Prevalence of Commonly Reported SNPs in Pakistani Obese Kindreds

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DECLARATION

I hereby declare that this thesis for the submission of Degree of Master of Subject is based upon my own work and to the best of my knowledge contains no material previously published. I have not previously presented any part of this work elsewhere for any other degree.

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PREVALENCE OF COMMONLY REPORTED SNPs IN PAKISTANI OBESE KINDREDS

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DEDICATION

My this humble effort is dedicated to My loving, kind, and friendly Parents Mr Hameed Gul, Mrs Guhari, and Uncle Mr Shakeel ur Rehman and Aunt Ms Andaleeb Hussain, whose love, affection, and devotion guided me throughout my this study. Their prayers and support made me able to complete this journey with ease.

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ABBREVIATION

(WHO) World Health Organization

(NFHS) National Family Health Survey

(T2D2) Type 2 Diabetes Mellitus

(CVD) Cardio Vascular Disease

(FTO) Fat Mass and Obesity related Gene

(GWAS) Genome Wide Association Study

(BMI) Body Mass Index

(NIH) National Institute of Health

(WHR) Waist to Hip Ratio

(NGS) Next Generation Sequencing

(MC4R) MelanoCortin 4 Receptor

(LEPR) Leptin Receptor

(LEP) Leptin Gene

(POMC) Pro-Opiomelanocortin

(PC1) Prohormone Convertase 1

(AGRP) Augoti Related Protien

(CART) Cocaine Amphetamine Related Transcript

(SNP) Single Nucleotide Polymorphism

(ARMS PCR) Amplification Refractory Mutation System Polymerase Chain Reaction

(EDTA) Ethylene Diamine Tetra Acetic Acid

(TAE) Tris Acetate EDTA

(db SNP) Single Nucleotide Polymorphism Database

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ABSTRACT

Obesity the accumulation of access fats or access adipose tissues is a major health problem affecting billions of people both in developed and developing countries. Recent report of the WHO reveal, that more than 1.9 billion adults are overweight, and more than 600 million are obese. Spending pattern of financial state and food especially processed food in Pakistan has an important role in the increase prevalence of obesity, type 2 diabetes and cardiovascular disease. Obesity is reported to be caused either due to environmental factors such as food habit, physical inactivity, sedentary life style, and socioeconomic status or due to genetic factors including FTO, MC4R, LEPR, and POMC. Their variants have also been found associated with obesity. Variants of the FTO rs9939609, rs1558902, and rs8050136, Variants of the MC4R rs17782313, rs6567160, and rs12970134, and variant of the POMC rs713586 were analyzed in the present study using ARMS PCR approach. Their prevalence has been investigated to understand the mechanism and causes of obesity in obese individuals from Pakistani population. It was found that none of the already reported variant in our sampled kindred is associated with obesity. It is calculated that environmental and lifestyle factors play more important role in obesity in Pakistani population than genetics.

CHAPTER 1
INTRODUCTION

1. Introduction

Obesity the accumulation of access fats or access adipose tissues is a major health problem affecting billions of people both in developed and developing countries (Yazdi, Clee, & Meyre, 2015). According to the survey of World Health Organization (WHO, 2015), worldwide obesity has dramatically increased since 1980. In 2014, more than 1.9 billion adults, 18 years and more youngsters, were overweight. Of these more than 600 million were obese. In 2013, 42 million young individuals less than 5 years old were overweight or obese. Additionally, a large portion of the total population lives in countries where overweight and obesity affects a larger number of individuals than underweight ("WHO | Obesity and overweight," 2016). WHO alerts that more significant future increase in obesity and diabetes will affect developing countries, and the expacted values of new instances of obesity and diabetes can reach to millions in the comming decades. The obesity pandemic begun in the US and crossed to Europe and the world's other rich countries. Now it infiltrated even the world's poorest nations particularly in their urban zones (Prentice, 2005).

It were estimated in 2010 that overweight and obesity causing 3.4 million deaths, 3.9% of years of life lost, worldwide ("Global, regional and national prevalence of overweight and obesity in children and adults 1980-2013: A systematic analysis Europe PMC Funders Group," 2014). world health organization (WHO) declared it as chronic disease because of its association with other major health abnormalities which reduces life expectancy like type2 diabetes, coronary heart disease, hypertension, stroke, gall bladder, and other disorders (Hubbard, 2000).

Obesity is rising day by day because of significant changes in environment and society and these are considered as its causing elements. A term that can cover every one of these factors is 'obesogenic'.The changes that are coming in the circle of obesogenic are Urbanization, a considerable measure of utilization of energy dense food substances, and physical inactivity (Yazdi, Clee, & Meyre, 2015).

The issue of obesity and overweight has gotten greater attention by the health authorities. They recorded those dramatic changes that are causing obesity in Asian people, and the resultant changes in the foods and nutrition issues. Information retrieved from different studies conducted on Asian population indicates considerably higher prevalence. The prevalence is 5% to 9% among a few urban communities in Asia. In a few other developing countries in the area, the predominance is most likely low, with prevalence of less than 1%. Considerable variation in the prevalence of obesity has been found in different countries of the region. Different studies have paid attention to the seriousness of the issue of obesity. Various reports have shown prevalence of overweight of more than 20% and obesity of more than 5% (Tee, 2002).

The Asia-Pacific area which is mostly consists of Southeast Asia, and Oceania. Countries of this locality have wide differences in socio-cultural background and are at various levels of economic and technological advancement. Expanding financial improvement in a number of the lower to middle income countries of this region has been playing an important role in the increase prevalence of obesity, type 2 diabetes and cardiovascular disease (Ranasinghe, Mathangasinghe, Jayawardena, Hills, & Misra, 2017).

Information from the India National Family Health Survey (NFHS) demonstrated that overweight and obesity is more prevalent among the urban and high financial status population in India. A higher prevalence of obesity found in the urban regions in developing countries is related with the change from rural environment to urban way of life like decrease level of physical activity and usage of energy rich foods in daily routine (Ramachandran & Snehalatha, 2010).

The pandemics of obesity in South Asians differs by age, sex, place of living arrangement, financial status, and criteria utilized for the estimation of overweight and obesity. Expanding predominance of overweight and obesity has been found in all the studies done in India and other South Asian countries. South Asians are becoming increasingly wealthier and hence progressively using food materials high in saturated fats, cholesterol, and refined sugars and low in unsaturated oil and fibers.

Urbanization is also expanding quickly and is presently about 38%, and is expected to be doubled in 2020. Urbanization exposes individuals to various difficulties, imbalanced use of food products, physical inactivity, long working hours and other urban anxiety making them obese. The pandemic of obesity, T2DM and CVD in South Asia is more in the upper financial strata due to various dietary choices and "western foods (Misra & Shrivastava, 2013).

In Sri Lanka, the predominance of obesity in urban regions is three times more than other regions of the country and such a situation have been found in India and Pakistan. However, Shah et al reported that in urban areas of Pakistan, the predominance of overweight and obesity expanded from 13.9% in 1995 to 19.4% in 2007. These outcomes recommend that the rate of obesity may increase in the population that are more urbanized (Jayawardena, Byrne, Soares, Katulanda, & Hills, 2013). The prevalence of overweight and obesity in general Pakistani population was 25.0%. The pandemics were most elevated, among ladies of 35–54 years. The factors contributing to obesity and overweight included age, being female, urban living arrangement, and having a high or very low financial status and a high consumption of meat (Jafar, Chaturvedi, & Pappas, 2006).

Research has proved that obesity is responsible for cardiovascular disease and unexpected losses so this general medical issue needs critical consideration. Different components like genetic predisposition, ethnicity, hormonal, metabolic, dietary, eating practices and sedentary life style all these are contributing to the pathogenesis of obesity. However the main consideration is caloric imbalances because of overabundance intake of energy and less utilization of that energy. Pakistan is a nation where the idea of eating routine among the overall population is completely changed. Pakistani food materials are "energy rich" with higher extent of saturated fats, trans-unsaturated fats and free sugar adding to high caloric intake. Utilization of "desi ghee", high consumption of meat and over eating is normal dietary practices in Pakistan. Because of increasing costs of staple foods like oats, fruits, vegetables and natural products, ordinary citizens utilizing the less expensive methods for energy like fats and sugar, due to these practices they are becoming obese (Sherin, 2014).

Obesity has been thought to be identified with imbalance between energy intake and consumption. Energy is consumed in the eating routine through protein, starch and fats. Within the sight of overabundance calories, the body will in this way change over and store these energy supplements as triglycerides in fat tissue. After some time, if abundance calories are consumed without increase in energy use, overabundance of fats will occur which leads to obesity. Exess consumption of energy supplements are responsible for increasing the size and number of adipocytes at different phases of the life (Wilborn , 2005).

The accessible proof proposes that a complex physiological control framework is involved in energy balancing mechanism of the body. This framework incorporates afferent signals from the periphery about the condition of energy stores and efferent signals that influence energy intake and consumption. In the event that energy balance was not controlled by such a framework and were subject just to behavioral components controlling food consumption and energy use the vast majority would cause wide variety changes in body weight over time. Thus the relative stability of body weight from daily life is reliable with the view that energy balance is due to physiological control (Hill, Wyatt, & Peters, 2012).

Some metabolic processes are also involved in energy control, metabolism and fat storage are playing critical role in obesity. Metabolic basis of obesity has a long history, early discoveries shows that the hormone insulin control digestion and aid in the accumulation of fat in fat tissue. The hormone leptin playing role in energy stores signals to the hypothalamus, and influence hunger while expanding energy use. Different studies have identified other signaling hormones required in the direction of energy digestion (Wells & Siervo, 2011).

The prevalence and occurrence of obesity inside and between ethnically different populations living in comparable and differentiating environments recommend that some ethnic groups are more vulnerable than others to obesity. More than 150 basic hereditary variations (Genetic variants) have been vigorously associated with obesity however the individual effect of every variation is little. There is currently persuading

epidemiological proof of interaction between common variations in the FTO (Fat mass and weight related protein) gene and lifestyle concerning obesity (Romieu et al., 2017).

GWAS is the root of identification for novel loci responsible for a disease or phenotype of interest, causal variants and their mechanism of action. Recent advances in human genetics, epigenomics, and genome editing equipping our range of knowledge that is necessary for understanding the complex phenomena of involvement of a single specific variant in disease pathophysiology. These approaches were used by the scientists to identify the causative effect of intronic variants in the Fat Mass and Obesity gene and their association with obesity (Wu & Arora, 2016).

GWAS have been identified thousands of single nucleotide polymorphism (SNPs) and more than 90 loci associated with Body Mass Index. Most of these SNPs are located in regions of the genome that are non-coding or intronic hence they do not directly influence protein sequences but some GWAS regions have been involved in histone modification so they may regulated genes (Laber & Cox, 2015).A range of genomic techniques were also used to examine three SNPs of the FTO gene rs1558902, rs9930506, and rs1421085 in intron 1 and 2 that are associated with obesity. The significant SNP rs1421085 alteration C to T causes the repression of the genes responsible for mitochondrial thermogenesis which increases energy expenditure as a result of repression decrease in mitochondrial thermogenesis occur lead to the storage of lipids. These three SNPs of the FTO involved in pathway that is responsible for adipose thermogenesis shows association of the FTO with obesity (Claussnitzer et al., 2015).

Additionally reversible epigenetic changes specifically in alteration in DNA methylation could serve as biomarkers of energy balance and involved in mediation of gene–environment interaction in obesity. A few 'epigenome wide studies' have now been led and have distinguished some gene loci where methylation levels fundamentally vary in obese and normal individuals. Such discoveries could give novel bits of knowledge into how energy balance and its determinants impact obesity

pathogenesis, connection with eating habits environmental factors and subsequent metabolic deregulation (Romieu et al., 2017).

1.1 Aim of the Study

The study is designed to determine the genetic basis of obesity in Pakistan. In this regard prevalence and functional analysis of already reported SNPs associated with obesity is determined.

1.2 Objectives

1. To elucidate the common genetic variants of obesity among Pakistani kindred.
2. To determine the prevalence of already reported SNPs in sampled kindred.

CHAPTER 2
LITERATURE REVIEW

2. Literature Review

2.1 Prevalence and Epidemiology of obesity

The term "obesity" refers to an overabundance of fat. However, the strategies used to measure body fat ratios are not accessible in routine practice. Hence, obesity generally is evaluated by the relation among weight and height (anthropometrics), which gives an estimate of body fat ratio that is adequately precise for clinical purposes (“Definition; epidemiology; and etiology of obesity in children and adolescents,” 2008.).

Obesity has gotten impressive consideration as a negative health impact and it can be measured by Body Mass Index. Body mass Index (BMI), characterized as the weight in kilograms divided by the height in meters squared (kg/m2). It is the most generally utilized measure of obesity because of its ease and simplicity. The World Health Organization (WHO) and the National Institutes of Health (NIH) have characterized overweight as having a BMI in the vicinity of 25.0 and 29.9 kg/m2; and obesity having a BMI more than 30.0 kg/m2.The most increase rate of obesity has been accounted in the Pacific Islands and the least rates have been found in Asia. The rates in Europe and North American are large high, while the rates in Africa and Middle Eastern nations are variable(Nguyen & El-Serag, 2010).

According the report of WHO, the over fed population has expanded to 1.2 billion. WHO informations demonstrate, there are more than 1 billion people overweight and 300 million obese globally. The issue of obesity is expanding in the developing countries with more than 115 million individuals that are experiencing obesity related issues. Obesity rates have expanded 3-times or more since 1980 in Middle East, the Pacific Islands, Australasia, and China (Ellulu, Abed, Rahmat, Ranneh, & Ali, 2014).

Obesity rates are rising around the world. The main outstanding locality of the world where obesity is not regular is sub-Saharan Africa. Overweight and obesity are the fifth driving danger for worldwide deaths. At laest 2.8 million adults die every year

thus of being overweight or obese. Additionally, 44% of the diabetes load, 23% of the ischaemic coronary illness trouble and in the vicinity of 7% and 41% of certain cancer burdens are inferable from overweight and obesity (Ellulu et al., 2014).

The load of obesity and obesity related diseases is especially higher in the center locality of Eastern Europe, Latin America and Asia, where obesity position is just beneath underweight. It is the fifth most regular reason for disease load. National Health Survey and studies in Pakistan demonstrate that while obesity and diabetes are more pervasive in urban areas, yet the commonness is likewise high in rural territories. In Metroville health study, 34% men and 49% ladies were observed to be over-weight/fat, while expanded WHR (waist to Hip Ratio) was seen in 41% and 72% of men and ladies respectively. There is a general observation that obesity or overweight is pervasive more in urban population but there is presently proof that obesity is arising even in the poor population(Amin et al., 2015).

2.2 Genetics of obesity

As it is a heritable complex disorder so it is very important to understand about its molecular mechanism to overcome the prevalence of this epidemic (Bessesen, 2008). A considerable effort has been made by conducting study on rodents and humans to identify genes which are responsible for obesity (Yazdi, Clee, & Meyre, 2015) .

Various family studies show that heritability of obesity is about 40% to 70%, its genetical studies like linkage analysis and candidate gene association studies have been conducted to reveal the involvement of genetic loci, and to dissect the biological mechanisms responsible for body-weight regulation; however, these studies have a little success. Another study was designed to identify the genetical bases of obesity is Genome-Wide Association study, and then linkage analysis and candidate gene studies were successfully shifted to GWAS after 2005 (Apalasamy & Mohamed, 2015).

In order to identify both monogenic and polygenetic factors of obesity a great effort has been made in the obesity genes identification which can emphasize our understanding about these two forms of obesity (Farooqi & O'Rahilly, 2006). The study of in vivo and invitro models is also helpful in identification of these two types (Nilsson, Raun, Yan, Larsen, & Tang-Christensen, 2012).

2.2.1 Monogenic obesity

It is the obesity causing by mutation in a single gene. Monogenic is the severe rare type of obesity and its effect is more prominent. This single gene mutation causes severe onset obesity including hyperphagia and other endocrine abnormalities. Several candidate genes have identified for monogenic obesity. These are MC4R, LEPR, POMC, and BDNF which led to understanding the biological pathway of the disease. Invitro cell line technique was used on rodents to identify these candidate genes. The arrival of new technologies like Next Generation Sequencing (NGS) is offering a new domain to study these causative agents for monogenic diseases (Apalasamy & Mohamed, 2015).

2.2.2 Polygenic obesity

Polygenic obesity is caused by multiple genes actions in combination with environmental risk factors (Hinney, 2008).Monogenic mutations were identified in several candidate genes but after the entrance of GWA study in the field of obesity it become clear that monogenic mutations are rare and in 2007 many variant were analyzed in noncandidate genes that were responsible for polygenic form of obesity (Zhao et al., 2011). In 2007 three GWA study shows that Fat Mass and Obesity (FTO) gene is associated with most common form of obesity (Frayling et al., 2007). Variant responsible for polygenic obesity has least effect on phenotype but in combination with other variants it can develop a distinct and prominent phenotype.

2.3 Obesity related genes

More than 30 genome-wide scans for phenotypes related with obesity have been reported and 70 commonly accepted loci affecting phenotypes related with obesity have been found (Loos & Bouchard, 2003). Heritability of obesity in children is higher than adults. Studies conducted on French and Germen population found two loci rs121458332 in SDCCAG8 and rs13278851 near TNKS responsible for childhood obesity. Asia is inhabited by about 60% of the world population and they have high percentage of obesity, BMI, and metabolic disorders. Genetic study on Asian population not only provide information about genetic bases of obesity but it provides a better understanding in the identification of genetic variants in Asians.28 SNPs near 32 loci have been found in east Asians among which 4 were confirmed near BDNF, PCSK1,TMEM18 in chinese (Wang et al., 2016) .

2.3.1 LEP and LEPR

Mutation in the LEP gene were found in two cousins from Pakistani origin they had truncated leptin and were severely obese with the complication of hyperphagia. LEPR can also cause obesity and hyperphagia in humans after birth. Mutation of LEP gene causes low serum level of leptin and hypogonadism because of its relation with AdrinoCorticoTrophic Hormone ACTH. Mutation in extracellular LEPR limited bindings of the leptin and as result leptin signaling reduced (Loos & Bouchard, 2003). It was also found that homozygous mutation in LEPR is present in 3% and mutation in LEP is 17% in Pakistani obese children from consanguineous families (Saeed et al., 2014).

2.3.2 POMC

It was observed in transgenic animals that POMC gene is playing role in energy homeostasis and its mutation causing early onset obesity in humans (Loos & Bouchard, 2003). A study conducted on Mexican Americans shows that variation occur in level of serum leptin due to genetic variation in the region of 2p21 where

POMC gene is present. Variation in leptin level leads to accumulation of body fats. Another study of the French population reported the same situation (Pritchard, Turnbull, & White, 2002).

2.3.3 MC4R

It is related with neuroendocrine system and involved in controlling feeding behavior. The knock out mechanism of MC4R in mice shows severe hypophagia and obesity. Specifically it plays role in appetite regulation (Pritchard et al., 2002). It was identified in a study on Pakistani population that early cohort of 62 obese children have 3% homozygous mutation in MC4R. Mutation of this gene is more common in west (5.8%) (Saeed et al., 2014).

2.3.4 FTO

FTO gene and its association with type 2 diabetes, BMI, and obesity have been studied in different Asian populations. It has been found in association with type 2 diabetes, BMI, and obesity in Indians and in East Asians. Association of this gene has been reported in Pakistani population with BMI and obesity (Shahid et al., 2013). It plays role in body fat deposition which is a factor of obesity. It also plays role in energy regulation and can alter lipid profile (Shabana, Hasnain, Shabana, & Hasnain, 2015).

2.3.5 PC1

PC1 is prohormon convertase1 and its mutation causes severe onset obesity, hypoglycemia, endocrine dysfunction, and hyperphagia (Jackson et al., 2003). It was studied in 1997 for the first time in obese child with severe hyperphagia. PC1 enzymes have many substrates involved in energy homeostasis including POMC. Its deficiency causes impaired processing of the neuropeptides involved in intake of food like POMC (Farooqi et al., 2007).

2.3.6 SH2B

SH2B is involved in energy balance and it is a key regulator of the leptin sensitivity. Its mutation is responsible for leptin resistivity. Body weight is regulated by the cycle of energy expenditure and energy intake, leptin increases energy expenditure and decreases energy intake helping in weight maintenance. SH2B gene mutation causes hyperphagia, and obesity in mice. This reveals that elevated energy intake and leptin resistivity is the primary cause of obesity (Ren, Li, Duan, & Rui, 2005). It is an adopter key regulator protein of metabolism and impaired function can cause obesity and type2 diabetes in humans (Sheng et al., 2013). It have studied recently that deletion in SH2B is associated with severe onset obesity. A SNP of this gene rs7498665 have been found responsible for this association and it was suggested that SH2B is likely an obesity gene (Volckmar et al., 2012).

Table 2.1: Obesity related genes and their products (Yazdi, Clee, & Meyre, 2015).

Gene	Gene Name	Location	Gene Product
LEP	**Leptin Gene**	**7q31.3**	**Produced by Fat cells**
LEPR	**Leptin Receptor**	**1p31**	**When bound by leptin it inhibit Appetite**
MC4R	**Melanocortin4 Receptor**	**18q22**	**When bound MSH it stimulate food intake**
PCSK1	**Proprotien convertase**	**5q15-q21**	**Regulates Insulin Biosynthesis**
FTO	**Fat Mass Obesity Assciated Gene**	**16p12.2**	**Promote food intake**
POMC	**Pro-Opiomelanocortin**	**2p23.3**	**Its derived peptides control food intake and body weight**
SH2B	**SH2 Adopter Protein coding gene**	**16p**	**Involved in GH and Insulin signaling**

2.4 Reconstruction of Functional pathways of Obesity Associated Genes

All the reported genes were analyzed from literature. Leptin secreted by adipose tissue bind with their receptors in hypothalamus and start production of Pro-opiomelanocrtin which is responsible for the production of Alpha and Beta melanocyte stimulating hormones by the process of prohormone convertase 1.Alpha and beta melanocyte stimulating hormines bind with melanocortin 4 receptor to perform their activity like reduction of food intake and increase in energy expenditure. On the other hand leptin binding can also activate the jenus/kinase signaling and transcription Jak/state signaling this pathway further activate State 3 by the action of SH2B protein. State 3 will then migrate to nucleus and activates its target genes responsible for energy homeostasis (Yazdi, Clee, & Meyre, 2015). All the genes and their pathogenic role in obesity were reconstructed in figure 2.1.

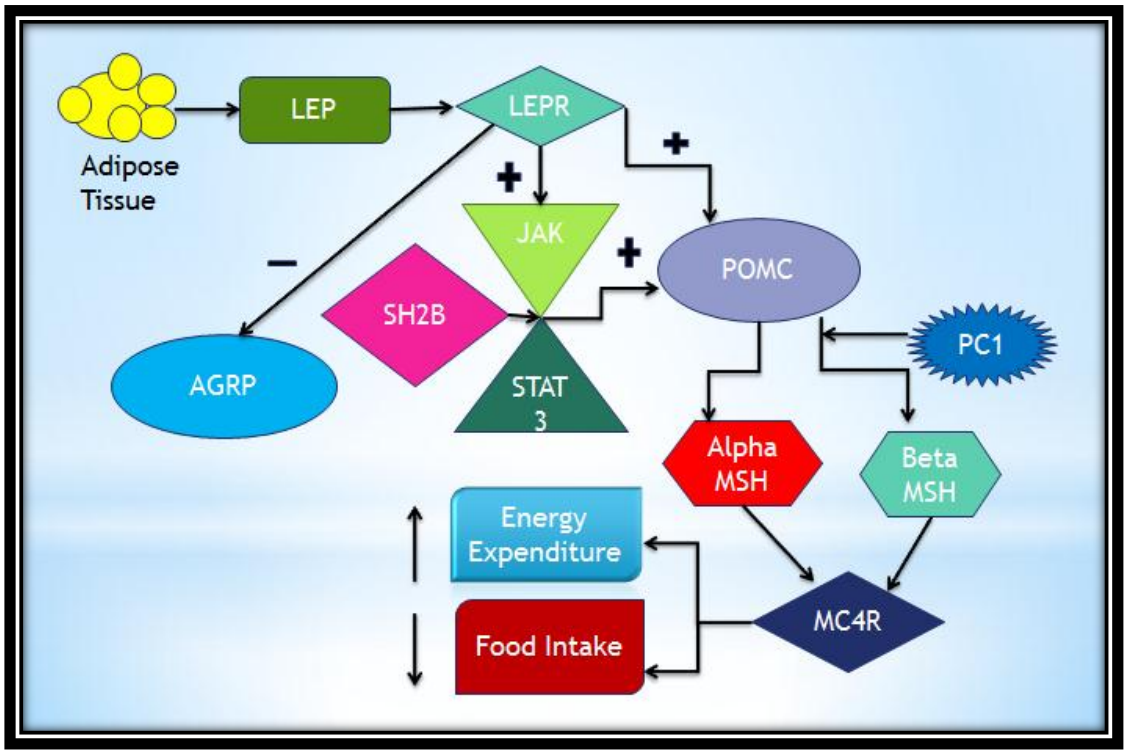

Figure 2.1: Genes involved in energy intake and expenditure in Leptin-melanocortin pathway that lead to obesity.

2.5 Variants of the obesity related Genes

Frayling et al., 2007 distinguished a typical variant, rs9939609, in the FTO (fat mass and related weight) gene that was emphatically associated with BMI and obesity in European populace. This affiliation was thus affirmed by other individual studies in view of European companions. However, it stays disputable whether the affiliation is replicable in other ethnic populaces, particularly when there is confirmation to show ethnic contrasts in the allele frequencies of FTO variations (Liu et al., 2010).

Common variants of the FTO gene resulted in obesity and elevated Bio Mass Index are rs9939609, rs1558902 and rs8050136 (Hinney, Carla, Vogel, & Hebebrand, 2011). Recent studies identified that rs1778231 and rs129070134 SNPs of MC4R is responsible for obesity in Europeans, Asians, children and adults (Xi, Chandak, Shen, Wang, & Zhou, 2012). A variant of POMC rs713586 was also found in the complication of BMI and elevated body weight (Graff et al., 2013).SNP of LEPR rs11208659 is also involved in childhood obesity, biomass index, and overweight (Yazdi, Clee, & Meyre, 2015).

2.6 Physiological factors responsible for obesity

Physiological system which is regulated by the brain is also involved in obesity. Various organs are involved in this process, signals that are related to adiposity integrating with signals from gastrointestinal system to form signals which control energy homeostasis (George. J. Schwartz, 2010). Hypothalamus performs adiposity by receiving signals from different endocrine hormones like leptin, ghrelin, and peptide. Two types of neurons are involved in the interpretation of these signals Augoti related protein neurons (AGRP) and cocaine Amphetamine related transcript neurons (CART). AGRP is related with promoting intake of food while CART is responsible for reducing the appetite (M. W. Schwartz et al., 2000). Insulin can decrease food intake by the stimulation of CART (Druce, Small, & Bloom, 2004). Leptin has also an anorexeginic effect providing long term regulation of adiposity (Lee et al., 1996). Ghrelin is the short term regulator of appetite produced in stomach

and duodenum which has inhibitory function of the CART neurons. Its increases level in serum can be observed in hunger and decrease after eating. A peptide YY secreted by the distal part of gastrointestinal system is found in decreasing food intake by inhibition of the AGRP neurons (Druce et al., 2004). Orexin neurons are second effectors include Y1R and MC4R receptors that are receiving modified signals from dopamine and serotonin to control the overall balance between food intake and energy usage (Spiegelman et al., 2001) The process have shown in Figure 2.2.

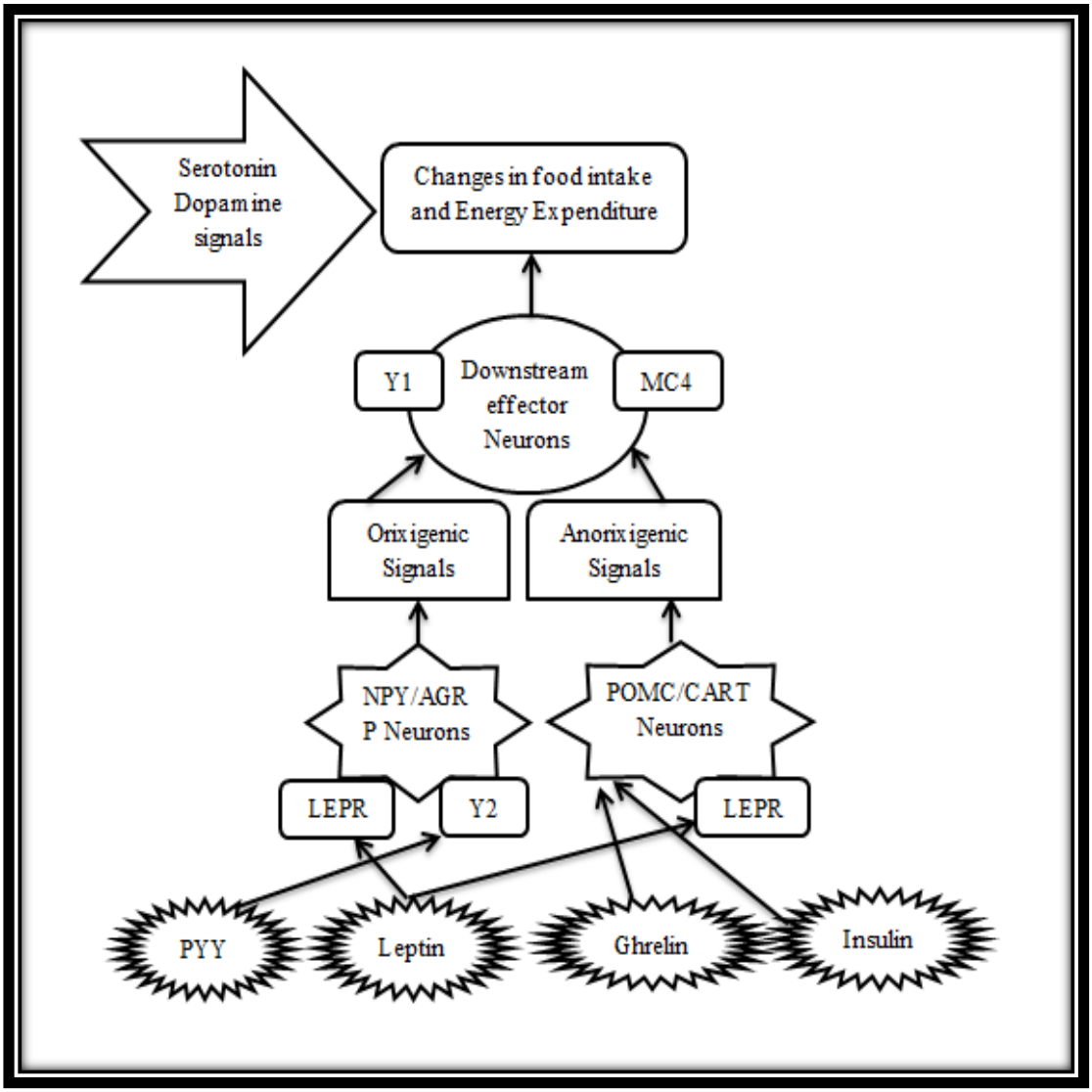

Figure 2.2: Physiological central pathways responsible for energy homeostasis and their relation with obesity (Kim et al., 2011).

The hormone ghrelin, favor adiposity by expanding food intake and diminishing fat usage in rodents, an impact that is autonomous from the capacity of the hormone to empower GH discharge. Intracerebroventricular administration of ghrelin antiserum diminishes food intake in rodents, demonstrating a focal part of endogenous ghrelin in the control of energy balance. Plasma ghrelin concentration increases before each meal and declines after supplement consumption, recommending a role in meal starting. Plasma ghrelin level is down in obese people as compared to lean people, recommending that both intense and unending changes in energy balance influence flowing ghrelin concentration. peripheral ghrelin administration at dose closes to the physiological range increases food intake in people, showing that ghrelin is a peripheral signal to the mind to invigorate food consumption. The pathway is summarized in figure 4.2.(Delparigi et al., 2002).

2.7 Gut Microbiota and obesity

Microbiota is also involved in obesity, in a healthy symbiotic state; the colonic microbiota communicates with our ingested food, specifically with dietary fiber, permitting energy derivation from inedible dietary mixes. They interact with cells, including immune cells, and in addition with the metabolic and sensory systems (nervous system); and provide protection against pathogens. Research on gut flora has been identified that they are required in energy digestion. Different people have different gut flora so they have variation in their ability to absorb energy from a given dietary food. Two bacterial colonizers of the human gut, the Bacteroidetes and the Firmicutes, vary in their metabolic productivity. Obese people have a higher extent of Firmicutes, have an improved ability to reap dietary energy and subsequently increase weight more readily (Wells & Siervo, 2011),(Romieu et al., 2017).

CHAPTER 3
MATERIAL AND METHODS

3. MATERIAL AND METHODS

3.1 Selection of Genes and SNPs

A biomedical text mining tool i.e. PolySearch2 (http://polysearch.ca) has been used to find out the set of obesity-associated genes and SNPs in Pakistani population. Two types of search options quick search and advanced search provide by the tool. From that advanced search was chosen. The search category was set as "Given Disease, Find all associated genes/proteins". Input to the tool consisted of "Obesity" provided in the search keyword field. The term "Pakistani population" was used as custom filter words. Databases were selected according to research requirements such as Pubmed, PubmedCentral, NCBI books, OMIM, and MedlinePlus etc. List of genes and list of SNPs were generated as a result with reference to articles including the query words.

3.2 Study Area

All the Samples were collected from rural areas of Khyber Pakhtunkhwa, central Punjab and Azad Jammu Kashmir. Sampling was carried out by the criteria that each family should have at least three obese individuals and obese individuals have no other comorbidity like mental retardation etc.

3.3 Sample Collection

Blood samples were collected from families having at least three obese members. Families were visited and complete clinical information was collected. Blood samples were taken using sterile syringes and immediately were transferred to 5 ml EDTA tubes which contain anticoagulant agents Ethylene Diamine Tetra Acetic Acid. All the samples were kept at 4°C till DNA extraction.

3.4 Primer Designing For ARMS PCR

Insilco primer designing was performed by using bioinformatics tool. Sequence of genes containing the SNP responsible for obesity was taken from NCBI db SNP and db var where all the necessary information related to SNP of interest is present. Sequences containing SNPs of interest were selected and primers for normal and

mutant SNPs were designed in such a way that the forward primers had our SNP at its last nucleotide. It can design many primers but the best suitable primers were selected on the basis of position of SNP, GC content and annealing temperature. Primers were designed for ARMs PCR. ARMS PCR is used to detect single base change mutation and small deletions. Only sequence-specific PCR primers can used to perform ARMS PCR that can amplify the region of test DNA only when the target allele is contained within the sample. After the designing of primers *In-silico* PCR were performed for the proper working of primers and its validation. List of the primers for SNPs of obesity related genes are shown in Figure 3.1.

Table 3.1: List of primers for SNPs of Obesity related genes.

Gene	SNP (variant ID)	Forward primer	Tm	Reverse primer	Product length
FTO	rs9939609	TTGCGACTGCTGTGAATTTTG	60	GTTTGCTTTTATGCTCTCCCACT	100bp
	Rs1558902	GTGTCTAGCCCTGTGGGTTT	59.6	ATCATATCAAGTTAGGGTACGTTGC	100bp
MC4R	rs17782313	GAAGTTTAAAGCAGGAGAGATTGT	58	AATGTCACCTTCCCCCTGAAG	70bp
	rs6567160	CACAGCCTGCCCTAATGGTAT	59.86	ACTTAAACTGGCAGCTCAGACA	70bp
	rs12970134	ACTGACTCTTACCAAACAAAGCA	58.48	AATACAGGTACTAACAAGCACCCT	53bp
Pomc	rs713586	CTCCCAAGGGAGCCAGTTT	60.61	CAACCTCTCAGGCTGCTTCA	92bp

3.5 DNA Extraction

3.5.1 Chemical used in DNA Extraction

.TKM 1 Buffer or low salt buffer (500 ml) consist of 0.605g of Tris HCl (10mM) pH 7.6, 0.372 g of KCl (10mm), 1.016 g of MgCl2 (10mM), 0.372 g of EDTA (2mM), and 500ml of distilled water.

TKM 2 Buffer or High Salt Buffer (100 ml) composed of 0.121 g of Tris HCl (10mM) pH 7.6, 0.074 g of KCl (10mM), 1.203 g of MgCl2 (10mM), 0.074 g of EDTA (2mM) 0.467 g of NaCl (0.4M), and 100 ml of distilled water.

Triton-X (10 ml) composed of 0.1ml of 100% Triton-X and 9.9 ml of distilled water, SDS consists of one g of sodium dodecyl sulphate and 10 ml of distilled water, 6M NaCl contain 8.765 g of NaCl and 25 ml of distilled water, TE Buffer contain 0.03 g of Tris HCl (10mM) pH 8.0, 0.009 g of EDTA, and 100 ml of distilled water.

3.5.2 DNA extraction Method

Non enzymatic salting out method was used to extract the DNA of all samples. Heparinized blood of 300 micro liter were added to 1.5 ml of eppendorf, 900 micro liter of TKM1 and 50 micro liter of 1x triton-x were added to that blood. The eppendorf tubes were then incubated at 37°C for 5 min for RBC lyses. All the samples were then centrifuge at 800 rpm for 3 min to pellet down the cells supernatant was discarded. This step was repeated 2-3 times with 10 time decreasing amount of triton-x till the complete lyses of RBCs and at the end white pellet of WBCs were obtained.

After the obtaining of the white pellet 300 micro liter of TKM2 and 40 micro liter of SDS were added to all the samples and after thoroughly mixing they were incubated at 37°C for 5 min. 100 microliter of 6M NaCl was added at the end of incubation and then vertexed to precipitate the proteins. All samples were then vertexed at 800 rpm for 5 min to pellet down the proteins.The supernatant were transferred to new labeled 1.5 ml of eppendorf tubes containing 300 micro liter of Isopropanol. After inverting the tubes slowly DNA were precipitated then the samples were centrifuge at 800 rpm for 10 min to get the DNA. The supernatant was discarded and 70% ethanol was

added to the DNA to remove access salts and purify the DNA. The supernatant was discarded and the DNA was air dried. After through drying 50 micro liter of TE buffer were added for the dissolution of the DNA.

3.6 Agarose Gel Analyses for the Confirmation of the Extracted DNA

1.2 gram of agarose powder was dissolved in 60 ml of TAE buffer and was kept in oven for 1 min till the transparency of the solution. 20 micro liter of Ethedium Bromide were added to the solution. After through mixing the solution were transferred to the Gel Assembly.

5 micro liter of DNA was mixed with 2 micro liter of DNA loading buffer and loaded into gel. The gel was run at 200 voltage for 30 min and the DNA was visualized under the UV light by using Photo Doc.

3.7 ARMS PCR

1.5 microliter of isolated DNA of the samples was taken into autoclaved PCR tubes. 1 microliter of 10x buffers, 1 microliter of 2 milimolar DNTPs, and 1 microliter of MgCl2 were added to the DNA. 0.3 microliter of Taq Polymerase, 0.3 microliter 0f forward and Reverse primers, and 4.6 microliter of Double Distilled water were also added to PCR tubes. After addition all of these materials were mixed thoroughly and placed into thermal cycling which was fixed for the required settings summarizing in the table 3.2.

Table 3.2: Conditions of thermal cycling that were used for ARMS PCR.

Step	Temperature	Time	Number of cycles
Initial denaturation	95 degree Celsius	1-3 min	1
Denaturation	95 degree Celsius	30 sec	25-40
Annealing	Tm of primer	30 sec	
Extension	72 degree Celsius	1 min	
Final extension	72 degree Celsius	5-15 min	1

3.8 Agarose Gel Analyses for the Amplicon

3% agarose gel was used to for loading the amplicon. 3 gram of agarose powder was dissolved in 100 ml of TAE buffer and was oven for 1 min 20 micriliter of Ethidium Bromide solution was added to agarose solution and pure into gel assembly. After its solidification 10 microliter of amplicon was mixed with 2 microliter of gel loading dye and was loaded in the gel. The gel was run at 120 volt for 15 min and the amplified product was visualized under the UV light.

CHAPTER 4

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1 Selection of Genes and SNPs

A text mining tool PolySearch2 (http://polysearch.ca) has been used to construct a table of obesity related genes and SNPs commonly reported in Pakistani population. Word "Obesity" was provided as search key word and "Pakistani population" was used as custom filter words. Table 4.1 and 4.2 summarize the list of genes and SNPs respectively.

Gene name	Gene symbol	Gene ID	Gene product's role in energy balance	Co-morbidity	Sampling area	Reference	Year
FTO, alpha-ketoglutarate dependent dioxygenase	FTO	79068	Promotes food intake.(Shabana et al. (2015))	Cardiovascular diseases.(Lie et al. (2017))	Lahore, Sheikhupura, Bhakkar, and Burewala	(Shabana et al. (2015))(Shahid, Saleem Ullah et al. (2016)),(Shahid, Adeela et al. (2013)), (Fawwad et al. (2016))	2013, 2015, 2016
Leptin receptor	Lepr	3953	When bound by leptin, inhibits appetite.(Saeed,Sadia et al. (2012))	Cardiovascular diseases (Koh et al.(2017)) Entamoeba histolytica infections. (P,Duggal et al. (2011))	Lahore,Central Punjab Pakistan	(Saeed,Sadia et al. (2012)),(S. Saeed et al.(2013)), (Shabana et al. (2016)) (S. Hasnain et al.(2016)) (S. Saeed et al.(2017))	2012, 2013, 2016, 2017
Melanocortin 4 receptor	Mc4r	4160	When bound by alpha-melanocyte stimulating hormone, stimulates appetite.(R, Adan et al. (2006))	Hypertriglycerid-emia(Fernandis et al. (2015))	Central Punjab Pakistan	(Sadia et al.(2012)) (S.Saeed et al.(2017))	2012, 2017
Proopio-melanocortin	POMC	5443	Regulates feeding behavior.(K, Simpson et al.(2008))	Adrenal insufficiency, and red hair pigmentation (Krude, Heiko et al. (2011))	Punjab Pakistan	(Shabana et al. (2016))	2016

Table 4.1: Table of selected genes associated with obesity and BMI in Pakistani population.

Table 4.2: Table of variants (SNPs) of the Obesity related genes.

Gene	SNP	Position	Band	Summary	Function	Reference
FTO	rs9939609	Chr16:53786615	16q12.2	A>A/T	Intron Variant	(Shabana et al. (2015))
	rs1558902	Chr16:53769662	16q12.2	T>A/T	Intron Variant	(Shahid, Saleem Ullah et al. (2016))
	rs8050136	Chr16:53782363	16q12.2	A>A/C	Intron Variant	(Shahid, Saleem Ullah et al. (2016))
MC4R	rs17782313	Chr18:60183864	18q21.32	T>C/T	Intergenic	(Yazdi et al. (2015))
	rs6567160	Chr18:60161902	18q21.32	T>C/T	Intergenic	(Sadia et al.(2012))
	rs12970134	Chr18:60217517	18q21.32	G>A/G	Intergenic	(S.Saeed et al.(2017))
POMC	rs713586	Chr2:24935139	2p23.3	C>C/T	Unknown	(Shabana et al. (2016))

4.2 Positional and Functional Information of SNPs

All the SNPs of the obesity related genes in this study are intronic or intergenic, and these findings have taken from the UCSC Genome Browser (https://genome.ucsc.edu). The intronic regions have regulatory sequences of RNAs that can affect the stability and translation of mRNA. Mutation in the intronic region can also affect splicing because introns contain splice factors binding regions, and changes in these regions can lead to truncated products (Ward & Cooper, 2009). Positional information of functional SNPs have shown in figure 4.1 to 4.7.

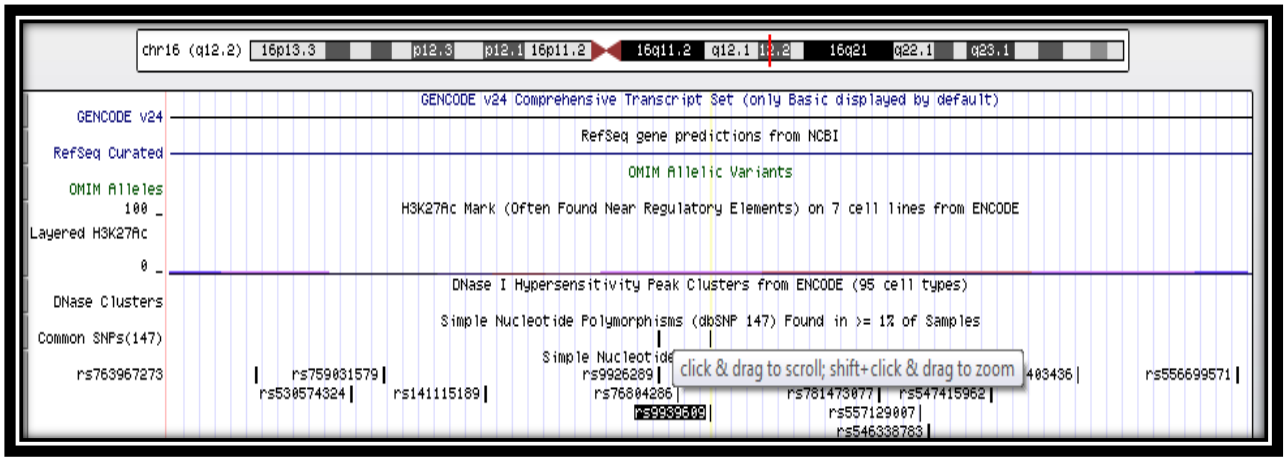

Figure 4.1: Positional information of FTO rs9939609 from Ucsc Genome Browser.

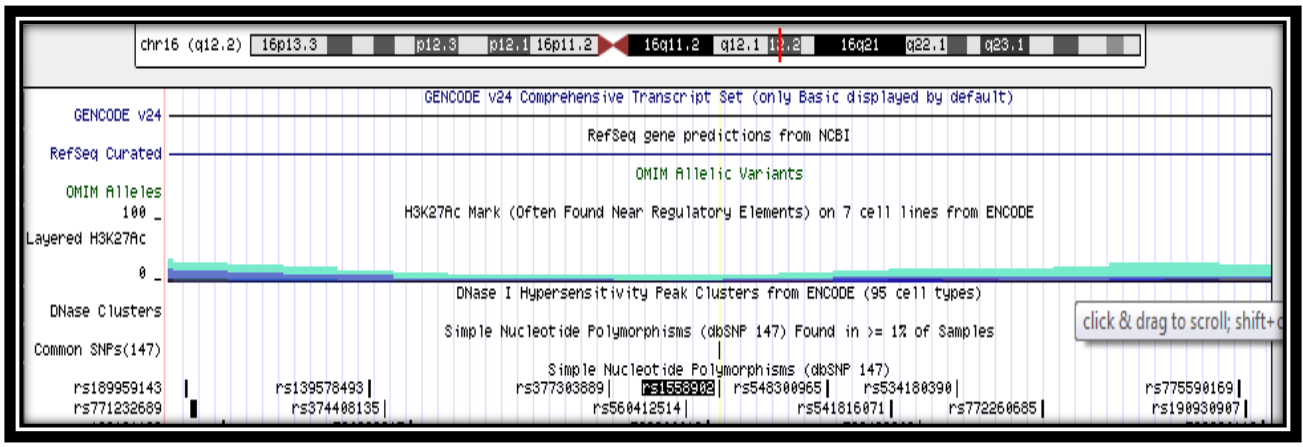

Figure 4.2: Positional information of FTO rs15589602 from Ucsc Genome Browser.

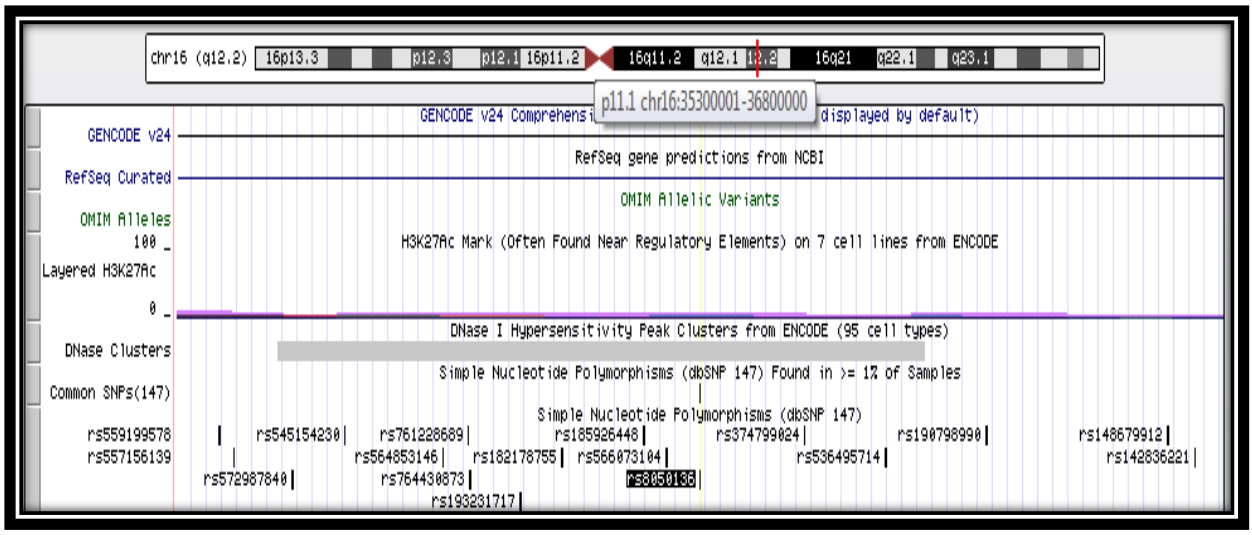

Figure 4.3: Positional information of FTO rs8050136 from Ucsc Genome Browser.

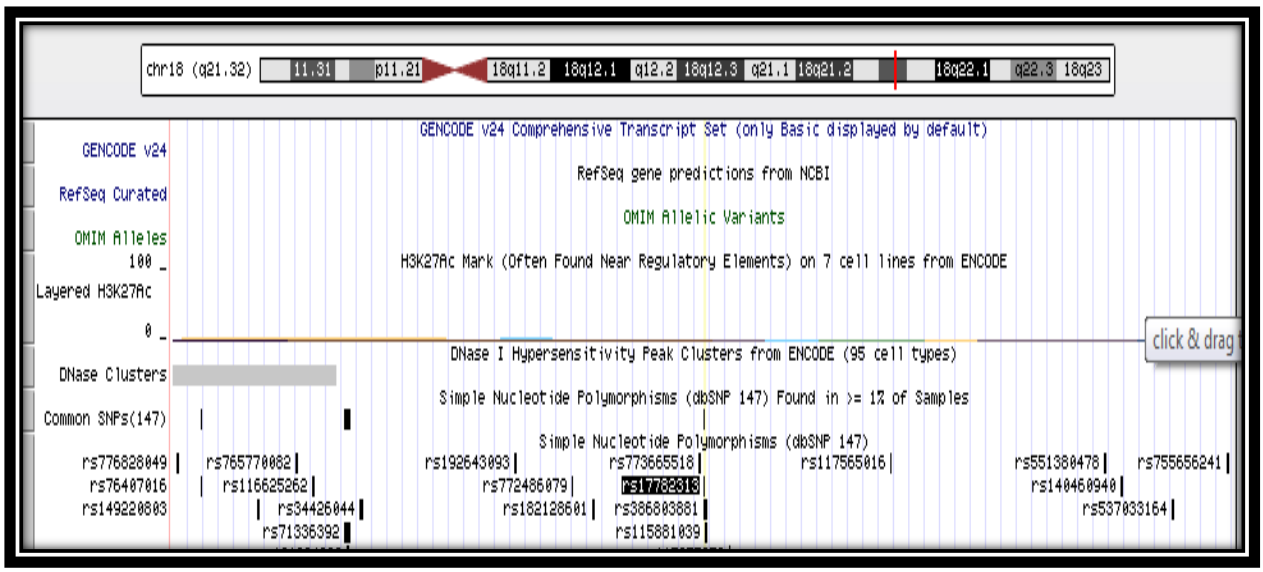

Figure 4.4: positional information of MC4R rs17782313 from Ucsc Genome Browser.

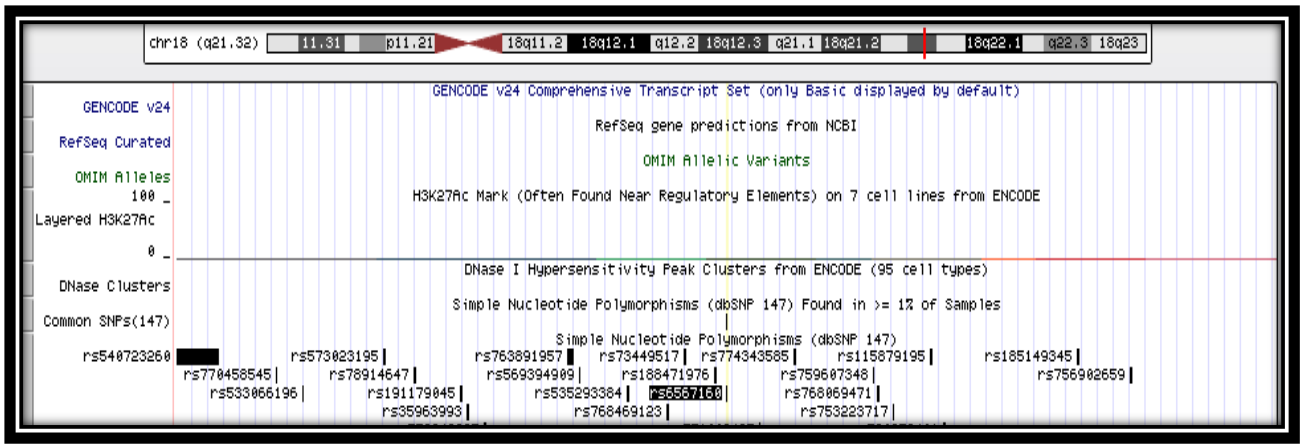

Figure 4.5: positional information of MC4R rs6567160 from Ucsc Genome Browser.

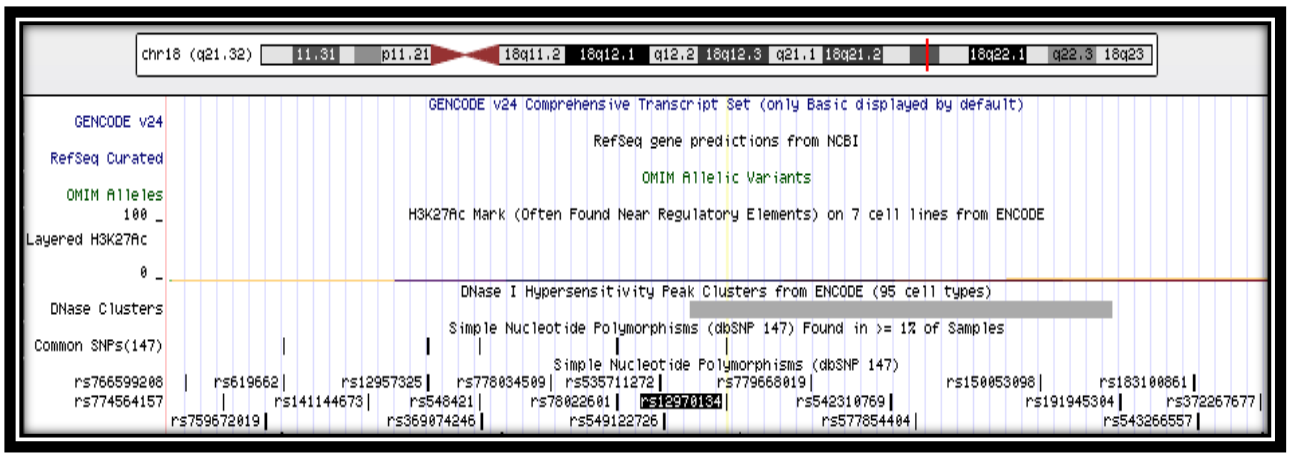

Figure 4.6: positional information of MC4R rs12970134 from Ucsc Genome Browser.

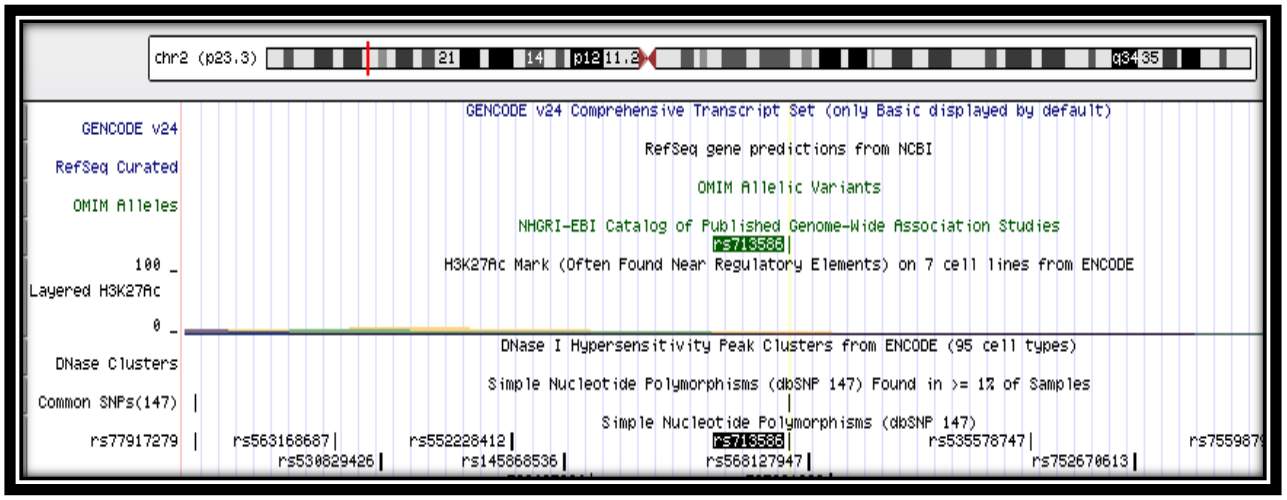

Figure 4.7: positional information of POMC rs713586 from Ucsc Genome Browser.

4.3 Data Collection

4.3.1 Family A

Family A was sampled from urban area of Kashmir. This family was identified by a proband that was obese and had BMI 39.48.This family has total 9 obese individuals out of which we were able to sample 6 members, out of these 6, two were obtained with BMI 39.48 and 29.94. standard deviation of their BMI was 4.77.This family shows no other comorbidities. Pedigree of the family shown in Figure 4.8 and other information is summarizing in table 4.3.

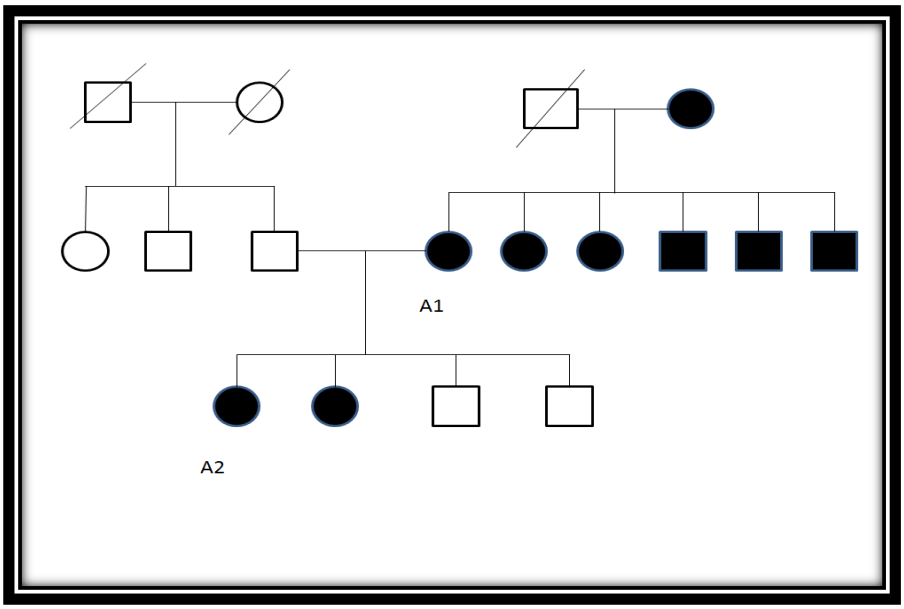

Figure 4.8: Figure 4.10 shows Pedigree of family A. Solid filled shapes show obese and open shapes show normal individuals. Square indicating males circle indicating females and diagonal lines show dead individuals.

Table 4.3: Body Mass Index, Blood pressure and other observations of individuals sampled from Family A.

Serial No	Sample ID	BMI	BP	Diabetes	Eating Disorder
1	A1	39.48	Yes	Yes	No
2	A2	29.94	No	No	No

4.3.2 Family B

Family B was sampled from urban area of Kashmir. This family was identified by a proband who was obese and had BMI 32.44.This family has total 9 obese individuals out of which we were able to sample 6 members , out of these 6 , 5 were obtained with BMI 39.48, 29.94,20.19,24.14,16.10 and 27.91. The standard deviation of their BMI was 5.71. This family shows no other comorbidities. The Pedigree of the family shown in Figure 4.9 and other information is summarizing in table 4.4.

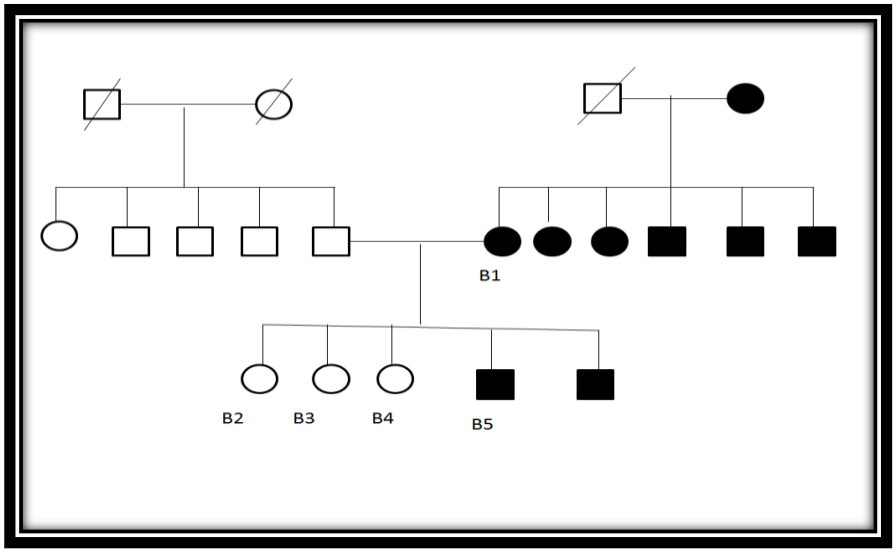

Figure 4.9: Figure 4.11 shows pedigree of family B. Solid filled shapes show obese and open shapes show normal individuals. Square indicating males circle indicating females and diagonal lines show dead individuals.

Table: 4.5 Body Mass Index, Blood pressure and other observations of individuals sampled from Family B.

Serial No	Sample ID	BMI	BP	Diabetes	Eating Disorder
1	B1	32.44	Yes	No	Yes
2	B2	20.19	Yes	No	No
3	B3	24.14	No	No	No
4	B4	16.10	No	No	No
5	B5	27.91	No	No	Yes

4.3.3 Family C

Family C was sampled from urban area of Kashmir. This family was identified by a proband that was obese and had BMI 49.26.This family has total 17 obese individuals out of which we were able to sample 4 members, out of these 4, three we obtained with BMI 49.26, 32.73 and 37.28. standard deviation of their BMI was 6.97. This family shows no other comorbidities. Pedigree of the family shown in Figure 4.10 and other information is summarizing in table 4.6.

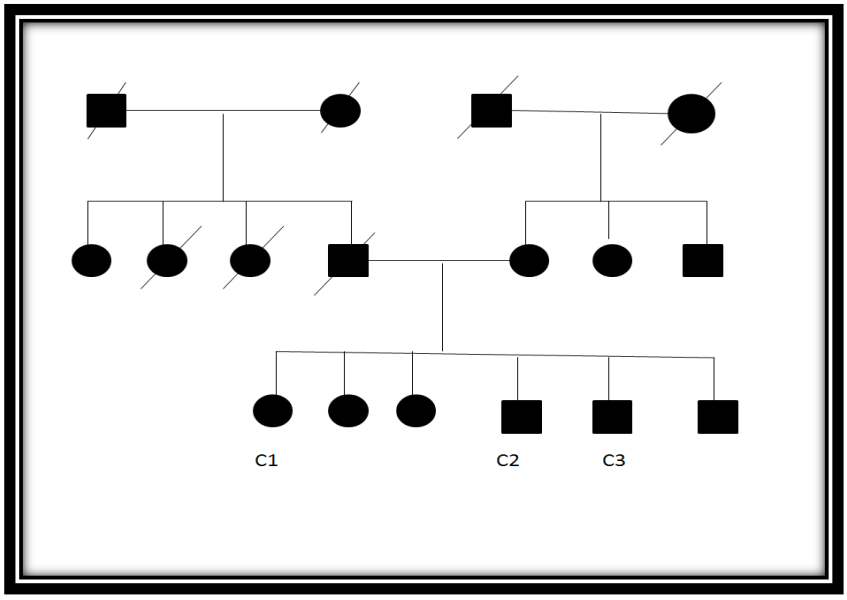

Figure 4.10: The figure 4.12 shows Pedigree of Family C. Filled squares shows obese males while filled circles show obese females, and diagonal lines shows dead individuals.

Table 4.6: Body Mass Index, Blood pressure and other observations of individuals sampled from Family A.

Serial No	Sample ID	BMI	BP	Diabetes	Eating Disorder
1	C1	49.26	Yes	No	Yes
2	C2	32.73	Yes	No	No
3	C3	37.28	Yes	No	No

4.3.4 Family D

Family D sampled from rural area of district Malakand of KP. This family was identified by a proband that was obese and had BMI 36.1.This family had total 21 individuals out of which 10 were affected and we were able to sample 6 members. All these 6 members born from consanguineous marriages and were obese with BMI D1 35.1, D2 36.3, D3 36.1, D4 46.2, D5 43, and D6 31. The standard deviation of their BMI was SD 5.10pedigree of this family has shown in figure 4.11 and other information related to this family has shown in the Table: 4.7.

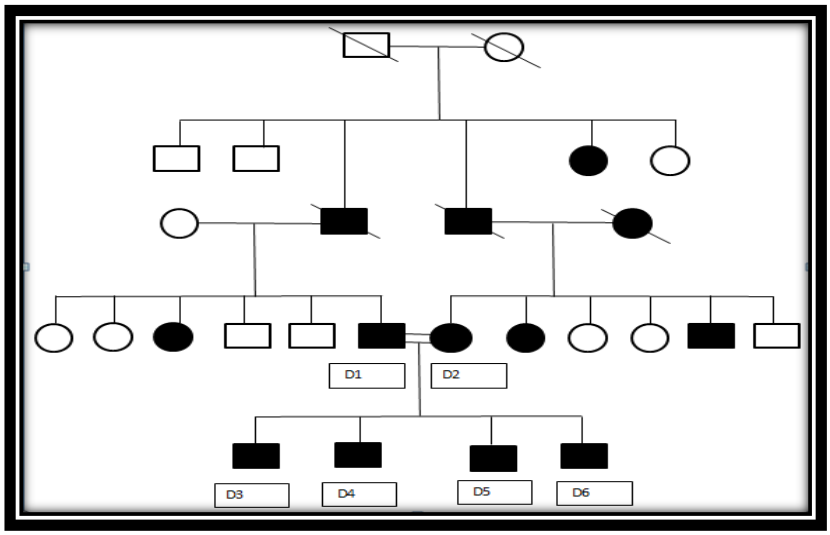

Figure 4.11: The figure shows Pedigree of family D. Filled squares shows obese males while filled circles show obese females, and diagonal lines shows dead individuals.

Table: 4.7 Body Mass Index, Blood pressure and other observations of individuals sampled from Family D.

Serial No	Sample ID	BMI	BP	Diabetes	Eating Disorder
1	D1	35.1	Yes	Yes	No
2	D2	36.3	Yes	Yes	No
3	D3	36.1	No	No	No
4	D4	46.2	Yes	No	No
5	D5	43	No	No	No
6	D6	31	No	No	No

4.3.5 Family E

Family E was sampled from rural area of district Malakand of KP. The family consist total of 30 members out of which 15 were obese. We were able to sample 8 individual of this family whose mother E2 was obese with BMI 39.2 her three daughters were also obese with BMI E5 36.9 and E6 34.1, and BMI E3 25.6.One son was also obese with BMI, E7 27.2.one son and one daughter were normal. Standard deviation of their BMI was 7.31.The pedigree of this family has shown in figure 4.12 and other information related to this family shown in table 4.8.

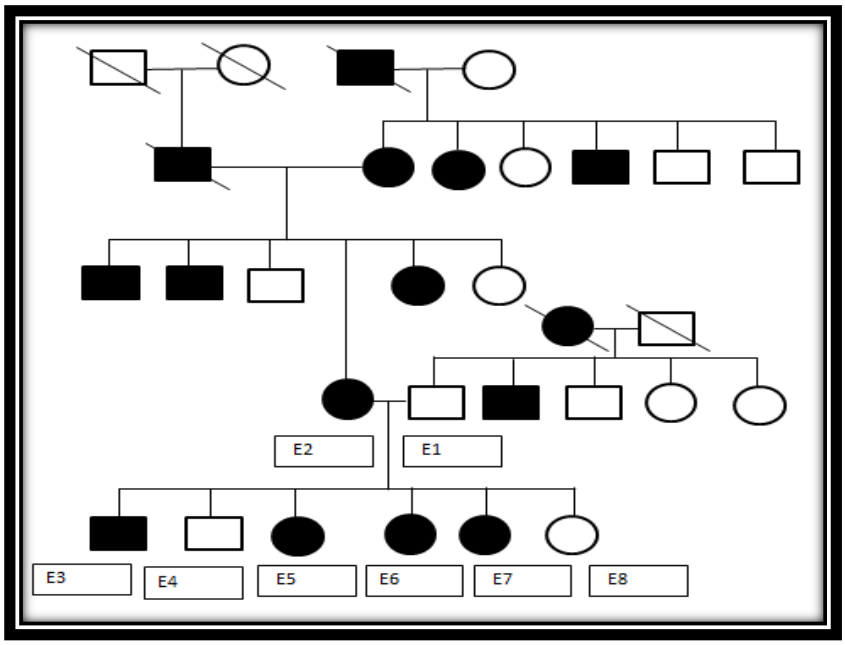

Figure 4.12: The figure shows Pedigree of Family E. Filled squares shows obese males while filled circles show obese females, and diagonal lines shows dead individuals.

Table: 4.8 Body Mass Index, Blood pressure and other observations of individuals sampled from Family E.

Serial No	Sample ID	BMI	BP	Diabetes	Eating Disorder
1	E1	25.4	Yes	No	No
2	E2	39.2	Yes	No	No
3	E3	25.6	No	No	No
4	E4	19.4	No	No	Yes
5	E5	36.9	No	No	No
6	E6	34.1	No	No	No
7	E7	27.2	No	No	No
8	E8	18	No	No	No

4.3.6 Family F

Family F was sampled from Dargai area of KP which is a rural area of district Malakand. Family F had total 21 members out of which 10 were obese. Only five members of this family were sampled in which three members were obese. One obese member was 13 year old boy with BMI F4 26.9, his father had BMI F1 35.1 and his mother had BMI 26. Other 2 family members were normal. Standard deviation of their BMI was 6.5.The Pedigree of this family has shown in figure 4.13 and their other information of BMI and obesity related diseases have shown in table 4.9.

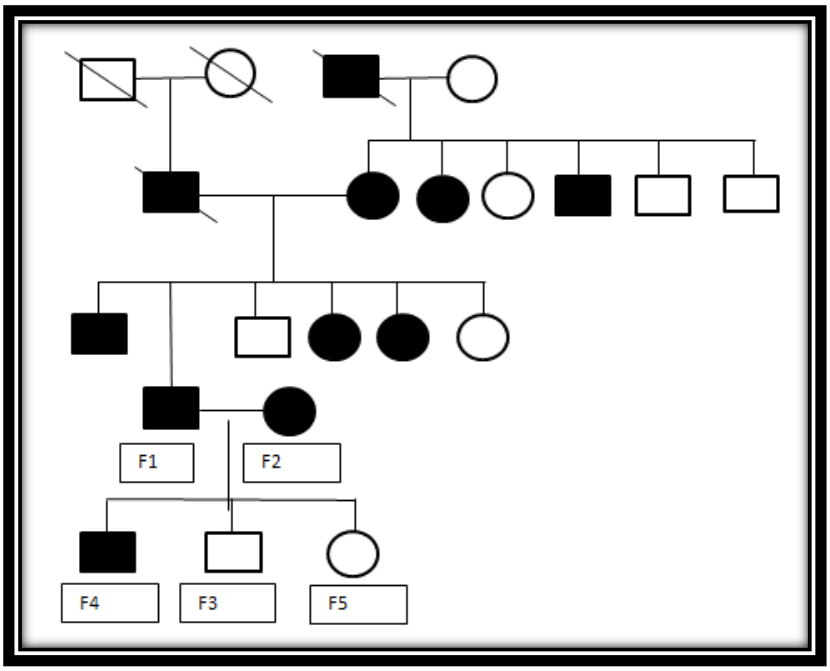

Figure 4.13: The figure shows Pedigree of family F. Solid filled shapes show obese and open shapes show normal individuals. Square indicating males circle indicating females and diagonal lines show dead individuals.

Table: 4.9 Body Mass Index, Blood pressure and other observations of individuals sampled from Family F.

Serial No	Sample ID	BMI	BP	Diabetes	Eating Disorder
1	F1	35.1	No	No	No
2	F2	26	No	No	No
3	F3	14.6	No	No	No
4	F4	26.9	No	No	No
5	F5	24.2	No	No	No

4.3.7 Family G

Family G was Sampled from Malakand district of KP which is rural area. Family G had 9 obese individuals out of 15 members. Five individuals were able to sampled in which five were obese with BMI G2 34.9, G3 30.2, BMI G1 28.4, G4 29.7, and G5 25.8. The standard deviation of their BMI was 2.97. The pedigree of family G has shown in figure 4.14 and information of BMI and diseases related to obesity have summarizing in Table 4.10.

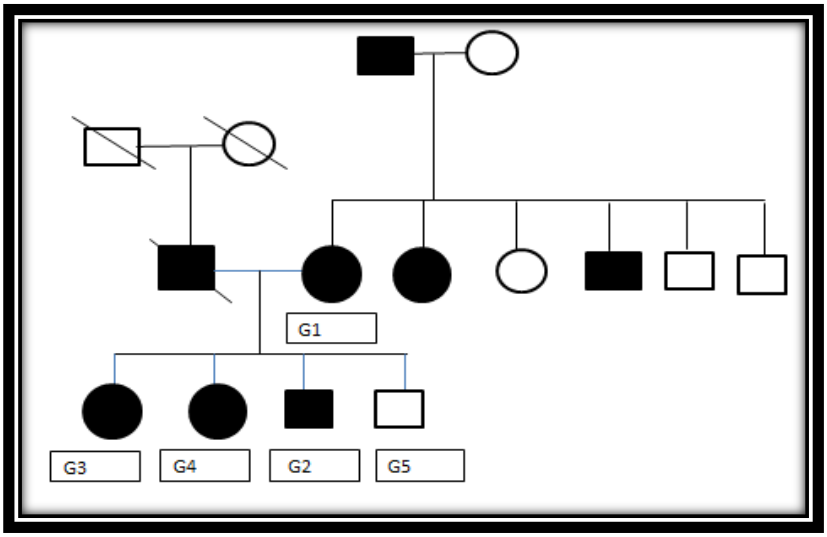

Figure 4.14: The figure shows Pedigree of Family G. Solid filled shapes show obese and open shapes show normal individuals. Square indicating males circle indicating females and diagonal lines show dead individuals.

Table: 4.10 Body Mass Index, Blood pressure and other observations of individuals sampled from Family G.

Serial No	Sample ID	BMI	BP	Diabetes	Eating Disorder
1	G1	28.4	Yes	No	No
2	G2	34.9	No	No	No
3	G3	30.2	No	No	No
4	G4	29.7	No	No	No
5	G5	25.8	No	No	No

4.3.8 Family H

Family H originated from District of Azad Kashmir. This family consists of 15 individuals. Out of 15 individuals, 7 were obese and others were healthy. Out of 7 individuals, 3 individuals were sampled that were obese with BMI 49.1, 40.2, and 29.8. The standard deviation of this family was 7.8. The Pedigree of this family has shown in Figure 4.15. Information related to these individuals is summarized in table 4.11

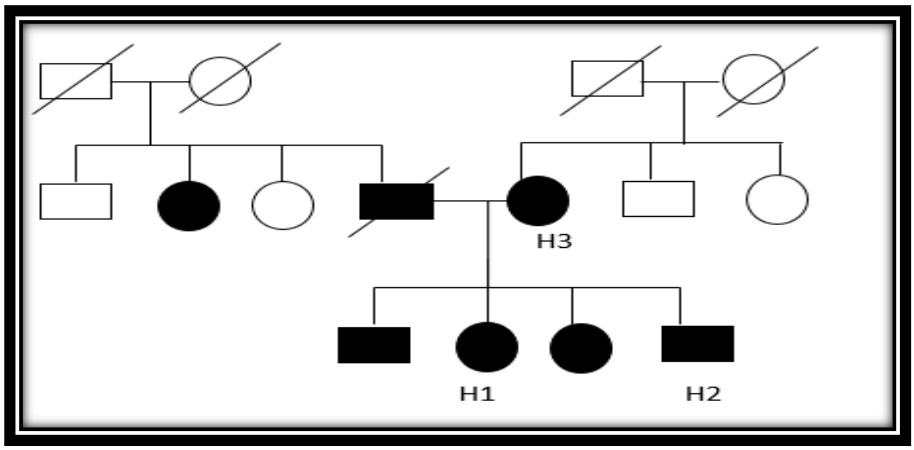

Figure 4.15: The figure shows Pedigree of family H. Solid filled shapes show obese and open shapes show normal individuals. Square indicating males circle indicating females and diagonal lines show dead individuals.

Table 4.11: Body Mass Index, Blood pressure and other observations of individuals sampled from Family H.

Serial No	Sample ID	BMI	BP	Diabetes	Eating Disorder
1	H1	40.2	No	No	No
2	H2	29.8	No	No	No
3	H3	49.1	Yes	No	Yes

4.3.9 Family I

Family I was sampled from District of Azad Kashmir. This family consists of 21 individuals. Out of which 15 were obese. Out of 14 individuals, 4 individuals were sampled in which 3 were obese with BMI 60.1, 41.8, and 40.2 and one was normal with BMI 20.5. Standard deviation of their BMI was 14.0. Pedigree of this family has shown in Figure 4.16. Information related to these individuals is summarized in table 4.12.

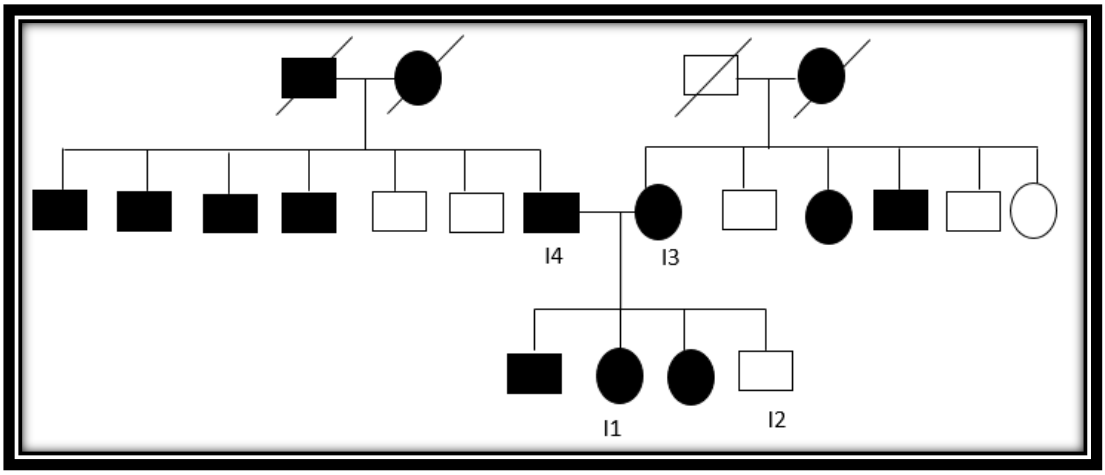

Figure 4.16: The figure shows Pedigree of Family I. Solid filled shapes show obese and open shapes show normal individuals. Square indicating males circle indicating females and diagonal lines show dead individuals.

Table 4.12: Body Mass Index, Blood pressure and other observations of individuals sampled from Family I.

Serial No	Sample ID	BMI	BP	Diabetes	Eating Disorder
1	I1	40.2	No	No	No
2	I2	20.5	No	No	No
3	I3	60.1	No	Yes	Yes
4	I4	41.8	No	No	Yes

4.3.10 Family J

Family J was sampled from District of Azad Kashmir. This family was identified by a proband having BMI 51.6. This family consists of 13 individuals. Out of which 9 were obese and others were normal. Out of 13 individuals, 4 individuals were sampled in which two were obese with BMI 51.6 and 43 and two were normal with BMI 17.9. The standard deviation of this family was 15.0. Pedigree of this family has shown in Figure 4.17. Information related to these individuals is summarized in table 4.13.

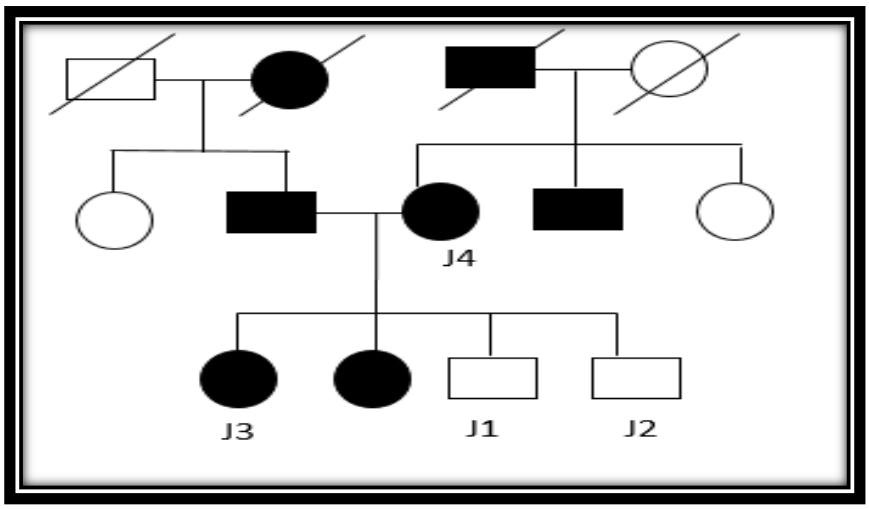

Figure 4.17: The figure shows pedigree of Family J. Solid filled shapes show obese and open shapes show normal individuals. Square indicating males circle indicating females and diagonal lines show dead individuals.

Table 4.13: Body Mass Index, Blood pressure and other observations of individuals sampled from Family J.

Serial No	Sample ID	BMI	BP	Diabetes	Eating Disorder
1	J1	17.9	No	No	No
2	J2	17.9	No	No	No
3	J3	43	No	No	Yes
4	J4	51.6	No	No	Yes

4.3.11 Family K

Family k originated from District of Azad Kashmir. This family consists of 18 individuals. Out of 18 individuals, 10 were obese and others were normal. Out of these 10 individuals, 3 individuals were sampled. All of these three members were obese with BMI 55.8, 51.6, and 55.8. The standard deviation was 1.97. Pedigree of this family has shown in Figure 4.18. Information related to these individuals is summarized in table 4.14.

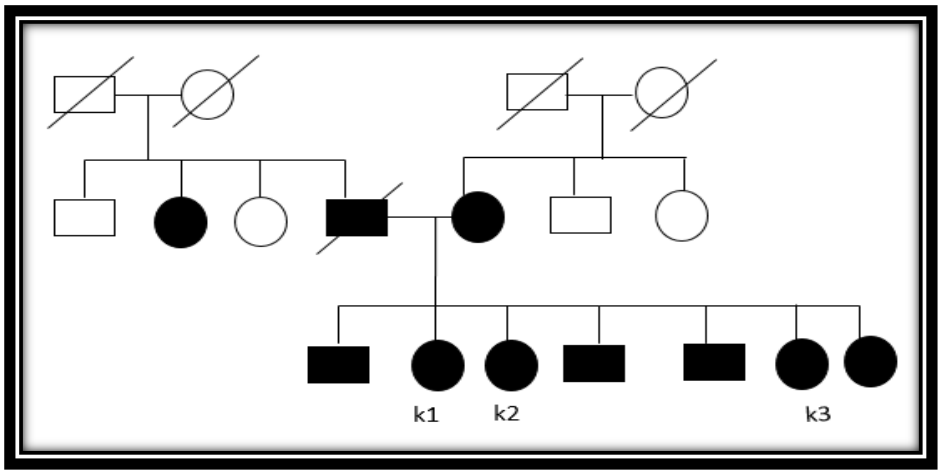

Figure 4.18: The figure shows Pedigree of family K. Solid filled shapes show obese and open shapes show normal individuals. Square indicating males circle indicating females and diagonal lines show dead individuals.

Table 4.14: Body Mass Index, Blood pressure and other observations of individuals sampled from Family K

Serial No	Sample ID	BMI	BP	Diabetes	Eating Disorder
1	K1	55.8	Yes	No	No
2	K2	55.8	No	No	No
3	K3	51.6	Yes	Yes	No

4.3.12 Family L

Family L originated from District of Gujrat. This family consists of 12 individuals. Out of 12 individuals, 8 were obese. Out of 8 individuals, 5 individuals were sampled that were obese had BMI 62.3, 43.0, 61.9, and 51.6. The standard deviation was 5.8 of this family. Pedigree of this family has shown in Figure 4.19. Information related to these individuals is summarized in table 4.15.

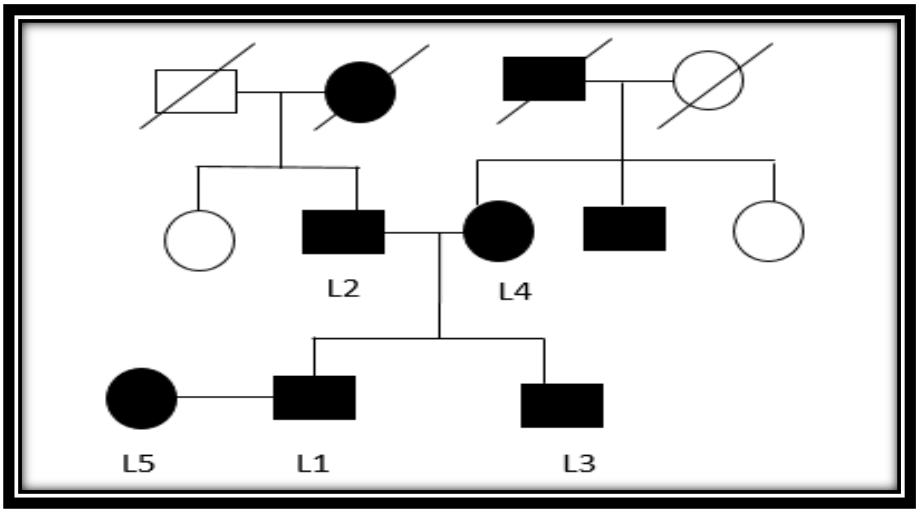

Figure 4.19: The figure shows Pedigree of family L. Solid filled shapes show obese and open shapes show normal individuals. Square indicating males circle indicating females and diagonal lines show dead individuals.

Table 4.15: Body Mass Index, Blood pressure and other observations of individuals sampled from Family L.

Serial No	Sample ID	BMI	BP	Diabetes	Eating Disorder
1	L1	62.3	No	No	Yes
2	L2	43.0	Yes	No	No
3	L3	61.9	No	No	Yes
4	L4	51.6	No	No	Yes
5	L5	51.6	No	No	No

4.3.13 Family M

Family M originated from District of Gujrat. This family consists of 15 individuals. Out of 15 individuals, 9 were obese and others were normal. Out of these 9 individuals, 4 individuals were sampled all of these four were obese had BMI 43, 25, 29, and 43. The standard deviation of this family was 10.5. Pedigree of this family has shown in Figure 4.20. Information related to these individuals is summarized in table 4.16.

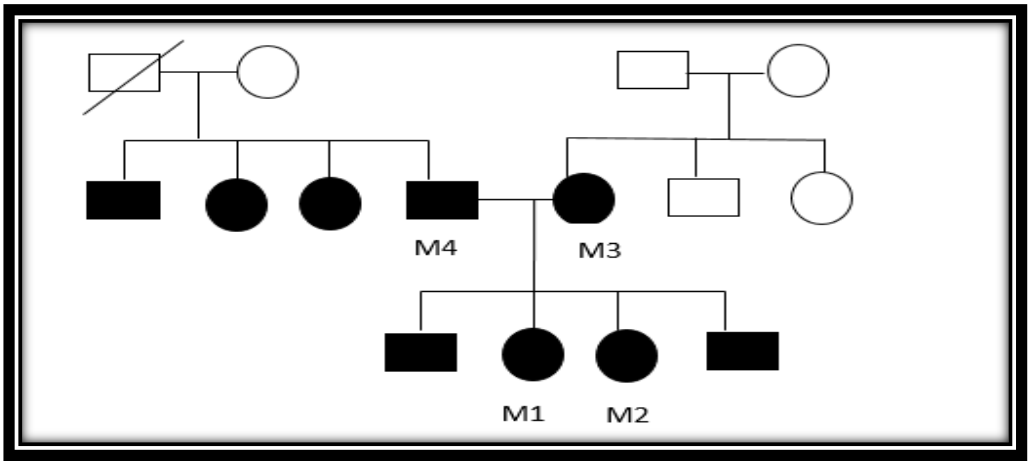

Figure 4.20: The figure shows pedigree of family M. Solid filled shapes show obese and open shapes show normal individuals. Square indicating males circle indicating females and diagonal lines show dead individuals.

Table 4.16: Body Mass Index, Blood pressure and other observations of individuals sampled from Family M.

Serial No	Sample ID	BMI	BP	Diabetes	Eating Disorder
1	M1	25	No	No	No
2	M2	29.5	No	No	Yes
3	M3	43	Yes	No	No
4	M4	43	Yes	No	No

4.3.14 Family N

Family N was originated from District of Gujrat. This family consists of 14 individuals. Out of 14 individuals, 10 were obese out of 10 individuals, 6 individuals were sampled that were obese had BMI 46.7, 30.3, 39, 44, and 31. The standard deviation was 5.9. Pedigree of this family has shown in Figure 4.21. Information related to these individuals is summarized in table 4.17.

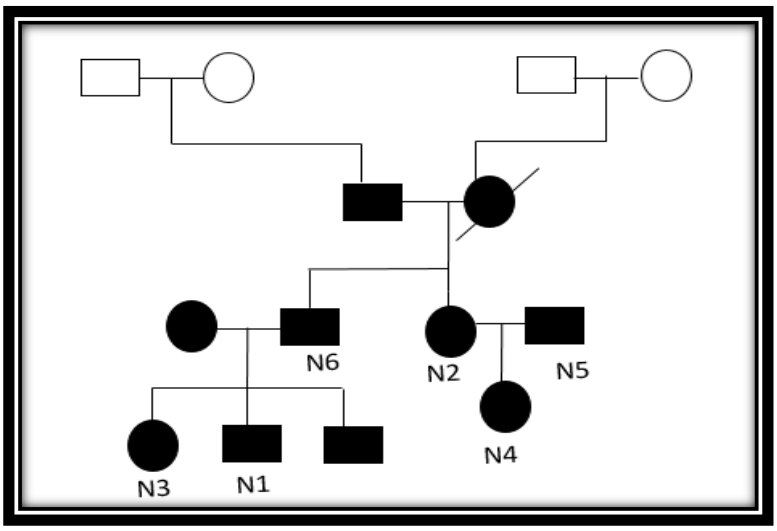

Figure 4.21: The figure shows Pedigree of Family N. Solid filled shapes show obese and open shapes show normal individuals. Square indicating males circle indicating females and diagonal lines show dead individuals.

Table 4.17: Body Mass Index, Blood pressure and other observations of individuals sampled from Family N

Serial No	Sample ID	BMI	BP	Diabetes	Eating Disorder
1	N1	35.2	No	No	Yes
2	N2	46.7	No	Yes	Yes
3	N3	30.3	No	No	Yes
4	N4	31.6	No	No	Yes
5	N5	39	Yes	Yes	Yes
6	N6	44.3	Yes	Yes	Yes

4.4 Prevalence of SNPs

4.4.1 FTO rs9939609

The variant rs9939609 is a SNP of the FTO gene at position chr16:53786615 on band 16q12.2 and is responsible for obesity phenotype. It is an intron variant SNP and it was checked for its association with obesity and BMI in Pakistani population. Their genotype results have shown in figure 4.22.

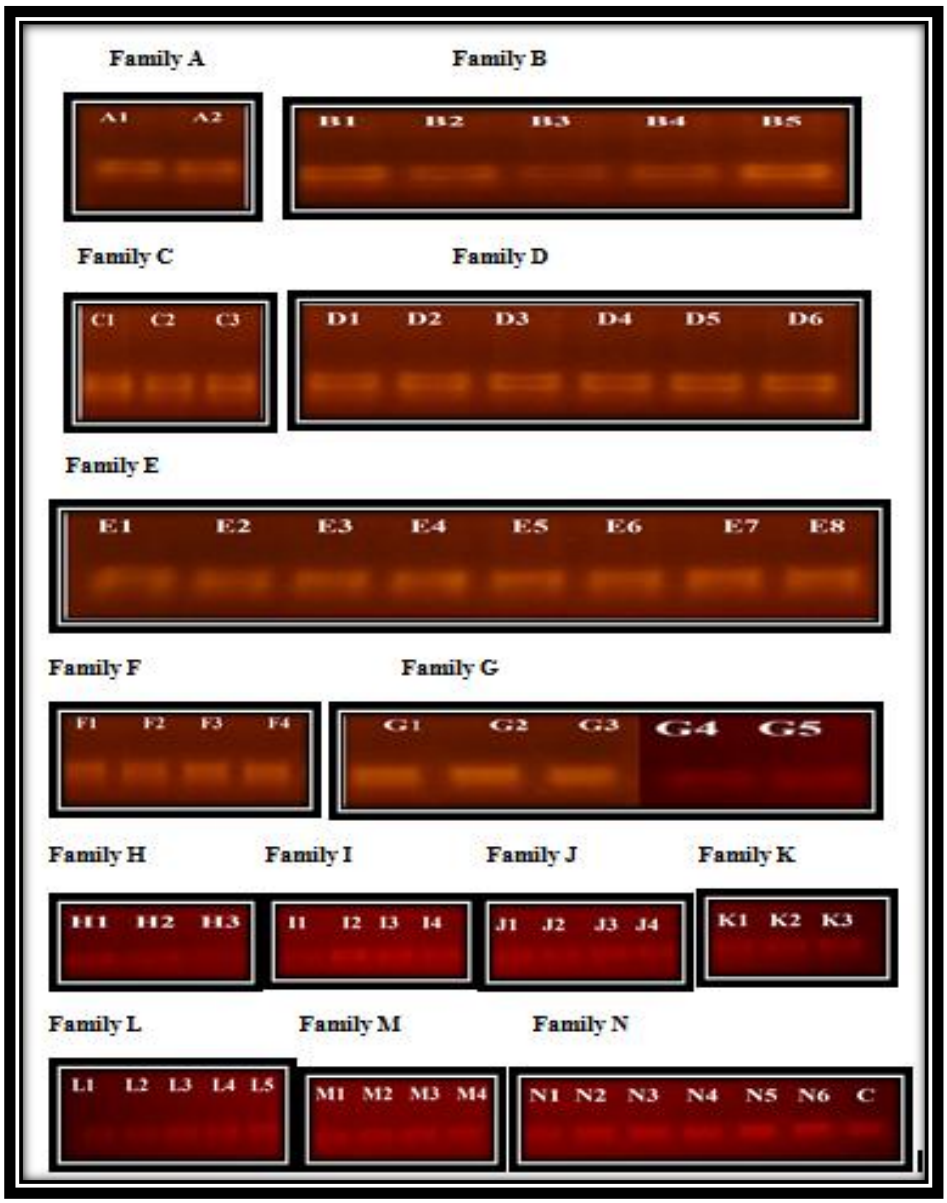

Figure 4.22: Agarose gel Electrophoresis indicating Genotyping of FTO rs9939609 for all the sampled families.

A significant association of FTO rs9939609 with obesity have been observed in a North Indian population (Prakash, Mittal, Srivastava, Awasthi, & Srivastava, 2016). In south Asian population association between FTO rs9939609 and obesity have also observed (Rees et al., 2011).

Previous studies that is comparable to our study, which shows no association of the FTO rs9939609 SNP with obesity. The present study has found no association between FTO rs9939609 and obesity. A study conducted on some of the Pakistani population have found no association between FTO rs9939609 SNP and obesity (Fawwad et al., 2016). Another study conducted on Chinese population have also found no association of the SNP rs9939609 with obesity and BMI (Li et al., 2008). Association of rs 9939609 with obesity phenotype in Oceanic population have also not found (Ohashi et al., 2007).

4.4.2 FTO rs1558902

FTO rs1558902 is the intronic variant on position Chr16:53769662 and responsible for obesity phenotype. It has investigated for the association with obesity in Pakistani kindreds . Its genotype results have mentioned in the figure 4.23.

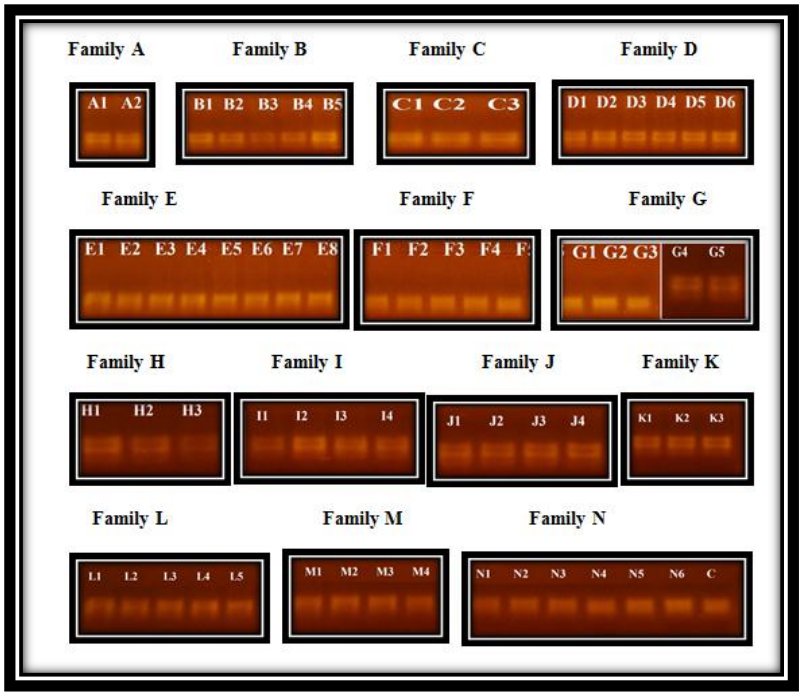

Figure 4.23: Agarose gel Electrophoresis indicating Genotyping of FTO rs1558902 for all the sampled families.

Significant association have been found between FTO rs1558902 and BMI by William Paul T (Williams, 2012). A study also found FTO rs1558902 in linkage disequilibrium (Claussnitzer et al., 2015). It was demonstrated that FTO rs1558902 may be associated with visceral fat accumulation in Japanese population (Hotta et al., 2010). A study conducted on Japanese population found no association of the FTO rs1558902, and rs9939609 with obesity phenotype (Shimaoka et al., 2010). The present investigation also indicating no association of FTO rs1558902 with obesity related phenotype. Another study conducted on Japanese contradicting the present study, have found significant association of the FTO rs1558902 with obesity (Hotta et al., 2008).

4.4.3 FTO rs8050136

FTO variant rs8050136 on position Chr16:53782363 is associated with obesity and BMI. It has been checked for its association in Pakistani population sampled from central Punjab, Azad Jamu Kashmir, and Khayber Pakhtun Khwa. Its genotyping results have shown in figure 4.24

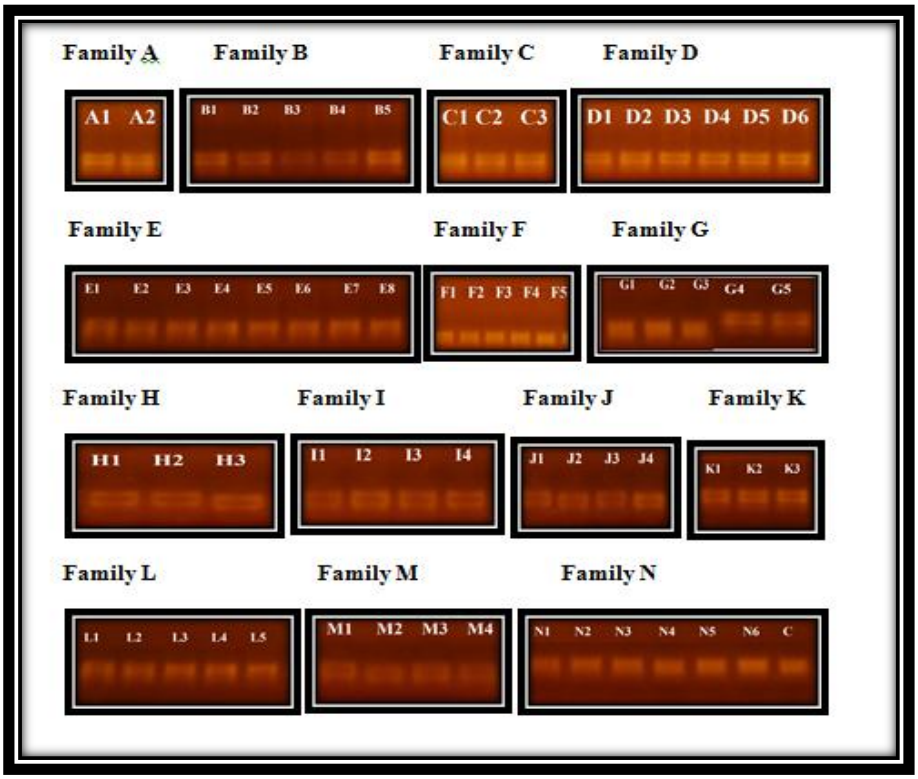

Figure 4.24: Agarose gel Electrophoresis indicating Genotyping of FTO rs8050136 for all the sampled families.

A study conducted on Indian population have found association of FTO rs8050136 and obesity (Chauhan et al., 2011). FTO rs8050136 shows no association with BMI and obesity in African-American population, and is significantly associated in Non-Hispanic white population (Bollepalli, Dolan, Deka, & Martin, 2010). The present study also indicates no association between the variant of FTO rs8050136. A study have found association of this SNP with fat intake that led to obesity (Park et al., 2013). A study that is similar to present study, have also found no association of this variant with overweight in Asian population (Liu et al., 2010).

4.4.4 MC4R rs17782313

The variant of MC4R on Chr18:6060183864 which is responsible for obesity phenotype. It was investigated for its association with BMI and obesity in Pakistani population. Its genotype results have shown in figure 4.25.

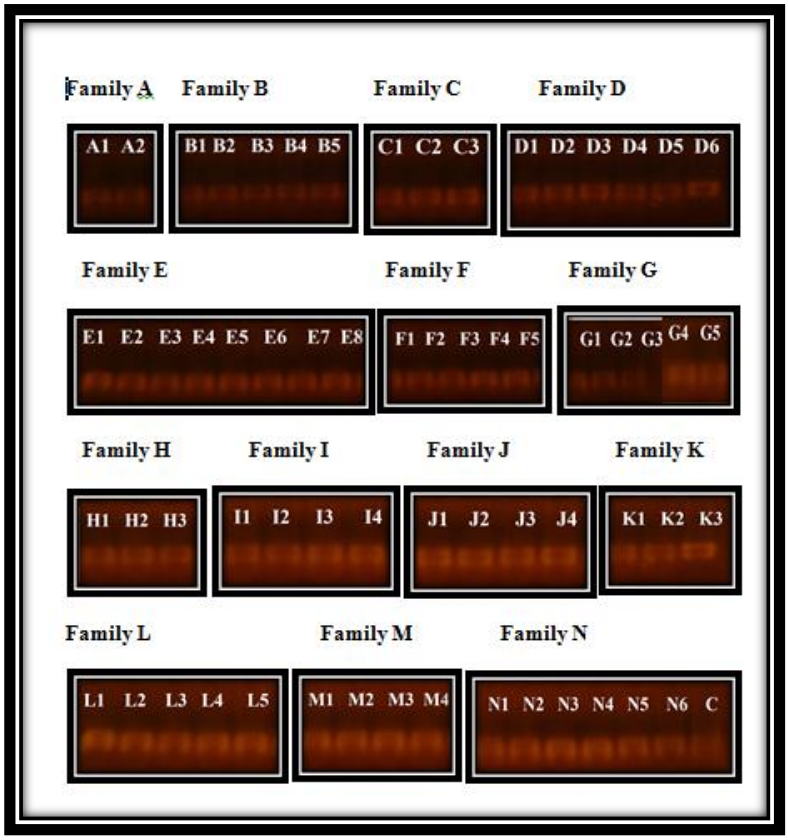

Figure 4.25: Agarose gel Electrophoresis indicating Genotyping of MC4R rs17782313 for all the sampled families.

Association between MC4R rs17782313 and obesity have found by a study in North India (Srivastava et al., 2014). Another study have also showed association of this SNP with obesity (Resende et al., 2017). It was also found by a study that MC4R is associated with BMI and overeating (Yilmaz et al., 2015). Some studies showed that MC4R rs17782313 is not associated with macronutrient intake (Park et al., 2013). The present study has not found any linkage of the MC4R rs17782313 with obesity. A study that is comparable to present findings shows no association of the MC4R SNP rs17782313 with obesity and BMI (Vasan et al., 2012). It was also found by a study that this SNP is not associated with BMI (Evans et al., 2014).

4.4.5 MC4R rs6567160

The variant of MC4R rs6567160 on chromosome position Chr18:60161902 is involved in obesity and BMI. It has checked for its role in obesity with Pakistani population. Its genotype result has shown in the figure 4.26.

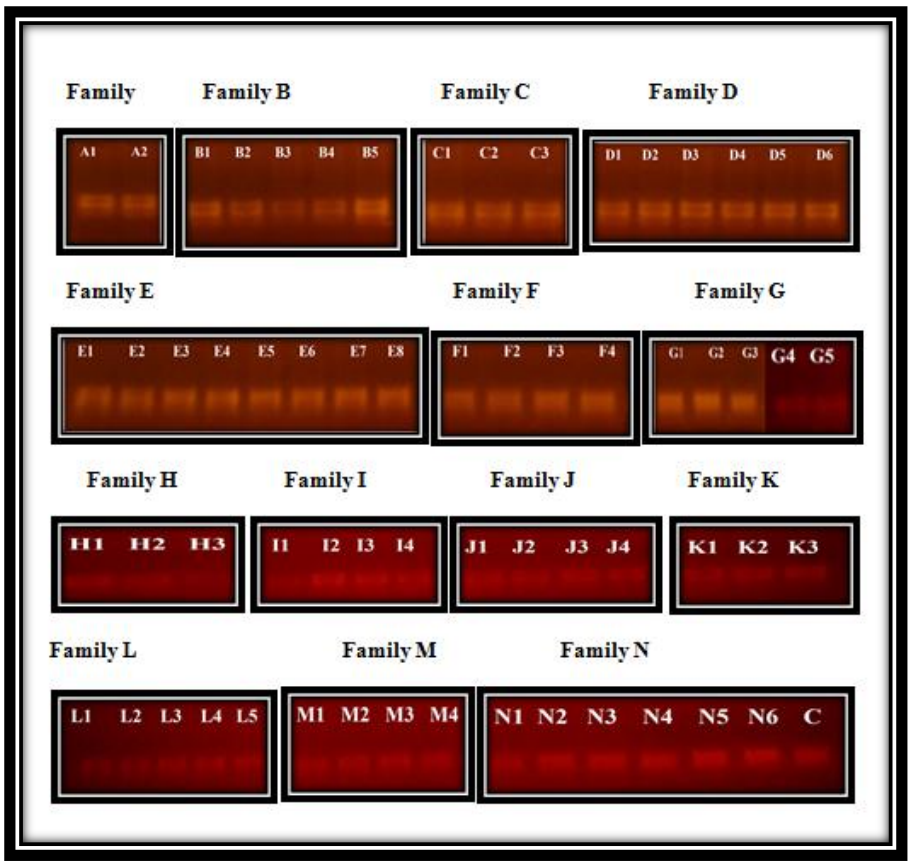

Figure 4.26: Agarose gel Electrophoresis indicating Genotyping of MC4R rs6567160 for all the sampled families.

SNP MC4R rs6567160 is found in association with BMI in African and American population but allelic heterogeneity exist (Evans et al., 2014). Another study which investigate this SNP for its association and they found significant association between this SNP and BMI (Sandholt et al., 2010). A meta-analysis of genome wide association study also identified this SNP in association with BMI (Pei et al., 2014). This SNP had identified in Asian Indians and in European ancestors by GWAS. It has also investigated that this SNP has relatively smaller effect on BMI and obesity and is less frequent (Hassanein et al., 2010). The present study identified no association of this SNP MC4R rs6567160 with obesity.

4.4.6 MC4R rs12970134

The intergenic SNP of MC4R rs12970134 on position Chr18:60217517 is also responsible for obesity and obesity related phenotype. It has investigated for its role in obesity in Pakistani population in this study. Its genotype result has shown in figure 4.27.

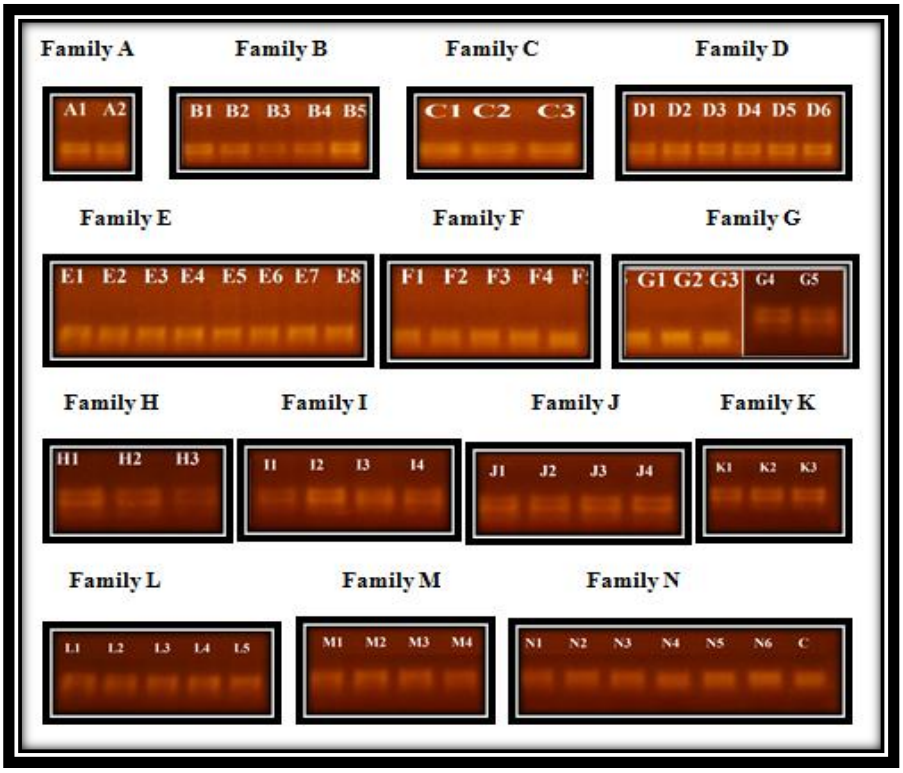

Figure 4.27: Agarose gel Electrophoresis indicating Genotyping of MC4R rs12970134 for all the sampled families.

A study conducted on Portuguese children demonstrated association of MC4R rs12970134 with BMI (Albuquerque, Nóbrega, Rodríguez-López, & Manco, 2014). Association of this SNP with BMI have successfully replicated by a study on Asian Sheikhs (Been et al., 2010). (Chambers et al., 2008) also found association between MC4R rs12970134 and obesity. A study that can be related to present study have not found association of MC4R rs12970134 with BMI (Evans et al., 2014). In present study this SNP also shows no linkage with obesity.

4.4.7 POMC rs713586

The SNP of gene POMC on position Chr2:24935139 is also playing role in obesity susceptibility. It has investigated for its Association with obesity in Pakistani kindreds. Its genotype result has shown in the figure 4.28.

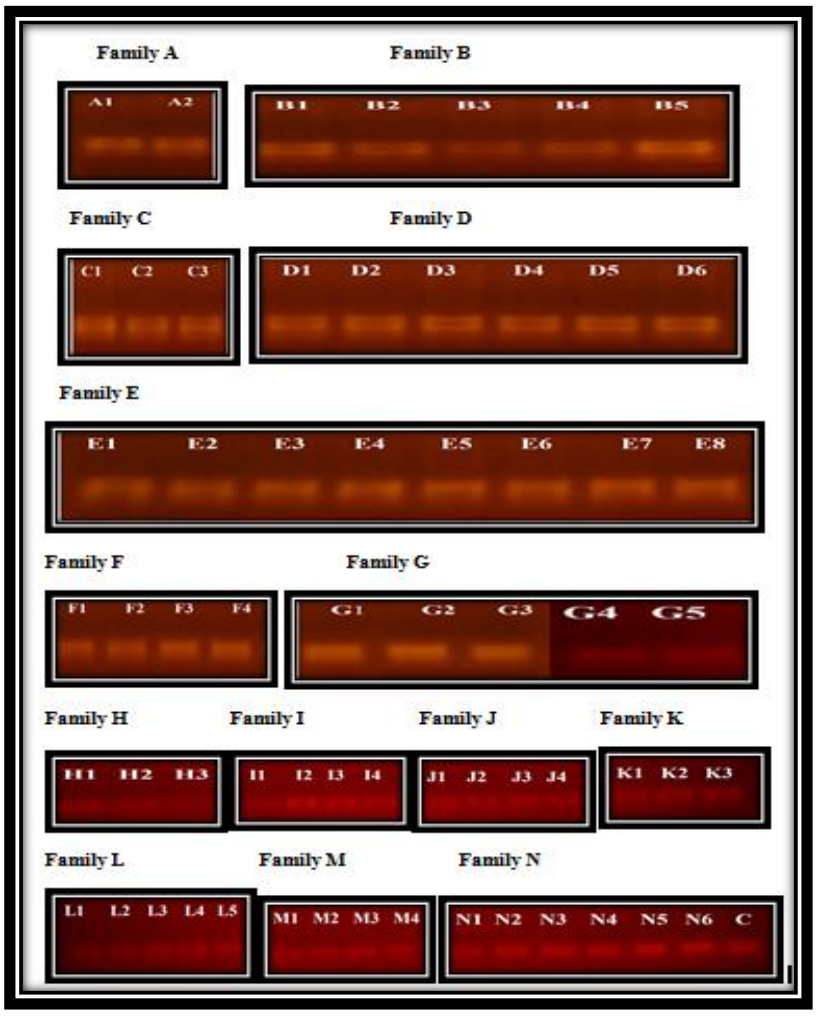

Figure 4.28: Agarose gel Electrophoresis indicating Genotyping of POMC rs713586 for all the sampled families.

The SNP of POMC rs713586 is playing role in the susceptibility of obesity and involved in the regulation of energy balance (Speliotes et al., 2010). Another study has identified no significance deference in the distribution of the variants of POMC between lean and obese persons (Raffan, Becker, Yeo, & O'Rahilly, 2014). The present study found no association of POMC rs713586.

CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

5. CONCLUSION AND FUTURE DIRECTIONS

Results of ARMS PCR of the present study demonstrated that SNPs of the FTO gene, rs9939609, rs1558902, rs8050136, SNPs of the MC4R gene, rs17782313, rs6567160, rs12970134, SNP of the LEPR rs11208659, and SNP of the POMC rs713586 have no association with obesity and BMI in Pakistani population. Further studies should perform to elucidate other causes of obesity in Pakistani population. Other SNPs, STR markers of these genes or other genes for obesity should be identified to explore the heterogenecity of the obesity in this population. Studies on geographical differences in relation with genetics should conduct to find out the causing agents of obesity due to this reason. Comorbidities and environmental factors related to obesity also needed to explore this multifactorial complex disease. The stutdy of these SNPs evaluate the value of genetic studies in geographically and ethnically different populations and suggest that more common and rare variants, epigenetic effect like gene-gene and gene environment interaction should identified for further elucidation of this heterogenetic phenotype.

As obesity is multigenetic complex multifactorial disease so further genetic studies are need to conduct to identify the causing agents of this phenotype. GWAS can be used for the identification of novel loci, variants, and their mechanism of action related with phenotype of obesity. Recent advanced methods like Epigenomics and Genome Editing can also be performed to understand the complex phenomena involvement of a single specific SNP and intron variants in obesity pathophysiology. Epigenome wide studies can also lead to the elucidation of this complex, multigenic, and multifactorial phenotype. Microarray Analysis can also be found helpful in the study and exploration of the causing effect of obesity.

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6. REFERENCES

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ANNEXURE I

PREVALENCE OF COMMONLY REPORTED SNPS IN PAKISTANI OBESE KINDREDS

QUESTIONARE

Project Title: **Association of obesity and commonly reported SNPs in Pakistani families**

Sponsor: Pakistan Science Foundation (PSF)

Investigator(s): Capital University of Science & Technology, Expressway, Kahuta Road, Zone-V, Islamabad. PHONES: +92-51-2512800-1, +92-51-4486700-4, FAX NUMBER: +92-51-4486705 UAN: +92-51-111-555-666 Extensions: 123,280,0

Instructions:

1. This survey form may contain words that are new to you. If you read any words that are not clear to you, please ask the person who gave you this form to explain them to you.
2. Your records will be kept confidential and will not be released without your consent except as required by law.
3. Your identity will be kept private.
4. If the results of this study are written in a scientific journal or presented at a scientific meeting, your name will not be used.
5. Your initials _____ indicate your permission to be identified by name in any publications or presentations.
6. If you do not want to be acknowledged by name in any publications or presentations, please initial here _____.
7. The data will be stored in a locked file cabinet.
8. Your signed consent form will be stored in a cabinet separate from the data.
9. Your decision to take part in this research study is entirely voluntary.
10. You may refuse to take part in or you may withdraw from the study at any time without penalty or loss of benefits to which you are normally entitled.
11. You may be asked to leave the study for any of the following reasons:
12. Failure to follow the Project Director's instructions;
13. A serious adverse reaction which may require evaluation;
14. The Project Director thinks it is in the best interest of your health and welfare; or
15. The study is terminated.
16. You may wish to discuss this with others before you agree to take part in this study.
17. If you have any questions about the research now or during the study, please contact: _____

1. **BIODATA:** (This information provided by Patient will be confidencial)

First Name: _____ Middle Name: _____

Last Name/Surname: _____ Date of Birth_____

Age:_____Gender:_____ _____

Contact No:

(Office)_____Home:_____

Cell:_____Email:_____ _____

Permenant Address:

Address:

_____

City:_____

Province:_____

Temporary Address:

Address:_____

_____

City: _____ **Province:**

_____

1. ANTHROPOMETRIC MEASUREMENT:

Weight (kg)	
Height (m)	
BMI (kg/m^2)	
HC (cm)	
WHR (WC/HC)	
Total cholesterol (TC)	
Triglycerides (TG) (mmol/l)	
HDL-C (mmol/l)	
LDL-C (mmol/l)	

WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

2. OBESITY RELATED COMPLAINTS

- High blood pressure Yes / No
  _____
- Diabetes Yes / No
  _____
- Heart disease Yes / No
  _____
- Eating disorder Yes / No
  _____

3. FAMILY HISTORY

	Severe Obesity	Heavy	Normal Weight	Diabetes	Heart Problem	Eating disorder
Father						
Paternal GrandFather						
Paternal GrandMother						
Father's Brothers						
Father's Sisters						
Mother						
Maternal GrandFather						
Maternal GrandMother						
Mother's brothers						
Mother's Sisters						
Your brothers						
Your sisters						
Yours sons						
Yours daughters						

4. PROGRESSION OF WEIGHT GAIN PATTERN

☐ No pattern

☐ Steady and gradual increase of weight over the years

☐ Sudden increases of weight with pregnancies

☐ Variable weight gain/loss due to intermittent diet and exercise

5. PHYSICAL ACTIVITY:

What is your exercise program?

- I am unable to exercise due to ☐ Severe joint pain
  ☐ Shortness of breath
  ☐ Wheelchair/bed
- I am able to exercise but I do not have a regular routine
- I walk / run _____ times per week for ____ minutes
- I swim _____ times per week for _____ minutes
- I lift weights ____ times per week for ____ minutes
- How often I climb up stairs ____daily____weekly____monthly
- How often I lift my arms up ____daily____weekly____monthly
- Walk for doing work at home/office_____
- Other

  _____

  _____

6. DIETARY HISTORY

What do you consider to be your daily eating pattern?

☐ than normal ☐ Normal ☐ Overeat

☐

☐ Binge ☐ Serious eating disorder Excessive snacking

- Do you eat/snack just before bedtime? Yes/No
- Which meals do you eat each day?

☐ Breakfast ☐ Lunch ☐ Supper ☐ Snacks

- What and how much do you usually eat for breakfast?

  _____

- What and how much do you usually eat for lunch?

  _____

- What and how much do you usually eat for supper?

  _____

- What are your favorite snacks?

  _____

- How much of them do you eat per sitting?

  _____

7. SOCIAL AND PERSONAL HISTORY

- Highest level of education:

  _____

- Occupation: _____

  Part time /Full time

- Do you have children? No / Yes - How many? _____
- Marital status:

  Single / Married /Separated / Divorced

8. MEDICAL/CLINICAL HISTORY

- Medication to control

  obesity_____

- Diet plan to control

  obesity_____

- Any surgery if yes when or for

  what_____

- Medicins using for any other

  diesease_____

9. **SAMPLES:**

- **Blood Sample:** ☐
- **Stool Sample:** ☐

Thank you for completing the questionnaire please return it to_____ **Faculty of Computing, Department of Bioinformatics and Biosciences, Capital University of Science and Technology, Islamabad.** If you have any concerns regarding this research please contact me or my supervisor in the first instance.

Consent

I have read the above description of this research study. I have been informed of the risks and benefits involved, and all my questions have been answered to my satisfaction. Furthermore, I have been assured that any future questions I may have will also be answered by a member of the research team. I voluntarily agree to take part in this study. I understand I will receive a copy of this consent form.

_____

_____

Subject's Signature Date

ANNEXURE II

Table 1: Table Of information of Physical and dietary History of family A

Serial No	Sample ID	Weight gain Pattern	Physical Activity	Dietary History	Exercise
1	A1	Steady	Yes	Normal	No
2	A2	Steady	Yes	Normal	No

Table 2: Table Of information of Physical and dietary History of family B

Serial No	Sample ID	Weight gain Pattern	Physical Activity	Dietary History	Exercise
1	B1	Steady	Yes	Normal	No
2	B2	Variable	No	Normal	No
3	B3	Variable	Yes	Normal	No
4	B4	Steady	No	Normal	No
5	B5	Steady	No	Over Eating	No

Table 3: Table Of information of Physical and dietary History of family C

Serial No	Sample ID	Weight gain Pattern	Physical Activity	Dietary History	Exercise
1	C1	Steady	No	Normal	No
2	C2	Steady	No	Normal	Yes
3	C3	Steady	No	Normal	Yes

Table 4: Table of information of physical activity and dietary history.of family D

Serial No	Sample ID	Weight gain Pattern	Physical Activity	Dietary History	Exercise
1	D1	Gradual	No	Normal	No
2	D2	Gradual	No	Normal	No
3	D3	Gradual	Yes	Normal	No
4	D4	Gradual	No	Normal	No
5	D5	Gradual	No	Normal	No
6	D6	Gradual	Yes	Normal	No

Table 5: Data of the family E about their dietry history, physical activity, and weight gain pattern.

Serial No	Sample ID	Weight gain Pattern	Physical Activity	Dietary History	Exercise
1	E1	Gradual	No	Normal	No
2	E2	Gradual	Yes	Normal	No
3	E3	Variable	Yes	Normal	No
4	E4	Gradual	Yes	Over Eating	No
5	E5	Gradual	No	Normal	No
6	E6	Gradual	No	Normal	No
7	E7	Gradual	Yes	Normal	No
8	E8	Normal	Yes	Normal	No

Table 6: Table shows dietry history, physical activity, and weight gain pattern of Family F.

Serial No	Sample ID	Weight gain Pattern	Physical Activity	Dietary History	Exercise
1	F1	Variable	Yes	Normal	Yes
2	F2	Sudden increase	No	Normal	No
3	F3	Normal weight	Yes	Normal	Yes
4	F4	Steady	Yes	Normal	No
5	F5	Steady	Yes	Normal	No

Table 7: Information of weight gain pattern, exercise, and diet related with family G.

Serial No	Sample ID	Weight gain Pattern	Physical Activity	Dietary History	Exercise
1	G1	Steady	Yes	Normal	No
2	G2	Steady	Yes	Normal	No
3	G3	Variable	Yes	Normal	No
4	G4	Steady	Yes	Normal	No
5	G5	Steady	Yes	Normal	No

Table 8: Physical and Dietary History of family H

Serial No	Sample ID	Weight gain Pattern	Physical Activity	Dietary History	Exercise
1	H1	Steady	No	Over eat	No
2	H2	No pattern	Yes	Normal	Yes
3	H3	Sudden increase due to pregnancy	No	Serious eating disorder	No

Table 9: Physical and Dietary History of family I

Serial No	Sample ID	Weight gain Pattern	Physical Activity	Dietary History	Exercise
1	I1	Steady	Yes	Overeat	Yes
2	I2	Steady	Yes	Normal	Yes
3	I3	Steady	No	Overeat	No
4	I4	Steady	No	Overeat	No

Table 10: Physical and Dietary History of family J

Serial No	Sample ID	Weight gain Pattern	Physical Activity	Dietary History	Exercise
1	J1	No pattern	Yes	Normal	Yes
2	J2	No pattern	Yes	Normal	Yes
3	J3	Steady	No	Overeat	No
4	J4	Steady	Yes	Overeat	Yes

Table 11: Physical and Dietary History of family K

Serial No	Sample ID	Weight gain Pattern	Physical Activity	Dietary History	Exercise
1	K1	Steady	Yes	Normal	Yes
2	K2	Steady	No	Normal	No
3	K3	Steady	No	Normal	No

Table 12: Physical and Dietary History of family L

Serial No	Sample ID	Weight gain Pattern	Physical Activity	Dietary History	Exercise
1	L1	Steady	No	Overeat	No
2	L2	Steady	No	Normal	No
3	L3	Steady	No	Overeat	No
4	L4	Steady	Yes	Normal	No
5	L5	Steady	No	Overeat	No

Table 13: Physical and Dietary History of family M

Serial No	Sample ID	Weight gain Pattern	Physical Activity	Dietary History	Exercise
1	M1	No pattern	Yes	Normal	Yes
2	M2	Steady	Yes	Normal	Yes
3	M3	Sudden increase with pregnancy	Yes	Normal	Yes
4	M4	Steady	Yes	Normal	Yes

Table 14: Physical and Dietary History of family N

Serial No	Sample ID	Weight gain Pattern	Physical Activity	Dietary History	Exercise
1	N1	Steady	Yes	Overeat	Yes
2	N2	Steady	Yes	Overeat	Yes
3	N3	Steady	Yes	Overeat	No
4	N4	Steady	Yes	Overeat	No
5	N5	Steady	Yes	Overeat	No
6	N6	Steady	No	Normal	No