Risk Assessment of HHEX & SLC30A8 gene Common variants in T2DM with Microvascular Complications

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Risk Assessment of HHEX & SLC30A8 gene Common variants in T2DM with Microvascular Complications

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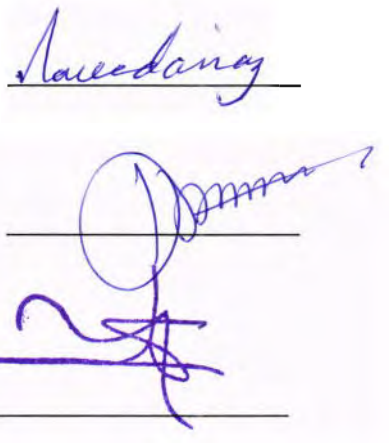

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as a partial fulfilment of requirement for the award

of the degree of MS in Biotechnology

Special dedication to

The Lord of the Lords; Allah the Almighty and His beloved Prophet

(PBUH)

And

Also to my parents, family and friends for supporting me right

throughout this research project

DECLARATION

I hereby declare that the present work in the following thesis is my own effort except, where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Date 19-9-2015

Shamaila Akram

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I pray to Almighty Lord that may He bestow me with true success in all fields and shower the knowledge upon me which is beneficial for me, for all Muslims and whole mankind.

Amin

Shamaila Akram

LIST OF ABBREVIATIONS

ACD	Acid Citrate Dextrose
ARMS	Amplification Refractory Mutation system
BMI	Body Mass Index
BSF	Blood Sugar Fasting
BSR	Blood Sugar Random
CAN	Cardiac Autonomic Neuropathy
CHD	Coronary Heart Disease
CI	Confidence Interval
DBP	Diastolic Blood pressure
DMC	Diabetic Microvascular Complications
DN	Diabetic Nephropathy
DR	Retinopathy
DN	Diabetic Neuropathy
dNTP	Deoxynucleoside Triphosphate
ESRD	End Stage Renal Disease
FI	Forward Inner
FO	Forward Outer
HDL	High Density Lipoprotein
$KHCO_3$	Potassium bicarbonate
LDL	Low Density Lipoprotein
$MgCl_2$	Magnesium Chloride
MODY	Maturity Onset Diabetes of Youth
MS	Metabolic Syndrome
NaCl	Sodium Chloride
NH_4Cl	Ammonium Chloride
NIDDM	Non Insulin Dependent Diabetes Mellitus

OGTT	Oral Glucose Tolerance Test
OD	Optical Density
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
RI	Reverse Inner
RO	Reverse Outer
SBP	Systolic Blood Pressure
SDS	Sodium Dodecyl Sulfate
SNP	Single Nucleotide Polymorphism
SPSS	Statistical Procedure for Social Sciences
STE	Saline Tris EDTA
TBE	Tris-Borate- Ethylene diamine tetra acetic acid
TG	Triglyceride
UN	Uremic Neuropathy
WC	Waist Circumference
H-W	Hardy Weinberg
$\chi 2$	Chi square value

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Introduction and Review of literature

Diabetes mellitus is a multifaceted and genomically complex disease and rapidly emerging experimental evidence has started to shed light on wide ranging mechanisms which are contributory in insulin resistance or insulin deficiency (Davidson`s Principles., 2010). Type 2 Diabetes Mellitus (T2DM) was previously known as NIDDM or adult-onset diabetes (Kumar *et al.*, 2005). Pancreatic β-Cell continuously secretes insulin despite of what is the level of glucose. Detailed mechanistic insights revealed that insulin release is triggered by a rise in glucose level. Physiologically insulin has a vital role to play as it controls glucose uptake from blood into different cells e.g., skeletal muscle cells and adipocytes. Conversion of glucose to glycogen for internal storage in liver and skeletal muscle is a major function of insulin. Maintenance of glucose is strictly controlled by diametrically opposed role of insulin and glucagon context dependently. Glucose is produced by gluconeogenesis and glycogenolysis following an overnight fast. In T2DM, 3 defects in the beginning of elevated level of glucose (hyperglycemia) are: (De Feronze *et al.*, 1992 and Stumvoll *et al.*, 2005).

a) Impaired insulin action

b) Diminished insulin secretion

c) Hepatic glucose production

1.1 Symptoms of T2DM

The classic symptoms of T2DM (Cooke and Plotnick, 2008) are ;

a) Polydipsia which is increased thirst

b) Polyuria means frequent urination

c) Polyphagia which is increased hunger.

d) Weight lost and fatigue

In type 1 diabetes symptoms may start to exist very quickly (weeks or month) but in case of type 2 diabetes symptoms appears very slowly or difficult to detect or may be absent. In T2DM more common but a rare and equally severe condition is present known as hyperosmolar nonketotic state, is mainly the result of dehydration.

1.2 Causes of T2DM

TDM is caused by combination of lifestyle (Risérus *et al.*, 2009) and genetic factors (Ripsin *et al.*, 2009). Several changes are involved in life and food habit modifications. The other causes (Ripsin *et al.*, 2010) include ;

a) Age

b) Obesity

c) Food habit modification

1.2.1 Food habit modification

Balanced and healthy diet and regular physical activity is essential and lowers risk of diabetes (Mozaffarian *et al.*, 2009).

1.2.2 Obesity and BMI

Obesity has been found the other major risk factor of T2DM to contribute to approximately 55% of cases of type 2 diabetes (Centers for Disease Control and Prevention (CDC., 2002). Certain hints have emerged suggesting that greater weight gain, increasing body mass index and risk of diabetes are tightly interconnected in Asians (Shai *et al.*, 2006). A study done in urban adult populations from six cities in India reported a BMI less than 23 kg/m^2 and WC of 85 cm for men and 80 cm for women as the optimal cut point values.

1.2.3 Inactive life style

It is relevant to mention that occupational stress is severely disturbing routine of persons. In accordance with his approach, there is a rapidly declining attention towards

regular physical activity. In between 1960s 2000s the increased rate of childhood obesity believed a leading cause of increase rate of T2DM in children and adolescents (Ramachandran *et al.*, 2002). Some other causes like Genes significantly associated with developing type 2 diabetes.

1.2.4 Central obesity and metabolic syndrome

Central obesity is associated with insulin resistance and the metabolic syndrome. Hyperglycaemia, is independently associated with higher CVD risk (Hu *et al.*, 2004; Zimmet *et al.*, 2001 and Carey *et al.*, 1997). The risk of serious health consequences in the form of type 2 diabetes, CHD and a range of other conditions, including some forms of cancer, has been shown to rise with an increase in body mass index (Lee *et al.*, 1993). But it is an excess of body fat in the abdomen, measured simply by waist circumference that is more indicative of the metabolic syndrome profile than BMI (Poulio *et al.*, 1994; Ohlsone et *al.*, 1998; Rexrode *et al.*, 1998).

Table 1.1: Worldwide definition of metabolic syndrome according to IDF

According to the International Diabetic Federation (The IDF consensus worldwide definition of the metabolic syndrome) metabolic syndrome is a condition characterized by three or more of the following signs	
Elevated triglycerides	Triglycerides equal to or higher than 150mg/dL (1.7mmol/L)
Low HDL cholesterol	Men <40mg/dL(1.03 mmol/L) and women <50mg/dL(1.29mmol/L)
Elevated blood pressure	Blood Pressure equal to or higher than 130/85mmHg
Elevated fasting plasma glucose	Fasting blood glucose equal to or higher than 100mg/dl (5.6mmol/L) If level is above 5.6mmol/L or 100mg/dl, It's not necessary to define presence of disease, however, OGTT is strongly suggested.

1.3 Prevalence of T2DM

Rate of T2DM have increased significantly over the last 50 years as obesity risk factor increasing among peoples. There were 285 million people with diabetes (90% cases of diabetes are of T2DM) worldwide as of 2010 (Williams., 2009). Majority of affected individuals live in the developing world were 30 million in 1985, increased to 135 million in 1995 and 217 million in 2005 (Smyth and Heron, 2006) this is equivalent to about 6.4% of the world's adult population (Shaw *et al.*, 2010)

Diabetes is common both in the developed and the developing world (Vijan., 2010; Williams., 2009). By the World Health Organization it is identified as a global epidemic (Wild *et al.*, 2004). In certain ethnic groups (Williams.,2009), such as South Asians, Pacific Islanders, Latinos, and Native Americans (Abate *et al.*,2001) womens are at a higher risk. Western lifestyle is a major risk factor in certain ethnic groups (Carulli *et al.*, 2005).

1.4 Complications of Diabetes

T2DM in patients leads to progression of microvascular complications (contribute to cardiovascular diseases to which atherosclerosis is a contributor, coronary artery disease leads to myocardial infarction, peripheral vascular disease, stroke and microvascular complications contribute to diabetic neuropathy, diabetic nephropathy, diabetic retinopathy. Both complications associated with T2DM known as diabetic vascular complications and associated with a seven year shorter life expectancy. End stage renal disease is linked with a 4 fold reduction in life expectancy (Haffner *et al.*, 1998; Goodkin *et al.*, 2003). T2DM patients are more susceptible to different forms of both long and short term complications as shown in Figure 1.2.

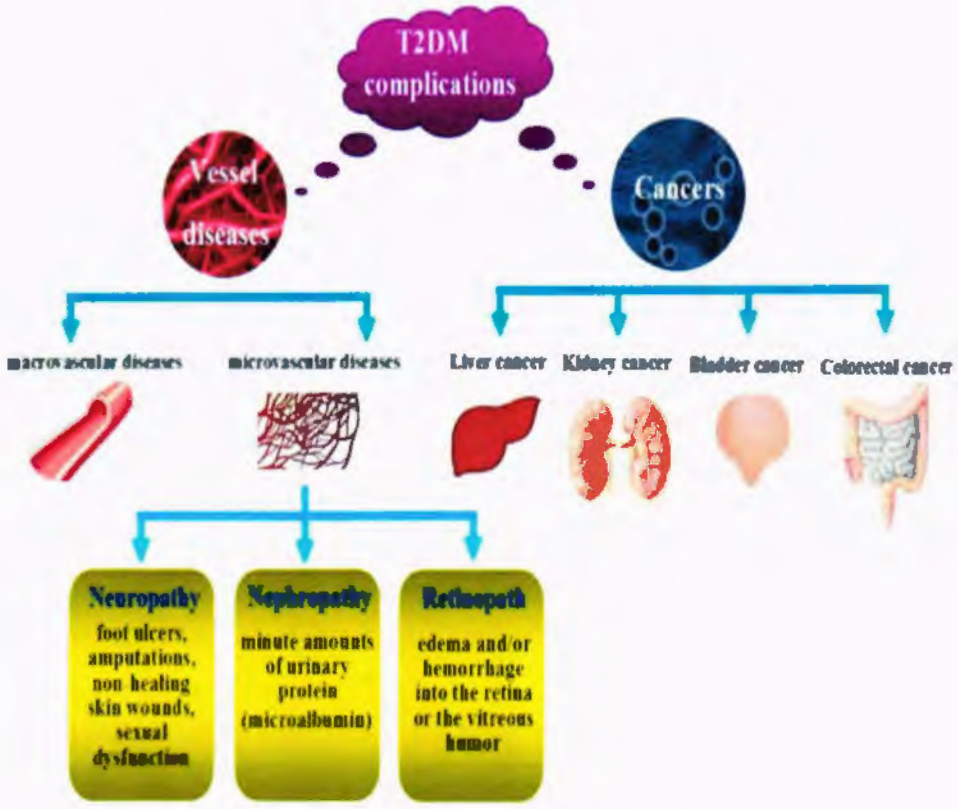

Figure 1.2: Long and short term Complications of T2DM adopted from International journal of medical sciences (Yanling *et al*., 2014)

1.4.2 Diabetic nephropathy and genetic linkage

DN is one of the most serious among the long term complications of T2DM and characterized by proteinuria which leads to end stage renal disease Rise in the excretion of proteins in urine leads to a major decrease in glomerular filtration rate with increased blood pressure and risk of cardiovascular diseases in DN (Bouchard *et al.*, 2010).

First genome linkage scans was conduct in Pima Indians for DN. They came up with strongest evidence of linkage to chromosome 7q and further evidence of linkage to chromosomes 3, 9 and 20 (Imperatore *et al.*, 1998). A Further genome wide linkage study was conducted on a large Turkish kindred sample having multiple individuals with (T2DM) and show a strong linkage of DN to chromosome18(Vardarlietal.,2002). A genome wide scan was conducted in African American families and show Evidence for nephropathy loci on chromosomes 3, 7p and 18q.

1.4.3 Diabetic Retinopathy and genetic linkage

Diabetic Retinopathy could result in irreversible blindness if left untreated and most dreadful related to ocular complications. Hyperglycemia and longer duration of diabetes have been most risk factors contributing in proceedings of DR (Frank., 2004). Chronic hyperglycemia leads to oxidative stress and capillary damage in the retina. It is contributing to an overall 4.8% blindness across the globe and second leading cause of blindness due to retinal degeneration (Resnikoff *et al.*, 2004). India feared to end up with an alarming of 11.4 million type 2 diabetes mellitus (T2DM) being the diabetic capital of the world (Rema *et al.*, 2005).

Genome wide linkage analysis for DR in Pima Indians provided that chromosome 1 may harbor genes contributing in susceptibility to DR (Hallman *et al.*, 2007). Looker *et al*, conducted a genomewide linkage scans in 2007 for DR in Mexican–Americans and come to know with findings that DR linked to chromosomes 3 and 12 (Looker *et al.*, 2007).

A genome wide linkage study was conducted in 2006 and reported that genes for

susceptibility to diabetic retinopathy may be different from those for diabetes itself, emphasizing the highlighting the complexity of multifactorial interactions in the pathogenesis of diabetes and the development of its major complication (Hallman *et al.*,2007). In one candidate gene study a weak association of polymorphisms in *TCF7L2* with diabetic retinopathy was reported (Melzer *et al.*, 2006) and this was not conformed in another study (Buchbinder *et al.*, 2008). The genes *IGF2BP2, CDKAL1, CDKN2A, CDKN2B, TCF7L2, SLC30A8, HHEX, FTO, PPARG,* and *KCNJ11* are consistently associated with risk of type 2 diabetes as reported in recent genome-wide association studies (Sladek *et al.*, 2007; Scott *et al.*, 2007).

1.4.4 Diabetic Neuropathy and genetic linkage

Chronic Ulcers, pain, numbness are major characteristic of neuropathy and leading cause of amputation (Ahmedan *et al.*, 2007). Hyperglycemia is a major cause of damage to the peripheral nerves. Oxidative stress, involvement of polyol accumulation, injury from advanced glycation end-product was found in great parsimony analysis (wada *et al.*, 2005). Polymorphisms within *ACE, AKR1B1, APOE, MTHFR, NOS3,* and *VEGF* may be associated with diabetic neuropathy in T2DM as a genetic risk factors (Davi *et al.*, 2009). The mechanisms for the development of these neuropathies is multifactorial and involve environmental and lifestyle factors, genetic predisposition (Wilson and Wright., 2011). The clinically most important types of diabetic neuropathies are uremic neuropathy, diabetic peripheral neuropathy and cardiac autonomic neuropathy as shown in Fig 1.3

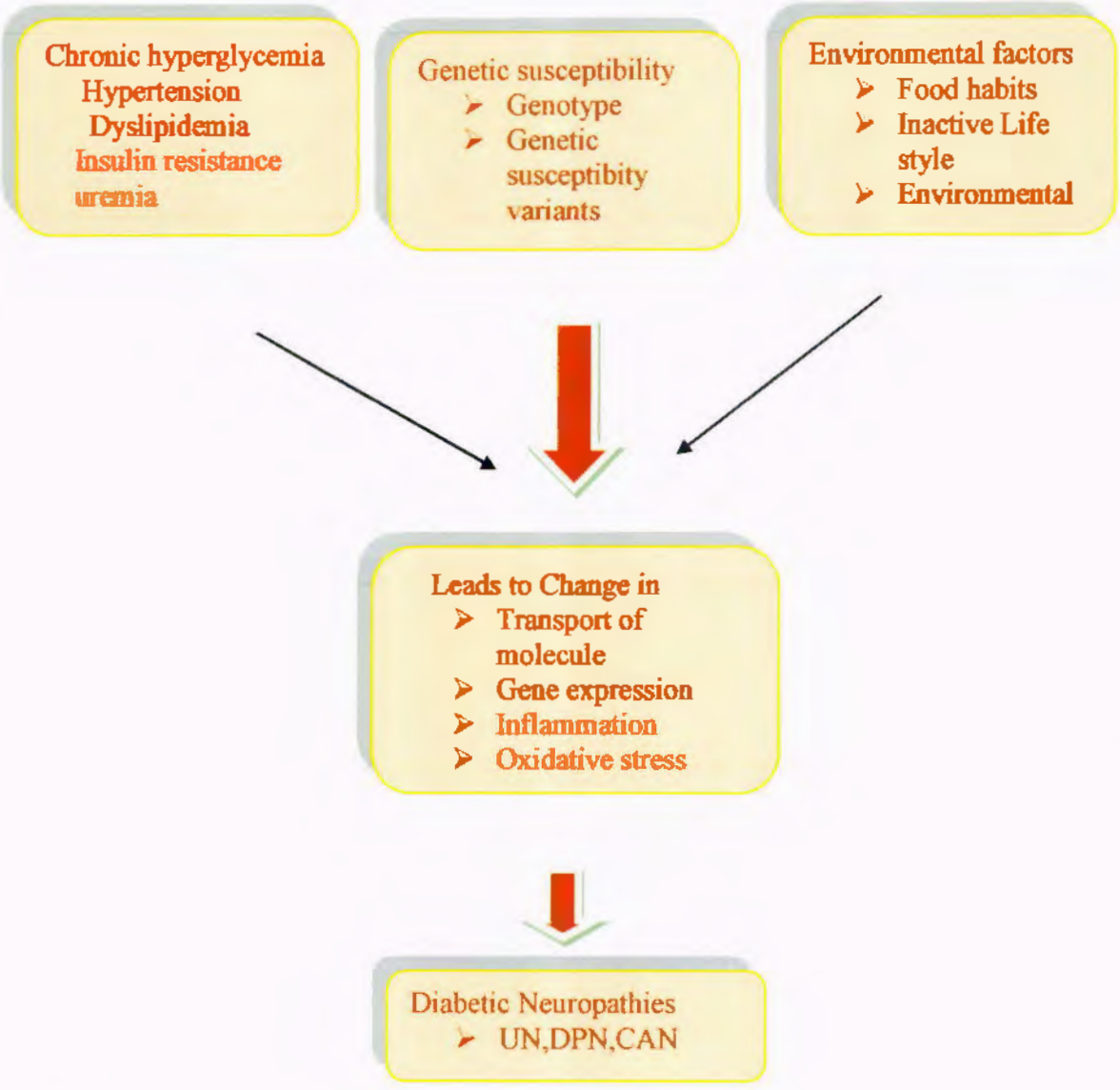

Figure 1.3: Multifactorial risk factor of the diabetic neuropathies (Tesfaye *et al.*, 2010)

1.5 Heritability and environment in type 2 diabetes

T2DM is inherited and has a genetic origin. The risk of developing T2DM is determined by both genetic and environmental factors. The mode of transmission of T2DM does not follow Mendelian pattern unlike monogenic diabetes. The lifetime risk of developing T2DM is 40% for individual who have 1 parent with T2D and almost 70% if both parents are affected (Meigs *et al.*, 2000). There is a approximately 3 or 4 times higher risk for first degree relatives of a diabetic patients compared with a population having no family history of diabetes. Subjects with family history of diabetes show 3 or 4 times higher risk of developing T2D in first degree relatives as compared to subjects having no family history of diabetes (Van *et al.*, 2001) approximately 40% of the variability of the diabetic phenotype may be heritable withy familial genetic. Twin studies have evaluated the importance of heredity and environment on the cause of T2D has been a compelling evidence of a significant genetic component in T2DM (Kaprio *et al.*, 1992).

1.6 Genetic association for T2DM

T2DM is a polygenic, heterogeneous disorder and 5 to 10% of patients may have maturity onset diabetes of youth (Fajans., 1989) Environmental factors and multiple genes are responsible for the development of disease (Hamman, 1992; Kahn, 1994; Bouchard, 1995; Yki, 1995; Hales and Barker, 1992) and exhibits a polygenic mode of inheritance (Turner *et al.*,1995; Granner *et al.*,1992; Aitman*et al.*,1995). Polymorphism, insulin secretion, each one is under genetic control and presence of multiple abnormal genes is responsible for development of the disease (Iselius*et al.*, 1985; Bogardus*et al.*, 1989; Martin *et al.*,1992).

Meta-analysis in diverse ethnic groups and genome wide association studies shows that hundreds of genes contribute for development, progression to T2DM. Genes associated with developing T2DM include *SLC30A8, PPARG, FTO, KCNJ11, HHEX,*

NOTCH2, WFS1, GF2BP2, SLC30A8, JAZF1,TCF7L2 (Lyssenko *et al.*, 2008). Most cases of DM2 involved many genes contributing small amount to the overall condition (Roden., 2011).

1.7 *H*HEX gene and locus

HHEX stands for hematopoietically expressed homeobox. This gene is located on chromosome 10 at position q24. *HHEX* contain 4 exons and spans 5.7kb of genomic sequence (Morgutti *et al.*, 2001). *HHEX* gene repeatedly found as a promising candidate gene in susceptibility and development of T2DM by Genome wide association studies.). Hundreds of association studies are triggered by development of hyperglycemia which act as a genetic determinants of *HHEX* (Saxena *et al.*, 2007; Voight *et al.*, 2011). This gene encodes a member of homeobox family of transcription factor, many of which are involved in developmental processes. *HHEX* gene involved in a Wnt signaling pathway for cell growth and development (Foley *et al.*, 2005).

1.7.1 HHEX ROLE

Ten genes are reported as strong candidates act as a susceptibility genes modulating the pathogenesis of type 2 diabetes as reflected in genome wide association studies (Sladek*et al.*, 2007; Steinthorsdottir*et al.*, 2007). *HHEX* act as a transcription factor perform important functions both in the liver and ventral pancreas development and expressed in embryonic ventral-lateral foregut. Loss of function of this gene was associated with alterations in budding and morphogenesis of the ventral pancreas, Positioning of ventral foregut related to mesoderm, proliferation of endodermal epithelial cells (Bort *et al.*, 2004). HHEX knockout in mice is associated with embryonic lethal, with impaired forebrain, liver, and thyroid development. It was necessary to discover whether the two SNPS rs7923837, rs5015480 near the HHEX locus responsible for impairment of cell function because of developmental defects of the HHEX knockout (sladek *et al.*, 2007). For this reason this was evaluated in depth and characterized by an intravenous glucose tolerance test in five nondiabetic European populations.

1.7.2 rs7923837 SNP in HHEX gene

rs7923837 located to 270kb linkage disequilibrium block located near of telomeric end. This LD block contains three genes: insulin-degrading enzyme (IDE), kinesin family member (*K1F11*) and hematopoietically expressed homeobox (Sladek *et al.*, 2007). SNP rs7923837 present at chromosome 10q23.33 on 92722160 position in the flanking region of *HHEX* gene (Tanaka *et al.*, 2005). *HHEX* is critical for insulin signaling and islet function and regulation of β-cell development (xu *et al.*, 2010).

1.7.3 Association of rs7923837 polymorphism with the risk of T2DM

The G allele of rs7923837 in *HHEX* was a minor allele (23.8%) in the Japanese population, although it was a major allele (66.5%) in the French population (Sladek *et al.*, 2007). It was investigated that the G allele of rs7923837 was significantly more frequent in the diabetes patients than in the controls [odds ratio 1.66, 95% confidence interval (CI) 1.28–2.15; P = 0.00014 proceed in a same way as in the French population of the previous report. Odd ratios of Heterozygous and homozygous carriers of the risk allele was 1.57 (95% CI 1.15–2.16; P = 0.0050) and 3.16 (95% CI 1.40–7.16; P = 0.0038) in comparison to heterozygous carriers (sladek *et al.*, 2007).

The rs7923837 SNP in *HHEX* showed the strongest association with type 2 diabetes as reported in Japan earlier, came up with finding that the frequencies of risk alleles of rs7923837 in *HHEX* were even lower in the Japanese samples than European populations (Horikoshi *et al.*, 2007; Furukawa *et al.*, 2008).

A significant association between rs7923837 and type 2 diabetes is mediated through decreased β-cell secretory capacity or decreased β-cell mass as reported in several studies (Pivovarova *et al.*, 2009; Pascoe *et al.*, 2007). In a Chinese population, rs7923837 and rs1111875 were independently associated with risk of type 2 diabetes as reported in previous fine-mapping study (Zhao *et al.*, 2010) It was demonstrated that in European American children, type 2 diabetes risk-associated G allele of rs7923837 was associated with higher pediatric BMI.

1.7.5 rs5015480 SNP in HHEX gene

SNP rs5015480 is present upstream of the *HHEX* (hematopoietically expressed homeobox) gene at chromosome 10q23.33 on 92705802 position (Bort *et al.*, 2004). rs5015480 was associated with beta-cell glucose sensitivity and decrease insulin response (Pascoe *et al.*, 2007). GWAS shown that the risk allele for the *HHEX* SNP (rs5015480) analogous to the protective allele in the sample population is common in genetic studies (Lin *et al.*, 2007) identified SNP is probable not the causal variants and may be attributed to population-unique linkage-disequilibrium patterns. Both *CDKAL1* and *HHEX* variants has strong susceptibility and association with T2DM and insulin secretion in several Southeast Asian cohorts and among Pima Indians, respectively (Rong *et al.*, 2009). Cruz *et al,* in 2010 investigated *HHEX* rs5015480 as a metabolic syndrome-susceptibility variant and association between the rs5015480 and risk of metabolic syndrome (MS) in a case-control study from Mexico city and came up with the finding that rs5015480 show a significant association with MS. Collectively this affirmation recommended that SNPS on the *IDE- KIF11-HHEX* gene cluster at 10q23.33 may contribute to metabolism-related traits and diseases, including circulating lipid levels and diabetes risk.

1.8 SLC30A8 gene

SLC30A8 is a Solute carrier family 30 (zinc transporter), member 8 gene located on 8q24.11.The SLC30A8 gene encodes a zinc transporter which is expressed in pancreatic β-cells expressed solely. This protein is comprised of ten members that share a similar six transmembrane domain structure having a histidine rich loop which is located between helices IV and V and contain 369 amino acid residues like other members of cation diffusion facilitator (CDFs) (Gold *et al.*, 1984; Dodson *et al.*, 1998). This gene is expressed in insulin secreting beta cells, transfer the zinc from cytoplasm into insulin secretary vesicles. Within beta cells ZnT-8 localizes to insulin secretary granules and insulin is stored as a hexamer bound to two zinc ion. ZnT 8 is an important protein for regulation of zinc homeostasis and insulin secretion. Zinc is an important metal for insulin secretion into intracellular vesicles and storage (Qian *et al.*, 2000).

1.8.1 Association of rs13266634 polymorphism with the risk of T2DM

An Arg325Trp polymorphism in *SLC30A8* (rs13266634) associated to an increase susceptibility to the development and progression of type 2 diabetes with an OR of 1.52±0.31, p=5×10−7 in a genome-wide association study (Sladek *et al*, 2007). Other independent genome-wide association studies provided the similar findings (Steinthorsdottir *et at*., 2007) and replicated in four case–control studies (Saxena *et al*., 2007). One study showed that the major risk C allele of rs13266634, which confers increased risk of type 2 diabetes, was linked with reduction in insulin release during an OGTT in 3,982 middle-aged people of the general Danish population.

1.9 Objective of the present Study

The objective of the current study was to find the association of rs7923837, rs5015480 of *HHEX* gene and rs13266634 of *SLC30A8* gene with microvascular complications (diabetic Nephropathy, diabetic Retinopathy, and diabetic Neuropathy) of T2DM in Pakistani population.

No research has been reported on association between *HHEX, SLC30A8* gene and Microvacular Complications of T2DM in Pakistan.

OD and DNA concentration was created. DNA extraction was carried out in thermo control centrifuge by using standard phenol chloroform (Sambrook and Russell,2001) DNA extraction protocol. It is an organic method and recruits two organic chemicals namely Phenol and Chloroform. The DNA extraction was performed in strict explicated

Day 1

- Blood sample from each tub was transferred to the appropriately labeled falcon tube.
- Freshly prepared cell lysis buffer -1 (NH_4Cl, $KHCO_3$ and 0.5 M EDTA) was added to each blood sample in 3:1 ratio. Then sample tubes were placed on ice for 30 min.
- Samples were centrifuged at 1200 rpm at 4°C for 10 minutes in a refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130R).
- The pellet was re-suspend after discarding the supernatant; if the pellet was reddish then 10 ml of cell lysis buffer was added. Again samples were centrifuge for 10 min at 1200 rpm at 4°C.
- The supernatant was discarded and the pellet was re-suspended.
- To the pellet, 4.75ml of STE (Saline Tris EDTA) and 250µL of 10% SDS (Sodium Dodecile Sulphate) were added.
- To the mixture, 10µl of proteinase K (20mg/ml) enzyme (Fermentas, Lithuania) was added. Sample tubes were placed in water bath (Orbit Shaker Bath, Lab-Line, USA). at 55°C for overnight.

Day 2

- Samples were extracted with 5ml (equal quantity) of equilibrated phenol (pH=8), Samples were kept on ice for 10 minutes and gentle shaking with intervals. After that centrifugation was performed for 30min at 4°C using the speed of 3200 rpm in refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130R). The aqueous layer

1.9 Objective of the present Study

The objective of the current study was to find the association of rs7923837, rs5015480 of *HHEX* gene and rs13266634 of *SLC30A8* gene with microvascular complications (diabetic Nephropathy, diabetic Retinopathy, and diabetic Neuropathy) of T2DM in Pakistani population.

No research has been reported on association between *HHEX, SLC30A8* gene and Microvacular Complications of T2DM in Pakistan.

MATERIALS AND METHODS

2.1 Blood Sampling

Following the approval of Institutional ethical board, Pakistan Institute of Medical Sciences (PIMS) and Federal Government Hospital Polyclinic Islamabad, peripheral. The patients were evaluated by personal information, family history, clinical, laboratory investigations and diabetes related complications of the patient; all information was included in a clinical cum consent form. The T2DM were informed about the objectives of the study and consent form was signed by every T2DM participant. Then 5 ml blood was drawn from each participant and collected in Acid Citrate Dextrose (ACD) vacutainer (BD Franklin Lakes, NJ, USA). This ACD vacutainer serve as preservative and anticoagulant for the fresh blood sample. Each sample was assigned unique sample number. Before the DNA extraction was started the blood samples were stored at 4°C. Study subjects were divided into 2 groups: 92 healthy individuals as control, 192 DM patients with nephropathy (DN), Retinopathy (DR), and Neuropathy (DN). The T2DM patients were informed about the objectives of the study and consent form was signed by every T2DM participant.

Demographic features and clinical parameters of control and patients were recorded. Demographic features include age (year), Gender (Male, Female), BMI (body mass index), and Family History. The clinical characteristics include systolic blood pressure (SBP) (mmHg), diastolic blood pressure (DBP) (mmHg), glycemic status, sugar profile, blood sugar fasting (BSF)(mg/dl), blood sugar random (BSR)(mg/dl), lipid profile a) TG (Triglyceride)(mg/dl) b) LDL (low density lipoprotein) (mg/dl) c)HDL (high density lipoprotein) (mg/dl) d) serum cholesterol and renal functions (mg/dl) a)urine R/E b)patients with ESRD c)urea/creatinine level (mg/dl).

2.2DNA Extraction from whole blood samples

Before the DNA extraction, a long shee having columns for sample ID, , age, sex,

OD and DNA concentration was created. DNA extraction was carried out in thermo control centrifuge by using standard phenol chloroform (Sambrook and Russell,2001) DNA extraction protocol. It is an organic method and recruits two organic chemicals namely Phenol and Chloroform. The DNA extraction was performed in strict explicated

Day 1

- Blood sample from each tub was transferred to the appropriately labeled falcon tube.
- Freshly prepared cell lysis buffer -1 (NH_4Cl, $KHCO_3$ and 0.5 M EDTA) was added to each blood sample in 3:1 ratio. Then sample tubes were placed on ice for 30 min.
- Samples were centrifuged at 1200 rpm at 4°C for 10 minutes in a refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130R).
- The pellet was re-suspend after discarding the supernatant; if the pellet was reddish then 10 ml of cell lysis buffer was added. Again samples were centrifuge for 10 min at 1200 rpm at 4°C.
- The supernatant was discarded and the pellet was re-suspended.
- To the pellet, 4.75ml of STE (Saline Tris EDTA) and 250µL of 10% SDS (Sodium Dodecile Sulphate) were added.
- To the mixture, 10µl of proteinase K (20mg/ml) enzyme (Fermentas, Lithuania) was added. Sample tubes were placed in water bath (Orbit Shaker Bath, Lab-Line, USA). at 55°C for overnight.

Day 2

- Samples were extracted with 5ml (equal quantity) of equilibrated phenol (pH=8), Samples were kept on ice for 10 minutes and gentle shaking with intervals. After that, centrifugation was performed for 30min at 4°C using the speed of 3200 rpm in refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130R). The aqueous layer

from each sample tube was removed with 1ml cut tips in to 15ml new appropriately labeled centrifuge tubes.

- To the supernatant was removed & 5ml of chilled chloroform-isoamylalcohol (24:1) was added. Samples tubes were shaked for 10 min on ice. After centrifugation was performed for 30 min at 4°C using the speed of 3200 rpm in refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130R). By cut- tips, aqueous layer was removed from each tube in to new appropriately labeled 15ml centrifuge tubes.
- To this aqueous layer, 500µl of 10M ammonium acetate and 5ml of chilled isopropanol (or 10ml of chilled absolute ethanol) was added and gently shaked until DNA precipitated as visible white threads. Precipitated DNA was placed overnight at -20°C (or for 15 min at -70°C).

Day 3

- Precipitated DNA Samples were centrifuged at 3200 rpm for 60min at the temperature of 4°C in refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130R) and then the pellet was re-suspended after discarding the supernatant.
- DNA was washed with 5ml of chilled 70% ethanol for 40 minutes at 3200rpm at 4°C in refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130R).
- Then the pellet was dried after discarding the supernatant and samples were re-suspend in 10mM Tris-HCl (10mM Tris; 1mM EDTA) buffer (PH 8.0, volume of Tris added according to the size of pellet).
- The complete DNA dissolving in TE buffer was achieved by incubating the sample tube in shaking water bath at 55°C (Orbit Shaker Bath, Lab-Line, USA).
- Re-suspended samples were transferred into appropriately labeled 1.5ml eppendorf tubes. DNA samples were stored at 4°C as Stock.

2.3 Composition of the solutions used in DNA Extraction

2.3.1 Cell-lysis buffer

This buffer is composed of three chemicals as:

i. $KHCO_3$ (Potassium Carbonate) 1gm/L
ii. NH_4Cl (Ammonium Chloride) 8.29gm/L
iii. 0.5 M EDTA (Ethylene Diamine Tetra Acetate) 0.34gm

Cell lysis buffer cleaves the cell membranes and exposes the chromatin material of the nucleated cells.

2.3.2 STE buffer

This buffer provides a saline environment to the newly exposed chromatin material

i. 3M NaCl (Sodium Chloride) 33.3ml
ii. 1M Tris-HCl buffer (pH8.0) 4.0ml
iii. 0.5M EDTA (pH8.0) 2.0ml

The above reagents were mixed and volume was made up to 1 liter with dH_2O.

2.3.3 DNA Dissolving Buffer

This buffer is used for dissolving the DNA and is composed of

i. 10mM Tris-HCl (pH8.0)
ii. 0.1mM EDTA

2.3.4 SDS solution

10 % solution of sodium dodecyl sulfate or sodium lauryl sulfate helps in protein degradation.

2.3.5 Chloroform-Isoamyl Alcohol

This solution is made in 24:1 v/v for 500 ml total volume as:

i. Chloroform 480ml (24)
ii. Isoamyl alcohol 20ml (1)

2.4 Optical Densities of DNA Samples

Optical densities (ODs) of stock DNA were analyzed on UV spectrophotometer (U-3210, Hitachi, Japan) at 260nm and 280nm wavelength by diluting 6µl DNA sample to 294 µl distilled water and DNA concentration were calculated for each stock sample in µg/µl. The stock sample was prepared for tetra primer ARMS-PCR (Amplification Refractory Mutation system-PCR) and RFLP (Restriction Fragment Length Polymorphism).

Optical density (OD) ratio for each sample was calculated as:

OD=Absorbance at 260nm / Absorbance at 280nm

The ratio from above formula should lie between 1.7-1.9 for a good quality DNA.

The concentration of DNA samples was calculated as:

DNA concentration (µg/ml) =Absorbance at 260nm × dilution factor ×correction factor

2.5 Primers for rs5015480 and rs7923837 genotyping

Analysis of SNP has been a very dynamic discipline in genetics to find the genetic determinants of complex diseases like T2DM. Such studies are approached through rapid, convenient high-throughput and cost effective methodologies for SNP genotyping. One such SNP genotyping method is Tetra-primer ARMS PCR which recruits two primer pairs to amply the two different alleles of a SNP in a single PCR (Shu *et al.*, 2001). The targeted and allele specific amplification of rs5015480 and rs7923837 SNPs in *HHEX* gene was done through Tetra-primer ARMS- PCR. Upstream and downstream sequence of rs5015480 and rs7923837 was obtained from human Genome at ensemble genome browser (http://asia.ensembl.org/Homo_sapiens/Search/Details?db= core; end= *1;idx=Variation=rs5015480 and rs7923837;species=Homo_sapiens).* Allele Specific Tetra primer ARMS PCR primers for *rs5015480* C>T Polymorphism in the *HHEX* gene Was designed at the following internet link:http://cedar.genetics.soton.ac.uk/public_html/primer1.html

The detail of primers with allele specifications is given below in Table 2.1

Table 2.1: Sequence of the primers used for the genotyping of rs5015480.

Primer	Sequence
Forward inner primer (FI) (C allele)	5'- TGAAGTAAACTCGAATGTTGATTAGAC -'3
Reverse inner primer (RI) (T allele)	5'- TGAATACTTGAATAATTTGATAGAAACCA -'3
Forward outer primer (FO)	5'- TCAGTCTCTATCAAAGCCTATAAAAGC -'3
Reverse outer primer (RO)	5'- ATAATTATGCTTAGGGTGAAAATAGGTT -'3

Allele Specific Tetra primer ARMS-PCR primers for *rs7923837* A>G Polymorphism in the *HHEX* gene was designed at the following internet link:http://cedar.genetics.soton.ac.uk/public_html/primer1.html

The detail of primers with allele specifications is given below in Table 2.2

Table 2.2: Sequence of the primers used for the genotyping of rs7923837.

Primer	Sequence
Forward inner primer (FI) (A allele)	5'- TAGGCAAGAAACTTTGTGGCACTGGGTA -'3
Reverse inner primer (RI) (G allele)	5'- CCTTTTTTTTTACGTTTTAAAATGGTACAC -'3
Forward outer primer (FO)	5'- AAAAGGTTGTTGCAGAAGTCCAATAAGA -'3
Reverse outer primer (RO)	5'- ATGTGATGAGTTCAGGGCTTACCAATAC -'3

2.5.1 Specificity of the primers and PCR product size

The forward outer (FO) and reverse outer (RO) primers amplified the 322bp region including the rs5015480 in all PCR reactions irrespective of C/T genotypes. However forward inner (FI) primer amplified the T allele with combination of reverse outer (RO) primer; product size 162bp. The C allele was amplified with combination of reverse inner (RI) primer and forward outer (FO) primer; product size 216bp. The four primer set are included in single PCR reaction.

In case of rs7923837 the FO and RO primers amplified the 329bp region in all PCR reactions irrespective of G/A genotypes. However, forward inner (FI) primer amplified the A allele with combination of RO primer; product size 203. The G allele was amplified with combination of reverse inner RI primer and forward outer FO primer; product size 184bp. The four primer set are included in single PCR reaction.

2.5.2 Reconstitution of primers

Lyophilized primers were mixed with calculated amount of deionized water to make a stock solution of 100µM of each primer. From these 100µM stock primer solutions, 20µM working solutions for each primer were prepared by taking 20µl stock solution and 80µl of distilled water. PCR amplifications were performed by using 20µM primer solutions.

PCR-RFLP:

2.6 PCR analysis of rs5015480 and rs7923837

After the optimization, all the samples were amplified for target DNA fragment of rs5015480 and rs7923837. The PCR reaction was done for each sample in final volume of 25µl containing 2.5µl of 10X PCR buffer without $MgCl_2$ (Fermentas, Lithuania), 1.2µl of 25mM $MgCl_2$ (Fermentas, Lithuania), 1µl 2mM dNTPs (Fermentas, Lithuania), 0.2µl of 0.04U *Taq* DNA polymerase enzyme (Fermentas, Lithuania),1µl of 20µM of each forward and reverse outer primers, 1µl of 20µM of each forward and reverse inner primers, 5µl of 40ng/ µl human genomic DNA and volume of the reaction was made up to 25µl with autoclaved deionized water. The initial and final concentrations of the reagent (optimized amounts/concentrations) used in PCR are shown in table 2.3.

Table 2.3: PCR reagents with their concentrations used for PCR of rs7923837,rs5015480

S.no	Reagents with initial concentration.	Reagents with final concentration.	Quantity used in µl
1	d.H_2O	---	11.1 µl
2	10x PCR buffer	1x buffer	2.5 µl
3	25 mM $MgCl_2$	1.5 mM $MgCl_2$	1.2 µl
5	2mM dNTPs	200µM dNTPs	1 µl
6	5U/µl *Taq* DNA polymerase	0.04U *Taq* DNA polymerase	0.2 µl
7	20µM Forward outer primer	1.0 µM	1 µl
8	20µM Reverse outer primer	1.0 µM	1 µl
9	20µM Forward inner primer	1.0 µM	1 µl
10	20µM Reverse inner primer	1.0 µM	1 µl
11	40ng Sample Genomic DNA	ng/µl	5 µl
---	Total reaction volume	---	25 µl

2.6.1 RFLP:

For *SLC30A8* genotyping the amplified DNA fragments harbouring the SNP was digested with AluI restriction enzymes at 37°C for 16 hours in a final volume of 30µL containing 0.5µL AluI enzyme, 3µL of digestion buffer 20µL of the PCR product adjusted with d.H_2O for final volume of 30µL. The RFLP products were checked on 2% w/v agarose gel containing 10 µL of 10mg/ml of ethidium bromide. The agarose gel electrophoresis was done at 200 volts for 30 minutes using Bio-Rad Power PAC 3000. The forward and reverse primers for *SLC30A8* gene are mentioned in table 2.4

Table 2.4: Sequence of the primers used for the genotyping of rs13266634

Regions	**Primer**	**Sequence**
SLC30A8 C/T	Forward primer	5'- CAAACGTGGCTTCCTCTGAG3
	Reverse r primer	5'- TACGATGCACTCACTCACCA-'3

2.6.2 PCR analysis of rs13266634

The PCR reaction was carried out in a total 25µl volume containing 2.5µl of 10X PCR buffer without $MgCl_2$ (Fermentas, Lithuania), 1.2µl of 25mM MgCl2 (Fermentas, Lithuania), 1µl 2mM dNTPs (Fermentas, Lithuania), 0.2µl of 0.04U *Taq* DNA polymerase enzyme (Fermentas, Lithuania), 1µl of 20µM of each forward and reverse primers, , 5µl of 40ng/ µl human genomic DNA and volume of the reaction was made up to 25µl with autoclaved deionized water. The initial and final concentrations of the reagent (optimized amounts/concentrations) used in PCR are shown in table 2.5.

Table 2.5: PCR reagents with their concentrations used for PCR of rs13266634

S.no	Reagents with initial concentration.	Reagents with final concentration.	Quantity used in µl
1	d.H2O	---	12.1 µl
2	10x PCR buffer	1x buffer	2.5 µl
3	25 mM $MgCl_2$	2.5 mM $MgCl_2$	1.2 µl
4	2.mM dNTPs	200µM dNTPs	1 µl
5	5U/µl *Taq* DNA polymerase	0.04U *Taq* DNA polymerase	0.2 µl
6	20µM Forward primer	1.0 µM	1 µl
7	20µM Reverse primer	1.0 µM	1 µl
8	40ng Sample Genomic DNA	ng/µl	5µl
9	Triton X	----	1 µl
---	Total reaction volume	---	25 µl

2.6.3 Thermal Profile for the PCR

The above PCR was carried out in 96 well thermal cycler (Thermo Electron Corporation, Mill ford, USA) at the following thermal stages, steps and cycles as shown in table 2.6

Table 2.6: PCR stages and cycles

No. of Stage	No. of Step	Temperature	Time	No. of Cycles
Stage 1	Step 1 Initial (denaturation)	94 °C	4 Minutes	01 Cycle
Stage 2	Step 1 (denaturation)	94 °C	45 Seconds	35 Cycles
	Step 2 (Annealing)	60 °C	45 Seconds	
	Step 3 (extension)	72 °C	45 Seconds	
Stage 3	Step 1 Final extension	72 °C	10 Minutes	01 Cycle

2.7Analysis of PCR products by using Agarose Gel Electrophoresis

Analysis of PCR product was performed by using 2% agarose gel through electrophoresis.

Following reagents were used in the process of agarose gel electrophoresis:

a) 10x Tris-Borate- Ethylene diamine tetra acetic acid (TBE) buffer
b) 6X Gel loading dye (Fermentas, Lithuania)
c) 10mg/ml Ethidium Bromide
d) Agarose(Promega)
e) 100bp DNA Ladder (Ferments, Lithuania).

2.7.1 Preparation of 10x TBE buffer stock

i. Tris-base (promega) = 107.8gm/liter
ii. Boric acid (promega) = 55.02gm/liter
iii. EDTA (Ethylene diamine tetra acetic acid) (Bio Rad) = 9.04gm/liter

All reagents were dissolved in 800ml deionized water using magnetic stirrer and finally 1000ml volume was made in measuring cylinder and stirred to mix well.

2.7.2 Preparation of 2% agarose gel

For 20cm×20cm agarose gel pouring plate 300 ml of 2% agarose was made as:

i. Ion free fine agarose (Promega) = 6gm
ii. 10 X TBE = 30ml
iii. Deionized water = 270ml
iv. Ethidium bromide (10mg/ml) = 5µl

Mix 6gm agarose, 30ml 10X TBE and 270ml deionized water in Pyrex bottle and heat in oven with loose bottle cap until agarose boils to mix in liquids. Add 5µl Ethidium bromide in the agarose gel solution and shake to mix. Set the 20cm×20cm gel plate with combs at suitable distance and pour the gel solution.

2.7.3 Gel electrophoresis and gel documentation

Before loading the sample in the gel, 5µl of the PCR product was mixed with 6X gel loading buffer (Fermentas, Lituania). Then samples were loaded on agarose gel. In the first well of gel, 100bp DNA marker was loaded as size reference for amplified target DNA.. The gel was run for 40 minutes using the Maxicell EC360-M electrophoretic gel system (EC Apparatus Corporation, St. Petersburg, Florida, USA) at 200 constant volts by using BioRad Power Pack 3000 (BioRad, USA) in 1X TBE buffer. DNA bands were visualized under UV illumination and photographed using Syngene gel documentation system (GeneGenius, Syngene, UK).

2.8 Statistical analysis:

The statistical analysis was done by managing and interpreting the data in SPSS (version 14.0, USA). The following statistical tests were performed for the data.

2.8.1 Hardy-Weinberg Equilibrium:

The frequency of each allele of rs5015480, rs7923837 and rs13266634 was calculated by Hardy-Weinberg Equilibrium equation:

$$p^2 + 2pq + q^2 = 1$$

The allele frequency of the study groups was calculated by using the following formula:

$$\text{Allele Frequency} = \frac{\text{No. of that allele in study group}}{\text{Total number of alleles}}$$

The expected H-W frequencies for the two alleles A and T; three genotype AA, TT and AT were calculated to determine any significant deviation of the observed allele and genotype frequencies of rs5015480,rs7923837 and rs13266634 in T2DM and control group using an internet source (*http://www.had2know.com/academics/hardy-weinberg-equilibrium-calculator-2-alleles.html).*

2.8.2 P value

To find the association of the rs5015480, rs7923837 and rs13266634 genotype and allele with the T2DM the *p* value and *Chi* square test was performed by using statistical program SPSS (Version 20) and as described by Preacher (Preacher, 2001). A *p* value ≤ 0.05 was considered as statistically significant whereas *p* value >0.05 was taken as non-significant.

RESULTS

192 patients having T2DM were inducted in this study having micro vascular complications along with age, sex-matched, non-obese healthy, non-hypertensive individuals above 40 as controlled. T2DM patients were assessed for age, gender, height, weight, BSF, BSR and hypertension. The features/parameters recorded for the T2DM patients who partaken in this study are summarized in the table 3.1

Table 3.1 Anthropometric and clinical profile of patients

Parameter	T2DM	Normal Range
Age(years)	53.9 ±12.461	-
Mean BMI (kg/m2)	27.804 ± 6.769	-
Systolic blood pressure(mmHg)	137.92 ±29.891	-
Diastolic blood pressure (mmHg)	85.78± 16.971	-
Male	39.8%	-
Female	56.3%	-
BSF (mg/dl)	178.80±75.966	50-100mg/dl
BSR (mg/dl)	291.184±130.700	60-140mg/dl
Triglyceride (mg/dl)	172.62±124.159	150-199mg/dl
LDL (mg/dl)	105.53±104.655	100-129mg/dl
HDL (mg/dl)	38.59±12.085	40-45mg/dl
Cholesterol (mg/dl)	162.03±50.591	Less than 180mg/dl
Urea (mg/dl)	97.42±71.381	0.5-1.2mg/dl
Creatinine (mg/dl)	3.37±3.449	6-20mg/dl

From the table it is clear that patients had elevated level of BSF 178.80±75.966 (blood sugar fasting) and BSR 291.184±130.700 (blood sugar random) so patients were in hyperglycemic condition. Cholesterol lies in the normal range so cholesterol was not disturbed among the patients. The mean BMI value was 27.804 ± 6.769kg/m2 which shows that patients were non obese. The mean values of cholesterol, HDL, LDL and TG were 162.03±50.591) mmol/L), 38.59(±12.085) mmol/L, 105.53(±104.655) mmol/L, 172.62±124.159mmol/L, respectively. The mean value of creatinine and urea were 97.42±71.38, 3.37±3.449. Creatinine and urea values lie in a normal range.

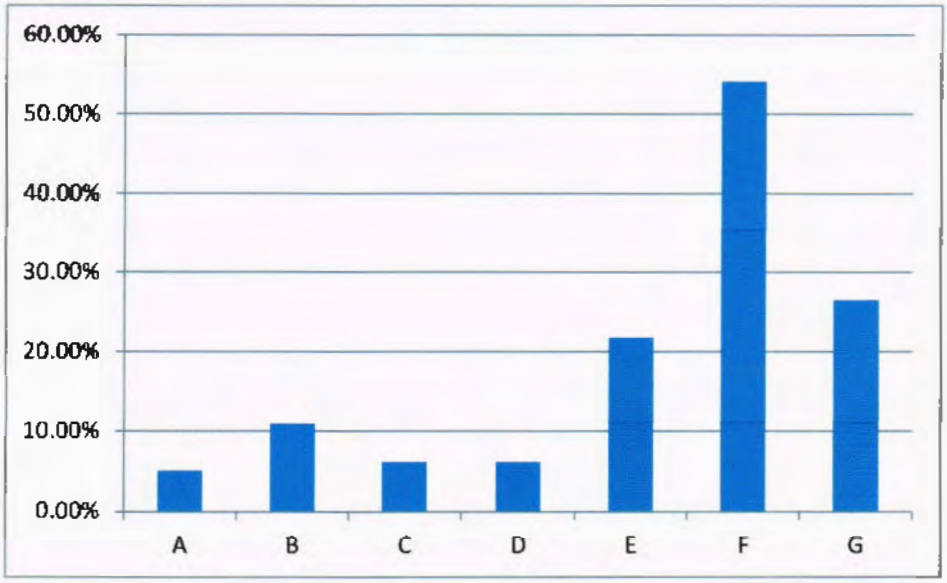

Fig3.1: Percentage of diabetic microvascular complications (Nephropathy, Neuropathy, retinopathy) in 192 patients.

A= **Nephropathy, Neuropathy, Retinopathy** 5.2%

B=Neuropathy, Nephropathy 10.93%

C=Retinopathy and Neuropathy 6.25%

D=**Nephropathy and Retinopathy** 6.25%

E=Neuropathy 21.8%

F=Nephropathy 54.16%

G=**Retinopathy** 26.56%

This distribution is based on the featured microvascular complications / or set of microvascular complications present in individual(s).

Nephropathy was most prevalent microvascular complications in T2DM patients with 54.16% as shown in figure 3.1 and 2nd most prevalent complication was retinopathy with 26.56% and 3rd one is neuropathy with 21.8% respectively. Three diabetic microvascular complications were present in T2DM patients with 5.2 % collectively. Both Neuropathy and Nephropathy were present with 10.93% collectively in patients while Retinopathy and Neuropathy both were present with 6.25% collectively and Nephropathy and Retinopathy were also with 6.25% collectively as well.

3.2: genotyping analysis of rs7923837on agarose gel

After the agarose gel electrophoresis of the PCR products and gel documentation, the DNA fragments' size was located on the gel of three SNPS. Three different DNA bands; one internal control and A and G allele specific of rs7923837 were present as shown in representative gel picture figure 3.1

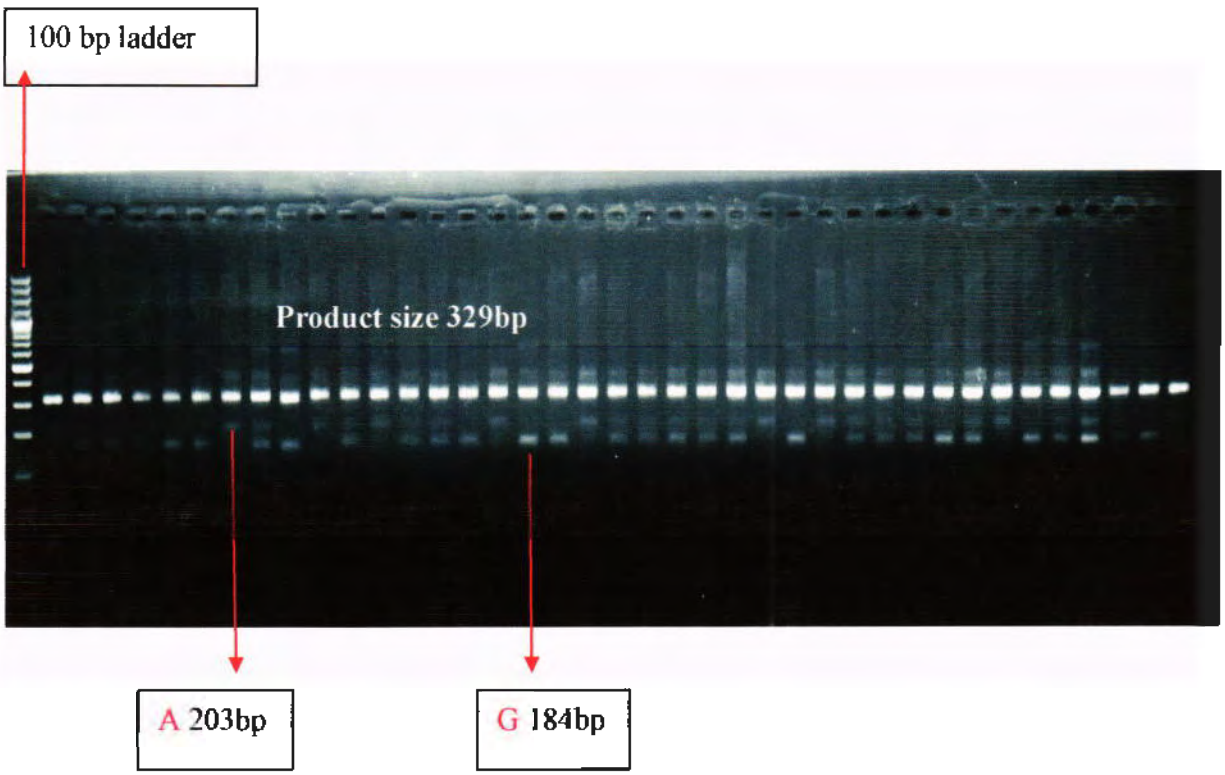

Figure 3.2: RepresentativePCR results for rs7923837 genotyping in type 2 diabetic patients: Molecular marker/Ladder 100bp; Each lane represents a single individual. The 329 bp, 203bp A and 184bp G bands are evident and it was measured by running 100bp ladder (Fermentas, Lithuania). All the bands are explained through arrows and the concerned band type. Next to the first lane with 100bp marker; on the left side of the above figure, each adjacent line towards the right side represents a single individual participated in this study.

3.3: genotyping analysis of rs5015480 on agarose gel

Three different DNA bands; one internal control and C and T allele specific of rs7923837 were present as shown in representative gel picture figure 3.3

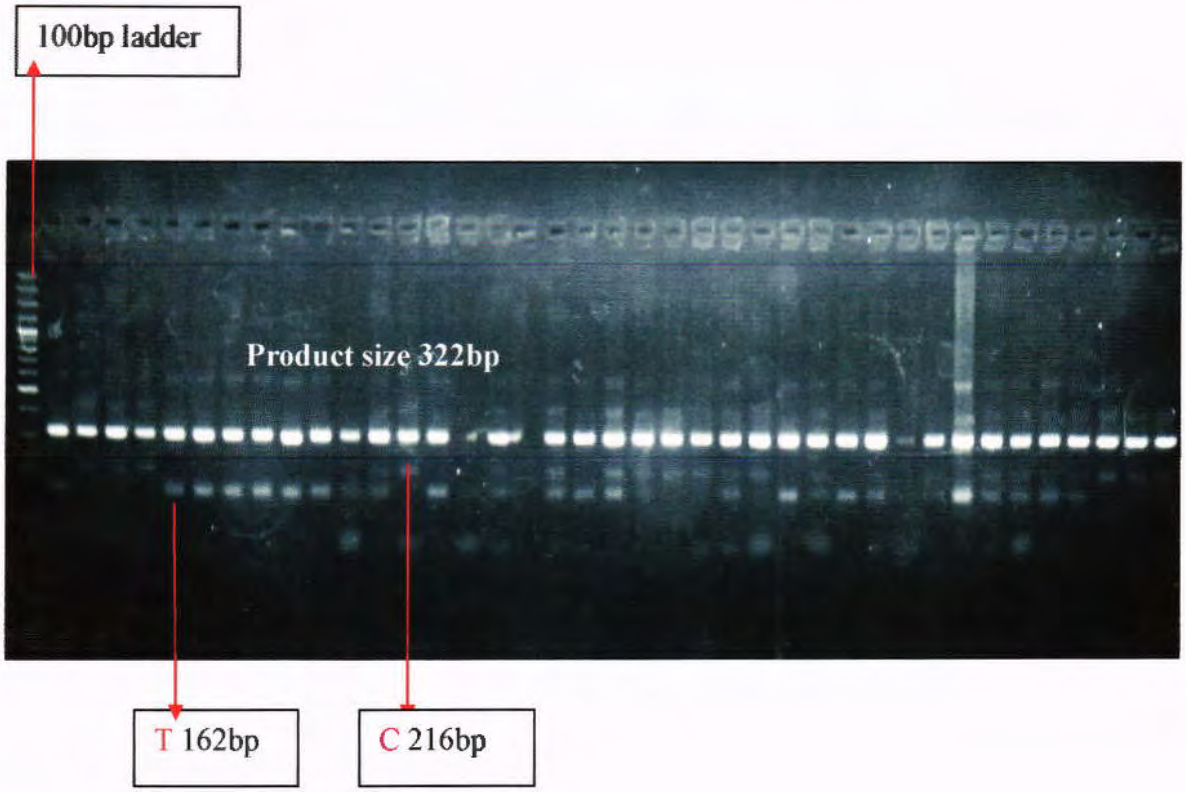

Figure 3.3: RepresentativePCR results for rs5015480 genotyping in type 2 diabetic patients: Molecular marker/Ladder 100bp; each lane represents a single individual. The 322 bp, C216bp and T162bp bands are evident and it was measured by running 100bp ladder (Fermentas, Lithuania). All the bands are explained through arrows and the concerned band type. Next to the first lane with 100bp marker; on the left side of the above figure, each adjacent line towards the right side represents a single individual participated in this study.

3.4: genotyping analysis of rs13266634 on agarose gel

Three different DNA bands; one internal control and C and T allele specific of rs13266634 were present as shown in representative gel picture figure 3.3

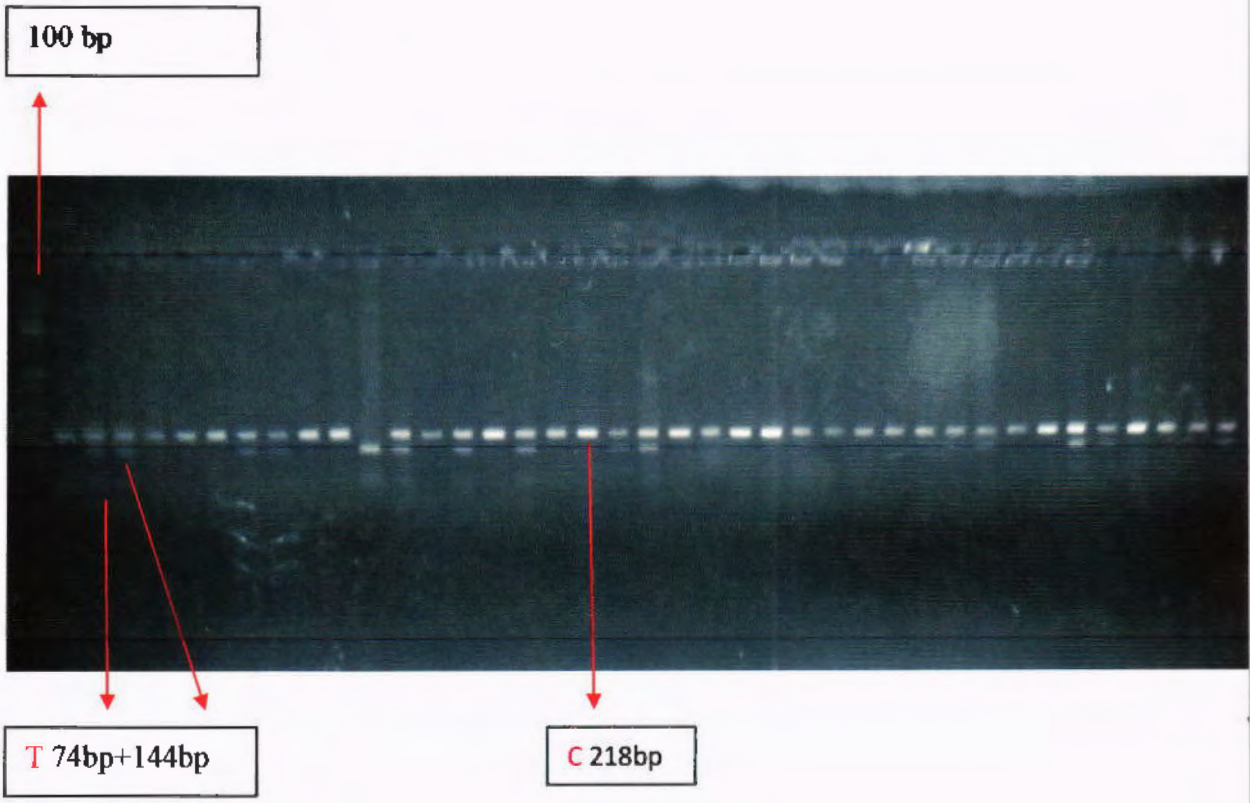

Figure 3.4: RepresentativePCR results for rs13266634 genotyping in type 2 diabetic patients: Molecular marker/Ladder 100bp; Each lane represents a single individual. The 218 bp, 144bp C and 74bp T bands are evident and it was measured by running 100bp ladder (Fermentas, Lithuania). All the bands are explained through arrows and the concerned band type. Next to the first lane with 100bp marker; on the left side of the above figure, each adjacent line towards the right side represents a single individual participated in this study.

3.5 Internal Control Band

The DNA band amplified by HHEX forward outer primer and Reverse outer primer resolved at329bp and 322bp according to the 100bp standard marker. This band was amplified in every individual and serves as the internal control.

3.6Allele specific Bands

The two SNPs of HHEX rs792383 A/G, rs5015480 C/T allele specific amplification came up with two different size bands.

3.6.1 A allele

A allele of rs7923837 with forward outer primer and reverse inner primer amplified and resolved at position 203bp on the agarose gel.

3.6.2 G allele

The G allele of rs7923837 amplified with forward inner and reverse outer primer amplified and resolved at position 184bp on agarose gel.

3.6.3 C allele

C allele of rs5015480 with forward outer primer and reverse inner primer amplified and resolved at position 216bp on the agarose gel. The C allele of rs13266634 amplified and resolved at position 144 on the agarose gel

3.6.4 T allele

The T allele amplified with forward inner and reverse outer primer amplified and resolved at position 162bp on agarose gel. The T allele of rs13266634 amplified and resolved at position 74 on the agarose gel.

The 329 bp band of rs7923837 amplified in every individual and represents the internal control. The presence of two bands of A (203bp) and g(184bp) represent the three different genotypes of rs7923837 SNP i.e. AA (only internal control band 329bp and 203bp band for A allele), GG(329bp band and 184bp band for G allele) and AG (329bp; 203bp and 184bp for A and G allele respectively). The 322 bp, C216bpand T162bp of rs5015480 .

The 322bp band of rs5015480 amplified in every individual and represents the internal control. The presence of two bands of C (203bp) and T (184bp) represent the three different genotypes of rs5015480 SNP i.e. CC(only internal control band 322bp and 216bp band for C allele), TT(322bp band and 162bp band for T allele) and CT(322bp; 216bp and 162bp for C and T allele respectively.

The 218bp band of rs13266634 amplified in every individual and represents the internal control. The presence of two bands of C (144bp) and T (74bp) represent the three different genotypes of rs513266634 SNP i.e. CC (only internal control band 218bp and 144bp band for C allele), TT (218bp band and 74bp band for T allele) and CT (218bp; 144bp and 74bp for C and T allele) respectively.The genotypes were assigned to the individuals participated in this study by the agarose gel analysis.

3.7 Genotype analysis of rs7923837, rs5015480, rs13266634 polymorphism

It is clear from the agarose gel electrophoresis and gel documentation for rs7923837, rs5015480, rs13266634 PCR products that there are three different genotypes in the studied groups of T2DM patients and controls. In order to find the genotype percentages, allele frequency and other statistical evaluation of the SNPs in the groups, all the results were entered in SPSS Version 20.

3.7.1 Genotype distribution

The genotype distribution of the rs7923837, rs5015480, rs13266634 in T2DM patients and controls was analyzed. The frequencies for expected H-W equilibrium were calculated on an internet calculator for Hardy-Weinberg calculator for 2 alleles *(http://www.had2know.com/academics/hardy-weinberg-equilibrium-calculator-2-alleles.html).*

Table 3.2: Distribution of rs7923837 genotypes and allele frequency in T2DM patients and control with p value

Group studied	rs7923837			A	G
	AA	AG	GG		
Patients (N=192)	**80 (41.8%)**	**85 (44.4%)**	**26 (13.7%)**	**0.640**	**0.36**
Expected H-W frequency	41.33%	45.92%	12.76%	64.29	35.71
Control (N=92)	**36 (39.5%)**	**41 (44.2%)**	**15 (16.3%)**	**0.616**	**0.38**
Expected H-W frequency	37.97%	47.3%	14.73%	61.62	38.38
p-value of patients & controls	**0 .80**	**1**	**0.63**	**0.97**	**0.97**
χ2value	**0.06**	**0**	**0.22**	**.001**	**.001**

There was no significant genotype difference between the T2DM patients and control groups which could show the association of rs7923837 polymorphism for the risk of microvascular complications of T2DM, *p* value (*p* value > 0.05). Of the 192 T2DM patients have AA genotype which is 41.8%. The percentage of this genotype is 39.5% in control group, there was no significant difference for this genotype in T2DM and controls as evaluated by *p* value = 0.80 (*p* value ≥ 0.05), Chi square value = 0.06 with degree of freedom 1. The percentage of major allele's genotype AG was 44.4% and 44.2% for T2DM and controls respectively, was also found to be non-significant, *p* value = 1 (*p* value ≥ 0.05), Chi square value = 0 with degree of freedom 1 for any significant association or risk for diabetes mellitus in the studied group. The percentage of GG genotype of rs7923837 in T2DM 13.7% and controls 16.2% did not show any significance in the two groups *p* value = 0.66(*p* value > 0.05), Chi square value = 0.22 with degree of freedom 1.

3.7.2 Allele frequencies

The frequency of A and G allele of rs7923837 are shown in table 3.2 along with expected H-W frequency. The allele frequencies were not significantly deviating from H-W equilibrium. The frequency of A allele was higher than the G allele in both the T2DM patients and controls. There was no significant difference in the frequency of the major allele G between T2DM and controls *p* value =0.97 (*p* value > 0.05), Chi square value = .001 with degree of freedom 1alsono significant difference for A allele was observed in the groups, *p* value 0.97 (*p* value > 0.05),Chi square value = .001 with degree of freedom 1.For *HHEX* gene polymorphism the genotype of rs5015480 (CC, CT, TT) and allele (C and T) Frequencies are described in Table 3.3

Table 3.3: Distribution of rs5015480 genotypes and allele frequency in T2DM patients and control with p value

Group Studied	rs5015480			C	T
	CC	CT	TT		
Patients (N=192)	**83 (43.0%)**	**102 (53.3%)**	**7 (3.7%)**	**0.70**	**0.30**
Expected H-W frequency	49.28%	41.84%	8.88%	70.2	29.8
Control (N=92)	**32 (34.9%)**	**41 (44.2%)**	**19 (20.9%)**	0.57	0.43
Expected H-W frequency	32.65%	48.98%	18.37%	57.14	42.86
p-value of patients & controls	**0.36**	**0.36**	**0.00052**	**0.91**	**0.88**
$\chi 2$ value	**.84**	**.84**	**12.06**	**.01**	**.02**

There was a genotype difference between the T2DM patients and control groups which could show the association of rs5015480 polymorphism for the risk of microvascular complications of T2DM, *p* value (*p* value > 0.05) of the 192 T2DM patients have TT genotype which is 3.7%. The percentage of this genotype is 20.9% in control group, there was a difference for this genotype in T2DM and controls as evaluated by *p* value = 0.000524 (*p* value < 0.05),Chi square value = 12.6 with degree of freedom 1. The percentage of major allele's genotype CC was 43.0% and 34.9% for T2DM and controls respectively, was also found to be non- significant, *p* value = 0.36(*p* value > 0.05)),Chi square value = 0.84 with degree of freedom 1 for any significant association or risk for diabetes mellitus in the studied group. The percentage of CT genotype of rs5015480 in T2DM 53.3% and controls 44.2% did not show any significance in the two groups *p* value =0.36, (*p* value > 0.05),Chi square value = 0.84 with degree of freedom 1.

3.7.3 Allele frequencies

The frequency of C and T allele of rs5015480 are shown in table 3.5 along with expected H-W frequency. The allele frequencies were merely deviating from H-W equilibrium. The frequency of C allele was higher than the T allele in both the T2DM patients and controls. There was no significant difference in the frequency of the major C allele between T2DM and controls *p* value =0.91 (*p* value > 0.05),Chi square value = .01 with degree of freedom 1 also no significant difference for T allele was observed in the groups, *p* value 0.88 (*p* value > 0.05),Chi square value = .02 with degree of freedom 1.

Table 3.4: Distribution of rs13266634 genotypes and allele frequency in T2DM patients and control with p value

Group studied	rs13266634			C	T
	CC	CT	TT		
Patients (N=192)	**133 (69.1%)**	**50 (25.8%)**	**10 (5.1%)**	**0.82**	**0.18**
Expected H-W Frequency	67.66%	29.5%	3.12%	82.32	17.68
Control (N=92)	**64 (69.2%)**	**26 (28.8%)**	**2 (1.9%)**	**0.84**	**0.16**
Expected H-W Frequency	71.73%	25.93%	2.34%	84.69	15.31
p-value of patients & controls	**1**	**0.68**	**0.22**	**1**	**0.97**
$\chi 2$ value	**0**	**0.16**	**1.46**		**.001**

There was no significant genotype difference between the T2DM patients and control groups which could show the association of rs13266634 polymorphism for the risk of microvascular complications of T2DM, *p* value (*p* value > 0.05). Of the 192 T2DM patients have TT genotype which is 5.1%. The percentage of this genotype is 1.9% in control group, there was no significant difference for this genotype in T2DM and controls as evaluated by *p* value =0.22 (*p* value > 0.05)Chi square value = 1.46 with degree of freedom 1. The percentage of major allele's genotype CC was 69.1% and 69.2% for T2DM and controls respectively, was also found to be non significant, *p* value = 0 (*p* value> 0.05),Chi square value = 0 with degree of freedom 1 for any significant association or risk for diabetes mellitus in the studied group. The percentage of CT genotype of rs13266634 in T2DM was 25.8% and controls 28.8% did not show any significance in the two groups *p* value =0.68 (*p* value > 0.05),Chi square value = 0.16 with degree of freedom 1.

3.7.4 Allele frequencies

The frequency of C and T allele of rs13266634 are shown in table 3.4 along with expected H-W frequency. The allele frequencies were not significantly deviating from H-W equilibrium. The frequency of C allele was higher than the T allele in both the T2DM patients and controls. There was no significant difference in the frequency of the major C allele between T2DM and controls *p* value =1(*p* value > 0.05),Chi square value = 0.16 with degree of freedom 1 also no significant difference for T allele was observed in the groups, *p* value 0.97 (*p* value > 0.05),Chi square value = .001 with degree of freedom 1

DISCUSSION

T2DM is a heterogeneous group of disease which is accelerating worldwide. The pathogenesis of T2DM seems to operate through complex genetic triggers influenced by environmental factors. T2DM is a major contributor of microvascular complications Nephropathy. Retinopathy, Neuropathy, peripheral vascular and cardiovascular disease (Mealey *et al.*, 2000). In this study, we have conducted a genetic association study to detect the genetic susceptibility of *HHEX & SLC30A8* for microvascular complications of T2DM in a Pakistani population. To achieve the objective 192 T2DM patients were selected from two different hospitals of Islamabad. 96 control samples were also taken for comparison. T2DM patients were assessed for age, gender, height, weight, BSF, BSR and hypertension, Triglyceride, HDL, LDL, Cholesterol, Urea, Creatinine. Patients had elevated level of BSF and BSR so patients were in hyperglycemic condition along with raised triglyceride level in our study. Nephropathy was most prevalent complications of T2DM with highest percentage of 54.16% than Retinopathy and Neuropathy in this study. Chan *et al* reported that prevalence of metabolic syndrome was 50.5% as all the patients had T2DM and incidence of diabetic nephropathy was 30 (17.4%) in 192 patients (Chan *et al.*, 2013)

In **rs7923837** AA genotype was 41.33% and 37.97.0%; GG genotype was 12.76% and 14.73%; AG genotype was 45.92% and 47.3% in T2DM patients and controls respectively. It was observed that all the genotypes were slightly deviating from expected H-W equilibrium. When the genotypes were observed statistically for any association with microvascular complications of T2DM, it was found that there was no statistically significant difference between AA (p value 0.80,), GG (p value 0.63,) and AG (p value,1) genotypes in patient and control groups. Also there was statistically insignificant difference for the risk allele G (p value, 0.97) and the major allele A between T2DM and control group (p value 0.97).

In **rs5015480** CC genotype was 49.28% and 32.65%; TT genotype was 8.88% and 18.37%; CT genotype was 41.84% and 48.98% in T2DM patients and controls respectively. It was observed that all the genotypes were merely deviating from expected H-W equilibrium. When the genotypes were observed statistically for any association with microvascular complications of T2DM, it was found that there was statistically insignificant difference between CC (*p* value, 0.355), TT (*p* value 0.000524,) and CT (*p* value 0.356,) genotypes in patient and control groups .Also there was statistically significant difference for the risk allele T (*p* value, 0.88) and the major allele C between T2DM and control group (*p* value 0.91). either there was a significant difference 0f TT, CC genotype in patients and controls but the percentage 0f TT genotype was higher in controls than patients and percentage of CC genotype was higher in patients than controls.

In **rs13266634** CC genotype was 67.77% and 71.73%; TT genotype was 3.12% and 2.34%; CT genotype was 29.1% and 25.93% in T2DM patients and controls respectively. It was observed that all the genotypes were merely deviating from expected H-W equilibrium. When the genotypes were observed statistically for possible association with microvascular complications of T2DM, it was found that there was no statistically significant difference between CC (*p* value,1), TT (*p* value 0.226,) and CT (*p* value, 0.67) genotypes in patient and control groups. Also there was statistically insignificant difference for the risk allele T (*p* value, 0.97) but the percentage of minor allele is higher in patients than control. This may suggest that T allele may a risk allele for microvascular complications of T2DM in Pakistani population. In this study group rs31266634 although not statistically significant but the risk allele has a higher percentage in patients. Fu *et al* in 2012 reported rs13266634 SNP being not a risk factor in Chinese population (Fu *et al*., 2012).

In humans the rs13266634 polymorphism in the *SLC30A8* gene has been linked to not only type 2 diabetes but also impaired glucose tolerance (Xu K *et al.,* 2011), impaired

proinsulin to insulin conversion (Kirchhoff *et al.*, 2008) and reduced first phase insulin secretion (Boesgaard *et al.*, 2008). rs13266634, *SLC30A8* variant is associated with type 2 diabetes risk in Asians and Europeans, no association is observed in Africans (Xu *et al.*, 2011). High glucose concentrations produce specific cellular effects, which influence various resident kidney cells including endothelial cells, smooth muscle cells, mesangial cells, podocytes, cells of the tubular and collecting duct system, and inflammatory cells and myofibroblast (Xu *et al.*, 2011).

Conclusion

The SNPs studied for the study subjects revealed that rs13266634 risk allele genotype TT may be a strong contributor towards diabetic microvascular complications development and or progression. However, we also found that raised CC genotype of rs5015480 in patients which makes it as a risk concern for DMC. This study also highlights the fact that hyperglycemia along with raised triglycerides are very important factors in development of diabetic microvascular complications.

Future prospects

- The present genetic research focusing on the identification of genes and the polymorphism responsible for diabetic microvascular complications might prove fruitful for the affected families to formulate future strategies to control and prevent the disease to reduce the prevalence of T2DM in Pakistan.
- Polymorphism once established in a particular population can be used for an accurate diagnosis through use of markers.
- Understanding of genetic basis can help to understand cellular pathways.
- This information can be supplied or shared to pharmacological industries and can be used for therapeutic purposes.

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