

**CALPAIN 10 GENE SNP-44 T>C POLYMORPHISM
IN TYPE 2 DIABETES MELLITUS
OF BANGLADESHI ORIGIN**



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DECLARATION OF ORIGINALITY OF THE WORK

This is to confirm that the Thesis entitled '**Calpain 10 gene SNP-44 T>C polymorphism in type 2 diabetes mellitus of Bangladeshi origin**' is submitted in partial fulfillment for the degree of Masters in Biotechnology, Department of Mathematics and Natural Sciences, BRAC University, Dhaka, was carried out in the Laboratory of BMRG, BIRDEM during the period of June 2009 to March 2010.

No part of the work has been submitted for another degree or qualification in any other institutes at home or in abroad.



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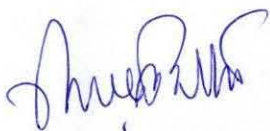
CERTIFICATION OF ORIGINALITY OF THE WORK

This is to confirm that the Thesis entitled '**Calpain 10 gene SNP-44 T>C polymorphism in type 2 diabetes mellitus of Bangladeshi origin**', a requirement for partial fulfillment of the degree of Masters of Science (MS) in Biotechnology under the Dept of Mathematics and Natural Science, BRAC University was carried out in the Laboratory of Biomedical Research Group, BIRDEM, Dhaka during the period of June 2009 to March 2010, under our joint supervision.

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TO

MY BELOVED PARENTS

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ABSTRACT

Insulin resistance and/or B cell secretory defect have been accepted as the fundamental features in the pathogenesis of type 2 diabetes mellitus, though the exact molecular defect affecting the insulin secretion or sensitivity still to be clearly elucidated. The present study was undertaken to determine CAPN 10 gene SNP-44 T>C polymorphism in Bangladesh type 2 diabetes mellitus and explore its relationship with its insulin secretory capacity and insulin sensitivity. A total number of 65 T2DM subjects were recruited and 91 subjects served as control. Glucose was measured by glucose-oxidase, lipids by enzymatic-colorimetric, insulin by ELISA methods. Homeostatic model assessment was used to calculate pancreatic B cell secretion, expressed on HOMA%B and insulin sensitivity, HOMA%S. DNA was extracted using QIAGEN spin column blood DNA kit. CAPN 10 gene polymorphic marker was determined by mutation specific PCR. Data were managed by statistical package for social science (SPSS). $P < 0.5$ was taken as level significance. T2DM and Control subjects were age and BMI matched. Fasting serum insulin ($\mu\text{U/ml}$, mean $\pm$ SD) in the T2DM subjects was significantly higher compared to controls (7.97 ± 3.83 vs 5.01 ± 0.57). Insulin secretory capacity (HOMA%B) in the T2DM group and the controls did not show any significant difference ($p = 0.240$). Insulin sensitivity (HOMA%S) was significantly lower in the T2DM group compared to the controls (48.9 ± 21.7 vs 79.9 ± 20.5) ($p < 0.001$). CAPN10 gene SNP-44 genotype frequencies for the T2DM were 0.662, 0.323 and 0.150 for Wild, Ht and Hz variant respectively and in the controls the frequencies were 0.670, 0.297 and 0.330 respectively, which did not show any significant association with diabetes mellitus. Subjects with variant (TC and TT) genotype did not show significant difference regarding glucose, BMI, HOMA%B, HOMA%S either in the T2DM or control group. In conclusion (i) CAPN 10 SNP 44 T>C polymorphism was not associated with T2DM of Bangladeshi origin. (ii) Insulin sensitivity appeared to be predominant in the subsets of T2DM subjects, and (iii) CAPN 10 SNP 44 T>C variant did not show any relation with pancreatic B cell secretion and insulin sensitivity of the study subjects.

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LIST OF ABBREVIATIONS

ADA	American Diabetes Association
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
BFM%	Body Fat Mass
BIRDEM	Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders
BMI	Body Mass Index
<i>CAPN 10</i>	Calpain 10
DBP	Diastolic Blood Pressure
DM	Diabetes Mellitus
DZ	Dizygotic Twin
EDTA	Ethylene Diamine Tetracetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
FFA	Free Fatty Acid
FSG	Fasting Serum Glucose
GDM	Gestational Diabetes Mellitus
GLUT 2	Glucose Transporter 2
GLUT 4	Glucose Transporter 4
HDL	High Density Lipoprotein
HDL-c	High Density Lipoprotein Cholesterol
HLA	Human Leukocyte Antigen
HOMA %B	Homeostasis Model Assessment B-cell Function
HOMA %S	Homeostasis Model Assessment Insulin Sensitivity
Ht	Heterozygous
Hz	Homozygous
IDF	International Diabetes Federation
IGT	Impaired glucose tolerance
Kg	Kilogram
LDL	Low Density Lipoprotein
MAC	Mid Arm Circumference
MHC	Major Histocompatibility Complex
μl	Microliter
MIM	Mendelian Inheritance in Man
MODY	Maturity Onset Diabetes in Young

MZ	Monozygotic Twin
mRNA	Messenger Ribonucleic Acid
NGT	Normal Glucose Tolerance
NIDDM	Non-insulin Dependent Diabetes Mellitus
OGTT	Oral Glucose Tolerance Test
OR	Odds Ratio
PCR	Polymerase Chain Reaction
PPG	Post Prandial Glucose
RFLP	Restriction Fragment Length Polymorphism
SBP	Systolic Blood Pressure
SD	Standard Deviation
SGPT	Serum Glutamic Pyruvic Transaminase
SNARE	Soluble N-ethyl Maleimide Sensitive Fusion Protein Attachment Receptor
SNP	Single Nucleotide Polymorphism
SPSS	Statistical Package for Social Sciences
SSF	Sub-scapular Skin Fold Thickness
STR	Sub Scapular Triceps Ratio
T Chol	Total Cholesterol
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TG	Triglycerides
TSF	Triceps Skin Fold
UCSNP	Unique Combination Single Nucleotide Polymorphism
VLDL	Very Low Density Lipoprotein
WHO	World Health Organization
WHR	Waist Hip Ratio

Chapter 1

INTRODUCTION

INTRODUCTION

Diabetes Mellitus (DM) is chronic metabolic disorders mainly characterized by high rise in blood sugar levels, affecting multiple organ systems (WHO, 1999). This condition may arise mainly due to impairment of insulin action, insulin secretion or both (ADA, 2005). The World Health Organization (WHO) sponsored estimate, showed in 1995 that there were 135 million diabetic individuals and projected the number to increase around 300 million by the year 2025 (King *et al.*, 1998). Subsequent study estimated the global prevalence of diabetes for all age-groups to rise from 2.8% in 2000 to 4.4% in 2030 and also demonstrated the total number of diabetics to rise from 171 million in 2000 to 366 million in 2030. It was also shown that about two third of the rise in total diabetic patients would be constituted from those of the developing countries particularly China, India, Indonesia, Pakistan and Bangladesh (Wild *et al.*, 2004). Based on recent community based study, prevalence of diabetes in Bangladesh was found to be about 7%. Studies involving rural and urban population revealed the prevalence of diabetes 4% and 11.4% respectively (Sayeed *et al.*, 2007).

Classification of Diabetes Mellitus

Classification of diabetic patients has been a difficult task. In early days it used to be classified only the basis of age of onset and need for insulin of the individual to control blood glucose. In early 50's two major types of diabetes was introduced – Type 1 Diabetes Mellitus (T1DM) and Type 2 Diabetes Mellitus (T2DM) (Lawrence, 1951). However, this did not solve the variations showed by the diabetics over the years of diagnosis. Thus in late 70's National Institute of Health (NIH) and United States of America (USA) collectively introduced National Diabetes Data Group for formulation of classification and diagnosis of diabetes (NDDG, 1979). Subsequently there have been major revisions in diagnostic criteria and classification of diabetes by committees from both ADA and WHO (WHO 1985 and 1999; ADA 1997). The present diabetes classification, originally proposed by ADA (1997) and later endorsed by WHO (1999) mainly considered etiological basis in classifying diabetes.

According to present classification diabetes are mainly four types which are shown in table 1.

Table 1: Etiological Classification of Diabetes Mellitus (WHO, 1999)

-
- A. Type 1 (beta- cell destruction, usually leading to absolute insulin deficiency)
- Type 1A- Autoimmune mediated type
 - Type 1B- Non- immune mediated idiopathic type
- B. Type 2 (may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with or without insulin resistance).
- C. Other specific types
- D. Gestational diabetes
-

Type 1 Diabetes Mellitus (T1DM)

T1DM is characterized by abrupt onset, mainly occurs before the age of 20 years (i.e. in childhood or adolescence), but may be diagnosed at any age, usually to patients who are lean. It contributes to about 10% of the diabetics' worldwide (ADA 1997). The incidence of T1DM has been found to be lowest in Asia (0.1 per 1000,000 in China) and highest in Scandinavia (35.5 per 1000,000 in Finland). The underlying pathophysiologic defect in T1DM is mainly thought to be resulting from selective B cells. These patients usually have very low level of endogenous insulin and need insulin supplementation for survival.

Type 1A DM: T1DM are found to be associated with number of islet specific autoantibodies. These include insulin autoantibody (IAA), Islet cell antibody (ICA), glutamic acid decarboxylase (GAD65) antibody and anti tyrosine phosphatases (Anti-IA₂) antibody. One or more of these autoantibodies are found to be present in 85–90% of individuals when fasting hyperglycemia is initially detected. It also has strong HLA associations, with linkage to the DQA and DQB genes (Donner *et al.*, 2000), and it is influenced by the DRB genes. These HLA-DR/DQ alleles can be either predisposing or protective.

Type 1B DM: Some of these patients have permanent insulinopenia and are prone to ketoacidosis, but have no evidence of association of autoimmunity in its pathogenesis. Although only a minority of patients (usually obese teen) with T1DM falls into this

category, of which are mostly African and Asian in ancestry (McLarty *et al.*, 1990; Tanaka *et al.*, 2000).

Type 2 Diabetes Mellitus

T2DM is characterized by impairment in insulin secretion and resistance to insulin action/or both. It is the most common form of diabetes and one of the most frequent metabolic disorders worldwide that contributes to more than 95% of all diabetics in most populations.

In American Indians and South Pacific Islanders, T2DM is the only form of the disorder. Overall prevalence of diabetes varies between 15-20%. The highest prevalence of T2DM (50%) was found among Pima Indians (49.4% in male and 51.1% in female) in USA and Nauru (41%; male 40.0% and female 42%) and a very low (0-1.4%; male 0% and female 1.4%) was observed among the Mapuches population in Chile and the prevalence was almost nil in rural and peri-rural population of Papua New Guinea (WHO 1994). In the non-Euripides population the prevalence has been shown to vary from <1% in rural Bantu of Tanzania and Mainland China to 40-50% in Pima Indians (USA) and Nauru (King and Rewers, 1993). Age-standardized prevalence of T2DM among native Asians and non-Indian Asians demonstrated an increased trend from rural to urban and markedly increased trend among the migrants to the west and affluent countries. A similar trend also has been observed among native and migrant Pacific Island populations (Coughlan *et al.*, 1997). In multiethnic US society the prevalence of diabetes has shown to vary among different ethnic groups; 5% in the Caucasian origin, 10% in African-Americans, 16% in Cuban origin and 26% in Puerto Ricans (Harris 1991; Flegal *et al.*, 1991). Studies involving rural and suburban population in Bangladesh revealed the prevalence of T2D to be 4.3% and 4.1% respectively (Sayeed *et al.*, 1997 and 2003).

Pathological basis of Diabetes Mellitus

Environmental factors are found to play strong role in genetically susceptible cases in the pathogenesis of diabetes in both type 1 and type 2 variety.

Environmental factors

A number of environmental factors implicated in the pathogenesis of type 1 and type 2 varieties, which include age, diet, and viral infection and stress (Knip and Akerblom 1999; Dahlquist 1998).

Age: Glucose tolerance has been shown to decrease with age (Shimokata *et al.*, 1991). However, whether the deterioration is caused by increased chronological age or secondary age related factors, such as decreased physical activity, is still to be clearly understood.

Virus: Coxsackie virus B (CVB), rubella, cytomegalovirus (CMV) and Epstein-Barr viruses (EPV) are the most important viruses that have been implicated in the pathogenesis of diabetes. Infections through these viruses are known to have systemic effects on the pancreas. Coxsackie B4 virus is strongly associated with the development of T1DM in humans and shares sequence similarity with the islet GAD65 (Horwitz *et al.*, 1998). About 12-20% patients with congenital rubella syndrome (CRS) develop T1DM within 5-20 years of age. CMV and EPV have also been associated with newly diagnosed T1DM (Hassan, 2006).

Diet: Diets with high-fat, low-carbohydrate impair the action of insulin on endogenous glucose production, glucose oxidation, and probably lipolysis but it does not clearly affect the action of insulin on total glucose disposal and tend to enhance the action of insulin on non-oxidative glucose disposal (Bisschop *et al.*, 2001). Intakes of excessive amounts of saturated fats have lead to obesity in individuals, who were found to be more susceptible to develop insulin resistance (Lovejoy *et al.*, 2001).

Obesity: The major environmental risk factor for T2DM is obesity and a sedentary lifestyle (Bleich *et al.*, 2007). Thus, the tremendous increase in the rates of T2DM in recent years has been attributed, primarily, to the dramatic rise in obesity worldwide (Zimmet *et al.*, 2001). It has been estimated that approximately 80% of all new T2DM cases are due to obesity (Lean, 2000). This is true for adults and children. In the Pima Indians, 85% of the T2DM children were either overweight or obese (Fagot-Campana *et al.*, 2000). Although excess weight and obesity (BMI>25 kg/m²) account for 64% cases of diabetes in men and 74% in women, many cases of diabetes occur in lean subjects (Chan *et al.*, 1994; Colditz *et al.*, 1995).

Sex: Some studies had showed a higher age-adjusted prevalence in women (Eriksson *et al.*, 1993), where as some investigators demonstrated male preponderance (Lintott *et al.*, 1992). Variation in T2DM prevalence between men and women also has been shown to vary in different age group in the same community.

Stress: Stress has long been suspected as having important effects on the development of diabetes. Slawson et al (1963) showed that 80% of a group of 25 adult diabetes patients gave a history of antecedent stress mainly in terms of losses, 1-48 months prior to the onset of diabetes. These were mostly patient of depression. Metabolic stress may develop due to disproportionate amount of free radical productions in diabetes by glucose autoxidation, polyol pathway and non-enzymatic glycation of proteins. In a study, oxidative stress pathway markers in the diabetic patients, type 1 and type 2, was compared to normoglycemic subjects. The increased level of glycosylated hemoglobin was observed in the diabetic patients and this increase is directly proportional to the blood glucose level (Strain *et al.* 1991). This suggests the increase in oxidative stress due to hyperglycemia.

Hypoinsulinaemia in diabetes increases the activity of the enzyme fatty acyl coenzyme A oxidase, which initiates β -oxidation of fatty acids, resulting in lipid peroxidation, which impairs membrane function changing the activity of membrane-bound enzymes and receptors (Halliwell *et al.*, 1997).

Genetic factors

Genetic susceptibility has been implicated in the pathogenesis of both Type 1 and Type 2 diabetes. T1DM accounts for approximately 5–10% of all cases of diabetes with the major susceptibility gene mapping to the HLA region of chromosome 6 (Herr *et al.*, 2000). It has been estimated that HLA provides up to 40-50% of familial clustering in T1DM (Noble *et al.*, 1996). The HLA region is a cluster of genes located with major histocompatibility complex (MHC) on chromosome 6p21. The HLA complex is classically divided into four regions, known as class I, class II, class III and class IV (Gruen and Weissman, 1997). The class II MHC region encodes the class II HLA molecules which are involved in immune process and turn out to be important candidate in the pathogenesis of T1DM. Among other gene locus found to be implicated with T1DM are in chromosome 4p15 (Sawicki *et al.*, 1997; Hayes *et al.*, 1998), chromosome 2q13, chromosome 2q3-2q34 (Morahan *et al.*, 1996; Larsen *et al.*, 1999) and chromosome 6q21 (Temple *et al.*, 2000).

Family aggregation of the disease showed higher prevalence for T2DM. In T1DM cases more than 80% of the newly diagnosed individuals had no familial diabetic history (WHO 1999). However, the children of T1DM mothers showed lower risk to diabetes

compared to T1DM fathers ($1.7 \pm 0.6\%$ vs $4.1 \pm 0.9\%$) (Tilli and Koberling, 1987). In a study, siblings were shown to have 10.4% prevalence to T2DM, but if one or both parents were affected the prevalence was 17.8% and 25.2% respectively (Klein *et al.*, 1996). In another study, life-time risk of developing T2DM in offsprings was 40% with one diabetic parent and 70% with both parents have the disease (Groop *et al.*, 1996). The CODIAB Study in France showed that 66% T2DM patients had at least one relative with diabetes. Diabetes was found to be 4-fold increased risk for T2DM in siblings of a diabetic proband compared with the general population (λ_S of 3.5 to 4), the odds ratio (OR) of 3.4–3.5 with only a single affected parent, and the increase in the OR to 6.1 if both parents are affected (Meigs and Cupples 2000).

Multiple studies of twin concordance rates have been undertaken in understanding of the genetic basis of diabetes. The concordance rate for T1DM among monozygotic twins was reported between 21% to 70% (Barnett *et al.*, 1981) and 13% in dizygotic twins (Kumar *et al.*, 1997). In the same study by Barnett *et al.*, (1981) showed concordance rate for T2DM in monozygotic twins to be 91%. Estimates for concordance rates have ranged from 0.29 to 1.00 in monozygotic (MZ) twins, while in dizygotic (DZ) twins the range was 0.10–0.43 (Barnett *et al.*, 1981; Newman *et al.*, 1987; Poulsen *et al.*, 1999). Concordance among both MZ and DZ twins increases with the duration of follow up period (Medici *et al.*, 1999). In spite of several caveats in twin studies, the high concordance in MZ twins and the 50% fall in DZ twins provide compelling evidence for a genetic component of T2DM.

Monogenic form accounts for approximately 10% of all cases of the condition constitute a field where substantial progress has been made in the dissection of the molecular background of T2DM. Monogenic form of T2DM with profound defect in insulin secretion include subtypes of maturity onset diabetes of the young (MODY), maternally inherited diabetes with deafness (MIDD) caused by mitochondrial mutations, and rare cases resulting from insulin gene mutations. The majority of proteins associated with MODY are transcription factors, such as hepatocyte nuclear factor 4 α (HNF-4 α), HNF-1 α , insulin promoter factor-1 (IPF-1), HNF-1 β , and NEUROD1. They influence expression of the other genes through regulation of mRNA synthesis. Only MODY2 form is associated with glucokinase, a key regulatory enzyme of the β cell. There are striking differences in the clinical picture of MODY associated with glucokinase and MODY associated with transcription factors. Three monogenic forms of T2DM

characterized by severe insulin resistance are the consequence of mutations in the PPAR γ , ATK2, and insulin receptor genes. These forms of T2DM have a middle/late age of onset and occur with both impaired insulin secretion and insulin resistance. Their clinical picture is created by the interaction of environmental and genetic factors, such as frequent polymorphisms of many genes, not just of one. These polymorphisms may be localized in the coding or regulatory parts of the genes and are present, although with different frequencies, in T2DM patients as well as in healthy populations. Sequence differences in a few genes have been associated, so far, with complex, polygenic forms of T2DM, for example, calpain 10, PPAR γ , KCJN11, and insulin. In addition, some evidence exists that genes, such as adiponectin, IRS-1, and some others may also influence the susceptibility to T2DM (Malecki, 2005).

No T2DM gene susceptibility has been identified as that of the magnitude of HLA in T1DM. However, several chromosomal regions have been found in strong linkage with T2DM. Some of the loci have shown linkage in more than one population. Important hotspots for T2DM have been identified on chromosome 2q37 (Hanis *et al.*, 1996), chromosome 12q24 (Mahtani *et al.* 1996), chromosome 20q12-q13 (Bowden *et al.*, 1997), chromosome 1q21-q23, chromosome 3q27 (Vionnet *et al.*, 2000) and chromosome 8q21.3-22 (Wiltshire *et al.*, 2001). These factors have been primarily sought using the candidate gene approach and the genome-wide scan approach.

To date, more than 50 candidate genes for T2DM have been studied in various populations worldwide. However, results for essentially all candidate genes have been conflicting. Use of this approach has identified a number of candidate T2DM susceptibility genes such as PPARG, KCJN11, ABCC8 and CAPN 10 (Barroso, 2005). Positional cloning of genes have identified CAPN10 as the only candidate till date, in the T1DM region of chromosome 2 to be associated with T2DM (Horikawa *et al.*, 2000).

The first such study conducted a genome wide scan on 170 Mexican-American families and found linkage near the terminus of chromosome 2q37 (lod = 3.2, $P < 10^{-4}$). This susceptibility locus, NIDDM1, originally encompassed 10–20 cM, but the target region was subsequently narrowed to 7cM. A genomic contig was constructed encompassing most of the 7 cM NIDDM1 interval that is likely to include a predisposing gene for T2DM (Horikawa *et al.*, 2000). The authors identified a total of 214 polymorphic sites across this region and tested for association with T2DM and for evidence of linkage. At

NIDDM1, five singlenucleotide polymorphisms (SNPs) showed a difference for allelic frequencies between patients and controls, so Horikawa et al (2000) sequenced approximately 60 kb in this region, revealing two genes and one expressed sequence tag. One gene, designated calpain 10 (CAPN10), encodes a new member of the cysteine protease family, and the other encodes G protein-coupled receptor 35 (GPR35). Both of these genes were shown to be ubiquitously expressed in tissues, including pancreatic islets and skeletal muscle. None of the coding region SNPs in CAPN10 or in GPR35 was associated with the disease or with the evidence for linkage. However, one SNP (UCSNP-43) in intron 3 of CAPN10 showed an increase in the frequency of the common

CALPAIN 10

Calpains are a family of calcium-activated, neutral, nonlysosomal proteases. They have been proposed to be implicated in the regulation of a variety of cellular functions and may be responsible for adipocyte differentiation (Patel and Lane, 1999) as well as insulin-induced down-regulation of insulin receptor substrate-1, a key mediator of insulin action (Smith *et al.*, 1996).

CAPN10 is a member of the calpain superfamily (Guroff, 1964; Dayton *et al.*, 1976). Calpains are often subdivided into three separate classes, the typical calpains (calpain 1, 2, 3, 8a, 9, 11, 12 and 13), the calpain small subunits (calpain 4 and 14) and the atypical calpains (calpain 5, 6, 7, 8b, 10 and 15). This classification is based upon domain structure (Figure 1). The large subunit contains domains (D) I, II, III and IV. Domain II comprises the active proteolytic domain, domain III contains the C2-like region, and domain IV contains multiple Ca^{2+} /calmodulin-like (CaM) EF hands. The smaller subunit consists of domains V and VI, the latter also containing multiple Ca^{2+} /calmodulin-like EF hands. Domain structure resembles that of the large subunit of typical calpain, with the notable exception of the presence of a T-domain of an unknown function in place of domain IV. Atypical calpains are monomeric, and hence have no small subunit. Domains which contain Ca^{2+} -binding sites are present in both typical and atypical calpains.

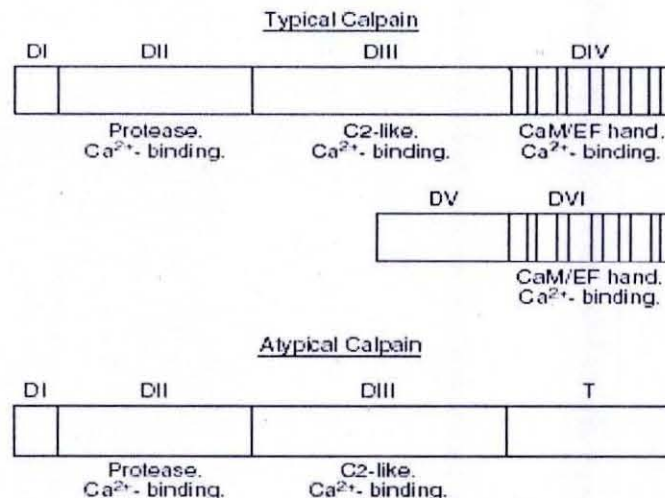


Figure 1. Calpain protein domain structure and function (Turner *et al.*, 2005).

Ma et al (2000) first cloned calpain 10 in 2001 and proposed that this calpain isoform was localized to the cytosol and translocated to the nucleus after increases in cellular Ca^{2+} . Arrington et al (2006) revealed calpain 10 to be present in the cytosol and mitochondria, while significant nuclear staining is absent under nonstimulated conditions.

Calpain 10 levels in tissues vary; are highest in the heart, followed by the brain, liver, kidney, and pancreas in humans. At least eight splice variants of calpain 10 (calpains 10a through 10h) have been identified, including three variants that lack intact domain II and thus have no protease activity (Horikawa *et al.*, 2000). Some calpain 10 is detectable in the soluble fraction by Western blotting using an antibody raised against a synthetic peptide, but it is preferentially found in the water-insoluble fraction in rat tissues (Ma *et al.*, 2000).

Short-term exposure of mouse pancreatic islets to calpain inhibitors enhanced glucose-induced insulin secretion but reduced insulin-stimulated glucose uptake into adipocytes and skeletal muscle, and reduced glycogen synthesis rates in muscle. This partly supports the above consideration, but the exact site of calpain action in the insulin-signaling pathway is not clear. But as calpain inhibition showed similar reduced effects on a hypoxia-associated increase in glucose uptake, which is not mediated by the insulin receptor system, the calpain regulated step might not be directly involved in this pathway (Itani *et al.*, 2000). The protease activity of calpain 10 has not yet been analyzed. Its

calcium sensitivity appears to be significantly different from typical calpains on the basis of the structure.

CAPN10 polymorphism has been associated with T2DM as well as insulin action, insulin secretion, aspects of adipocyte biology and microvascular function. However, this has not always been with the same single nucleotide polymorphism (SNP) or haplotype or the same phenotype, suggesting that there might be more than one disease-associated CAPN10 variant and that these might vary between ethnic groups and the phenotype under study. Calpain-10 is also an important molecule in the beta-cell. It is likely to be a determinant of fuel sensing and insulin exocytosis, with actions at the mitochondria and plasma membrane respectively. The multiple actions of calpain-10 may relate to its different protein isoforms.

Calpain plays roles in membrane fusion (Barnoy *et al.*, 1999) and hydrolyzes various proteins that participate in cellular signaling, such as kinases, receptors, and transcription factors. Further, calpain is also important for differentiation of preadipocytes into adipocytes. These facts raise the possibility that calpain modulates both insulin secretion and action. In skeletal muscle, liver, and pancreas, several calpain species are expressed, including typical and atypical calpains. Studies on the action of calpain using calpain inhibitors suggest that calpain species other than calpain 10 also participate in insulin secretion and action and susceptibility to T2DM. Because many steps could be modulated by calpain, further precise experiments are essential to clarify the molecular and physiological mechanism explaining the association of calpain with type 2 diabetes.

CALPAIN 10 (CAPN 10) GENE

The human CAPN 10 located on chromosome 2q37.3 comprises 15 exons and spans 31Kb of genomic sequence. This sequence encodes the 672 amino acids of the full-length calcium-dependent cysteine proteases, participating in hydrolysis of specific substrates important in calcium-regulating signaling pathways. A genetic variant of CAPN10 may alter insulin secretion, insulin action, and the production of glucose by the liver (Horikawa *et al.*, 2002).

Genetic variants in the CAPN 10 found to be associated with elevated free fatty acids and insulin resistance (Ortho-Malender, 2002). In vitro studies have shown that free fatty

acids activate protein kinase C, which results in hyperphosphorylation of insulin receptors, leading to reduction in the kinase activity of insulin receptors and thus enhancing insulin resistance (Griffin *et al.*, 1999). Therefore, downregulation of protein kinase C activity appears to be an important factor to maintain proper phosphorylation levels of insulin receptors (Itani *et al.*, 2000).

Polymorphism within intron 3 of CAPN 10 (SNP-44) has been suggested to be located as an enhancer element and might regulate CAPN 10 expression. A meta-analysis showed that the SNP-44 allele 2 was associated with the increased risk of T2DM (Song *et al.*, 2007).

SNP-44 results by a T504A substitution in domain III (domain T) of calpain 10 amino acid sequence, suggesting that the synthesis of a mutant protein and/or altered transcriptional regulation could contribute to diabetes risk. A combination study comparing SNP-44 with SNP-19, -43, -63, showed greater transmission of diabetes. A significant increase ($P=0.033$) was observed in transmission of C allele at SNP-44, to affected offspring in parents-offspring trios (odds ratio 1.6). It revealed a significant over difference of an excess of allele 2 of SNP-44 in the trios probands, compared with that in the population controls (0.23 vs. 0.16; $P=0.005$) (Evans *et al.*, 2001).

A study showed that the 1112/1221 diplotype defined by SNP-44/43/19/63 in CAPN 10 gene was associated with the reduced risk of diabetes and hypertension in this studied population. Since the allele 2 of SNP-44 almost wholly occurred on the common haplotype 2111, with frequencies of the 2112, 2121 and 2221 haplotypes less than 0.5% in this population, the 1112/1221 diplotype in the present study is equivalent to the 112/221 diplotype in Mexican-American of Horikawa's study. This finding was consistent with the result of a small-size sample study in southern Chinese and the first examined group of Mexican-American. However, the study in South Indians suggested that this diplotype appeared to increase risk for impaired fasting glucose/impaired glucose tolerance and T2DM (Chan *et al.*, 2007).

Other meta-analyses suggested that increased risk of T2DM is associated with both SNP-43 and SNP-44 and that these polymorphisms may form the basis of an approach to predict the condition. Studies on nondiabetic British subjects with high risk haplotype (112/121) had increased plasma glucose levels both at fasting and after a 2-h oral glucose tolerance test concomitant with a decrease in the insulin secretory response. Consistent

with these data, polymorphism at SNP44 was found to modify the plasma glucose curve obtained from an oral glucose tolerance test in a manner that was indicative of T2DM association (Harris *et al.*, 2006).

DIABETES IN BANGLADESH

Diabetes in Bangladesh follows a similar pattern that to worldwide. Overall prevalence of diabetes in Bangladesh was found to be 7% (Sayeed *et al.*, 2007). However in their previous report it was about 6.6% (Sayeed *et al.*, 2004). The group reported higher prevalence of diabetes mellitus among the tribal population compared to the mainstream Bangladesh. As expected prevalence of diabetes found to be much higher in the Urban community (11%) compared to the rural counterpart (4%). However, surprisingly much higher impaired glucose tolerance was observed among the rural dwellers.

Another study based on rural community revealed higher prevalence in men compared to women (9.4% vs 8.0% respectively) (Rahman *et al.*, 2007). About 7% of all diabetics registered at BIRDEM are reported to be of young onset type (below 30 years of age). They do not fit either to type 1 or type 2 diabetes mellitus. About 13% of these young onset diabetic patients have features of pancreatitis which are termed as fibrocalculus pancreatic diabetes (FCPD) (Azad and Ali, 1997).

Studies were undertaken to understand the pathophysiology of T2DM in Bangladeshi population. Newly diagnosed, both normal weight and overweight patients were reported to have both insulin secretory dysfunction and insulin resistance with form being predominant in pathophysiology T2DM (Al-Mahmood, 2000). The extent of insulin secretory dysfunction was higher compared to loss of insulin sensitivity in obese type 2 subjects (Junaid, 2000).

Both insulin secretory defect and insulin resistance are predominantly present in the pathogenesis of these T2DM. However the basis of the pathogenesis of the T2DM is to be clearly understood. As a part of series of experiments to elucidate pathological basis of T2DM in Bangladeshi population, the present study was undertaken to investigate CAPN10 gene common polymorphism in the Bangladeshi T2DM patients. It is of importance to determine novel genetic variants in the Bangladeshi diabetic subjects to understand its etiopathogenesis.

Chapter 2

REVIEW OF LITERATURE

Review of Literature

Calpain is a cytoplasmic cysteine protease requiring calcium ions for its activity. Although its physiological function is still not fully understood, it is implicated in a variety of calcium-regulated cellular processes such as signal transduction, cell proliferation, cell cycle progression, differentiation, apoptosis, membrane fusion, and platelet activation (Sorimachi *et al.*, 1997; Huang *et al.*, 2001). Deregulation of its activity has been implicated in various pathological conditions such as neuronal degeneration, Alzheimer's disease, metastasis, and cataract.

Calpain has attracted much attention because of the recent discovery of correlations between calpain gene mutations and human diseases, together with elucidation of its three-dimensional structure and calcium-induced activation mechanisms (Hosfield *et al.*, 1999; Strobl *et al.*, 2000). Positional cloning of genes responsible for diseases has revealed association of the calpain 10 gene (*CAPN10*) with type 2 diabetes, but information on molecular and physiological mechanisms explaining the association of the calpain 10 gene with type 2 diabetes remains unclear and limited (Hirokawa *et al.*, 2000).

Properties and structure of Calpain

Calpain is not specific for certain amino acid residues or sequences but recognizes bonds between domains. As a consequence, calpain hydrolyzes substrate proteins in a limited manner, and large fragments retaining intact domains are produced by hydrolysis. Calpain is regarded as a bio-modulator, because properties of the substrate proteins are often modulated upon hydrolysis by calpain (Sorimachi *et al.*, 1997; Huang *et al.*, 2001).

Two calpain (μ -calpain and m-calpain) activities are detected when tissue extracts are separated by Diethylaminoethyl column chromatography. These correspond to two conventional calpain species in mammals: μ -calpain and m-calpain. They are heterodimers composed of a large 80-kDa catalytic subunit and a common 30-kDa regulatory subunit. These contain four (I–IV) and two (V and VI) domains, respectively. The protease domain II, like other cysteine proteases such as cathepsins B and L, is composed of two subdomains (IIa and IIb) with its substrate binding cleft in-between. The active site Cys-105 on IIa is 10 angstrom apart from other catalytic triad residues (His-262 and Asn-286) on IIb, indicating that IIa and IIb are slightly too separated and

open to constitute a functional catalytic triad (Hosfield *et al.*, 1999; Strobl *et al.*, 2000). This inactive conformation of domain II is stabilized by interaction of domain I with domain VI on one side and interaction of IIb with domain III, especially an acidic loop, on the other side (Strobl *et al.*, 2000). Domain III has no apparent sequence homology to other proteins, but its higher order structure resembles C2 domains found in phospholipase C, protein kinase C, synaptotagmin, etc., and it binds Ca^{2+} and phospholipids (Hosfield *et al.*, 1999). Both domains IV and VI contain five sets of EF-hand Ca^{2+} -binding motifs similar to those found in calmodulin. The extreme COOH-terminal fifth EF-hand motif in IV and VI cannot bind Ca^{2+} but interacts with each other to form a heterodimer comprising 80K and 30K. The NH₂-terminal domain V of 30K is rich in glycine and cannot be seen in the crystal structure because of its mobile conformation.

This dissociation theory of calpain, although a full consensus has not yet been reached, is apparently consistent with the existence of various 80K homologues that appear to function without 30K. Dissociated 30K might have a function different from proteolysis after forming a homodimer (Pal and Elce, 2001). Both μ - and m-calpains and 80K homologues are inhibited by reagents that react with the active site cysteine residues, such as E64, leupeptin, and N-Ac-Leu-Leu-norleucinal (Sorimachi *et al.*, 1997). These inhibitors also react with other cysteine proteases and are not specific for calpain. The nonpeptidic inhibitor PD150606 inhibits calpain by interacting with the calmodulin domain. This inhibitor is relatively specific for calpain, and the inhibition mechanism is different from other inhibitors (Wang *et al.*, 1996).

An endogenous calpain inhibitor, calpastatin, exists in the cytosol. It contains four equivalent inhibitory domains of 140 residues, having three conserved regions (A, B, and C) that are important for inhibition. A and C interact with IV and VI, respectively, in a Ca^{2+} -dependent manner, and B shows inhibitory activity by itself, probably by binding at or near the active site (Tomba *et al.*, 2002). Presence of the two calmodulin-like domains IV and VI are necessary for effective inhibition by calpastatin. Thus, calpastatin inhibits only dimeric calpain (namely μ -calpain, m-calpain, and calpain 9) with 30K. Calpain 80K homologues including calpains 1 and 2 are not inhibited and thus escape from the regulatory actions of calpastatin.

All members of calpain family abundant in human cells are now known, many of which are associated with human disease (Huang *et al.*, 2001). Calpains 1 and 2 are implicated in causing injury to the brain after a stroke and also have been linked to the pathology seen in Alzheimer's disease. Calpain 3 is mainly found in the muscle, and mutations cause limb-girdle muscular dystrophy. Mutations of calpains in the worm *Caenorhabditis elegans* affect sexual development (Barnes *et al.*, 1996), and mutations of a calpain-like gene in the fly cause a degeneration of parts of the nervous system (Delaney *et al.*, 1991).

Activation mechanism of Calpain

Calpain exists in the cytosol as an inactive enzyme and translocates to membranes in response to increases in the cellular Ca^{2+} level.

At the membrane, calpain is activated in the presence of Ca^{2+} and phospholipids. Autocatalytic hydrolysis of domain I take place during activation, and dissociation of 30K from 80K occurs as a result. Activated calpain or 80K hydrolyzes substrate proteins at membranes or in cytosol after release from membranes (Suzuki *et al.*, 1995).

In the absence of Ca^{2+} , two protease subdomains IIa and IIb are separated by structural constraints imposed by domain interaction. Ca^{2+} -induced structural changes that release the constraints are prerequisite for activation to form a functional catalytic site. Recent X-ray structural analyses have revealed a Ca^{2+} -induced activation mechanism at the molecular level. There are at least three different Ca^{2+} -binding sites in m-calpain, two calmodulin-like domains IV and VI, an acidic loop region in C2-like domain III, and a protease domain II. A proposed mechanism for activity regulation of m-calpain by Ca^{2+} comprises two stages (Fig. 2). The first stage is the release of constraints imposed by domain interactions. Binding of Ca^{2+} to domains IV, VI, and III releases domain I from VI and domain II from III and leads to dissociation of 30K from 80K. The second stage is the rearrangement of the active site cleft caused by binding of two Ca^{2+} atoms to the protease domain (one each to the IIa and IIb subdomains). Activation by the second stage occurs only after release of the constraints freed by the first stage (Moldveanu, 2002).

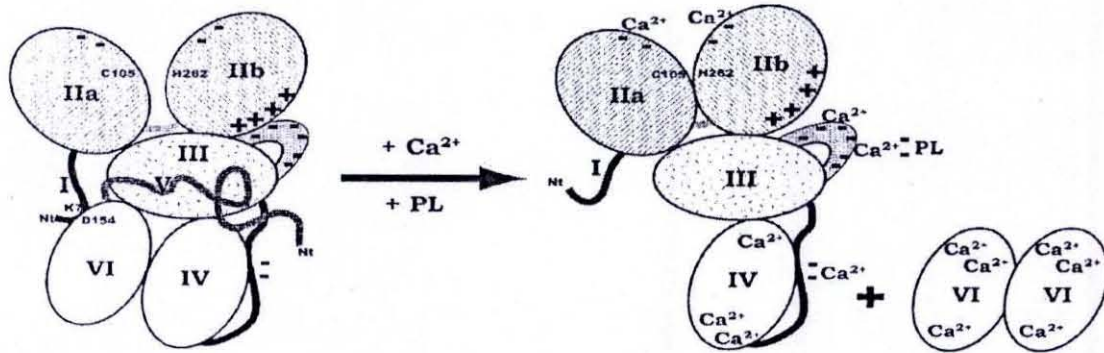


Figure 2. Activation mechanism of calpain by Ca^{2+} . Binding of Ca^{2+} and phospholipids (PL) to m-calpain induces conformational changes, which brings IIa and IIb closer together to form a functional catalytic site and causes dissociation of 30K from 80K, resulting in 30K homodimer formation. There are at least three different calcium-binding sites in m-calpain, two calmodulin like EF-hand structures in domains IV and VI, an acidic loop in domain III, and two non-EF hand calcium-binding sites in IIa and IIb. C105 and H262 are catalytic residues. K7 and D154 form a salt bridge in the absence of calcium ions. Ca^{2+} , calcium atom bound to calpain; Nt, NH2-terminal residue; + and -, basic and acidic amino acid residues important for binding of calcium ions. (Suzuki *et al.*, 2004)

The two-stage activation mechanism is a general mechanism applicable to calpains without 30K or atypical calpains, because the protease domains are always composed of IIa and IIb, and the calcium-coordination residues for the two Ca^{2+} -binding sites in IIa and IIb are conserved among calpains (Sorimachi *et al.*, 2001, Moldveanu, 2002). In the cases of calpains without 30K or without domain III or IV, the first stage of activation might be bypassed and they are activated directly by the second stage. Autolysis of domain I facilitates dissociation of 30K from 80K (Nakagawa *et al.*, 2001), and binding of Ca^{2+} at domain III is responsible for translocation of calpain to membranes, as observed with protein kinase C and phospholipase C (Tompa *et al.*, 2001).

Activity of calpain is tightly regulated both temporally and spatially mainly by Ca^{2+} , because deregulation of calpain activity causes excessive degradation or accumulation of coexisting cellular proteins resulting in serious cellular damage and pathological conditions. Phosphorylation of calpain might be another important mechanism for activity regulation. Phosphorylation of calpain at Ser-369 in domain III by protein kinase A restricts domain movement and freezes m-calpain in an inactive state (Shiraha *et al.*, 2002).

Function of Calpains

Calpains are cytosolic enzymes implicated in numerous cellular functions and pathologies. Arrington et al (2006) identified a mitochondrial Ca^{2+} -inducible protease that hydrolyzed a calpain substrate (SLLVY-AMC) and was inhibited by active site-directed calpain inhibitors as calpain 10. Immunoblot analysis and activity assays revealed calpain 10 in the mitochondrial outer membrane, intermembrane space, inner membrane, and matrix fractions.

Mitochondrial staining was observed when COOH-terminal green fluorescent protein-tagged calpain 10 was overexpressed in NIH-3T3 cells and the mitochondrial targeting sequence was localized to the NH₂-terminal 15 amino acids. Overexpression of mitochondrial calpain 10 resulted in mitochondrial swelling and autophagy that was blocked by the mitochondrial permeability transition (MPT) inhibitor cyclosporine A. With the use of isolated mitochondria, Ca^{2+} -induced MPT was partially decreased by calpain inhibitors. Ca^{2+} -induced inhibition of Complex I of the electron transport chain was blocked by calpain inhibitors and two Complex I proteins were identified as targets of mitochondrial calpain 10, NDUFV2, and ND6. Calpain 10 was the first to be reported mitochondrially targeted calpain and a mediator of mitochondrial dysfunction through the cleavage of Complex I subunits and activation of MPT (Arrington *et al.*, 2006). Their proposed model is shown in figure below:

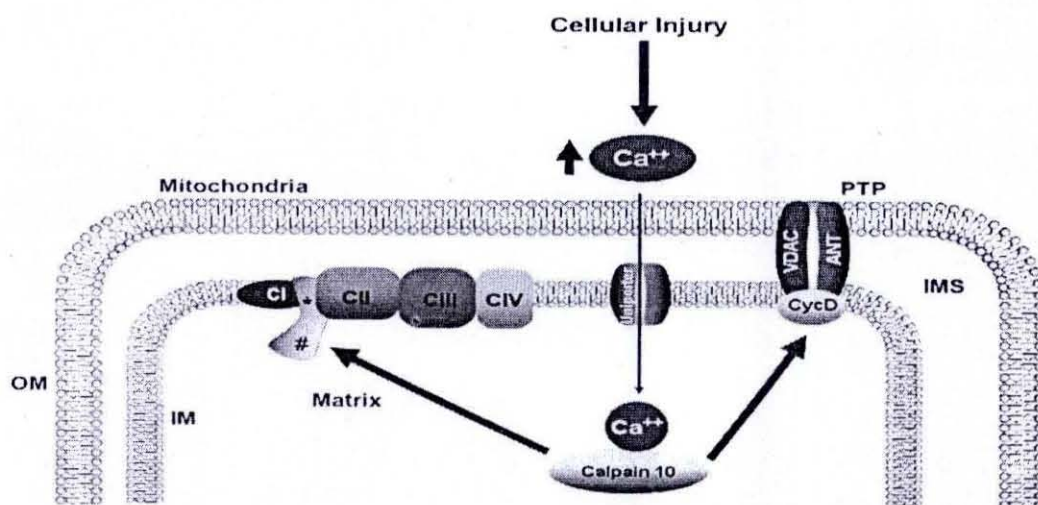


Figure. 3. Proposed mechanism of action for calpain 10-mediated ETC dysfunction. Depicted above is a schematic diagram of the proposed mechanism of calpain 10 action after Ca^{2+} -induced mitochondrial dysfunction. After injury, increases in cytosolic Ca^{2+} are translated to the mitochondria via the ruthenium red-sensitive calcium channel uniporter. Increases in matrix Ca^{2+} activate mitochondrial calpain 10, inducing cleavage of two critical subunits of Complex I, ND6 (*) and NDUFV2 (#), leading to subsequent respiratory deficits. Increases in mitochondrial calcium also contribute to the induction of MPT, possibly through proteolysis of membrane pore proteins. (Arrington *et al.*, 2006)

In another study, (Paul *et al.*, 2003) inhibition of calpain activity has been shown to reduce insulin stimulated glucose uptake in isolated rat-muscle strips and adipocytes. They examined the mechanism by which calpain affects insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Inhibition of calpain activity resulted in approx. a 60% decrease in insulin-stimulated glucose uptake. Furthermore, inhibition of calpain activity prevented the translocation of insulin-responsive glucose transporter 4 (GLUT4) vesicles to the plasma membrane, as demonstrated by fluorescent microscopy of whole cells and isolated plasma membranes; it did not, however, alter the total GLUT4 protein content. While inhibition of calpain did not affect the insulin-mediated proximal steps of the phosphoinositide 3-kinase pathway, it did prevent the insulin-stimulated cortical actin reorganization required for GLUT4 translocation. Specific inhibition of calpain 10 by antisense expression reduced insulin-stimulated GLUT4 translocation and actin reorganization. Based on these findings, a role was proposed for calpain in the actin reorganization required for insulin-stimulated GLUT4 translocation to the plasma membrane in 3T3-L1 adipocytes. These studies identify calpain as a novel factor involved in GLUT4 vesicle trafficking and suggest a link between calpain activity and the development of type 2 diabetes. The overall summary is briefly described below:

(1) Glucose enters B cells through GLUT2 transporters. The ensuing result is glycolysis and an elevated ATP: ADP ratio generated by mitochondria. The ability of mitochondria to perform this function has been shown to be severely diminished by calpain inhibition, and calpain-10 is thus hypothesized to function as a regulator of mitochondrial fuel sensing (Zhou *et al.*, 2003).

(2) With the exception of pre-docked granules primed for 1st phase secretion, secretory granules are for the most part found at sites distal to the plasma membrane. Following the initial burst of secretion from pre-docked granules, subsequent exocytotic granule fusion requires the recruitment of granules from deep within the cell. This is a process that requires cytoskeletal rearrangement and the concomitant transport along microtubules of secretory granules (Burgoyne and Morgan, 2003; Goldstein and Yang, 2000; Tang, 2001), or in the case of skeletal muscle and adipocytes GLUT4-containing vesicles (Kanzaki and Pessin, 2003; Watson *et al.*, 2004), to active sites of exocytosis at the plasma membrane. The process of actin cytoskeletal rearrangement is sensitive to calpain inhibition, both pharmacologically or through use of specific CAPN10 antisense oligonucleotides (Paul *et al.*, 2003) and the actin cytoskeleton is a common target for calpain-mediated proteolysis in numerous cell types.

Brown *et al* (2007) have shown that targeted suppression of calpain-10 expression in cultured human skeletal muscle cells impaired insulin-stimulated glucose uptake. The degree of suppression of CAPN10 gene expression was in line with that observed in insulin resistant subjects at increased risk of T2DM. This suggests that the observed degree of suppression in these subjects may contribute directly to the skeletal muscle insulin resistance.

(3) The final step in the secretory pathway is the actual exocytotic fusion event. This is mediated by the SNARE family of proteins. Soluble N-ethyl maleimide sensitive fusion protein attachment receptors (SNAREs) have been recognized as key components of protein complexes that drive membrane fusion in vesicular transport (Jahn and Scheller, 2006). One isoform of calpain-10 has been shown to associate with the t-SNAREs, syntaxin 1 and SNAP-25 (Marshall *et al.*, 2005). Furthermore, insulin secretion is accompanied by a Ca^{2+} -dependent partial proteolysis of SNAP-25. As both events are strongly inhibited by calpain inhibition, and no other calpain has thus far been found to

be associated with SNAP-25, calpain-10 has been proposed to be a Ca^{2+} -sensor central to exocytosis in B cells.

In B cells only the 54 kDa isoform of calpain-10 has been shown to be associated with the cytoskeleton (Turner *et al.*, 2007). As this is the same isoform that was shown to associate with and cleave the t-SNARE SNAP-25 (Marshall *et al.*, 2005), it is tempting to speculate that CAPN 10 might regulate insulin secretion as part of a single complex at the plasma membrane. Support for this hypothesis comes from the finding that there is dynamic interaction between t-SNAREs and F-actin during glucose-stimulated insulin secretion (Thurmond *et al.*, 2003). Therefore, activation of this one isoform of CAPN 10 could result in simultaneous actions on both the exocytotic machinery and the cytoskeleton.

Calpain 10 and Type 2 Diabetes Mellitus

Calpain 10 was an unexpected find in the search for a putative diabetes susceptibility gene. Its link with diabetes is complex; susceptibility is not attributable to a single variation but to several variations of DNA that interact to either increase, decrease, or have no effect on the risk of developing diabetes. Calpain 10 is an atypical member of the calpain family, and its biological role is unknown. Because CAPN10 mRNA is expressed in the pancreas, muscle, and liver, its role in diabetes may involve insulin secretion, insulin action and the production of glucose by the liver (Horikawa *et al.*, 2002)

At first genome-wide scan studies on 330 sibling pairs identified NIDDM1, the putative T2DM susceptibility locus, to the region D2S125– D2S140 on chromosome 2q37.3 of Mexican Americans (Harris *et al.*, 1996). Four years later positional cloning studies revealed that NIDDM1 was a single gene, CAPN10, and that genetic variation in this gene accounted for 14% of the population attributable risk to T2DM in this ethnic group (Horikawa *et al.*, 2002). An intronic single nucleotide polymorphism (SNP) in CAPN10 (FIG. 4) was significantly increased in affected individuals but the highest overall risk for T2DM was determined by a heterozygous combination of two haplotypes, which were defined by UCSNP43 and two further intronic SNPs: UCSNP19 and UCSNP63. These high-risk haplotypes are generally referred to as “112” (UCSNP43, allele 1; UCSNP19, allele 1; UCSNP63, allele 2) and “121” (UCSNP43, allele 1; UCSNP19, allele 2; UCSNP63, allele 1). This haplotype combination was found to confer an overall

increased risk of T2DM in Mexican Americans of 2.8-fold, in Botnian Finns of 2.5-fold, and in Germans of almost 5-fold (Horikawa *et al.*, 2002). Since this initial work, there have been a number of follow-up studies on a diverse range of ethnic populations, which either confirmed or extended the associations of CAPN10 with T2DM (Garant *et al.*, 2002) or disputed the original findings (Tsai *et al.*, 2001).

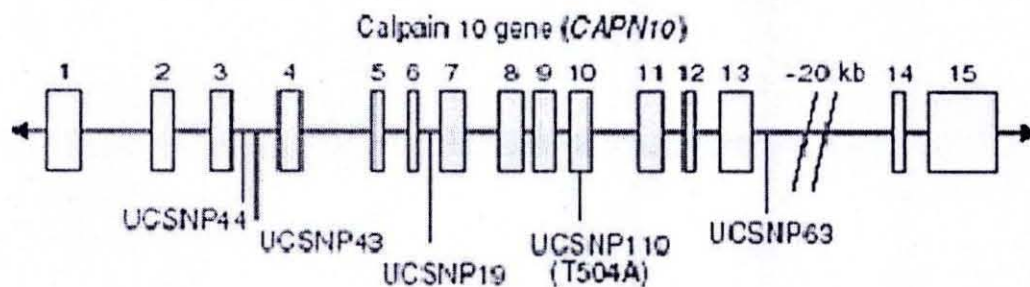


Figure 4. This figure shows the structure of CAPN10, the gene encoding human calpain 10 along with associated SNPs. The gene is located on chromosome 2q37.3 and spans 31 Kb of genomic sequence. The gene contains 15 exons and encodes a protease with a full length of 672 amino acid residues. Annotated are UCSNP19, UCSNP43, and UCSNP63, which are the SNPs that form the risk haplotypes for T2DM. Along with UCSNP44, which is in almost perfect linkage disequilibrium with the missense coding mutation UCSNP110, these SNPs have also been associated with a variety of T2DM associated phenotypes and disorders. (Turner *et al.* 2005)

Genetic variations in the CAPN10 gene at UCSNP43, UCSNP-63, and haplotype combinations involving these polymorphisms along with those at UCSNP19 and UCSNP63 have been shown to be associated with elevated free fatty acid levels. Studies on a Chinese population found that the haplotype combination 112/121 was a potential risk factor for increased plasma cholesterol while studies on a Japanese population found that the UCSNP43 G allele was associated with increased levels of plasma cholesterol (Daimon *et al.*, 2002) and increased free fatty acid levels and dyslipidemia (Sugimoto *et al.*, 2003).

Similar to the Mexican Americans, the Pima Indians of Arizona had a high prevalence of type 2 diabetes. However, in a study among the Pima Indians, no association was found between the high-risk genotype SNP-43 G/G and an increased prevalence of diabetes, although G/G individuals did have reduced expression of CAPN10 mRNA and showed signs of insulin resistance, which may increase susceptibility to diabetes (Baier *et al.*, 2007).

In Europe, CAPN10 appears to contribute less to T2DM. In Britain, there was no association between SNP-43, SNP-19, and SNP-63 and diabetes, but it is possible that SNPs at other sites in the calpain gene may increase T2DM risk (Evans *et al.*, 2001). In a large study in Scandinavians, no association was found between these three SNPs and diabetes (Rasmussen *et al.*, 2002). In Asia, genetic variation in CAPN10 did not contribute to diabetes in Japan (Horikawa *et al.*, 2003) or in the Samoans of Polynesia (Tsai *et al.*, 2001), although variations of CAPN10 may play a role in the risk of diabetes in the Chinese (Wang *et al.*, 2002).

Obesity is a key determinant in diabetes development, with increased lipolysis from the increased fat cell mass being suggested to lead to the development of skeletal muscle insulin resistance (Beck-Nielsen and Groop, 1994; Boden, 1997). Genetic variations in the CAPN10 gene at SNP-43, SNP-63 and the haplotype combination SNP-44/43/19/63 1121/1121 have been shown to be associated with elevated free fatty acid levels and insulin resistance in a Finnish population (Orho-Melander *et al.*, 2002). They found that fasting FFA levels were significantly higher in carriers of the SNP-43 genotypes 11 or 12 compared to genotype 22 carriers. This difference was evident across both sexes in patients with type 2 diabetes as well as in normoglycemic individuals. Mildly obese individuals with the SNP-43 G/G genotype have been shown to have both elevated triglyceride levels and reduced adipose tissue CAPN10 mRNA levels (Carlsson *et al.*, 2004). The effect of insulin to stimulate glucose uptake into peripheral tissues is essential for the maintenance of normal glucose homeostasis (Cushman and Wardzala, 1980). Impaired glucose uptake into skeletal muscle and adipose tissue is a major feature of T2DM (Rothman *et al.*, 1992). One of the important rate-limiting steps for insulin action that has been found to be impaired in T2DM is that of glucose transport mediated by the glucose transporter-4 (GLUT4) (Cline *et al.*, 1999). GLUT4 is normally translocated from an intracellular pool to the plasma membrane in response to insulin (Watson *et al.*, 2004), but inhibition of calpain activity has been shown to cause a reduction in insulin-stimulated glucose uptake into isolated rat muscle strips and adipocytes (Sreenan *et al.*, 2001). Overexpression of the calpain inhibitor calpastatin in skeletal muscle leads to an intracellular accumulation of GLUT4, but with no concomitant increase in transporter at the plasma membrane or upregulation of glucose metabolism (Otani *et al.*, 2004).

CAPN10 gene polymorphisms have been found to correlate with several aspects of insulin secretion (Lynn *et al.*, 2002). Genetic variation in the CAPN10 gene accounted

for 14% of the population attributable risk to T2DM in Mexican Americans, with the common G allele of an intronic SNP, UCSNP-43, significantly increased in affected subjects (Oda *et al.*, 2000). In Mexican American, the frequency of the common SNP-43 allele 1 was significantly higher in the patient group than that in random sample (Horikawa *et al.*, 2000). The most common diplotype of SNP-43, -19, and -63 observed in Mexican Americans with T2DM was 112/121, which was associated with a 2.80-fold increased risk of diabetes (95% CI, 1.23–6.34) (Horikawa *et al.*, 2000), whereas this was 2.55 and 4.97 fold in Botnian Finns and Germans respectively (Oda *et al.*, 2000). In a study in Northern Sweden, this chromosomal region was also demonstrated to be linked to diabetes while there was no association of the variations in CAPN10 gene (Einarsdottir *et al.*, 2006).

Other variants apart from UCSNP-43 may have a role in determining disease susceptibility. The UCSNP-43 association with T2DM appears so far to be almost unique to Mexican Americans (Oda *et al.*, 2000), whereas UCSNP-44 may dictate an underlying low level risk across many ethnic groups (Weedon *et al.*, 2003). In contrast, the UCSNP-63 variant was more important for disease association in both Botnian Finns and South Indians (Cassell *et al.*, 2002) but not in Mexican Americans. In addition, a borderline association was detected with the UCSNP-63 locus when family data was pooled (Song *et al.*, 2004). Furthermore, analysis of the South Indian data using a novel statistical approach suggested that the UCSNP-19 genotype may additionally contribute to the disease susceptibility within this ethnic group and obesity in Europeans (Hoffstedt *et al.*, 2002).

A study of non-diabetic German subjects showed that SNP-43 was associated with increased insulin secretion and proinsulin processing (Stumvoll *et al.*, 2001). Polymorphism at SNP-44 has been shown to alter the shape of the plasma glucose curve during oral glucose tolerance test (Tschritter *et al.*, 2003), indicative of disease association.

Variation in CAPN10 gene has similarly been associated with quantitative traits related to diabetes. Thus, the SNP43 has in some studies been related to fasting and post-oral glucose tolerance test (OGTT) plasma glucose levels (Lynn *et al.*, 2002) and other investigators have reported associations with different estimates of insulin resistance and insulin secretion (Orho-Melander *et al.*, 2002). A genotype combination of

SNP43/SNP44 GG/TT has been reported to associate with elevated fasting serum cholesterol levels (Daimon *et al.*, 2002), whereas SNP43 alone was associated with elevated fasting serum triglyceride levels in obese subjects (Carlsson *et al.*, 2004). Association of the CAPN10 SNPs and obesity has been reported in one study (Pihlajamäki *et al.*, 2006), whereas no association with obesity has been found in other studies (Carlsson *et al.*, 2004).

Another study found that haplotype combination 111/121 of CAPN10 was related (Kang *et al.*, 2006) with an increased risk of metabolic syndrome in patients with T2DM. These results suggest that the genotype combination SNP-43, -19, and -63 is a risk factor for metabolic syndrome. Intronic variation in CAPN10 was associated with elevated triglyceride levels and reduced adipose tissue mRNA expression (Carlsson *et al.*, 2004) in morbidly obese Swedish subjects, suggesting that a low CAPN10 gene expression might be a risk factor for conditions related to the metabolic syndrome. Taking these data together, it appears that polymorphisms of CAPN10 gene do influence susceptibility to T2DM and insulin resistance across populations of different racial backgrounds. The strength of this influence does seem to differ between populations. Some of these studies have been shown with a modest increase in risk to T2DM due to the CAPN10 gene.

HYPOTHESIS

CAPN 10 gene SNP 44 T>C polymorphic is not associated with type 2 diabetes in Bangladeshi population.

OBJECTIVES

General objects

The general objective of the present study was to determine CAPN 10 gene SNP 44 T>C variant in TDM patients of Bangladeshi origin to explore in association with insulin secretory capacity and insulin sensitivity.

Specific objectives

The specific objectives of the present study were to:

- To determine CAPN 10 gene SNP 44 (T>C) in the TDM subjects of Bangladeshi origin.
- To measure the insulin level of the TDM subjects and association their secretory capacity and insulin sensitivity.
- To explore the relationship, if any, between CAPN 10 SNP 44 T>C polymorphism and of the T2DM subjects.

Chapter 3

SUBJECTS & METHODS

SUBJECTS AND METHODS

Place of the study

The study was conducted in the laboratory of Biomedical Research Group (BMRG), Research Division, Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM), Dhaka, Bangladesh.

Study period

This study was done during the period of June 2009 to March 2010.

Study design

This was a case control study. CAPN10 SNP-44 T>C polymorphic marker was taken as the exposure factor.

Subjects

A total number of 156 (One hundred and fifty six) subjects were purposively recruited in the study irrespective of race, religion and socioeconomic status. Diabetes was diagnosed following WHO criteria (WHO1999). Of the total 91 subjects were healthy control and 65 type 2 diabetes mellitus (T2DM) subjects.

Development of Questionnaire

A predesigned case record form was used to document detail medical records, anthropometric measurements (*Appendix I*). Diabetic patients were recruited from the Out-patient department (OPD), BIRDEM. Control subjects were recruited from the community through personal communication.

Purpose and method of the study was briefed to each individual and informed consent (*Appendix II*) was obtained.

Collection of the subjects

Subjects were collected on every working day of the week in the laboratory of BMRG, BIRDEM. Subjects recruited through personal communication from the community. Subjects willing to have blood glucose check were also invited to volunteer the study. They were advised to be on their usual diet for 3 days and requested to report BMRG Lab between 8.00-10.00 am in fasting state (8-10 hours).

Selection Criteria

Inclusion Criteria

- Healthy subjects with age range 30 to 60 years.
- Voluntarily agreed to participate in this study.

Exclusion criteria

- Subjects with co-morbid diseases (infection, stroke, myocardial infarction, major surgery, essential hypertension, malabsorption etc.).
- History of using drugs which may significantly affect glucose metabolism (glucocorticoids, high-dose estrogen, phenytoin, high-dose thiazide diuretics etc.).
- Pregnancy.

Anthropometric measurements

Height (m)

Standing height was measured using appropriate scales (Detect-Medic, Detect scales INC, USA) following standard procedure. Height was recorded to the nearest 5 mm.

Weight (kg)

The balance was placed on a hard flat surface and checked for zero balance before measurement. The subjects were in the center of the platform wearing light cloths without shoes. Weight was recorded to the nearest 0.5 kg.

Calculation of BMI (kg/m²)

Body mass indexes (BMI) of the subjects were calculated using following formula

$$\text{BMI} = \frac{\text{Weight (kg)}}{[\text{Height (m)}]^2}$$

MAC (cm)

Mid Upper Arm Circumference (MAC) was measured at a point mid way between the acromial process of scapula and olecranon process of ulna of right arm hanging relaxed. A measuring tape was used to record the circumference following the above technique at the nearest millimeter.

Waist circumference (cm)

Waist circumference was measured to the nearest 0.5 cm with a soft non-elastic measuring tape. The tape was snug, but not so tight as to cause skin indentation or pinching. The waist circumference was taken to the nearest standing horizontal circumference between the lower border of the 12th rib and the highest point of the iliac crest on the mid-axillary line at the end of normal expiration.

Skin fold thickness (mm)

Skin fold thickness was measured using Harpenden Skinfold Calipers. Triceps skin fold thickness was measured 1 cm above the mid point between the acromial process of scapula and olecranon process of ulna at mid-line posteriorly with skin fold lying along the long axis of right arm hanging relaxed. Subscapular skin fold thickness was measured 1 cm below the inferior angle of right scapula with skin fold lying at 45 degree. Values were recorded at the nearest millimeter.

Body fat mass (%)

Body fat mass was measured by Omron Body Fat Monitor (Model HBF-302). Height in cm, weight in kg, age in yrs and gender at the individuals enter to the body fat monitor. The subjects were asked to hold the monitor by both hands with upper limbs horizontal in standing position. The machine was then initiated and body fat mass (%) calculated by the unit was flashed on the display screen and value was recorded.

Measurement of blood pressure

Blood pressure was measured in sitting position, with calf at the level of the heart. After 10 minutes of rest a second reading was taken. Recorded Korotkoff sound I (the first sound) and V (the disappearance of sound) denoted the systolic blood pressure (SBP) and diastolic blood pressure (DBP), respectively (according to WHO-IHS).

METHODS

Collection of blood samples

Overnight fasting (8-10 hours) blood was collected between 8.00-9.00 am. Venous blood (10 ml) was obtained by venepuncture following standard procedure. Subjects were then given glucose to drink (75 g in 300 ml of water). They were requested not to take any food and be rested for two hours. The second blood sample was then obtained.

A portion of blood (5 ml) sample was taken into a tube containing EDTA (1 mg/ml), mixed thoroughly and preserved in at -30°C for future DNA extraction and subsequent experimentation. Fasting and postprandial blood sample were taken into plain tube allowed to clot for 30 minutes and serum was separated by centrifugation for 10 min at 3000 rpm using refrigerated centrifuge and preserved at -25°C for further biochemical analyses.

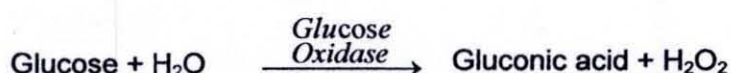
Biochemical methods

Estimation of Glucose

Glucose was estimated by enzymatic colorimetric (GOD-PAP) method in the Hitachi 704 Automatic Analyzer, Hitachi Ltd., Tokyo, Japan using reagents of RANDOX Laboratories Ltd., UK.

Principle

Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts with phenol and 4-aminophenazone under catalysis of peroxidase to form a red violet quinoneimine dye as indicator (Trinder, 1969).



Reagents

Contents	Initial concentration of solution
Phosphate Buffer	0.1 mol/l, pH 7.0
Phenol	11 mol/l
4-aminophenazone	0.77 mmol/l
Glucose oxidase	≥1.5 kU/l
Peroxidase	≥1.5 kU/l
Glucose Standard	5.55 mmol/l (100 mg/dl)
Uranyl Acetate	0.16%

Procedure

The method determines glucose without deproteinization. The instrument was calibrated before estimation. Serum and reagent were taken in specific cup. They were arranged serially into the Auto analyzer. The Auto analyzer was programmed for the estimation of glucose and allowed to run with following procedure:

5 µl sample and 500 µl reagent were mixed and incubated at 37° C for 10 minutes. The reaction occurred in reaction cell or cup. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

Calculation of the result

Optical densities or absorbances were fed into a computer and calculation was done using the software program. Values for the unknown samples were calculated by extrapolating the absorbance for the standard using following formula.

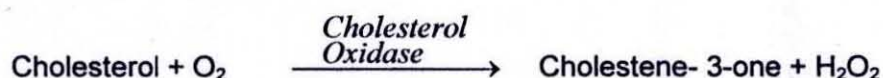
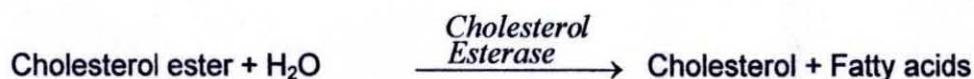
$$\text{Glucose concentration (mmol/l)} = \frac{A_{\text{Sample}}}{A_{\text{Standard}}} \times 5.55$$

Estimation of Total Cholesterol

Total cholesterol was measured by enzymatic endpoint method (cholesterol Oxidase/Peroxidase) method in auto analyzer Auto analyzer HITACHI 704, Hitachi Ltd Tokyo, Japan.

Principle

The cholesterol was determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase (Richmond, 1973).



Reagents

Contents	Initial Concentration of Solution
4-Aminoantipyrine	0.30 mmol/l
Phenol	6 mmol/l
Peroxidase	≥ 0.5 U/ml
Cholesterol esterase	≥ 0.15 U/ml
Cholesterol oxides	≥0.1 U/ml
Pipes Buffer	80 mmol/l; pH 6.8
Standard	5.17 mmol/l (200 mg/dl)

Procedure

Serum and reagents were taken in specific cup or cell. They were arranged serially. Then ID number for each test was entered in the Auto analyzer. 5 µl sample and 500 µl reagent were mixed and incubated at 37°C for 5 minutes within the Auto lab. The reaction occurred in reaction cell or cup. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

Calculation of result

Concentration of cholesterol in sample was calculated by using software program with the following formula.

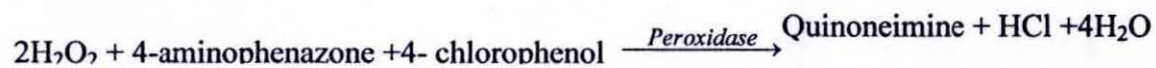
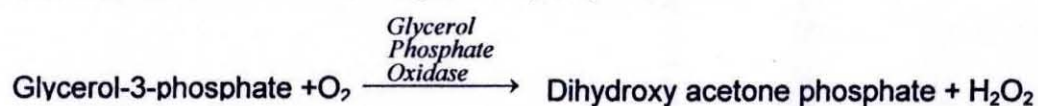
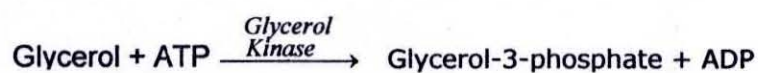
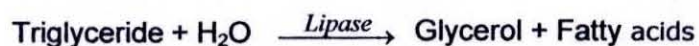
$$\text{Cholesterol concentration (mg/dl)} = \frac{A_{\text{Sample}}}{A_{\text{Standard}}} \times \text{concentration of standard.}$$

Estimation of Triglyceride

Triglyceride was measured by enzymatic colorimetric (GPO-PAP) method in the Hitachi 704 Automatic Analyzer, Hitachi Ltd., Tokyo, Japan using reagents of RANDOX Laboratories Ltd., UK.

Principle

The triglyceride is determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen- peroxide, 4- aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase (Fossati and Prencipe, 1982).



Reagents

Contents

Concentrations in the Test

Buffer

Pipes Buffer 40 mmol/l, pH 7.6

4-choloro-phenol 5.5 mmol/l

Magnesium-ions 17.5 mmol/l

Enzyme Reagent

4-aminophenazone 0.5 mmol/l

ATP 1.0 mmol/l

Lipases >150 U/ml

Glycerol-3-phosphate oxidase 1.5 U/ml

Peroxidase 0.5 U/ml

Standard 2.29 mmol/l (200 mg/dl)

Procedure

Serum and reagents were taken in specific cup. They were arranged serially. Then ID number for test was entered in the analyzer. Five (5) µl sample and 500 µl reagent were mixed and incubated at 37°C for 5 minutes within the cell. Reading was taken at 500 nm.

Calculation of result

Triglyceride concentration was calculated by following formula:

$$\text{Triglyceride concentration (mg/dl)} = \frac{A_{\text{Sample}}}{A_{\text{Standard}}} \times \text{Concentration of standard.}$$

Estimation of high density lipoprotein (HDL) cholesterol

High density lipoprotein cholesterol (HDLc) was measured by enzymatic colometric method using reagent of Randox laboratories, UK.

Principle

HDL (High Density Lipoproteins) is separated from chylomicrons, VLDL (very low density lipoproteins) and LDL (Low density lipoproteins) by precipitating reagent (phosphotungstic acid-magnesium chloride). After centrifugation, the cholesterol contents of HDL fraction, which remains in the supernatant, was determined by the enzymatic colorimetric method using CHOD- PAP (Friedwald *et al.*, 1972).

Materials and reagents

1. Precipitant Buffer
2. Lipid Controls
3. Randox aqueous Cholesterol Standard: 200 mg/dl
4. Reagent solution for cholesterol CHOD-PAP assay.
5. Pipettes (5 µl –50 µl, 100 µl-1000 µl) and Pipette Tips.
6. Multi-Channel Pipettes and Pipette Tips: 50-300 µl
7. Buffer and Reagent Reservoirs

8. Vortex Mixture
9. Deionized Water
10. Microtiter Plate Reader capable of reading absorbency at 450 nm 590 nm
11. Orbital Microtiter Plate Shaker
12. Absorbant Paper

Reagents composition

Phosphotungstic Acid: 0.55 mmol/l

Magnesium Chloride: 25 mmol/l

Standard Preparation

Dilute Randox aqueous cholesterol standard (200mg/dl) with deionized water by volume of 0, 20, 40, 50, and 100 μ l. The final volume was 200 μ l.

Assay Procedure

1. 100 ml serum samples taken in microcentrifuge tube
2. Add 250 μ l HDL-c Precipitant.
3. Mix well and allow sitting for 10 minutes
4. Vortex the mix components and centrifuged for 15 minutes at 4000 rpm.
5. Transfer 30 μ l of each Standard in first six wells.
6. Transfer 30 μ l of clear supernatant into the other wells
7. 250 μ l of cholesterol reagent was then added into all the 96 wells quickly using multi-channel pipettes.
8. Incubated for 5 minutes at 37°C on orbital microtiter plate shaker.
9. Absorbance was read at 490 nm.

Calculation

The HDL-c value of each sample was obtained as follows:

The net absorbance value for each level obtained, by subtracting the value for the HDL-c concentration (mg/dl) from the value of individual. The smooth linear curve was drawn and the results of unknown samples were calculated using logistic function.

Estimation of LDL-cholesterol

The LDL-Cholesterol level in serum was calculated by using by Friedewald formula (Friedewald *et al.*, 1972).

Formula

$$\text{LDL cholesterol} = \left\{ \text{Total cholesterol} - \left(\text{HDL Cholesterol} + \frac{1}{5} \times \text{Triglyceride} \right) \right\}$$

Estimation of Creatinine

Estimation of creatinine was done by alkaline-picric acid methods using reagents from Randox Laboratories, UK.

Principle

Creatinine in alkaline solution reacts with picric acid to form a colored complex. The amount of the complex formed is directly proportional to the Creatinine concentration

Sample: Serum.

Reagents

Standard	177umol/l (2mg/dl)
Picric acid surfactant	35 mmol/L
Sodium hydroxide	0.32 mol/L

Preparation of reagent

All reagents are supplied ready to use, stable to expiry date when stored at +15 to 25°C.

Preparation of working reagent

Mix Equal volumes of Solutions 2+3, Stable for 3 Days At + 15 to+25°C.

Procedure

Wavelength	492 (490-510) nm
Cuvette	1 Cm Light Path
Temperature	25/30/37 C
Measurement	against Air

Pipette into cuvette	Standard	Sample
Working reagent	1.0 ml	1.0 ml
Standard solutions	0.1 ml	---
Sample	---	0.1ml

Mix and after 30 sec, read the absorbance A1 of the standard and sample Exactly 2 min. later read the absorbance A2 of the standard and sample

Calculation

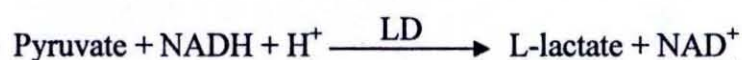
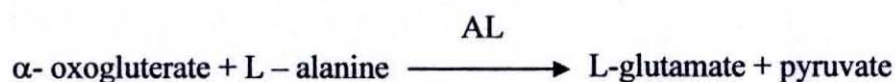
$$A2-A1 = \Delta A \text{ sample or } \Delta A \text{ standard}$$

$$\text{Concentration of Creatinine in Serum (mg/dl)} = \frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times 100$$

Estimation of SGPT

SGPT was estimated by UV method using ALT (GPT) opt. kit (RANDOX) (IFCC, 1980).

Principle



Reagents

Contents	Concentration in the test
1. Buffer/Substrate	
Tris biffer	100 mmol/l, pH 7.5
L-alanine	0.6 mol/l
2. Enzyme/Coenzyme/ α -oxoglutarate	
α -oxoglutarate	15 mmol/l
LD	≥ 1.2 U/ml
NADH	0.18 mmol/l

Preparation of Solutions

1. Buffer/Substrate: Buffer/Substrate supplied in the kit was used as it is.
2. Enzyme/Coenzyme/ α -oxoglutarate: One vial of Enzyme/Coenzyme/ α -oxoglutarate² was reconstituted with the appropriate volume of Buffer/Substrate 1:

2 ml	for the	20 x	2 ml kit (AL 1200)
10 ml	for the	20 x	10 ml kit (AL 1205)
20 ml	for the	5 x	20 ml kit (AL 1268)

Cat No AL 2360 5 x 100 ml

One vial of Enzyme/Coenzyme/ α -oxoglutarate 2 was reconstituted with a portion of Buffer/Substrate 1 and then the entire content was transferred to bottle 1 rinsing bottle 2 several times.

Procedure

Wavelength:	340 nm (Hg 334 nm or Hg 365 nm)
Cuvette:	1 cm light path
Temperature:	25/30/37°C

Measurement:	against air	
Pipetted into cuvette:	Macro	Micro
Serum	0.2 ml	0.1 ml
Enzyme/Coenzyme/ α -oxoglu-tarate 2	2.0 ml	1.0 ml

Mixed and initial absorbance was read after 1 minute. Again after 1, 2 and 3 minutes the absorbance was read. The absorbance change per minute was noted and if the value is between:

0.11 and 0.16 at 340 nm/Hg 340 nm

0.06 and 0.08 at Hg 365 nm

Only then the values for the first 2 minutes were used for the calculation.

Calculation

To calculate the ALT activity the following formulae was used:

$$\text{U/l} = 1746 \times \Delta A \text{ 340 nm/min}$$

$$\text{U/l} = 1780 \times \Delta A \text{ Hg 334 nm/min}$$

$$\text{U/l} = 3235 \times \Delta A \text{ Hg 365 nm/min}$$

Normal values in serum

	25°C	30°C	37°C
Men	Up to 22 U/l	Up to 29 U/l	Up to 40 U/l
Women	Up to 17 U/l	Up to 22 U/l	Up to 31 U/l

Linearity

If the absorbance change per minute exceeds

0.16 at 340 nm/Hg 334 nm

0.08 at Hg 365 nm

0.1 ml of sample was diluted with 0.9 ml of 0.9% NaCl solution and reassessed. The result was multiplied by 10.

Estimation of insulin

Insulin by enzyme linked immunosorbent assay (ELISA) method using kit from Linco Research Inc., USA.

Principle

This assay is a sandwich ELISA based, sequentially, on: 1) concurrent capture of human insulin molecules from samples to the wells of a microtiter plate coated by a pre-titered amount of monoclonal anti-human insulin antibodies and the binding of a second biotinylated monoclonal mouse anti- antibody to the captured insulin, 2) washing of unbound materials 3) binding of horseradish peroxidase to the immobilized biotinylated antibodies, 4) washing of free enzyme conjugates, and 5) quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5'-tetramethylbenzidine. The enzyme activity is measured spectrophotometrically by the increased absorbance at 450 nm after acidification of formed products. Since the increase in absorbance is directly proportional to the amount of captured human insulin in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of human insulin.

Reagents supplied

Each kit was sufficient to run one 96-well plate and contains the following reagents:

1. Microtiter plate: Coated with mouse monoclonal anti-human insulin antibodies.
2. Adhesive plate sealer
3. 10X concentrate HRP wash buffer (2×50ml).

4. Standards: Human insulin in buffer 2, 5, 10, 20, 50, 100 and 200 $\mu\text{U/ml}$; 0.5 ml/bottle, ready to use.
5. Quality Controls 1 and 2: Purified recombinant human insulin in assay buffer, ready to use.
6. Matrix Solution: Heat-treated charcoal stripped off the clot human insulin serum (2 $\times$ 1 ml), ready to use.
7. Assay Buffer: 0.05 M Phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.08% Sodium azide, 1% BSA, 2 $\times$ 9 ml, ready for use.
8. Human insulin detection antibody: Pre-titered biotinylated monoclonal mouse anti-human insulin antibody (2 $\times$ 12 ml), ready to use.
9. Enzyme solution: Pre-titered streptavidin-horseradish peroxidase. Conjugation Buffer (2 $\times$ 12 ml), ready to use.
10. Substrate: 3,3',5,5'-tetramethylbenzidine in buffer (12 ml), ready to use. Minimize exposure to light.
11. Stop solution: 0.3 M HCl (12 ml), ready to use.

Materials required

1. Pipettes and pipette tips: 10 μl –20 μl or 20 μl -100 μl
2. Multi-channel pipettes: 5~50 μl and 50~300 μl
3. Reagent reservoirs
4. Vortex mixture
5. Deionized water
6. Microtiter plate reader capable of reading absorbency at 450 nm 590 nm
7. Orbital microtiter plate shaker
8. Absorbant paper
9. Standard refrigerator

Reagent preparation

HRP Wash Buffer: Diluted 10X HRP washing buffer concentrate 10 fold by mixing the entire content of each bottle with 450 ml deionized water.

Storage and Stability

All components of the kit were stored at 2-8° C. Multiple freeze/thaw cycles of the insulin standards and Matrix solution were avoided.

Assay Procedure

1. The reagents were allowed to come to room temperature. Then each well filled with 300µl of diluted HRP wash buffer and incubated at room temperature for 5 minutes. Then wash buffer was decanted and the residual amount was removed from all wells by inverting the plate and tapping it smartly onto the absorbent towels several times.
2. The microtitre plate was marked. A layout of the plate for blank, standards, QCs and unknown samples were made and recorded on a paper.
3. 20 µl assay buffer was added to the NSB (Non Specific Binding) wells and each of the sample wells.
4. 20 µl matrix solution was added to the NSB, Standard and Control wells.
5. 20 µl human insulin standard in the order of ascending concentration was added to the appropriate wells.
6. 20 µl of QC 1 and QC 2 was added to the appropriate wells.
7. Then 20 µl of unknown samples were added sequentially in wells according to the plate layout.
8. After that 20 µl of detection antibody was added to all wells. All procedures were performed within 30 minutes. Plates were covered using plate sealer and incubated at room temperature for 1 hour on an orbital microtitre plate shaker to rotate at moderate speed approximately 400 rpm.
9. At the end of incubation, plate sealer was removed and solutions were decanted. Residual solutions were removed by tapping as before. Each wells was then

- washed for 3 times with 300 μ l diluted HRP wash buffer. After each wash the plate was tapped on a pad of tissue to dry it off.
10. 100 μ l of enzyme solution was added to each well using a multichannel pipette. Plates were covered with sealer and incubated again with moderate shaking at room temperature for 30 minutes on microtitre plate shaker.
 11. Then sealer was removed and solutions were decanted from the plate and tapped to remove the residual fluid. Then wells were washed for 5 times using 300 μ l HRP wash buffer per well per wash and after each wash the plate was tapped smartly to remove the residual buffer.
 12. 100 μ l of substrate solution was added to each well and covered with sealer and incubated with moderate shaking at room temperature for 10 minutes on microtitre plate shaker. Blue color was formed in the wells of insulin standards with intensity proportional to increasing concentrations of insulin.
 13. Finally sealer was removed and 100 μ l of stop solution was added. Plate was placed on a microtitre plate shaker for gentle shaking (400 rpm).
 14. Reading was taken using a microplate reader at 450 nm.

Calculation of results for unknown samples

Optical densities of standard and unknown samples were fed in to a computer programme. A result of unknown samples was calculated extrapolating standard four parameter logistic curves using a Software Kinetical 3.

Determination of insulin secretory capacity and insulin sensitivity

Homeostasis Model Assessment (HOMA%S) is a simple widely used method which derives separate indices of B cell secretion (HOMA%B) and insulin sensitivity (HOMA% S) from the serum glucose and insulin concentrations under basal conditions by using mathematical formula or software (Levy *et al.*, 1998). The HOMA model has been incorporated in a simple MS-DOS-based computer program (HOMA-CIGMA software) that allows rapid determination of % B (B cell secretion) and % S (insulin sensitivity) from measured values. Although the simple equation gives a qualitatively useful approximation of the model prediction, most authors prefer the computer model. In this study HOMA-CIGMA software was used.

DNA extraction

Extraction of DNA was performed using GenElute DNA extraction kit (QIAGEN, USA). The kit uses the principal of silica gel DNA isolation from whole blood adapted in spin column.

Equipment, reagent and accessories

- Water bath
- Vortex
- Centrifuge
- Microcentrifuge tubes (1.5 ml)
- Pipette tips at different capacities (10 µl, 20 µl, 100 µl, 200 µl and 1000 µl)
- Micropipettes (4-50 µl, 100-1000 µl)
- Absolute ethanol (95-100 %)

Content of DNA kit

- Extraction column
- Collection tubes
- Proteinase K
- RNase-A solution
- Lyses Buffer AL
- Wash Buffer AW1
- Pre-Wash Buffer AW2
- Elution Buffer AE

Preparation of reagents

Wash buffer AW1: 125 ml ethanol (96-100%) was added to obtain 125 ml Buffer AW1 (95 ml concentrated).

Wash buffer AW2: 160 ml ethanol (96-100%) was added to obtain 226 ml Buffer AW2 (66 ml concentrated).

Extraction procedure

1. Frozen blood was brought to room temperature and made homogenous by brief vortexing. Aliquot of blood (200 µl) was transferred into 1.5 ml microcentrifuge tube.
2. 20 µl QIAGEN proteinase K was added inside the cap of the microcentrifuge tube.
3. 4 µl of an RNase-A stock solution (100mg/ml) was added to the sample before adding of Buffer AL.
4. 200 µl Buffer AL was added to the sample. In order to ensure efficient lyses, pulse-vortexed for 15 sec to make a homogenous solution.
5. Incubated at 56°C for 10 min.
6. To remove drops from the inside of the lid at the tube briefly centrifuged.
7. 200 µl ethanol (96-100%) was added to the sample, and mixed by pulse-vortexing for 15 sec and briefly spanned.
8. The mixture from the step 7 was transferred to the QIAamp Mini Spin Column (mounted on 2 ml collection tube) without wetting the rim and after closing the cap, centrifuged at 6000 x g for 1 min. The QIAamp Mini Spin column was placed on a fresh 2 ml collection tube.
9. The QIAamp Mini Spin column was opened carefully and 500 µl Buffer AW1 was added without wetting the rim. Centrifuged at 6000 x g for 1 min. the collection tube was discarded and the spin column was placed on fresh collection tube.
10. The QIAamp Mini Spin column was opened carefully and 500 µl Buffer AW2 was added without wetting the rim. Cap was screwed carefully and the centrifuged at 20000 x g for 3 minutes. At this stage the column appeared to be clean.
11. The QIAamp Mini spin column placed again on a 2 ml collection tube. 200 µl Buffer AE was added to the column. Incubated at room temperature for 5 minutes. Centrifuged at 6000 x g for 1 minute at 25°C. This elute supposed to contain DNA.

Check for DNA extraction

DNA yield for each sample was checked by agarose gel (1%) electrophoresis. To prepare agarose gel, appropriate amount of agarose was taken into polypropylene conical flasks containing required volume of working tris-borate EDTA (TBE) buffer. Agarose and buffer solution was mixed by swirling of the flasks. It was then heated to boiling point in a microwave oven with intermittent mixing until agarose was properly boiled. The gel was cooled nearer to the gelling point and ethidium bromide (0.5 μ g/ml) was added. The gel was then poured into horizontal gel mould, combs inserted, and allowed to polymerize. The gel was subsequently placed in horizontal electrophoresis tank filled with working TBE buffer.

To resolve DNA extract 3 μ l of DNA elute mixed with appropriate amount of loading buffer and then the mixer was loaded in agarose gel prepared earlier. The gel was run for at medium voltage for required time. DNA presence was visualized under UV light and gel image was captured.

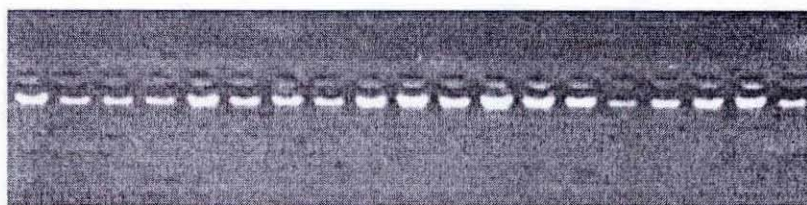


Figure 5: Gel image of DNA electrophoresis to check the extraction yield

CAPN 10 gene SNP-44 T>C polymorphic marker analyses

Subjects were genotyped for SNP-44 T>C polymorphic marker by a mutagenically separated PCR (MS-PCR) method using a common reverse primer and two allele-specific forward primers of different lengths. The primers used were as follows:

Forward primer:

T allele specific: 5'-CAGGGCGCTCACGCTTGCTAT-3'

C allele specific: 5'-GTGGGCAGAGGACTGGTGGGCGCTCACGCTTGCTTC-3'

Reverse primer: 5'-CTCATCCTCACCAAGTCAAGGC-3'

PCR was carried out in 10 µl reaction volume. Product size for the T allele is 60 bp and for C allele is 75 bp. The PCR protocol is as follows:

Name of the component	Volume (µl)	Concentration
DNA	5.0 µl	10-50 ng/ml
Buffer	1.00 µl	10 X
dNTPs	0.10 µl	200 µmol
Forward Primer (T)	1.00 µl	10 µmol
(C)	0.067µl	10 µmol
Reverse Primer	1.00 µl	10 µmol
HotStart Taq DNA polymerase	0.08 µl	5U/µl
ddH ₂ O	1.753µl	
Total	10.0 µl	

PCR conditions

PCR was carried out using HotStart Taq polymerase. Conditions for the amplification of the above mention product include initial step of denaturation 95°C for 15 min followed

by 34 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and elongation at 72°C for 30 seconds and the 34th cycle was followed by a step of final elongation 72°C for 10 minutes.

The PCR products were resolved in 3% agarose gel and visualized using gel documentation system following ethidium bromide staining. The PCR products were detected as 60 bp for T allele and 75 bp for C allele.

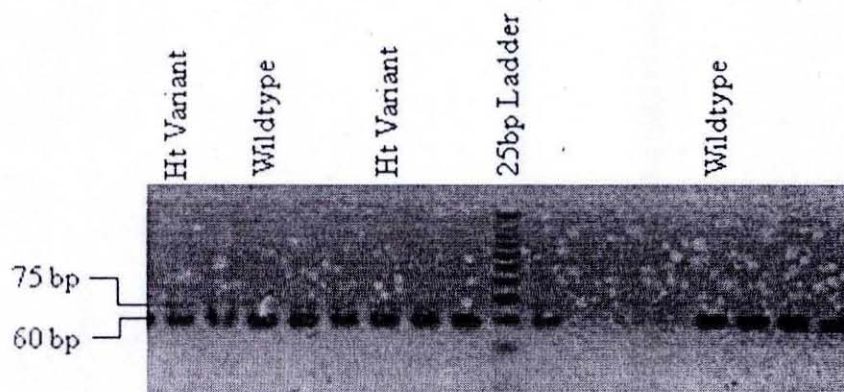


Figure 6: Gel image of PCR products for CAPN10 SNP-44 polymorphic marker analysis

Statistical Methods

Data were expressed as mean ($\pm$ SD) and number (percentage) as appropriate. Difference between two groups was determined by unpaired Student's 't' test, Chi-square test where applicable. Risk for outcome event of candidate gene maker was estimated by Odds ratio. Data were managed using statistical package for social science (SPSS) for Windows Version 10.

Chapter 4

RESULTS

RESULTS

A total number of 156 unrelated subjects were screened for glycemic status. Of them 91 were healthy controls and 65 were type 2 diabetes mellitus (T2DM).

Age, BMI and blood pressure of the study subjects

Mean ($\pm$ SD) age (yrs) of the control and T2DM subjects was 41.0 ± 8.0 and 43.0 ± 7.0 respectively which appeared to be statistically significant different ($p=0.058$) (Table 2).

Mean ($\pm$ SD) body mass index (BMI) of the control and T2DM subjects was 25.9 ± 3.9 and 24.9 ± 4.0 respectively which did not show statistical significant difference ($p=0.163$) (Table 2).

Mean ($\pm$ SD) systolic blood pressure (SBP, mmHg) of the control and T2DM subjects was 112 ± 12 and 122 ± 15 respectively which showed statistical significant difference ($p<0.001$) (Table 2).

Mean ($\pm$ SD) diastolic blood pressure (DBP, mmHg) of the control and T2DM subjects was 75.0 ± 7.9 and 77.9 ± 8.8 respectively which showed statistical significant difference ($p=0.039$) (Table 2).

Mean ($\pm$ SD) creatinine (mg/dl) of the control and T2DM subjects was 0.99 ± 0.12 and 0.95 ± 0.15 respectively which did not show statistical significant difference ($p=0.099$) between the groups (Table 2).

Mean ($\pm$ SD) S GPT (U/L) of the control and T2DM subjects was 26.3 ± 14.1 and 29.3 ± 28.4 respectively which did not show statistical significant difference ($p=0.434$) (Table 2).

Anthropometric measurements of the study subjects

Mean ($\pm$ SD) mid arm circumference (MAC, cm) of the control and T2DM subjects was 29.8 ± 4.2 and 29.2 ± 5.4 respectively which did not show significant difference ($p=0.396$) (Table 3).

Mean ($\pm$ SD) triceps skin fold (TSF, mm) of the control and T2DM subjects was 23.17 ± 13.1 and 19.5 ± 9.7 respectively which showed statistically significant difference ($p=0.064$) (Table 3).

Mean ($\pm$ SD) subscapular skin fold thickness (SSF, mm) of the control and T2DM subjects was 30.8 ± 11.0 and 32.7 ± 10.9 respectively, which did not show statistical significance difference ($p=0.291$) compared to T2DM (Table 3).

Mean ($\pm$ SD) body fat mass (BFM, %) of the control and T2DM subjects was 30.0 ± 6.6 and 28.5 ± 6.5 respectively which did not show significant difference ($p=0.161$) (Table 3).

Mean ($\pm$ SD) waist hip ratio (WHR) of the control and T2DM subjects was 0.92 ± 0.06 and 0.94 ± 0.06 respectively. WHR value of T2DM group was significantly higher compared to the controls ($p=0.083$) (Table 3).

Serum glucose levels and insulinemic status of the study subjects

Mean ($\pm$ SD) fasting serum glucose (FSG, mmol/l) of the control and T2DM subjects was 5.0 ± 0.6 and 8.0 ± 3.8 respectively. Mean ($\pm$ SD) postprandial glucose (PPG, mmol/l) of control and T2DM subjects was 5.8 ± 1.1 and 15.3 ± 4.9 respectively (Table 4).

Mean ($\pm$ SD) fasting serum insulin (μ U/ml) in the control and T2DM subjects was 10.5 ± 5.3 and 17.2 ± 10.9 respectively. Mean serum insulin in the T2DM group was significantly higher compared to the Controls ($p<0.001$) (Table 4).

Mean ($\pm$ SD) of HOMA%B in the Control and T2DM subjects was 115.0 ± 52.0 and 99.8 ± 57.3 respectively. Mean HOMA%B values did not show any statistically significant difference between the groups ($p=0.176$) (Table 4).

Mean ($\pm$ SD) HOMA% S values in the control and T2DM subjects was 79.9 ± 20.5 and 48.9 ± 21.7 respectively. Mean HOMA%S value in T2DM group was significantly lower than Control group ($p<0.001$) (Table 4).

Lipid levels of study subjects

Mean ($\pm$ SD) serum triglycerides (TG, mg/dl) of the control and T2DM subjects was 134.1 ± 60.2 and 197.4 ± 84.0 respectively which showed statistical significant difference ($p<0.001$) (Table 5).

Mean ($\pm$ SD) serum total cholesterol (mg/dl) of the control and T2DM subjects was 192.4 ± 60.2 and 229.0 ± 50.8 respectively which showed significantly higher in the T2DM group compared to the controls ($p<0.001$) (Table 5).

Mean ($\pm$ SD) high density lipoprotein cholesterol (HDL-c, mg/dl) of the control and T2DM subjects was 34.6 ± 10.3 and 32.0 ± 8.1 respectively which did not show significant difference ($p=0.100$) (Table 5).

Mean ($\pm$ SD) low density lipoprotein cholesterol (LDL-c, mg/dl) of the control and T2DM subjects were 132.0 ± 38.0 and 155.6 ± 50.7 respectively which showed significant difference ($p=0.003$) (Table 5).

Table 2: Age, BMI, blood pressure, S Creatinine, SGPT level of the study subjects

Variables	Control (n=91)	T2DM (n=65)	<i>t/p values</i>
			Control vs T2DM
Age (yrs)	40.53 ± 8.41	42.86 ± 6.80	1.913/0.058
BMI (kg/m^2)	25.92 ± 3.95	24.99 ± 4.09	1.403/0.163
SBP (mmHg)	112.12 ± 12.25	122.53 ± 14.50	4.157/<0.001
DBP (mmHg)	75.00 ± 7.93	77.90 ± 8.76	2.091/0.093
S Creatinine (mg/dl)	0.99 ± 0.16	0.95 ± 0.15	1.658/0.099
SGPT (U/L)	26.39 ± 14.12	29.39 ± 28.39	0.786/0.434

Results were expressed as mean $\pm$ SD.

Statistical comparison between groups was performed using unpaired Student's 't' test.

BMI, Body Mass Index; SBP, Systolic Blood Pressure DBP, Diastolic Blood Pressure; S Creatinine, Serum Creatinine; SGPT, Serum Glutamic Pyruvic Transaminase; T2DM, Type 2 Diabetes.

Table 3: Anthropometric measurements of the study subjects

Variables	Control (n=91)	T2DM (n=65)	<i>t/p values</i>
			Control vs T2DM
MAC (cm)	29.83±4.17	29.12±5.44	0.851/0.396
TSF (mm)	23.17±13.05	19.55±9.66	1.867/0.064
SSF (mm)	30.79±11.01	32.70±10.88	1.063/0.291
BFM (%)	30.03±6.60	28.51±6.52	1.408/0.161
WHR	0.92±0.06	0.94±0.06	1.746/0.083

Results were expressed as mean±SD.

Statistical comparison between groups was performed using unpaired Student's 't' test.

MAC, Mid Arm Circumferences; TSF, Triceps Skin Fold; SSF, Sub Scapular Skin fold; BFM, Body Fat Mass; WHR, Waist Hip Ratio; T2DM, Type 2 Diabetes Mellitus.

Table 4: Glycemic and insulinemic status of the study subjects

Variables	Control (n=91)	T2DM (n=65)	<i>t/p values</i>
			Control vs T2DM
FSG (mmol/l)	5.01±0.57	7.97±3.83	
PPG (mmol/l)	5.75±1.11	15.26±4.86	
Insulin (μU/ml)*	10.55±5.31	17.19±10.87	4.068/<0.001
HOMA % B*	115±52	100±57	1.363/0.176
HOMA % S*	79.9±20.5	48.9±21.7	7.248/<0.001

Results were expressed as mean±SD.

Statistical comparison between groups was performed using unpaired Student's 't' test.

FSG, Fasting Serum Glucose; PPG, Postprandial Glucose; HOMA%S, Insulin sensitivity assessed by Homeostatic Model Assessment; HOMA%B, B-cell function assessed by Homeostatic Model Assessment; T2DM, type 2 diabetes.

* Was determined in 101 (42 Controls and 59 T2DM) subjects

Table 5: Lipid levels of the study subjects

Variables	Control (n=91)	T2DM (n=65)	<i>t/p values</i>
			Control vs T2DM
TG (mg/dl)	134.07±60.16	197.38±84.04	5.107/<0.001
T Chol (mg/dl)	192.42±39.18	229.02±50.80	4.866/0.001
HDL-c (mg/dl)	34.62±10.25	32.02±8.13	1.655/0.119
LDL-c (mg/dl)	131.98±37.90	155.54±50.70	3.043/0.027

Results were expressed as mean±SD.

Statistical comparison between groups was performed using unpaired Student's 't' test.

TG, Triglycerides; T chol, total cholesterol; HDL-c, High-density lipoprotein cholesterol; LDL-c, Low density lipoprotein cholesterol, T2DM, type 2 diabetes mellitus.

CANDIDATE GENE MARKER ANALYSES

CAPN 10 SNP-44 (T>C) genotype of the study subjects

Genotype frequencies of the CAPN 10 gene SNP-44 (T>C) control variants were 0.670, 0.297 and 0.330 for wild type, heterozygous (Ht) variant and homozygous (Hz) variant respectively. In the T2DM group the frequencies were 0.662, 0.323 and 0.150 respectively. This genotype frequency distribution in the two groups did not show statistical significant association ($\chi^2=0.547$; $p=0.761$) (Table 6).

When heterozygous and homozygous variant genotypes were grouped together, the distribution was as following: Control- 0.670, and 0.330 for wild and variant respectively; T2DM- 0.663 and 0.338 respectively. This distribution also did not show significant association ($\chi^2=0.013$; $p=0.909$) for the genotype frequencies (Table 7).

Table 6: CAPN 10 SNP-44 (T>C) variant genotype of the study subjects

CPN 10 (T>C) marker Genotype	Control (n=91)	T2D M (n=65)	Total (n=156)
Wild (T,T)	0.670 (61)	0.662 (43)	0.667 (104)
Ht variant (T,C)	0.297 (27)	0.323 (21)	0.308 (48)
Hz variant (C,C)	0.330 (3)	0.150 (1)	0.260(4)
	$\chi^2=0.547$	$P=0.761$	

Results were expressed as frequency (number).

Chi-square test was performed to calculate statistical association.

Hz Wild, homozygous wild; Ht variant, heterozygous variant; Hz variant, homozygous variant; T2DM, type 2 diabetes.

Table 7: CAPN 10 SNP-44 genotype (Ht and Hz variant together) of the study subjects

CPN 10 SNP-44 (T>C)	Control (n=91)	T2DM (n=65)	Total (n=156)
Wild (C,C)	0.670(61)	0.663(43)	0.667(104)
Variant	0.330(30)	0.338(22)	0.333(52)
	$\chi^2=0.013$	$p=0.909$	

Results were expressed as frequency (number).

Chi-square test was performed to calculate statistical association.

Glycemic and insulinemic status of study subjects on the basis of CAPN10 SNP-44 T>C variant

Mean ($\pm$ SD) fasting glucose levels in subjects with wild and variant (heterozygous and homozygous together) genotype was found to be almost similar in the control (5.0 ± 0.6 and 5.0 ± 0.5 respectively; $p=0.633$) as well as in T2DM subjects (7.4 ± 3.2 and 9.2 ± 4.7 respectively; $p=0.116$) (Table 8).

Mean ($\pm$ SD) postprandial glucose levels in subjects with wild and variant (heterozygous and homozygous together) genotype was found to be almost similar in the control (5.7 ± 1.1 and 5.8 ± 1.2 respectively; $p=0.920$) as well as in T2DM subjects (14.7 ± 4.5 and 16.3 ± 5.4 respectively; $p=0.268$) (Table 8).

Mean ($\pm$ SD) insulin levels in subjects with wild and variant (heterozygous and homozygous together) genotype was found to be almost similar in the control (9.8 ± 2.5 and 12.1 ± 8.8 respectively; $p=0.377$) as well as in T2DM subjects (18.6 ± 12.3 and 13.7 ± 4.9 respectively; $p=0.114$) (Table 8).

Mean ($\pm$ SD) HOMA%B in subjects with wild and variant (heterozygous and homozygous together) genotype was found to be almost similar in the control (107.2 ± 24.7 and 132.3 ± 85.8 respectively; $p=0.320$) as well as in T2DM subjects (105.6 ± 58.8 and 85.5 ± 52.3 respectively; $p=0.226$) (Table 8).

Mean ($\pm$ SD) HOMA%S in subjects with wild and variant (heterozygous and homozygous together) genotype was found to be almost similar in the control (80.6 ± 18.8 and 78.2 ± 24.6 respectively; $p=0.721$) as well as in T2DM subjects (47.3 ± 21.6 and 52.8 ± 21.9 respectively; $p=0.388$) (Table 8).

BMI, WHR and Blood pressure of study subjects on the basis of CAPN10 SNP-44 T>C variant

Mean ($\pm$ SD) BMI in subjects with wild and variant (heterozygous and homozygous together) genotype was found to be almost similar in the control (25.8 ± 4.2 and 26.1 ± 3.4 respectively; $p=0.423$) as well as in T2DM subjects (24.8 ± 3.9 and 25.4 ± 4.4 respectively; $p=0.560$) (Table 9).

Mean ($\pm$ SD) waist to hip ratio in subjects with wild and variant (heterozygous and homozygous together) genotype was found to be almost similar in the control (0.92 ± 0.05 and 0.92 ± 0.06 respectively; $p=0.850$) as well as in T2DM subjects (0.93 ± 0.05 and 0.94 ± 0.06 respectively; $p=0.539$) (Table 9).

Mean ($\pm$ SD) SBP in subjects with wild and variant (heterozygous and homozygous together) genotype was found to be almost similar in the control (10.3 ± 11.1 and 110.3 ± 11.1 respectively; $p=0.055$) as well as in T2DM subjects (121 ± 15.1 and 122.5 ± 13.5 respectively; $p=0.691$) (Table 9).

Mean ($\pm$ SD) DBP in subjects with wild and variant (heterozygous and homozygous together) genotype was found to be almost similar in the control (73.7 ± 7.3 and 73.7 ± 7.2 respectively; $p=0.037$) as well as in T2DM subjects (77.8 ± 8.7 and 78.2 ± 9.1 respectively; $p=0.656$) (Table 9).

Lipid levels of study subjects on the basis of CAPN10 SNP-44 T>C variant

Mean ($\pm$ SD) triglyceride in subjects with wild and variant (heterozygous and homozygous together) genotype was found to be almost similar in the control (131.8 ± 61.3 and 138.8 ± 58.6 respectively; $p=0.599$) as well as in T2DM subjects (195.9 ± 82.7 and 200.6 ± 88.9 respectively; $p=0.842$) (Table 10).

Mean ($\pm$ SD) total cholesterol in subjects with wild and variant (heterozygous and homozygous together) genotype was found to be almost similar in the control (196.0 ± 42.3 and 197 ± 38 respectively; $p=0.894$) as well as in T2DM subjects (225.0 ± 50.0 and 185.1 ± 31.3 respectively; $p=0.393$) (Table 10).

Mean ($\pm$ SD) HDL-c in subjects with wild and variant (heterozygous and homozygous together) genotype was found to be almost similar in the control (35.8 ± 11.8 and 32.1 ± 4.3 respectively; $p=1.29$) as well as in T2DM subjects (31.4 ± 9.27 and 33.3 ± 5.1 respectively; $p=0.292$) (Table 10).

Mean ($\pm$ SD) LDL-c in subjects with wild and variant (heterozygous and homozygous together) genotype was found to be almost similar in the control (133.6 ± 41.1 and 128.4 ± 30.0 respectively; $p=0.565$) as well as in T2DM subjects (153.3 ± 50.3 and 160.4 ± 52.7 respectively; $p=0.628$) (Table 10).

Table 8: Glycemic and insulinemic status of the study subjects on the basis of CAPN 10 SNP-44 T>C variant genotype

	FSG (mmol/l)	PPG (mmol/l)	F Insulin (pmol/l)*	HOMA%B*	HOMA%S*
<i>Control subjects</i>					
Wild (n=61)	5.0±0.6	5.7±1.1	9.8±2.5	107.2±24.7	80.6±18.8
Variant (n=43)	5.0±0.5	5.8±1.2	12.1±8.8	132.3±85.8	78.2±24.6
<i>t/p values</i>	0.479/0.633	0.101/0.920	0.916/0.377	1.035/0.320	0.360/0.721
<i>T2DM subjects</i>					
Wild (n=30)	7.4±3.2	14.7±4.5	18.6±12.3	105.6±58.8	47.3±21.6
Variant (n=22)	9.2±4.7	16.3±5.4	13.7±4.9	85.5±52.3	52.8±21.9
<i>t/p values</i>	1.635/0.116	1.126/0.268	1.607/0.114	1.224/0.226	0.876/0.388

Results were expressed as mean ± SD.

Statistical comparison between groups was performed using unpaired Student's 't' test.

Variant, heterozygous and homozygous variant together; FSG, fasting serum glucose; PPG, postprandial glucose; F insulin, Fasting Insulin; HOMA %B, Homeostasis model assessment B-cell Function; HOMA %S, Homeostasis model assessment insulin sensitivity; T2DM, type 2 diabetes.

*Determined in 42 control (Wild vs Variant, 29 vs 13) and 59 T2DM (Wild vs Variant, 42 vs 17) subjects.

Table 9: BMI, WHR and blood pressure of the study subjects on the basis of CAPN 10 SNP-44 T>C variant genotype

	BMI	WHR	SBP	DBP
<i>Control subjects</i>				
Wild (n=61)	25.8±4.2	0.92±0.05	113.3±11.1	73.7±7.3
Variant (n=43)	26.1±3.4	0.92±0.06	110.3±11.1	74.7±7.2
<i>t/p values</i>	0.512/0.423	0.190/0.850	1.964/0.065	2.148/0.067
<i>T2DM subjects</i>				
Wild (n=30)	24.8±3.9	0.93±0.05	121±15.1	77.8±8.7
Variant (n=22)	25.4±4.4	0.94±0.06	122.5±13.5	78.2±9.1
<i>t/p values</i>	0.502/0.560	0.619/0.539	0.400/0.691	0.182/0.656

Results were expressed as mean ± SD.

Statistical comparison between groups was performed using unpaired Student's 't' test.

Variant, heterozygous and homozygous variant together; BMI, Body Mass Index; SBP, Systolic Blood Pressure DBP, Diastolic Blood Pressure; T2DM, Type 2 Diabetes.

Table 10: Lipid levels of the study subjects on the basis of CAPN 10 SNP-44 T>C variant genotype

	TG	Cholesterol	HDL-c	LDL-c
<i>Control subjects</i>				
Wild (n=61)	131.8±61.3	196.0±42.3	35.8±11.8	133.6±41.1
Variant (n=43)	138.8±58.6	185.1±31.3	32.1±4.3	128.4±30.0
<i>t/p values</i>	0.529/0.599	1.253/0.213	1.534/1.29	0.587/0.565
<i>T2DM subjects</i>				
Wild (n=30)	195.9±82.7	225.0±50.0	31.4±9.27	153.3±50.3
Variant (n=22)	200.6±88.9	236.7±56.8	33.3±5.1	160.4±52.7
<i>t/p values</i>	0.201/0.842	0.863/0.393	1.063/0.292	0.490/0.628

Results were expressed as mean ± SD.

Statistical comparison between groups was performed using unpaired Student's 't' test.

Variant, heterozygous and homozygous variant together; TG, Triglycerides; T chol, total cholesterol; HDL-c, High-density lipoprotein cholesterol; LDL-c, Low density lipoprotein cholesterol, T2DM, type 2 diabetes.

Chapter 5

DISCUSSION

DISCUSSION

Insulin resistance and/or progressive B cell dysfunction have been implicated in the pathogenesis of type 2 diabetes (Khan 1992). However, the specific molecular defects affecting insulin sensitivity and/or B cell secretory dysfunction still remained to be clearly understood.

Prevalence of T2DM found to vary mostly around 10-15% in different populations. However, in certain ethnic groups' prevalence of diabetes found to be very high (Lillioja *et al.*, 1991). Moreover high concordance for diabetes (63-90%) was observed in monozygotic twin pairs, which have strongly suggested a heritable genetic component creates susceptibility for the development of T2DM.

Relentless search continued for diabetogenic gene(s) by different research groups. Linkage analyses showed some breakthrough in this regard, which has identified several specific gene loci in a few number of families. Diabetes found to follow autosomal dominant Mendelian pattern of inheritance. These groups of patients are relatively young, normal to moderate obese, who are termed as maturity onset of the diabetes young (MODY) which accounts for 10% of all T2DM patients in the world. However genetic determinants for majority of the late onset T2DM remain to be elusive.

For sometimes researchers have started genome wide scan in search of different disease related genes. Some of the groups have completed first stage of genome scan. The first gene discovery from genome wide scan was identification of calpain 10 gene in Mexican American T2DM patients. Calpain 10 was found to be account for 14% of the population attributed risk for T2DM (Horikawa *et al.*, 2000). Later on the CAPN10 gene polymorphisms were investigated in different other populations of European origin for its role in the development of T2DM (Evans *et al.*, 2001). However, these studies have failed to replicate the association at the CAPN10 polymorphic allele (Horikawa *et al.*, 2000), but suggested that other alleles at this locus may increase the risk for T2DM in the UK population.

Single nucleotide polymorphism (SNP's) in the CAPN10 gene was found not to be associated in the Finish cohort (Fingerlin *et al.*, 2002). However, Rasmussen *et al* (2002) have shown that different haplotypes of CAPN10 gene variants confer low risk for T2DM in Scandinavian population. Earlier Song *et al* (2004) suggested that both large-scale and well-designed association studies are needed to explain the findings regarding the role of CAPN 10 gene polymorphism and the pathogenesis of T2DM. Functional studies have been suggested to reliably confirm or conclusively refute the initial hypothesis regarding the role of CAPN10 in T2DM risk in the Europoid population (Einarsdottir *et. al.*, 2006).

The candidate CAPN10 gene polymorphisms were also investigated in different Asian populations. Of these most notable ones are carried out by Cassell *et al* (2002) in South Indian and Kang *et al* (2006) in Korean ethnic population. Cassell *et al* (2002) in their study identified a particular haplotype conferring increased risk for both IFG/IGT and type 2 diabetes mellitus. However owing to the relative infrequency of at risk combination in the studied subjects they have suggested that CAPN10 gene was not a common determinant at susceptibility to T2DM.

In the present study T2DM subjects found to have significantly low insulin sensitivity (48.9 ± 21.7), as evaluated by HOMA%S, compared to the BMI matched controls (Table 5). However, the lower insulin sensitivity was found to be consistent with lower value (67.6 ± 1.7) and (57.7 ± 16.8) by Roy *et al* (2007) and Rahman (2008) respectively. This lower HOMA%S value in this subset of T2DM subjects further strengthen the notion of both insulin secretory dysfunction and lower insulin sensitivity are involved in the pathogenesis of T2DM in Bangladesh population.

Risk factors for T2DM like BMI and WHR between the T2DM and control- group did not show statistical difference. This feature was also in common with the previous studies. Variations in Calpain 10 gene have shown to affect blood glucose levels in British population. This effect has been attributed, at least partly, to its effect on the early insulin secretory response (Lynn *et al.*, 2002). A positive effect of haplotypic combination was also shown by Cassell *et al* (2002) on South Indian population.

Since both secretory defect and tendency to insulin resistance were observed in T2DM patients of Bangladesh, attempts were made to explore its pathological basis. As a part of ongoing research activities to investigate the molecular basis of pathogenesis at T2DM subjects, the present study was undertaken to determine insulin secretory capacity and sensitivity and their relationship with CAPN 10 gene SNP-44 T>C genotype frequencies.

The genotype frequency did not show significant association with T2DM. This finding of the present study was in agreement with the study of Orho-Melander et al (2002). Studies involving determination of CAPN 10 gene SNP -44 T>C genotype was however rare. In most of the cases the marker was analyzed with other polymorphism. In the present study the determination of the marker was confined to only SNP-44 T>C.

CAPN 10 gene SNP-63 C>T and In/Del 19 polymorphisms were also determined which did not show significant association with T2DM of Bangladeshi origin (Sultana 2009). The findings strongly suggest that CAPN 10 gene common polymorphism may not be causally associated with T2DM in this population.

Although, it is consistent with finding of Roy et al (2007) who found predominant insulin secretory defect. In the present study individuals possessing variant genotypes in either T2DM or control group did not show any statistical difference. Waist to hip ratio has recently been considered as important indicator of diabetes associated risk. In this subset of T2DM subjects WHR did not show significant difference compared to the age- and BMI-match control.

Conclusions

The present data concluded that: (i) CAPN 10 SNP 44 T>C polymorphism was not associated with T2DM of Bangladeshi origin. (ii) Insulin sensitivity appeared to be predominant in the subsets of T2DM subjects, and (iii) CAPN 10 SNP 44 T>C variant did not show any insulin sensitivity of the study subjects.

Limitations of the study

- i. Number of T2DM subjects in the study is small;
- ii. For this particular study only one polymorphic marker was determined.

Recommendations

- i. Study involving larger number of samples to achieve the statistical power for analysis, and
- ii. Inclusion of other polymorphic markers is recommended to conclusively comment on the effect of CAPN 10 gene variants in the pathogenesis of T2DM of Bangladeshi origin.

Chapter 6

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APPENDICES

Appendix I

Data Collection Sheet

MS Thesis

1. Identification No:

2. Particulars of the Subjects:

i) Name: _____

ii) Father's /Husband's Name: _____

iii) Age: _____ years

iv) Sex: Male / Female

v) Address:

Present: _____

Permanent: _____

vi) Phone/Cell: _____

vii) Date of 1st Examination: _____

3. Past Medical history:

4. Drug history:

Anti-HTN:

Lipid Lowering:

Anti-obesity:

Anti-ischemic:

5. Family history:

- i) Family history of Diabetes
- ii) Family history of HTN

6. Social history:

i) **Marital status:** Married / Unmarried / Widow.

ii) **Scio-economics Status:**

Total members of the family:

Income of the family (Monthly / Yearly):

iii) **Educational Status:**

Class I-VIII / SSC-HSC / Graduate / Others (_____)

iv) **Occupation:**

Current Designation

Duration:

Type of Work

Professional / Housewife / Sales Person / Domestic help /

Laborer / Others (_____)

v) **Habit:**

Exercise:

Present (Type_____, Duration_____)

Past (Type_____, Duration_____)

Not

Smoking:

Present (Duration_____, Sticks/day_____)

Past (Duration_____, Sticks/day_____)

Not

Betel Leaf: Yes / No (_____)

7. Physical Examination:

(b) Anthropometry:

- (ii) Height in meter:
- (iii) Weight in Kg:
- (iv) Waist circumference:
- (v) Hip circumference:
- (vi) Mid-upper arm circumference (MUAC):
- (vii) Skin fold thickness:
Triceps:
Sub-scapular skin folds thickness:
- (viii) Body fat mass:

(b) Blood pressure:

	1 st reading	2 nd reading	Average reading
Systolic			
Diastolic			

Appendix II

ক্রমিক নং

নাম:.....

বয়স:.....

ঠিকানা:.....

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খালী পেটে এবং ৭৫ গ্রাম গ্লুকোজ ড্রিঙ্ক পান করার পর পরিমানমত রক্ত নিয়ে পরীক্ষা করে,
ডায়াবেটিসের আক্রান্তের পূর্বের এবং শরীরে ইনসুলিনের অভাব অথবা কার্যক্ষমতা হ্রাসের
পরিমাপ করা হবে।

স্বাক্ষর:.....

তারিখ:.....