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Association between Interleukin-10 promoter polymorphism with type 2 Diabetes mellitus

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Abstract

Type 2 diabetes mellitus is a multifactorial illness triggered by a complicated interplay of various genetic variants with various environmental variables. The quantity of replicated common genetic variants associated with type 2 diabetes mellitus has increased rapidly with the recent genome-wide association (GWA) research. Major health issue in the public are type 2 diabetes mellitus (T2DM) is common throughout the world. Diabetes mellitus incidence is growing and is anticipated to affect 300 million individuals by 2025. Diabetes has been suggested to alter patterns of cytokine expression as an immune-dependent illness. Insulin resistance (IR) is a disease that results in less than anticipated biological impact of a specified insulin concentration. Insulin resistance and insulin secretion decreased are both defined pathophysiology of T2DM. One of the most alarming health issues of the 21st century is the spread of diabetes around the globe. Our goal in this study was to identify the role of IL-10 polymorphism in T2DM patients. The average age of 60 median patients with type 2 diabetes mellitus (31 males and 29 females) ±SD (45.91667 ±16.08799), fasting blood sugar (FBS) is ±SD (184.25 ±57.76387), hypertension (35 positive/25 negative) and 60 non-diabetic controls (32 males and 28 females) is ± SD (47.31667 ±15.13722). The group (T2DM patients and their control) had not a substantial distinction (P=0.33) and in each group (CC, TT, CT) there was a comparison between IL-10 gene polymorphism. T2DM patients and healthy individuals are not associated with the polymorphism of the gene IL-10 (SNP rs 3021097 (C/T).

Keywords: Insulin, Diabetes Mellitis Type 2, Polymorphism, Interleukin-10, Genotype frequencies

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1. INTRODUCTION

Diabetes is a non-communicable disease associated with hyperglycemia and chronic carbohydrates, lipid and protein metabolism due to insulin secretion deficiencies and their effects[1]. The most prevalent form of diabetes is type 2 diabetes mellitus, often caused by reduced manufacturing of pancreatic acid (NIDDM) or adult cancer[2,3]. Due to the complex interplay of distinct genetic and environmental variables, T2DM is also recognized as a multifactorial disease. Different genes affect the high glucose tolerance and thus contribute to T2DM's general susceptibility[4,5]. Analysis of genetic connections and studies of associations have

identified several genes contributing to T2DM. However, polymorphisms associated with T2DM are crucial for each ethnic group due to differences in ethnic lifestyle, environmental variables and genetic background[6,7]. Interleukin-10 (IL-10) is often believed to play an important role in controlling inflammatory and cytotoxic responses to cells, making it a powerful anti-inflammatory mediator. Anti-β cell immunity caused by Th2 has been suggested to be primarily mediated by IL-10[8,9]. In a study 1q was assigned to the IL-10 gene encoding chromosome. The promoter region outlined several polymorphic sites from the transcription point of departure, including two microsatellite polymorphisms and three biallelic polymorphisms: -1082, -819 and -592[10,11]. The genotype promoter IL-10 SNP and the haplotype frequencies appear to have separate racial distributions[12,13]. T2DM has been recognized as an immune-mediated disease resulting in insulin signaling deficiencies and selective destruction of beta-cell-producing insulin in which cytokines play an important role[14]. IL-10 is a significant T-cell-activated immunoregulatory cytokine[15]. IL-10 plays an important role in activating and suppressing the modulator of immune reaction[16,17]. Type 2 diabetes mellitus (T2DM) is a pro-inflammatory metabolic disease linked to acute hyperglycemia and enhanced concentrations of cytokines that show inflammatory causal function. The polymorphism of the cytokine gene was studied in patients with T2DM and standard health-care monitoring, including interleukin-6 (IL-6), factor-α (TNF-α) tumor necrosis and interleukin-10 (IL-10). Genomic DNA and T2DM controls were separated and the polymorphic fragment primers with adequate reaction restrictions (PCR-RFLP) were subsequently measured and genotyped. Patients and controls have been assessed for the distribution of genotypical and transportation frequencies. To identify the authority of the logistical regression model association, odd ratios with a confidence interval of 95 percent were recognized. Double and triple combinations of genotyping have been explored. Tests were performed for gene-gene interaction and disequilibrium association. There was no single IL-6, TNF-α, and IL-10 relationship observed. In a dual combination, the genotype IL-6 -597GA and TNF-α -30GG increased the risk by21 times, while the risk of T2DM increased 314 times in a triple combination of IL-6 -597 AA, TNF-α -308GG and IL-10 -592 CA. The risk of T2DM was 1,41 times higher in all polymorphisms studied by gene-gene communication alone A. These results suggest the increased risk of developing T2DM for the IL-6, TNF-α and IL-10 polymorphisms of people with a haplotype mixture of AA, GG, and CA. Polymorphism is a variation in the DNA sequencing within the individual or populations[18]. A gene is said to show polymorphism if there are currently more than one allele at the same locus. If a change in the sequence of DNA between people is found to be correlated with disease, it is frequently referred to as genetic mutation[19].

2. MATERIALS AND METHODS

2.1 Samples collection

Blood samples of 60 patients were collected of T2DM from the OPD of three hospitals in Lahore city. Consent was taken from all the patients on a Performa of agreement allowing blood drawing for research purposes. All the patients were asked some questions which collected the blood sample and fill the consent form from the patients which give the blood. This consent form is conducted the name of patients, gender, age, glucose level in blood, blood pressure level and the family history of this disease or any other person in this family suffering from this disease. 2 ml blood was collected from the patients in 0.5M EDTA vials and stored at -20°C. Sugar level in blood was noted to determine diabetic status. A person was considered non-diabetic if blood sugar level is less than 100mg/dL (5.6 to 6.9mmol/L). If sugar level was noted between 100 to 125mg/dL (5.6 to 6.9mmol/L) is considering pre-diabetes. If fasting sugar level is 126 mg/dL (7mmol/L) or higher than it is considered diabetic. The collected blood samples from different hospitals are transferred to the University of Lahore and stored at -20°C. Blood samples of normal 60 persons were collected who did not any previous record of the T2DM or any other disease those blood samples were considered as control group in his study.

2.2 Samples collection

DNA was extracted from peripheral blood using the sodium iodide method. Blood was added to equal volumes of 6 M sodium iodide and chloroform: alcohol and centrifuged at 5000 g for 5 minutes. After centrifugation the aqueous layer was removed and addition of isopropanol to the pellet was done to deposit DNA sample. Isolated DNA was rinsed three times with 70% alcohol and then resuspended in 40 ml of TE buffer having pH 8.0[20].

2.3 Agarose gel electrophoresis

For the detection of genomic DNA horizontal gel electrophoresis was used. Amplicons were electrophoresed on 3% agarose with ethidium bromide staining was used. Under the UV light bands of DNA were observed[21].

2.4 Polymerase chain reaction

Primer designing

For the amplification of our genome region -819, allele specific PCR was designed. Genomic sequence of our desired part of the genome was retrieved from NCBI. The properties of primers such as Tm, size, hairpin loop formation, GC content and self-complementation were checked by using **OligoCalc software**. Characteristics of primers are described in **Table 1.**

Table 1: Characteristics of Primers

Sr. No.	Name of Primer	SEQUENCE OF PRIMER 5' to 3'	Product size
1.	T-allele specific primer IL-10-819F1	CCC TTG TAC AGG TGA TGT ATT	232bp
2.	C-allele specific primer IL-10-819F2	CCC TTG TAC AGG TGA TGT ATC	232bp
3.	Reverse (common primer) IL-10-819R	GGA TGT GTT CCA GGC TCC	Common primer

Primer dilution

The primers were first passed through a short spin and melted. Then in 30nmol of primers the inventory solution (100pmol/µl) was prepared by adding 300µl nuclease-free water. In the 90µl nuclease-free water ratio, 10µl of stock solution was taken and dissolved (1:10) which was used as a working solution (10pmol/µl). Primers were well mixed and kept at-20°C.

Optimization of PCR

Initially gradient PCR was done with a range of annealing temperature (53˚C, 54˚C and 55˚C), 54˚C showed accurate result. Concentration of $MgCl_2$ and 10x Taq buffer were also optimized. Two buffers (KCL and $NH_4(SO_4)_2$) were tested and a suitable amplification was noted by using KCL buffer and different concentrations of $MgCl_2$ were tested include 2mM, 2.5mM and 3mM. 3mM was the final concentration. After tested and observed all optimizing conditions like temperature and type of buffer were applied to run the polymerase chain reaction. Optimization conditions of PCR is described in **Table 2.**

Table 2: Optimization conditions for the polymerase chain reaction

Sr. No.	Steps	Temperature	Time	Cycles
1.	Initial denaturation	95˚C	3 min	
2.	Denaturation	95˚C	1 min	30 Cycles
3.	Annealing	54˚C	45 sec	
4.	Extension	72˚C	45 sec	
5.	Final extension	72˚C	7 min	

PCR reaction components

All of these components were used to amplify the specific region of genomic DNA. Chemicals used in PCR are described in **Table 3.**

Table 3: Chemicals used in PCR

Sr.No.	Chemicals	Quantity	Final Concentration
1.	dNTP mix (2mM)	2µl	0.2Mm
2.	$MgCl_2$ (25mM)	2.4µl	3mM
3.	Template DNA	2µl	100ng
4.	Taq Polymerase 5U/µl	0.5µl	-
5.	Forward primer (10pmol/µL)	2µl	1pmol/µL
6.	Reverse primer (10pmol/µl)	2µl	1pmol/µL
7.	10X Taq buffer with KCL	2µl	1x
8.	Nuclease free water	7.1µl	-
	Total volume	20µl	-

PCR procedure

The solution of polymerase chain reaction was prepared according to the quantities of the components and the PCR on all extracted DNA were performed on Applied Biosystems 2720 thermal cycler.

Gel electrophoresis

Gel electrophoresis was used for the conformation of PCR product and analyzes under the UV light. 1.5% (1.5g in 100ml of 1x TAE buffer) agarose gel was used to detect the PCR product[21].

2.5 Statistical Analysis

All the data obtained were analyzed using SPSS statistics software (version 20) for allele-specific genotypes. It was estimated that both sexes (control and case) were genotyped. Using the Chi-square (X2) test, the Hardy Weinberg equilibrium in both control and patient genotypes were evaluated. The P value was found >0.05.

3. RESULTS

3.1 Sample collection

Collection of samples is briefly explained previously in sample collection. DNA extracted from all collected the samples and run the allele specific PCR. Noted some information of all collected samples. In the control group samples are lableled L1, L2 etc. In the control group information like age and gender were noticed. The age between 21 to 75 and gender (32 male and 28 female) were found. Some other information of control group like mean, median and SD are shown in table 4. On the other hand case group 60 samples were labeled H1, H2 etc. In the case group age between 19 to 80 and gender (31 male and 29 female) were found. In the case group hypertension (35 positive and 25 negative) was noticed.

Table 4: Mean, Median, SD, Max and Min information present in this table of both group control and case group.

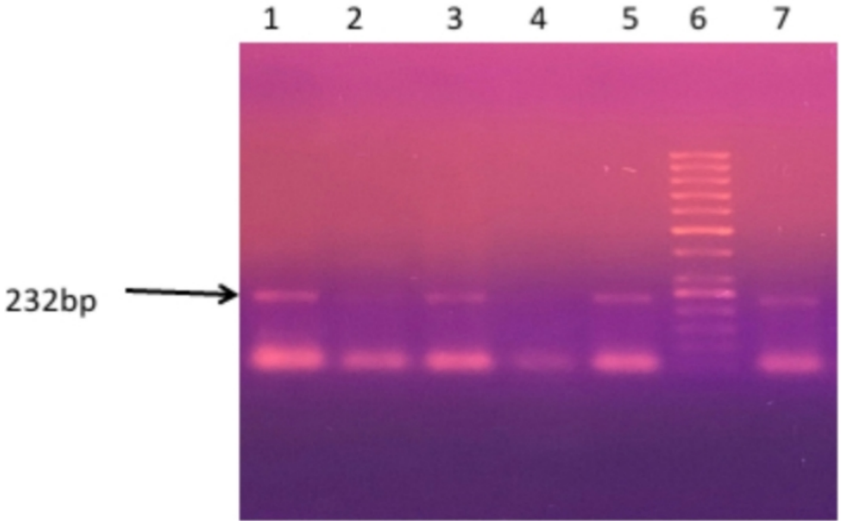
Sr. No.	Group		Mean	Median	S.D	Max	Min
1.	Control	Age	47.316	48	±15.137	75	21
		Glucose level in blood	117.89	102	±39.635	123	104
2.	Case	Age	45.916	45	±16.087	80	19
		Glucose level in blood	184.25	160	±57.763	375	127

3.2 Genomic DNA isolation

Extraction of DNA from whole blood using the salting out method was done and 1% agarose gel was run to confirm isolated genomic DNA. DNA extraction of all 60 cases and 60 controls was done and confirmed through 1% agarose gel under UV light.

3.3 Optimization of allele specific PCR

After the completion of PCR and analyze the PCR products on 1.5% agarose gel it was seen the results. Different annealing temperatures were examined and 54˚C was best suitable annealing temperature for our primers. Therefore, it was decided that annealing temperature 54˚C is the one at which the binding of primers with DNA strand at our desire region (Figure 1).



Figures 1: 1.5% Agarose gel represent PCR results. Lane 1 and 3 show the C allele specific fragment and lane 5 and 7 showing C allele specific and T allele specific. Lane 6: DNA ladder.

This result showed both heterozygous and homozygous genotype. Lane 1 and 3 represent homozygous and lane 5 and 6 represent heterozygous genotype. Two samples showed CC genotype and one sample showed CT genotype. Figure 2is showing band of 232bp of C allele specific PCR product.

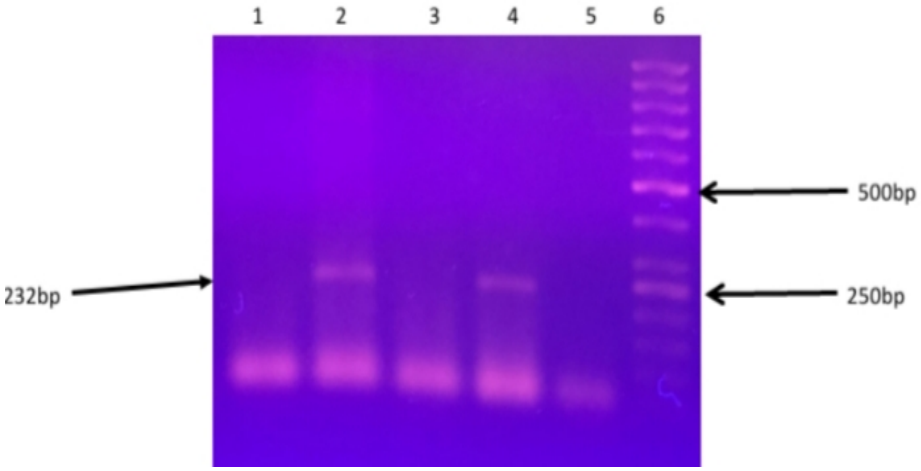


Figure 2: 1.5% Agarose gel represent the results of PCR product. Lane1, 2, 3 and 4 showing the result of T allele specific genotype and lane 6 showing DNA ladder.

Figure 3 showed the result of PCR products are homozygous. It represented TT genotype at 232bp band. That sample gave both C allele and T allele specific fragment. The result showed the both homozygous and heterozygous genotypes. In Figure 3 lane 2 and 13 showed the T allele specific fragment and it was homozygous. Lane 3, 4, 5, 6, 7, 8, 9, 11, 14 and 15 showing heterozygous genotype, CT allele specific fragments and it was 232bp size.

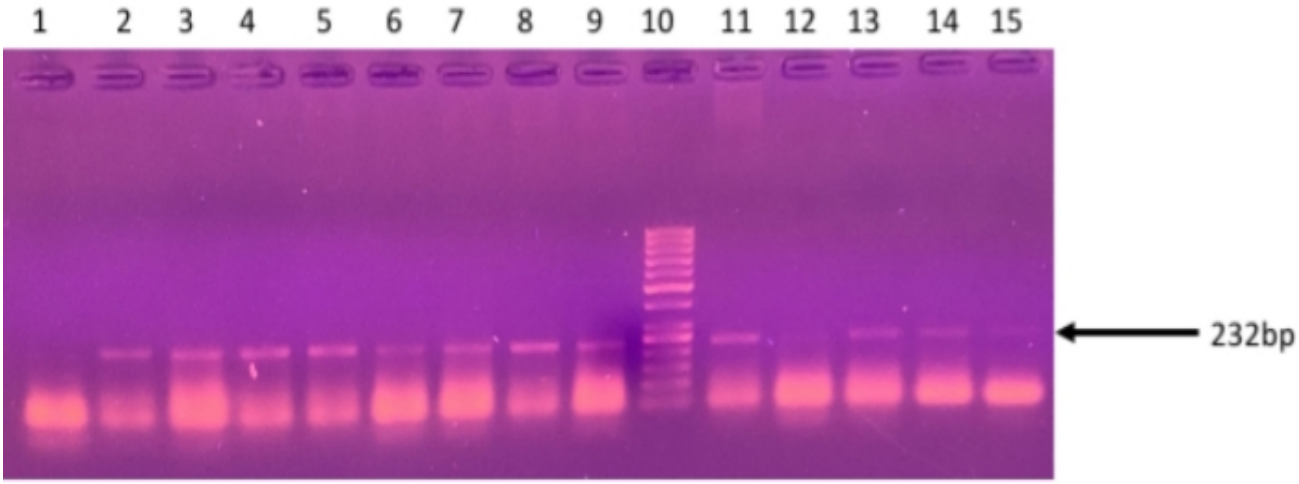


Figure 3: 1.5% Agarose gel showing PCR products. Lane 1 and 2: T allele specific fragments and Lane 12 and 13: T allele specific fragment. Lane 3, 4, 5, 6, 7, 8, 9, 11, 14 and 15: C allele specific fragment and T allele specific fragment. Lane 10: DNA ladder.

3.4 Genotype determination

Genotypes of control groups are described in Table 5 and genotypes of case groups are described in Table 6.

Table 5: Genotypes of control group

Sr. No.	Sample Name	Genotype	Sr. No.	Sample Name	Genotype
1.	L1	CC	31.	L31	CT
2.	L2	CT	32.	L32	TT
3.	L3	CT	33.	L33	CT
4.	L4	CC	34.	L34	CC
5.	L5	CC	35.	L35	TT
6.	L6	CT	36.	L36	TT
7.	L7	TT	37.	L37	CC
8.	L8	CT	38.	L38	TT
9.	L9	TT	39.	L39	TT
10.	L10	CC	40.	L40	CT
11.	L11	TT	41.	L41	CT
12.	L12	CT	42.	L42	TT
13.	L13	TT	43.	L43	CC
14.	L14	CC	44.	L44	CT
15.	L15	CT	45.	L45	CT
16.	L16	CC	46.	L46	CC
17.	L17	TT	47.	L47	TT
18.	L18	CT	48.	L48	TT
19.	L19	CC	49.	L49	CC
20.	L20	TT	50.	L50	TT
21.	L21	TT	51.	L51	TT
22.	L22	CC	52.	L52	TT
23.	L23	TT	53.	L53	CC
24.	L24	CT	54.	L54	TT
25.	L25	TT	55.	L55	CT
26.	L26	CC	56.	L56	CT
27.	L27	TT	57.	L57	CC
28.	L28	CT	58.	L58	CT
29.	L29	CC	59.	L59	CT
30.	L30	CT	60.	L60	CC

Table 6: *Genotypes of case group*

Sr. No.	Sample Name	Genotype	Sr. No.	Sample Name	Genotype
1.	H1	TT	31.	H31	CC
2.	H2	CT	32.	H32	CC
3.	H3	CT	33.	H33	CT
4.	H4	CC	34.	H34	CC
5.	H5	CT	35.	H35	TT
6.	H6	TT	36.	H36	CT
7.	H7	CT	37.	H37	CC
8.	H8	CT	38.	H38	CT
9.	H9	CT	39.	H39	CT
10.	H10	CC	40.	H40	CT
11.	H11	CC	41.	H41	TT
12.	H12	CT	42.	H42	CC
13.	H13	TT	43.	H43	CT
14.	H14	CT	44.	H44	CC
15.	H15	CC	45.	H45	CT
16.	H16	CT	46.	H46	CT
17.	H17	CT	47.	H47	CT
18.	H18	TT	48.	H48	CC
19.	H19	CC	49.	H49	TT
20.	H20	CT	50.	H50	CT
21.	H21	CT	51.	H51	CT
22.	H22	CT	52.	H52	CC
23.	H23	CC	53.	H53	CT
24.	H24	CT	54.	H54	CC
25.	H25	TT	55.	H55	CT
26.	H26	CT	56.	H56	CT
27.	H27	CT	57.	H57	CC
28.	H28	CT	58.	H58	CT
29.	H29	CT	59.	H59	CT
30.	H30	TT	60.	H60	TT

3.5 Genotype frequencies of control group

The results showed that the frequencies of homozygous TT is higher of all other frequency of CT and CC allele specific genotype were 20 and 18 respectively. Percentages of TT, CT and CC were 18.3, 16.7 and 15.0 respectively. Genotype frequencies of control group are described in Table 7 and shown in Figure 4.

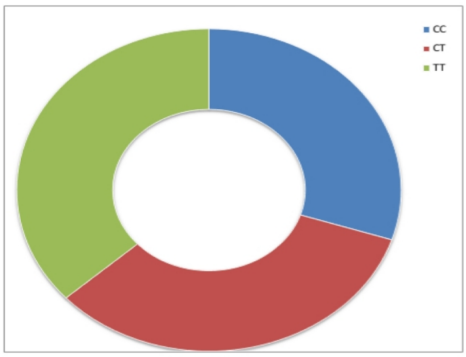


Figure 4: Graphical representation of control group genotypes. Green color represents TT homozygous genotype. Blue color represents CC homozygous genotype and Red color are represents CT heterozygous genotype Percentages of TT, CT and CC were 18.3, 16.7 and 15.0 respectively.

Table 7: Genotype frequencies of Control Group

Genotype	Frequency	Percent
CC	18	15.0
CT	20	16.7
TT	22	18.3
Total	60	100.0

3.6 Genotype frequencies of case group

The results show that the CT heterozygous genotype is higher for all whereas the frequency of TT and CC allele specific genotype is 10 and 16 respectively. Genotype frequencies of case group are described in Table 8 and shown in Figure 5.

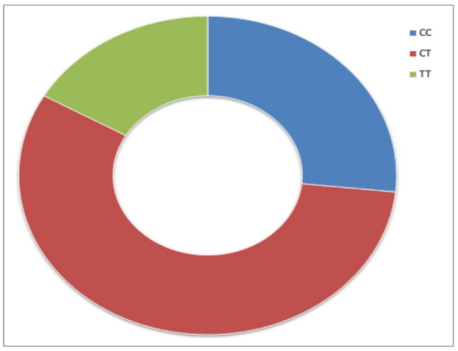


Figure 5: Graphical representation of case group genotypes. Green color represents TT homozygous genotype. Blue color represents CC homozygous genotype and Red color are represents CT heterozygous genotype.

3.7 Chi-Square test

Pearson Chi-Square value was 8.247 (P=0.33). The results of Chi-square approved that there was not a statistically significant between genotype frequency of the two sample groups for the IL-10 and -819 SNP C/T.

4. DISCUSSIONS

Type 2 diabetes mellitus was not considered the risk factor in Pakistan. Extracted the DNA from 60 normal and 60 patients group and all the samples proceed allele specific PCR. Observed the Genotype in the allele specific PCR. In the statistical analysis and find the p value is (P=0.33) and this value is shown the CT genotype are not significant between the control and case group. Type 2 diabetes mellitus (T2DM) identified as an immune-mediated disease that results in deficiencies in insulin signaling and selective destruction of beta-cell insulin that plays an important role in cytokines[14]. It plays an important role in activating and suppressing the immune response and acts as a modulator of the immune reaction[16,17]. Previous research demonstrates that IL-10 genetic polymorphisms can contribute to the determination of diabetes susceptibility, but do not appear significant in clinical manifestations of diabetes[22]. On the other hand, the incidence of (mutant) -819 T allele and -819C/T genotype was reduced in Tunisian patients with diabetes nephropathy; neither polymorphism -1082G/A and also not the polymorphism -592C/A was related with asthma nephropathy[23]. In the gene promoter area of IL10 -1082A/G, -819C/T and -592A/C, several polymorphic upstream sites have previously been reported[24]. Other studies found an association between genetic polymorphism of IL 10 and the risk of T2DM[25,26,27,28]. Patients with T2DM and controls have shown clinical and laboratory characteristics that patients with T2DM have higher proportions of waist and hip than controls. These findings represent the understanding that not only obesity (mainly obesity) but in particular the distribution of body fat (mainly obesity) influence glucose metabolism[29]. Increase in cytokine manufacturing, decreased expression, an inhibited matrix-degrading metalloproteinase and encouragement of phenotypic lymphocyte shift into Th2 phenotype is an anti-inflammatory cytokine that plays an significant role in controlling immune systems. The genetics of the cytokine are T cells, B cells, monocytes and macrophages and is estimated to be 75% of the variation in IL-10 manufacturing[30]. An important study found that in people with impoverished glucose tolerance or T2DM IL-10 concentrations were smaller than those with ordinary glucose tolerance and inverted BMI correlation[31]. Higher IL-10 concentrations were discovered in T2DM patients with the resulting checks. It is not clear from the overall perspective whether higher IL-10 levels protect against T2DM by reducing pro-inflammatory cytokine production and increased T2DM IL-10 levels by compensating for pro-inflammatory mediators, mainly IL-6, TNF-α and T2DM[32]. There was no polymorphism that showed any difference between patients of T2DM and the control group in all and frequencies of genotype. Therefore, this research did not involve T2DM related polymorphism. But how these polymorphisms are linked to T2DM remains unsure. In 2011, when considering the Caucasian and Asian populations, the meta-analysis found no significant correlation between TNF-α-308G/A polymorphism and T2DM risk[33]. A meta-analysis showed, however, that TNF-α-308A allele can be a risk factor in Asian patients developing T2DM and No polymorphism showed any difference between T2DM patients and control group in all genotype frequencies. Consequently, this research did not involve polymorphism related to T2DM but it remains uncertain how these polymorphisms are linked to T2DM[34]. In 2011, meta-analyzing failed to detect significant links in the consideration of Caucasian and Asian populations between TNF-α-308 G/A polymorphism and T2DM. However, the authors found a connection with T2DM, respectively, between the genotype -1082GA and -1082 G allele[33]. In addition, a case-control study by another author found a higher risk of T2DM -1082 GA+GG and -592 AC+AA genotypes. These conflicting results are likely to be associated with the sample size and separate population genetic background. In order to further assess these connections, greater genome studies are required[35]. Plasma levels were significantly differing between the 174 C/G genotypes, but this difference was not maintained after Bonferroni's correction. The concentrations of IL-10 -819TT for patients of the genotype were greater than those of the -819 CC-genotype. Previous studies have demonstrated that ATA haplotypes (-1082A, -819 T, -592A), as well as the -819T-allele, are associated with decreased transcription activity. However, no further research in patients with diabetic diseases showed that IL-10 is associated with serum/plasma and polymorphism with serum/plasma concentrations and IL-10[11,36]. Interleukin 10 is a significant cytokine that stimulates and suppresses the immune reaction[16]. The low-capacity association of IL-10 has been created with metabolic syndrome and T2DM. Co-treatment has been recorded in rats with IL-10 to prevent deficiencies in hepatic and skeletal muscle insulin[37]. Interleukin 10 is a significant cytokine that stimulates and suppresses the immune reaction[16]. The low-capacity association of IL-10 has been created with metabolic syndrome and T2DM. Co-treatment has been recorded in rats with IL-10 to prevent deficiencies in skeletal muscle and hepatic insulin[37].

5. CONCLUSIONS

This research analyzed polymorphism of IL-10 -819C/T in type 2 diabetes mellitus. After performing an allele specific PCR on 60 case group and 60 controls for the -819C/T genotyping. Our findings have been evaluated by means of statistical analysis. IL-10 and SNP -819C/T were found not associated with T2DM in the geneIL-10. As a consequence, gene IL-10 CC and CT were not discovered in Pakistan as risk factors for genetic susceptibility for type 2 diabetes mellitus.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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