls were statistically significant. (OR 2.286; 95%CI 0.804 to 6.945; P=0.118), and the CTLA-4 +49A/G polymorphism was recognized as a risk susceptibility factor for T1DM and T2DM. The study concluded that significant association between CTLA-4 +49A/G polymorphism and T1DM and T2DM. So, the study suggested that the CTLA4 gene polymorphism is closely linked to the pathogenesis of diabetes.

Further studies are necessary to investigate how genetic and environmental factors affect the pathogenesis of DM to provide a basis for early diagnosis, genotyping and treatment of DM.

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