NLRP3-Inflammasome Gene Variations in the Risk of Type 2 Diabetes

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ABSTRACT: Inflammation is the natural immunological response of an organism against any harmful, foreign or destructive effect to heal and repair damaged tissue. The nod-like receptor pyrin domain-containing-3 (NLRP3) inflammasome is one of the main components of the inflammatory mechanism and is associated with many inflammatory diseases, but it is also closely related to metabolic abnormalities, such as type 2 diabetes mellitus (T2DM), insulin resistance and obesity. NLRP3 activates inflammation and causes interleukin-1β release, exogenous and endogenous danger signals, as well as insulin resistance. In this direction, we focus on the gene structure of NLRP3 in diabetes and accordingly, we aim to determine the relationship between eight gene variations in the NLRP3 gene and T2DM. We investigated the rs10802501, rs10733113, rs10754558, rs10925026, rs10925027, rs35829419, rs4612666 and rs4925659 single-nucleotide polymorphisms of NLRP3 gene using the Sequenom MassARRAY system in 100 T2DM patients and 100 control individuals. There were no significant differences between T2DM risk and the genotype frequencies of rs10802501, rs10733113, rs35829419 and rs10925026 variants (p > 0.05). However, significant genotype frequencies were determined for rs10925027 (p = 0.0013) and rs4925659 (p < 0.001). For the risk allele G of the rs10754558 variant, significant differences were found in the heterozygous and dominant model (p = 0.036, p = 0.033). The genotype distribution of the rs4612666 variant was significant only in the heterozygous model (p = 0.047). In this hospital-based case-control study, rs10925027, rs4925659 and rs10754558 variants were found to be closely related to T2DM risk. The rs10925027 CC genotype, rs4925659 GG genotype, rs10754558 GG and GC+GG genotypes of the NLRP3 were determined as important risk factors for the T2DM.

KEY WORDS: inflammasome, NLRP3, type 2 diabetes, genetic variation

I. INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a metabolic disease that results from impaired insulin secretion and insulin effects. This leads to a loss of metabolic control by resulting in an increase in circulating glucose and lipid concentrations.1 The insulin resistance syndrome can be defined as a decrease or impairment in insulin action and is the most important characteristic feature of T2DM. Insulin resistance is caused by a variety of factors, such as the direct harmful effects of lipid increase on organs and tissues, and activation of inflammatory signals.1,2

Insulin is a hormone synthesized by β-islets in the pancreas. If the insulin produced by the pancreas does not produce the necessary or sufficient response in fat, muscle and liver cells, blood glucose is reduced by secreting more insulin (hyperinsulinemia). Fasting glycaemia and glucose tolerance remain at normal levels when the compensatory hyperinsulinemia is sufficient to overcome insulin resistance. In patients who are candidates for T2DM, the ability to compensate for β-cells decreases and insulin failure, which causes T2DM to develop. In other words, the body cannot respond to or use insulin. Although there has been controversy over whether insulin resistance or β-cell defect is a priority, recent epidemiological studies have emphasized that insulin resistance is a primary abnormality in T2DM.3–5

It has been pointed out that activated inflammatory response has an important role in the pathogenesis of T2DM. Additionally, β-cell death and chronic hyperglycemia can be triggered by
pro-inflammatory cytokines and chemokines. Though insulin secretion is the main cause of insulin resistance, inflammation has been reported to be an important cause of insulin resistance. The most important link between these two important mechanisms is the adipose tissue.

Adipose tissue has an endocrine function and many of the pro-inflammatory adipokines, cytokines and chemokines are expressed by adipose tissue. These cytokines and chemokines prevent insulin sensitivity and insulin action. Therefore, obesity and high-fat nutrition play an important role in triggering inflammation. In summary, high-fat mass or nutrition with a high-fat diet causes insulin resistance and T2DM in relation to inflammation.

Inflammation is an immunological response to fight invaders, such as pathogens, damaged cells, irritants and toxic chemicals. At a basic level, an acute and controlled inflammatory response, triggered by conditions, such as infection or tissue injury, is beneficial for metabolism and restoring homeostasis occurs physiologically. However, chronic and irregular inflammation is harmful and may play a role in the pathogenesis of chronic diseases, such as obesity, cardiovascular diseases, insulin resistance and diabetes.

Inflammasome is a protein complex that recognizes stimuli with the potential to produce inflammation and which, in turn, manages a process responsible for the production and secretion of pro-inflammatory cytokines. It recognizes pathogen-associated molecular patterns (PAMPs) and cell damage-associated molecular patterns (DAMPs). There are different types of inflammasomes, but nod-like receptor pyrin domain-containing-3 (NLRP3) inflammasome is mainly associated with T2DM, insulin resistance and obesity.

Dangerous endogenous and exogenous signals, such as ceramide and saturated fatty acids from high-fat diets in macrophages, activate NLRP3 inflammasome and cause interleukin-1β (IL-1β) release. IL-1β disrupts the insulin signal, which causes insulin resistance in cells that are targeted by insulin; both tumor necrosis factor (TNF) dependent and TNF independent. The NLRP3 expression in adipose tissue macrophages increases in obesity. In adipose tissue, interferon (IFN-γ) is produced, and T-cells are activated. This increases macrophage activation and systemic inflammation.

In recent studies, it is emphasized that NLRP3 inflammasome plays an important role in T2DM and insulin resistance mechanisms. However, these studies have focused on gene expression and protein analysis.

In our study, we focus on the gene structure of NLRP3 in diabetes and, accordingly, we aim to determine the relationship between the eight gene variations on the NLRP3 gene and T2DM.

II. MATERIALS AND METHODS

In our study, NLRP3 gene variants are investigated in relation to T2DM. Within the scope of our research, eight single-nucleotide polymorphisms (SNPs) located on the NLRP3 gene are selected using current databases (http://www.ncbi.nlm.nih.gov/gene and http://www.ensembl.org). The accession numbers of these SNPs are as follows: rs10733113, rs10754558, rs10802501, rs10925026, rs10925027, rs35829419, rs4612666, and rs4925659.

A. Ethics Statement

This study was approved by the Clinical Research Ethics Committee of Eskisehir Osmangazi University, Turkey (Approval No. 80558721/G-107) and, according to the Helsinki Declaration, all participants signed informed consent forms prior to participation in the study.

B. Subjects

This case-control study consisted of one hundred T2DM patients and one hundred control individuals who were recruited from the Department of Endocrinology, Eskisehir Osmangazi University, Medical Faculty in Turkey, between January 2014 and February 2015.

The control group consisted of individuals who did not have any chronic diseases and who were not receiving medication. The T2DM group included patients who were newly diagnosed and whose treatments had not yet begun.
T2DM has been defined considering the following criteria: hemoglobin A1C (HbA1C) levels greater than or equal to 6.5%; fasting plasma glucose (FPG) levels greater than or equal to 126 mg/dl; and oral glucose tolerance test (OGTT) levels (75 gr glucose/120 min) greater than or equal to 200 mg/dl. Control subjects were included in the study from outpatients according to the following criteria: HbA1C levels less than 5.7%, FPG levels less than 100 mg/dl, OGTT level less than 140 mg/dl. Subjects providing these criteria and who had agreed to participate in the study were selected.

The mean ages of the controls and patients were 47.29 ± 8.14 (with 49 males and 51 females) and 58.21 ± 8.89 (with 47 males and 53 females), respectively. The mean BMI for the control individual was 25.85 ± 2.87, and 31.65 ± 5.65 for the T2DM patients. The mean HbA1C levels in the controls were 5.28 ± 0.64 and 7.74 ± 1.89 in the T2DM patients. The FGP levels were 95.94 ± 5.28, and 31.65 ± 5.65 for the T2DM patients.

C. DNA Preparation

Two ml of whole blood samples were collected in EDTA tubes from all of the participants for DNA isolation and stored at –80°C. Genomic DNA was extracted from frozen peripheral whole blood using a PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific, USA) according to the kit procedure. Absorbances at 260 and 280 nm (A260/A280) were measured using Multiskan™ GO Microplate Spectrophotometer µDrop (Thermo Fisher Scientific, USA) to determine the amount and purity of the DNA samples. Absorbance levels between 1.7 and 2.0 indicate the lowest protein contamination. For this reason, DNA samples with an A260/A280 value of less than 1.7 were isolated again. After providing 1.7 ≤ A260/A280 ≤ 2.0 for all of the DNA samples, the DNA samples were diluted with an elution buffer at a concentration of 50 ng/µl.

D. Genotyping

The iPLEX GOLD SNP protocol on a Sequenom MassARRAY® System was used for genotyping of the rs10733113, rs10754558, rs10802501, rs10925026, rs10925027, rs35829419, rs4612666, and rs4925659 variants of the NLRP3 gene. The MassARRAY genotyping protocol consisted of the following five steps.

E. Multiplex DNA Amplification

The first step in the iPLEX GOLD genotyping is the design of amplification and elongation primers suitable for variation for targeted SNPs. A 10-mer tag sequence (5’-ACGTTGGATG-3’) is added to the 5’ ends of the amplification primers to increase the mass of the primers. These primers are therefore, outside of the detection range of the MALDI-TOF mass spectrometer. Sequenom Assay Design v3.1 software (Sequenom Incorporation, San Diego, CA) was used for primer sequence design and designed oligonucleotide sequences were purchased from Metabion (Martinsried, Germany).

In the second step, 1 µl of DNA samples were loaded into 384-well polymerase chain reaction (PCR) plates for multiplex PCR. The PCR mixture for each sample was prepared as follows:

- 0.5 µM primer mix, 1.0 µl
- 25 mM dNTP mix (Applied Biosystems®, Foster City, USA), 0.1 µl
- 10X PCR Buffer (with 20 mM MgCl2) (Qiagen GmbH), 0.5 µl
- 5 U/µl HotStarTaq DNA polymerase (Qiagen GmbH), 0.2 µl
- 25 mM MgCl2 (Qiagen GmbH), 0.4 µl
- Ultra-purified water (Invitrogen™ Life Technologies, Carlsbad, CA), 0.8 µl

A PCR was performed on Bio-Rad C1000 Thermal Cycler (CA, USA) under the following conditions: 95°C for 2 min, 45 cycles X (95°C for 30 sec, 56°C for 30 sec, 72°C for 60 sec), and 72°C for 5 min.

F. Shrimp Alkaline Phosphatase (SAP) Reaction

SAP reaction is used to neutralize unbound dNTPs in PCR amplification. For this purpose, after the PCR amplification, the 2 µl of SAP mixture consisting of 0.17 µl 10XSAP buffer, 0.3 µl SAP
enzyme (Sequenom Inc., San Diego, CA, USA) and 1.53 µl ddH2O was pipetted onto the PCR plate. The SAP enzyme shows the most effective reaction at 37°C. Therefore, 37°C for 40 min, 85°C for 5 min, and 4°C to hold protocol was used for the SAP reaction.

G. iPLEX GOLD Extension Reaction

The iPLEX GOLD reaction is defined as the primer elongation reaction. After the SAP reaction, an extension reaction was performed with the iPLEX GOLD reaction mix, 2 µl per well, including 0.2 µl iPLEX termination mix, 0.94 µl extension primer mix, 0.619 µl ddH2O and 0.041 µl iPLEX enzyme (Sequenom Inc., San Diego, CA, USA) under the following conditions; [94°C for 30 sec, 94°C for 5 sec, (52°C for 5 sec and 80°C for 5 sec) × 5 cycles] × 40 cycles, 72°C for 3 and final 4°C hold.

H. Resin Purifying

Cationic resin provides the elimination of ions, such as Na⁺, K⁺, and Mg²⁺. For this purpose, approximately 6 mg of sterile Spectro CLEAN Resin (Sequenom Inc., San Diego, CA, USA) was transferred onto a 384-well PCR plate and incubated at room temperature for 35 minutes.

I. SpectroCHIP Transferring and MALDI-TOF Mass Spectrometry Reading

After a clean-up step with resin, the reaction products were nano-pipetted into SpectroCHIP with Nanodispenser RS1000 (Sequenom Inc., San Diego, CA, USA). After the transfer was completed, the SpectroCHIP was analyzed with a MALDI-TOF mass spectrometry device and the results were reported by a MassARRAY analyzer.

J. Statistical Analysis

The genotype distributions and odds ratios (ORs) were analyzed using IBM SPSS Statistics 21 software (SPSS Inc., Chicago, IL, USA). The FINNETI program (https://ihg.gsf.de/cgi-bin/hw/hwa1.pl) was also used for deviation from the Hardy-Weinberg equilibrium (HWE), differences in genetic models (χ² test), and ORs. A power analysis was performed by Power Analysis and Sample Size (PASS) software version 11.0.10. p < 0.05 was considered significant for all of the analyses.

III. RESULTS

The genotype frequencies and statistical evaluations of the NLRP3 gene variants that we identified in patients and control individuals within the scope of our research are presented in Tables 1 and 2.

When the genotype frequencies of the rs4612666, rs10754558 (Table 1), rs10733113, rs10802501, rs10925026, and rs35829419 (Table 2) variants are considered, there is no statistically significant difference between the patients and the control subjects (p > 0.05). However, when the genotype frequencies of rs10925027 (p = 0.0013) and rs4925659 (p < 0.001) variants were evaluated, statistically significant genotype frequencies were determined for control individuals and T2DM patients (Table 1).

Allele frequencies, HWE values, and allele positivity for dominant and recessive inheritance were evaluated for the NLRP3 gene rs10925027 variant and the statistical values are presented in Table 1. While the T allele of the rs10925027 variant of the NLRP3 gene has been found to be significantly higher in control subjects, the C allele was found to be significantly higher in T2DM patients (p = 0.002). Although there is no statistical significance in terms of allele positivity for the heterozygous and dominant models, a significant difference in genotype distributions for homozygous and recessive models has been determined (Table 1). For the risk allele C of NLRP3 gene rs10925027 variant, the TT genotype in the control subjects and the CC genotype frequency in individuals with T2DM are significantly higher (p = 0.029; Table 1). Similarly, the CC genotype in the recessive model for risk allele C shows high frequency compared to the TT+TC genotype in individuals with T2DM (p < 0.001; Table 1). For the rs10925027, a sample size of 200 achieves 92% power to detect an effect size (W) of 0.2583, using a 2 degrees of freedom chi-squared test with a significance level (alpha) of 0.05000.
### TABLE 1: Statistical comparison of rs10925027, rs4925659, rs10754558, and rs4612666 variations

<table>
<thead>
<tr>
<th>SNP</th>
<th>Allele</th>
<th>Statistic</th>
<th>OR (95% CI)</th>
<th>Genotype</th>
<th>Statistic</th>
<th>HWE</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs10925027</td>
<td>T</td>
<td>C</td>
<td>Statistic P</td>
<td>Genotype TT</td>
<td>Genotype TC</td>
<td>Genotype CC</td>
</tr>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>0.002</td>
<td>1.878 (1.243–2.837)</td>
<td>13</td>
<td>62</td>
</tr>
<tr>
<td>Control</td>
<td>88 (44)</td>
<td>112 (56)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2DM</td>
<td>59 (29.5)</td>
<td>141 (70.5)</td>
<td>0.029</td>
<td>1.511 (0.615–3.713)</td>
<td>0.366</td>
<td>0.333</td>
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<td></td>
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<tr>
<td>Test for association OR (95% CI) (risk allele C)</td>
<td></td>
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<tr>
<td>Heterozygous TT vs. TC</td>
<td>Homozygous TT vs. CC</td>
<td>Dominant TT vs. TC+CC</td>
<td>Recessive TT+TC vs. CC</td>
<td>Armitage’s trend test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.955 (0.374–2.438)</td>
<td>2.889 (1.088–7.668)</td>
<td>1.511 (0.615–3.713)</td>
<td>0.333 (0.183–0.607)</td>
<td>Common OR: 1.865 p = 0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SNP</th>
<th>Allele</th>
<th>Statistic P</th>
<th>OR (95% CI)</th>
<th>Genotype</th>
<th>Statistic</th>
<th>HWE</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs4925659</td>
<td>A</td>
<td>G</td>
<td>Statistic P</td>
<td>OR (95% CI)</td>
<td>Genotype AA</td>
<td>Genotype GA</td>
</tr>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>&lt; 0.001</td>
<td>2.795 (1.853–4.216)</td>
<td>26</td>
<td>57</td>
</tr>
<tr>
<td>Control</td>
<td>109 (54.5)</td>
<td>91 (45.5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2DM</td>
<td>60 (30)</td>
<td>140 (70)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Test for association OR (95% CI) (risk allele G)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Heterozygous AA vs. AG</td>
<td>Homozygous AA vs. GG</td>
<td>Dominant AA vs. AG+GG</td>
<td>Recessive AA+AG vs. GG</td>
<td>Armitage’s trend test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.193 (0.542–2.628)</td>
<td>6.235 (2.635–14.75)</td>
<td>2.351 (1.128–4.901)</td>
<td>0.182 (0.095–0.349)</td>
<td>Common OR: 2.577 p &lt; 0.001</td>
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</table>
TABLE 1: (continued)

<table>
<thead>
<tr>
<th>SNP rs10754558</th>
<th>Allele</th>
<th>Statistic</th>
<th>OR (95% CI)</th>
<th>Genotype</th>
<th>Statistic</th>
<th>HWE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>G</td>
<td>P</td>
<td>CC</td>
<td>GC</td>
<td>GG</td>
</tr>
<tr>
<td>Control</td>
<td>116 (58)</td>
<td>84 (42)</td>
<td>0.088</td>
<td>1.409 (0.950–2.090)</td>
<td>39</td>
<td>38</td>
</tr>
<tr>
<td>T2DM</td>
<td>99 (49.5)</td>
<td>101 (50.5)</td>
<td>25</td>
<td>49</td>
<td>26</td>
<td>0.842</td>
</tr>
</tbody>
</table>

Test for association OR (95% CI) (risk allele G)

<table>
<thead>
<tr>
<th>SNP rs4612666</th>
<th>Allele</th>
<th>Statistic</th>
<th>OR (95% CI)</th>
<th>Genotype</th>
<th>Statistic</th>
<th>HWE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>T</td>
<td>P</td>
<td>CC</td>
<td>CT</td>
<td>TT</td>
</tr>
<tr>
<td>Control</td>
<td>139 (69.5)</td>
<td>61 (30.5)</td>
<td>0.439</td>
<td>0.843 (0.546–1.300)</td>
<td>46</td>
<td>47</td>
</tr>
<tr>
<td>T2DM</td>
<td>146 (73)</td>
<td>54 (27)</td>
<td>57</td>
<td>32</td>
<td>11</td>
<td>0.059</td>
</tr>
</tbody>
</table>

Test for association OR (95% CI) (risk allele T)

CI, confidence interval. Statistically significant p values were made in bold.
Allele frequencies, HWE values, and allele positivity in terms of dominant and recessive inheritance were evaluated for the rs4925659 variant of the NLRP3 gene and their statistical values are presented in Table 1. In the control and T2DM individuals, allele frequencies for the rs4925659 variant of the NLRP3 gene showed a statistically significant difference ($p < 0.001$; Table 1). In an evaluation of the allele positivity analysis, which is made for the risk allele G, while statistical significance was determined for the homozygous, recessive and dominant model, no difference was found for the heterozygous model ($p > 0.05$; Table 1).

For the risk allele G of the NLRP3 gene rs4925659 variant, the AA genotype in the control subjects and the GG genotype frequency in individuals with T2DM were found to be significantly higher in the homozygous model ($p < 0.001$). Similarly, the GG genotype in the recessive model for risk allele G showed high frequency compared to the AA+AG genotype in individuals with T2DM ($p < 0.001$). In the dominant model, the AG+GG genotype showed significantly higher frequency compared to the AA in T2DM subjects ($p = 0.020$) (Table 1). For the rs4925659, a sample size of 200 achieves 100% power to detect an effect size ($W$) of 0.3786 using a 2 degrees of freedom chi-squared test with a significance level (alpha) of 0.0500.

When the allele positivity is evaluated in terms of HWE values, allele frequencies, dominant and recessive inheritance for the NLRP3 gene rs10754558 variant, a significant difference is found for the risk allele G in the heterozygous and dominant model ($p = 0.036$, $p = 0.033$). While the GC genotype was significantly higher in diabetic subjects compared to the CC genotype ($p = 0.036$), GC+GG genotype frequency was also higher in T2DM individuals in the dominant model ($p = 0.033$) (Table 1). The rs4612666 variant of the NLRP3 gene was significant only in the heterozygous model ($p = 0.047$) and the CT genotype was less frequent compared with the CC genotype in diabetic individuals (Table 1).

The rs10802501, rs10925027, rs10733113, rs35829419, rs4612666, and rs4925659 genotype frequencies were in HWE, whereas the rs10733113, rs10754558, and rs10925027 genotypes in the control individuals deviated from HWE, contrary to expectations.

The statistical power of the rs4925659 and rs10925027 results was more than 90%, but the power of the rs10802501, rs10925026, rs35829419, rs4612666, and rs4925659 genotype frequencies in the control individuals deviated from HWE, contrary to expectations.

The statistical power of the rs4925659 and rs10925027 results was more than 90%, but the power of the rs10802501, rs10925026, rs35829419, rs4612666, rs10733113, and rs10754558 results was less than 50%.

### IV. DISCUSSION

Eukaryotic genomes contain exons, which are protein-coding regions, and introns, 5’ and 3’ untranslated regions, which are non-protein-coding regions, in addition to regulatory regions, such as promoters and enhancers. Introns are non-protein coding
A. The rs10925027 (rs60294761) Variation of the NLRP3 Gene

There was limited data in the literature on the rs10925027 variant and no study was found investigating the rs10925027 variant and T2DM. The rs10925027 variation is located at 5’ UTR of the NLRP gene, contains the C/T conversion and does not lead to a change in the amino acid encoded.25

Granell et al. examine the frequency of rs10925027 variation in 133 patients with allogeneic stem cell transplantation and report that the donor TT genotype is associated with a relapse of the disease. They found that NLRP3 variants are the most important prognostic factor for clinical outcomes after allogeneic stem cell transplantation.25

Kukkonen et al. report that the NLRP3 rs35829419 variant is associated with interstitial lung fibrosis, while the rs10925027 variant is not related to disease pathogenesis.26

The data which we obtained regarding the rs10925027 variant is a remarkable part of our study. In our study, the CC genotype is determined as an important factor related to T2DM risk. The CC genotype frequency is significantly higher in the T2DM group, and evaluation of allele frequency also shows that the C allele is an important risk factor for the disease. In the recessive model, individuals with CC genotypes are associated with an increased risk for T2DM. In the dominant model, there is no relationship between individuals carrying at least one copy of the C allele and the risk of T2DM.

B. The rs4925659 (rs57137043) Variation of the NLRP3 Gene

The NLRP3 gene rs4925659 variant is another variant with limited data in the literature. Additionally, there has been no study investigating the rs4925659 variant of the NLRP3 gene and T2DM.

The rs4925659 is an intronic sequence variant of the NLRP3 gene and occurs with G/A transition at position chr1:247440161 (https://www.ncbi.nlm.nih.gov/snp/rs4925659).

Wassell et al. investigate the SNPs of several gene loci associated with fibrinogen levels in cardiovascular diseases and determine that the rs4925659
minor allele (G) is associated with high fibrinogen levels, but that the rs4925659 AA is not associated with fibrinogen phenotypes.\(^{27}\)

In another study that investigates the relationship between anti-TNF treatment responses and genetic changes in NLRP3-inflammasome in patients with rheumatoid arthritis, Sode et al. determined that there is no relation between the NLRP3 gene rs4925659 variant and disease pathogenesis.\(^{28}\)

The data obtained from the NLRP3 gene rs4925659 variant is another remarkable part of our study. In our study, rs4925659 GG genotype frequency was found to be significantly higher in the T2DM group and that it is determined as an important factor related to T2DM risk. The allele frequency evaluation also shows that the G allele is more frequent compared to A allele in T2DM individuals and that the G allele is an important risk factor for the disease. In the dominant model, individuals with at least one copy of the G allele were found to have an increased T2DM risk.

C. The rs10754558 Variation of the NLRP3 Gene

The rs10754558 variation is located at 3’ UTR of the NLRP gene, contains the C/G conversion and does not lead to a substitution in the amino acid encoded.\(^{25}\)

Zheng et al. evaluate the efficacy of the rs10754558 variant of the NLRP3 gene in 952 untreated T2DM patients and 871 healthy controls and report that the GG genotype and G allele are associated with an increased T2DM risk and predisposition to insulin resistance.\(^{29}\)

In another study, the rs10754558 variant of the NLRP3 gene was evaluated for the risk of Type 1 Diabetes in the Brazilian population. It has been reported that the NLRP3 rs10754558 G allele was less frequent in T1DM and this variant could play a protective role in the development of the disease.\(^{30}\)

Bai et al., in their study with 286 T2DM patients and 306 healthy control subjects, observe that carriers of homozygous NLRP3 rs10754558 CC exhibit a significantly higher risk of developing T2DM compared to homozygous GG carriers.\(^{31}\)

Wang et al. determine that the risk of T2DM increased significantly in individuals with GG and GC+GG genotypes of the rs10754558 variant of the NLRP3 gene in their study.\(^{32}\)

In parallel with the study by Wang et al.,\(^{32}\) significant differences have been found for the risk allele G in the heterozygous and dominant model in our study when the genotype frequencies of the rs10754558 variant are compared. The frequency of the GG and GC+GG genotype of the rs10754558 variant is found to be associated with an increased T2DM risk.

D. The rs4612666 (rs58794905) Variation of the NLRP3 Gene

The rs4612666 is intronic sequence variant of the NLRP3 gene and occurs with a T/C transition at position chr1:247435768 (https://www.ncbi.nlm.nih.gov/snp/rs4612666).

Zheng et al. evaluate the efficacy of the NLRP3 gene rs4612666 variant in their study with 952 untreated T2DM patients and 871 healthy control patients and report that rs4612666 genotypes and allele frequencies have no significant difference between the groups.\(^{29}\)

In another study, the efficacy of the NLRP3 (rs4612666) and IL-1B (rs1143634) single nucleotide polymorphisms in the etiopathogenesis of chronic periodontitis (CP) is evaluated in the Colombian population. Although it is not associated with CP risk and the rs4612666 variant of the NLRP3 gene, the rs4612666 CC genotype is reported to be associated with the smoking habit.\(^{33}\)

Bai et al. performed a study in the Chinese population with 286 T2DM and 306 healthy controls and evaluate the genetic variant of NLRP3 rs4612666 in terms of T2DM risk. However, they observe that there is no significant relationship between this variation and the risk of T2DM.\(^{31}\)

In our study, the rs4612666 variant of the NLRP3 gene is significant only in the heterozygous model and the CC genotype is more frequent compared with the heterozygote CT genotype in individuals with T2DM. This result suggests that the CC genotype may be associated with disease risk, but the results should be confirmed in larger populations.
E. The rs10733113 Variation of the NLRP3 Gene

The rs10733113 is an intergenic variant of the NLRP3 gene and occurs with A/G transition (https://www.ensembl.org/Homo_sapiens/Variation/Explore?db=core;r=1:247458555-247459555;v=rs10733113;vdb=variation;vf=4461511).

Pihl et al. investigate the effect of common polymorphisms of NLRP3 on T1DM sensitivity and the effectiveness of GAD-alum treatment. In the rs10733113 variant of the NLRP3 gene evaluated by the study they identified that patients with at least one G allele are more likely to produce auto-antibodies against two or more of the GAD, insulin or IA-2 from islet antigens.34

In a study investigating the genetic role of NLRP3 inflammasome in susceptibility to psoriasis, the NLRP3 rs10733113 variant is investigated using a transmission imbalance test (TST) and the G genotype is found to be higher in patients with psoriasis.35

Kim et al. investigated the relationship between NALP3 and CARD8 genetic polymorphisms and anti-tuberculosis drug-induced hepatitis, finding that there is no relationship between rs10733113 variant of the NLRP3 gene and anti-tuberculosis drug-induced hepatitis.36

In our study, no correlation is found between the NLRP3 gene rs10733113 variant and T2DM risk. However, gene frequencies may differ from studies in larger populations.

F. The rs10802501 Variation of the NLRP3 Gene

The rs10802501 variation is located at 3' UTR of the NLRP gene, contains the A/T conversion and does not lead to a change in the amino acid encoded.35

Hitomi et al. report that the rs10802501 variant is found in the 3'UTR of NLRP3 and has recently been associated with NLRP3 mRNA stability.37

The rs10802501 variant was evaluated for the risk of T1DM in the Brazilian population and no significant relationship was found between the risk of T1DM and the rs10802501 variation.38

The effect of the rs10802501 variant on Crohn’s disease and ulcerative colitis patients in the Korean population was evaluated and it has been reported that the rs10802501 variation is not associated with the risk of Crohn’s disease and ulcerative colitis.38

In our study, no relation was found between the rs10802501 variant and T2DM risk in individuals of Turkish origin, but our study is the first study to investigate the relationship of this variant with T2DM. In this respect, it is thought that it will bring new information to the literature.

G. The rs35829419 (rs61753367) Variation of the NLRP3 Gene

The rs35829419 variation is a missense variant of the NLRP gene, located at chr1:24742556 leading to Q(Gln) > K(Lys) substitution in the amino acid sequence (https://www.ncbi.nlm.nih.gov/snp/rs35829419).

The rs35829419 variant has been evaluated for macrovascular and microvascular complications in 181 clinically well-characterized T2DM patients and it is especially associated with an increased risk of myocardial infarction and macrovascular complications. In this study, 88.4% of patients for the rs35829419 variant are identified as homozygous for a wild-type CC genotype, and this variation is defined as a rare variation.39

Pontillo et al. evaluate the rs35829419 variant in terms of the risk of T1DM and Celiac disease in the Brazilian population and report a relationship with Celiac disease risk, although there is no relationship between the risk of T1DM and the rs35829419 variation.40

In a meta-analysis study, Zhang et al. investigate the association between the NLRP3 rs35829419 polymorphism and increased susceptibility to various diseases in humans determining that the NLRP3 rs35829419 polymorphism significantly increases sensitivity to various diseases, such as leprosy, colorectal cancer, and rheumatoid arthritis.40

In another study, the NLRP3 rs35829419 variant is evaluated for the risk of myocardial infarction, but no significant relationship is found between this variant and myocardial infarction risk. However, it is shown that the minor A allele of the variant rs35829419 provides a protective effect against the
risk of developing MI in women and is also associated with increased CRP levels in men.\textsuperscript{41}

In our study, no relationship is found between the NLRP3 rs35829419 variant and T2DM risk. There is no AA genotype in the patient and control groups, whereas the CC and CA genotype distributions are similar. According to this data, it can be suggested that the variant NLRP3 rs35829419 is not associated with the risk of T2DM.

H. The rs10925026 (rs58762256) Variation of the NLRP3 Gene

The rs10925026 is an intronic variant of the NLRP3 gene and occurs with C/A transition (https://www.ncbi.nlm.nih.gov/snp/rs10925026).

In the literature review, we did not find any studies investigating the risk of T2DM with the rs10925026 variant. However, Mathews et al. emphasize the importance of NLRP3-inflammasome activation in rheumatoid arthritis. In their study, they investigate the response to rheumatoid arthritis sensitivity and anti-TNF treatment with genetic variants in the NLRP3-inflammasome complex and correlate rs10925026 C allele with individuals who do not respond to anti-TNF therapy.\textsuperscript{42}

Sode et al. determined that there is no relationship between the rs10925026 variant and disease pathogenesis in a study that investigates anti-TNF treatment responses and genetic changes on the NLRP3-inflammasome in patients with rheumatoid arthritis.\textsuperscript{28}

In our study, there is no statistically significant difference in genotype frequencies of the rs10925026 variant in patients and controls. It is thought that this variation is not associated with the pathogenesis of T2DM.

V. CONCLUSIONS

In conclusion, the rs10925027, rs4925659 and rs10754558 variants have been found to be closely related to T2DM risk in patients of Turkish origin. The rs10925027 CC genotype, rs4925659 GG genotype, rs10754558 GG and GC+GG genotypes of the NLRP3-inflammasome gene have been determined as important risk factors for T2DM.

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