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Association Study between T2DM and *CAPN10* SNP-19 (rs3842570) Polymorphism in Navi Mumbai Population

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Abstract

Genetic research has brought a lot of new knowledge in the area of genetic predisposition of type 2 diabetes mellitus (T2DM). It has been proposed that excessive insulin resistance and obesity are also responsible for the higher incidence of type 2 diabetes. Calpain-10 (CAPN10) is a member of a large family of intracellular proteases. The polymorphism at deletion/insertion SNP19 of this gene influences susceptibility to T2DM. The aim of the study was to determine whether calpain-10 (ins/del SNP19) polymorphism contributes significantly to susceptibility to T2DM in population of Navi Mumbai. The study included randomly selected 75 patients of which 33 had T2DM and 42 served as control subjects. Mean waist-to-hip ratio, HDL, LDL, VLDL, cholesterol, and triglyceride showed no difference whereas mean of age, FBS, and body mass index showed significant differences between the control and diabetes subjects. Genotyping of calpain-10 (ins/del SNP19) polymorphism was performed by polymerase chain reaction method. Among 75 participants, for allele-specific SNP19, genotype frequencies of allele1 (2R-32 bp), heterozygous allele (2R-3R 32 bp), and allele 2 (3R-32 bp) were 20 (26.6%), 36 (48%), and 19 (25.3%) observed, respectively. The results from the present study have indicated that *CAPN10* (SNP19) shows no significant association with T2DM and more extensive studies on T2DM using candidate gene approach may provide better preventive measures and potential disease diagnostic tools.

Keywords: Calpain-10, Diabetes mellitus, Genotyping, Polymerase chain reaction, Single-nucleotide polymorphism

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Introduction

Diabetes mellitus is a metabolic illness that can have a variety of etiologies. Type 2 diabetes mellitus (T2DM), alternatively referred to as non-insulin-dependent diabetes mellitus, is characterized by insulin resistance or inefficiency. According to Wild *et al*., the "top" three nations in terms of T2DM individuals with diabetes in 2000 were India (31.7 million), China (20.8 million), and the United States (17.7 million in 2000). Clearly, T2D has become an epidemic in the 21st century and is accelerating throughout India.[1] India has the highest number of diabetic patients in the world. Moreover, diabetes is expected to affect 79.4 million individuals in India by 2030. Prevalence rates are increasing in cities, while recent numbers indicate an unexpected increase in rural areas.[2] It is apparent that over the previous two decades, the prevalence of diabetes has increased significantly among both urban and rural Indians. In addition, there is a shift in age of onset to younger age groups, which is alarming and this could have adverse effects on the nation's economy.[3]

Recent technology improvements enable large-scale sequence-based analysis, which aids in the understanding of the genetic underpinnings of complex disorders. Researchers use population-based genetic association studies to characterize disease-associated genetic variants. This involves analysis of a sequence, such as a chromosome region, a haplotype, a gene, or an allele, for its involvement in controlling the phenotype of a certain trait, metabolic pathway, or disease. These investigations establish a link between genetic sequence data from unrelated individuals and a measure of disease progression or state. Association study-based genotype analysis can be aided in identifying the T2DM associated genes which may hold preventive, predictive, and therapeutic value.

Calpains are a multidomain family of non-lysosomal calcium-activated intracellular cysteine proteases.[4] They induce partial proteolysis rather than total degradation of multiple substrates, hence changing the substrate structure and function.[5] There are 16 calpains, some of which are expressed ubiquitously and others with tissue-specific expression.[6] *CAPN10* (calpain family protease) was identified as the first potential susceptibility gene through genome-wide association studies for T2DM.[7] Numerous association studies, but not all, have frequently discovered a relationship between the CAPN10 polymorphism and type 2 diabetes.

Thus, the objective of the present research investigation is to reveal any possible potential association between the *CAPN10* gene variant and T2DM in the population of Navi Mumbai.

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Materials and Methods

Materials

DNA extraction from blood was done using affinity columns provided by Thermo Scientific, USA. Polymerase chain reaction (PCR) primers used in this study were procured from Eurofins, Operon, India. Taq Ready Master Mix was obtained from Kappa Biosystems. Restriction enzymes AvaII were acquired from Thermo Fischer Scientific, USA**.** ELISA kit for serum insulin detection was procured from NovaTec, Immundiagnostica GMBH, Germany. Kits for blood glucose, cholesterol, triglyceride, LDL, HDL, and VLDL were bought from Cogent, Span Diagnostics, India.

Methods

Subject selection

The study was conducted in 75 subjects, 33 subjects with T2DM and 42 controls (without diabetes) attending the medical outpatient department at Dr. D. Y. Patil Hospital and Research Centre, Navi Mumbai. Subjects with fasting plasma glucose greater than 126 mg/dl were diagnosed as diabetic. Before participation in the study, approval from the ethics committee of the hospital and a written informed consent was obtained from each subject. Subjects were carefully selected on basis of pre-decided inclusion and exclusion criteria. Subjects of either gender, 18 years or above with or without T2D, were included in the study. Whereas subjects with complications such as renal failure, heart failure, and diabetic ketoacidosis or subjects taking lipid-lowering agents or corticosteroids/antidepressants were excluded from the study.

Anthropometric analysis

Selected subjects were considered for anthropometric assessments (weight, height, body mass index [BMI], and waist and hip circumference assessments). Height and weight were measured by standard procedures. Waist and hip circumferences were measured using a flexible measuring tape, midway between the xiphoid and the umbilicus during the mid-inspiratory phase, and at the maximum circumference in the hip area, respectively. The waist-to-hip circumference ratio was also calculated for each subject.

Biochemical analysis

Blood sample from the patients was collected after 12 h of fasting in plain bulb for biochemical analysis and EDTA bulb for DNA extraction and genotyping studies. *In vitro* quantitative estimation of fasting blood glucose, cholesterol, triglyceride, HDL, LDL, and VLDL was carried out at the Department of Biochemistry, D. Y. Patil Medical College and Research Centre, Navi Mumbai.

Serum insulin estimation by ELISA

Collected blood sample was allowed to clot and serum was separated by centrifugation. A 100 µl of standards, control and serum samples, were added into the respective 96 wells of ELISA plate. A 100 µl conjugated antibody was added to each well other than substrate blank and plate was covered and kept at room temperature (25° C) for 2 h. After incubation, contents in the well were removed and wells were washed with 300 µl of diluted wash buffer thrice with an interval of 5 s. A 100 µl of TMB substrate was then added to all the wells and plate was further incubated at room temperature for 15 min in dark. Post-incubation 100 µl of stop solution was added in all the wells and absorbance was recorded at 450 nm on ELISA plate reader.

DNA extraction from whole blood sample

DNA extraction was done from whole blood using affinity columns kit provided by Thermo Scientific. A 1.5 ml of whole blood was taken in Eppendorf tube and centrifuged for 10 min at 5000 rpm. A 200 µL of intermediate buffy layer was taken using a micropipette and added with 20 µl of proteinase K. Then, 400 µL of lysis solution was added, mixed, and incubated at 56°C for 10 min while vortexing occasionally until the cells were completely lysed. Further, absolute ethanol was added and mixed by pipetting. Solution was then transferred to spin column and centrifuged at 8000 rpm for 2 min at room temperature. Further, collection tube was discarded and spin column was transferred to new collection tube. A 500 µL of wash buffer was added and centrifuged for 1 min at 10,000 rpm. Flow-through was then discarded and column was placed in collection tube. A 500 µL of wash buffer II was added to the column, centrifuged for 3 min at 14,000 rpm after which the flow-through was removed and column was again spun at 14,000 rpm for a minute. Collection tube containing the flow-through solution was discarded and spin column was transferred to a sterile 1.5 ml microcentrifuge tube. To elute the genomic DNA, 200 µL of elution buffer was added to the center of the column membrane, incubated for 2 min at room temperature, and centrifuged at 10,000 rpm for 1 min. The collection was stored at −20°C till further use. The integrity of the isolated DNA was estimated on 0.8% agarose gel with 0.5 µg/ml ethidium bromide.

PCR for CAPN10 SNP19 gene amplification

Primers were selected on basis of *in silico* testing and were reconstituted using nuclease-free water to final concentration of 10 µM. PCR conditions were estimated with the help of *in silico* data and GC% of primers was calculated to decide annealing temperature. The insertion/deletion polymorphism SNP19 was amplified by forward primers 5′-GTTTGGTTCTCTTCAGCGTGGAG-3′ and reverse primer 5′-CATGAACCCTGGCAGGGTCTAAG-3′. A 10 µl reaction was set up with, 2.5 µl of nuclease-free water, 0.5 µl of each primer, 1.5 µl template DNA, and 5 µl of kappa master mix. Conditions used for PCR were initial denaturation at 94°C for 5 min, denaturation at 94°C for 40 s; primer annealing was done at 54.3°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 8 min. These conditions were repeated for 35 cycles to obtain desired product. Qualitative estimation of allele-specific PCR product was performed using 2.5% agarose gel with 0.5 µg/ml ethidium bromide. Electrophoresis was carried out at 63 V until the loading dye migrated about three-fourth of the gel. A DNA molecular marker of 100 bp was run alongside the samples. The PCR product was visualized by observing the gel using gel documentation system (Syngene).

Statistical analysis

Data for anthropometric assessment and biochemical assay are presented as Mean±SEM. The significance of difference among the

groups was assessed by unpaired Student's t-test using GraphPad Prism software version 8. In figures and tables, symbols represent statistical significance as indicated: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. The genotype frequencies of control and case samples for calpain SNP 19 genes were confirmed with the Hardy-Weinberg equilibrium. Chi-squared test and Fisher's exact test were used to reveal any possible significant association of gene SNP with T2DM.

Results and conclusion

This study was conducted in total 75 subjects selected on basis of inclusion and exclusion criteria. Out of 75 subjects, 42 were non-diabetic subjects with blood glucose level below 126 mg/dl and 33 were classified as diabetics.

Anthropometric Assessment

Of the 75 subjects assessed for anthropometric and biochemical parameters, 42 subjects were control with an average age of 37.6±2.72. Percentage of female participation in the study found in control was 67% and that of male was 33%. Rest of the 33 subjects was classified as diabetic with an average age of 54.7±2.03. Percentage of female and male involved as diabetic was 45% and 55%, respectively. The mean age was revealed to be significantly higher (*P* < 0.001) in diabetic patients when compared with controls confirming the possible susceptibility of an individual to diabetes with increase in the age [Table 1].

Significant difference in the control and diabetic groups was also recorded for other anthropometric parameter like BMI (*P* < 0.01), whereas the mean difference of waist-to-hip ratio in control and diabetics was found to be non-significant, thereby indicating relevance of BMI in susceptibility to T2DM [Table 1]. Data based on gender classification showed significance difference between diabetic and non-diabetic females (*P* < 0.05), whereas mean BMI did not significantly differ in male control versus diabetic population. For waist-to-hip ratio, no significant difference was observed in both the groups of both the genders [Table 2]. The previous reports also have presented similar results affirming the association of BMI with the risk of type 2 diabetes.[8]

Biochemical Analysis

Biochemical profiling of T2DM cases aids in identifying parameter combinations that can serve as T2DM risk markers. It helps in recognizing the lifestyle changes that can reduce T2DM risk as well as influences the method of treatment employed in the specific population. More importantly, hyperlipidemia can develop in pre-diabetic phase of T2DM (as a consequence of insulin resistance) and lead to macrovascular complications. This emphasizes on the importance of screening lipid abnormalities and also encourages early interventions. Hence, in our study, mean levels of FBS, total cholesterol, TG, HDL, LDL, and VLDL were analyzed for diabetic and non-diabetic individuals. A high significant difference was observed in fasting blood glucose levels of the control and diabetic groups (*P* < 0.001) as expressed in Table 3 as blood sugar level served as one of the important inclusion criteria that highlight the segregation of both the groups evidently. A similar trend of significance was observed on gender classification. Cholesterol and triglyceride levels were observed to be increased in the diabetic subjects albeit the mean difference was statistically non-significant when compared to control [Table 3]. The classification of the population on the basis of gender also revealed non-significant difference between female control versus female diabetics and male control versus male diabetics [Table 4]. Data for other biochemical parameters such as HDL, LDL, and VLDL also remained statistically insignificant between the control and diabetic groups with and without gender classification [Table 5 and 6]. Our biochemical parameter results replicate those of Samatha *et al*. who found a strong significance in the level of FBS although significant difference in the levels of cholesterol and triglyceride was not observed in our study.[9]

Insulin resistance is present and precedes the development of T2DM in the majority of patients. The insulin resistance can be related to genetic abnormalities in a few individuals, but in most it appears to be related to obesity and in particular to central or visceral obesity.[10] After hyperglycemia is present, an additional component of insulin resistance occurs that is caused by the effects of hyperglycemia itself (glucose toxicity or desensitization).[11] To investigate insulin resistance in the population of Mumbaikar's, we performed quantitative estimation of serum insulin by ELISA method. The results reveled increased insulin levels although statistical analysis of the difference in serum insulin of patients and controls showed no significance difference (*P* > 0.50), indicating that the possibility of impaired glucose utilization is the presence of increased insulin resistance. The difference in serum insulin means of male and female patients and their respective control also followed the same trend of showing non-significant difference [Figure 1].

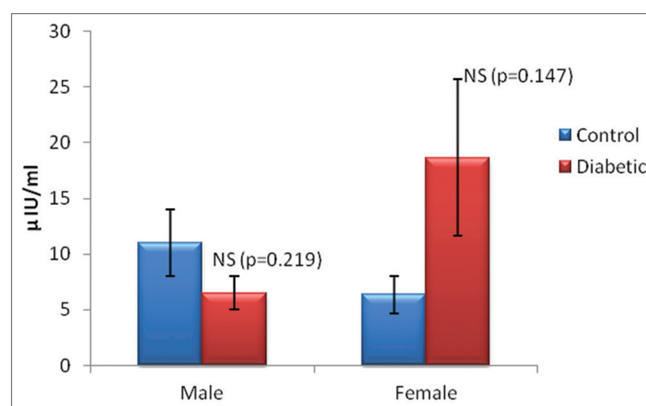

Figure 1: Graphical representation of serum insulin levels in the control and diabetics on gender classification. Values are expressed as Mean ± SEM, symbols in the table indicate statistical significance, NS: Non-significant

Table 1: Characteristics of T2DM patients and control group with various anthropometric data

Group	N	Female, n (%)	Male, n (%)	Age	BMI	Waist: hip ratio
Control	42 (56)	28 (67)	14 (33)	37.6±2.72	22.2±0.55	0.92±0.006
Diabetic	33 (44)	15 (45)	18 (55)	54.7±2.03***	25.2±0.88**	0.94±0.012^{NS}

The data represent Mean±SEM and the symbols in the table represent statistical significance as: ***P*<0.01, *P*<0.001 and NS: Non-significant

DNA Extraction and Amplification of CAPN10 SNP19 (rs3842570) Gene

DNA extraction was performed for 75 blood samples. Integrity of the isolated DNA was evaluated on 2% agarose gel and visualized on an UV transilluminator [Figure 2]. PCR amplification of CAPN10 SNP19 gene was performed using designed primers and isolated DNA samples as templates. Figure 3 shows a representative gel image of PCR amplification. The PCR product as inferred from previous studies was of homozygous allele1 (155 bp), homozygous allele2 (187 bp), and heterozygous allele (155 bp and 187 bp).

A photograph of ethidium bromide-stained 2.5% agarose gel showing the allele-specific PCR product for SNP19. L = 25 bp DNA ladder, lanes 1, 2, and 7 indicate a homozygous sample for allele 2 (187 bp); lanes 4, 5, 6, 9, and13 indicate heterozygous samples for allele 12 (155 bp and 187 bp); and lanes 8, 10, and 11 represent homozygous sample for allele 1 (155 bp).

Amplified PCR products of CAPN10 SNP 19 variant were used to decipher the genotypic and allelic frequencies. The genotypic frequency observed of the homozygous "2 repeats of 32 bp sequence" was 20, heterozygous "2 repeats/3 repeats" was 36, while the homozygous for "3 repeats of 32 bp repeat" was 19. The frequency of allele 1 "2 repeats 32 bp" at del/ins-19 was 0.506 and the frequencies of allele 2 "3 repeats 32 bp" at del/ins-19 were 0.494. The comparison between diabetics and controls revealed no significant difference between genotype frequency distribution ($\chi 2 = 0.3449$, $P = 0.8415$) on performing Chi-squared test with two degree of freedom and did not follow Hardy-Weinberg equilibrium. Our results suggest no association of CAPN10/SNP19 with T2DM in the current population.

An inconsistency has been observed in the pattern of association of *CAPN10 SNP19* (rs3842570) polymorphism with T2DM and its attributes. In the most recent 2019 study on association of this polymorphism to metabolic syndrome, susceptibility in four different ethnic groups (Goun, Nago, Tori, and Yoruba) constituting the South Benin population was analyzed. In Goun and Nago ethnic groups, the "del" allele (deletion of 32 bp repeat) was concluded to be a potential risk factor and the "ins" allele (insertion of the repetition 32 bp) was found to be protective against the metabolic abnormalities. However, in the Tori and Yoruba population, the polymorphism was indicated as a neutral marker.[12] A similar study on different ethnic groups in the Northwest Indian (Punjab) population, indicated a strong association between ins/ins genotype and Brahmin ethnic group. No such association was found in Banias and Sikhs ethnicities. These conclusions affirm that genetic predispositions to MetS vary between ethnic groups.[13] Discordances are also observed in the association between the polymorphism and anthropometric and biochemical entities related to T2DM. Association between BMI and ins/ins genotype in Turkish males and Brahmin Indians is significant.[13,14] Similarly, significant association also exists between obesity and ins/ins genotype in the random Japanese population,[15] Mexican children,[16] and Columbian youngsters.[17] The del/del genotype is associated to Tunisian Arab population.[18] On the contrary, an association study on Mexican population (2019) revealed correlation in waist circumference and triglycerides; both

Table 2: Anthropometric assessment in the control and diabetic subjects subclassified on the basis of gender

Group	*N*	*Gender (%)*	*Age*	*BMI*	*Waist: hip ratio*
Control	42	Female (67)	37.6±3.59	21.9±0.73	0.91±0.007
		Male (33)	37.6±4.06	22.9±0.77	0.92±0.01
Diabetic	33	Female (45)	54.7±2.92**	25.7±1.52*	0.94±0.01^{NS}
		Male (55)	54.7±2.89**	24.7±1.04^{NS}	0.94±0.02^{NS}

Values are expressed as Mean±SEM, N stands for total number of subjects. Comparison is made between control female versus diabetic female, and control male versus diabetes male group. Symbols in the table indicate statistical significance, *P<0.05, **P<0.01 and NS: Non-significant

Table 3: Biochemical parameter analysis in the control and diabetic subjects

Group	*N*	*Blood sugar*	*Cholesterol*	*Triglyceride*
Control	42	93.1±1.66	171.7±5.62	116.4±12.0
Diabetic	33	174.03±15.3***	178.6±6.87^{NS}	140.8±9.52^{NS}

Values are expressed as Mean±SEM, N stands for total number of subjects. Comparison is made between the control and diabetes groups. Symbols in the table indicate statistical significance, ***P<0.001 and NS: Non-significant

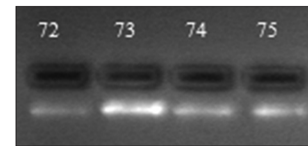

Figure 2: Representative gel image of isolated DNA

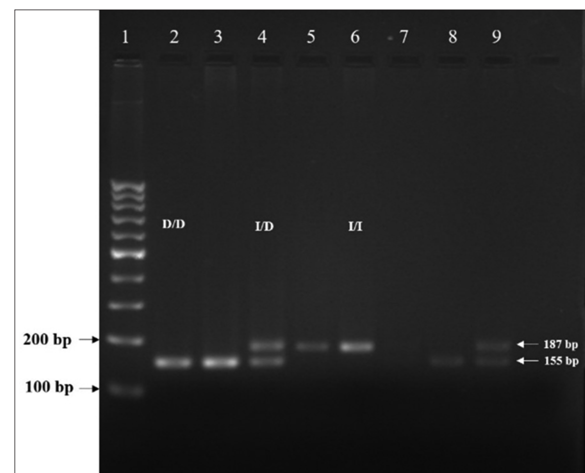

Figure 3: A representative gel image of PCR product for calpain SNP 19 (rs3842570) in different subjects

Table 4: Biochemical parameter analysis in the control and diabetic subjects subclassified on the basis of gender

Group	*N*	*Sex (%)*	*Blood sugar*	*Cholesterol*	*Triglyceride*
Control	42	Female (67)	93.7±2.19	171.1±7.19	114.1±16.95
		Male (33)	91.9±2.43	173±9.15	120.9±12.85
Diabetic	33	Female (45)	147±19.6***	170.7±7.39^{NS}	129±15.2^{NS}
		Male (55)	196.6±21.96***	185.2±10.95^{NS}	150.6±11.90^{NS}

Values are expressed as Mean±SEM, N stands for total number of subjects. Comparison is made between "a" control female versus diabetic female, "b" control male versus diabetes male group. Symbols in the table indicate statistical significance, ***P<0.001 and NS: Non-significant

Table 5: Biochemical estimation of HDL, LDL, and VLDL in the control and diabetic subjects

Biochemical parameters	*Control (n=42)*	*Diabetic (n=33)*
HDL	40.6±1.98	45.4±6.48[NS]
LDL	107.3±4.99	99.1±7.33[NS]
VLDL	29.8±3.92	32.39±3.98 [NS]

NS: Non-significant, HDL: High-density lipoprotein, LDL: Low-density lipoprotein, VLDL: Very low-density lipoprotein

Table 6: Biochemical parameter analysis in the control and diabetic subjects subclassified on the basis of gender

Group	*N*	*Sex (%)*	*HDL*	*LDL*	*VLDL*
Control	42	Female (67)	41.07±2.53	107.89±5.94	29.74±5.0
		Male (33)	39.57±3.2	106.1±9.42	30±6.2
Diabetic	33	Female (45)	38.46±2.49[NS]	88±9.33[NS]	24.8±3.1[NS]
		Male (55)	51.16±11.6 [NS]	108.3±10.7[NS]	38.7±6.52[NS]

Values are expressed as Mean±SEM, N stands for total number of subjects. Comparison is made between "a" control female versus diabetic female, "b" control male versus diabetes male group. Symbols in the table indicate statistical significance, NS: Non-significant, HDL: High-density lipoprotein, LDL: Low-density lipoprotein, VLDL: Very low-density lipoprotein

were found to be higher in individuals with del/del and del/ins genotypes. However, there was no link with BMI found.[19] These inconsistencies between studies are attributed to the differences in genetic background, sample size, age groups considered, dietary habits, and statistical analysis criteria. Overall, our study presents no association of calpain-10 SNP 19 in genetic predisposition to T2DM in Navi Mumbai population.

Conclusion

Our data show no association of *calpain-10* SNP 19 (rs3842570) with T2DM in the sample population of Navi Mumbai. The results indicate that our current status of knowledge regarding association of T2DM with specific genetic markers is still incomplete to explain most of the inherited risk. However, as data become available and better statistical techniques are applied to analyze gene-gene and gene-environment interaction, this predictive ability is likely to improve. More extensive studies on T2DM using candidate gene approach may provide better preventive measures, potential disease diagnostic tools, and personalized medicine initiatives in future treatments of T2DM.

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