Investigation of *Endothelial Nitric Oxide Synthase Gene* Polymorphisms in Type 2 Diabetes Mellitus and Coronary Artery Disease

By

Sadia Nasr

2009-BS-V&I-34

Atta urRahman School of Applied Biosciences (ASAB)

National University of Sciences and Technology (NUST)

H-12, Islamabad, Pakistan, 2013

Investigation of *Endothelial Nitric Oxide Synthase Gene* Polymorphisms in Type 2 Diabetes Mellitus and Coronary Artery Disease

By

Sadia Nasr

2009-NUST-BS-V&I-34

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelors of Sciences

In

Applied Biosciences

Project Supervisor: Dr. AttyaBhatti

Asst. Professor,

ASAB, NUST

National University of Sciences and Technology

(NUST)

BS Thesis Work

Certificate

This is to certify that work in this dissertation/report has been carried out by Ms. Sadia Nasr and completed under my supervision in Functional Genomics Laboratory, Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST), H-12, Islamabad, Pakistan.

Supervisor: ______________________

Assistant professor Dr. AttyaBhatti

Atta-ur-Rahman School of Applied Biosciences (ASAB)

National University of Sciences and Technology (NUST)

H-12, Islamabad, Pakistan

Principal/Dean: ___________________

Professor Dr. Muhammad Ashraf

Atta-ur-Rehman School of Applied Biosciences (ASAB)

National University of Sciences and Technology (NUST)

H-12, Islamabad, Pakistan

Atta urRahman School of Applied Biosciences (ASAB)

National University of Sciences and Technology (NUST)

H-12, Islamabad, Pakistan

2013

ACKNOWLEDGEMENTS

First of all, I would like to thank ALMIGHTY ALLAH, all praises for Him for providing me the strength and patience to accomplish this effort.

It is an honor for me to express my deepest sense of gratitude and acknowledgement to my worthy and knowledgeable supervisor Dr. AttyaBhatti, Assistant Professor ASAB, NUST, H-12 Islamabad, for her continuous support, personal attention, immaculate devotion and guidance that kept my morale high. Her diligent and scholarly advices helped me a lot in solving my research problems and attaining my objectives.

I am also grateful to, Dr. Peter John, Assistant Professor, ASAB, NUST, H-12 Islamabad, whose guidance and support has rendered this venture the way it is. My special thanks for Miss SadafMoeez, Miss AminaSiddiqa and Mr. Jalil for assisting me in the laboratory work enabling me to learn all the skills which were required for carrying out this project. Myriads of thanks to all my friends and laboratory fellows, who were always there to help and encourage me through this project and provided me moral and scientific support during my research work.

I owe for gratitude and reference for Dr. Muhammad Ashraf, Principal, ASAB, NUST, H-12 Islamabad, for keeping the environment congenial and providing all possible research facilities. I also owe my acknowledgements to my friends and lab fellows for providing me with scientific help.

TABLE OF CONTENTS

Title.. **Page No.**

Acknowledgements…………………………………………………………………………....iii

Table of Contents………………………………………………………………………………iv

List of Acronyms……………………………………………………………………………...vii

List of Tables…………………………………………………………………………………..ix

List of Figures……………………………………………………………………………………x

Abstract……………………………………………………………………………………......xii

Chapter 1

Introduction……………………………………………………………………………...1

1.1- Type 2 Diabetes Mellitus and Coronary Artery Disease………………………1

1.2- Prevalence ……………………………………………………………………...1

1.3- Diagnosis Criteria of T2DM and CAD………….……………………………..2

1.4- Complications Associated with T2DM…………………………………………3

1.5- Increased Incidence Of Cardiovascular Complication With T2DM……,………4

1.6- Management and Treatment…………………………………………………….4

1.7- Risk Factors……………………………………………………………………...4

1.8- Single Nucleotide Polymorphisms……………………………………….…..5

1.9- Endothelial Nitric oxide Synthase Gene…………………………………………6

1.10- Objective of the Study…………………………………………………………7

Chapter 2

Review of Literature………………………………………………………………9

Chapter 3

Materials and Methods………………………………………………………..15

3.1- Study Subjects…………………………………………………………...…15

3.2- Blood Sample Collection………………………………………………..15

3.3- Sample Storage and Transport…………………………………………..16

3.4- Preparation of solutions for DNA extraction………………………………16

3.5- Tris-EDTA Buffer Solution (TE Buffer)…………………………………..17

3.6- DNA Extraction……………………………………………………………17

3.7- Dilution of Extracted DNA……………………………………………...18

3.8- Primer Designing for Allele Specific Polymerase Chain Reaction (PCR)...18

3.9- Allele specific PCR Amplification…………………………………………19

3.10- Agarose Gel Electrophoresis………………………………………………20

3.11- Statistical Analysis…………………………………………………………21

3.12- Bioinformatics Analysis……………………………………………...….21

Chapter 4

Results……………………………………………………………………………………24

4.1- Characteristics of Study Subjects for 786 T/C Polymorphism…………………24

4.2- PCR Amplification of eNOS Gene…………………………………………..…..24

4.3- Statistical Analysis……………………………………………………………….25

4.4- Bioinformatics Analysis…………………………………………………………..25

Chapter 5

Discussion………………………………………………………………………………41

Chapter 6

References…………………………………………………………………………… 45

LIST OF ACRONYMS

Atta urRehman School of Applied Biosciences	ASAB
Centers for Disease Control and Prevention	CDC
Base pair	bp
Nitric oxide	NO
Endothelial nitric oxide synthase	eNOS
Type 2 Diabetes Mellitus	T2DM
Coronary Artery Disease	CAD
Plasminogen Activator Inhibitor	PAI-I
Nitrate Preparations	Nitroglycerin
Gestational Diabates	GD
Nicotinamide Adenine Dinucleotide Phosphate	NADPH
DNA-binding protein 1	A1
Double-Knockout	DKO
Impaired Glucose tolerance	IGT
Deoxyribonucleic Acid	DNA
Deoxyribonucleotide Triphosphates	dNTPs
Ethylenediaminnetetraacetic Acid	EDTA
Figure	Fig.
World Heath Organization	WHO
Microliter	µl
Milliliter	ml

Millimeter	mm
Millimolar	mM
Nanogram	ng
Percent	%
Polymerase Chain Reaction	PCR
Body Mass Index	BMI
Blood Pressure	B.P
Single Nucleotide Polymorphism	SNP
Glutamate	Glu
Aspartate	Asp
Statistical Practices for Social Sciences	SPSS

LIST OF TABLES

Table No. **Title** **Page No.**

Table 1.1- Diabetes Diagnostic Criteria…………………..3

Table 3.1-Bioinformatics Tools and Databases Used………………………………………...23

Table 4.1- Hardy-Weinberg equilibrium (HWE) calculations on test population for
786T/C Polymorphism……………………………………………………………….37

Table 4.2- Observed genotype and allele frequencies of cases and controls…………………..37

Table 4.3- Statistical analysis of T2DM group and CAD plus T2DM group using
Chi-Square Test……………………………………………………………………….38

Table 4.4- Properties of wild type vs. mutant protein obtained from PROTPRAM……………39

Table 4.5- Structural attributes of wild type amino acid and its neighboring residues…………40

Table 4.6- Structural attributes of wild type amino acid and its neighboring residues……......40

LIST OF FIGURES

Figure No.Title **Page No.**

Figure 1.1- Functions of eNOS Gene………………………………………..8

Figure 3.1- Profile of PCR for SNP, 786T/C Polymorphism…………………20

Figure 4.1- Electropherogram of Ethidium Bromide Stained 3% Agarose Gel Showing Allele Pattern Obtained for 786T/C Polymorphism in Healthy Controls………………………………………………..28

Figure 4.2-Electropherogram of Ethidium Bromide Stained 3% Agrose Gel Showing Allele Pattern Obtained for 786T/C Polymorphism in T2DM Patients…………………………………………………………………..29

Figure 4.3-Electropherogram of Ethidium Bromide Stained 3% Agarose Gel Showing Allele Pattern obtained for 786T/C Polymorphism in CAD plus T2DM Patients……………………………………………..30

Figure 4.4- Genotype frequency distribution of 786T/C polymorphism in cases and controls……………………………………………………31

Figure 4.5- Distribution of gender across genotypes for 786T/C polymorphism……………………………………………….…32

Figure No.Title **Page No.**

Figure 4.6- Wild Type Protein Secondary Structure…………………………....33

Figure 4.7- Mutated Protein Secondary Structure……………………………34

Figure 4.8- Wild type protein tertiary structure……………………………...35

Figure 4.9- Mutated protein tertiary structure………………………………..36

ABSTRACT

Diabetes is a metabolic disorder, characterized by recurrent or persistent hyperglycemia, affecting body`s vital organs and resulting in life threatening complications. One of such complications is coronary artery disease (CAD). It results in the constriction of arteries which prevent blood from reaching the heart muscles, thereby, increasing strain on heart. Among several candidate genes, endothelial nitric oxide synthase (eNOS) is known to play vital role in the pathogenesis of diabetes and atherosclerosis. The two most studied single nucleotide variants of eNOS gene are 786T/C and 894 G/T polymorphisms. This study was aimed to investigate the association of 786T/C and 894G/T nucleotide polymorphisms of endothelial nitric oxide synthase gene in type 2 diabetes and coronary artery disease patients from Pakistan. Total sample size of 105 was grouped into 35 controls, 35 T2DM patients and 35 CAD plus T2DM patients. Allele specific PCR was carried out to investigate the effect of 786T/C polymorphism in cases and healthy controls. The genotype 786 T/T was significantly more increased in the two disease groups than controls X^2 and P-value of (7.2, 0.0002) in T2DM group and (18.11, 0.0001) in CAD plus T2DM group respectively. Bioinformatics analysis was carried out for 894 G/T polymorphism by modeling and comparing normal vs. mutant protein. Significant differences were observed in the parameters of two models, indicating the effect of 894 G/T polymorphism on protein structure and hence function. Thus, it can be concluded that 786 T/C polymorphism in the promoter region is significantly associated with T2DM and CAD in Pakistani patients.

CHAPTER 1

INTRODUCTION

1.1- Type 2 Diabetes Mellitus and Coronary Artery Disease:

Diabetes is a metabolic disorder which can be defined as body's inability to process glucose. Normally glucose is converted to glycogen for storage in liver and muscle cells. The hormone which carries out this conversion is known as insulin. The deficiency of insulin or receptors` insensitivity is responsible for all forms of diabetes.

Coronary artery disease is caused by thickening of the walls of coronary arteries. It involves build-up of plaque in the blood vessels that supply blood, oxygen and nutrition to heart and rest of the body. Consistent high blood glucose levels cause blood vessel constriction leading to atherosclerosis and CAD.Plaque is made up of cholesterol, fats, fibrin and calcium and can block vital blood vessels. Constricted arteries prevent blood from reaching the heart muscles, thereby, increasing strain on heart. In patients with diabetes, symptoms of CAD may go undetected for years and after years of progression, a sudden heart attack might occur. Heart disease is often established prior to the diagnosis of diabetes.

1.2- Prevalence:

It is estimated that 347 million people in the world have diabetes. It is the seventh leading cause of death worldwide. According to national survey of Centers for Disease

Control and Prevention (CDC), diabetes in Asian Americans is found to be 8.4 percent. Gender wise 13.0 million men and 12.6 million women have diabetes. Pakistan stands on

number sixth among the top ten countries with increasing burden of this disease. According to a recent estimate by World Health Organization, total deaths from diabetes are projected to rise by more than 50% in the coming 10 years.

The most prevalent form is T2DM which accounts for approximately 90% of all affected individuals. Onset of this disease usually takes place in the later age. The main focus of this study would be on type 2 diabetes because of its high prevalence and its association with heart disease.

1.3- Diagnosis Criteria of Type 2 Diabetes Mellitus and Coronary Artery Disease:

The diagnosis of T2DM is made on the basis of medical history, serological laboratory tests and clinical findings. Criteria for the diagnosis of T2DM developed by Word Health Organization (table 1.1). CAD is diagnosed on the basis of detailed history, risk factor assessment and focused physical examination. Electrocardiographic evidence of changes in ST-T wave and left ventricular hypertrophy are highly suggestive of underlying CAD. In addition to this, diagnosis is supported by the evidence of angiography (scores > 70%).

Table 1.1:

Diabetes Diagnostic Criteria			
Condition	**2 hour glucose** mmol/l (mg/dl)	**Fasting glucose** mmol/l (mg/dl)	**HbA_{1c}** %
Normal	<7.8 (<140)	<6.1 (<110)	<6.0
Impaired fasting glycemia	<7.8 (<140)	≥ 6.1(≥110) &<7.0(<126)	6.0–6.4
Impaired glucose tolerance	≥7.8 (≥140)	<7.0 (<126)	6.0–6.4
Diabetes mellitus	≥11.1 (≥200)	≥7.0 (≥126)	≥6.5

1.4- Complications Associated with Type 2 Diabetes Mellitus:

Diabetes is a devastating disorder in terms of its serious complications. Life-threatening consequences of uncontrolled T2DM are hypertension, renal failure, stroke, retinopathy and autonomic neuropathy. Endothelial dysfunction resulting from T2DM is the root cause of all these complications. Dysregulation of the endothelium causes narrowing of the vessels that transport blood in the body. Result is peripheral vascular disease which causes loss of blood supply to the leg and arm muscles. Constriction of the peripheral vessels ultimately leads to foot ulcers, gangrene and amputation of the lower extremities in patients suffering from diabetes.

1.5- Increased Incidence Cardiovascular Complications Type 2 Diabetics:

Heart problems are usually associated with diabetes and account for 50% of all diabetic fatalities. These include atherosclerosis, hypertension, angina, myocardial infarction and the most common being coronary artery disease (CAD). It is the most common cause of morbidity and mortality in patients with T2DM.CAD accounts for 80% of the death toll of people with diabetes.

1.6- Management and Treatment:

Management of T2DM and CAD is typically done with diet and exercise. Exercise improves glucose and lipid metabolism which decreases the risk of T2DM. Physical activity, such as daily walk for more than 30 minutes, has been shown to significantly reduce the risk of T2DM. Some of the currently available oral medications for T2DM include metformin, sulfonylureas, repaglinide, alpha- glucosidase inhibitors, thiazolidinedione, DPP4 inhibitors, incretinmimetics and amylin analogues. Exogenous insulin may be given either alone or in combination with oral hypoglycemic.

1.7- Risk Factors:

Risk factors contributing to T2DM and CAD include both environmental and genetic factors as described below.

1.71- Environmental Risk Factors of T2DM and CAD:

The major environmental risk factors for T2DM are obesity, family history and sedentary lifestyle. It has been estimated that approximately 80% of all new T2DM cases are due to central or abdominal obesity (Murthy*et al.*, 2012). The risk of T2DM and CAD

also increases in older age. Smoking, high levels of cholesterol and hypertension are some of the major factors contributing to CAD. Certain drugs like long term use of exogenous steroids and thiazides may increase the risk of diabetes.

1.72- Genetic Risk Factors of T2DM and CAD:

Genetic factors also play major role in the pathogenesis of T2DM and CAD. More than 50 candidate genes have been reported whose malfunction can disrupt pancreatic β cell function and glucose metabolism. Family studies reveal that T2DM is inherited and first degree relatives of individuals with diabetes are 3 times more likely to develop the disease. CAD also has multiple genetic predispositions. Up till now 250 genes have been identified to be involved in CAD. This disease often results from the blended effects of multiple gene like MRPS6, APOE, LDLR, ADAMTS7,eNOS and PDGFD .

1.8- Single Nucleotide Polymorphisms and Genes Associated:

Insights into Genome Wide Association Studies reveal the importance of single nucleotide polymorphisms (SNPs) in increasing or decreasing the susceptibility to certain diseases. SNPs predict person’s response to certain drugs, toxins and can be used to track the inheritance of disease genes within families. Mutations in some of the genes like those coding for LDL receptor, apolipoprotein E, CBS, MTHFR and eNOS have roles in the combined onset of T2DM and CAD.

1.9- Endothelial Nitric Oxide Synthase Gene:

One of the most important candidate genes in CAD and T2DM is the eNOS gene. This gene located on chromosome 7q36 consisting of 26 exons spanning 21 kb. It encodes 1.5 kDaenzyme which acts on L-arginine to produce nitric oxide (NO). NO is a free radical gas and a powerful vasodilator. It is naturally produced in our body and helps in blood pressure regulation. NO also acts as a neurotransmitter and helps in the processing of nerve signals as they cross synapses. NO functions are given in figure 1.1.

There is general agreement that hyperglycemia and diabetes lead to defective NO production and its activity. This in turn becomes a major cause of CAD. Thus, impairment in NO productions involved in endothelial dysfunction and insulin resistance, T2DM and cardiovascular complications. This study would be focusing on some of the reported eNOS gene polymorphisms which have role in T2DM and CAD.

Single nucleotide polymorphism in the promoter region (T-786C) reduces promoter activity and protein expression. G to T substitution at nucleotide +894 in exon7 results in amino acid change from Glutamate to Aspartate at 298position. This causes structural changes in protein hence affecting normal function.It has been reported that these two variants are major risk factors in certain ethnic groups like Hispanics, Native Americans and particularly South Asians. Positive association of G894T polymorphism has been found among South Indians.This suggests the probability of finding the association in Pakistan too.

1.10- Objectives of The Study:

1- The aim of this study is to investigate the association of T-786C and 894G/T single nucleotide polymorphismsofeNOS gene inT2DM and CAD patients from Pakistani population.

2- Bioinformatics analysis willbe carried out involving homology modeling of normal vs. mutant protein.

This study is novel in its kind because no work has yet been done on the eNOS gene in Pakistan. We are employing allele specific PCR for determining any possible association of these SNPs in disease susceptibility. This study would pave way for the development of new treatment regimens and improvement of already existing drug targets.

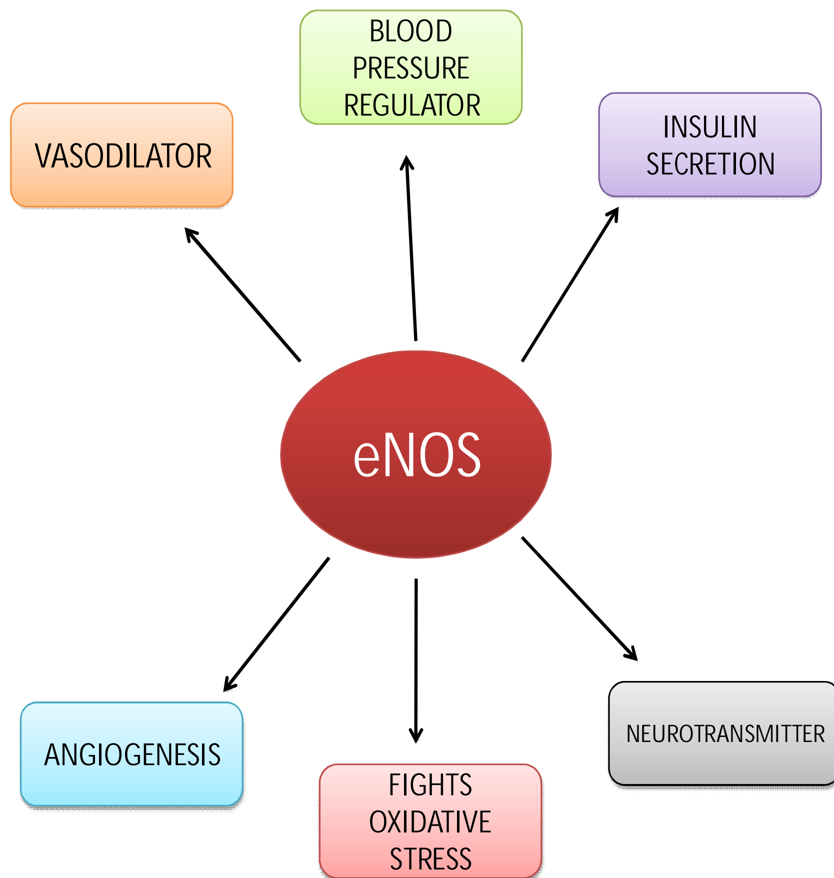

Figure 1.1: Functions of eNOS gene.

Functions of nitric oxide produced by endothelial nitric oxide synthase enzyme (eNOS) have been shown in this figure. eNOS gene codes for nitric oxide which is wonder molecule in terms of its roles in human body. Nitric oxide is a potent vasodilator and blood pressure regulator which causes widening of the blood vessels. It also has role in the formation of new blood vessels (angiogenesis), thus protecting against the risk of CAD. Literature suggests its role in insulin secretion and having protective role against T2DM.

CHAPTER 2

LITERATUREREVIEW

Diabetes mellitus is characterized by recurrent or persistent high blood sugar.Insulin deficiency reduces assimilation and storage of glucose in muscle adipose tissues and liver. Thus produces the classical symptoms of frequent urination, increased thirst, hunger and sudden weight loss (Rossi*et al*., 2003).Incomplete oxidation of fats produces ketone bodies and overwhelming amount of strong organic acids lower the blood pH and which leads to severe and potentially fatal ketoacidosis.T2DM is associated with reduced life expectancy and diminished quality of life.

Global estimate shows that 285 million people suffered from diabetes in the year 2010 and this number is expected to double by 2030 particularly in Asia and Africa .T2DM affects 3.8 million people in United Kingdom.More than 50 million Indians are affected with diabetes, 7.1% of the nation's adults.

American Heart Association (AHA)reports that every 25 seconds, an American has a coronary event. In the year 2005, more than 150,000 Americans killed by CAD were of age <65.In India, CAD prevalence rates were 3.0%, 23.5% and 11.3% in people with normal glucose tolerance, impaired glucose tolerance and diabetes mellitus respectively (Murthy *et al*., 2009).

Diabetes is classified into different types. Type 1 diabetes which is an autoimmune disorder caused by destruction of insulin producing pancreatic beta cells. Gestational diabetes (GD) temporarily occurs during pregnancy and 35–60 % of such individuals have chance of developing T2DM.In pre-diabetes, blood sugar levels remain higher than normal and affects 35% of adults with the age of 20.The major type accounting for up to 90% of the cases is type 2 diabetes (T2DM). It appears at an average age of 42.5 years. In T2DM, symptoms appear gradually, over a period of several months and lead to complications likeheart disease, stroke, blindness and chronic kidney disease. It accounted for 44% of kidney failure cases in 2008. In 2006, more than 60% diabetics underwent leg and foot amputations. Diabetic nephropathy is

the main cause of end-stage renal disease over the world and is the second cause of blood dialysis in China (13.5%).

The prevalence of hypertension and CAD in patients with T2DM is considerably higher as compared to non-diabetics (40–50% vs. 20%).A recently published research showed that aspirin therapy protects against heart muscle death and stroke. American Diabetes Association (ADA) recommends the use of aspirin in T2DM patients for secondary prevention of CAD. Nitrate preparations (nitroglycerin) and beta-blocker therapy is often given to treat the symptoms of angina in T2DM. Use of simvastatin significantly reduces the major coronary events and revascularizations. According to a study conducted in Ireland, atorvastatin 10 mg daily is safe and effective against the above mentioned signs by reducing the level of CRP (Ford *et al.*, 2002).

Environmental factors like increasing age, obesity, and physical inactivity are some of the common risk factors.US National Diabetes Data Group introduced the category of Impaired Glucose tolerance (IGT) as a risk of diabetes (WHO, 2006). CAD is associated with smoking, diabetes, and hypertension. Recent studies show that high levels of homocysteine, calcium, CRP, lipoprotein, family history and dietary habits are important predictors of CAD.

Using approaches like positional mapping, candidate gene experiments and genetic association studies, several genes linked to T2DM have been identified. Mutations in the insulin gene, its receptor as well as genetic defects ofpancreatic beta cell function and proinsulin conversion are the major contributing factors.D2S725 is involved in the development of T2DM in Mexican Americans. In A Genome-Wide Association Study of T2DM carried out in Finns, multiple susceptibility variants were detected including TCFIL2, SLC30A8, HHEX, FTO and PPARG.

The common clustering of the above mentioned risk factors might be due to single nucleotide polymorphisms (SNPs). One of the key genes involved in T2DM and CAD is endothelial nitric oxide synthase eNOS gene. Its product is nitric oxide synthase enzyme which is homodimeric in nature. It is composed of 1204 amino acids with the size of 1.5 kDa. Its C-terminal region contains flavin mononucleotide,

flavineadenine dinucleotide and reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) binding regions, whereas the N-terminal region contains the oxygenase fragment and the arginine-binding site. Active form of eNOS is produced by phosphorylation through PI3-K/Akt pathway. This cascade of reactions leads to protein kinase G activation thus, increasing the diameter of the blood vessels. As a consequence blood pressure decreases.

Endothelium is involved in vascular regulation by producing certain biochemical mediators with vasodilatory and vasoconstrictive properties, nitric oxide (NO) being one of them. In response to bradykinin,acetylcholineand substance P, it causes vessel dilation and blood pressure regulation. eNOS scavenges superoxide radicals and has been shown to have vasoprotective effects by inhibiting platelet aggregation .

Nitric oxide is a major mediator of endothelial function. In patients with T2DM and CAD, endothelial dysfunction seems to be a consistent finding. Vascular endothelial cells maintain cardiovascular homeostasis and act as physical barrier between the vessel wall and lumen. It releases secretes mediators involved in platelet regulation, fibrinolysis, coagulation and vascular tone. Abnormalities in the production of NO are implicated in atherosclerosis, diabetes and hypertension.

Animal studies and some clinical investigations have revealed that aberrant NO production may be an important contributing factor towards T2DM and CAD. Scientists from United States disrupted the gene encoding eNOS in mice and found that acetylcholine-induced relaxation, is absent, and eNOSmutant mice are hypertensive. It was seen that eNOS double-knockout (DKO) mice model developed fat deposition, hyperglycemia, and high blood pressure. Tissue analysis from DKO mice showed large islets in the pancreas and fat droplets in liver cells. Significant glomerular capillary damage in the kidney was identified, with DKO mice demonstrating a robust diabetic nephropathy similar to human disease. These findings indicate that inactivation of eNOS exacerbates renal injury in diabetic in these mice and provide direct evidence for a protective role of eNOS-derived NO in diabetic nephropathy (Mohan *et al.*, 2008).Literature suggests that SNPs in eNOS gene might be a common factor

underlying the combined onset of T2DM and CHD. Two polymorphisms, T-786C (rs2070744) and 894G/T (rs1799983), have been suspected to have role in altered metabolism and may contribute to vascular complications associated with diabetes.

Substitution from G to T at nucleotide +894 in exon7 has been identified in eNOS gene which results in Glu being replaced by Asp at codon 298 causing structural change ineNOS protein. It appears that replacement of one nucleotide alters quality rather than the quantity of eNOS gene product (Imamura*et al*., 2008). It has been seen that this eNOS variant increases its propensity towards cleavage resulting in the generation of 100 and 35 kDa fragments, causing a tight turn of the alpha-helix.

A luciferase reporter construct demonstrated that C/C genotype was associated with a 52% and 62% decrease in promoter activity. Carriers of the T allele appear to have higher levels of ox-LDL and IL-6, associated with a higher risk for premature myocardial infarction. However, G894T polymorphism does not affect eNOS activity in cell cultures.

Another important polymorphism of eNOS gene, 786T/C, is located in the promoter region and is important regulator of eNOS gene transcription.This variant is a result of a thymidine being replaced by a cytosine at position 786. Literature shows that 786 C allele affects promoter activity and leads to compromised production of the eNOS enzyme in vivo.DNA-binding protein (A1), essential for DNA repair and replication, binds to this eNOS gene variant, thus reducing promoter activity. A luciferase promoter assay indicated that a constructwith the C–786 allele decreased eNOS enzyme production by 50% (30). T-7863C transition is reported to be associated with development of CAD, infarction, hypertension, and endothelial dysfunction in adults.

CHAPTER 3

MATRERIALS AND METHODS

3.1 Study Subjects:

The study design was case control and individuals were recruited from different hospitals of Rawalpindi, Islamabad, Gujranwala and KPK. The individuals included, were divided into three groups; patients clinically diagnosed with T2DM, patients affected by CAD and those affected by both T2DM and CAD.

Inclusion and exclusion criteria were designed for the subjects of interest. Patients over 30 and less than 70 years of age were included in the study. Smokers, alcohol consumers, hypertensive and obese individuals were excluded from the study. Written consent was taken from patients and controls before drawing blood.

3.2 Blood Sample Collection:

Venous blood from both patients and healthy controls were drawn by using 5 ml (BD 0.6 mm X 25 mm, 23 G X 1 TW) and 10 ml (BD 0.8 mm X 38 mm, 21 G X 1 ½ TW) clean and sterilized syringes. Samples were then collected in 10 ml ethylenediaminetetraacetic acid (EDTA) tubes (BD vacutainer TM, Frankin Lakes, New Jersey, USA). EDTA tubes were then properly labeled. The label included identification (ID) number of the sample, date and time of the sample collection and name and age of the individual from which sample was being collected.

3.3 - Sample Storage and Transportation:

The samples were immediately transported from hospitals to the Immuno Genetics Laboratory (IGL), Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST), Islamabad, Pakistan. These blood samples were stored in EDTA tubes at 4 degree Celsius (°C) before any further processing and genomic DNA extraction.

3.4- Preparation of Solutions for DNA Extraction:

For DNA extraction, four different types of solutions named A,B,C,D were prepared as described below.

3.4.1- Solution A:

The purpose of adding solution A is lysisofthewhite blood cells. Solution A is made by adding 0.32 M Sucrose, 10 mMTris (pH 7.5) and 5mM magnesium chloride (MgCl2) this solution was then autoclaved followed by the addition of Triton X-100 (1%volume by volume) to the distilled water.

3.4.2- Solution B:

Solution B is used for protein separation and DNA precipitation. It is composed of 10 mMTris (pH 7.5), 400mM of sodium chloride (NaCl), 2 mMEthylenediaminetetraccetic acid (pH7.5). Distilled water is then added to make solution upto the desired volume.

3.4.3- Solution C:

Phenol is used as solution C which helps in the separation of DNA into separate a layer.

3.4.4- Solution D:

Mixture of 720ul chloroform and 250 ulisoamylalcohol was used per sample as solution D. This solution helps in the purification of DNA.

3.5- 1X Tris Borate Ethylenediamineteteraacetic (TBE) Buffer:

1X tris Borate Ethylenediaminetetraacetic acid (TBE) buffer is used for maintaining pH during agarose gel electriphoresis. It was initially prepared as 10x stock solution by dissolving 890 mMTris base, 890 mM boric acid and 20 nM of EDTA (pH 8.0) in distilled water.

3.6 - DNA Extraction:

Phenol chloroform method was used for the extraction of DNA from blood samples. We followed a two days protocol.

3.6.1- First Day of DNA Extraction:

For DNA extraction, the stored blood samples were incubated for a short interval of time at room temperature and 750 µl of blood was added into 1.5ml centrifuge tube (Axygen, California, USA). It was then mixed with 750 µl of solution A. Centrifuge tubes were then inverted 4-6 times followed by incubation at room temperature for 5-10 minutes. Centrifugation was carried out at 13,000 revolutions per minute (rpm) for 1 minute in a microcentrifuge (Spectrafuge 24D Labnet, Edison, New Jersy, USA). Supernatant was discarded and nuclear pellet was resuspended in 400 µl of solution A. It was then centrifuged at 13,000 rpm for 1minute. Supernatant was discarded again and nuclear pellet was resuspended in 400 µl of solution B, 12 µl of 20% sodium dodecyl sulphate (SDS) and 5 ul of proteinase K. This mixture was subjected to overnight incubation at $37^{o}C$

3.6.2- Second Day of DNA Extraction:

A fresh mixture of equal volumes of solution C and solution D was made next day and 500 µl of this mixture was added to the tube. The tube was then centrifuged for 10 minutes at 13,000 rpm. After centrifugation, the upper aqueous phase was collected in a clean appropriately labelled tube. To this aqueous layer, 500 µl of solution D was added and was centrifuged again for 10 minutes at 13,000 rpm. The upper aqueous layer was again collected in clean microfuge tube. To precipitate the DNA, 55 µl of sodium acetate (3 M, pH 6.0) and 500 µl of isopropyl alcohol was added. DNA was precipitated by inverting the tubes several times. This mixture was centrifuged again for 10 minutes at 13,000 rpm and the supernatant was discarded. For DNA washing, 200 µl of 70% ethanol was added and centrifuged for 7 minutes at 13,000 rpm. Ethanol was discarded leaving the DNA settled to the bottom of the tube. DNA pellet was dried by keeping the microfuge tubes at 37 °C until all the ethanol had evaporated off. To the pellet, 100 µl of TE buffer was finally added and left for 4-5 hours for the DNA to dissolve.

3.7- Dilution of Extracted DNA:

To confirm the purity and concentration of DNA, nano-drop was used. Stock with high concentration of DNA was diluted by adding 50 µl of the dilution buffer.

3.8- Primer designing for Arms PCR:

eNOS gene sequence was obtained from Gen Bank. Two forward and one reverse allele specific primers were designed for each of the SNP. Forward primers were designed to have polymorphic nucleotide at 3'end. One of the forward primers was designed to contain wild type allele while the other was to amplify mutant allele.

Reverse primer was designed in a way to amplify product size 250 -400 base pairs. Allele specific primers amplify the product only in case of a perfect match. It tells whether a person is homozygous, heterozygous or mutant for a particular SNP.

3.8.1- 786T/C Polymorphism:

This variant is located in the promoter region of eNOS gene.

3.8.2- 894 G/T Polymorphism:

This variant is located in the exon 7 of eNOS gene.

3.9 - Allele specific PCR Amplification:

In order to identify polymorphisms in the population of interest, ARMs PCR was carried out. For each of the SNP two reactions were run separately in two different PCR tubes. Each tube contained a wild type or a mutant primer with reverse common in both tubes.

Master mix for PCR was prepared in eppendorf tube (Axygen, California, USA) by adding 3µl of sample DNA(50 ng/ ul), 2.5 ul (10X) of PCR buffer (Fermentas, Burlington, Canada), 1.5 µl of 25 mM magnesium chloride (MgCl2) (Fermentas , Burlington, Canada), 1.5 µl of of 2mM deoxyribonucleotide triphosphates (dNTPs) (Fermentas , Burlington, Canada), 1 µl of forward and reverse primers 20pm/ul), 0.2 µl of Taq polymerase (1unit) (Fermentas , Burlington, Canada) and 16.3 ul of PCR water. Reaction mixture was mixed properly and air bubbles were removed by gentle tapping.

The reaction mixture was processed in 2720 thermocycler (Applied Biosystem, Foster City, USA). Thermocycling conditions consisted of template denaturation at 94°C for 5 minutes followed by 40 cycles of PCR amplification. Each PCR cycle

further consisted of 3 steps: 45 seconds at 94 for denaturation of template DNA strands into single strands, 45 seconds at 57°C to allow annealing of primers to their target sites on template DNA and 30 seconds for extension of DNA. Final extension of unextended strands was carried out after 40 cycles by TaqPolymerase at 72 °C for 7 minutes. Amplified PCR products were stored at -20°C. PCR conditions are given in figure 3.1.

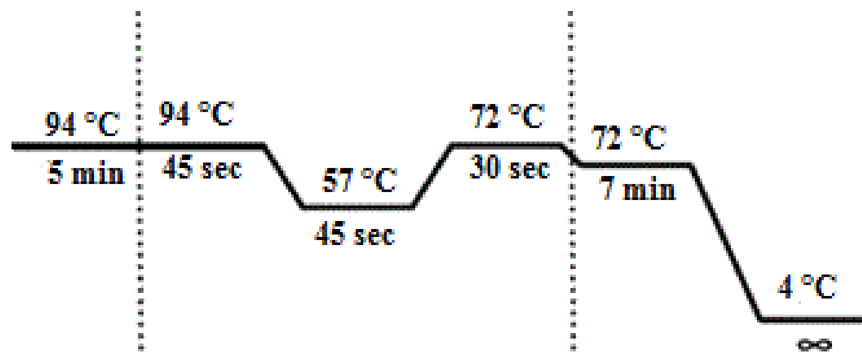

Figure 3.1: PCR Profile

3.10- Agrose Gel Electrophoresis:

In order to confirm the amplified PCR products, agarose gel electrophoresis was carried out. 1% (w/v) agrose gel was prepared by melting 0.5 g of agrose in 50 ml of 1X TBE buffer in a microwave oven for 2 minutes. Ethidium bromide solution (0.5 ug/ml final concentration) was added for the staining of DNA. The gel was then allowed to solidify at room temperature in a gel casting tray. After solidification gel was immersed in electrophoretic tank containing buffer. 3 ul loading dye (0.25% bromophenol blue in 40% sucrose solution), mixed with 6 ul of DNA was loaded into the wells of the agarose gel. Electrophoresis was performed at 100 V for almost half an hour in 1X TBE buffer. Gel was visualized under Ultraviolet Transilluminator (Biomretra, Geottingen, Germany).

3.11 - Statistical Analysis:

Statistical analysis was performed using Statistical Practices for Social Sciences (SPSS) and Graph Pad Prism. Chi square and T-test were applied for the association analysis of polymorphisms in the study population.

3.12- Bioinformatics Analysis:

In order to understand the role of common genetic variants in susceptibility to common diseases association studies are conducted. Bioinformatics analysis can be performed to aid these studies in multiple ways. One aspect is to predict the possible phenotypic changes associated with SNP. We in this study investigated the possible effect of the SNP under investigation on the respective protein structure. 894T/C variant lies in the exon 7 of eNOSgene and leads to the replacement of Glu by Asp at 298 position. This SNP has also been reported to affect the normal functioning. We therefore investigated the possible involvement of this SNP in causing structural aberrations which would in turn cause malfunctioning of target protein.

Various structural bioinformatics analysis tools and databases were utilized to analyze the primary, secondary and quaternary structures of both wild type and mutated proteins (Table 3.1). The methodology deployed in this analysis includes various steps. First of all a structural template was searched from PDB. The NMR structure of protein domain in which our SNP lies was retrieved from PDB. Amino acid sequence of normal protein in Fasta format was obtained from PIR. Then Swiss PDB was used to

mutate the specific amino acid (298) in the structure which was stored in PDB format. Next primary structure analysis was performed using PROTPARAM. Various attributes like isoelectric point, hydrophobicityetc were measured from it. Secondary structure

prediction was performed using Psipred. Secondary structure for wildtype was taken from UNIPROT. Tertiary structure analysis was performed using Swiss PBD view.Various attributes like E-value bonds, torsion angles etc were scrutinized and compared for all neighboring amino acid residues of the mutant amino acid. The neighboring amino acid residues were compared because these residues can contribute in distorted structure.

CHAPTER 4

RESULTS

4.1- Characteristics of Study Subjects:

In this case control study, genetic association analysis of 786 T/C polymorphism was performed on a total sample size of 105 which was divided into 35 controls, 35 CAD plus T2DM patients and 35controls. Patients and controls above 30 and below 70 were included in this study. Patients were screened for CAD on the basis of angiography scores > 70%, 2 to 3 loof vessels and positive electrocardiogram results. Diabetes patients with fasting glucose level ≥11.1 (≥200) mmol/l(mg/dl), random blood sugar level ≥7.0 (≥126) mmol/l(mg/dl) and HbA_{1c} values ≥6.5 % were recruited for the study. All three groups were screened for the presence of 786T/C polymorphism using allele specific PCR based analysis. Resulting data was statistically analyzed for any significant association of this polymorphism with mentioned diseases.

4.2- PCR Amplification:

The 786 T/C variant located in promoter region was analyzed in patients and controls using allele specific PCR. Gene products with band size of 315 have been shown (Figure 4.1, 4.2 and 4.3). All possible allele combinations i.e. TT, TC and CC are tabulated. All observed allele frequencies and genotype frequencies are illustrated in Table 4.2.

Hardy-Weinberg Equilibrium (HWE) calculations were made for each group (Table 4.1). There was no significant difference between observed and expected values. P-values for control, diabetes and CAD plus T2DM groups were found to be 0.5, 0.7 and 0.3 respectively. This indicated that there is no significant drift in the allele frequencies of patients when compared to the allele frequency of random healthy population. All groups were suitable for further analysis and association studies.

4.3- Statistical Analysis:

Chi-Square (X^2) test was used for the association analysis 786 T/C polymorphism with T2DM and CAD plus T2DM groups separately. Data was analyzed with Graph Pad Prism Software using two tailed analysis. The value of significant association(X^2) was found to be 7.2 and 18.11 with a probability error (*p*-value) 0.0001 in case of T2DM group CAD plus T2DM group respectively. The observed results as described in the table 4.3 indicated statistically significant association of the SNP with T2DM and CAD. Moreover, Pearson test was applied to find out correlation between genotype frequency and various characteristic features like age, gender, systolic and diastolic blood pressure. There was no significant association of age and gender with genotype frequency.

4.4- Bioinformatics Analysis of 894 G/T polymorphism:

The analysis of mutated and wildtype structures of 894T/C variant was performed at various levels of structural details. These include primary, secondary and tertiary structure analysis.

4.4.1- Primary Structure Analysis of Normal vs. Mutant Protein:

PROTPRAM revealed the differences in properties calculated on the basis of primary structure. Properties of both wild type and mutant proteins are given below (Table 4.4). A very slight difference in the stability index is observed where 3 atoms were found to be removed.

4.4.2- Prediction of Protein Secondary Structure:

The secondary structure of wild type protein was retrieved from PDB entry eNOS for Homo sapiens (Figure 4.6). It is reported to be calculated through SCOP. Next the mutated sequence retrieved from Swiss PDB view was subjected for prediction of protein secondary structure using PSIPRED (Figure 4.7). No significant difference was found in the secondary structure position of amino acid in mutant. It was found to be present in the coil region in both wild type and mutated sequences.

4.4.3- Analysis of Protein Tertiary Structure:

From reported NMR and X-ray diffraction based templates, available on protein data bank, a fragmented NMR structure was chosen with highest resolution. The reported crystal structure fragment did not cover region of our SNP. The chosen region covers 415 amino acids residues ranging from 67 to 480 amino acid. The desired change (Glu/Asp) lies in this region i.e., 298. On the basis of this template, we inserted a mutation and modeled SNP based mutant protein.

The structure was analyzed using SPDB Viewer. The glutamate had seven neighboring residues. All of them are displayed in figure 4.8. Next the energy minim-

-izationwas performed and different structural properties were calculated. However the structural attributes of the wildtype residue and only its neighboring residues are presented below (Table 4.5) for comparison.

Similarly the Aspartate alongwith its neighboring residues aredisplayed in figure 4.9. The structural attributes of the wildtype residue and only its neighboring residues are presented in table 4.6 for comparison. Significant differences are observed between both wild type and variant's structural attributes. The torsion, bonds , angles and electrostatic potential of ach amino acid is changed in neighborhood. This is likely due to bulky glutamate is being replaced with less bulky side group containing amino acid. The electrostatic constraint of various amino acids is altered including Gln 294 and Asp 297. The electrostatic interactions are very critical and have been reported to play significant role in structure activation/inactivation properties. (Critical role of electrostatic interactions of amino acids at the cytoplasmic region of helices 3 and 6 in rhodopsin conformational properties and activation.(Ramon, E et al., 2005). The double mutant and single mutants both have been found to play such critical roles in structural stability. Rest the differences in torsions and bonds might play role in relaxing/contracting the connected B sheets thus altering the slight conformation between them.

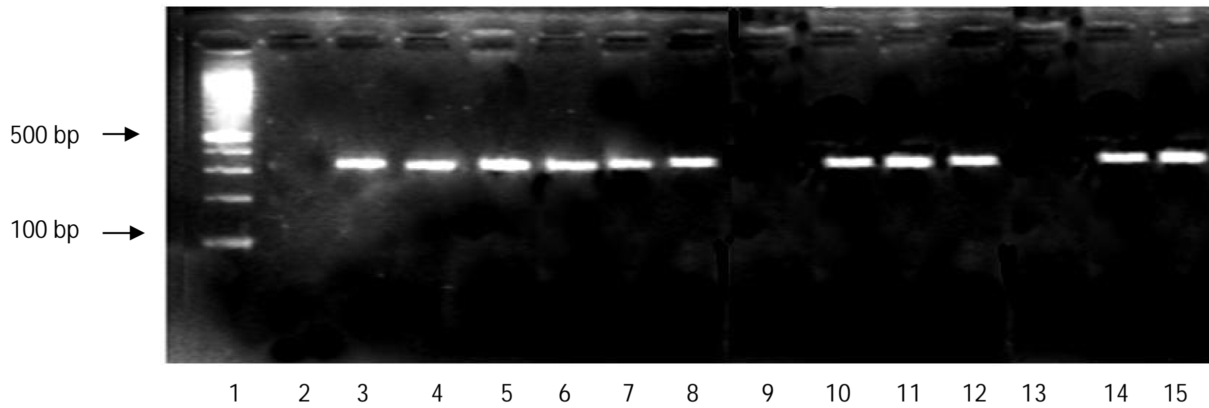

Figure 4.1: Electropherogram of ethidium bromide stained 3% agarose gel showing allele pattern obtained for 786 T/C polymorphism in healthy controls.

Lane 1: Ladder (100bp), Lane 2: Negative control, Lane 3: Positive control

Lane 4 and 5: Sample 1 (heterozygous TC)

Lane 6 and 7: Sample 2 (heterozygous TC)

Lane 8 and 9: Sample 3 (homozygous for C)

Lane 10 and 11: Sample 5 (heterozygous TC)

Lane 12 and 13: Sample 6 (homozygous for C)

Lane 14 and 15: Sample 7 (heterozygous TC)

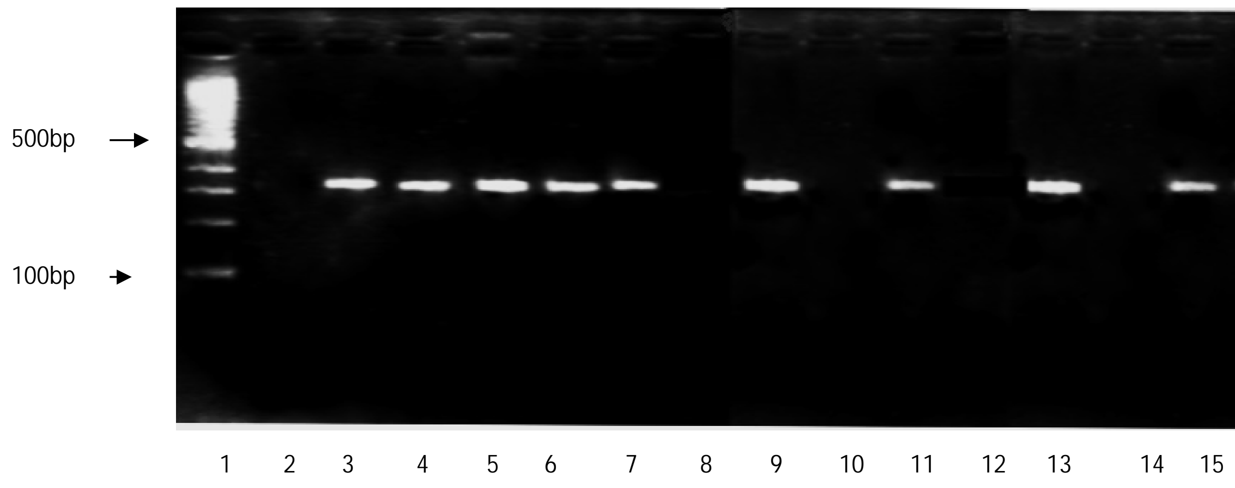

Figure 4.2: Electropherogram of ethidium bromide stained 3% agarose gel showing allele pattern obtained for 786 T/C polymorphism in T2DM patients.

Lane 1: Ladder (100bp), Lane 2: Negative control, Lane 3: Positive control

Lane 4 and 5: Sample 1 (heterozygous TC)

Lane 6 and 7: Sample 2 (heterozygous TC)

Lane 8 and 9: Sample 3 (homozygous for T)

Lane 10 and 11: Sample 5 (homozygous for T)

Lane 12 and 13: Sample 6 (homozygous for T)

Lane 14 and 15: Sample 7 (homozygous for T)

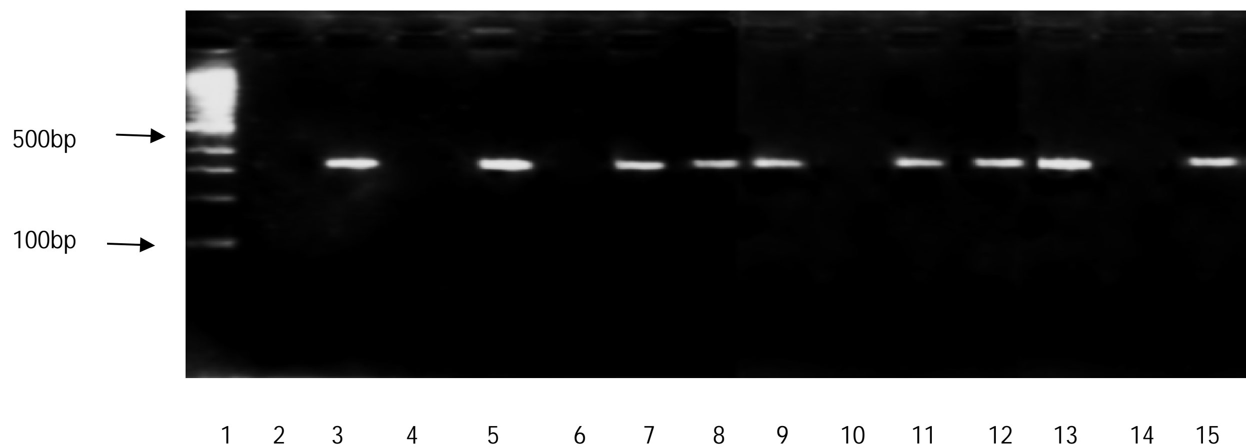

Figure 4.3: Electropherogram of ethidium bromide stained 3% agarose gel showing allele pattern obtained for 786 T/C polymorphism in CAD plus T2DM group of patients.

Lane 1: Ladder (100bp), Lane 2: Negative control, Lane 3: Positive control

Lane 4 and 5: Sample 1 (homozygous for T)

Lane 6 and 7: Sample 2 (homozygous for T)

Lane 8 and 9: Sample 3 (heterozygous TC)

Lane 10 and 11: Sample 5 (homozygous for T)

Lane 12 and 13: Sample 6 (heterozygous TC)

Lane 14 and 15: Sample 7 (homozygous for T)

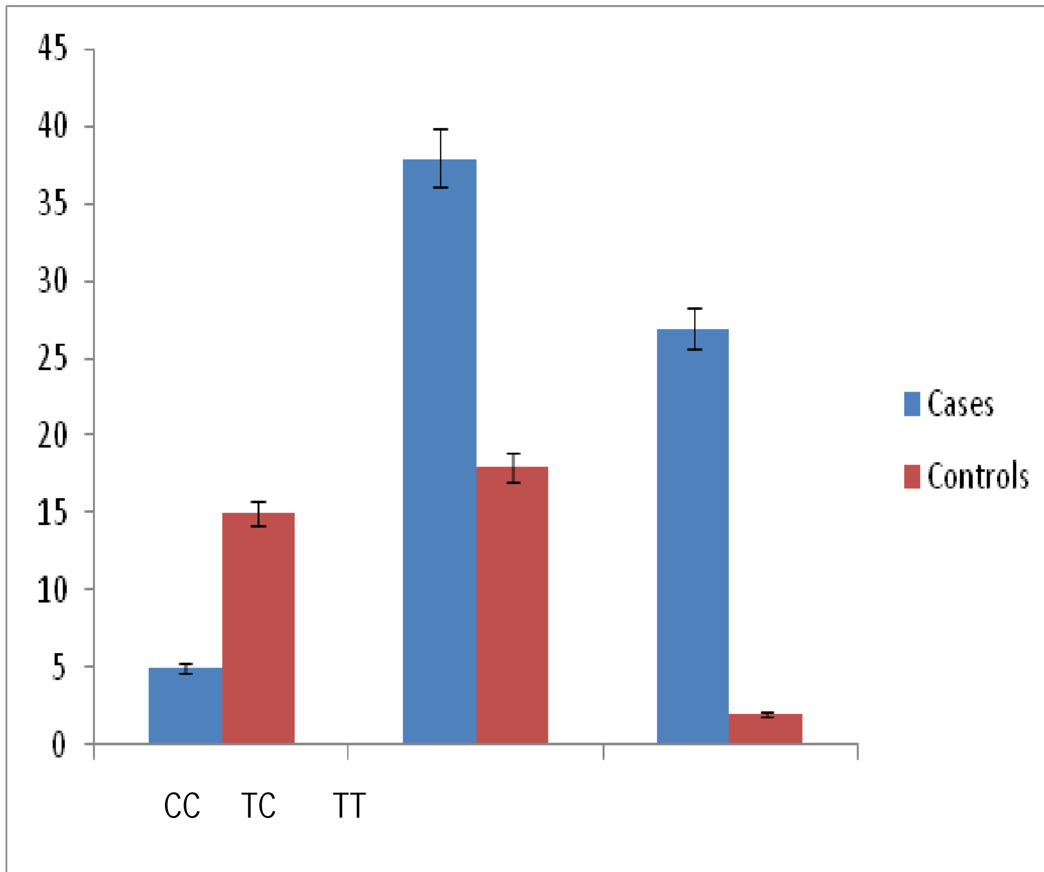

Figure 4.4: Genotype frequency distribution of 786T/C polymorphism

in cases andcontrols.

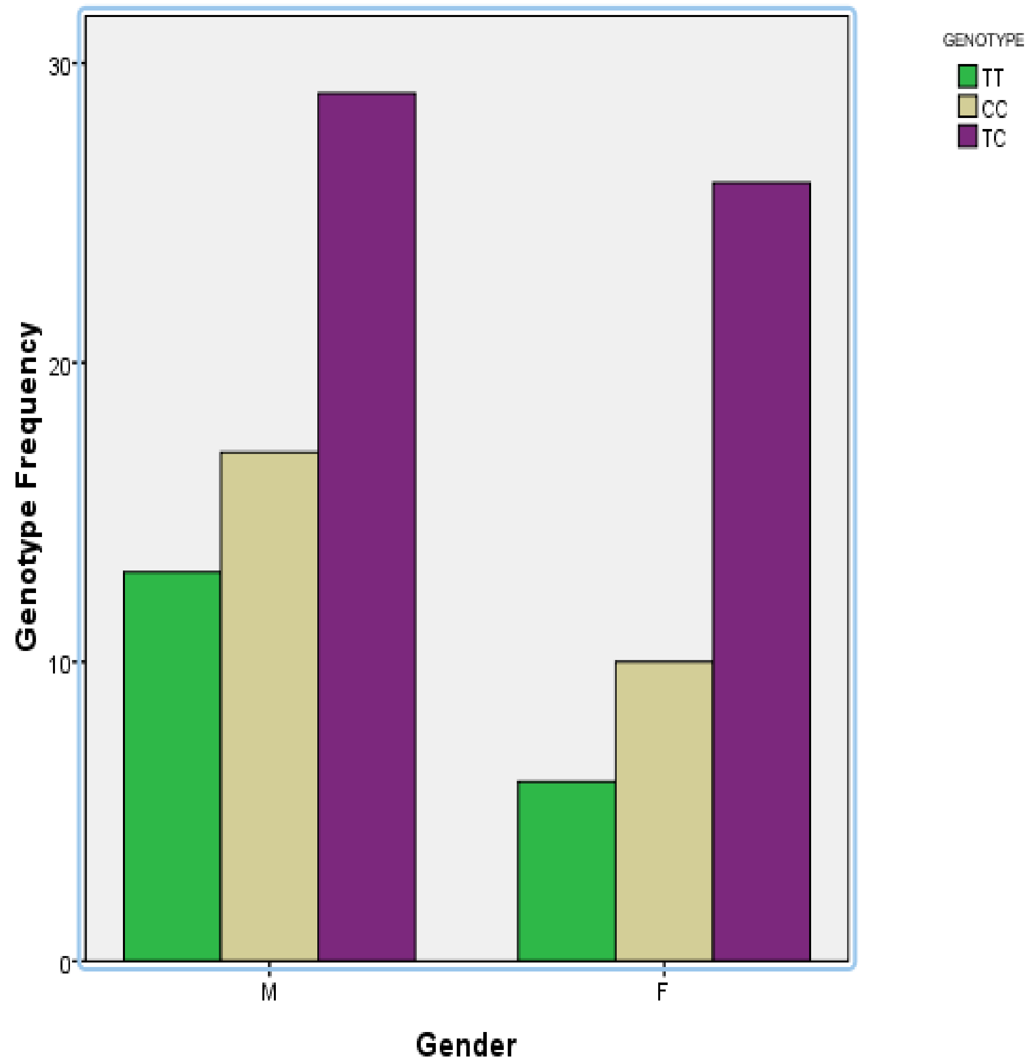

Figure 4.5: Distribution of gender across genotypes for 786T/C polymorphism

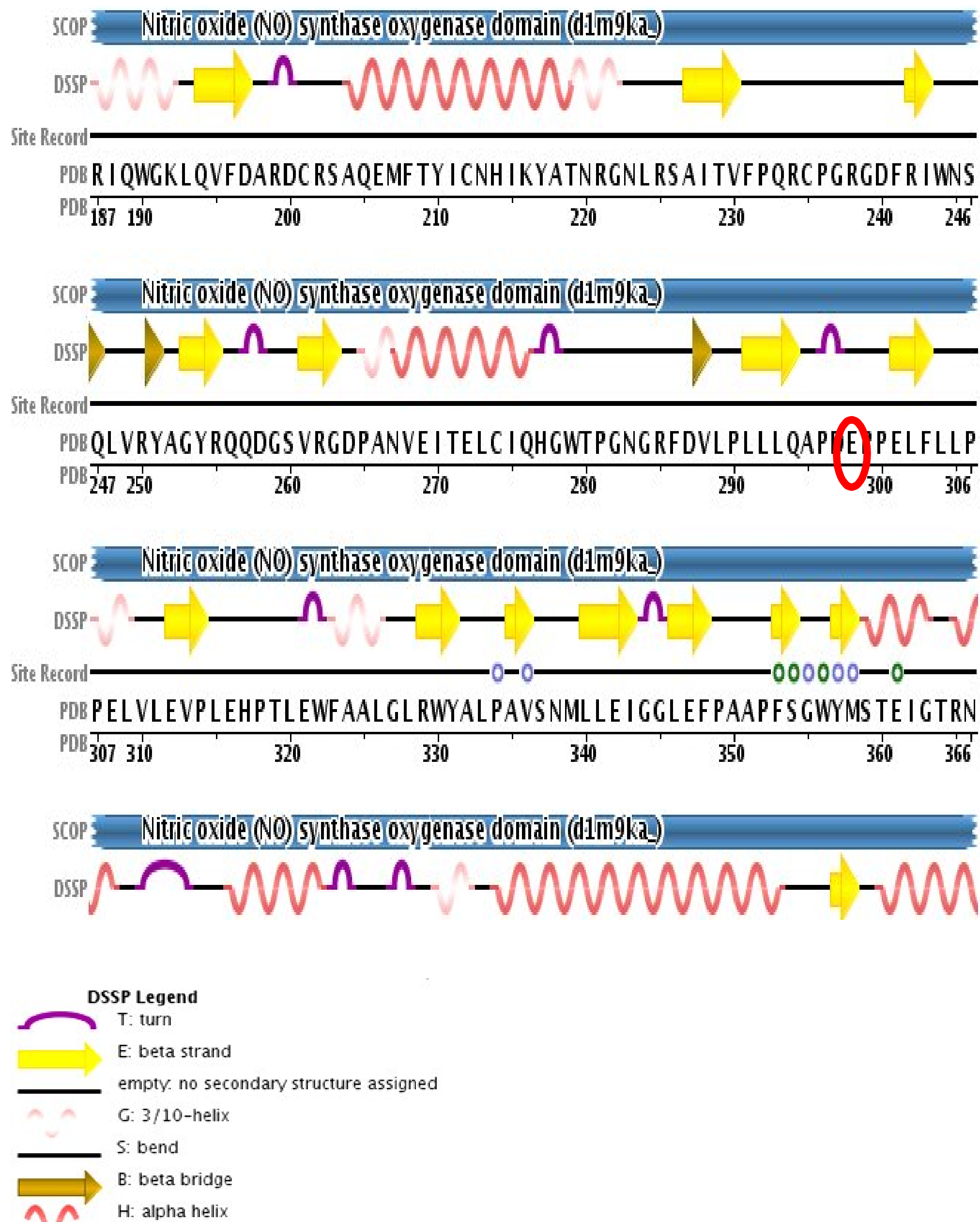

Figure 4.6:Wild Type Protein Secondary Structure.

Highlighted portion shows that amino acid Glu (298) lies in the coil region.

```
Conf:
Pred:
Pred: CCCCCCCCCEEEEEECCCCCCCEEECCCCCEEEEECCCCCC
  AA: PGNGRFDVLPLLLQAPDDPPELFLLPPELVLEVPLEHPTL
                        290       300       310       320

Conf:
Pred:
Pred: CHHHHCCCCCCCCCCCCCCCEEECCEECCCCCCCCCCCCC
  AA: EWFAALGLRWYALPAVSNMLLEIGGLEFPAAPFSGWYMST
                        330       340       350       360

Conf:
Pred:
Pred: CCCCCCCCCCHHHHCHHHHHHHCCCCCCCCCCCCHHHHHH
  AA: EIGTRNLCDPHRYNILEDVAVCMDLDTRTTSSLWKDKAAV
                        370       380       390       400

Conf:
Pred:
Pred: HHHHHHHCCCCCCEEEECCHHHHHHHHHHHHHHHHHHCCC
  AA: EINVAVLHSYQLAKVTIVDHHAATASFMKHLENEQKARGG
                        410       420       430       440

---

Legend:
  = helix     Conf: - + = confidence of prediction
  = strand    Pred: predicted secondary structure
  = coil      AA: target sequence
```

Figure 4.7: Mutated Protein Secondary Structure.

Highlighted portion shows that Asp 298 also lies in the coil region.

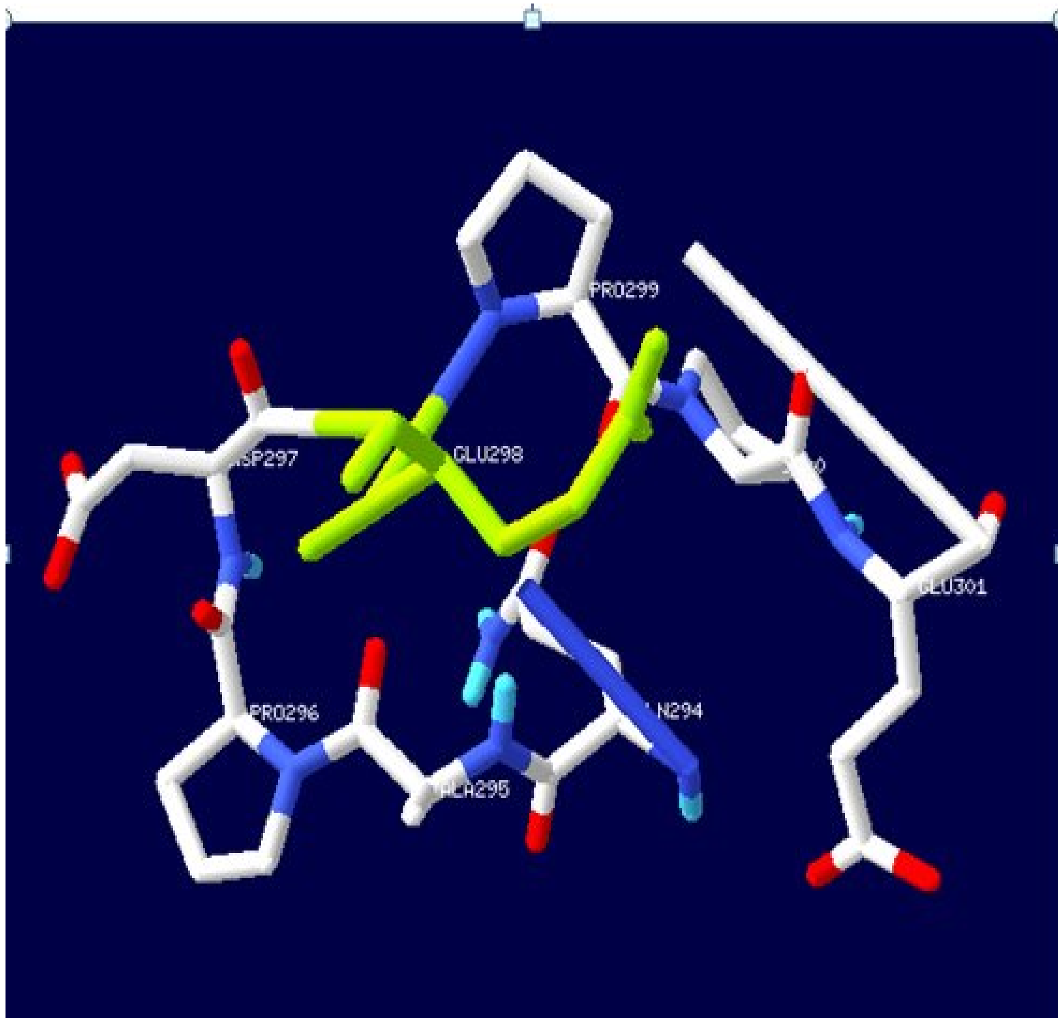

Figure 4.8: Wild Type Protein Tertiary Structure.

In wild type protein Glu 298 is shown in green colour and its neighboring residues are selected for display.

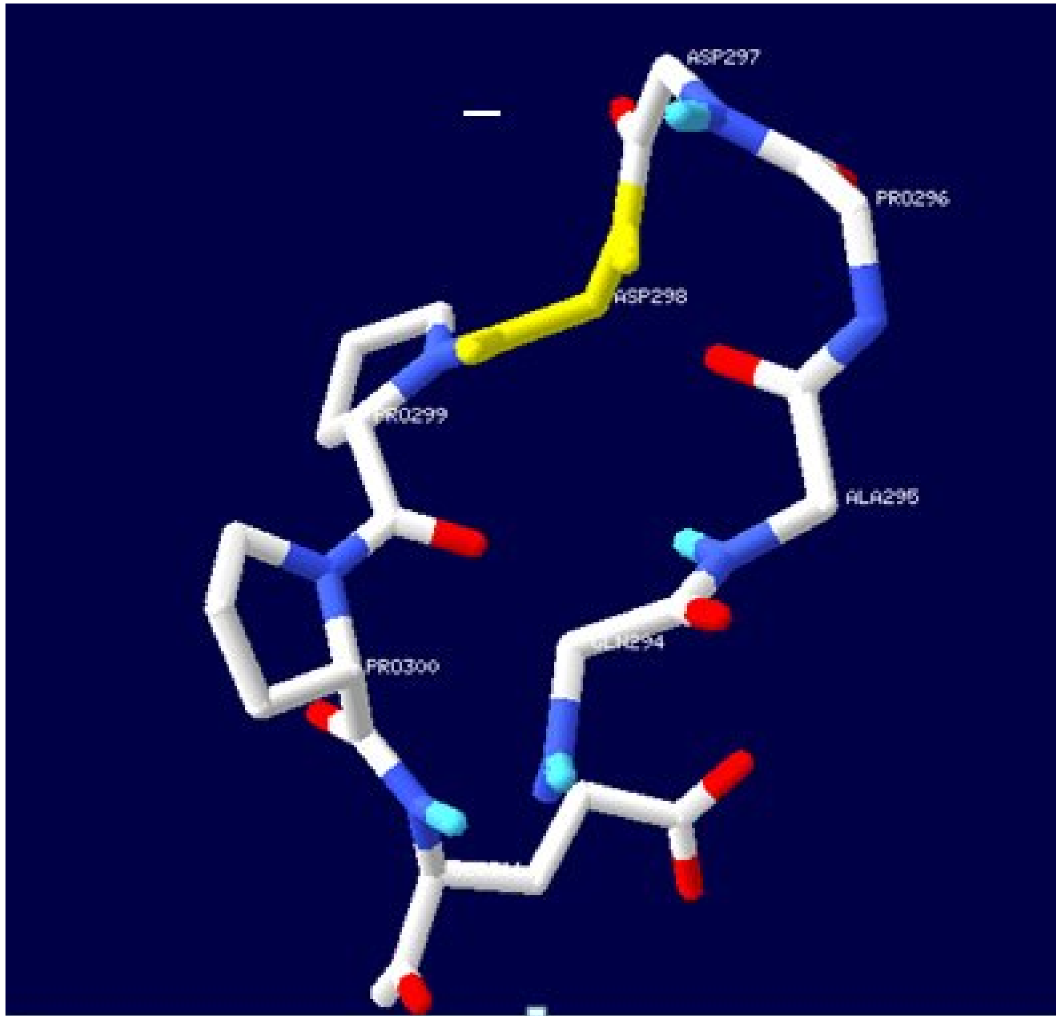

Figure 4.9: Mutated Protein Tertiary Structure.

In mutated protein Asp 298 is shown in yellow colour and its neighboring residues are selected for display.

Table 4.1:Hardy-Weinberg equilibrium (HWE) calculations on test population

for 786 T/C polymorphism

Cases and Controls	Genotype frequency			Allele frequency		Expected genotype counts under HWE			X^2	df	*p*-value
	CC	TC	TT	T	C	CC	TC	TT			
Controls (Healthy Individuals)	15	18	2	48	22	16.46	15.0	3.46	1.3	2	0.5
Diabetes Patients	3	18	14	24	46	4.11	15.7	15.1	0.7	2	0.7
CAD Plus Diabetes Patients	2	20	13	24	46	4.11	15.7	15.1	2.5	2	0.3

Table 4.2:Observed genotype and allele frequencies of cases and controls

Cases and Controls	Genotype frequency			Allele frequency	
	CC	TC	TT	C	T
Controls (Healthy Individuals)	15	18	2	48	22
Diabetes Patients	3	18	14	24	46
CAD Plus Diabetes Patients	2	20	13	24	46

Table 4.3:Statistical analysis of T2DM group and CAD plus T2DMgroup using Chi-Square Test

Disease	Genotype			Allele		
	Chi-Square Test Values	***P*-value**	**Significance**	**Chi-Square Test Values**	***P*-value**	**Significance**
T2DM group with controls	7.2	0.0002	Significant	16.47	0.0001	Significant
T2DM plus CAD group with controls	18.11	0.0001	Significant	16.47	0.0001	Significant

Table 4.4: Properties of wild type vs. mutant protein obtained from PROTPRAM

Properties	Values for wild type	Values for mutant	Difference
Molecular Weight	133288.8	133274.7	14.1
Hydrophobicity	-0.349	-0.349	0.0
Isoelectric Point	6.94	6.94	0.0
Instability Index	53.65	53.49	0.16
Atomic Composition:			
H	9271	9269	2.0
N	1677	1677	0.0
O	1734	1734	0.0
S	46	46	0.0
Total Number Of Atoms	18659	18656	3.0

Table 4.5:Structural Attributes of Wild Type amino acid and its Neighboring Residues

Residue	Bonds	Angles	Torsion	Improper	Non-bonded	Electrostatic Constraint	Total
Gln294	2.602	2.687	4.593	0.606	-43.15	-172.18	-204.846
Ala295	1.430	1.651	1.390	0.683	-12.98	17.31	9.486
Pro296	4.806	21.949	15.205	0.841	-23.02	-21.04	-1.258
Asp297	0.658	5.556	1.962	0.071	-13.37	0.60	-4.519
Glu298	2.631	2.934	1.031	0.909	-19.49	23.53	11.543
Pro299	4.842	23.639	21.153	0.712	-14.17	-9.52	26.652
Pro300	7.766	18.783	16.537	0.980	-33.02	-23.85	-12.805
Glu301	4.115	6.601	1.227	0.517	-34.49	-15.20	-37.228

Table 4.6:Structural Attributes of Mutant amino acid and its Neighboring Residues

Residue	Bonds	Angles	Torsion	Improper	Non-bonded	Electrostatic Constraint	Total
Gln294	1.102	3.206	2.429	0.827	-50.28	179.30	-222.020
Ala295	0.845	2.846	2.066	1.045	-19.04	-16.95	4.718
Pro296	0.168	17.006	16.660	1.018	-28.22	-21.53	-14.917
Asp297	1.032	2.637	2.162	0.931	-16.07	-1.62	-9.944
Asp298	1.067	4.247	1.686	0.367	-19.05	24.76	12.939
Pro299	0.496	14.396	26.014	1.417	-11.15	-9.81	21.360
Pro300	0.603	14.367	16.722	1.346	-31.19	-23.82	-22.085
Glu301	0.191	4.350	1.866	0.609	-37.68	-16.77	-47.431

CHAPTER 5

DISCUSSION

Diabetes is a metabolic disorder associated with a number of other serious complications. One of them is CAD which accompanies diabetes most of the time. Various genes are involved in the key pathways of disease pathogenesis, most notable being eNOS. It plays vital role in angiogenesis, blood pressure regulation and vasodilation. It also serves as a neurotransmitter and protects against the risk of heart related problems including CAD. Its role in T2DM is evident from the fact that eNOS knockout mice develop pancreatic malfunction and exhibit signs of diabetes. Two most studied polymorphisms of eNOS gene are 786 T/C and 894G/T. To date, the prevalence of eNOS polymorphisms has been established for Caucasian, African American, Hispanic, Korean and Japanese populations.The aim of this study was to evaluate the role of −786 T/C and 893 G/TeNOS gene polymorphisms in the etiology of T2DM and CAD. Also the correlation between various parameters and genotype frequency of disease were assessed.

The results of this study show strong association of eNOS786 T/T genotype with CAD(p-value < 0.05, X^2 value 7.2) and T2DM (p-value < 0.05, X^2 value 18.11) in Pakistani patients, suggesting that 786 T/C polymorphism in the promoter region has important clinical implications. The 786 C/C genotype was significantly higher in healthy controls. Contrasting results have been found in a number of other studies performed on different populations, covering different ethnic groups.C-allele is less common in Koreans (6%), Japanese (2%) and African Americans (5%).The highest frequency of this allele has been reported to be 38% in Caucasians. These findings

support the role of ethnicity in determining the prevalence of eNOS gene polymorphisms. However, in our population C-allele seems to protect against the risk of CAD and T2DM.

Role of eNOS in B.P regulation is supported the fact that eNOS knockout mice have significantly elevated B.P (Matthew *et al*., 2002). Our data in conformity with the previous findings suggests that the T-allele in eNOS may be an important contributor to essential hypertension, given that patients with the T/T genotype have a significantly elevated systolic BP. In our study mean blood pressure (B.P) of 130/90 (*p*-value 0.05) or higher in the homozygous T/T subjects has been observed as compared to C/C genotype. However, in a similar study this polymorphism was not found to be associated with hypertension. This contrast in allelic frequencies between ethnic groups requires further investigation.

The interaction between cigarette smoking and polymorphism was assessed in CAD group. It has already been reported that smoking increases the chance of coronary spasm in the individuals with the C/C genotype (Yoshimura *et al*., 2000). In present study we were unable to find any such association (*p*-value 0.79). This difference in findings might be due to small sample size used in this study.

The 894 G/T polymorphism results in change of amino acid from Glu to Asp and is reported to be involved in the pathogenesis of CAD and T2DM. Allele specific primers were designed for this SNP but due to shortage of time we could not proceed with the genotyping of this polymorphism. However, we carried out with the bioinformatics analysis of this variant. Primary structure analysis showed no significant differences except for a few properties like carbon content and total number of atoms which were found to be less in the mutant as compared to wild type. Tertiary protein

structure was modeled for both wild type and mutant templates and compared using Swiss PDB Viewer. The values of electrostatic constraints were significantly different in case of mutant and wild type. Electrostatic interactions are very critical and have been reported to play significant role in structural activation/inactivation. Effect of electrostatic interactions of amino acids at the cytoplasmic region of helices 3 and 6 has been seen on rhodopsin conformational properties and activation. Double and single mutants both have been found to play such critical roles in structural stability. In addition, differences in torsions and bonds might play role in relaxing/contracting the connected B sheets thus altering slight conformation between them. We propose that these changes might be involved in the relaxation of the overall mutant molecule exposing its cleavage site, thus increasing the susceptibility to ubiquitination.

Millions of people die each year due to inappropriate diagnosis facilities available. In future, this study might pave way towards the development of a diagnostic marker based on these SNPs. This would save mankind from the pain and suffering resulting from serious inappropriate diagnosis. Moreover, this study would lead to the improvement of already existing drug targets and development of new treatment regimens.

Thus, it can be concluded from this study that 786T/C polymorphism is a major risk factor towards the progression of T2DM and CAD in Pakistani patients. It is recommendable to replicate this study on a larger sample size to increase the level of significance.

CHAPTER 6

REFRENCES

Antoniades, C., Tousoulis, D., Vasiliadou, C., Pitsavos, C., Chrysochoou, C., Panagiotakos, D., Tentolouris, C., Marinou, K., Koumallos, N., Stefanadis, C.(2005). Genetic polymorphism on endothelial nitric oxide synthase affects endothelial activation and inflammatory response during the acute phase of myocardial infarction. Journal of the American College of Cardiology, 46(6): 101-110

Avogaro, A., Albeiro, M., Menegazzo, I., Kreutzenberg, S., Fadini, G.(2011). Endothelial dysfunction in diabetes. Diabetes Care, 34(2):105-115.

Braun, R.E. Genetics and coronary artery disease.(2006).Journal of Insurance Medicine, 27(3): 22-30.

Colberg, S.R., Sigal, R.J., Regensteiner, J.G., Blissmer, Richard, R.R., Braun, B.(2010). Exercise and Type 2 Diabetes. Diabetes Care, 33(12): 2692–2696.

Duplain, H., Burcelin, R., Sartori, C., Cook, S., Egli, M., Lepori, M., Vollenweider, P., Pedrazzini, T., Nicod, P., Thorens, B., Scherrer, U. (2001). Insulin resistance, hyperlipidemia, and hypertension in mice lacking endothelial nitric oxide synthase. Journal of American Heart Association, 104: 342-345.

Ford, E.S., Giles, W.H., Dietz, W.H., (2002). Prevalence of the metabolic syndrome among US adults. Journal of American Medical Association, 287:356–9.

Gao, F., Gao, E., Yue, T.L., Eliot, H.O., Bernard, L.L., Theodore, A., Christopher, T.A., Liang, X.M. (2002). Nitric oxide mediates the antiapoptotic effect of insulin in myocardial ischemia-reperfusion: the roles of PI3-kinase, Akt, and endothelial nitric oxide synthase phosphorylation. Journal of American Heart Association, 105: 1497-1502.

He, Y., Fany, Z., Zhang, J., Zheng, Q. Z. M., Li, Y., Zhang, D., Gu1, S., Yang, H.(2011). Polymorphisms of eNOS gene are associated with diabetic nephropathy: a meta-analysis. Mutagenesis, 26(2): 339–349.

Heltianu, C., Costache, G., Gafencu, A., Diaconu a, M., Cristea, C., Azibi, K., Poenaru, L., Simionescu, M. (2005). Relationship of eNOS gene variants to diseases that have in common an endothelial cell dysfunction. Journal of Cellular and Molecular Medicine, 9(1): 135-143.

Imamura, A., Takahashi, R., Murakami, R., Kataoka, H., Cheng, X.W., Numaguchi, Y., Murohara, T., Okumura, K. (2008). The effects of endothelial nitric oxide synthase gene polymorphisms on endothelial function and metabolic risk factors in healthy subjects: the significance of plasma adiponectin levels. European Journal of Endocrinology, 158: 189–195.

Iqbal, S. ,Dodani, S., Qureshi, R. (2004). Risk Factors and Behaviours for coronary artery disease among ambulatory Pakistanis. J Pak Med Assoc, 54: 105-110

Kuboki, K., Zhen, Y. J., Takahara, N., Ha S.W., Igarashi, M., Yamauchi, T., Edward, P. F., Terrance, P. H., Christopher, J. R., George, L. K. (2000). Regulation of endothelial constitutive nitric oxide synthase gene expression in endothelial

cells and in vivo: a specific vascular action of insulin. Journal of American Heart Association, 101: 676-681.

Lin, J.P., Johannes P., Schwaiger.,Lingenhel, A., Hunt, S.C., Yang, S., Kronenberg, F. Association between the ugt1a1 28 allele, bilirubin levels, and coronary heart disease in the framingham heart study. (2006).Circulation, 114:1476-1481.

Mackawy, M.A., Badawy, M.(2012).Endothelial nitric oxide synthase (enos) -786 t>c and +894 g>t gene polymorphisms and their association with coronary artery disease risk factors in type 2 diabetic Egyptian patients.Z.U.M.J, 18:20-35

Matthew, E. H., Howard G. P., Verma, S., Peter, J. B., Edworthy, S., Jones, C., Lonn, E., Charbonneau, F., Todd J. A. (2002). The T-786→C mutation in endothelial nitric oxide synthase is associated with hypertension. Journal of American Heart Association, 39: 919-922.

Meroufel, D., Benchekor, S.M., Dumont, J., Soraya. (2009). Relationship between endothelial nitric oxide synthase gene polymorphisms and the risk of myocardial infarction in the Algerian population. Egyptian Journal of Medicine and Human Genetics, 10 (1): 89-93.

Murthy, P.D., Prasad, K.T., Gopal, P.V., Rao, K.V, Rao, R.M. (2012). A survey for prevalence of coronary artery disease and its risk factors in an urban population in Andhra Pradesh. Journal of Associate Physicians India, 60: 17-20.

Philip, A. M., Henry, H. Q. H., Stephen, W. S., Robert, J. S., Anne, V. X., Keith, T. S. (1993). Structure and chromosomal localization of the human constitutive

endothelial nitric oxide synthase gene. Journal of Biological Chemistry, 68 (23): 17478-17488.

Richard, G. K., Salvador, M. (1994).Nitric oxide synthases in mammals. Journal of Biochemistry, 298: 249-258.

Rossi, G.P., MD, Cesari, M., Zanchetta, M., Colonna, S., Maiolino, G., Pedon, L., Cavallin, M., Maiolino, P., Pessina, A.C. (2003). T-786C Endothelial Nitric Oxide Synthase genotype is a novel risk factor for coronary artery disease in Caucasian patients of the Genica study. Journal of the American College of Cardiology, 41(6): 55-68.

Stoney, R. M., O'Dea, K., Herbert, K. E.(2001). Insulin resistance as a major determinant of increased coronary heart disease risk in postmenopausal women with Type 2 diabetes mellitus. Diabetic Medical Journal, 18: 476–82.

Tamemoto, H., Ishikawab, S., Kawakami, M. (2008). Association of the Glu298Asp polymorphism of the eNOS gene with ischemic heart disease in Japanese diabetic subjects. Diabetes Research and Clinical Practice, 80: 275–279.

Thameem, F., Puppala, S., Nedal, H.A., Stern, P.S, Blangero, J., Duggirala, R., Abboud, H.E. (2008). Endothelial nitric oxide synthase (eNOS) gene polymorphisms and their association with type 2 diabetes-related traits in Mexican Americans. Journal of Diabetes and Vascular Disease Research, 5: 109-112.

Yaghoubi, R and Khatibi, F.K.(2012). T-786C single-nucleotide polymorphism (SNP) of endothelial nitric oxide synthase gene and serum level of vascular endothelial

relaxant factor (VERF) in non-diabetic patients with coronary artery disease. African Journal of Biotechnology, 11(93): 145-159.

Zafar J, Bhatti F, Akhtar N, Rasheed U, Humayun S, Waheed A, Younus F, Nazar M, Umaima ,Bashir R. (2011). Prevalence and risk factors for diabetes mellitus in a selected urban population of a city in Punjab. Journal of Pakistan Medical Association, 61:40-50

.