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Determination of individual type 2 diabetes risk profile in the North East Indian population & its association with anthropometric parameters

Purabi Sarkar[1,†], Ananya Bhowmick[1,†], Manash P. Baruah[3], Sahana Bhattacharjee[2], Poornima Subhadra[4] & Sofia Banu[1]

[1]Departments of Bioengineering & Technology, [2]Statistics, Gauhati University, [3]Department of Endocrinology, Excelcare Hospitals, Guwahati, Assam & [4]Department of Genetics & Molecular Medicine, Kamineni Academy of Medical Sciences & Research Center, Hyderabad, Telangana, India

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***Background & objectives*: Diabetes genomics research has illuminated single nucleotide polymorphism (SNP) in several genes including, fat mass and obesity associated (*FTO*) (rs9939609 and rs9926289), potassium voltage-gated channel subfamily J member 11 (rs5219), *SLC30A*8 (rs13266634) and peroxisome proliferator-activated receptor gamma 2 (rs1805192). The present study was conducted to investigate the involvement of these polymorphisms in conferring susceptibility to type 2 diabetes (T2D) in the North East Indian population, and also to establish their association with anthropometric parameters.**

***Methods*: DNA was extracted from blood samples of 155 patients with T2D and 100 controls. Genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism and DNA sequencing. To confirm the association between the inheritance of SNP and T2D development, logistic regression analysis was performed.**

***Results*: For the rs9939609 variant (FTO), the dominant model AA/(AT+TT) revealed significant association with T2D [odds ratio (OR)=2.03, *P*=0.021], but was non-significant post correction for multiple testing (*P*=0.002). For the rs13266634 variant (*SLC30A*8), there was considerable but non-significant difference in the distribution pattern of genotypic polymorphisms between the patients and the controls (*P*=0.004). Significant association was observed in case of the recessive model (CC+CT)/TT (OR=4.56 *P*=0.001), after adjusting for age, gender and body mass index. In addition, a significant association (*P*=0.001) of low-density lipoprotein (mg/dl) could be established with the *FTO* (rs9926289) polymorphism assuming dominant model.**

***Interpretation & conclusions*: The current study demonstrated a modest but significant effect of *SLC30A8* (rs13266634) polymorphisms on T2D predisposition. Considering the burgeoning prevalence of T2D in the Indian population, the contribution of these genetic variants studied, to the ever-increasing number of T2D cases, appears to be relatively low. This study may serve as a foundation for performing future genome-wide association studies (GWAS) involving larger populations.**

Key words Body mass index - HbA_{1c} - hyperglycaemia - obesity - polymorphism - type 2 diabetes

[†]Equal contribution

Type 2 diabetes (T2D) is a condition of multiple metabolic disorders leading to abnormally high blood glucose levels (hyperglycaemia); disruption of insulin resistance-associated signalling pathways and defects in insulin-mediated glucose uptake in muscle cells. T2D is a classical example of multifactorial trait, where individual risk is defined by the complex interplay of genes and environmental factors[1]. The knowledge gap about the genetic architecture of T2D is often referred to as 'missing heritability'[2].

Genes play a pivotal role in susceptibility to T2D development. Genetic polymorphisms may augment or reduce a person's risk for developing the disease[3]. An example for this is the appreciable rate of T2D in families and in between identical twins and also the wide disparity in diabetes occurrence by ethnicity. Some of the broadly studied genes include peroxisome proliferator-activated receptor gamma 2 (*PPARγ*2), fat mass and obesity associated (*FTO*), potassium voltage-gated channel subfamily J member 11 (*KCNJ*11) and solute carrier family 30 (zinc transporter), member 8 (*SLC30A*8)[4,5]. Although majority of the loci illustrated the association pattern in certain populations, but the same pattern did not replicate in other ethnicities. This most plausibly is a result of genetic heterogeneity in T2D.

India, comprising one-sixth of the world's population, rapidly undergoing socio-economic evolution and containing high-risk phenotypic traits, provides an important resource for understanding the pathogenesis of T2D[6]. It is imperative to replicate and evaluate the distribution of the previously associated T2D markers in different Indian populations, as well as to identify novel genetic polymorphisms. This study was undertaken to investigate four previously reported genetic markers *PPARγ*2 (rs1805192), *FTO* (rs9939609 and rs9926289), *SLC30A*8 (rs13266634) and *KCNJ*11 (rs5219) to elucidate their effect on T2D disposition in North East Indian population and establish a probable association with the anthropometric parameters.

Material & Methods

A total of 155 well-characterized consecutive adult diabetic patients and 100 non-diabetic controls belonging to non-tribal Northeastern population of India, of age group ranging from 30 to 64 yr, visiting the department of Endocrinology, Excelcare Hospitals, Ulubari, Guwahati, India, between March and May, 2016, were included in this study. Individuals below 30 yr and above 70 yr of age were excluded. The age group of the control individuals was determined from the following formula: mean age of onset of T2D in patients±standard deviation[7]. The diagnosis of T2D was made according to the criteria of the World Health Organization[8]. T2D cases were referred to as patients having a fasting plasma glucose level of >126 mg/dl and/or those who were being treated as T2D with antidiabetic medication or other standard modality of treatment after a confirmed diagnosis. Patients with a history of ketoacidosis/requiring continuous insulin treatment since diagnosis and having exocrine pancreatic disease were excluded. Patients with severe liver or renal dysfunction were also excluded. Non-diabetic individuals with no known positive family history of diabetes for three generations attending the clinic for routine health checkups were included in the study as control population (glucose level <110 mg/dl). In addition, as per the plasma glucose estimation (WHO criteria)[8], diagnosis of diabetes was ruled out in controls if the post glucose value was <140 mg/dl after oral glucose tolerance test. Data from each patient were collected in a questionnaire form for recording clinical variables and lifestyle parameters including age, age of onset of T2D, body mass index (BMI), HbA_{1c} levels, low-density lipoprotein (LDL)-cholesterol levels and family history.

A written informed consent was obtained from all the participants. The study was approved by the Institutional Ethics Committee of Gauhati University (GUEC-01/2015).

Assessment of the clinical parameters: Three quantitative traits were studied namely BMI, LDL-cholesterol and family history. Obese patients were characterized by BMI >26 kg/m^2. Individuals with HbA_{1c} levels >6.4 per cent (44 mmol/mol) were considered as diabetic during enrolment. Individuals with LDL-cholesterol levels >130 mg/dl were categorized as high LDL. LDL-cholesterol levels and HbA_{1c} were estimated in serum and whole blood, respectively. LDL-cholesterol levels were estimated by using standard enzymatic methods[9] using Hitachi-912 autoanalyzer (Hitachi, Mannheim, Germany). The HbA_{1c} levels were determined using high-performance liquid chromatography[10] employing the Variant machine (Bio-Rad, Hercules, CA, USA). Individuals with a history of T2D amongst ≥25 per cent of their family members within previous three generations were considered to be having 'strong' family history[11].

DNA extraction SNP selection and genotyping: Two ml of peripheral blood was collected from each individual in 0.5 M EDTA tube. Genomic DNA was isolated from the whole blood using the standard phenol-chloroform extraction method[12]. The quantity and quality of DNA were determined using Nano Drop quantifier (Thermo Fisher Scientific, USA) and by agarose gel electrophoresis. DNA was stored at −20°C till subsequent use.

Restriction enzymes were purchased from New England Biolabs, USA. Genotyping for the *PPARγ*2 (rs1805192), *SLC30A*8 (rs13266634) and *KCNJ*11 (rs5219) genes was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)[13] with in-house designed primers procured from BioServe Biotechnologies Pvt. Ltd., Hyderabad (Table I), whereas the *FTO* (rs9939609 and rs9926289) gene was sequenced using the same set of primers for amplification, as these occur within the same locus. Genomic DNA (50 ng) was amplified in a 15 µl PCR reaction, PCR buffer containing 3 mM $MgCl_2$, 0.25 mM deoxynucleotide triphosphates, 5 pmol of each primer and 0.6 U Taq polymerase (Bangalore Genei, Bengaluru). As regards the PCR cycle, primary denaturation was executed at 94°C (4 min), followed by 36 cycles at 94°C for 30 sec, 50-60°C for 30 sec, 72°C for 50 sec and final extension at 72°C for 10 min. PCR amplicons digested with 1 U restriction enzymes were visualized in a three per cent high-resolution agarose gel under an ultraviolet transilluminator. To validate the PCR-RFLP genotyping, experiments were conducted by the two authors individually and the results were matched (Fig. 1). To confirm the results, genotype of 10 per cent of the participants was confirmed by direct sequencing. Genotyping of the *FTO* single nucleotide polymorphisms (SNPs) (rs9939609 and rs9926289) was performed using 3730xl DNA Analyzer (Applied Biosystems, USA) (Fig. 1). The genotype call accuracy rates were determined to be 99.99 per cent for the SNPs rs9939609 and rs9926289.

Statistical analysis: The data were analyzed using Statistical Package for Social Sciences (PASW Statistics 18.0.0 (SPSS Inc., Chicago, IL, USA). Quantitative variables such as LDL-cholesterol and BMI were expressed as mean±standard error (SE) of mean. Hardy–Weinberg equilibrium (HWE) test was applied to determine the disparity in the distribution of alleles and genotypes; 2×3 Fisher's exact test was used to determine the significant association of polymorphisms with the overall allelic and genotypic frequency distribution. Association analysis was further confirmed by conditional logistic regression for anthropometric parameters adjusted for age, gender and BMI, using MedCalc, version 7.4.1.0 (MedCalc Software, Belgium). Power calculation was performed using odds ratio (OR), by using the GAS Power Calculator (*http://csg.sph.umich.edu/abecasis/cats/gas_power_calculator/index.html*). Our study yielded 22.1, 32.7 and 8.01 per cent power (P=0.05) for *FTO* (rs9930609), *KCNJ*11 (rs5219) and *SLC30A*8 (rs13266634) polymorphisms, respectively, and hence was not adequately powered to detect the true association of the SNPs.

Results

A description of the study population stratified by T2D status is summarized in Table II. The average values of clinical characteristics including age (P<0.001), LDL-cholesterol (P<0.01) and HbA_{1c}

Table I. List of primers and restriction enzymes used

Gene	Primer	Sequence of primer	Amplicon size (bp)	Restriction enzyme
KCNJ11 (rs5219)	Forward Reverse	5'-TTCATGAAGATGCAGCCAAG-3' 5'-ACCCAGGTGGAGGTAAGGAA-3'	689	*Ban* II
SLC3OA8 (rs13266634)	Forward Reverse	5'-TCTGTGGGGAGCTCTAAACG-3' 5'-AGCTGTTACTTCGGCTCCAC-3'	845	*Msp* I
FTO (rs9939609)	Forward Reverse	5'-GGCGAGATATGGTGAGTGGT-3' 5'-TCCCAAAGTCCTGGAAACAC-3'	845	NA
FTO (rs9926289)	Forward Reverse	5'-GGCGAGATATGGTGAGTGGT-3' 5'-TCCCAAAGTCCTGGAAACAC-3'	845	NA
*PPARγ*2 (rs1805192)	Forward Reverse	5'-ACCTGGGTAAAGGGTGACTTC-3' 5'-AAGGCAAGATTGACCTCGTG-3'	632	*Hae* III

NA, not applicable

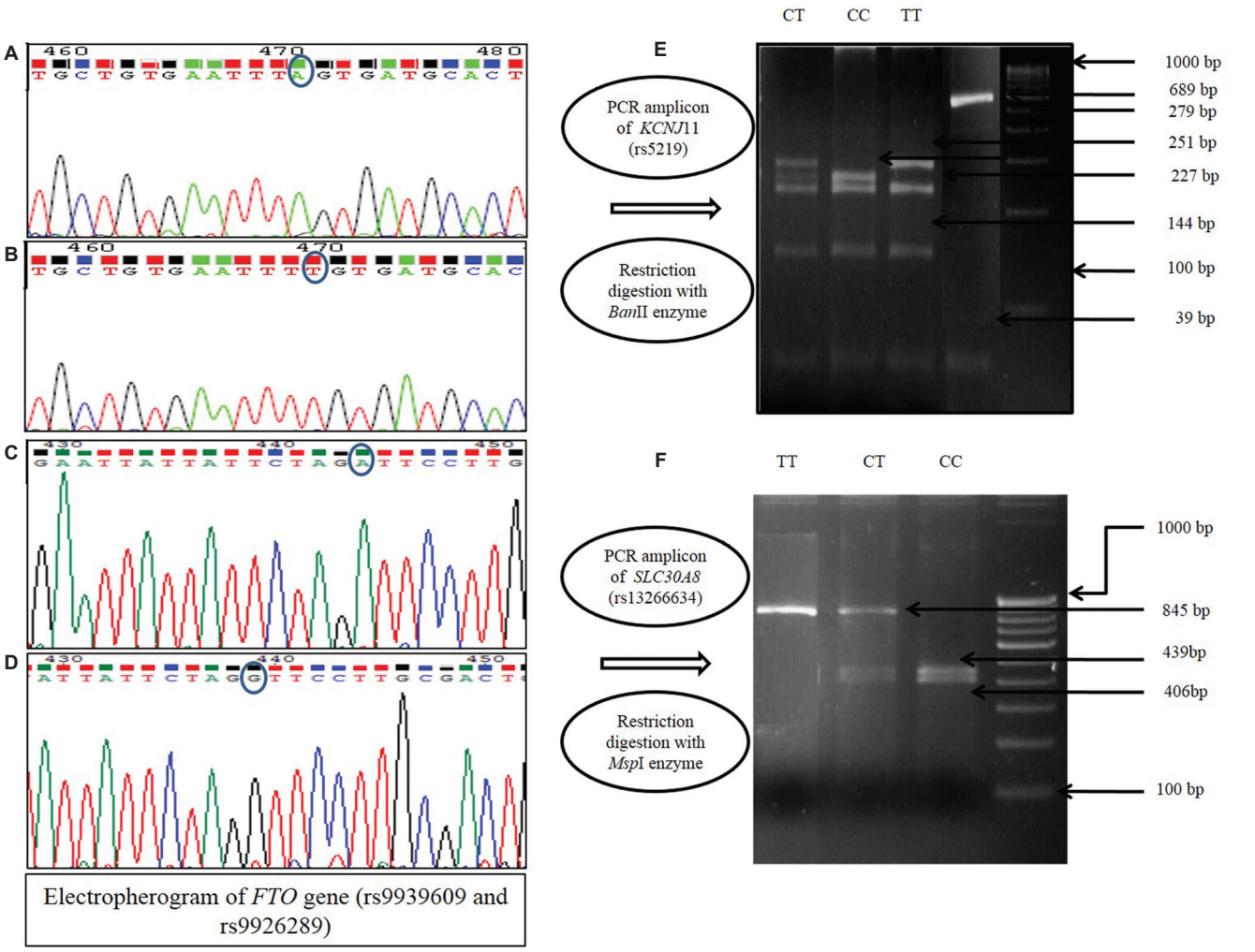

Fig. 1. Chromatogram depicting *FTO* SNPs [rs9939609: (**A**) wild type, (**B**) homozygote]; [rs9926289: (**C**) wild type, (**D**) homozygote] and restriction digestion patterns in *KCNJ11* (**E**) and *SLC30A8* (**F**). *FTO*, fat mass and obesity associated; *KCNJ11*, potassium voltage-gated channel subfamily J member 11; SNPs, single nucleotide polymorphisms.

Table II. Descriptive characteristics of the study population

Characteristics	Mean±SD	
	Patients (n=155)	Controls (n=100)
Age (yr)	47.81±8.40***	39.69±8.06
BMI (kg/m^2)	25.84±4.21	27.19±26.60
LDL cholesterol (mg/dl)	95.88±45.47**	112.59±39.51
HbA_{1c} (%)	7.55±2.10***	4.85±1.39
Family history (%)	16.68±16.63	12.79±16.81
Sex		
Male (%)	20 (20.0)	89 (57.4)
Female (%)	80 (80.0)	66 (42.6)

*P<0.05, **<0.01, ***<0.001 compared to controls. BMI, body mass index; LDL, low-density lipoprotein; HbA_{1c}, glycosylated haemoglobin; SD, standard deviation

(P<0.001) differed significantly between patients and controls.

The genotypes of all the variants were in HWE (P>0.05). The linkage disequilibrium (LD) plot for two SNPs of the *FTO* gene (Fig. 2A and B) suggested strong LD (Patients: D'=1.00, r^2 =0.727; controls: D'=1.00, r^2=0.887). On comparing the distribution of genotype and allele frequencies (Table III), the frequency of T allele and the TT genotype for rs9939609 variant (*FTO*) was significantly higher in T2D patients when compared with the controls (P=0.013 and 0.027, respectively). This association pattern did not remain significant after Bonferroni's correction for multiple testing was applied, with the threshold for significance being 0.05/25=0.002 [5 SNPs × 5 tests (genotype frequency, allele frequency and three models of inheritance)]. Similarly, for the

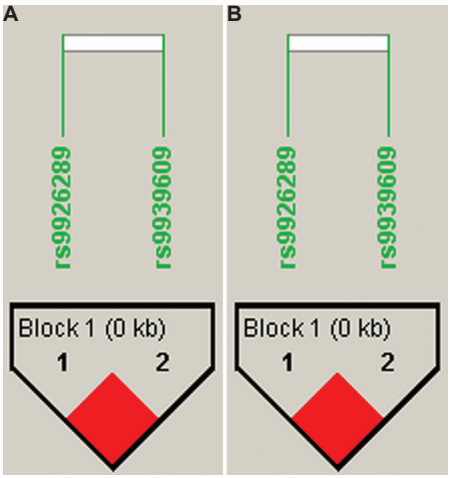

Fig. 2. Linkage disequilibrium plots for *FTO* SNP rs9939609 and rs9926289 in patients (**A**) and controls (**B**).

rs13266634 variant (*SLC30A8*), there was considerable but non-significant difference in the distribution pattern of genotypic polymorphisms between the patients and the controls (P=0.004). None of the other SNPs could establish a positive association with T2D predisposition and not a single case of polymorphism was reported for the rs1805192 variant (*PPARγ2*).

From the logistic regression analysis, the highest odds and significant risk for T2D development was conferred by the recessive model (CC+CT/TT) of *SLC30A8* (rs13266634) variant (OR=4.56, P=0.001) (Table IV).

The anthropometric parameters were compared with SNPs based on dominant model (Table V). A significant association (P<0.001) of LDL

Table III. Association of single nucleotide polymorphisms (SNPs) with genotypic and allelic traits

SNP (Gene)	Genotype/allele	Patients (n=155), n (%)	Controls (n=100), n (%)	*P*
rs9939609 (*FTO*)	AA	22 (14.19)	21 (21.00)	
	AT	73 (47.09)	56 (56.00)	
	TT	60 (38.70)	23 (23.00)	0.027
	A	117 (37.74)	98 (49.00)	
	T	193 (63.25)	102 (51.00)	0.013
rs9926289 (*FTO*)	AA	33 (21.29)	19 (19.00)	
	AG	75 (48.38)	54 (54.00)	
	GG	47 (30.32)	27 (27.00)	0.688
	A	141 (45.40)	92 (46.00)	
	G	169 (54.50)	108 (54.00)	0.927
rs5219 (*KCNJ11*)	CC	54 (34.80)	46 (46.00)	
	CT	81 (52.20)	42 (42.00)	
	TT	20 (12.90)	12 (12.00)	0.191
	C	189 (60.96)	134 (67.00)	
	T	121 (39.04)	66 (33.00)	0.187
rs13266634 (*SLC30A8*)	CC	74 (47.74)	44 (44.00)	
	CT	75 (48.38)	40 (40.00)	
	TT	6 (3.80)	16 (16.00)	0.004
	C	223 (71.90)	128 (64.00)	
	T	87 (28.00)	72 (36.00)	0.063
rs1805192 (*PPARγ2*)	CC	155 (100.00)	100 (100.00)	
	CG	0 (0.00)	0 (0.00)	
	GG	0 (0.00)	0 (0.00)	
	C	310 (100.00)	200 (100.00)	
	G	0 (0.00)	0 (0.00)	

KCNJ11, potassium voltage-gated channel subfamily J member 11; *FTO*, fat mass and obesity associated

Table IV. Association analysis of single nucleotide polymorphisms (SNPs) by logistic regression

SNP ID	Genetic model	Genotype	OR (95% CI)	*P**
FTO (rs9939609) (A/T)	Dominant	AA/(AT+TT)	2.03/(1.14-3.32)	0.021
	Co-dominant	AT/(AA+TT)	0.45/(0.29-0.84)	0.035
	Recessive	(AA+AT)/TT	1.50/(0.79-2.90)	0.136
	Allelic	T/A	1.52/(1.07-2.04)	0.015
FTO (rs9926289) (A/G)	Dominant	AA/(AG+GG)	0.84/(0.44-0.15)	0.586
	Co-dominant	AG/(AA+GG)	0.77/(0.44-1.30)	0.362
	Recessive	(AA+AG)/GG	1.12/(0.64-1.99)	0.613
	Allelic	G/A	1.00/(0.70-1.25)	0.896
*KCNJ*11 (C/T)	Dominant	CC/(CT+TT)	1.57/(0.94-2.65)	0.064
	Co-dominant	CT/(CC+TT)	1.50/(0.90-2.30)	0.101
	Recessive	(CC+CT)/TT	1.06/(0.50-2.20)	0.752
	Allelic	T/C	1.10/(0.78-1.66)	0.143
*SLC30A*8 (C/T)	Dominant	CC/(CT+TT)	1.14/(0.69-1.79)	0.487
	Co-dominant	CT/(CC+TT)	1.39/(0.79-2.23)	0.164
	Recessive	(CC+CT)/TT	4.56/(1.56-11.45)	0.001
	Allelic	T/C	1.42/(0.87-1.98)	0.041

*Adjusted for age, gender and BMI. OR, odds ratio; CI, confidence interval

Table V. Association analysis of single nucleotide polymorphisms (SNPs) with quantitative phenotypes based on dominant model

SNP ID	Trait associated with T2D	Genotype	OR (95% CI)	*P**
FTO (rs9939609)	BMI (kg/m^2)	AA/(AT+TT)	1.52 (0.79-2.93)	0.205
FTO (rs9926289)		AA/(AG+GG)	0.86 (0.46-1.62)	0.657
*KCNJ*11		CC/(CT+TT)	1.59 (0.95-2.66)	0.075
SLC		CC/(CT+TT)	0.86 (0.51-1.42)	0.558
FTO (rs9939609)	LDL (mg/dl)	AA/(AT+TT)	0.70 (0.32-1.51)	0.364
FTO (rs9926289)		AA/(AG+GG)	0.25 (0.14-0.43)	0.001
*KCNJ*11		CC/(CT+TT)	1.59 (0.95-2.66)	0.075
SLC		CC/(CT+TT)	0.77 (0.49-1.23)	0.284

*Adjusted for age, gender and BMI. T2D, type 2 diabetes

(mg/dl) could be established with the *FTO* (rs9926289) polymorphism, highlighting that measures of obesity were related to T2D predisposition in our study population.

Discussion

FTO gene codes for a protein termed 2-oxoglutarate-dependent nucleic acid demethylase, which is involved in the regulation of body fat masses by lipolysis and fatty acid metabolism[14]. Polymorphisms of the *FTO* gene have been reported to be strongly associated with increased BMI in Europeans and Japanese, but have shown variable results in other ethnicities including Hispanics, Asian and African-Americans[15-17]. A study on Asian Indian Sikhs demonstrated a strong association of *FTO* variants with T2D[18]. A few other studies also identified a positive association of rs9939609 with T2D[19,20]. No association was found between *FTO* rs9939609 SNP and the risk of obesity in Pakistani population[21]. The

frequency of the minor 'A' allele of the rs9939609 variant in our population was 0.38, which was near to or comparable with Pakistani (0.40) patients. Low minor allele frequency (MAF) was reported in Chinese (0.12) Han population, indicating that the association pattern of *FTO* variants with BMI and T2D could vary within Asian population[22]. The dominant model of SNP rs9939609 yielded considerably high OR (2.03, *P*=0.021) and thus could validate the findings from a Punjabi population [*P*=0.001, OR=1.30, confidence interval (CI)=1.10–1.54][6].

The *KCNJ*11 encodes the Kir 6.2 subunit of the ATP-sensitive potassium channel of β-cells. This channel regulates insulin formation and release via glucose metabolism, and the E23K variant has been found to be associated with glucose intolerance and impaired glucose tolerance among Caucasians[23]. Our findings (MAF=0.39) did not complement the study reported by Qiu *et al*[23], where the rs5219 polymorphism (MAF=0.61) was significantly associated with T2D (OR=1.12; 95% CI=1.09–1.16; *P*<0.05), and Rizvi *et al*[24], where both dominant and additive models in KCNJ11 (rs5219) were significantly associated with T2D. Our findings were similar to that of Phani *et al*[25], where south Indians showed no association on susceptibility to T2D, and a study by Qin *et al*[26], where no association was reported in Chinese Han population.

The *SLC30A*8 gene encodes zinc transporter protein member 8 (ZnT-8), and rs13266634 variant is one of the most religiously replicated diabetes risk mutants (with an OR of 1.14 for the mutant R allele)[27]. The MAFs for rs13266634 from Indian studies reported 0.22[28] and 0.21[29], which were comparable to our population with a frequency of 0.28. Our findings were at par with the study by Chauhan *et al*[30], who reported a positive association of the SNP in north Indian populations. In contrast, studies on south Indians[28,29] did not reveal any significant association of the gene with T2D. This inconsistent pattern of genetic association with T2D between north and south Indians could be due to the dissimilar ethnic and genetic architectural backgrounds.

*PPARγ*2 is a transcription factor which regulates adipogenesis and insulin function presumably by increasing the ability of the *PPARγ*2 receptors to bind to DNA response elements and modulate the transcription of the corresponding genes[5]. A positive association between the substitution of G (alanine) allele for C (proline) allele at codon 12 of *PPARγ*2 gene and T2D has been reported in populations from North America and Asia[31,32]. No polymorphism of *PPARγ*2 gene has been observed in our study population. Our results corroborated with the findings from a north Indian population[6] and West Bengal population[33], which showed no association of *PPARγ*2 (rs1801282) gene with T2D.

Our study showed a strong association of *SLC30A*8 (rs13266634) observed with BMI levels. Hence, this biomarker could serve as a potential tool to tailor therapy for T2D prevention and management in the North East Indian population and would have important implications in the early detection of T2D. The lack of association of the remaining SNPs with T2D could probably be due to the small sample size. The present study had certain limitations to be addressed such as small sample size, samples were not gender matched and the study was underpowered to detect difference in the MAF between the two study groups at a significance of 5 per cent in case of all the four SNPs. A positive association between the inheritance patterns of polymorphisms with T2D could be established only for the *SLC30A*8 gene (rs13266634) variant. Though our study supported a few earlier Indian genome-wide association studies (GWAS) which illuminated the concept of genetic heterogeneity existing between Indo-Europeans and Dravidians, the present study provided information about the distribution of SNPs associated with T2D in the North East population, which can be a foundation for performing GWAS in larger populations with more sensitive techniques. Despite all the limitations, the high OR observed in the recessive model of *SLC30A*8 (rs13266634) gene variant indicates a probable association of T2D and SNP. Further studies need to be done involving larger populations from different geographic regions of the country.

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***Conflicts of Interest*:** None.

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For correspondence: Dr Sofia Banu, Department of Bioengineering & Technology, Gauhati University, Guwahati 781 014, Assam, India
e-mail: sofiabanu2@gmail.com