Gene polymorphism of transcription factor 7/Like 2 (*TCF7/L2 rs7903146*) in type 2 diabetic subjects with and without retinopathy.

Running Title: *TCF7/L2* polymorphism in T2DM subjects with and without retinopathy

Authors: Shazia Azhar, Asher Fawwad, Kahkashan Perveen, Iftikhar Ahmed Siddiqui, Nazish Waris, Ruqaya Nangrejo, Abdul Basit

Ms. Shazia Azhar, PhD Scholar.
Assistant Professor and Deputy Director
Baqai Institute of Medical Technology, Baqai Medical University

Prof. Dr. Asher Fawwad, PhD
Professor of Biochemistry
Dean, Faculty of Basic Medical Sciences, Baqai Medical University (BMU)
Chairman, Biochemistry Department, BMU

Dr. Kahkashan Perveen, PhD
Associate professor
Department of Biochemistry,
Baqai Medical College, Baqai Medical University

Prof. Dr. Iftikhar Ahmed Siddiqui, PhD
Professor of Biochemistry
Vice Chancellor, Baqai Medical University

Dr. Nazish Waris, PhD
Assistant Professor
Department of Biochemistry,
Baqai Medical College, Baqai Medical University

Dr. Ruqaya Nangrejo, PhD
Associate Professor
Department of Physiology,
Baqai Medical College, Baqai Medical University

Prof. Dr. Abdul Basit, PhD
Director,
Baqai Institute of Diabetology and Endocrinology,
Baqai Medical University

Author for Correspondence

Ms. Shazia Azhar, PhD Scholar.
Assistant Professor and Deputy Director
Baqai Institute of Medical Technology, Baqai Medical University
Address: 51, Deh Tor, Gadap Road, Super Highway, Karachi-Pakistan

Abstract

Objectives: To find out the gene polymorphism of transcription factor 7/Like2 (*TCF7/L2 rs 7903146)* in type 2 diabetic subjects with and without retinopathy.

Method: This prospective cross sectional study was designed in Biochemistry Department, Baqai Medical University from March 2021 to April 2022. Subjects were identified from outpatient department of Baqai Institute of Diabetology and Endocrinology (BIDE) and categorized into three groups: Group A (healthy subjects without diabetes), Group B (T2DM subjects with retinopathy), Group C (T2DM subjects without retinopathy). Predesigned proforma was used for baseline characteristics and blood sample was collected for biochemical parameters and genetic analysis. Biochemical analysis was done at BIDE and genetic analysis at University of Karachi.

Results: Total of 148 subjects were recruited, 74 in Group A, 25 in Group B and 49 in Group C. Mean age (years) of group A, group B and group C was 43.27±9.45, 54.0±13.2 and 51.2±10.8, respectively. In all the groups, males and females ratio were almost equal. Results for systolic blood pressure, fasting blood glucose, HbA1c, triglycerides and low density lipoprotein cholesterol were found significant ($p<0.05$) between the groups. The TT genotype of TCF7/L2 rs 7903146 was observed to be abundant in group B (0.80) than group C (0.39) and group A (0.1). While, the CT genotype was found higher in group A (0.77) and group C (0.59) than in group A

(0.20). Moreover, the proportion of mutant T allele was predominantly higher in group B (0.90) and group C (0.68) than group A (0.40).

Conclusion: Overall, homozygous TT genotype of *TCF7L2 rs7903146* polymorphism was found dominant in T2DM subjects with retinopathy and CT genotype was found higher in T2DM subjects without retinopathy and healthy individuals. We also found mutant T allele as predominantly higher in T2DM subjects with and without retinopathy.

Keywords: *TCF7L2 rs7903146*, gene polymorphism, T2DM, retinopathy

Introduction

Diabetes Mellitus Type 2 (T2DM) is increasing with high prevalence in Pakistan (30.8%) followed by French Polynesia (25.2%) and Kuwait (24.9%) around the globe [1]. T2DM is associated with both macrovascular (ischemic heart disease, peripheral vascular disease, and cerebrovascular disease) and microvascular (retinopathy, nephropathy, and neuropathy) complications [2]. Environmental factors like oxidative stress, polyol pathway accumulation, Wingless/Integrated (Wnt) signaling pathway and advanced glycation of end products (AGEs) contribute to the development of diabetic angiopathy [3]. Changes in vessel lining alter the endothelial permeability and blood flow in microvasculature, resulting in diabetic nephropathy, neuropathy, and retinopathy [4].

Diabetic retinopathy (DR) in people with T2DM may cause damage to the eye vessels, formation of micro aneurysms and angiogenesis which result in changes in the retinal and vitreous vasculature and partial or total blindness [5]. Vessel occlusions occur due to the formation of hard exudates that are yellow-white intra-retinal deposits of variable size made up of extracellular lipids leaked from abnormal retinal capillaries. Micro aneurysms are deep red dots

that are usually found in the posterior poles of the eye due to pericyte degeneration [6]. Hyperglycemia, hypertension, ageing, and dyslipidemia are all known potential cardio metabolic risk factors for DR [7]. Findings of retinal hemorrhage; cotton wool patches, proliferative retinal vessels, and micro aneurysms are used as diagnostic criteria in the retinal examination of DR [8]. It was found that transcription factor 7/Like2 (TCF7/L2) rs-7903146 gene polymorphism increases the susceptibility of developing T2DM [9]. Association between DR and polymorphism of TCF7L2 expression and alteration in vascular endothelial growth factor (VEGF) expression levels have also been found in certain populations [10]. A high-mobility group TCF7L2 plays a role at the endpoint of the canonical Wnt signaling transduction cascade. TCF7L2 is activated by ligands activation Wnt or growth factors that are insulin and insulin growth factor (IGF)-1 to participate in many biological processes related to Wnt [11]. The Wnt signaling pathway is the key effector of TCF7/L2 that plays a central role in directing glucose homeostasis in the pancreas and is required for maintaining glucose-stimulated insulin secretion (GSIS) and beta-cell survival [12]. The interaction of the Wnt receptor complex releases β-catenin which then enters the nucleus and binds to the promoter region, aiding gene expression of TCF7L2 gene [13]. The TCF7L2 controls the expression of glucagon-derived hormones like glucagon like peptide-1 (GLP-1) and binds with its receptor GLP-1R to limit the food intake by activating GLP-1R in the hypothalamus and stomach, which inhibits gastric intestine emptying and lowers plasma glucose levels [14].

Though, TCF7L2 variations in association with diabetes susceptibility in different ethnic descent were found in literature, however, TCF7L2 association in T2DM with and without retinopathy subjects are scarce in Pakistan. Therefore, this study aims to explore the gene polymorphism of transcription factor 7/Like2 (TCF7/L2 rs 7903146) in T2DM with and without retinopathy.

Methodology

This prospective cross sectional study was designed in the Department of Biochemistry, Baqai Medical University (BMU). The duration of study was from March 2021 to April 2022. Subjects of age group 18 years and above were recruited who visited to outpatient department of Baqai Institute of Diabetology and Endocrinology (BIDE), BMU. Subjects with already known T2DM with or without retinopathy, and/or subjects without T2DM (healthy) were included. Participants were divided into three groups: Group A (healthy without diabetes), Group B (T2DM subjects with retinopathy), Group C (T2DM subjects without retinopathy). Healthy subjects were recruited using oral glucose tolerance test (OGTT).

Preliminary data was collected using predesigned questionnaire that includes age, gender, systolic and/or diastolic blood pressure (SBP/DBP), body mass index (BMI) and details of retinopathy complications (blindness, micro aneurysm and blot hemorrhage). Direct ophthalmic examination and/or early treatment for diabetic retinopathy study (ETDRS) was used to confirm fundus examination. Subjects with type 1 diabetes, gestational diabetes, diabetic subjects with other complications such as diabetic foot, diabetic nephropathy, diabetic neuropathy, cataracts, subject with any disability and subject with any serious complications were excluded from the study.

Blood sample for biochemical parameters were collected and analyzed at BIDE. Biochemical parameters include fasting blood glucose (FBG), 2-hour post glucose load (only for healthy subjects), glycated hemoglobin A1c (HbA1c) and lipid profile. FBG and 2-hour post glucose load was performed by glucose oxidase peroxidase method, HbA1c by high performance liquid chromatography (HPLC) method, total cholesterol by cholesterol oxidase dehydrogenase

peroxidase (CHOD-PAP) method, triglycerides by glycerol phosphate oxidase - peroxidase (GPO-PAP) method and high-density lipoprotein (HDL) and low-density lipoprotein (LDL) was performed by direct method.

As per the American Diabetes Association (ADA) criteria, healthy subjects with FBG < 100 mg/dl and plasma glucose after 75gm glucose load <140 mg/dl were considered as normal (fasting defined as no caloric intake for minimal 8 hours) [15]. Good glycemic control is considered if found HbA1c <7% and poor HbA1c >7% [16]. Serum total cholesterol >200 mg/dl, LDL >130 mg/dl, HDL <40 mg/dl (for males) and <50 mg/dl (for females), and triglycerides >150 mg/dl was considered as participant is suffering from dyslipidemia [17]. Height was measured in cm and weight in kilogram. BMI was calculated by dividing weight (kg)/Height $(m)^2$. BMI <23 kg/m^2 was considered as normal, ≥23-24.9 kg/m^2 as overweight and ≥25 kg/m^2 as obese as per World Health Organization Asia Pacific Guideline [18]. SBP/DBP ≥ 140/90 mmHg was considered as hypertension [19].

Genetic Analysis

Genetic analysis was performed in University of Karachi. In genetic analysis, DNA was purified by Quaigen method. A 400 µL of lysis solution and 20 µL of proteinase K solution were added thoroughly by vortexing or pipetting to obtain a uniform suspension. Samples at 56ºC were incubated while vortexing occasionally and shaking water bath and thermo-mixer was used until the cells were completely lysed (10 minutes). Then, 200 µL of ethanol (96-100%) was added and mixed by pipetting or vortexing. Further, the bag was closed with Gene Jet Genomic DNA purification columns. Purified DNA was used immediately in downstream applications or stored at -20 ºC. Whole Genomic DNA of group A, B and C is shown in figure 1.

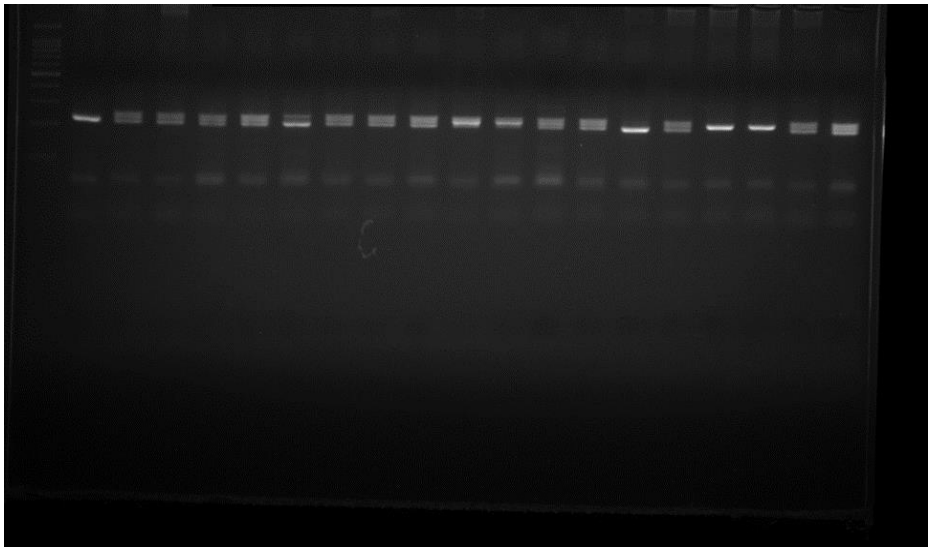

Figure 1: Whole Genomic DNA of group A, B and C

The primer sequences of polymorphisms were designed for allele specific polymerase chain reaction (AS-PCR) using online WASP (Web-based Allele Specific PCR) software.[20] Primers of TCF7L2 gene (rs 7903146) include product size of 103 wildtype forward primer 5' TGGAGGAATGCATAGGCAAGATAAC 3', mutant forward primer 5' TGGAGGAATGCATAGGCAAGATAAG 3' and common reverse primer of 5' GCGAAATTTGTCTTTACACC 3'. The general rules standard for primer designing rules were strictly followed including high specificity, ratio of GC content, introduction of mismatches at specific places, length of oligonucleotide sequence and melting temperature of the primer set. The designed pair of primers were also confirmed using online matching bioinformatics software BLAST (Basic Local Alignment Search Tool) [21].

Reagents for optimized AS-PCR for 1 x 20 µL reaction are 7 µL Dream Taq Green Master Mix, 1µ Forward Primer (F1 or F2), 1µl Reverse Primer (R), 4µ DNA and 7µl Deionized water. An optimized program condition for amplification is based on 5 steps. Step-1 involve 1cycle at 94°C for 3 minutes, step-2 will be followed at 94°C for 30 seconds, step-3 consisting of 35 number of

cycles at 59.3°C for 30 seconds, step-4 at 72°C for 1 minutes and step-5 involve 1 no. of cycle for 72°C at 5 minutes. For 100ml of 1.5 % agarose gel, 1.5 g of agarose powder was weighed. The volume was raised up to 100ml with 1X TBE buffer. The mixture was heated in order to dissolve the agarose powder. A clear solution was formed with no particles of agarose remain un-dissolved. VisualaNa DNA stain of 6ml was added. Then the solution was poured slowly in to the gel-casting tray. Bubbles were pushed away to the side with the help of disposable tip. The combs were inserted, and its positioned was double checked. The gel was left to polymerize approximately for 15-20 minutes depending on the surrounding temperature.

Statistical Analysis

The statistical package social science (SPSS) version 22 was used for the analysis of data. The description of the continuous variables was carried out by the calculation of means and standard deviations. Frequencies and percentages were used for the categorical variables. To test the normal distribution of continuous variables, the Kolmogorov-Smirnov test was used. Initially, in order to analyze the mean difference between the groups One-Way ANOVA was performed. For categorical variable chi-square was performed. The p-value <0.05 indicated as significant. For gene polymorphism odd ratio and 95% confidence interval (95% CI) were calculated by using online software MedCalc.

Result

Total of 148 subjects were recruited, 74 in Group A, 25 in Group B and 49 in Group C. Table I presents the baseline demographic, anthropometric and biochemical parameters of studied participants. Mean age (years) of group A, group B and group C was 43.27±9.45, 54.0±13.2 and 51.2±10.8, respectively. In all the groups, males and females ratio were almost equal. BMI (kg/m2) in group A, B and C was 29.42±6.4, 28.9±4.8, and 29.4±6.2, respectively but found

non-significant between the groups. Results for systolic blood pressure, FBG, HbA1c, triglycerides and LDL cholesterol were found significant ($p<0.05$) between the groups. Characteristics of register blindness (24%), microdot (32%), blot hemorrhage (40%), exude hard (48%), exude soft (44%), and vitreous hemorrhage (28%) were commonly observed in diabetic subjects with retinopathy.

Interpretation of gene sequencing

Genotyping of TCF7/L2 rs 7903146 variant of group A, B and C using AS-PCR are shown in figure 2(a), 2(b) and 2(c), respectively.

TCF7/L2 genetic variant rs 7903146 between group A and B was explored in table II. The TT genotype of this variant was observed to be abundant in group B (0.80) than group A (0.1). While the CT variant was found higher in group A (0.77) in comparison to group B (0.20). Moreover, the proportion of mutant T allele was predominantly higher in group B (0.90) than group A (0.40). Chi-square revealed a strong significant association of this variant ($\chi2= 6.447$, $p=0.011$). A significant role of mutant T allele was also found by odds ratio ($OR=0.07$, $p<0.001$).

TCF7/L2 genetic variant rs 7903146 between group A and C was presented in table III. In group A (0.77) and group C (0.59) the CT genotype of this variant was found higher. Moreover, in group C, genotype variant TT (0.39) was also significantly found higher than in group A (0.1). The proportion of mutant T allele was also predominantly found higher in group C (0.68) than group A (0.40).

Table IV shows the TCF7/L2 genetic variant rs 7903146 between group B and C. The TT variant in group B (0.80) was found higher than group C (0.39). In group C, the CT genotype as

compared to CC genotype was found dominant. The proportion of mutant T allele was predominantly found higher in group B (0.90) than group C (0.68).

Discussion

T2DM is known as a complex metabolic disorder associated with various environmental risk factors and genetic susceptibility. We found homozygous TT genotype of TCF7L2 rs7903146 polymorphism as dominant in T2DM subjects with retinopathy and CT genotype was found higher in T2DM subjects without retinopathy and healthy individuals. We also found mutant T allele as predominantly higher in T2DM subjects with and without retinopathy. Significant results for SBP, FBG, HbA1c, triglycerides and LDL were also observed between the groups.

Our findings with dominant frequency of mutant T alleles in T2DM subjects with and without retinopathy are somehow in line with Bahaaeldin et al study who also reported that TT genotype play role in development of T2DM [22]. In literature, very scarce of data for TCF7L2 rs7903146 polymorphism in T2DM subjects with retinopathy was found, that make difficult to compare our data with other ethnic groups [23],[24]. However, considering T2DM subjects without retinopathy our study also in line with Cropano et al who showed that the T risk allele confers the strongest risk of T2DM known to date in Caucasians and other ethnic groups [25]. The occurrence of T alleles in subjects with T2DM is also consistent with Anjum et al and Assmann et al study who also found a significant association of CT and TT polymorphisms [26],[27]. In meta-analysis of different ethnic groups a positive association of the CT genotype with T2DM was also reported [28].

We found poor control of glycaemia, triglycerides and LDL more prominent in T2DM subjects with retinopathy. Ahmed et al., reported that poor glycemic control is highly associated with DR [29]. Our findings are in agreement with Gunavathy et al study who reported dyslipidemia in

T2DM subjects [30]. Our findings support Dornan et al., study who first time reported an association between DR and LDL cholesterol [31]. It support the postulate that rise in viscosity and alterations in the fibrinolytic system which forms hard exudates occur with dyslipidemia. Additionally, alteration in membrane fluidity and leakage of plasma occur with incorporation of TG into the cell membrane that results in retinal hemorrhage and endothelial dysfunction which also worsens the retinopathy [32]. We observed most of the individuals were obese in all the groups though the findings are non-significant. It may because the prevalence of obesity was found with rising trends in Pakistan [33]. It is also an established fact that obesity is a risk factor for several systemic diseases such as hypertension, stroke, dyslipidemia, and DR as well [34].

We observed significantly high SBP in T2DM subjects with and without retinopathy. Recently, it was also reported that hypertension is an independent risk factor and strongly associated with DR [35]. High blood pressure and diabetes may cause optic neuropathy, systemic disorders, hemorrhages, blur vision, congestion retinal veins, and also form hard exudates that deposit in the macula that harm the retina including by reducing the inner retina and impairing the microcirculation [36]. Hypertension and diabetes are also responsible for focal arteriolar narrowing. The Wnt/β-catenin/T-cell factor (TCF) (canonical) signaling pathway inhibits the adipogenic differentiation of pericyte (a contractile cell in small retinal arterioles), and later effect in regulating retinal microvascular function [37]. This pathway also regulates vascular smooth muscle cell proliferation; it may be involved in thickening, hyperplasia and arteriosclerosis manifesting causes focal arteriolar narrowing [38].

Small sample size as per our population due to low resources is the limitation of our study. In literature, TCF7L2 rs7903146 polymorphism with retinopathy in our population was not found is

our study strength. Future research on large sample size to determine an association of TCF7L2 rs7903146 polymorphisms with DR and other associated risk factors are highly recommended for this population.

Conclusion

Overall, homozygous TT genotype of TCF7L2 rs7903146 polymorphism was found dominant in T2DM subjects with retinopathy and CT genotype was found higher in T2DM subjects without retinopathy and healthy individuals. We also found mutant T allele as predominantly higher in T2DM subjects with and without retinopathy.

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Ethical Approval and Informed Consent

Ethical approval of the study was obtained from the ethical review committee (ERB) of BMU (ERB no: BMU EC/02/2020-04(0L). Written informed consent was obtained from each subject prior to enrolment in the study.

Competing Interests Statement

We authors declare that we do not have any competing interest statement.

Conflict of Interests

We, all authors have no conflict of interest to declare.

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Author's Contribution

All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

SA: Data analysis, interpretation, and wrote the manuscript.

AF: Concept and design, edit, review and approved the manuscript.

KP: Concept and design, edit, review and approved the manuscript.

IAS: Review and approved the manuscript.

NW: Data analysis and wrote the manuscript

RN: Interpretation of data, edits, review and approved the manuscript.

AB: Review and approved the manuscript.

Results:

Table I: Baseline demographic, anthropometric and biochemical parameters of studied participants

Variables	Group A	Group B	Group C	P-value
n	**74**	**25**	**49**	
Age	43.27±9.45	54.0±13.2	51.2± 10.8	0.000*
Gender				
Male	37(50.0)	11(44.0)	24(49.0)	0.87
Female	37(50.0)	14(56.0)	25(51.0)	
Body mass index (kg/m2)	29.42±6.4	28.9±4.8	29.4±6.2	0.52
Blood Pressure				
Systolic	118.9±21.4	138.8±21.6	142.5±17.7	0.03*
Diastolic	81.7±16.5	83.2±10.5	86.3±19.7	0.09
Fasting Blood Glucose (mg/dl)	91.54±11.86	146.33 ±18.69	135.06±22.53	0.001*
2-hour glucose load (mg/dl)	119.51±23.4	NA	NA	NA
HbA1c (%)	5.7±0.5	9.2±2.1	8.6±2.7	0.000*
Cholesterol (mg/dl)	181.1±34.8	178.2±47.9	189.6±44.3	0.699
Triglyceriods (mg/dl)	181.4±20.1	229.7±35.1	208.1±36.56	0.017*
Low density lipoprotein (mg/dl)	104.27±31.6	152.0±39.96	141.95±36.61	0.000*
High density lipoprotein (mg/dl)	26.6±7.39	32.33±6.95	34.93±8.54	0.065

Data presented as mean ± SD and/or n (%), * indicates $p<0.05$ is statistically significant

Groups: Group A is healthy individuals; Group B is T2DM subjects with retinopathy; Group C is T2DM subjects without retinopathy

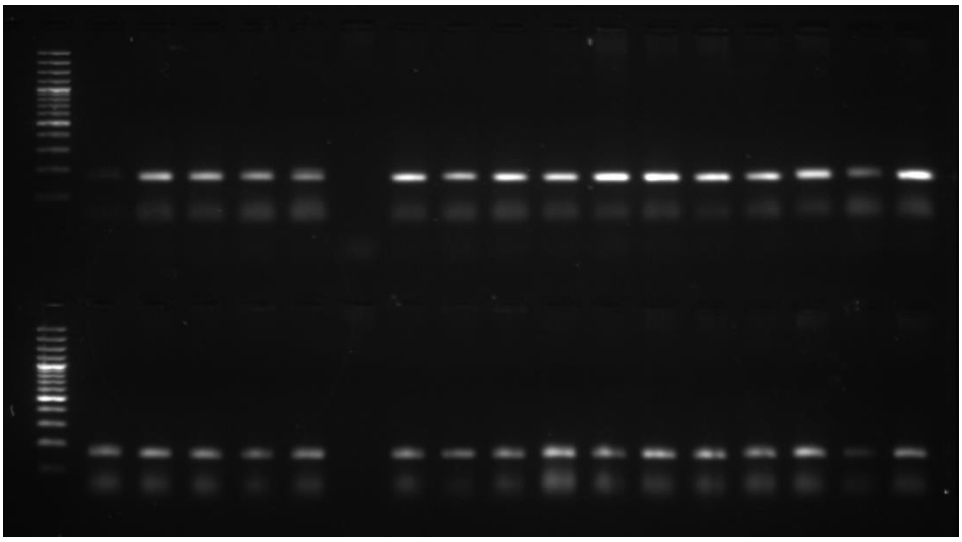

Figure 2(a): Genotyping of TCF7/L2 rs 7903146 variant of group A using AS-PCR

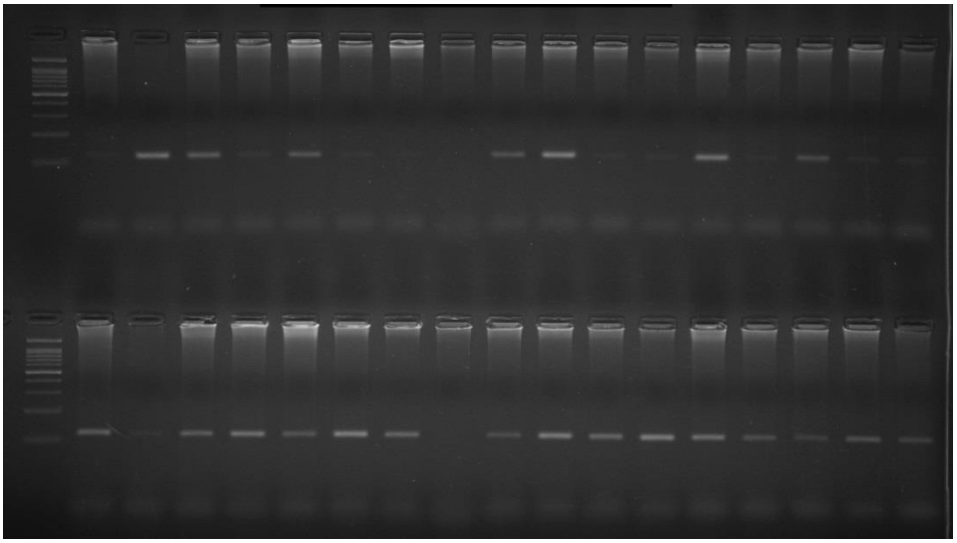

Figure 2(b): Genotyping of TCF7/L2 rs 7903146 variant of group B using AS-PCR

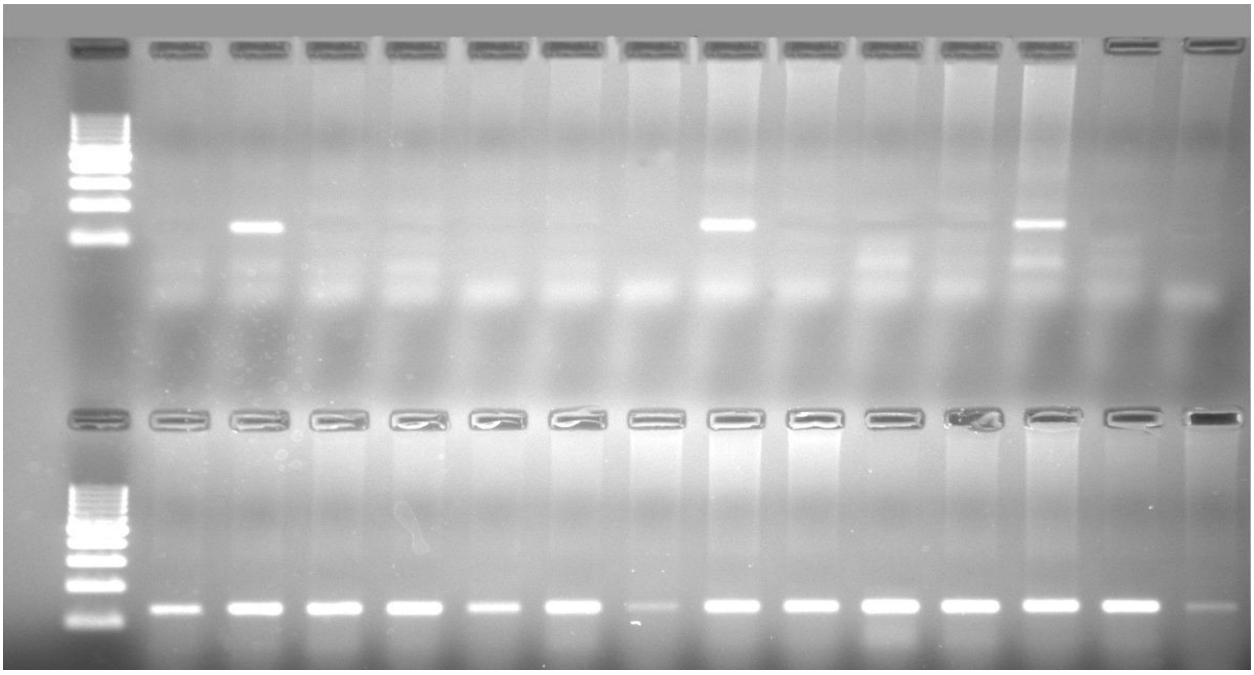

Figure 2(c): Genotyping of TCF7/L2 rs 7903146 variant of group C using AS-PCR

Table II: Genotype of TCF7/L2 rs 7903146 variant distribution between group A and group B

Genotype n = 99	**Group A n = 74**	**Group B n = 25**	**Chi-square p-value**
CC	16(0.22)	0(0.0)	
CT	57(0.77)	5(0.20)	6.447
TT	1(0.1)	20(0.80)	p = 0.011
Alleles n = 198	**Group A n =148**	**Group B n = 50**	**OR (95% CI) p-value**
C	89(0.60)	5(0.10)	0.07 (0.027, 0.1964)
T	59(0.40)	45(0.90)	p<0.001

Data presented as number (proportion) and odd ratio (95% CI), P<0.05 indicates significant. Chi-square test and odds ratios with 95% confidence intervals (95% CI) were calculated to determine the association between variables.

Groups: Group A is healthy individuals; Group B is T2DM subjects with retinopathy

Table III: Genotype of TCF7/L2 rs 7903146 variant distribution between group A and group C

Genotype n = 99	Group A n = 74	Group C n = 49	Chi-square p-value
CC	16(0.22)	1(0.2)	69.751 p=0.002
CT	57(0.77)	29(0.59)	
TT	1(0.1)	19(0.39)	
Alleles n = 198	**Group A n =148**	**Group C n = 98**	**OR (95% CI) p-value**
C	89(0.60)	31(0.32)	0.31(0.179 , 0.52) p<0.001
T	59(0.40)	67(0.68)	

Data presented as number (proportion) and odd ratio (95% CI), p<0.05 indicates significant. Chi-square test and odds ratios with 95% confidence intervals (95% CI) were calculated to determine the association between variables.

Groups: Group A is healthy individuals; Group C is T2DM subjects without retinopathy

Table IV: Genotype of TCF7/L2 rs 7903146 variant distribution between group B and group C

Genotype n = 74	Group B n = 25	Group C n = 49	Chi-square, p-value
CC	0(0.0)	1(2.0)	11.38, p=0.003
CT	5(0.20)	29(0.59)	
TT	20(0.80)	19(0.39)	
Alleles n = 148	**Group B n = 50**	**Group C n = 98**	**OR (95% CI) p-value**
C	5(0.10)	31(0.32)	0.2401(0.0868 , 0.6642) p<0.006
T	45(0.90)	67(0.68)	

Data presented as number (proportion) and odd ratio (95% CI), p<0.05 indicates significant. Chi-square test and odds ratios with 95% confidence intervals (95% CI) were calculated to determine the association between variables.

Groups: Group A is healthy individuals; Group C is T2DM subjects without retinopathy

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