Study of STK11 Gene Polymorphism in Selected Type 2 Diabetic Population of Pakistan

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Gene polymorphism

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Selected Type2 Diabetic Population of Pakistan

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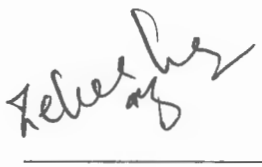

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Dedicated to
My Beloved Parents

DECLARATION

I hereby declare that the work present 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.

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LIST OF ABBREVIATIONS

Acronym	Abbreviation
AMPK	Adenosine monophosphate activated protein kinase
BMI	Body mass index
bp	Base pair
β	Beta
CVD	Cardiovascular Diseases
°C	Degree Celsius
DPP	Diabetes Prevention Programme
DM	Diabetes Mellitus
dH20	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose nucleotides Triphosphate
EDTA	Ethylene Diamine Tetra Acetic Acid
FBS	Fasting Blood sugar
Fig	Figure
kDa	kilo Dalton
KCNJ11	Potassium inwardly rectifying channel, subfamily J, member 11
M	Molar
mM	milli Molar
MgCl2	Magnesium chloride
mg/ml	milli gram per milli liter

min	minute
NaCl	Sodium Chloride
ng/µl	nano gram per micro liter
NH_4Cl	Ammonium chloride
PKB	Protein kinase B
pH	Power of Hydrogen
PASA	PCR Amplification of Specific Alleles
PCR	Polymerase chain reaction
rpm	Revolution per minute
rs	Reference SNP ID Number
SDS	Sodium dodecyl sulphate
SNPs	Single nucleotide polymorphism
SPSS	Software package Used for Statistical Analysis
STE	Sodium chloride, tris-HCl and EDTA
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TBE	Tris borate-ethylene diamine tetra acetic acid
TCF7L2	Transcription factor 7–like 2
TE	Tris EDTA
µL	Microliter
$\chi 2$	Chi-square value

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ABSTRACT

Diabetes mellitus is usually a complex, long-term illness characterized by increased glucose levels in blood. It is a condition primarily defined by the level of hyperglycaemia giving rise to risk of microvascular damage. Probable mechanisms through which STK11 variants influence type 2 diabetes integrate its part in pancreatic islet expansion, beta cell persistence and insulin secretory granule. Diabetes prevention programme found that variants in STK11 are intricate in development of type 2 diabetes and some variants are also accompanying with a positive reaction to metformin therapy and action.

Diabetes mellitus type 2 is often a metabolic dysfunction that is considered as a hyperglycemic condition inside the framework connected with insulin level of resistance. In the present research, we studied A/G polymorphism in STK11 gene in 100 diabetes type 2 patients and 50 healthy individuals. For rs375622587 A>G genotypes, the homozygous AA was 14% in controls and 20% in patients that were on sulphonylureas medication, and 6% in patients that were on metformin medication. The heterozygous AG was 23% in controls and 9% in patients that were on sulphonylureas medication, and 15% in patients that were on metformin medication. The homozygous GG genotype was higher in patients (14%) that were on metformin medication as compared to the control (10%).

Allele frequency of the major allele A was 0.5% in controls and 0.3% in patients that were on metformin medication and 0.6% in patients that were on sulphonylureas medication. The percentage of the minor allele G was 0.7% in patients that were on metformin medication as compared to the controls 0.41%. This study will help in exact therapeutic targets for drug development and pattern of mutation in humans.

CHAPTER 1

INTRODUCTION

1.0 Introduction

Diabetes mellitus is defined as a group of metabolic illnesses that are characterized by prolonged hyperglycemia. Diabetes mellitus is a long-lasting disease that happens either when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces.(American Diabetes Association, 2014). There are many types of diabetes mellitus, and T2DM is the most common form. Type 1 diabetes mellitus is diagnosed in up to 5–10% of DM patients, T2D is the most common form of DM (up to 90–95% of all cases) (Canivell *et al.,* 2014) and more rare forms account for MODY (maturity-onset diabetes of the young), gestational diabetes (altogether up to 4%), pre diabetes, neonatal diabetes, diabetes inspidus, latent autoimmune diabetes of adults (Zimmet *et al.,* 2001).

Type 2 diabetes is the most common form and usually sets as the form of a disease with the commencement of adult life. Almost 90-95% of individuals are diagnosed as type 2 diabetes. It is related to the resistance of insulin comparatively to the deficiency of insulin. As far as public health is concerned T2D is found of excessive significance as compared to the type 1 diabetes, because of the encumbrance of sickness and transience (Kraemer *et al.,* 2014).

Presently Diabetes Mellitus affects 387 million people globally (International Diabetes Federation, 2014), creating up about 90% of the cases with Type-2 diabetes, which is equal to 8.3% of the adult inhabitants, with identical tariff in both male and female (Vos *et al.,* 2012). Diabetes mellitus is assessed to have caused 1.5 to 4.9 million deaths per year, subsequently in the years 2012-2014 (International Diabetes Federation, 2014). In 2014, the worldwide financial budget of diabetes was expected to be $612 billion USD (IDF Diabetes Atlas, 2013). By 2035, the figure of people associated with diabetes is estimated to rise to 592 million. Pakistan is considered as an excessive epidemic region, presently involving 6.9 million people affected with expected estimations likely to double and affect 11.5 million people by 2025 (International Diabetes Federation, 2014) In most advanced countries, diabetes is considered to be the fourth foremost basis of demise (International Diabetic Federation Diabetes, 2006), Pakistan at present, stands at 7th position among the countries affected by DM and if situation prevails it is anticipated to move to forth position. Type 2 diabetes is the most common form as compared to

the type 1 diabetes and it includes90% of individuals with diabetes around the world (International diabetes federation,2013)

Occurrence of Type 2 diabetes mellitus between the adult population (>25 years) is 13.9% in Sindh and considered to be 8.6% in Baluchistan, and a incidence rate of type 2 diabetes mellitus in NWFP is 11.6% in adult women and 9.2% in adult men (Shaw *et al.,* 2010).

Being a complex disorder, many environmental and genetic reasons may contribute to the development of T2D, and that is why immense hard work is needed in order to find out the genetic origins of this compound disorder. Nowadays it is rather easy to recognize the functioning of glucose and energy particularly at the level of molecules (Brunetti *et el.,* 2014). Various momentous hard works are being made in order to find out the mechanism of the action of insulin and its secretion in the body that allow us to have complete responsiveness about energy equilibrium and hunger control (Ahlqvist *et al.,* 2011).

The main imperfections in subsidizing to the pathogenesis of type 2 diabetes are reduced insulin secretion and insulin resistance in peripheral tissues, such as adipose, muscle, and the liver. Indeed, some data propose that, at the time of identification or diagnosis a sheer 20% of beta-cell function will leftover. The progress of long-lasting and prolonged hyperglycemia will additionally damage the function of the discharge of insulin and beta cell. Several factors e.g enlarged production of the hepatic glucose, and unnecessary secretion of glucagon and also the lessened effect of incretin, together play a significant part in the progression of type 2 diabetes (Fronzo *et al.*, 2010). The level of the glucose in the body is enhanced or gets enlarged in the case of type 2 diabetes, since the body responds inadequately to the insulin, and the cells will not be able to achieve the optimal glucose level. Other factors that may contribute to the development of 90-95% occurrences of type 2 diabetes includes the insulin secretion and insulin resistance. Unfortunately due to certain limitations the absolute hereditary inadequacies are not predicted and are also not considered markedly. It is likely anticipated that multiple factors like age, obesity, physical inactivity, smoking and dietary consumption can proliferate the hazard of emerging the type 2 diabetes. In addition other causes in developing the type 2 diabetes can also be the certain medications, prescriptions or sensitivity to different chemicals, hereditary deficiencies in insulin activity, pancreatic illness, undergoing surgical procedures and also the infections and contamination.(American Diabetes Association, 2010). The achievement of

attaining the maximum level of glucose in the case of type 2 diabetes is not possible due to various reasons which includes failure to lose weight, weight gain overtime, the progressive nature of the disease or a mixture of these factors. Maximum number of patients will consequently require pharmacotherapy to retain the prerequisite glycemic control (Nathan *et al.,* 2009).

The most common and first line of therapy for type 2 diabetes is metformin which is considered to be an eldest, and undeniably one of the harmless medications used in the management of preventing the type 2 diabetes. It is usually the suggested initial pharmacotherapy, which has the ability to apply the glucose-lowering actions with a moderate risk of hypoglycemia. In addition it will decrease the probability of emerging macrovascular and microvascular difficulties or complexities. It is considered to be one of the most effective target for type 2 diabetes with no weight gain or modest weight loss, these makings are what make it striking as initially recommended pharmacotherapy for type 2 diabetic patients (Lamanna *et al.,* 2011).

It is not absorbed or metabolized in the body but is expelled unchanged in the urine by certain active tubular secretion system. Various carrier proteins are responsible for the oral absorption of metformin, its uptake in the liver and its elimination by the renal system. It is proposed through certain evidences that the main action of metformin is exerted in the liver, principally from the triggering of AMPK. Stimulation of AMPK is done by serine-threonine kinase 11 (STK11/LKB1), which suppresses hepatic gluconeogenesis, thereby, reducing glucagon-mediated glucose output by the liver. Serine/threonine kinase 11 (STK11) which is also known as (LKB1) is a protein kinase that in humans is determined by the *STK11* gene. TheSTK11/LKB1 gene, which encodes a member of the serine/threonine kinase family, controls the polarity of a cell and also functions as a tumor suppressor. STK11/LKB1 is the basic upstream kinase of (AMPK), an essential component in cell metabolism that is mandatory for retaining energy homeostasis (Schwartz *et al.,* 2006).

Possible mechanisms through which STK11 variations influence type 2 diabetes integrate its part in pancreatic islet expansion, beta cell persistence and insulin secretory granule. Diabetes prevention programme originate that variants in STK11 gene is involved in development of type 2 diabetes and some variants are also concomitant with a positive reaction to the therapeutic effects of metformin and its action. The Peutz-Jegher syndrome tumor-suppressor gene encodes a protein-threonine kinase, LKB1, which results in the phosphorylation and stimulation of

AMPK (adenosine monophosphate (AMP)–activated protein kinase). In mice, who isSTK11deficient, results with hyperglycemia, increased gluconeogenic and lipogenic gene expression.Single nucleotide polymorphisms (SNPs), are variations in DNA sequences that occur when a single nucleotide in the sequence of genome sequence gets changed. It is anticipated by certain evidences that in patients with type 2 diabetes, variants in STK11 genee.grs741765,were examined in relation to the metformin treatment which is defined by attaining HbA1c <7% and a completedecline in HbAlc after 6-monthsof metformin pharmacotherapy (Shaw *et al.*, 2010). Another variant in STK11 gene rs8111699 SNP influences both insulin sensitivity and metformin efficacy in hyperinsulinemic girls with androgen excess (Hardie, 2006).

Due to comparatively commendable safety profile of metformin, it is widely used as a primarily therapeutic agent in the treatment of type 2 diabetes when it seems impossible to attain the optimal glucose level through lifestyle alterations. Guidelines in ADA agreement have been recommended initiating all appropriate newly identified type 2 diabetic patients on metformin in combination with exertions to modify lifestyle. When the immune system of a patient will not respond to metformin, the physicians will prescribe another drug in addition with metformin i-e sulphonylureas which is considered to be the second line of therapy for type 2 diabetes. Sulfonylureas comprise several prescriptions that act on b-cells to upsurge the insulin release. They bind to the sulfonylurea receptor on the exterior of the b-cell and hinder potassium efflux, thus depolarizing the b-cells and aiding insulin release (Nathan *et al.,* 2006).Patients with type 1 diabetes, or advanced stages of type 2 diabetes, do not react to the sulphonylureas treatments. Additional drawback of using sulfonylureas is the possibility of weight gain. Many patients practice an increase $\geq$ 2 kg after commencement of these treatments. Elder patients may be at greater threat for hypoglycemia after treating with sulphonylureas (Gangii *et al.,* 2007).

For the research compiled in this thesis blood samples from the diabetic patients and healthy individuals of different ages were collected and tested. The purpose of this study was to investigate the association between the STK11 gene polymorphism in selected type 2 diabetic population of Pakistan. Blood samples of diabetic patients were collected and several methods were performed to evaluate the risk of type 2 diabetes including the DNA extraction, PCR and agarose gel electrophoresis. After the gel electrophoresis of all the reactions products, specific banding patterns were obtained. The current study is based on the need to know more about

genetic constitution of the selected Pakistani population in correspondence with type 2 diabetes and to study the effects of single nucleotide polymorphism of the STK11 gene in type 2 diabetic patients. The present study was aimed to assess that how variations in STK11 gene may control efficacy or toxicity of metformin drug in selected T2DM Pakistani population. The purpose was to address the genetics of T2DM on the basis of a collection of blood samples of patients treated with metformin. Genotyping ofSTK11gene polymorphism was carried out and then investigates the potential relationship between the SNP and clinical response of metformin in T2DM patients.

Aims and Objectives

The aims and objectives of the present study are to evaluate:

1. The find out the association of single nucleotide polymorphism in STK11 gene with type 2 diabetes.
2. To evaluate the potential role of variants in SKT11 gene with metformin efficacy.

CHAPTER 2

LITERATURE REVIEW

2.0 Literature Review

2.1 Diabetes Mellitus

Diabetes mellitus, or simply diabetes, is a group of diseases characterized by high blood glucose levels that result from defects in the body's ability to produce and/or use insulin. It is a condition primarily defined by the level of hyperglycaemia giving rise to risk of microvascular damage. It is associated with reduced life expectancy, significant morbidity due to specific diabetes related microvascular complications, increased risk of macrovascular complications (ischaemic heart disease, stroke and peripheral vascular disease), and diminished quality of life. Several pathogenetic processes are involved in the development of diabetes. These include processes, which destroy the beta cells of the pancreas with consequent insulin deficiency, and others that result in resistance to insulin action. The abnormalities of carbohydrate, fat and protein metabolism are due to deficient action of insulin on target tissues resulting from insensitivity or lack of insulin (Galtier, 2010).

Diabetes mellitus may present with characteristic symptoms such as dehydration, polyuria, distorting of visualization, and weight loss. Impediments owed to diabetes are the foremost basis of infirmity, low eminence of life, and decease. The difficulties that may arise due to diabetes can disturb numerous portions of the body and displaying in dissimilar means for diverse people. Diabetes mellitus can significantly multiply the threat for numerous severe health complications. It is one of the main factors in causing erectile dysfunction, decrease level of testosterone production, hopelessness and nervousness that can hinder with sensual approaches in men. Diabetes mellitus can increase the risk of gestational diabetes during pregnancy in women. Cardiovascular diseases are the primary basis of death in diabetic women (Galtier, 2010).

Diabetes can also increase the risk of depression, anxiety and eating complaints in women. Diabetes mellitus can disturb or distress every part of the body e.g feet, eyes and the skin. In some cases these complications are occasionally the principal sign that an individual has diabetes. Foot impediments can get poorer and lead to severe problems, such as neuropathy, skin deviations, nodules as well as foot sores (Craig *et al.,* 2009).

2.2 Types of diabetes mellitus

2.2.1 Type 1 diabetes: It is usually diagnosed in children and young adults and was previously known as juvenile diabetes (Dabelea *et al.,* 2014).

2.2.2 Type 2 diabetes: It is related to the resistance of insulin and moderately the insufficiency of insulin. It is currently the most common type of diabetes mellitus world-wide. (Kraemer *et al.,* 2014).

2.2.3 Gestational Diabetes: During pregnancy, some women experience heightened blood sugar levels and can't produce enough insulin to absorb it all. In most cases it develops between the 14th and 26th week of pregnancy, known as the second trimester, and disappears after the baby is born (Roglic and Colagiuri , 2014).

2.2.4 Neonatal diabetes: It is very rare type of diabetes which is caused by an alteration in a gene that primarily affects the production of insulin in the body (Yorifujii *et al.,* 2004).

2.2.5 Maturity onset diabetes of the young (MODY): It is also very rare form of diabetes triggered by a transmutation in a single gene (Barry *et al.,* 2008).

2.2.6 Prediabetes: It indicates a condition that occurs when a person's blood glucose levels are higher than normal but not high enough for a diagnosis of type 2 DM. Many people destined to develop type 2 DM spend many years in a state of prediabetes (Huang *et al.,* 2014).

2.2.7 Latent autoimmune diabetes of adults (LADA): It is a complaint in which type 1 diabetes mellitus progresses in adults. Adults with latent autoimmune diabetes of adults

are often originally misdiagnosed as having T2DM, on the basis of an age relative to particular cause of the disease (Borg *et al.*, 2002).

2.2.8 Diabetes insipidus (DI): It is a situation categorized by unnecessary thirst and elimination of large amounts of rigorously dilute urine, with decrease of fluid intake having no consequence on the concentration of the urine (Babey *et al.*, 2011).

2.3 Type II Diabetes

Type 2 diabetes is known as non-insulin dependent or insulin independent diabetes mellitus. It is the most collective and mutual form of diabetes. Lots of individuals around the world have been identified with this type of diabetes, and several individuals remain undiagnosed. It is the most common form of diabetes and 90-95% of individuals are suffering from T2DM. Individuals are at a larger threat of emerging cardiovascular diseases such as heart attack and stroke if type 2 diabetes is left undiagnosed or measured poorly. These individuals are also at a greater risk for vision loss, foot and leg subtraction due to injury to the nerves and blood vessels, and renal failure demanding dialysis or organ relocation (Babey *et al.*, 2011).

T2D is found of excessive significance as compared to the type 1 diabetes, because of the encumbrance of sickness and transience (Dabelea *et al.*, 2014). Insulin is an essential component for our body, so that it will be able to use proper amount of glucose in order to provide the optimal quantity of energy to the immune system. Sugar and starches that we get after consuming the food are broken down into glucose, which is the foremost requirement for the cells. The role of Insulin is to take the sugar from the blood into the cells, but when glucose deposit in the blood rather than going into the cells, it can lead to diabetes complications and difficulties (Redondo, 2013).

Before the development of Type 2 diabetes, people mostly have "prediabetes" in which the blood glucose levels in the body are higher than the normal but not adequately high to be identified as diabetes In the case of type 2 diabetes, there is a less production of insulin in the body or the cells do not respond properly to insulin. Insulin resistance in type 2 diabetes patients upsurges the mandate for insulin in insulin-target tissues. In accumulation to insulin resistance, the improved request for insulin could not be encountered by the pancreatic β cells due to imperfections in the utility of these cells (Halban *et al.*, 2014).

2.4 Epidemiology of type 2 diabetes

Presently Diabetes Mellitus affects 387 million people globally (International Diabetes Federation, 2014), creating up about 90% of the cases with Type-2 diabetes, which is equal to 8.3% of the adult inhabitants, with identical tariff in both male and female (Vos *et al.,* 2012). Diabetes mellitus is assessed to have caused 1.5 to 4.9 million deaths per year, subsequently in the years 2012-2014 (World Health Organization, 2013). In 2014, the worldwide financial budget of diabetes was expected to be $612 billion USD (IDF Diabetes Atlas, 2013). By 2035, the figure of people associated with diabetes is estimated to rise to 592 million. Pakistan is considered as an excessive epidemic region, presently involving 6.9 million people affected with expected estimations likely to double and affect 11.5 million people by 2025 (International Diabetes Federation, 2014). Pakistan at present stands at the 7th position among the countries affected by DM and if situation prevails it is anticipated to move to forth position. Type 2 diabetes is the most common form as compared to the type 1 diabetes and it includes 90% of individuals with diabetes around the world. Occurrence of Type 2 diabetes mellitus between the adult population (>25 years) is 13.9% in Sindh and considered to be 8.6% in Baluchistan, and a incidence rate of type 2 diabetes mellitus in NWFP is 11.6% in adult women and 9.2% in adult men (Shaw *et al.,* 2010).

2.5 Risk factors for type 2 diabetes

There are certain environmental and biological factors which upsurge the risk of developing type 2 diabetes.

2.5.1 Environmental factors:

2.5.1.1 Physical inactivity:

The less dynamic the individual is, more prominent the danger of type 2 diabetes will be. Physical movement controls weight, uses glucose as an energy source and help body cells to become more subtle to insulin (Almdal *et al.,* 2008).

2.5.1.2 Smoking:

It has been shown in numerous studies that existing smoking is a hazardous factor for emerging type 2 diabetes. Newly, a meta- analysis comprising 25 probable studies showed that present smoking was accompanying with a 44% better risk of diabetes. The connotation between smoking and type 2 diabetes was sturdier for substantial smokers, 20 cigarettes per day associated with light smokers or earlier smokers (Willi *et al.,* 2007).

2.5.1.3 Stress:

It has been suggested that emotional stress plays a role in the etiology of type 2 diabetes mellitus. Because when someone is under stress, his blood sugar level rises. Interestingly, stress has long been suspected as having important effects on the development of diabetes. English physician Thomas Willis (1621-1675) noted that diabetes often appeared among persons who had experienced significant life stresses, sadness, or long sorrow (Kato *et al.,* 2009).

2.5.2 Biological Factors:

2.5.2.1 Weight

Being overweight is a primary risk factor for type 2 diabetes. Obese people, are at high risk of developing Type-2 diabetes, if their body weight is beyond 20% for their height and it is associated with medical impediments. Obesity is one of the main factor which is accompanying with improved danger of developing insulin resistance and type 2 diabetes mellitus. Obese people mainly suffer from insulin resistance. The pancrea, with insulin resistance, has to work excessively hard to yield surplus insulin (Dansinger, 2014).

2.5.2.2 Fat Distribution

If the body stores fat in belly, danger of type 2 diabetes is more prominent than if body stores fat somewhere else, for example, hips and thighs (Jacobsen *et al.,* 2002).

2.5.2.3 Family History

The danger of type 2 diabetes increments if parent or kin has T2DM (Hu *et al.,* 2001).

2.5.2.4 Race

Individuals of certain races, together with Blacks, Hispanics, American Indians and Asian-Americans, are more likely to develop type 2 diabetes than whites are, even though the reason is unclear (Zimmet *et al.,* 2001).

2.5.2.5 Age

The risk of type 2 diabetes increases as person gets older, especially after age of 45. That's probably because people tend to exercise less, lose muscle mass and gain weight as they age. But type 2 diabetes is also increasing dramatically among children, adolescents and younger adults (Kumari *et al.,* 2004).

2.5.2.6 Impaired Glucose Tolerance (IGT)

IGT is a condition in which blood sugar level is higher than normal, but not high enough to be classified as diabetes. Left untreated, prediabetes often progresses to type 2 diabetes (International Diabetes Federation, 2015).

2.5.2.7 Gestational Diabetes

Pregnant women who are overweight, have been diagnosed with IGT, or have a family history of diabetes are all at increased risk of developing gestational diabetes mellitus (GDM). In addition, having been previously diagnosed with gestational diabetes or being of certain ethnic groups puts women at increased risk of developing GDM (International Diabetes Federation, 2015).

2.5.2.8 Polycystic Ovarian Syndrome

For women, having polycystic ovarian syndrome, a common condition characterized by irregular menstrual periods, excess hair growth and obesity, increases the risk of diabetes (Blake *et al.,* 2003).

Increased consumption and not doing the proper physical activities are shared factors in many current civilizations and are considered to be the main issues in subsidizing to obesity. Being overweight and obese are the main factors in contributing to the progression of the type 2 diabetes. It is likely anticipated that due to the modifications in life style e.g reduced caloric consumption and improved physical activity have a positive influence on optimal glycemic control and cardiovascular disease which are related to the development of type 2 diabetes (Ryan

et al., 2003). The achievement of attaining the maximum level of glucose in the case of type 2 diabetes is not possible due to various reasons which include failure to lose weight, weight gain overtime, the progressive nature of the disease or a mixture of these factors. Maximum number of patients will consequently require pharmacotherapy to retain the prerequisite glycemic control (Nathan *et al.,* 2009). T2DM patients are treated with nine major classes of approved drugs including insulin and its analogues, sulphonylureas, biguanides, TZD's, meglinitides, alpha glucosidase inhibitors, amylin analogues, incretin hormone mimetics, and DPP4 inhibitors (Thule, 2012). Metformin is considered to be an eldest, and undeniably one of the harmless medications used in the management of preventing the type 2 diabetes. It is usually the suggested initial pharmacotherapy, which has the ability to apply the glucose-lowering actions with a moderate risk of hypoglycemia. In addition it will decrease the probability of emerging macrovascular and microvascular difficulties or complexities. It is considered to be one of the most effective target for type 2 diabetes with no weight gain or modest weight loss, these makings are what make it striking as initially recommended pharmacotherapy for type 2 diabetic patients. It is not absorbed or metabolized in the body but is expelled unchanged in the urine by certain active tubular secretion system. Various carrier proteins are responsible for the oral absorption of metformin, its uptake in the liver and its elimination by the renal system (Viollet *et al.,* 2012).

STK11-adenosine monophosphate (AMP)-activated protein kinase (AMPK) motioning path is a leading controller of glucose and lipid metabolism with fundamental functions in liver, skeletal muscle, pancreas and brain. These roles of STK11 are supposed to be attained via uninterrupted phosphorylation of the adenosine monophosphate activated protein kinase family of proteins. It is proposed through certain indications that the main action of metformin is employed in the liver, predominantly from the prompting of AMPK. Stimulation of AMPK is done by (STK11/LKB1), which overpowers hepatic gluconeogenesis, thus, decreasing glucagon-mediated glucose production by the liver as shown in figure 2.1. In mice, LKB1 deficiency outcomes with hyperglycemia, enlarged gluconeogenic and lipogenic gene manifestation. The stimulation of AMPK in the liver is carried out by STK11 (Avery *et al.*, 2009).

AMPK has just established significant consideration because (i) AMPK stimulation is a main controller of both glucose and lipid metabolism associated with cellular energy status, and (ii) the antidiabetic drugs metformin, increase insulin sensitivity by triggering AMPK (Kola *et al.,*

2006). AMPK is extant in numerous tissues, comprising the liver and skeletal muscle, and subsists as a heterotrimer collected of a catalytic a-subunit and two regulatory b- and c-subunits. It has been pronounced that adenosine monophosphate activated protein kinase a2-knockout mice display improved compassion to diet-induced obesity and insulin resistance, while a1-knockout mice have no deceptive metabolic imperfections (Villena *et al.,* 2004). AMPK is recognized to be stimulated not only through an allosteric mechanism by AMP, but also through phosphorylation of a key threonine residue (Thr172) on the a-catalytic subunit, this is carried out by LKb11 in the liver. It has been recommended that primary metabolic modifications in peripheral tissues such as the liver, skeletal muscle and adipose could pledge the consequent growth of insulin resistance, obesity and type 2 diabetes. Subsequently, genes that encrypt mechanisms of the AMPK signaling path, including various AMPK subunits, LKB1 and TORC2, are captivating entrants which might clarify the hereditary foundation of type 2 diabetes (Kahn *et al.,* 2005).

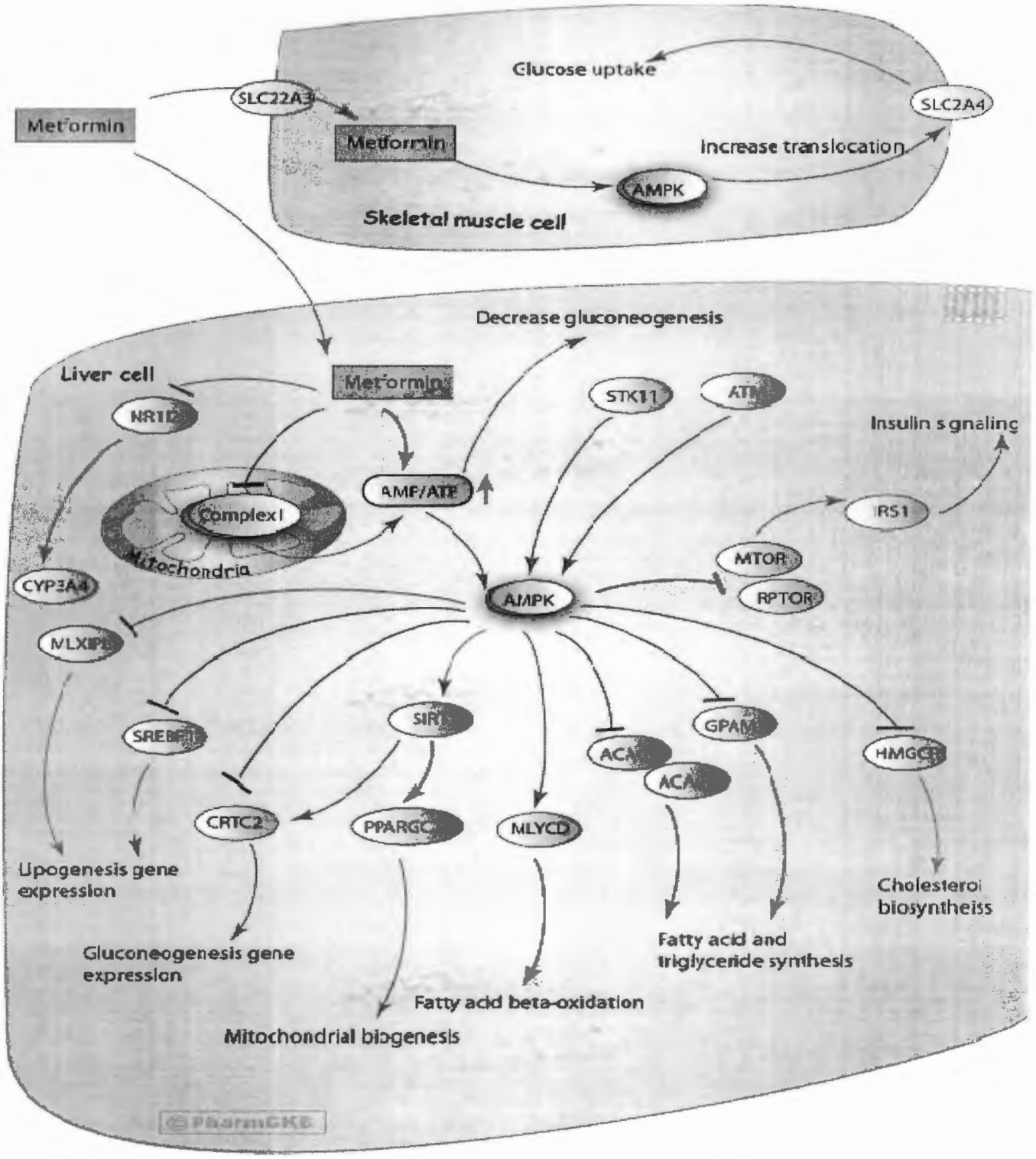

Figure2.1: Metformin Pathway-Pharmacodynamics (Gong *et al.*, 2012)

Despite the wide spread use of metformin, there is a considerable variation in its response with about 35% of patients fails to achieve optimal glycemic control on metformin monotherapy, so another commonly used class of anti-diabetic agent is sulphonylureas. Around 80-90% of patients respond well to this drug, but still some patients fail to achieve optimal glycemic control even with the use of high dose. Those patients who respond very well to sulphonylureas, loose the capability to maintain the required control of glucose in the body with the passage of time. Malfunction of sulphonylureas therapy in patients might be associated with a lot of factors but the most predominant factor is the failure of beta cell function (Nathan *et al.,* 2006).

Additional drawbacks of using sulfonylureas is the possibility of weight gain. Many patients practice an increase $\geq$ 2 kg after commencement of these treatments. Elder patients may be at greater threat for hypoglycemia after treating with sulphonylureas (Gangii *et al.,* 2007).

2.6 Genetic Factors of Type 2 Diabetes

Most cases of diabetes involve many genes, with each being a small contributor to an increased probability of becoming a type 2 diabetic (Williams's textbook of endocrinology, 12th edition). If one identical twin has diabetes, the chance of the other developing diabetes within his lifetime is greater than 90%, while the rate for non identical siblings is 25–50%. As of 2011, more than 36 genes had been found that contribute to the risk of type 2 diabetes. All of these genes together still only account for 10% of the total heritable component of the disease The TCF7L2 allele, for example, increases the risk of developing diabetes by 1.5 times and is the greatest risk of the common genetic variants. Most of the genes linked to diabetes are involved in beta cell functions (Gardner, 2011). There are a number of rare cases of diabetes that arise due to an abnormality in a single gene known as monogenic forms of diabetes or "other specific types of diabetes"). These include maturity onset diabetes of the young (MODY), Donohue syndrome, and Rabson Mendenhall syndrome. Maturity onset diabetes of the young constitutes 1–5% of all cases of diabetes in young people (Herder and Roden, 2011).

2.7 Genes Involved in Type-2 Diabetes

Development of Type-2 diabetes has been associated with genes, FTO, TCF7L2, PPARG, KCNJ11, WFS1, NOTCH2, IGF2BP2, HHEX, SLC30A8, JAZF1, among others (McCarthy, 2010). KCNJ11 (potassium inwardly rectifying channel, subfamily J, member 11), encodes the

islet ATP-sensitive potassium channel Kir6.2. TCF7L2 (transcription factor 7–like 2) adjusts pro-glucagon gene expression as well as the assembly of glucagon-like peptide-1 (Rother, 2007). Further, severe case of diabetes develops if there is a mutation in the Islet Amyloid Polypeptide gene that results in an earlier onset (Cho *et al.,* 2003).

2.8 STK11/LKB1

It is recognized and considered as a unique human gene encrypting the serine/threonine kinase STK11 within a region on chromosome 19p13.3 as shown in figure 2.2 which is identified as a locus for Peutz-Jeghers syndrome (PJS; 175200) and controls energy uptake and cell polarization (Xu *et al.,* 2013).

A structure similarity examine in Gene Bank with the genomic structure attained from the telomeric end of a cosmid from the Peutz-Jeghers syndrome region exposed individuality of 32 bp with a coding region of a human STK11, formerly named LKB1 but retitled STK11. The gene consists of 10 exons straddling 23 kb, the 10th exon occurs within the 3' untranslated region of the gene. The gene is recorded in telomere to centromere direction. STK11 which is also known as liver kinase B1 (LKB1) is a protein kinase that in humans is encrypted by the *STK11* gene. The STK11/LKB1 gene, which codes a member of the serine/threonine kinase family, regulates cell polarity and functions as a tumour suppressor (Nakada *et al.,* 2010). The STK11 gene delivers guidelines for creating an enzyme called serine/threonine kinase 11. This enzyme is a tumor suppressor, which means that it benefits retain cells from developing and distributing too fast or in an unrestrained way. This enzyme aids numerous kinds of cells properly orient themselves inside tissues and supports in defining the quantity of energy a cell utilizes. This kinase also indorses a type of involuntary cell death identified as apoptosis. In accumulating to its part as a tumor suppressor, STK11 role seems to be essential for normal growth before birth. Hereditary variations in the STK11 gene significantly upsurge the danger of emerging breast cancer, as well as other supplementary types of cancer.

STK11-adenosine monophosphate (AMP)-activated protein kinase (AMPK) motioning path is a leading controller of glucose and lipid metabolism with fundamental functions in liver, skeletal muscle, pancreas and brain. These roles of STK11 are supposed to be attained via uninterrupted phosphorylation of the adenosine monophosphate activated protein kinase family of proteins (Kola *et al.,* 2006). Previous studies specify that obliteration of STK11 in white adipose tissue

decreases the level of insulin receptor substrate 1 and leads to the diminished expression of several adipogenic genes. Dysfunctions in white adipose tissue are known to cause several metabolic diseases, such as obesity and type 2 diabetes (Zhang *et al.,* 2013). The quantity of shared genetic variants that are associated with type 2 diabetes is rising. Candidate gene association studies and genome-wide association studies have recognized more than two dozen loci strongly related with Type 2 diabetes and STK11 gene is one of those candidates (Jablonski *et al.,* 2010). LKB1 is a main upstream kinase of AMPK, an essential component in cell metabolism that is compulsory for upholding energy homeostasis. Probable mechanisms through which STK11 variants influence type 2 diabetes integrate its part in pancreatic islet expansion, beta cell persistence and insulin secretory granule. Diabetes prevention programme found that variants in STK11 are intricate in development of type 2 diabetes and some variants are also accompanying with a positive reaction to metformin therapy and action.In mice, who is STK11 deficient, results with hyperglycemia, increased gluconeogenic and lipogenic gene expression. Single nucleotide polymorphisms (SNPs), are variations in DNA sequences that occur when a single nucleotide in the sequence of genome sequence gets changed. It is anticipated by certain evidences that in patients with type 2 diabetes, variants in STK11 gene e.g rs741765,were examined in relation to the metformin treatment which is defined by attaining HbA1c <7% and a complete decline in HbAlc after 6-months of metformin pharmacotherapy (Shaw *et al.*, 2010). Another variant in STK11 gene rs8111699 SNP influences both insulin sensitivity and metformin efficacy in hyperinsulinemic girls with androgen excess (Hardie, 2006). An enriched understanding of genes and pathways that regulate reaction to metformin also has the probable to expose new drug targets for the management of diabetes.

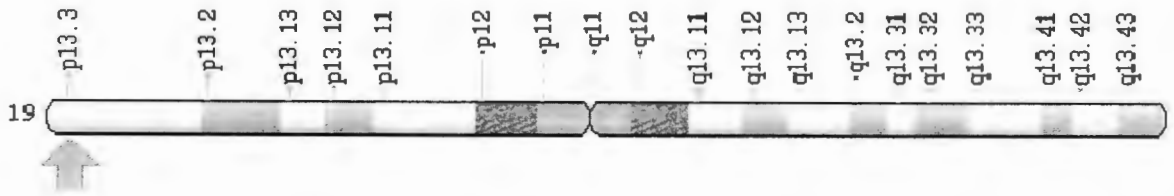

Fig 2.2: Cytogenetic Location of STK11 on 19p13.3, which is the short (p) arm of chromosome 19 at position 13.3(Xu *et al.*, 2013)

CHAPTER 3
METHODOLOGY

3.0 Methodology

3.1 Study Approval

This study has been approved by the Committee of International Islamic University Islamabad and was carried out at the department lab of Bioinformatics and Biotechnology (IIUI). In this study, single nucleotide polymorphism of STK11 gene in selected type 2 diabetic population of Pakistan was performed to evaluate the role of genetic variants in SKT11 gene with the disease progression and metformin efficacy. The patients were informed about the research, and they signed an informed consent.

3.2 Study Subjects

Study subjects consisted of 100 patients of type 2 diabetes and samples were collected with the help of collaborating hospitals of Pindi/Islamabad. Samples were collected as per following:

- One category consisted of 50 patients that were on metformin medication.
- Second category also consisted of 50 patients that were on metformin plus sulfonylureas medication.

3.3 Patient Inclusion Criteria

The diabetic patients who were taking the chosen drug were be enrolled from the collaborating hospitals. An interview regarding age, sex, family history of the disease, age at diagnosis, duration of diabetes, fasting glucose, BMI etc was conducted.

3.4 Patient Exclusion Criteria

All the patients were excluded from the study, who were not taking the chosen drug. Patients with type 1 diabetes, gestational diabetes, hypertension, risk of cardiovascular disease and pregnant women were also excluded.

3.5 Control group

This group consisted of 50 healthy individuals that were also taken from the collaborating hospitals and clinics. Individuals, included in this group were:

- Diabetes free
- They were not suffering from any other disease e.g blood pressure and cardiovascular disease.
- Their age and ethnicity was similar to the affected individuals that were included in the current study.

3.6 Family Information and Blood Samples Collection

All the samples for the current study were collected from the selected type 2 diabetic population of Pakistan. For this, questionnaires were given to the patients concerning health care, self-care and medical history information. Blood pressure and blood glucose levels were also measured. Blood samples were collected and histories were obtained by interviewing the patients. About 5-6 ml blood was drawn from the available affected and unaffected individuals in EDTA vaccutainers. The samples were kept at 4°C till the DNA extraction.

3.7 DNA Extraction

Phenol chloroform method (Sambrook et al., 1989) was used for extracting genomic DNA from intravenous blood samples. This method includes the following steps:

- 5ml of blood was occupied in an eppendorf tube and 15ml of cell lysis buffer (Solution A) was added and kept the tubes on ice for 30 minutes.

- The tubes were then centrifuged at 1200 rpm for 10 minutes at 4°C. Supernatant was discarded and the nuclear pallet was re-suspended in 15ml of cell lysis buffer (Solution A).

- Centrifuged was repeated at 1200 rpm at 4°C for 10 minutes, after discarding the supernatant the nuclear pallet was re-suspended in 5ml SDS drop wise while vortex and 10µl of protein kinase. The sample tubes were left overnight on a water bath at 55°C.

- On the following day 5ml of freshly prepared mixture of equal volume of Solution C (phenol) and Solution D was added, mixed and kept on ice for 10 minutes.

- The sample tubes were then centrifuged at 3200 rpm for 30 minutes at 4°C.

- The upper layer containing the aqueous phase was collected in a new tube. Equal quantity of Solution D was added and centrifuge was repeated at 3200 rpm for 30 minutes at 4°C.

- The upper layer having the aqueous phase was collected in a new tube and DNA was precipitated by adding 500µl of 10M ammonium acetate and 5ml of chilled Isopropanol and tubes were inverted several times to precipitate the DNA. Tubes were centrifuged at 3200 for 60 minutes at 4°C, supernatant was discarded.

- The obtained pallet was then washed with 5ml of chilled 70% ethanol and centrifuged again at 3200 for 40 minutes at 4°C and ethanol was discarded.

- The DNA was dried for half an hour by keeping the tube in a vaccum concentrator at 45°C for 10 minutes. After the evaporation of the residual ethanol, the DNA pallet was dissolved in 150µl of 10Mm Tris-EDTA and incubated overnight.

3.8 Composition and Preparation of different solutions:

3.8.1 Solution A:

Solution A contained a mixture of 10 Mm tris (pH-7.5), 0.32 mM sucrose and 5mM $mgCl_2$. This solution was autoclaved and finally 1% trition X-100 was added.

3.8.2 Solution B:

Solution B was prepared by mixing 10mM tris (pH-7,5). 400Mm NaCl and 2mM EDTA (pH-8)

3.8.3 Solution C:

Solution C contained only phenol and solution D was formed by mixing two chemicals i-e isoamylalcohol and chloroform in 1:24 volume.

3.8.4 20% SDS:

It was formed by dissolving 20 grams of SDS in 100 mL of distilled water.

3.9 Optical Density Measurement

The optical density of DNA samples was determined using Spectrophotometer (Thermo Scientific Nano drop 2000C). It can measure the concentration of the DNA. The DNA samples were transferred to 1.5ml Eppendorf tubes and stored at -20°C until further use. 50ng/200µl dilution of stock DNA was prepared for PCR amplification and stored at -20°C.

3.10 SNP Selection and Genotyping

NCBI's particular blast web index (www.ncbi.nlm.nih.gov) by method of utilizing SNP wings choice specified over there was used for the retrieval of all the single nucleotide polymorphisms described for STK11 gene. The SNP rs375622587 was choosen and sequence of SNP was also retrieved from the revealed rundown of all SNPs of STK11 gene. Single nucleotide polymorphism of the gene was selected on the basis of particular association of alleles or variants with the disease progression. Genotyping of SNP was done by using allele specific PCR.

3.11 Optimization of PCR conditions for genotyping of STK11 gene

Before moving towards PCR, 1% agarose gel was utilized for gel electrophoresis of genomic DNA in order to estimate the enough quantity of DNA and that was a minimum of 50 ng (fig 3.1).

3.12 Primer 3

By using Primer3 software (www.ncbi.nlm.nih.gov/tools/primer-blast) we designed primers to find the polymorphism in STK11 gene. In order to verify that the sequence of primer attaches on the intronic region of interest which has to be amplified, we used Blast (blast.ncbi.nlm.nih.gov/blast.cgi). After verification we got them designed from Integrated DNA Technologies, Inc., Coralville, Iowa, United States. In order to determine polymorphism in the STK11gene, PCR was performed by the following primers.

Primers for STK11 Gene

Forward Primer: 5'- CAACTA

CTGAGGAGGTTACGGCACA 3'

Reverse Primer: 3'- CATGGGGGTGTCCACAGATGAC-5'

3.13 Polymerase Chain Reaction

PCR amplification of specific alleles (PASA) is method for genotyping of single-base mutations. Genomic DNA is used as a template in two reactions, one with a primer that makes a perfect match to the wild-type allele, another with a primer matched to the mutated allele; both reactions contain a second primer that makes a perfect match to the opposite strand of either wild-type or mutant allele.

3.14 PCR Methodology

After optimization with reproducible results, all samples were amplified at optimized conditions of PCR. The master mix for all PCR reactions with specific primer pair was prepared in 1.5mL Eppendorf tube. Total reaction volume for a single PCR reaction was 25µL. All the PCR reagents with their concentrations are summarized in the table 3.1.

3.15 Thermal Profile of PCR Reaction for STK11

The PCR was carried out in 96 well thermal cycler (BIO RAD, Thermo Electron Corporation, Mill ford, USA) the thermal stages, steps and cycles of which are shown in table 3.2.

3.16 Analysis of PCR Products by Using Agarose Gel Electrophoresis

Analysis of PCR product was performed by using agarose through horizontal gel electrophoresis. Following reagents were used in the process of agarose gel electrophoresis:

a) 10x TBE buffer (Tris-Borate- Ethylene diamine tetra acetic acid)

b) 6X Gel loading dye (Fermentas, Lithuania)

c) 10mg/ml Ethidium Bromide

d) 2.5% Agarose (Promega)

e) 100bp DNA Ladder

3.16.1 Preparation of 10X TBE Buffer Stock

a) Tris-base (promega) = 107.8gm/liter

b) Boric acid (promega) = 55.02gm/liter

c) 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.

3.16.2 6X Gel loading dye (Fermentas, Lithuania)

Mix 100 mg of bromophenol blue with 10 ml of ddH_2O and mix it thoroughly. Then store at room temperature.

3.16.3 10mg/ml Ethidium Bromide

Dissolve 0.2g ethidium bromide to 20 ml water. Mix well and store at room 4°C in the dark.

3.16.4 Preparation of 2.5% Agarose Gel

For 20cm×20cm agarose gel pouring plate, 300 ml of 2.5% agarose was made as:

a) Ion free fine agarose (Promega) = 6.5gm

b) 1X TBE = 300ml

c) Glycerine = 300µl

d) Ethidium bromide (10mg/ml) = 5µl

6.5gm agarose, 300ml 1X TBE and 300µl glycerin in Pyrex bottle were mixed and heated in oven with loose bottle cap until agarose boiled. Then 5µl Ethidium bromide in the agarose gel solution was added and shaken to mix. The 20cm×20cm gel plate was set with combs at suitable distance and the gel solution was poured. The gel was polymerized in 30-45 minutes.

3.17 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 ladder CAT NO (15628-019), Invitrogen Life Technologies, USA was loaded as size reference for amplified target DNA. The gel was run for 35 minutes using the Maxicell EC360-M horizontal gel electrophoretic system (EC Apparatus Corporation, St. Petersburg, Florida, USA) at 120 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 (Gene Genius, Syngene, UK).

3.18 Statistical Analysis

The following statistical tests were performed for the data by using statistical program SPSS (Version 20.0).

3.19 Hardy-Weinberg Equilibrium

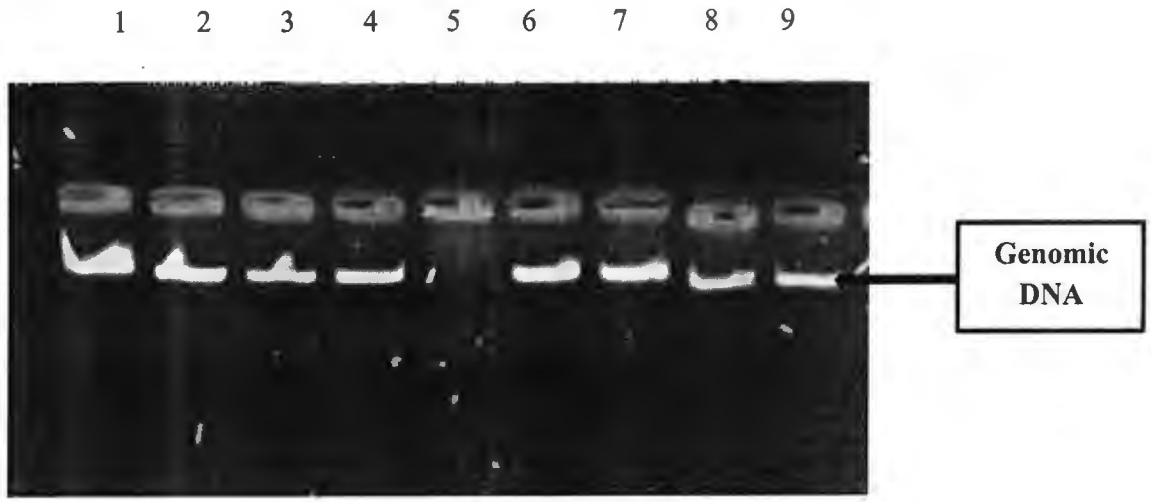

Figure 3.1: Genomic DNA of type 2 Diabetes Mellitus on 1 % Agarose Gel

Table 3.1: PCR reagents with their Concentrations Used for PCR

Sr. No.	Reagents with initial concentration.	Volume used (µl)	Total volume for 100 reactions
1	d.H2O	10.8 µl	1080 µl
2	10x PCR buffer (Fermentas, Lithuania)	2.5 µl	250 µl
3	MgCl2 (Fermentas, Lithuania)	1.5 µl	150 µl
4	2.0 mM dNTPs	1.0 µl	100 µl
5	5U/µl Taq DNA polymerase (Fermenta, Lithuania)	0.2 µl	20 µl
6	10 µM Forward primer	1 µl	100 µl
7	10 µM Reverse primer	1 µl	100 µl
8	25 ng Sample Genomic DNA	5 µl	5 µl
9	Triton/Glycerol	2.0 µl	200 µl

Table 3.2: Thermal Profile of PCR Reaction for STK11

No. of Stage	No. of Step	Temperature	Time	No. of Cycles
Stage 1	Step 1	95 °C	4Minutes	01 Cycle
Stage 2	Step 1	95 °C	45Seconds	35 Cycles
	Step 2	60 °C	45 Seconds	
	Step 3	72 °C	45 Seconds	
Stage 3	Step 1	72 °C	10 Minutes	01 Cycle

CHAPTER 4
RESULTS

4.0 Results

In this study a total number of 100 diabetic patients along with 50 healthy controls were participated. Diabetes type-2 patients were assessed for medication, age, gender, marital status and age group. The clinical and anthropometric parameters for the diabetes type-2 patients who participated in this study are summarized in the table 4.1.

4.1 Genotype Analysis of STK11 A/G Polymorphism

After the amplification, the PCR products had been subjected to gel electrophoresis and gel was documented in UV light and photographed, the DNA fragments were located on the gel according to their size and the band size was 449 bp. PCR gel image of STK11 gene A/G polymorphism of control samples are shown in figure 4.1. PCR gel image of STK11 gene A/G polymorphism of Type 2 Diabetic Patients that were on metformin medication are shown in figure 4.2. PCR gel image of STK11 gene A/G polymorphism of Type 2 Diabetic Patients that were on sulphonylureas medication are shown in figure 4.3.

The genotyping for STK11 gene was thus carried out. There are three different genotypes in the premeditated collections of diabetes type-2 patients and controls clear from the agarose gel electrophoresis and gel documentation for PCR products given in table 4.2, 4.3 and 4.4. In order to find the genotype percentages, allele frequency and other statistical evaluation of the STK11 A/G polymorphism among the collections, the outcomes were analyzed in SPSS Version 20.0.

The homozygous AA genotype of the major allele was higher 20% in patients that were on sulphonylureas medication than in controls 14%. This percentage was high and statistically this difference was insignificant $p = 0.07$ (p value > 0.05). The heterozygous AG genotype also showed statistically insignificant difference $p = 0.06$ (p value > 0.05) between two groups i.e 15% in patients that were on metformin medication and 9% in patients that were on sulphonylureas medication than in controls 23%. The homozygous GG genotype of the minor allele was critically higher in patients that were on metformin medication i.e 14% as compared to the control i.e 10% and showed statistically significant difference with value of $p = 0.03$ (p value < 0.05).

4.2 Allele Frequencies

The percentage of the major allele A was higher 0.5% in controls and 0.3% in patients that were on metformin medication and 0.6% in patients that were on sulphonylureas medication and this difference is statistically insignificant p=0.34 (P value > 0.05). The percentage of the minor allele G was higher 0.7% in patients that were on metformin medication as compared to the controls 0.41% and this difference is also statistically significant p= 0.006 (P value <0.05).The allele frequencies of A and G are shown in table 4.5.

Table4.1: Clinical and Anthropometric Parameters of T2DM

Clinical Parameters		**Respective values and incidence percentage**
Medication	Age Group	
Metformin	30-50	13.15%
	50-70	29.36%
	More than 70	30.26%
Sulphonylureas	30-50	20.19%
	50-70	23.68%
	More than 70	9.57%
Control Group	30-50	3.94%
	50-70	4.87%
	More than 70	6.57%
Gender	Male	42.1%
	female	57.89%
Age Group	30-50	27.4%
	50-70	35.9%
	More than 70	45.5%
Marital status	Married	88%
	Unmarried	12%

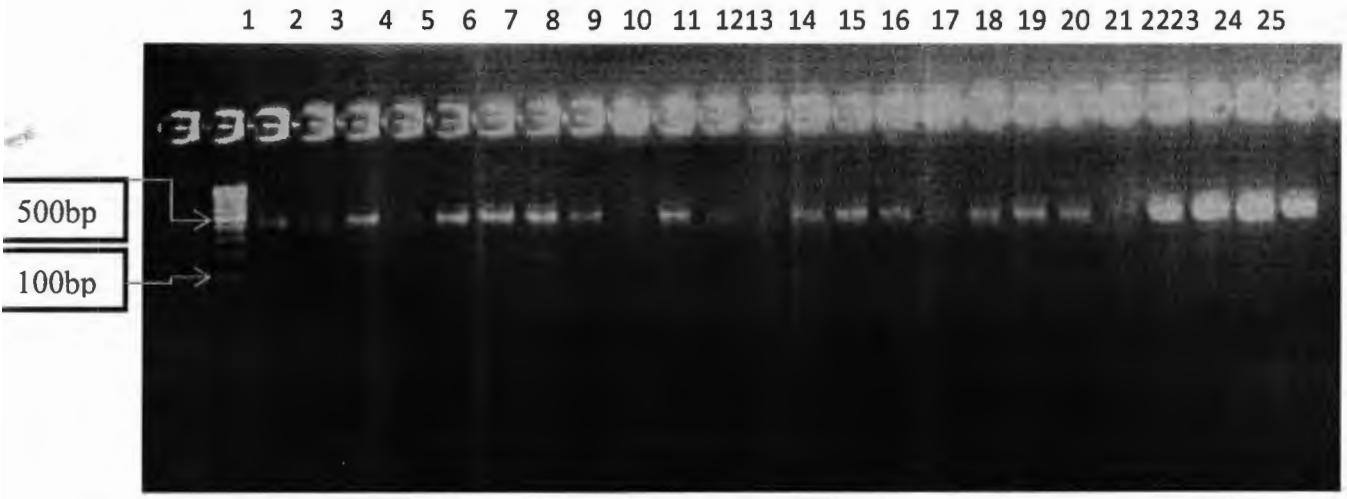

Fig 4.1: PCR Gel Image of STK11 Gene A/G Polymorphism of Control Samples

L1 (C1): DNA Ladder (100bp)	L2 (C2,C3): Heterozygous for both alleles
L3(C4,C5):Heterozygous for both alleles	L4(C6,C7)): Heterozygous for both alleles
L5(C8,C9): Heterozygous for both alleles	L6(C10,C11)): Homozygous for allele G
L7(C12,C13): Homozygous for allele A	L8(C14,C15):Heterozygous for both alleles
L9(C16,C17): Heterozygous for both alleles	L10(C18,C19):Heterozygous for both alleles
L11(C20,C21):Homozygous for A allele	L12(C22,C23):Heterozygous for both alleles
L13(C24,C25): Heterozygous for both alleles	

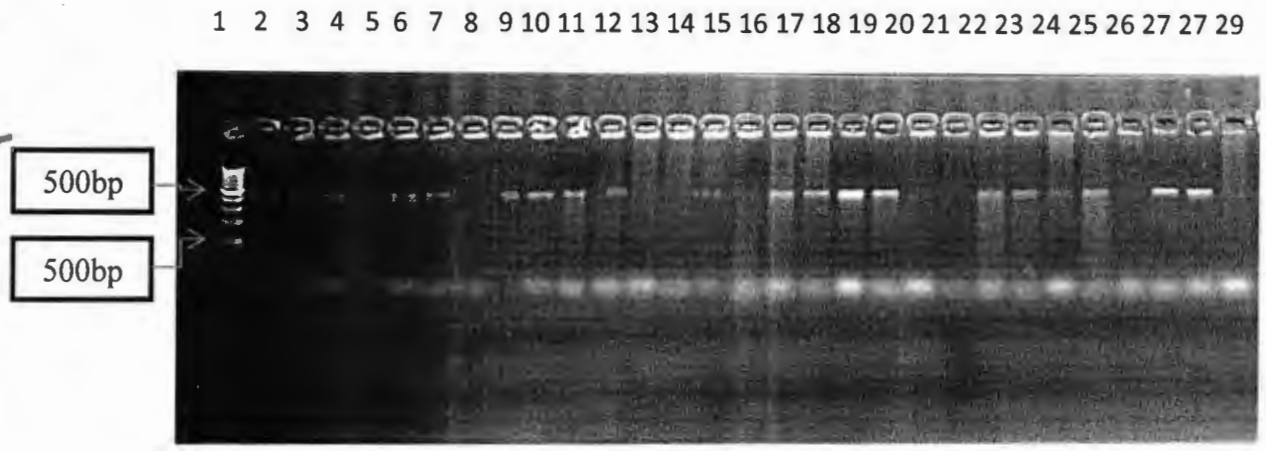

Fig 4.2: PCR Gel Image of STK11 Gene A/G Polymorphism of Type 2 Diabetic Patients that were on Metformin Medication

L1(M1): DNA Ladder (100bp)	L2(M2,M3): Heterozygous for both alleles
L3(M4,M5): Homozygous for allele A	L4(M6,M7): Heterozygous for both alleles
L5(M8,M9): Homozygous for allele G	L6(M10,M11): Heterozygous for both alleles
L7(M12,M13): Homozygous for allele A	L8(M14,M15): Homozygous for allele G
L9(M16,M17): Homozygous for allele G	L10(M18,M19): Heterozygous for both alleles
L11(M20,M21): Homozygous for allele A	L12(M22,M23): Heterozygous for both alleles
L13(M24,M25): Heterozygous for both alleles	L14(M26,M27): Homozygous for allele A
L15(M28,M29): Heterozygous for both alleles	

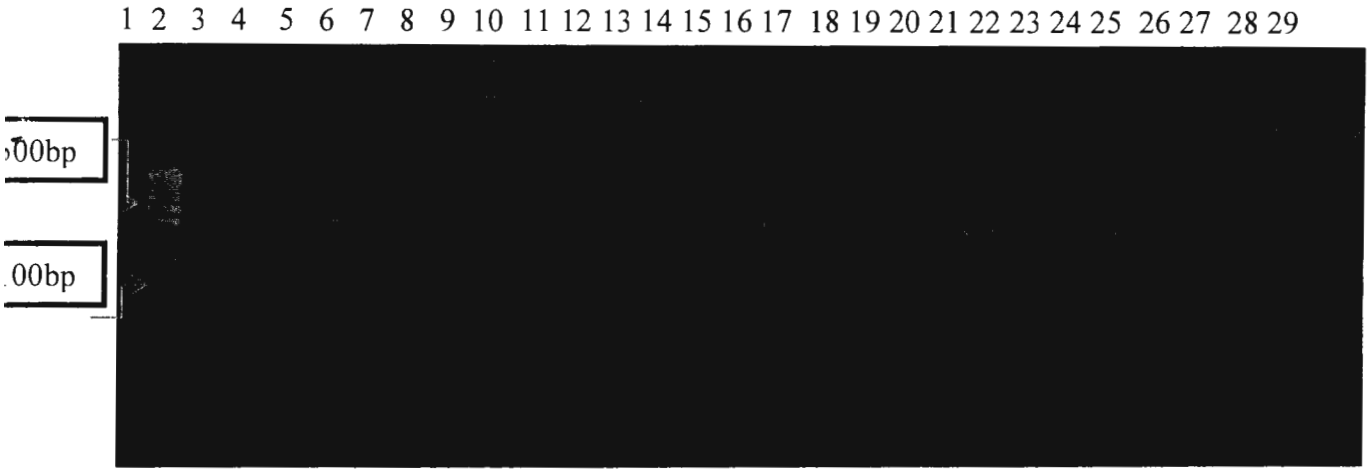

Fig 4.3: PCR Gel Image of STK11 Gene A/G Polymorphism of Type 2 Diabetic Patients that were on Sulphonylureas Medication

L1:DNA Ladder(100bp)	L2(S2,S3): Homozygous for allele A
L3(S4,S5): Homozygous for allele G	L4(S6,S7): Homozygous for allele A
L5(S8,S9): Heterozygous for both alleles	L6(S10,S11): Homozygous for allele G
L7(S12,S13): Homozygous for allele G	L8(S14,S15): Homozygous for allele G
L9(S16,S17): Homozygous for allele G	L10(S18,S19): Heterozygous for both alleles
L11(S20,S21): Homozygous for allele G	L12(S22,S23): Heterozygous for both alleles
L13(S24,S25):Homozygous for allele A	L14(S26,S27):Homozygous for allele G
L15(S28,S29): Homozygous for allele A	

Table 4.2: Number and Percentage of Patients (Metformin) and Controls with their Genotypes for STK11 A/G Polymorphism

Group Studied	STK11 Genotypes Percentage		
	AA	**AG**	**GG**
Controls	**14%**	**23%**	**10%**
Expected H-W frequency	0.359	0.479	0.196
Metformin	**6%**	**15%**	**14%**
Expected H-W frequency	0.09	0.42	0.49
P Value of Patients (Metformin) and Control	0.105	0.09	0.04

Table 4.3: Number and Percentage of Type 2 Diabetic Patients (Sulphonylureas) and Control with their Genotypes for STK11 A/G Polymorphism

Group Studied	STK11 Genotypes Percentage		
	AA	**AG**	**GG**
Controls	**14%**	**23%**	**10%**
Expected H-W frequency	0.359	0.479	0.196
Sulphonylureas	**20%**	**9%**	**12%**
Expected H-W frequency	0.45	0.510	0.130
P value of Patients (Sulphonylureas) and Control	0.07	0.06	0.02

Table4.4: Number and Percentage of Type 2 Diabetic Patients with their Genotypes for STK11 A/G Polymorphism

Group Studied	STK11 Genotypes Percentage		
	AA	**AG**	**GG**
Sulphonlureas	**20%**	**9%**	**12%**
Expected H-W frequency	0.45	0.510	0.130
Metformin	**6%**	**15%**	**14%**
Expected H-W frequency	0.09	0.42	0.49
P Value of Patients and Control	0.08	0.06	0.03

Table 4.5: The allele frequencies of A and G allele of STK11inControls and Patients

Group Studied	STK11 A/G Polymorphism	
	A	**G**
Controls	**0.5**	**0.41**
Metformin	**0.3**	**0.7**
P-value of Patient(Metformin) and Control	0.34	0.006
Control	**0.5**	**0.41**
Sulphonylureas	**0.6**	**0.4**
P-value of Patient (Sulphonylureas) and Control	0.27	0.002
Metformin	**0.3**	**0.7**
Sulphonylureas	**0.6**	**0.4**
P-value of Patient Sulphonylureas	0.3	0.006

CHAPTER 5
DISCUSSION

5.0 Discussion

Diabetes mellitus, or simply diabetes, is a group of diseases characterized by high blood glucose levels that result from defects in the body's ability to produce and/or use insulin. It is a condition primarily defined by the level of hyperglycaemia giving rise to risk of microvascular damage. It is associated with reduced life expectancy, significant morbidity due to specific diabetes related microvascular complications, increased risk of macrovascular complications (ischaemic heart disease, stroke and peripheral vascular disease), and diminished quality of life (American Diabetes Association, 2014). Several pathogenetic processes are involved in the development of diabetes. These include processes, which destroy the beta cells of the pancreas with consequent insulin deficiency, and others that result in resistance to insulin action. The abnormalities of carbohydrate, fat and protein metabolism are due to deficient action of insulin on target tissues resulting from insensitivity or lack of insulin (Galtier, 2010).

Type 2 diabetes is known as non-insulin dependent or insulin independent diabetes mellitus. It is the most collective and mutual form of diabetes. Lots of individuals around the world have been identified with this type of diabetes, and several individuals remain undiagnosed. It is the most common form of diabetes and 90-95% of individuals are suffering from T2DM. Individuals are at a larger threat of emerging cardiovascular diseases such as heart attack and stroke if type 2 diabetes is left undiagnosed or measured poorly. These individuals are also at a greater risk for vision loss, foot and leg subtraction due to injury to the nerves and blood vessels, and renal failure demanding dialysis or organ relocation. T2D is found of excessive significance as compared to the type 1 diabetes, because of the encumbrance of sickness and transience (Dabelea *et al.,* 2014).

Most cases of diabetes involve many genes, with each being a small contributor to an increased probability of becoming a type 2 diabetic. Most of the genes linked to diabetes are involved in beta cell functions. STK11-adenosine monophosphate (AMP)-activated protein kinase (AMPK) motioning path is a leading controller of glucose and lipid metabolism with fundamental functions in liver, skeletal muscle, pancreas and brain. These roles of STK11 are supposed to be attained via uninterrupted phosphorylation of the adenosine monophosphate activated protein kinase family of proteins (Kola *et al.,* 2006). Previous studies specify that obliteration

of *STK11* in white adipose tissue decreases the level of insulin receptor substrate 1 and leads to the diminished expression of several adipogenic genes. Dysfunctions in white adipose tissue are known to cause several metabolic diseases, such as obesity and type 2 diabetes (Zhang *et al.*, 2013). Probable mechanisms through which STK11 variants influence type 2 diabetes integrate its part in pancreatic islet expansion, beta cell persistence and insulin secretory granule. Diabetes prevention programme found that variants in STK11 are intricate in development of type 2 diabetes and some variants are also accompanying with a positive reaction to metformin therapy and action. In mice, who is STK11 deficient, results with hyperglycemia, increased gluconeogenic and lipogenic gene expression. It is anticipated by certain evidences that in patients with type 2 diabetes, variants in STK11 gene e.g rs741765,were examined in relation to the metformin treatment which is defined by attaining HbA1c <7% and a complete decline in HbAlc after 6-months of metformin pharmacotherapy. Another variant in STK11 gene rs8111699 SNP influences both insulin sensitivity and metformin efficacy in hyperinsulinemic girls with androgen excess (Hardie, 2006).

The novelty of this study is that there is no work done on humans before on this SNP of STK11 gene. In this study we examined the STK11gene polymorphism in selected type 2 diabetic population of Pakistan. The results showed that homozygous AA genotype of the major allele had a significantly higher percentage in patients than in controls and serves as a protective factor, whereas the heterozygous AG genotype had a significantly higher percentage in controls as compared to the patients and also serves as a protective factor. The homozygous GG genotype of the minor allele had a significantly higher percentage in patients as compared to the controls and is a risk factor. To summarize, our investigation on STK11 gene polymorphisms, homozygous GG genotype showed statistically significant association between diabetic patients and controls.

5.1 Conclusion

It is concluded from our current study that STK11 GG polymorphism was significantly associated with type 2 diabetes mellitus group comprised of Pakistani population.Even though many new drugs have been developed for type 2 diabetes mellitus, metformin is still extensively recognized as the first-line therapy because of its low occurrence of microvascular and macrovascularactions and its advantageous effects on the body.

5.2 Future Work

Investigation of gene–gene and gene–environment interactions may clarify significant polymorphisms, which are involved in triggering type 2 diabetes. Additionally, with progressions in sequencing platforms, whole exome or genome sequencing will simplify the exploration of rare variants, copy number variants, insertions, deletions, and other genetic variants that may play an important role in pharmacodynamics of metformin and its response.

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CHAPTER 6

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