Investigation of Calpain 10 (rs2975760) gene polymorphism in Asian Indians with Gestational Diabetes Mellitus

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Background: Type 2 Diabetes Mellitus (T2DM) and Gestational Diabetes Mellitus (GDM) are part of a heterogeneous and complex metabolic group of disorders that share common pathophysiological circumstances, including β-cell dysfunction and insulin resistance. The protein Calpain 10 (CAPN10) plays a role in glucose metabolism, pancreatic β-cell insulin secretion, and thermogenesis.

Objective: Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR–RFLP) based genotyping of CAPN10 (rs2975760) polymorphism was carried out in T2DM and GDM with suitable controls for each of the pathologies from the same population. Genomic DNA was isolated from 787 participants, including 250 cases of T2DM, 287 pregnant women, of which 137 were identified as having GDM and the remaining 150 were confirmed as non-GDM, and 250 healthy control volunteers, and association analysis was carried out for genotypes and alleles.

Results: In the present study, T2DM was compared with healthy controls and was not found to be associated with the CAPN10 C allele.
Introduction

India has the largest number of people with diabetes in the world. Diabetes refers to a group of common metabolic disorders that share the phenotype of hyperglycemia. There are several distinct types of diabetes, but the two imperative common types are Type 2 Diabetes Mellitus (T2DM) and Gestational Diabetes Mellitus (GDM), which have been developing rapidly over the last decade in India. T2DM constitutes more than 90% of the cases of diabetes, and the prevalence has been dramatically increasing particularly in developing countries, such as India, which has 50.8 million diabetics, and China, which has 43.2 million diabetics, with 40–59-year-old patients being the largest age group impacted by the disease (Bhaskar et al., 2011; Kota et al., 2012). T2DM is a complex metabolic and multifactorial disease in which impaired insulin secretion and insulin action affect target tissues such as muscle and liver, and environmental triggers interact with genetic variants as well as genetic predispositions to increase weight and obesity, a strong risk factor for the disease (Lyssenko et al., 2005).

GDM is defined as carbohydrate intolerance resulting in hyperglycemia of variable severity with the onset or first recognition occurring during pregnancy. It is also characterized by impaired insulin secretion and action, which indicates that GDM may share the same phenotypic characters and genetic susceptibilities with T2DM (Bajaj et al., 2013; Mao et al., 2012). It is believed that hormones produced during pregnancy reduce a woman’s sensitivity to insulin, which leads to high blood sugar levels in approximately 3.8–21% of pregnancies in different parts of India (Seshiah et al., 2009). This is important because GDM is classified as a pre-diabetic condition with increased maternal and perinatal risk to develop T2DM (Colomiere et al., 2009; Ferrara, 2007; Landon et al., 2009; Seshiah et al., 2009). Knowledge regarding the genetics of GDM is very limited so far, and its exact etiology is unknown (Buchanan and Xiang, 2005). Women with a history of GDM are at an increased risk of developing T2DM later in life, but the risk and time of onset have not been fully quantified and women with a family history of diabetes may be predisposed to an increased risk of GDM (Bellamy et al., 2009; Williams et al., 2003). A universal consensus on the appropriate diagnostic methods and thresholds for the diagnosis of GDM remains ambiguous (Chon et al., 2013).

Previous investigations have focused on candidate genes expressed in pancreatic β-cells, which modulate the insulin secretion and resistance rates that predispose individuals to GDM and T2DM later in life (Montazeri et al., 2010). The Calpain 10 (CAPN10) gene was identified by Horikawa et al. (2000a) in Mexican Americans through a linkage scan, which identified polymorphisms that were associated with altered CAPN10 expression. The highest expression of CAPN10 mRNA is found in the heart followed by the pancreas, brain, liver, and kidneys (Marshall et al., 2005). CAPN10 is involved in the regulation of glucose homeostasis through its actions in the pancreatic β islet cells, liver, skeletal muscle, and adipocytes. Located on chromosome 2q37.3, CAPN10 comprises 15 exons spanning 31 kb that encode a 672 amino acid intracellular protease. Several case–control and association studies indicated that polymorphisms in CAPN10 are associated with the development of T2DM and insulin resistance, more so in obese patients with an earlier age of disease onset (Carlsson et al., 2005; Ezzidi et al., 2010; Fullerton et al., 2002; Horikawa et al., 2000b; Pihlajamaki et al., 2006; Tsuchiya et al., 2006). The calcium-dependent, non-lysosomal cysteine protease CAPN10 is involved in the reorganization of the actin cytoskeleton, which is required for both insulin exocytosis in pancreatic β-cells and insulin-stimulated GLUT4 translocation to the plasma membrane in adipocytes (Diaz-Villasenor et al., 2013).

Patients with GDM have been reported to exhibit the same genetic susceptibility as patients with T2DM. Recent polymorphism studies have shown that several genes are related to T2DM and GDM. Our aim was to examine whether certain candidate genes, previously shown to be associated with T2DM, also offer a specific genetic predisposition to GDM in Asian Indian women. Therefore, this study sought to address the relationship...
between dissimilar forms of diabetes mellitus, such as T2DM and GDM, with the CAPN10 single nucleotide polymorphism (SNP)-44 (rs2975760) in the Asian Indian population and assess its risk along with the clinical factors.

Materials and methods

**T2DM patients and healthy controls**

A total of 250 unrelated, non-obese patients defined as having T2DM according to the American Diabetes Association (ADA) criteria were selected from the two hospitals from the Hyderabad and were involved in the study with prior informed consent. The T2DM cases were defined as patients having a fasting plasma glucose level of more than 126 mg/dL. The patients were selected based on the physicians’ recommendations, which were confirmed by the endocrinologists and nephrologists. The patient group was not using insulin and aged >40 years old with a Body Mass Index (BMI) <30. T2DM in the patient group had been clinically established for more than 10 years with normo-albuminuria and without a history of any renal complications (Movva et al., 2007).

Non-diabetic healthy controls (n = 250) with a BMI <30 (similar to the case group) were age matched with the case group. The healthy controls were defined as those with a fasting blood glucose level below 110 mg/dL, and none of the controls is receiving any medications at the time of participation. The data collection was performed for each patient on clinical variables including age and family history. The patients who were presented with T1DM, which is defined as diabetic ketoacidosis, acute presentation with heavy ketonuria (>3 +), or uninterrupted requirement of insulin within 1 year of diagnosis, were excluded. Ethical committee’s clearances were obtained from the respective departments prior to the recruitment of subjects in this study.

**Selection of pregnant women with and without GDM**

The study was carried out on 287 pregnant women: 137 had developed GDM during pregnancy, and 150 women that had normal glucose levels during pregnancy were selected as the non-GDM. All selected GDM women had a 58.4% family history of T2DM. The screening and management of diabetes during pregnancy were conducted by qualified physicians according to the ADA guidelines.

**GDM and diagnostic criteria for GDM**

GDM cases were identified after a glucose challenge test (GCT) between weeks 24 and 28 of gestation. Fifty grams (50 g) of glucose was given to patients that had a fasting plasma glucose value exceeding 130 mg/dL; otherwise, the standard oral glucose tolerance test (OGTT) was performed using 100 g of glucose after an overnight fast and three days of an unrestricted diet. Fasting plasma samples were drawn 1, 2, and 3 h after the administration of glucose. In this study, the GDM cases were defined as those patients who produced two or more glucose values that met or exceeded the threshold values (Bhat et al., 2010). Women with T1DM, T2DM, or any other diabetes diagnosed before the pregnancy were excluded from the study.

**Clinical and biochemical measurements**

Clinical and anthropometric parameters, including BMI, were calculated according to Quetelet’s equation by using the weight in kilograms divided by the height in meters square (kg/m²). Trait measures for both the patient groups (T2DM and GDM) and the control groups were fasting blood glucose and postprandial blood glucose (PPBG). The following lipid profile parameters were also measured which were obtained from the T2DM group as well as the healthy control group: triglycerides (TG), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), and low-density lipoprotein-cholesterol (LDL-C). GCT and OGTT were performed on the pregnant women.
Sample collection

From each patient, 5 mL of venous blood was collected; 3 mL of the serum sample was used for the biochemical analysis to confirm the disease, and 2 mL of the EDTA sample was used for the molecular analysis.

Molecular scrutiny

Genomic DNA was extracted from peripheral blood leukocytes using the salting-out technique as previously described (Movva et al., 2007). The DNA samples were stored at −80 °C. The genotyping was performed at the Department of Genetics and Molecular Medicine (NABL accreditation laboratory), Kamineni Hospitals, Hyderabad, India for the rs2975760 polymorphism in intron 3 of CAPN10 by Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR–RFLP) followed by agarose gel electrophoresis on the extracted DNA using a pair of oligonucleotide primers: (forward sequence) 5′-GATGTGGGCATCCATAGCTT-3′ and (reverse sequence) 5′-TGATCCCCATGTGTAGCA-3′. The primers were synthesized by Bioserve Biotechnology (Hyderabad, India) for PCR analysis. The DNA was denatured at 95 °C for 5 min, amplified by 35 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 45 s, and the final extension at 72 °C for 5 min. Initially, Fnu4HI (GC↓NGC) (Thermo Scientific, USA) enzyme was tested with λ DNA to confirm the restriction enzyme and the PCR products were digested for 4 h with Fnu4HI (GC↓NGC) (Thermo Scientific, USA) at 37 °C (2.5 μL of distilled water with 10 units of enzyme per 15 μL PCR product and 2 μL of buffer in a final volume of 20 μL). The digested PCR products (rs2975760) were electrophoresed on 3.5% agarose gel containing ethidium bromide.

Statistical analysis

The data are expressed as the mean ± SD. Student’s t-test for unpaired data was used. The allele and genotype frequencies were determined by allele counting, and the chi-square (χ²) test was used to compare the observed versus the expected outcomes. The qualitative data were compared by the χ² test. The allele frequencies were estimated by the gene counting method, and the χ² test was used to identify departures from Hardy–Weinberg equilibrium (HWE). The statistical significance was examined by two-sided tests, and the statistical analyses were performed with SPSS version 19.0 software. A p value of <0.05 was considered to be statistically significant.

Results

T2DM study subjects

In this study, the anthropometric and clinical characteristics of the study groups were compared between T2DM cases and the healthy control subjects. During the study period, 250 T2DM patients and 250 healthy

Table 1

| S. no | Characteristics | T2DM cases (n = 250) | Healthy controls (n = 250) | p value |
|-------|-----------------|---------------------|---------------------------|---------|
| 1     | Age (years)     | 41–82 (57.19 ± 8.22)| 41–60 (53.93 ± 6.32)     | 0.0003  |
| 2     | Males/females (%) | 138 (55.2%) / 112 (44.8%) | 144 (57.6%) / 106(42.4%) | 0.3461  |
| 3     | BMI (kg/m²)     | 27.5 ± 4.1          | 25.8 ± 3.9                 | 0.4306  |
| 4     | T2DM interval   | 13.1 ± 6.3          | NA                        | NA      |
| 5     | FBS (mg/dL)     | 143.61 ± 55.66      | 93.54 ± 12.13              | 0.0001  |
| 6     | PPBG (mg/dL)    | 201.29 ± 25.25      | 117.29 ± 19.07             | 0.0001  |
| 7     | Triglycerides (mg/dL) | 156.42 ± 78.97  | 138.77 ± 53.69             | 0.0001  |
| 8     | Total cholesterol (mg/dL) | 183.95 ± 51.54 | 175.06 ± 33.05             | 0.0001  |
| 9     | HDL-C (mg/dL)   | 88.72 ± 23.1        | 82.61 ± 20.6               | 0.01    |
| 10    | LDL-C (mg/dL)   | 38.76 ± 4.4         | 35.53 ± 4.1                | 0.2658  |
| 11    | Family antiquity, n (%) | 146 (58.4%) | 138 (55.2%)                 | 0.3745  |

NA = not analyzed/not applicable.
controls were included in the study. All of the patients and controls belonged to a South Indian population from Andhra Pradesh. The clinical characteristics of the T2DM patients and the controls are presented in Table 1. The mean age was 57.19 years for the T2DM patients and 53.93 years for the control group. There was a significant difference in the FBS, PPBG, TG, HDL-C, and TC between the patients and the controls (p < 0.05). The BMI and LDL-C were not associated when the T2DM cases were compared with the healthy control subjects (p > 0.05). 58.4% of the T2DM cases had a family history of T2DM: however, only 55.2% of the control group had a family history of T2DM.

Clinical characteristics of pregnant women

The phenotypic and biochemical characteristics of the study subjects (GDM cases versus non-GDM) were collected, analyzed, and are presented in Table 2. The GDM cases (n = 137) had an age range of 22–38 years with a mean age of 26.7 years, whereas the non-GDM subjects had an age range of 17–34 years with a mean age of 24.6 years. The pre-pregnancy BMI range of the GDM cases was 19.8–35.6 kg/m² with a mean of 26.8 ± 3.93 kg/m² versus 19–31.1 kg/m² for the control subjects with a mean of 24.1 ± 3.55 kg/m² (p = 0.31). The FBS and PPBG values were found to be higher in patients with GDM compared with the non-GDM subjects and found to be statistically significant (110.6 ± 3.93 vs. 99.24 ± 11.37, p < 0.0001; 158.80 ± 47.76 vs. 112.00 ± 39.70, p = 0.02). Of the pregnant women with GDM (n = 137), 40.9% (56/137) controlled their plasma glucose levels with only diet and exercise, whereas 59.1% (81/137) of the pregnant women required 4–8 units of insulin for the entire antenatal period.

CAPN10 allele and genotype frequencies

The genotype and allele distributions were in HWE in the T2DM and GDM subjects and both of the control groups (i.e., healthy controls (n = 250) and non-GDM (n = 150)). The genotype and allele distributions of the CAPN10 rs2975760 polymorphism in the T2DM subjects versus the healthy controls and the GDM subjects versus the non-GDM are displayed in Table 3.

The CAPN10 genotype in individuals with T2DM (n = 250) was 62.4% TT, 33.2% TC, and 4.4% CC, while the healthy control group (n = 250) consisted of 64.4% TT, 32% TC, and 3.6% CC. The GDM group (n = 137) consisted of 62% TT, 29.2% TC, and 8.8% CC, and non-GDM (n = 150) consisted of 64.7% TT, 28% TC, and 7.3% CC. The overall T-allele frequency in the T2DM group was 0.79, and the C-allele frequency was 0.21, whereas the healthy controls demonstrated overall T-allele and C-allele frequencies of 0.80 and 0.20, respectively. In the GDM subjects, the overall T-allele and C-allele frequencies were 0.77 and 0.23, respectively, whereas the non-GDM demonstrated overall T-allele and C-allele frequencies of 0.79 and 0.21, respectively.

There was no apparent difference in either the genotype or the allele frequencies between the T2DM subjects and the healthy controls and the GDM subjects and the non-GDM. However, there was no statistically significant difference in either the genotype or the allele frequencies between the T2DM group and the GDM group as well as between both of the control groups (for T2DM; C vs. T: OR = 1.09; 95% CI = 1.00–1.20; p = 0.5821, and for GDM; C vs. T: 1.12; 95% CI = 0.7585–1.667; p = 0.5606 (Table 4)).

Table 2
Clinical details of GDM as well as controls involved in the contemporary study.

| S. no | Factors               | GDM cases (n = 137)       | Non-GDM (n = 150)       | Statistical significance |
|-------|-----------------------|---------------------------|-------------------------|--------------------------|
| 1     | Age (years)           | 22–38 (26.7 ± 5.1)        | 17–34 (24.6 ± 3.55)     | p = 0.0001               |
| 2     | BMI (kg/m²)           | 26.8 ± 3.93               | 24.1 ± 3.55             | p = 0.2248               |
| 3     | Mean gestational age  | 10–34 (24.4 ± 5.0)        | NA                      | NA                       |
| 4     | FBS (mg/dL)           | 110.6 ± 3.93              | 99.24 ± 11.37           | p < 0.0001               |
| 5     | PPBG (mg/dL)          | 158.80 ± 47.76            | 112.00 ± 39.70          | **p = 0.02**             |
| 6     | Family history        | 80 (58.4%)                | 84 (56%)                | p = 0.9701               |
| 7     | Insulin/diet (Ri)     | 81 (59.1%)/56 (40.9%)     | NA                      | NA                       |

NA = not analyzed/not applicable. Bold values indicate significance at p < 0.05.
The present study is the first to appraise the distribution of the \textit{CAPN10} (rs2975760) polymorphism in GDM women from Asian Indian population, and we simultaneously conducted this assessment with T2DM patients from the same population. The silent T5019C substitution in intron 3 of \textit{CAPN10} was not significant for the two diseases. This study was carried out with T2DM patients versus healthy controls and GDM patients versus non-GDM. The results of our study do not demonstrate any association of the \textit{CAPN10} polymorphism with T2DM and GDM when compared with their individual controls in our study population.

T2DM and GDM are heterogeneous and chronic metabolism disorders characterized by insulin resistance and pancreatic \(\beta\)-cell dysfunction, which involves defects in various molecular pathways (Bao et al., 2012; Matharoo et al., 2013). In the former reports, candidate genes were reported to be involved in the signal pathways of insulin, glucose, and the differentiation of adipocytes, and from these genes, only a few polymorphisms are associated with T2DM in different populations (Martinez-Gomez et al., 2011).

Cassell et al. (2002) carried out the initial study in \textit{CAPN10} and T2DM in the South Indian population in India. Adak et al. (2010) conducted the first study that examined \textit{CAPN10} and T2DM in the East Indian population in India. The SNP-43, -19, and -63 polymorphisms were examined and the results were found to be unpredictable or non-significant. In previous studies, the association of the SNP-44, -43, -19, and -63 polymorphisms of \textit{CAPN10} with the risk of T2DM was reported to be inconsistent. This study was conducted to South Indian populations from Chennai (Bodhini et al., 2011). We have carried out our study to South Indian participants from Hyderabad, a city with a well-established diabetic population. We have selected the SNP-44 (rs2975760) polymorphism from \textit{CAPN10}, and we have demonstrated the results as described by Bodhini et al. (2011).

A universal consensus on the appropriate diagnostic methods and thresholds for the diagnosis of GDM remains ambiguous (Chon et al., 2013). During pregnancy, women are faced with increased adiposity and increased insulin resistance. The insulin resistance that develops during pregnancy is explained in part by the increased production of human placental lactogen, estrogen, and prolactin. Women with GDM are assumed to have decreased \(\beta\)-cell insulin secretory function, which is similar to women with T2DM (Mao et al., 2012).

The same polymorphism from \textit{CAPN10} was assessed in a GDM group along with non-GDM. There are no studies conducted in India regarding pregnant women who do or do not develop diabetes during pregnancy. In the pregnant women, the rs2975760 polymorphism was found to be inconsistent. Our results were similar to that from a study conducted by Shaat et al. (2005). This study was performed in GDM and non-GDM Scandinavian women from Sweden.

### Table 3
Allele and Genotype frequency of \textit{CAPN10} gene polymorphism studies.

| Genotypes & alleles | T2DM cases | Healthy controls | GDM cases | Non-GDM |
|---------------------|------------|------------------|-----------|---------|
|                     | Cases (n = 250) | Controls (n = 250) | Cases (n = 137) | Controls (n = 150) |
| Homozygous: TT      | 156 (62.4%) | 161 (64.4%) | 85 (62%) | 97 (64.7%) |
| Heterozygous: TC    | 83 (33.2%) | 80 (32%) | 40 (29.2%) | 42 (28%) |
| Homozygous: CC      | 11 (4.4%) | 9 (3.6%) | 12 (8.8%) | 11 (7.3%) |
| Wild allele: T      | 395 (0.79) | 402 (0.80) | 210 (0.77%) | 236 (0.79) |
| Mutant allele: C    | 105 (0.21) | 98 (0.20) | 64 (0.23%) | 64 (0.21) |

### Table 4
\textit{CAPN10} gene polymorphism genotype distribution and allele frequency in T2DM with healthy controls and GDM versus non-GDM.

| rs2975760 | Allele and genotype frequencies for T2DM vs. healthy controls | Allele and genotype frequencies for GDM vs. non-GDM |
|----------|---------------------------------------------------------------|-----------------------------------------------------|
| C vs. T  | OR = 1.09; 95% CI = 0.8011–1.484; \( p = 0.5821\) | OR = 1.124; 95% CI = 0.7585–1.667; \( p = 0.5606\) |
| TC + CC vs. TT | OR = 1.09; 95% CI = 0.7575–1.569; \( p = 0.6425\) | OR = 1.133; 95% CI = 0.8359–2.132; \( p = 0.2266\) |
| TC vs. TT + CC | OR = 1.056; 95% CI = 0.7266–1.535; \( p = 0.7747\) | OR = 1.06; 95% CI = 0.6352–1.77; \( p = 0.8226\) |
| CC vs. TC + TT | OR = 1.232; 95% CI = 0.5016–3.028; \( p = 0.6481\) | OR = 1.213; 95% CI = 0.5169–2.847; \( p = 0.6568\) |
GDM has been recognized for decades, but controversies remain regarding the screening tests and diagnostic criteria. There is a consensus that the incidence of GDM is increasing globally. A family history of diabetes has a strong correlation with the occurrence of GDM. A study in Iran (Soheilykhah et al., 2010) reported a positive family history of diabetes in 76% of women with GDM compared with 43% in the normal group. This result was consistent with the studies of Hadaegh et al. (2005) and Jawad and Irshaduddin (1996). The incidence of GDM is also influenced by previous pregnancy outcomes. Naylor et al. found glucose intolerance in 14.5% of women who had adverse obstetric outcomes (Naylor, 1989). A study from Iran determined that the incidence of GDM increased in women who had a previous abortion, stillbirth, history of macrosomia, and a previous history of GDM. Pre-pregnancy BMI was considered a predictor development of GDM (Soheilykhah et al., 2010).

A limitation of our study is that T2DM women before pregnancy were not included as a study group in our GDM study. The frequency of cesarean sections was significantly higher than that of normal spontaneous vaginal delivery in patients with GDM compared with the non-GDM subjects. Several studies have uncovered a significant correlation between maternal hyperglycemia and perinatal morbidity. Moreover, increases in adverse maternal–fetal outcomes have been documented across the spectrum of carbohydrate intolerance even for glucose values below the currently recommended cut-offs for the diagnosis of gestational diabetes. Previous studies have documented that an abnormal glucose screening test is solely an independent predictor of macrosomia (Landon, 2011) and advocates the usefulness of follow-up diagnostic testing or close surveillance for fetal overgrowth during the third trimester in women with abnormal screening tests between weeks 24 and 28 of gestation (Braissant et al., 1996).

Genetic association studies can be problematic to reproduce due to inadequate statistical power, multiple hypothesis testing, population stratification, publication bias, and phenotypic heterogeneity. Considering the lack of sufficient evidence regarding the effect of candidate genes of T2DM on GDM and the conflicting results, we performed a case–control study between T2DM and GDM diseases to assess the association between the most commonly studied ones of the polymorphism in CAPN10, and we found that it is not associated with either T2DM or GDM. Many studies have revealed that genetic variants are associated with a susceptibility to GDM and T2DM. These two disease entities share common pathophysiological backgrounds, including β-cell dysfunction and insulin resistance. A limitation of the current study includes a comparatively small number of selected subjects diagnosed with T2DM (n = 250) and GDM (n = 137). Because previous studies have attempted to evaluate and analyze other SNPs in the same gene, our selection of a single specific SNP may present another limitation of our study. Moreover, we conducted the current study with a single gene and did not consider the interactions between the gene and its protein, which would require further studies.

In conclusion, we propose that the CAPN10 polymorphism examined in this study is not of value in predicting the occurrence and diagnosis of T2DM and GDM. Functional studies remain to be performed to establish the precise roles of these variants and pathways.

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