

Dual mapping of *MTHFR* C677T (A1298C) and *BDNF* G196A (Val66Met) polymorphisms in patients with diabetic peripheral neuropathy

A. YEHYA¹, Z. ALTAANY², O. BENI-YONIS³

¹Department of Clinical Pharmacy and Pharmacy Practice, Faculty of Pharmacy, Yarmouk University, Irbid, Jordan

²Department of Basic Medical Sciences, Faculty of Medicine, Yarmouk University, Irbid, Jordan

³Department of Public Health and Family Medicine, Faculty of Medicine, Jordan University of Science and Technology (JUST), Irbid, Jordan

Abstract. – OBJECTIVE: Diabetic peripheral neuropathy (DPN) is a loss of distal sensory function in the lower limbs that is accompanied by pain and severe morbidity. The goal of this study was to perform a screening of the *MTHFR* C677T (A1298C) and *BDNF* G196A (Val66Met) polymorphisms and determine their possible relationships using biochemical blood tests and clinical presentations of symptoms in Jordanian patients with DPN.

PATIENTS AND METHODS: A cross-sectional study was conducted, and medical records were used to identify and recruit patients with DPN and collect their demographic and clinical characteristics. The total neuropathy score (TNSr) was used to assess the severity of sensory symptoms. In addition, direct sequencing was performed after Polymerase Chain Reaction (PCR) amplification to screen the two single nucleotide polymorphisms (SNPs) of interest.

RESULTS: Ninety patients with DPN participated in the study. The *MTHFR*-SNP variant (CT) and (TT) genotypes were identified in 39 (43.3%) and 19 (21.1%) patients, respectively. On the other hand, the *BDNF*-SNP variant (GA) and (AA) genotypes were identified in 22 (24.4%) and 11 (12.2%) patients, respectively. The distributions of the genotype frequencies of the *MTHFR*-SNP and *BDNF*-SNP variants statistically differed between patients with DPN and the control group ($p < 0.0001$, $p < 0.002$). Moreover, patients carrying variant genotypes of the two analyzed SNPs were more likely to have unsatisfactory HbA1c levels (> 7 mg/dl, $p = 0.029$) and moderate to severe symptoms (TNSr score 8-24).

CONCLUSIONS: The results of this study show that the *MTHFR* C>T-677 SNP and the *BDNF* G>A-196 SNP can be used as genetic risk markers for DPN. Assessing patients' genetic-metabolic risk profiles is recommended for providing personalized treatment.

Key Words:

Pharmacogenetics, Diabetic neuropathy, *MTHFR*, *BDNF*, Polymorphism, Jordan.

Introduction

Peripheral neuropathy is the most commonly encountered type of diabetic neuropathy and a major cause of morbidity and mortality in the diabetic population worldwide^{1,2}. Due to alterations in gene expression, molecular transport, inflammation, and oxidative stress, chronic hyperglycemia, hypertension, and dyslipidemia are regarded as the primary risk factors for diabetic peripheral neuropathy (DPN)¹. Patients may suffer from pain, loss of sensation, and even limb amputation, causing a deleterious impact on the overall quality of their lives and a significant increase in healthcare expenses^{3,4}.

Several investigations⁵ have revealed that even strict glycemic control cannot reverse diabetic neuropathy once it has developed. Nevertheless, early clinical diagnosis is key to decreasing the likelihood of patient deterioration and the burden of hospital admissions⁶. Combined screening for genetic variants and metabolic monitoring contribute to a better understanding of the etiology of DPN and improve clinical outcomes⁷. Several single nucleotide polymorphisms (SNPs) within key genes involved in the pathogenesis of DPN have been investigated in the literature^{8,9}. The rs1801133 (C>T-677) polymorphism of the methylenetetrahydrofolate reductase (*MTHFR*) gene and the rs6265 (G>A-196) polymorphism of the brain-derived neurotrophic factor (*BD-*

NF) gene are the two most frequently studied SNPs¹⁰⁻¹³. MTHFR is an enzyme that catalyzes the transition of the remethylation of homocysteine to methionine (gene *Ip36*)¹⁴. Moreover, the C677T mutation in the *MTHFR* gene results in an amino acid shift from alanine to valine. This variation reduces the enzyme's activity by 50%, lowers folate levels, and is believed to contribute to insulin resistance¹⁵. The *BDNF* gene is localized on chromosome 11 band p13 and comprises 11 exons and 9 functional promoters¹⁶. Furthermore, a naturally occurring amino acid change from valine (Val) to methionine (Met) at position 66 within the pro-region of BDNF may result in altered BDNF activity-dependent secretion¹⁷. BDNF is a major regulator of several types of neurons, including sensory neurons, retinal ganglion cells, spinal motor neurons, certain cholinergic neurons, and certain dopaminergic neurons^{18,19}.

Both the *MTHFR* and *BDNF* SNPs of interest have been associated^{12,20} with DPN in several populations. In Jordan, type 2 diabetes mellitus (T2DM) is the fourth leading cause of death. A recent analysis²¹ estimated that by 2050, approximately 3.4 million Jordanians are expected to suffer from T2DM, which is an increase of 28.8 percentage points from the number in 2020. Previous studies^{22,23} have evaluated the metabolic profiles and quality-of-life measures of patients with DPN, but there is a lack of genetic research. To target DPN more effectively, additional research needs to focus on screening for genetic variants and elucidating their association with DPN development and progression. The current study was guided by the following objectives: performing a screening for the *MTHFR* and *BDNF* SNPs and determining their possible relationships with biochemical blood tests and the clinical presentations of symptoms in Jordanian patients with DPN.

Patients and Methods

Study Participants and Setting

Ninety unrelated patients with DPN were registered at the outpatient clinics of King Abdullah University Hospital (KAUH), Irbid, Jordan, between April and June 2022. Patients were identified from medical records. Objective evaluation of the symptoms of DPN was done using the reduced version of the total neuropathy score (TNSr)²⁰. Patients with a score in the range of

1-8 were classified as having mild symptoms; those with a score of 8-16 were classified as having moderate symptoms; and those with a score in the range of 16-24 were classified as having severe symptoms. Moreover, the demographics and clinical characteristics of the patients were also recorded. One hundred and ten healthy controls were matched for age and gender with the DPN patients to compare the distribution of the SNPs of interest between the controls and the patients with DPN. All participants were asked to sign an informed consent form. The study protocol was approved by the Institutional Review Board (IRB) Ref number (2421113).

MTHFR C>T-677 and BDNF G>A-196 SNPs Genotyping

Genomic DNA was extracted from blood samples. 1-1.5 milliliters were collected from each participant (-20°C) using the G-spin™ Total DNA Extraction Mini Kit (iNtRON Biotechnology, Inc., Seongnam, Kyonggi-do, Korea). DNA samples were stored in EDTA-coated tubes at (-20°C) for further analysis. DNA quantity and quality were assessed using a Nano-drop spectrophotometer at $\lambda = 260$ nm, and 0.8% agarose gel electrophoreses (V = 100 volts) for 30 minutes. Amplification of the C677T region was performed using the forward primer: ACTCAGCGAACTCAGCACTC and the reverse primer AAGATCAGAGC-CCCCAAAGC. Polymerase Chain Reaction (PCR) was performed under the following conditions: 4 minutes of initial denaturation at 95°C, followed by 39 cycles of 95°C for 15 seconds, 60°C for 12 seconds, and 72°C for 15 seconds, with a final extension at 72°C for 10 minutes (Bio-Rad, S1000 Thermal cycler™, Hercules, CA, USA). The PCR products (410-bp) were visualized using a UV box transilluminator *via* 1.5% agarose gel electrophoresis. The specific genotypes were also identified by direct sequencing at Macrogen, Inc., (Seoul, Korea).

Amplification of the BDNF region was performed using the forward primer: 5'-ACTCTG-GAGAGCGTGAAT-3' and the reverse primer 5'-ATACTGTCACACACGCTC-3'. The PCR reaction was performed under the following conditions: one cycle of pre-denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 sec, annealing at 57°C for 45 sec, elongation at 72°C for 45 sec and one cycle of post-elongation at 72°C for 10 min. The PCR products (308-bp)

were visualized using a UV box transilluminator via 1.5% agarose gel electrophoresis. The specific genotypes were also identified by direct sequencing at Macrogen, Inc. (Seoul, Korea).

Statistical Analysis

Data were analyzed using the Statistical Package for Social Sciences (SPSS version 21, IBM Corp., Armonk, NY, USA). The Chi-square (χ^2) test was used to evaluate the distribution of the genotypes of the patients and the controls. The relationships between the *MTHFR*-SNP, *BDNF*-SNP genotypes and the demographic and baseline clinical characteristics of the patients were analyzed using the χ^2 test or analysis of variance (ANOVA) statistics. The χ^2 test and Fisher's exact test were used to compare categorical variables with odds ratios, and 95% confidence intervals were used to assess their relation to *MTHFR*-SNP, *BDNF*-SNP genotypes. A two-way ANOVA with multiple comparisons was used to evaluate the relation between TNSr score, the *MTHFR*-SNP and *BDNF*-SNP genotypes. All *p*-values were two-tailed, and those lower than 0.05 were considered statistically significant.

Results

Distribution of *MTHFR* C>T-677 and *BDNF* G>A-196 Polymorphisms

MTHFR and *BDNF* were genotyped in 90 DPN patients and 110 controls by direct sequencing were performed (Figure 1A-F). The frequency of the *MTHFR* wild-type (CC) genotype was identified in 32 DPN patients (53.6%), while the heterozygous mutant (CT) and homozygous mutant (TT) genotypes were identified in 39 (43.3%) and 19 (21.1%) DPN patients, respectively. These genotypes were also observed in the control group, in which (CC) genotype was found in 86 (78.1) controls, while (CT) and (TT) genotypes were found in 18 (16.3%) and 6 (5.4%) controls, respectively. The distributions of the genotype and allele frequencies were statistically different between the patients with DPN and the control group ($p = 0.003$ and $p < 0.0001$, respectively) (Table I).

With regard to *BDNF*-SNP, the *BDNF* wild-type (GG) genotype was identified in 57 patients (63.3%), while the heterozygous mutant (GA) and homozygous mutant (AA) genotypes were identified in 22 (24.4%) and

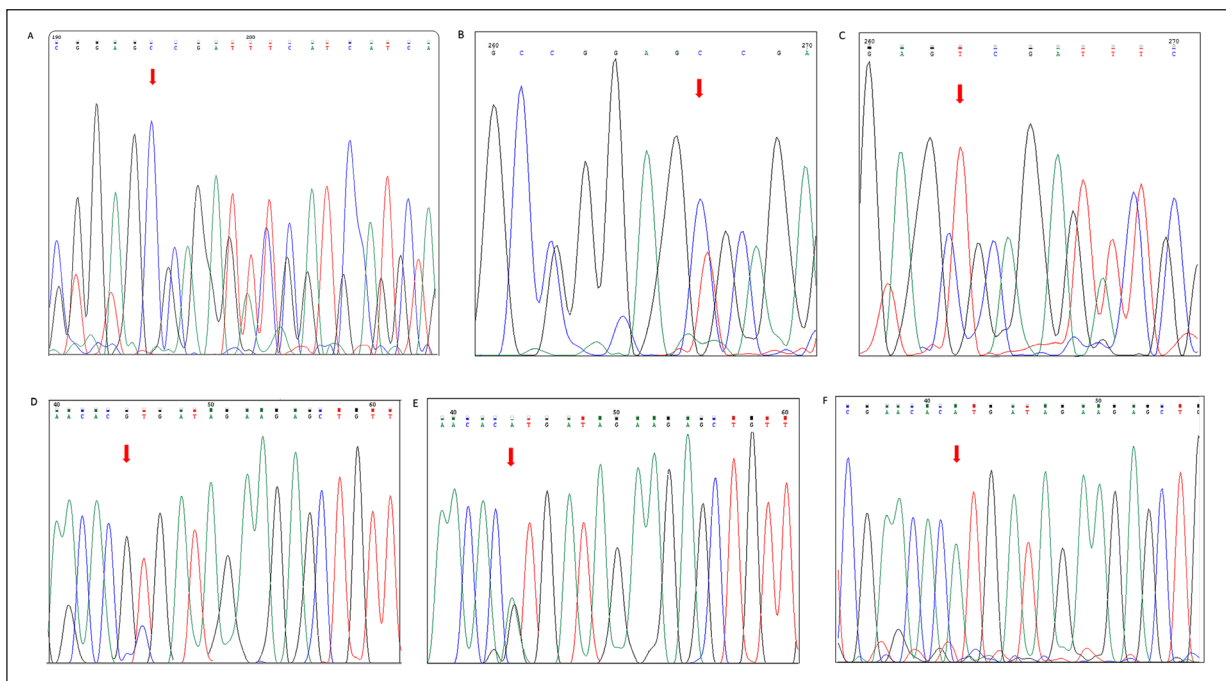


Figure 1. A-F, A representative chromatogram showing the sequence of the genomic DNA amplicon for randomly selected samples of the rs1801133 (C>T-677) polymorphism of the *MTHFR* gene for randomly selected samples. Arrows show the sites of the (A) CC, (B) CT, and (C) TT genotypes. The chromatogram for the rs6265 (G>A-196) polymorphism of the *BDNF* gene is also shown. Arrows show the sites of the (D) GG, (E) GA, and (F) AA genotypes.

Table I. Genotype frequencies of the *MTHFR* C>T-677 and *BDNF* G>A-196 polymorphisms in patients with DPN and control groups.

Genotype	DPN patients, (%), n = 90	Controls, (%) n = 110	p-value	OR (95% CI)
MTHFR C > T-677 SNP				
CC	32 (53.6)	86 (78.1)	< 0.0001	-
CT	39 (43.3)	18 (16.3)		1.8 (0.5-6.6)
TT	19 (21.1)	6 (5.4)		0.4 (0.08-2.3)
C	103 (57.2)	180 (82.0)	0.003	
T	77 (42.7)	30 (13.6)		
BDNF G > A-196 SNP				
GG	57 (63.3)	92 (83.6)	0.002	-
GA	25 (27.7)	14 (12.7)		2.1 (1.1-3.8)
AA	8 (8.8)	4 (3.6)		2.5 (0.9-4.9)
G	139 (77.2)	198 (90.0)	0.008	1.69 (1.17-2.14)
A	41 (23.0)	22 (10.0)		

11 (12.2%) patients, respectively. These genotypes were also identified in the control group, with (GG) genotype in 92 (83.6%) patients and (GA) and (AA) genotypes in 14 (12.7%) and 4 (3.6%) patients, respectively. The distributions of genotype and allele frequencies of the *BDNF*-SNP genotypes were statistically different between the patients with DPN and the control group ($p = 0.002$ and $p = 0.008$, respectively). The observed and expected frequencies of the two mutations were in Hardy-Weinberg equilibrium in the patient and control groups (Table I).

Baseline Characteristics of Patients with DPN According to the MTHFR C>T-677 and BDNF G>A-196 SNPs

The demographic and clinical data of patients with DPN are listed in Table II. The average age of the patients was 57.15 ± 6.590 (years), and approximately 54% were males. The average duration of T2DM was $8.71 \text{ years} \pm 5.007$, with glycated hemoglobin (HbA1C) of $7.44 \text{ mg/dl} \pm 2.443$. The majority of patients (84.4%) were treated with metformin, and over half of them suffered from hypertension (67.7%) and dyslipidemia (57.7%). Table II presents the baseline characteristics of the patients according to the *MTHFR*-SNP and *BDNF*-SNP genotypes. The analysis revealed that patients who carried the *MTHFR*-SNP (CT) and (TT) mutant genotypes or the *BDNF* (GA) and (AA) mutant genotypes had high HbA1c levels $> 8 \text{ mg/dl}$, compared to patients who carried the wild-type genotypes of the SNPs ($p = 0.029$) (Table II).

Association Between MTHFR C>T-677 SNP, BDNF G>A-196 SNP, and TNSr Scores in Patients with DPN

In the study, patients with DPN were classified according to the TNSr score to assess the signs and symptoms of neuropathy. More than half of the patients, 53%, reported mild symptoms, 38% reported moderate symptoms, and only 7% had severe symptoms (Table III). A comparison of the TNSr scores of the patients according to the expression of the *MTHFR*-SNP and *BDNF*-SNP genotypes revealed that the presence of the homozygous mutant genotypes correlated with an increase in the severity of the symptoms, as reflected by a higher TNSr score, compared to the presence of wild-type genotypes ($p < 0.01$) (Figure 2).

Discussion

Changes in glucose metabolism and inflammatory processes have been studied in relation to a number of genetic polymorphisms. This means that they can be linked to the development and progression of DPN²⁴. This study revealed that the frequencies of the mutant genotypes of *MTHFR* C>T-677 SNP and *BDNF* G>A-196 SNP were significantly higher among Jordanian patients with DPN compared to those in the control group ($p < 0.0001$, $p = 0.002$, respectively). These findings align with those of previous reports^{10,25,26} that proposed the implication of these mutations in the development of DPN. In addition, a strong association has been reported between the *MTHFR* C677T mutation and DPN in several populations, includ-

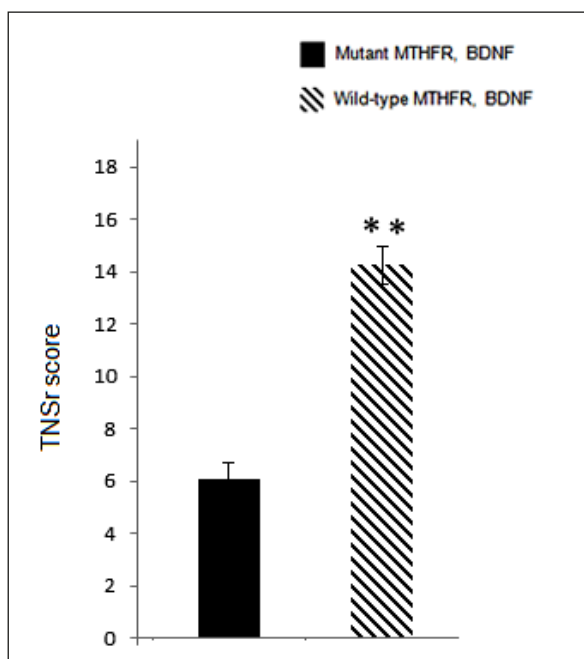
Table II. Baseline characteristics of patients with DPN according to MTHFR-SNP and BDNF-SNP genotypes.

Characteristic	Total, n = 90	CC, n = 32	MTHFR C>T-677 SNP		GG, n = 57	BDNF G>A-196 SNP		p-value
			CT, n = 39	TT, n = 19		GA, n = 22	AA, n = 11	
Gender: No. Male:Female (%)	49/41 (54.4/45.5)	21/11 (65.6/34.3)	16/23 (41.0/58.9)	12/7 (63.1/36.8)	27/30 (47.3/52.6)	14/8 (63.6/36.3)	8/3 (72.7/27.2)	0.857
Age (years): mean \pm SD	57.15 \pm 2.590	56.46 \pm 2.220	57.24 \pm 1.238	56.04 \pm 2.708	56.21 \pm 1.812	56.82 \pm 1.339	59.65 \pm 1.154	0.572
Disease duration (years): mean \pm SD	8.71 \pm 1.007	8.17 \pm 1.013	8.32 \pm 1.184	7.55 \pm 1.087	7.76 \pm 1.888	8.21 \pm 1.122	6.21 \pm 1.341	0.371
HbA1c (%): mean \pm SD	7.96 \pm 1.003	7.15 \pm 0.836	7.98 \pm 1.013	8.36 \pm 1.124	6.94 \pm 1.71	8.15 \pm 1.231	8.39 \pm 1.154	0.029
Metformin/Insulin treated: N (%)	76/14 (84.4/15.5)	20/12 (62.5/37.5)	37/2 (94.8/5.1)	19/0 (100/0)	48/9 (84.2/15.7)	17/5 (77.2/22.7)	11/0 (100/0)	0.242
History of hypertension: N (%)	61 (67.7)	19 (59.3)	31 (79.4)	11 (57.8)	33 (57.8)	19 (86.3)	9 (81.1)	0.744
History of dyslipidemia: N (%)	52 (57.7)	21 (65.6)	23 (58.9)	8 (42.1)	32 (56.1)	16 (72.7)	4 (36.3)	0.524

Table III. Classification of patients with DPN (n = 90) according to the TNSr score.

Patient-reported symptoms	
TNSr score	N (%)
Mild (< 8)	48 (53.3)
Moderate (9-12)	35 (38.8)
Severe (12-24)	7 (7.7)

ing European²⁷, Chinese²⁸, and Iranian patients²⁹. Moreover, previous reports^{30,31} have shown that plasma homocysteine levels and related increases in oxidative stress were significantly increased in patients with diabetic neuropathy compared with patients without diabetic neuropathy. To evaluate the effects of the variation in the *MTHFR* gene, Ukinc et al³² aimed to study the effect of hyperhomocysteinemia on the development of diabetic microvascular complications. No association was found between the gene variants, hyperhomocysteinemia, and neuropathy. These discrepancies could be assumed to be due to the effect of epigenetic mechanisms that control gene expression, which are also influenced by genetic and environmental factors, such as lifestyle-risk factors and diet.

**Figure 2.** The TNSr scores of patients carrying the wild-type MTHFR-SNP and BDNF-SNP genotypes (white bar), mutant *MTHFR*-SNP genotype (blue bar), or mutant *BDNF*-SNP genotype (mixed colors). Bars represent the mean (\pm SEM) values of the scores. * $p < 0.05$.

Previous research^{33,34} has shown that BDNF plays an important role in the etiology of T2DM by regulating food intake and energy homeostasis. In concurrence with the findings of the present study, previous studies^{12,13,35} have supported the association between the *BDNF* G196A mutation and DPN in several populations. However, we observed a lack of similar results in Arab countries. Notably, in the current study, none of the patients had mutant genotypes of both mutations; this may be due to the small number of patients. In addition, we observed that the frequency of the mutant *MTHFR*-SNP genotypes was higher than that of the *BDNF*-SNP genotypes (64.4% vs. 36.3%) among Jordanian patients with DPN. A previous study²³ included 103 Jordanian patients with T2DM who were identified as suffering from cardiovascular disease and dyslipidemia, which, along with the duration of diabetes, were the main predisposing risk factors for DPN among Jordanian patients. Ajlouni et al³⁶ evaluated the state of control of T2DM in Jordan in a sample of 1,121 Jordanians; the study revealed that more than half of the patients (54%) had unsatisfactory glycemic control. In the current study, the frequency of the patients carrying the mutant *MTHFR*-SNP and *BDNF*-SNP genotypes was higher in patients with poor glycemic control (HbA1C > 7.5%). The findings of the current study constitute a step toward defining the genetic-metabolic interrelationship among Jordanian patients with DPN.

Another key finding of this study was the possible link between *MTHFR*-SNP and *BDNF*-SNP and the progression of DPN. The frequency of the patients carrying the mutant genotypes of the two analyzed SNPs was higher among patients with moderate-severe symptoms, (TNSr score 8-24). A strong relationship was found between HbA1C variability and peripheral nerve amplitudes and velocities in patients with T2DM^{37,38}. Therefore, it is possible that this observation is partially related to the fact that patients carrying the mutations already had a higher tendency to have increased HbA1C levels. Given that, not all patients present typical signs and symptoms of neuropathy and clinical sensory examinations, and despite their genetic basis, blood glucose control is a major step to delay or reverse DPN.

The main strength of the current study is the dual analysis of the two relevant mutations compared to previous investigations³⁹⁻⁴³, which were dedicated only to single mutation analysis. Due to financial limitations, it was not possible to

include a larger number of patients in this study. Additional multicenter studies are needed to generalize the genetic analysis to the wider Jordanian population.

Conclusions

The findings of the current study support the use of the *MTHFR* C>T-677 and *BDNF* G>A-196 SNPs as genetic markers of the risk of DPN. This will create a deeper understanding of their complex disease etiologies, thereby enabling the development of a more comprehensive approach to assessing risk profiles and ultimately providing personalized treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Ethics Approval

The study protocol was approved by the Institutional Review Board (IRB) of Jordan University of Science and Technology (Ref. number 2281-1-13) and was conducted in accordance with the Declaration of Helsinki and good clinical practice guidelines.

Informed Consent

All participants were asked to sign an informed consent form.

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ORCID ID

Alaa Yehya: 0000-0001-5267-6406.

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