The Association of Nucleobindin 2 Gene (NUCB2) Variants with Type 2 Diabetes Mellitus Among Iranian Azeri-Turkish Population

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Nesfatin-1, which is derived from the NEFA/nucleobindin 2 (NUCB2) precursor, is a satiety factor secreted by several tissues, including the hypothalamus. NUCB2 gene expression positively correlates with insulin secretory capacity, as well as with insulin and glucagon gene expression in human islets. Therefore, we hypothesized that polymorphisms in the NUCB2 gene promoter influence the susceptibility for the development of diabetes. In the current study, we investigated the association of NUCB2 polymorphisms in the promoter region and 52 UTR, exon1, and 52 part of intron 1 of NUCB2 gene with type II diabetes. A total of 200 patients with type II diabetes and 200 healthy controls subjects were enrolled in this study. The 52 part of NUCB2 variants was assessed using PCR and direct sequencing. Twelve variants, including rs186174(c.-427A > C), rs214088 (c.-406C > G), rs4757506(c.254A > G), rs369209853 (c.- 205G > A), rs214087 (c.-166G > C), rs373592192 (c.-281G > A), rs182903196 (c.-308G > A), rs 370538176 (c.-426C > G), rs 190662423 (c.-612C > A), rs115148100 (c.-653G > A), rs374389403 (c -233T > c), and rs377756452 (c -207G > A), were identified. Analysis of the results showed that the frequency of seven of the polymorphisms [rs186174 (c.-427A > C), rs 4757506 (c.254A > G), rs369209853 (c.-205G > A), rs214087 (c.-166G > C), and rs182903196 (c.-308G > A)] is significantly different between patients and controls subjects (P< 0.05), which indicates the association of these polymorphisms with type 2 diabetes. Although the relationship between the promoter region of NUCB2 gene and T2D remains uncertain at present, These findings may support the role of NUCB2 gene in the regulation of the regulation of blood glucose and the increased type 2 diabetes in humans.

Keywords: NUCB2, type 2 diabetes, Single nucleotide polymorphism.

NUCB2/nesfatin and its proteolytically cleaved product nesfatin-1 [1] were originally identified as proteins secreted by the hypothalamus and involved in feeding regulation. NUCB2 consists of 14 exons, spanning 54785 nucleotides (accession number NC_000011.9). It codes for a precursor protein with a signal peptide containing 24 amino acids and a protein structure containing 396 amino acids[2] and is highly conserved among rodents and humans as well as within nonmammalian vertebrate species, indicating its importance[3]. It is post-translationally divided into three segments by the prohormone convertase (PC), which includes the N-terminal segment NUCB2/nesfatin-1 (1–86), two C-terminal peptide nesfatin-2 (85–163), and nesfatin-3 (166–396)[4].

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Nesfatin-1 is an anorexigenic and insulinotropic peptide found abundantly in the hypothalamus, pancreas, and gastric oxyntic mucosa[4]. It possesses anti-hyperglycemic properties, but these are likely to be insulin dependent[4]; therefore, the dysfunction of nesfatin-1 could be implicated in metabolic disorders, particularly type 2 diabetes (T2D), and consequently, it could act as an anti-diabetic factor by enhancing both insulin secretion and insulin action[5].

A major function of nesfatin-1 is the inhibition of food intake and regulation of blood glucose in time , dose , and insulin-dependent manners[6], and it also may have an important role in insulin resistance of T2D[7]. The relationship between plasma levels of nesfatin-1 and the presence of T2D correlates either negatively or positively[8,9]. The reasons for this discrepancy remain unclear, but it may be derived from the differences in the severity of disease; different patient characteristics, for example, body mass index; different assessment methods (ELISA recognizing NUCB2 and nesfatin-1 vs. sandwich-type ELISA recognizing only nesfatin-1); and gender differences (females vs. males) [2, 10]. However, the roles of nesfatin-1 in the pathogenesis of insulin resistance and T2D are presently not well understood[11].

As far as NUCB2 gene is concerned, the promoter of this gene has not yet been investigated. Therefore, we intended to investigate the known single nucleotide polymorphisms (SNPs) in the promoter region of this gene in T2D patients. On the other hand, the gene located on the short arm of chromosome 11 is associated with diabetes, and hence it is important to evaluate the promoter region. In the present study, we genotyped 12 SNPs in the NUCB2 gene in Azeri-Turkish population of Iran .The genotypes and alleles distributions of these 12 SNPs are shown in Table 1 and assessed the association between NUCB2 and T2D patients and control subjects.

METHODS AND MATERIALS

Subjects and Sample Collection

This case-control study was approved by the research ethics committee of Tabriz University of Medical Sciences, and each patient or control individual enrolled in this study gave a written informed consent and the approval form for genetic analysis. The study population consisted of 200 normoglycemic control subjects (105 men and 95 women; aged 47.30 ± 2.29 years; body mass index (BMI) 24.96 ± 0.92 kg/m^2) and 200 T2D patients (111 men and 89 women; aged 53.55 ± 1.80 years; body mass index (BMI) 26.46 ± 0.46 kg/m^2) from the outpatient Endocrinology clinic of Imam Reza university hospital, Tabriz, Iran. Table 2 shows the diabetes-related clinical and biochemical parameters of all the study subjects.

The control subjects consisted of healthy, unrelated, non-diabetic spouses of the T2D patients. None of them had a family history of diabetes or were taking any medication known to change glucose tolerance. Patients with type 1 diabetes mellitus, hypertension, heart failure, pregnancy, acute or chronic infectious disease, liver or kidney disease, and cancer were excluded from the study.

The T2D patients were recruited after a detailed investigation of their medical records for symptoms, use of an oral hypoglycemic agent and insulin, measurement of fasting blood glucose, and other related anthropometric parameters.

Primers were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and the NUCB2 genomic sequence, with the accession number NG_003180 [Homo sapiens cytochrome P450, family 2, subfamily D, polypeptide 6 genomic region (C YP2D6 on chromosome 22)].

There are 12 SNPs for the human NUCB2 gene listed in the SNP database of the National Center for Biotechnology Information [12]; therefore, we screened 700 bp of the NUCB2 gene in T2D patients and identified the 12 SNPs. To determine the SNPs, the DNA sequences of the variants were retrieved from the dbSNP database[12].The genotype and allele frequencies of all the SNPs were identified and determined in all the study participants. Data analysis was performed using the Statistical Package for Social Sciences-SPSS computer software for Windows (version 21.0).

DNA analysis

Genomic DNA was extracted from peripheral blood lymphocytes using the salting-out method, as previously described. According to the NUCB2 sequence, one pair of primers (F: 52

–GTGGGTGCGGTTACAAATG–32 and 52 TCTGCTCCACTCGCAGCT 32) was designed to amplify the 700 bp fragment consisting of the promoter, the exon 1, and the 5 µ end of the first intervening sequence of NUCB2 gene. Polymerase chain reaction (PCR) was performed with a final reaction volume of 25 µl, which included 100 ng of genomic DNA, 10 pM of each primer, 1.5 U of Taq DNA polymerase, 2.5 µl of the $10 \times$ buffer, 1.5 mM of $MgCl_2$, and 0.25 mM of dNTPs. The reaction was performed on a thermal cycler (Bio-Rad thermal cycler, My Cycler TM thermal cycler, USA), with initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 62°C for 1 min, and 72°C for 30 s, and a final extension at 72°C for 5 min. The PCR products were analyzed by sequencing on an ABI 3700 automatic sequencer machine (Macrogen, South Korea).

Statistical analysis

Data were analyzed using the general linear model by investigating the association between the variants and T2D. The differences between the genotype distribution and allele frequency in the study groups were analyzed using chi-square test. The odds ratios (OR) for each section were compared through the use of a fixed effects model. Statistical heterogeneity was evaluated using χ^2 to assess the appropriateness of pooling the individual study results.

RESULTS AND DISCUSSIONS

Results

To determine the sequence variation in the 52 region of nucb2 gene, we examined a 0.7 kb fragment comprising the promoter region, exon 1,

Table 1. Characteristics of study population

	Controls (200)	Patients (200)
Sex (M/F)	F:95M: 105	F:89M:111
Age (years)	47.30 ± 2.29	53.55 ± 1.80
Body mass index (kg/m²)	24.96 ± 0.92	26.46 ± 0.46
HbA1C (%)	5.4 ± 0.3	7.6 ± 0.29
Plasma glucose (mmol/L)	5.33 ± 0.26	8.41 ± 0.36
Total cholesterol (mmol/L)	5.2 ± 0.9	5.4 ± 0.32
Triglyceride (mmol/L)	1.2 ± 0.2	1.4 ± 0.8
Systolic blood pressure (mmHg)	128 ± 15	134 ± 18
Duration of T2D	-	6.44 ± 0.91
Treatment	-	
Diet only (%)		7.7
Oral agents (%)		66.7
Insulin (%)		25.6

Table 2. List of Single nucleotide polymorphism identified in this study

Polymorphism	Nucleotide position	Substitution	Location
rs115148100 (c.-653G > A)	-653	$G \rightarrow A$	Intronic
rs190662423 (c.-612C > A)	-612	$C \rightarrow A$	Intronic
rs186174 (c.-427A > C)	-427	$A \rightarrow C$	Intronic
rs370538176 (c.-426C > G)	-426	$C \rightarrow G$	Intronic
rs214088 (c.-406C > G)	-406	$C \rightarrow G$	Intronic
rs182903196 (c.-308G > A)	-308	$G \rightarrow A$	Intronic
rs4757506c.254A > G	254	$A \rightarrow G$	Intronic
rs374389403 (c -233T > C)	-233	$T \rightarrow C$	3´UTR/5´UTR
rs377756452 (c -207G > A)	-207	$G \rightarrow A$	3´UTR/5´UTR
rs369209853 (c.-205G > A)	-205	$G \rightarrow A$	3´UTR/5´UTR
rs214087 (c.-166G > C)	-166	$G \rightarrow C$	3´UTR/5´UTR
rs373592192 (c.-281G > A)	-281	$G \rightarrow A$	Intronic

Table 3. Allele distributions of NUCB2 gene in T2D patients and control subjects

	Genotype	Cases		Controls		P Value	OR (CI 95%)
		Frequency	(%)	Frequency	(%)		
rs115148100	AA	22	11%	27	5/13%	341/0	-
	AG	14	7%	8	4%		
	GG	164	82%	165	5/82%		
Allelic frequency	G	342	5/85%	338	5/84%	692/0	1.081
	A	58	5/14%	62	5/15%		(0.734–1.595)
rs190662423	AA	5	5/2%	6	3%	256/0	-
	CA	10	5%	4	2%		
	CC	185	5/92%	190	95%		
Allelic frequency	C	380	95%	384	96%	495/0	0.792
	A	20	5%	16	4%		(0.404–1.551)
rs186174	AA	0	0	0	0	001/0	-
	CA	35	5/17%	14	7%		
	CC	165	5/82%	186	93%		
Allelic frequency	A	35	8/8%	14	5/3%	002/0	2.644
	C	365	2/91%	386	5/96%		(1.4–4.994)
rs370538176	CC	182	91%	184	92%	225/0	-
	CG	14	7%	8	4%		
	GG	4	2%	8	4%		
Allelic frequency	G	22	5/5%	24	6%	761/0	0.912
	C	378	5/94%	376	94%		(0.502–1.655)
rs214088	CC	23	5/11%	19	5/9%	0001/0	-
	CG	98	49%	47	5/23%		
	GG	79	5/39%	134	67%		
Allelic frequency	C	256	64%	315	8/78%	0001/0	0.480
	G	144	36%	85	2/21%		(0.350–0.657)
rs182903196	AA	14	7%	7	5/3%	013/0	-
	AG	7	5/3%	20	10%		
	GG	179	5/89%	173	5/86%		
Allelic frequency	G	365	2/91%	366	5/91%	9/0	0.969
	A	35	8/8%	34	5/8%		(0.591 -1.587)
rs4757506	AA	102	51%	146	73%	0001/0	-
	AG	75	5/37%	29	5/14%		
	GG	23	5/11%	25	5/12%		
Allelic frequency	G	121	2/30%	79	8/19%	001/0	1.762
	A	279	8/69%	321	2/80%		(1.272–2.441)
rs374389403	CC	8	4%	17	5/8%	128/0	-
	TC	12	6%	8	4%		
	TT	180	90%	175	5/87%		
Allelic frequency	C	28	7%	42	5/10%	08/0	0.642
	T	372	93%	358	5/89%		(0.389–1.057)
rs377756452	AA	4	2%	6	3%	638/0	-
	AG	5	5/2%	3	5/1%		
	GG	191	5/95%	191	5/95%		
Allelic frequency	G	387	8/96%	385	2/96%	7/0	1.16
	A	13	2/3%	15	8/3%		(0.545–2.47)

|							
rs369209853	AA	6	3%	28	14%	0001/0	
	AG	21	5/10%	9	5/4%		
	GG	173	5/86%	163	5/81%		
Allelic frequency	G	367	8/91%	335	8/83%	001/0	2.158
	A	33	2/8%	65	2/16%		(1.365–3.365)
rs214087	CC	37	5/18%	33	5/16%	0001/0	-
	CG	105	5/52%	63	5/31%		
	GG	58	29%	104	52%		
Allelic frequency	G	221	2/55%	272	68%	0001/0	0.581
	C	179	8/44%	128	32%		(0.436–0.775)
rs373592192	AA	5	5/2%	25	5/12%	0001/0	-
	AG	19	5/9%	3	5/1%		
	GG	176	88%	172	86%		
Allelic frequency	G	371	8/92%	347	8/86%	005/0	1.954
	A	29	7/2%	53	13/2%		(1.214–3.145)

and first intron. Sequencing this region revealed 12 SNPs in all the 400 tested subjects, which included 7 SNPs in the promoter regions, 4 SNPs in exon 1, and 1 SNP in the first intron (Figure 1).

All variants were in agreement with the Hardy–Weinberg equilibrium. In total, 7 SNPs were observed in both T2D patients and healthy control subjects, while 5 SNPs showed a significant difference in the association between these two groups (Table 3).

Our results showed that rs186174 (c.-427A > C), rs 214088 (c.-406C > G), rs 4757506 (c.254A > G), rs369209853 (c.-205G > A), rs214087 (c.-166G > C), rs373592192, rs182903196 (c.-308G > A)]

variants are associated with T2D. The genotype and allele distributions of rs186174 (c.-427A > C) differed between the T2D patients and control subjects (P < 0.001). The C/C homozygote and C/A heterozygote genotypes of this variant were more frequent in the both groups. A comparison of the frequency of C/A genotypes between the two groups showed 10.5% higher occurrences of C/A heterozygote in T2D subjects than in control (17.5% vs.7.0%). The A/A homozygote genotype was not found in the case and control groups. These results confirm that rs 186174 variant has strong association with T2D (p = 0.001).

The genotype of rs 4757506 (c.254A > G)

(A)   (B)   (C)
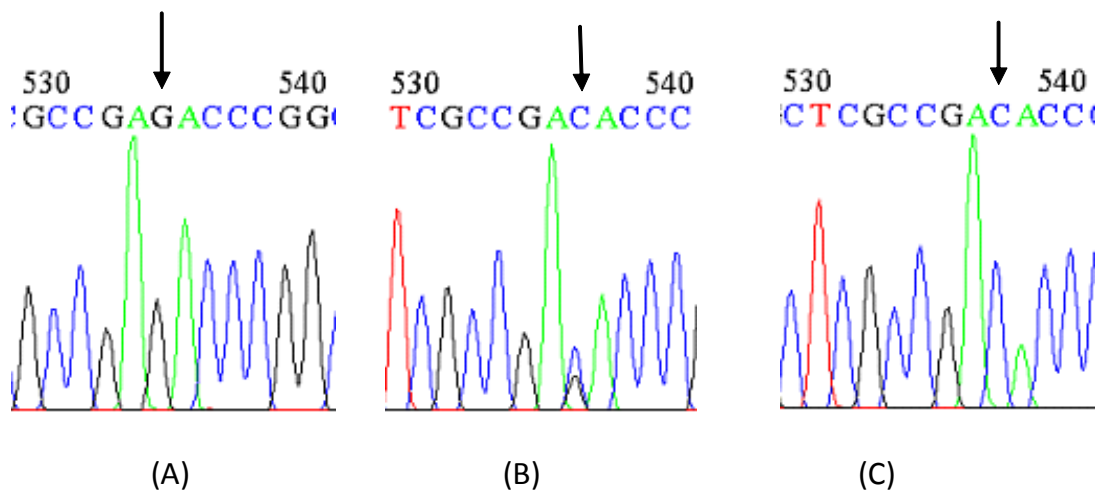
Fig. 1. Sequencing analysis of rs214087 *(c.-166G>C)* variants in human NUCB2 gene.
A representative chromatogram from (A) GG Homozygote, (B) GC Heterozygote and (C) CC Homozygote

differed significantly between the T2D patients and the control subjects (both P < 0.001). A comparison of the frequency of A/A genotypes in the rs 4757506 variant between the two groups showed 24.5% higher occurrences of rs 4757506 in control than in T2D subjects (53.0% vs.77.5%), while the A/G genotype was found more frequently in the T2D patients (39.0% in T2D) and the G/G genotype showed the same frequency (8.0%) in both the study groups.

The genotype and allele distributions of rs214087 (c.-166G > C) differed significantly between the T2D patients and control subjects (both P < 0.001), and the C/C and C/G genotypes were more frequent in T2D patients, while the G/G genotype was found more frequently in the control group. The genotype and allele distributions of rs369209853 (c.-205G>A) and rs373592192 (c.-281G > A) differed significantly between the two study groups (both P < 0.001), with an evident linkage to diabetes.

In rs182903196 (c.-308G > A)] variant, only genotype frequency differed significantly between the T2D patients and control subjects (P = 0.013), while allele distributions in this SNP were not different between the T2D patients and control subjects.

However, the genotype and allele distributions of the other five SNPs were not different between the T2D patients and control subjects. The SNPs including rs115148100 (c.-653G >A), rs190662423 (c.-612C>A), rs186174 (c.-427A >C), rs 370538176 (c.-426C > G), rs182903196 (c.-308G>A), rs374389403 (c -233T>c and rs377756452 (c -207G > A) did not show any significant association with diabetes (p> 0.05). The lowest correlation was observed for rs377756452 (c -207G >A), which showed an almost similar frequency in the two study groups. The remaining SNPs did not show any linkage with diabetes, and they were found in similar frequencies in both the groups.

DISCUSSIONS

As nesfatin-1 has been suggested to have insulin-dependent anti-hyperglycemic properties[4], dysfunction of nesfatin-1 could be implicated in T2D. To the best of our knowledge, no genetic research has been performed to investigate the association of NUCB2 genetic variants with the development of T2D. We performed sequencing analyses to assess the differential variants of NUCB2 promoter, exon 1, and the 52 end of the first intervening sequence of NUCB2 gene in T2D patients and normal controls subjects.

T2D is a genetically heterogeneous disorder and is associated with insulin resistance and impaired insulin secretion. The common forms of T2D are believed to be complex polygenic diseases[13]. Thus far, genetic association studies involving NUCB2 polymorphisms have been conducted in relation to obesity and metabolic syndrome. In 2011, Zegers et al. reported that three single nucleotide polymorphisms were associated with obesity in male Caucasian subjects[14]. In another cohort of 471 obese children, seven sequence variants of NUCB2 gene were detected[15]. However, these variants did not result in differences in the expression of NUCB2 and plasma nesfatin-1 levels from those observed in body weight matched controls subjects[16].

NUCB2 gene expression correlated positively with insulin secretory capacity, as well as with insulin and glucagon gene expression in human islets[17].

Zhang et al. reported a positive relationship between plasma nesfatin-1 levels and impaired glucose tolerance. In contrast, Li et al. reported that fasting plasma nesfatin-1 levels are lower in T2D mellitus than in normal controls subjects .The reasons for this discrepant results are still unclear; however it was suggested that the difference is due to the differences in disease severity or the different patient characteristics, such as body mass index[18].

The relationship between plasma levels of nesfatin-1 and the presence of T2D mellitus have been reported to correlate either negatively or positively[18] and associated with BMI, plasma insulin[19].

The chi-square analysis revealed significant polymorphisms of the rs186174 (c.-427A >C), rs4757506 (c.254A>G), rs369209853 (c.-205G >A), rs214087 (c.-166G>C), and , rs182903196 (c.-308G > A) variants of NUCB2, suggesting the association of these SNPs with T2D. These findings may support the role of nesfatin-1 or NUCB2 gene in the regulation of blood glucose levels and development of T2D in humans.

In the present study, we genotyped 12

SNPs in the NUCB2 gene in Iranian subjects and assessed the association between NUCB2 and T2DM using a case-control analysis. We demonstrated that some SNPs including rs115148100 (c.-653G > A), rs190662423 (c.-612C > A), rs186174 (c.-427A > C), rs370538176 (c.-426C > G), rs182903196 (c.-308G > A), rs374389403 (c -233T > c), and rs377756452 (c -207G > A) showed no association with T2D.

Although the relationship between the promoter region of NUCB2 gene and T2D remains uncertain at present, results of this study further suggest that NUCB2 has an important role in T2D. The nucleotides variants in the promoter region, 52 UTR and exon 1, the first part of intron 1 of Nucb2 gene, were examined by direct sequencing in patients with T2DM and control subjects, and the data were compared between the two groups. Understanding the relationship between SNP and mutation probability of Nucb2 gene promoter and T2D can be considered as a new therapeutic approach in patients with T2D, such as by reducing the amount of protein used.

CONCLUSIONS

Conclusion, we demonstrate, for the first time, the SNPs of the NUCB2 gene promoter region that are associated with T2D. Taken together, studies available so far support an expanding role of NUCB2, thus opening new fields of investigation. However, several questions remain unanswered, in particular, identifying all the SNPs of the promoter region of NUCB2 gene and the strongest SNP associated with T2D. In addition, it is necessary to understand the role of this gene in the development and progression of T2D and find new ways for treatments. Finally, further research on the promoter region of this gene appears to be essential.

List of abbreviations
SNP: single nucleotide polymorphism
OR: odds ratio
CI: confidence interval
T2D: type 2 diabetes
Competing interests
The authors report no financial and non-financial conflicts of interests.
Authors' contributions
Soodabeh khalili carried out the molecular

genetic studies, and drafted the manuscript. Mahmoud Shekari Khaniani participated in acquisition of data and analysis and interpretation of data. Naser Aghamohammdzade participated in case recognition and sample gathering. Sima Mansoori Derakhshan participated in the design of the study and performed the statistical analysis and has been involved in drafting the manuscript and revising it critically for important intellectual content. All authors read and approved the final manuscript.

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