The rs1043994 and rs3815188 genetic variations of the NOTCH3 gene and risk of type 2 diabetes mellitus

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ABSTRACT
NOTCH pathways are important regulators at the beginning of the genetic mechanisms controlling the embryonic development and cell differentiation required for normal pancreatic development during the embryonic period. Disruption of the NOTCH pathway induces apoptosis in pancreatic cells or corrupted pancreatic development that can lead to diabetes. Genetic mutations affecting the NOTCH pathway have been extensively studied in certain types of cancer, including solid tumours, but its metabolic functions are not well known. The objective of our study was to explore the relationship between NOTCH3 gene variants and the risk of developing type 2 diabetes mellitus in 100 patients and 100 healthy control subjects. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was carried out to identify the genotypes in 100 patients with diabetes and 100 control individuals. DNA extracted from peripheral blood samples was amplified and the rs1043994 variant was digested with MwoI, while the rs3815188 variant was digested with AciI. The products were evaluated by 3% agarose gel electrophoresis. The obtained results suggested that the rs1043994 variant was associated with the development of type 2 diabetes due to a significant ratio with the presence of the A allele. Similarly, the presence of the CC genotype and the absence of the T allele were determined to be associated with a notable risk of developing type 2 diabetes for individuals with the rs3815188 variant. In conclusion, we found a significant association between the rs1043994 and rs3815188 variants of the NOTCH3 gene and the risk of developing type 2 diabetes mellitus.

Introduction
Type 2 diabetes mellitus (T2DM) is characterized by defects in both insulin secretion from pancreatic β-cells and insulin action in target tissues such as fat, muscle and liver [1]. A decrease in islet number and/or increased β-cell death in the pancreas are important factors in the pathogenesis of T2DM [2,3]. One of the candidate pathways for the pathogenesis of T2DM is NOTCH signalling, which plays a key role in the differentiation of pancreatic cells and in pancreatic cell death [1,4].

NOTCH signalling has a critical part in embryonic development and is involved in important physiological and pathological processes such as cell cycle control and cell differentiation. At the same time, it is required for apoptosis. The disruption of the NOTCH signalling pathway leads to serious defects in embryonic development and tissue homeostasis. Correlatively, the importance of NOTCH signalling pathways for the normal development of the pancreas and pancreatic organogenesis has been reported. By influencing the differentiation of pancreatic progenitors probably impaired in NOTCH signalling causes premature differentiation of endocrine pancreas. Impaired NOTCH signalling in pancreatic progenitors leads to premature differentiation of endocrine pancreas [5,6].

There are four transmembrane receptors (NOTCH1–4) that belong to the NOTCH pathway and they interact with specific ligands to regulate cell fate [7]. Among these receptors, NOTCH3 is highly expressed in pancreatic mesenchyme and is closely associated with pancreatic epithelial proliferation and morphogenesis [8].

The NOTCH3 gene is located on chromosome 19p and encodes a large single-pass transmembrane protein. Mutations that lead to NOTCH malfunction have been associated with a variety of diseases [5,9,10]. A number of synonymous polymorphisms have been identified in the NOTCH3 gene and C381T (rs3815188) variants in exon 3 and A684G (rs1043994) variants in exon 4 have been found to be associated with migraine [9] and leukoencephalopathy [11].

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To the best of our knowledge, there are no studies so far assessing the possible relationship between NOTCH3 polymorphisms and T2DM. Thus, the aim of this study was to assess whether NOTCH3 gene variants might contribute to the risk of T2DM.

Materials and methods

Patient selection and sample collection

Peripheral blood samples were obtained from T2DM patients and healthy controls without T2DM that were enrolled at the Department of Endocrinology, Eskisehir Osmangazi University, Medical Faculty in Turkey. Demographic information (e.g., age, sex and family history of T2DM) was documented for all cases, and personal files were generated to record clinical data.

The study group consisted of 100 newly diagnosed and non-treated T2DM patients (47 men and 53 women) and the control group \( (n = 100) \) consisted of 49 men and 51 women ranging in age from 40 to 70 years. The prevalence of insulin resistance in the group of patients was 75%, whereas it was 22% in the control individuals.

Fasting plasma glucose (FPG) of greater than or equal to 126 mg/dL and haemoglobin A1C (HbA1C) of greater than or equal to 6.5% were used for diagnosis for T2DM patients. Oral glucose tolerance test (OGTT) (with 75 g glucose) was also used for diagnosis; 2 h (120 min) blood glucose of greater than or equal to 200 mg/dL was considered to indicate T2DM. Individuals who have FPG levels less than 100 mg/dL, HbA1C of less than 5.7% and OGTT of less than 140 mg/dL, HbA1C of less than 5.7% and glucose of greater than or equal to 200 mg/dL was considered to indicate T2DM. Individuals who have FPG levels less than 100 mg/dL, HbA1C of less than 5.7% and OGTT of less than 140 mg/dL, HbA1C of less than 5.7% and glucose of greater than or equal to 200 mg/dL was considered to indicate T2DM. Individuals who have FPG levels less than 100 mg/dL, HbA1C of less than 5.7% and OGTT of less than 140 mg/dL were included in the study to be control subjects. HOMA/IR evaluation was used to determine insulin resistance (fasting serum insulin (\( \mu U/mL \)) × fasting plasma glucose (mmol/L)/22.5) [12]. The HOMA/IR indexes of greater than or equal to 2.7 were attributed to insulin resistance syndrome.

This study was approved by the local ethics committee (Medical Faculty of the Eskisehir Osmangazi University, Turkey). According to the Helsinki Declaration, informed consent was obtained from all patients prior to inclusion in this study.

Genotyping analysis

Genomic DNA was extracted from peripheral blood using a PureLinkTM Genomic DNA Mini Kit (Invitrogen Corporation, Carlsbad, CA, USA).

The rs1043994 variant in exon 4 and the rs3815188 variant in exon 3 of the NOTCH3 gene were analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Polymerase chain reactions were performed in a total volume of 25 \( \mu L \) using OneTaq® Quick-Load® 2X Master Mix with Standard Buffer (New England Biolabs, Ipswich, MA, USA), according to the manufacturer’s instructions. Briefly, the 25-\( \mu L \) reaction volume consisted of 2 \( \mu L \) of genomic DNA, 0.5 \( \mu L \) of each primer (5 \( \mu mol/L \)), 12.5 \( \mu L \) of master mix and 9.5 \( \mu L \) of nuclease-free water. The sequences of the oligonucleotide primers obtained from Alpha DNA (Montreal, Canada) are shown in Table 1.

PCR amplification was performed in a Bio-Rad Thermal Cycler (T100™, Foster City, CA, USA), and the reaction conditions were as follows: initial denaturation at 94 °C for 30 s, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing for 1 min at 61.8 °C for rs1043994 or 59.4 °C for rs3815188, extension at 68 °C for 1 min, and then a final extension at 68 °C for 5 min and cooling down to 4 °C.

The amplification product of NOTCH3 A684G was 420 bp, and Mwol (New England Biolabs, Inc., Beverly, USA) digestion for 30 min at 60 °C yielded fragments of 168/60/57/44/37/30/15/9 bp when the A allele was present. In the presence of the G allele, the Mwol digestion eventuated in 107/61/60/57/44/37/30/15/9 bp fragments. Due to the small size of the fragments, the yields of 168 and 107 bp were used as the indicator bands.

For genotyping of the C381T polymorphism, 224 bp PCR products were digested for 1 h at 37 °C with Acil (New England Biolabs, Inc., Beverly, MA, USA), and the yields were analyzed by electrophoresis in a 3% agarose gel stained with RedSafe™ nucleic acid staining solution (Intron Biotechnology Inc., Seoul, Korea) and visualized by GeneGenius Gel Light Imaging System (Syngene, Cambridge, UK).

The presence of an Acil site (C allele) was indicated by cleavage of the 224 bp PCR product into fragments of 86/80/38/20 bp, whereas the TT genotype was indicated by cleavage of the product into 166/38/20 bp fragments (the fragments of 38 and 20 bp are not visible on the 3% agarose gel).

Statistical analysis

The comparisons of the studied genotypes between the groups and the odds ratios were evaluated using chi-
Results and discussion

Digestion of the PCR products for the rs1043994 variation of NOTCH3 yielded three genotypes: double bands of 168 and 60 bp in the AA homozygote, double bands of 107 bp and 60–61 bp (appears as a single band) in the GG homozygote, and all three bands (168/107/60–61) in GA heterozygotes. The genotype frequencies of the rs1043994 variation of the NOTCH3 gene are shown in Table 2. We found a significant difference in the genotype distribution between T2DM patients and controls ($p = 0.000$, $\chi^2 = 47.453$). The risk assessment for the genotypes showed that the AA genotype increased the risk of T2DM by 47.6-fold compared to the GG genotypes ($p < 0.05$) (Table 3).

Upon Acil digestion, the PCR product of the rs3815188 variation (224 bp) yielded four fragments (86/80/38/20 bp) when the C allele was present, although the 38 and 20 bp fragments were not visible on the 3% agarose gel. The genotype frequencies of the rs3815188 variation of the NOTCH3 gene are shown in Table 4. A significant difference in the genotype distribution of the rs3815188 variation was found between T2DM patients and controls ($p = 0.000$, $\chi^2 = 58.355$).

The risk assessment for the studied genotypes indicated that the CC genotype was associated with 11.3-fold higher risk of T2DM as compared to the CT genotypes ($p < 0.05$) (Table 5).

Table 2. Genotype frequencies of the NOTCH3 rs1043994 variant.

| Groups | n | AA | N % | AG | n % | GG | n % |
|--------|---|----|-----|----|-----|----|-----|
| Control | 100 | 43 | 43.0 | 53 | 53.0 | 4 | 4.0 |
| T2DM | 100 | 7 | 7.0 | 62 | 62.0 | 31 | 31.0 |
| Total | 200 | 50 | 25.0 | 115 | 57.5 | 35 | 17.5 |
| Statistics | | | $\chi^2 = 47.453; p = 0.000$ |

Table 3. Risk assessment for the NOTCH3 rs1043994 genotypes.

| Genotypes | Odds ratio | 95% CI | $p$ |
|-----------|------------|--------|-----|
| AA vs. AG | 7.186 | 2.984–17.307 | $< 0.05$ |
| AA vs. GG | 47.607 | 12.815–176.865 | $< 0.05$ |
| AG vs. GG | 6.625 | 2.197–19.982 | $< 0.05$ |

95% CI, 95% confidence interval.

Table 4. Genotype frequencies of the NOTCH3 rs3815188 variant.

| Groups | n | CC | N % | CT | n % | TT | n % |
|--------|---|----|-----|----|-----|----|-----|
| Control | 100 | 21 | 21.0 | 76 | 76.0 | 3 | 3.0 |
| T2DM | 100 | 69 | 69.0 | 22 | 22.0 | 9 | 9.0 |
| Total | 200 | 90 | 45.0 | 98 | 49.0 | 12 | 6.0 |
| Statistics | | | $\chi^2 = 58.355; p = 0.000$ |

NOTCH signalling has been widely studied for cell differentiation or cancerogenesis, but its metabolic functions are still not well known. However, some studies emphasize the importance of the NOTCH pathway in the control of cell fate during the development of both pancreatic endocrine and exocrine tissues [13,14]. In this regard, it has been demonstrated that reduced NOTCH signalling increases the expression of the pro-endocrine gene NGN3, which promotes the endocrine destiny. Conversely, at normal levels of NOTCH signalling, cells express HES-1 and p48 and adopt the exocrine fate. These findings have shed light on cell lineage in the pancreas and have shown that the NOTCH signalling pathway is critical in pancreas development [15]. It has been shown that, similar to the generation of neurons during neurogenesis, the endocrine cells of the pancreas are specified by lateral specification via NOTCH signalling [16].

Walsh et al. [17] reported that NOTCH signalling pathway genes, such as JAGGED1, NOTCH2 and ADAM10, are upregulated in biopsies from diabetic nephropathy patients. Dror et al. [7] examined whether the NOTCH pathway remains functional in adult islets and found that components and target genes of the NOTCH pathway are expressed in adult islets and block the NOTCH cleavage/activation-induced apoptosis in adult islet cells. Dror et al. [7] have also demonstrated that inhibition of growth factor signalling inhibits NOTCH activation in mouse and human islets.

Anastasi et al. [18] reported that injection of CD4+ T cells obtained from NOTCH3-transgenic mice inhibited hyperglycaemia and insulitis development after streptozotocin treatment in wild-type-mice, and this application indicated the in vitro suppressive activity. This study stated that the expansion and function of T regulatory cells are regulated and protected from experimental autoimmune diabetes by NOTCH3-mediated events. On account of these results, the NOTCH pathway is identified as a potential target for therapeutic intervention in diabetes [18].

Although there are a number of studies that have investigated the efficacy of NOTCH signalling on organogenesis and the function of the pancreas, there are limited data on the relationship between the NOTCH pathway and diabetes [4,5,17,18]. To date, to the best of our knowledge, there is no study on the relationship between variants of NOTCH genes and T2DM. However,
we know that genetic alterations may lead to immediate disruptions of protein function. Because of the importance of the NOTCH3 gene in pancreas function, we hypothesized that NOTCH3 gene variants might be associated with diabetes. Thus, the present study was undertaken to determine whether the NOTCH3 gene variants might contribute to the risk of T2DM.

Kavanagh et al. [19] assessed the key gene variants of the NOTCH signalling pathway for association with diabetic nephropathy and they found that gene variants of JAG1, HES1, NOTCH3 and ADAM10 are not strongly associated with diabetic nephropathy in type 1 diabetes. Although there are no studies that examine the effects of NOTCH3 variants in T2DM, the function of NOTCH3 variants has been investigated in various pathological conditions in humans, including migraine [20], breast cancer [10], ischaemic stroke [21], ischaemic cerebrovascular disease [22], gliomas [23] and schizophrenia [24].

Schwaag et al. [20] also studied the association between rs1043994 and rs3815188 variants of the NOTCH3 gene with migraine and suggested that mutations in the NOTCH3 gene do appear to play a major role in migraine. However, the rs1043994 variation was found to be associated with migraine susceptibility [20]. Cao et al. [10] reported that the rs1043994 and rs3815188 variants of the NOTCH3 gene are not associated with risk of ductal breast carcinoma in a Chinese population. Ross et al. [21] also investigated NOTCH3 rs1043994 and rs3815188 variants in ischaemic stroke patients and observed no association between these variants and ischaemic stroke risk. In another report, the most common polymorphism, T6746C, in the NOTCH3 coding region was not found to be associated with an increased risk for symptomatic ischaemic cerebrovascular disease [22].

An investigation of the 381C>T, 684G>A and 474C>A polymorphisms of the NOTCH3 gene in 266 glioma patients indicated that the 684G>A polymorphism was associated with the tumour NOTCH3 expression level, higher tumour grade and poorer tumour differentiation [23]. The 381C>T and 474C>A polymorphisms did not show any correlation with the clinical characteristics of gliomas [23].

In a case–control study, the genotype frequency of the rs1044009 variant of the NOTCH3 gene was not revealed to be significantly associated with susceptibility to schizophrenia [24].

In our study, we found that the rs1043994 and rs3815188 variants of the NOTCH3 gene were strongly associated with T2DM among Turkish individuals. The rs1043994 variant was found to be associated with T2DM due to a significant ratio with the presence of the A allele. The risk assessment for the rs1043994 genotypes showed that the AA genotype increased the risk of T2DM compared to the GG genotypes. Similarly, carriage of the rs3815188 CC genotype and absence of the T allele were observed in T2DM subjects. The genotype distribution of the rs3815188 variant suggested that the CC genotype was associated with an 11.351-fold increase in the risk of T2DM as compared to the CT genotype carriers.

Conclusions

The obtained results revealed that the rs1043994 and rs3815188 variants of the NOTCH3 gene could be considered to be associated with T2DM risk in the studied cohort. NOTCH3 variants may be considered as a prognostic marker for patients with T2DM. However, these results should be interpreted in other ethnic groups until confirmed by additional studies.

Disclosure statement

No potential conflict of interest was reported by the authors.

References

[1] Prokopenko I, McCarthy MI, Lindgren CM. Type 2 diabetes: new genes, new understanding. Trends Genet. 2008;24:613–621.
[2] Marchetti P, Bugliani M, Boggi U et al., The pancreatic β cells in human type 2 diabetes. Adv Exp Med Biol. 2012;771:288–309.
[3] Cnop M, Welsh N, Jonas J-C et al., Mechanisms of pancreatic β-cell death in type 1 and type 2 diabetes many differences, few similarities. Diabetes. 2005;54:S97–S107.
[4] Darville MI, Eizirik DL. Notch signaling: a mediator of β-cell de-differentiation in diabetes? Biochem Biophys Res Commun. 2006;339:1063–1068.
[5] Kim W, Shin Y-K, Kim B-J et al., Notch signaling in pancreatic endocrine cell and diabetes. Biochem Biophys Res Commun. 2010;392:247–251.
[6] Fujiyama J, Hasegawa K, Iwakura H et al., Notch/Rbp-j signaling prevents premature endocrine and ductal cell differentiation in the pancreas. Cell Metab. 2006;3:59–65.
[7] Dror V, Nguyen V, Walla P et al., Notch signalling suppresses apoptosis in adult human and mouse pancreatic islet cells. Diabetologia. 2007;50:2504–2515.
[8] Lammert E, Brown J, Melton DA. Notch gene expression during pancreatic organogenesis. Mech Dev. 2000;94:199–203.
[9] Menon S, Cox H, Kuwahata M et al., Association of a Notch 3 gene polymorphism with migraine susceptibility. Cephalalgia. 2011;31:264–270.
[10] Cao Y-W, Wan G-X, Zhao C-X et al., Notch1 single nucleotide polymorphism rs3124591 is associated with the risk of development of invasive ductal breast carcinoma in a Chinese population. Int J Clin Exp Pathol. 2014;7(7):4286–4294.
[11] Abramycheva N, Stepanova M, Kalashnikova L et al., New mutations in the Notch3 gene in patients with cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy (CADASIL). J Neurol Sci. 2015;349:196–201.

[12] Schianca GPC, Rossi A, Sainaghi PP et al., The significance of impaired fasting glucose versus impaired glucose tolerance importance of insulin secretion and resistance. Diabetes Care. 2003;26:1333–1337.

[13] Habener JF, Kemp DM, Thomas MK. Minireview: transcriptional regulation in pancreatic development. Endocrinology. 2005;146:1025–1034.

[14] Hald J, Hjorth JP, German MS et al., Activated Notch1 prevents differentiation of pancreatic acinar cells and attenuate endocrine development. Dev Biol. 2003;260:426–437.

[15] Apelqvist Å, Li H, Sommer L et al., Notch signalling controls pancreatic cell differentiation. Nature. 1999;400:877–881.

[16] Edlund H. Developmental biology of the pancreas. Diabetes. 2001;50:S5.

[17] Walsh DW, Roxburgh SA, McGettigan P et al., Co-regulation of Gremlin and Notch signalling in diabetic nephropathy. BBA Mol Basis Dis. 2008;1782:10–21.

[18] Anastasi E, Campese AF, Bellavia D et al., Expression of activated Notch3 in transgenic mice enhances generation of T regulatory cells and protects against experimental autoimmune diabetes. J Immunol. 2003;171:4504–4511.

[19] Kavanagh D, McKay G, Patterson C et al., Association analysis of Notch pathway signalling genes in diabetic nephropathy. Diabetologia. 2011;54:334–338.

[20] Schwaag S, Evers S, Schirmacher A et al., Genetic variants of the NOTCH3 gene in migraine—a mutation analysis and association study. Cephalalgia. 2006;26:158–161.

[21] Ross OA, Soto-Ortolaza Al, Heckman MG et al., NOTCH3 variants and risk of ischemic stroke. PloS One. 2013;8:e75035.

[22] Ito D, Tanahashi N, Murata M et al., Notch3 gene polymorphism and ischaemic cerebrovascular disease. J Neurol Neurosurg Psychiatry. 2002;72:382–384.

[23] Shen Z, Hou X, Chen B et al., NOTCH3 gene polymorphism is associated with the prognosis of gliomas in Chinese patients. Medicine (Baltimore). 2015;94(9):e482.

[24] Gregorio SP, Gattaz WF, Tavares H et al., Analysis of coding-polymorphisms in NOTCH-related genes reveals NUMBL poly-glutamine repeat to be associated with schizophrenia in Brazilian and Danish subjects. Schizophr Res. 2006;88:275–282.