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RESEARCH

Investigation of the relationship between NFKB1 polymorphisms and telomere length and apoptosis in patients with type-2 diabetes

Tip-2 diyabetli hastalarda NFKB1 polimorfizmleri ile telomer uzunluğu ve apoptoz arasındaki ilişkinin araştırılması

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Abstract

Purpose: Type 2 diabetes mellitus (T2DM) is a heterogeneous, chronic, and metabolic disease that affects a significant proportion of the global population. This study aimed to evaluate the effect of NFKB1 -94 ATTG ins/del polymorphisms on the expression of apoptosis genes and telomere length (TL) in patients with T2DM compared with healthy individuals.
Materials and Methods: Sixty-nine T2DM patients and sixty healthy people were enrolled in the study. DNA and RNA were isolated from the blood samples. NFKB1 genotypes were identified by Sanger sequencing. For TL analyses and to investigate the expression of the caspase-3, caspase-9, bax, and bcl2 genes, RT-PCR was utilized.
Results: There was a significant difference between the NFKB1 -94 ins/del genotype patients and the control group (OR:0.4792 (0.2345-1.011)). However, the distribution of other genotype/alleles (ins/ins and del/del) showed no difference between T2DM and control groups. The allelic frequency of NFKB1 -94 ins/del was 0.455/0.235 for the T2DM group and 0.435/0.165 for the control group. An increase in the mRNA expression of caspase-3, caspase-9 and Bax genes was observed in the T2DM group compared with the healthy group, while a decrease in the Bcl2 gene was found in the T2DM group. TL in T2DM patients was shorter than in healthy individuals.
Conclusion: NFKB1 -94 ins/del polymorphisms show significant differences in T2DM patients. We observed that apoptosis was activated and TL was shortened in patients with T2DM. However, no relationship between NFKB1 polymorphisms and apoptosis and TL could not be determined.
Keywords:. T2DM, NFKB1, apoptosis, telomere length, Sanger sequencing.

Öz

Amaç: Tip 2 diabetes mellitus (T2DM), dünya nüfusunun büyük bir bölümünü etkileyen kronik, metabolik ve heterojen bir hastalıktır. Bu çalışma, T2DM'li hastalarda NFKB1 -94 ATTG ins/del polimorfizmlerinin, apoptoz genlerinin ekspresyonu ve telomer uzunluğunu (TU) sağlıklı bireylerle karşılaştırmalı olarak değerlendirmeyi amaçladı.
Gereç ve Yöntem: Çalışmaya 69 T2DM hastası ve 60 sağlıklı kişi dahil edildi. Kan örneklerinden DNA ve RNA izole edildi. NFKB1 genotipleri, Sanger sekansı ile tanımlandı. TU analizleri için ve kaspaz-3, kaspaz-9, bax ve bcl2 genlerinin ekspresyonunu araştırmak için RT-PCR kullanıldı.
Bulgular: NFKB1 -94 ins/del genotipli hastalar ile kontrol grubu arasında anlamlı bir fark vardı (OR:0,4792 (0,2345-1,011)). Bununla birlikte, diğer genotip/alellerin (ins/ins ve del/del) dağılımı, T2DM ve kontrol grupları arasında fark göstermedi. NFKB1-94 ins/del'in alelik frekansı T2DM grubu için 0,455/0,235 ve kontrol grubu için 0,435/0,165'idi. T2DM grubunda kaspaz-3, kaspaz-9 ve Bax genlerinin mRNA ekspresyonunda sağlıklı gruba göre artış gözlenirken, T2DM grubunda Bcl2 geninde azalma saptandı. T2DM hastalarında TU, sağlıklı bireylerden daha kısaydı.
Sonuç: NFKB1 -94 ins/del polimorfizmleri, T2DM hastalarında anlamlı farklılık göstermektedir. T2DM'li hastalarda apoptozun aktive olduğunu ve TU'nun kısaldığını gözlemledik. Ancak NFKB1 polimorfizmleri ile apoptoz ve TU arasında ilişki saptanamadı.

Anahtar kelimeler: T2DM, NFKB1, apoptoz, telomere uzunluğu, Sanger sekans

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INTRODUCTION

T2DM is a common but serious and health problem that affects many people worldwide[1]. Many cases of diabetes go undetected until life-threatening complications arise, which can include heart disease, renal failure, retinopathy, neuropathy and can result in lower limb amputations[2,3]. It is reported that T2DM is strongly associated with age, obesity, and physical inactivity in people with a genetic predisposition[4]. The basic biology of insulin resistance and possible therapeutic roles of the genes expressed genes in T2DM are poorly understood[5].

Nuclear factor-kappa B (NFKB) pathway is a key player in the emergence of DM and a number of problems related to DM[6,7]. The NFKB is triggered by several pro-inflammatory cytokines in DM, and it controls both -cell survival and death, although it is mostly pro-apoptotic in cells[6]. The relationship between metabolism, inflammation, and insulin action depends on the NFKB signaling pathway[7]. In the immune system, NFKB performs its crucial and evolutionarily conserved function[8]. Activation of NFKB by oxidative stress from caused on by hyperglycaemia may increase the levels of inflammatory cytokines levels[9]. The transcription factor known as NFKB has been shown to regulate the expression of a number of genes involved in apoptosis, inflammation, immunological response, and cell proliferation[10]. In 2004, the insertion/deletion (rs28362491) variant of NFKB that regulates the NFKB1 gene was first reported[11]. Numerous inflammatory illnesses, autoimmune conditions, and malignancies have been linked to this polymorphism[12].

Telomeres situated at the ends of chromosomes, shorten with each cell cycle and serve as a biological indicator of cellular aging that is associated with smoking, obesity, insulin resistance and oxidative stress, and with increasing age, particularly in women[13-15]. Telomeres are essential for chromosomal integrity, protection, and help prevent chromosomal fusion[16]. People with metabolic syndrome and T2DM have shorter telomeres[17,18].

The goal of the this study was to examine the expression of apoptotic genes in correspondence to NFKB1 polymorphisms (-94 ATTG ins/del) (rs28362491), and TL in individuals with T2DM. Additionally, the study aimed to investigate into how these genetic alterations relate to demographic and clinical information.

MATERIALS AND METHODS

Sample

69 individuals diagnosed with T2DM and 60 healthy volunteers who were not diagnosed with diabetes, participated in this study. We aimed to include an equal number of patients and healthy individuals in the study. However, those who did not perform successful DNA and RNA isolation due to hemolysis in the blood of some of the individuals were excluded. All participants were over 18 years old and applied to the Department of Internal Medicine of Ordu University. The Clinical Ethics Committee of Atatürk University approved for sample collection (number: 2021/2-3) and informed consent was obtained from the individuals.

Procedure

Hemogram tubes (ethylenediamin tetra-acetic acid (EDTA)) were used to collect all blood samples. Haematological and biochemical parameters were calculated from using routine test results of the patients included in the study. The parameters used in this study were: aspartate aminotransferase (AST), alanine aminotransferase (ALT), haemoglobin (HB), insulin, white blood cells (WBC), triglyceride (TG), fasting blood sugar (FBS), platelets (PLT), blood urea nitrogen (BUN), HOMA-IR, creatinine (CRE), total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, HbA1c, thyroid stimulating hormone (TSH), free T4 (FT4), and erythrocyte sedimentation rate (ESR). Blood samples were taken from patients after 12 hours of fasting. Biochemical parameters were studied using the ARCHITECT c8000 clinical chemistry analyser (Abbott, IL, USA) and Hemogram parameters were analysed using the CELL-DYN Ruby automated haematology analyser (Abbott, IL, USA). ESR measurement was taken as the amount of the erythrocytes in the collected blood sample after 1 hour. The following method was used to compute the homeostatic model assessment of insulin resistance (HOMA-IR): fasting insulin (mIU/L) × fasting plasma glucose (mg/dL)/405 [19]. Body mass index (BMI) was computed using the formula (body weight (kg) / height^2 (m^2) after patient height and weight were recorded [20].

The mean and standard deviations of test data was determined to compare T2DM patients to healthy participants. Statistical analyses compared the clinical data of T2DM patients to healthy participants.

Detection of the NFKB1 (-94ins/del ATTG) (rs28362491) gene polymorphism

Genomic DNA was isolated from blood samples using an Eco-Tech DNA isolation kit according to the manufacturer protocol (Cat. No. EcoBGD-50x, TURKEY)[21]. The NanoDrop Take3 Plate (BioTek, USA) measured DNA sample concentration with an optical density at 260 nm and the purity at 260 nm/280 nm. Until PCR was carried out, all DNA samples were kept at -20°C.

For the PCR, a SensoQuest Labcycler instrument (thermal cycler) was used. Each PCR reaction were used 25 µl of EcoTaq 2X PCR Master Mix, 2 µl each of forward and reverse primers (10 M), 10 pg-500 g of template DNA, and ddH_2O[21]. Pre-optimised primers were preferred[22].

For NFKB1, the following PCR conditions were used: 5 minutes initialisation at 95 degrees, 30 seconds denaturation at 95 degrees, 30 seconds annealing at 60 degrees, 1 minute extension at 72 degrees, 5 minutes at 72 degrees, and held at 4 degrees until storage at -20 degrees. PCR products were run on a 2% gel using 3 µl ethidium bromide. NFKB1 PCR products were observed at 285 bp when visualised under UV light.

Sanger sequencing was performed using the ABI3500 sequencer from Applied Biosciences. PCR products were cleaned with exoSAP before sequencing and the Unipro UGENE v43.0 software was used for analysis. VectorBuilder was used to show the secondary structure of DNA.

Analysis of apoptosis gene expression levels

The EcoPURE total RNA isolation kit was used to isolate total RNA (catalog number: E2075; EcoTech Biotechnology, Erzurum, Turkey). 100 µl of blood was used for the RNA isolation. RNA of all T2DM and healthy individuals were kept at 20°C until it was time for RT-PCR.

The Bio-Rad iScript kit was used to cDNA synthesis from RNA (Bio- Rad, Hercules, CA, USA). The mix was prepared with 20 µl of 8 µl of RNA, 1 µl iScript reverse transcriptase, 4 µl 5x iScript reaction mix and 7 µl of nuclease-free water[23]. We performed the reaction as recommended by the manufacturer: priming: at 25°C for 5 minutes, reverse transcription at 46°C for 20 minutes, RT inactivation at 95°C for 1 minute, and hold at 4°C until storage at 20°C.

We utilized 3 µl of PCR grade water, 5 µl of cDNA, 1 µl of forward and reverse primers (300-500 nM), and 10 µl of TaqTM Universal SYBR Green Supermix (2X) (Bio-Rad) for the RT-PCR[23]. We used the previously primers for beta actin, caspase-3, caspase-9, Bax and Bcl2 that have been used before [24,25] (Table 1).

Using the Bio-Rad CFX RT-PCR device, a PCR was performed at the conditions recommended by the manufacturer (25 seconds at 95°C for polymerase activation, 3 seconds at 95°C for denaturation, 20 seconds at 60°C for annealing, for 35–40 cycles)[23]. The relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Telomere length analysis

RT-PCR was used as the T/S to determine the TL of samples' isolated DNA. 10 µl of iTaq™ Universal SYBR Green Supermix (2X) (Bio-Rad), 5 µl of genomic DNA (50 ng-5 pg), 1 µl of forward and 1 µl of reverse primer (300-500 nM), and 3 µl of PCR grade water were used to analyze TL[26].

The 36B4 gene, which has a single copy, was used as a reference gene for the traditional qPCR method of measuring telomeres (Table 1). For both telomere and 36B4 amplicons, the cycling conditions were adjusted to 95 °C for three minutes, 95 °C for three seconds, and 60 °C for 25 minutes[26,27]. The $2^{-\Delta\Delta Ct}$ method was used to calculate the samples' TL.

Statistical analysis

GraphPad Prism version 8.0.1 was used for all statistical analysis. The Kolmogorov-Smirnov (K-S) normality test was used to demonstrate a normal distribution data. To describe variables that were normally distributed, we used Mean±Sd. GNU pspp 1.4.1 was used to show the diagnostic value of FBS and HBA1C with ROC curve analysis. The Mann-Whitney U test or Student's t test was used to analyse the relationship between T2DM and healthy volunteers, also imcorporating clinical parameters. The association between apoptosis genes and TL incorporating clinical parameters was examined using Mann-Whitney U test and Student's t test. Using One-way ANOVA or the Kruskal-Wallis test, the relationship between NFKB1 polymorphisms and

clinical parameters, TL, and apoptotic genes was also investigated. The Chi- squared test was used for genotype/allele frequency comparation and Hardy-Weinberg Equilibrium (HWE). The Spearman Rho Correlation Coefficient test was used for correlation analysis. We considered p-values to be statistically significant if p< 0.05.

Table 1. Primer list

Beta-actin	Forward:5'-CCATAAACGATGCCGGA-3' Reverse: 5'-CACCACCCATAGAATCAAGA-3'
Caspase- 3	Forward: 5'- TTCATTATTCAGGCCTGCCGTGG -3' Reverse: 5'- TTATGACACGCCATGTCATCATCA -3'
Caspase -9	Forward: 5'-TCAGTGACGTCTGTGTTCAGGAGA-3' Reverse: 5'-TTGTTGATGATGAGGCAGTAGCCG-3'
Bax	Forward: 5'-TGGCAGCAGTGACAGCAGCG-3' Reverse: 5'-TACGGAGGTGGAGTGGGTGT-3'
Bcl2	Forward: 5'-TTCCGAGTGGCAGCTGAGATGTTT-3' Reverse: 5'-TGCTGGCAAAGTAGAAGAGGGCAA-3'
NFKB1	Forward: 5'-GGCTGAAAGAACATGGACTTG-3 Reverse: 5'-GTACACCATTTACAGGGAGGG-3'
Telo	Forward: 5'-GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT-3' Reverse:5'-TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA-3'
36B4	Forward: 5'-CAGCAAGTGGGAAGGTGTAATCC-3' Reverse: 5'- CCCATTCTATCATCAACGGGTACAA-3'

RESULTS

This study included 69 T2DM and 60 healthy individuals. The age of T2DM patients ranged from 36 to 65 years with a mean of 46.84 years. 79.71% of T2DM patients were female and 20.29% were male. Comparatively, the age of healthy individuals ranged from 19 to 67 years with a mean of 46.63 years. 86.67% of healthy individuals were female and 13.33 % were males.

BMI scores were significantly different between T2DM and healthy participants (p<0.0001). Significant differences were determined in the biochemistry results of insulin (p = 0.0109), fasting blood sugar (p < 0.0001), BUN (p = 0.001), HOMA-IR (p = 0.0075), CRE (p = 0.0238), ALT (p = 0.0081), TG (p = 0.0004), HDL cholesterol (p = 0.0111), HbA1c (p < 0.0001), WBC (p = 0.0410), FT4 (p = 0.0019) and ESR (p = 0.00601) between the two groups. These biochemical parameters with a significant difference (except HDL cholesterol in the patient group), were higher in T2DM patients compared to healthy individuals (Table 2).

The area under the ROC curve (AUC) value obtained from the HbA1c test, together with the confidence interval value, was 0.90 with confidence interval (CI) (0.85-0.95), and had an acceptable discrimination power. In addition, since the p-value was calculated as a very low value, the HbA1c test confirms that the discrimination result is at an acceptable power value (p<0.0001) (Figure 1A). Furthermore, the AUC value obtained from the FBS test, together with the CI value, was 0.85 with CI (0.80-0.91), and had an acceptable discrimination power. In addition, the FBS test confirms that the discrimination result is at an acceptable power value with a low p-value (p<0.0001) (Figure 1A). Also, a moderate positive correlation was determined between HbA1c level and FBS level (p<0.0001, r=0.5646) (Figure 1B).

Some participants had other diseases. Of the T2DM patients, 46 had hypertension, 18 had hyperlipidemia, 2 had coronary artery disease, 2 had goitre and 1 had chronic renal failure. Comparatively, of the healthy individuals, 19 had hypertension, 1 had hyperlipidemia and 3 had goitre.

Table 2. Comparison of demographic and various biochemical data between healthy individuals and Type 2 diabetes patients

Parameters	Healthy Group (n=60)	Patient Group (n=69)	P value
	mean±sd	mean±sd	
Age (years) ₤	46.63±13.12	46.84±8.55	0.9145
BMI₤	26.67±3.69	34.45±5.903	****<0.0001
Insulin#	11.38±7.11	17.65±6.84	*0.0109
Fasting blood sugar #	95.97±13.76	140.75±56.18	****<0.0001
BUN#	12.08±3.68	14.26±4.42	**0.001
HOMA-IR#	2.68±1.78	4.31±1.49	**0.0075
CRE#	0.69±0.13	0.75±0.16	*0.0238
ALT#	18.65±12.35	22.52±11.98	**0.0081
AST#	18.25±8.36	19.91±10.79	0.2328
TG#	137.79±96.14	170±70.49	***0.0004
Total cholesterol₤	197.79±38.73	204.28±44.92	0.3926
HDL cholesterol#	54.68±13.56	48.79±10.23	*0.0111
LDL cholesterol₤	114.53±27.56	119.15±33.17	0.4075
HbA1C#	5.76±0.99	7.50±1.49	****<0.0001
WBC#	6.72±2.07	7.39±2.16	*0.0410
HB#	12.90±1.69	12.69±1.61	0.2109
PLT#	252733.3±69167.17	272942±80214.58	0.2181
TSH#	2.18±.92	2.16±1.48	0.7215
FT4#	1.18±0.24	1.37±0.37	**0.0019
ESR#	19.17±9.68	25.08±12.22	**0.00601

Body Mass Index (BMI) (19 - 24.9 kg/m2), insulin (2.6-24.9 mlU/L), fasting blood sugar (FBS) (70-100 mg/dL), blood urea nitrogen (BUN) (0-18 mg/dL), HOMA-IR (0-2.5), creatinine (CRE) (0.50-0.90 mg/dL), alanine aminotransferase (ALT) (0-33 U/L), aspartate aminotransferase (AST) (0-32 U/L), triglyceride (TG) (0-200 mg/dL), total cholesterol (0-200 mg/dL), high-density lipoprotein (HDL) cholesterol (45-65 mg/dL), low-density lipoprotein (LDL) cholesterol (0-130 mg/dL), HbA1c (0-6.5 mg/dL), white blood cells (WBC) (4.49-12.58x109/L), haemoglobin (HB) (11.9-14.6 g/dL), platelets (PLT) (150-450x109 /L), thyroid stimulating hormone (TSH) (0-200 mlU/L), free T4 (FT4) (0.93-1.70 ng/dL), and erythrocyte sedimentation rate (ESR) (0-15 mm/h)

p<0.05 was considered statistically significant (: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001), sd: Standard deviation, # Mann-Whitney U test was used. ₤ Student's t-testi was used.

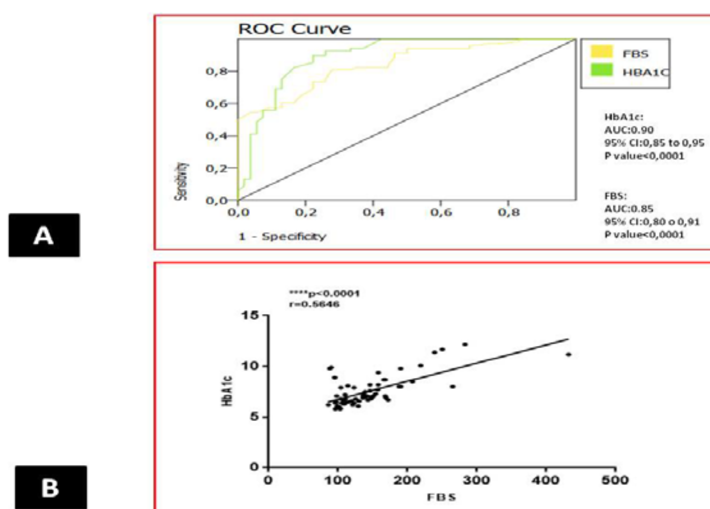

Figure 1. A) ROC curve for HbA1c test (95% Cl) (AUC: 0.90) and FBS (AUC: 0.85) B) Positive correlation between HbA1c and FBS.

NFKB1 gene was amplified by PCR and DNA bands were visualised under UV light using 2% obtained ethidium bromide (Figure 2). Variants of the NFKB1 gene were then identified by Sanger sequence analysis and the secondary structure of DNA was observed (Figure 3).

The frequency of NFKB1 genotypes in the participants are shown displayed in Table 3. In the T2DM patient group, genotypes frequency of NFKB1 gene were determined as 37.68% (26/69) ins/ins, 56.52% (39/69) ins/del and 5.80% (4/69) del/del. In the healthy group, genotype frequency of NFKB1 gene was determined as 53.33% (32/60) ins/ins, 38.33% (23/60) ins/del and 8.33% (5/60) del/del. The NFKB1 -94 ATTG ins/del frequencies were 0.455/0.235 for T2DM and 0.435/0.165 for healthy individuals. Significant differences of in ins/del genotype frequency were observed between T2DM patients and healthy individuals ($p = 0.0470$, O.R: 0.4792 (0.2345-1.011)). Chi-squared test was used for genotype/allele frequency comparison and Hardy-Weinberg Equilibrium (HWE) (Table 3). The HWE p-values of the healthy and T2DM patient groups were determined as 0.7646 and 0.0319, respectively (Table 3).

We found that T2DM patients had higher gene expression of caspase-3, caspase-9, and Bax compared with did healthy individuals ($p = 0.0480$, $p > 0.05$, and $p = 0.0312$ respectively) (Figure 4). However, Bcl2 gene expression was lower in T2DM patient ($p > 0.05$) (Figure 4). The intracellular ratio of Bax/Bcl2 proteins is defined as a cellular marker of apoptosis susceptibility.

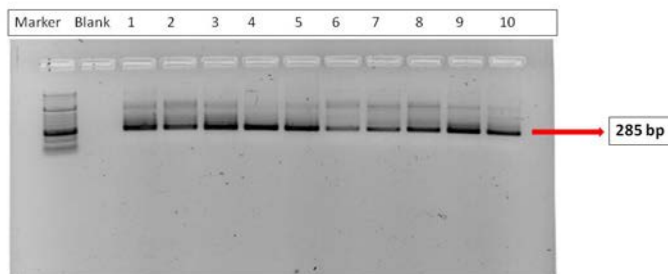

Figure 2. Gel electrophoresis for NFKB1.

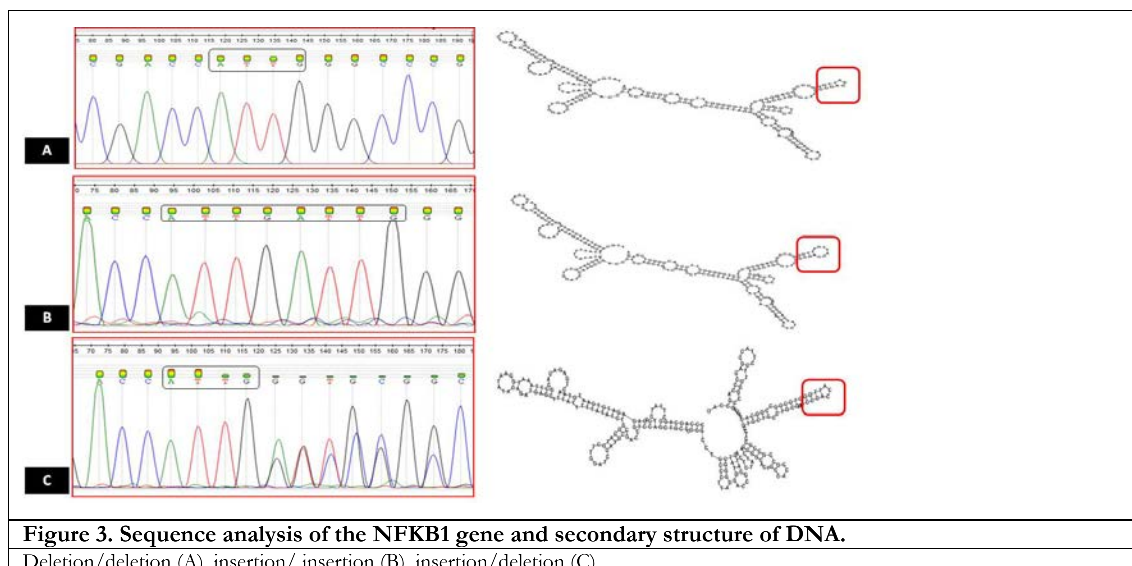

Figure 3. Sequence analysis of the NFKB1 gene and secondary structure of DNA.

Deletion/deletion (A), insertion/ insertion (B), insertion/deletion (C)

Table 3. Distribution of genotype and allele frequencies for NFKB1 insertion/deletion -94 ATTG polymorphism in Type 2 diabetes patients and control and HWE values

Genotype / allele	Type 2 DM n (%)	Control n (%)	Total n (%)	O.R (95% CI)	P value
	69	60			
II	26 (37.68)	32 (53.33)	58 (44.96)	Ref	
ID	39 (56.52)	23 (38.33)	62 (48.06)	0.4792 (0.2345-1.011)	*0.0470
DD	4 (5.80)	5 (8.33)	9 (6.68)	1.016 (0.2604-3.581)	0.9828
HWE p value	*0.0319	0.7646			
ID+DD	43 (62.32)	28 (46.66)		0.5291 (0.2554-1.062)	0.0747
I alleli	91(65.94)	87 (72.5)		Ref	
D alleli	47 (34.06)	33 (27.5)		0.7344 (0.4308-1.269)	0.2560

DM: Diabetes mellitus, **II:** insertion / insertion, **ID:** insertion / deletion, **DD:** deletion / deletion, Ref: deletion, **O.R:** odd ratio, Chi-square test was used.
***** p<0.05 was considered statistically significant.

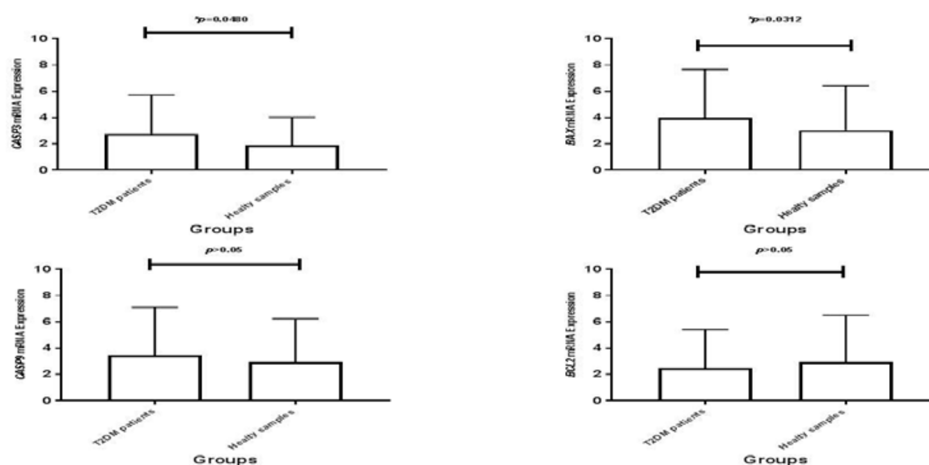

Figure 4. mRNA expression results of apoptosis genes (Casp3, Casp9, Bax and Bcl2) in type 2 diabetes patients versus and healthy individuals.

TL was shorter in T2DM patients compared with healthy individuals (p = 0.0350) (Figure 5). Age and TL were not appear to be significantly correlated, according to our findings (Figure 5).

A significant difference was found between caspase-3 gene expression in T2DM patients and healthy individuals with HbA1c, HB level and hypertension (p = 0.0165, p = 0.0136 and p = 0.0382 respectively). Furthermore, a statistically significant difference was found between Bcl2 gene expression and HbA1c level and individuals with hyperlipidemia (p=0.095 and p=0.048). Finally, Bax gene expression was significantly different in individuals with hyperlipidemia (p = 0.0466). Clinical-demographic parameters, TL and NFKB1 gene polymorphisms did not significantly differ between individuals (p > 0.05). When TL and expression distributions of apoptosis genes (caspase-3, caspase-9, Bax and Bcl2) were analysed according to NFKB1 polymorphisms, no significant difference was found according to genotype/alleles (p > 0.05).

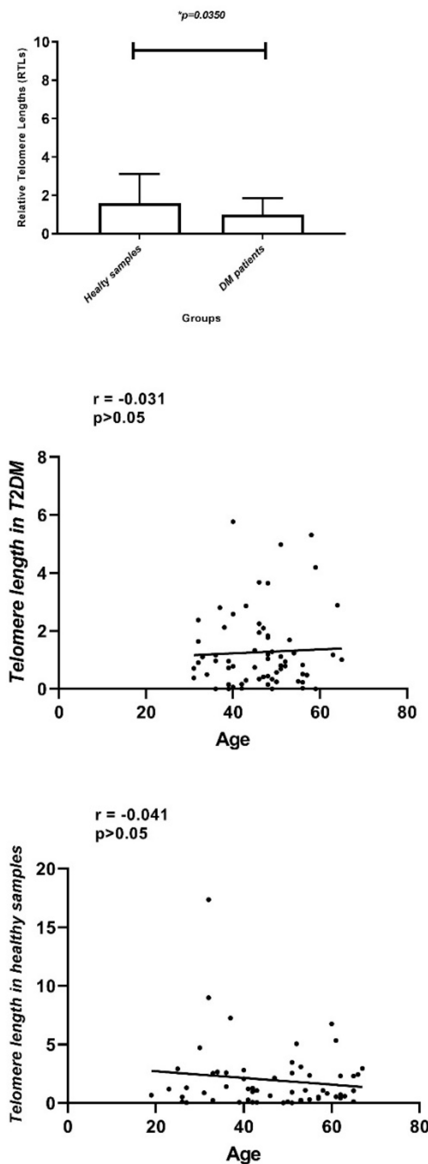

Figure 5. Telomere length analysis and correlation with age in type 2 diabetes patients and healthy individuals

DISCUSSION

In this study, we screened NFKB1 polymorphisms (ins/del -94 ATTG) in T2DM patients and healthy individuals to determinate the relationship between apoptosis gene expression and TL together. The NFKB pathway is thought to involved in the aetiology of T2DM and renal illness[28]. Through regulating the genes encoding pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors, and inducible enzymes, the NFKB plays a critical role in immunological and inflammatory responses[29]. Signals that cause cell growth, differentiation, death, and other processes activate transcription factors of the NFKB family[29]. Ageing and various age-related illnesses, such as cancer, coronary artery disease, heart failure, and diabetes, have been linked to telomere shortening[30]. In the pathophysiology of T2DM, apoptosis has crucial functions and several factors contribute to the aetiology of T2DM, including insulin resistance caused by obesity, poor insulin production, and loss of β-cell mass due to β-cell apoptosis[31].

This is the first research to investigate the relationship between NFKB1 polymorphisms, apoptosis and TL in in the Turkish T2DM patients. We detected that the frequency of ins/del frequency distribution was 56.52% in T2DM patients, 37.68% ins/ins and the 5.80% del/del distribution. Allele frequencies of NFKB1 -94 ATTG insertion/deletion were 0.455/0.235 in T2DM patients and 0.435/0.165 healthy individuals. Our findings show a statistically significant difference in the distribution of frequencies for NFKB1-94 ins/del polymorphism between T2DM patients and healthy individuals. Gautam et al. (2017) reported that the frequency of ins/del variant was highest in individuals with normoglycemic and T2DM mellitus and that the allele frequency of -94 ATTG ins/del was 0.62/0.38 for T2DM[10]. Another study showed not difference in the distribution of genotype and allele frequency for the NFKB1 gene polymorphism between T2DM and the control groups did not show any statistically significant differences[32].

There are two distinct pathways for apoptosis[33]: the internal pathway[34] mediated by the Bcl2 protein family and caspases and the external pathway associated with Fas or TNF death receptors (TNFR)[35,36]. The internal pathway, which is mediated by the Bcl2 family of proteins and is linked to the mitochondria[36-38]. The release of cytochrome c by mitochondria into the cytoplasm is an important step of mitochondrial apoptosis. The following activation phase of initiator caspase-9 is important, causing cleavage of executive caspases-3/7[36,39]. Proteins in the Bcl-2 family are involved in mitochondrial

integrity[36,40,41]. In the present study, we examined the mRNA expressions of caspase-3, caspase-9, Bax and Bcl2 apoptotisis genes and found that while the expression of the Bcl2 gene expression was lower in the T2DM group, caspase-3, Bax and caspase-9 genes were all higher in T2DM patients when compared to healthy individuals. These findings suggest that apoptosis is more active in T2DM patients and the expression profile of these apoptotic genes corresponds with previous studies. A significant difference was found between caspase-3 gene expression in T2DM patients and healthy individuals with HbA1c, HB level, and hypertension. Conversely, a statistically significant difference was found between Bcl2 gene expression and HbA1c level in individuals with hyperlipidemia. Finally, a significant difference was found between bax gene expression and hyperlipidemia status. Bax and Bcl2 are known regulators of apoptosis, and the antagonistic effect of these proteins in apoptosis control is reported therefore, the intracellular ratio of Bax/Bcl2 proteins serve as a cellular marker of apoptosis susceptibility[42]. High levels of Bcl2 and low levels of Bax described in this study are consistent with the literature. Apoptosis may be triggered by oxidative stress and is controlled by many signalling pathways[43,44]. Therefore, we hypothesise that high glucose may have led to the production reactive oxygen species, thus promoting apoptosis. NFKB plays a role in cellular events such as cell proliferation, inflammation, lymphocyte activation, cell growth and differentiation, cell survival, apoptosis necrosis, and is involved in the regulation of immunity[45]. Expression distributions of apoptosis genes (caspase-3, caspase-9, Bax and Bcl2) were analysed according to NFKB1 polymorphisms, and no significant difference was found according to genotype/alleles.

Telomere attrition is known to be enhanced in metabolic diseases[46]. T2DM is characterised by increased oxidative stress and oxidative DNA damage[47]. Telomeres length is shortened and DNA oxidative damage is increased when insulin levels are inadequate[48]. In T2DM patients, the anti-inflammatory adipokine adiponectin was found to positively correlate with TL, therefore adiponectin could potentially slow down aging[49]. Patients with ischaemic heart disease show a substantial correlation between telomere shortening and T2DM[50]. Age, known diabetes duration, blood pressure, glucose management, and baseline lipid levels were all linked with TL[51]. Our data shows that TL of T2DM patients was shorter compared with healthy individuals. However, there was no discernible correlation between TL and age or biochemical data. In addition, when we explored at the TL distribution according to NFKB1 polymorphisms, we could not find a significant difference.

In this study, we have compared NFKB1 polymorphisms with TL and apoptosis gene expression for the first time. However, the limited budget of our project and the low number of patients are among the main limitations of this study. Future studies should explore the mechanisms described in this study and investigate these in both *in vivo* and *in vitro.*

In summary, our study demonstrates a substantial difference between NFKB1 ins/del variations in T2DM patients and healthy individuals. We also demonstrated that T2DM patients have high expression levels of the apoptosis genes caspase-3, Bax and caspase-9 low levels of Bcl2. Finally, we show that TL in the T2DM patients was considerably shorter compared with healthy individuals. We suggest that studying these genetic mechanisms in a larger patient population can guide in both diagnosis and treatment.

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