

Preliminary investigation of two promoter region polymorphisms of the *TNFA* gene in patients with recurrent depressive disorder

AGNIESZKA JELEŃ¹, MARTA ŻEBROWSKA-NAWROCKA¹, DAGMARA SZMAJDA-KRYGIER¹,
KATARZYNA MAZUR¹, PIOTR GAŁECKI² and EWA BALCERCZAK¹

¹Laboratory of Molecular Diagnostics and Pharmacogenomics, Department of Pharmaceutical Biochemistry and Molecular Diagnostics, Medical University of Łódź, 90-151 Łódź, Łódź Voivodeship;

²Department of Adult Psychiatry, Medical University of Łódź, 91-229 Łódź, Łódź Voivodeship, Poland

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Abstract. Immune system dysregulation plays a role in the pathogenesis of complex human diseases, including psychiatric disorders. In addition, elevated levels of pro-inflammatory cytokines, including tumor necrosis factor α (TNF- α) may be conditioned by the presence of specific polymorphic variants. The present case-controlled preliminary study evaluated the prevalence of *TNFA* gene single nucleotide polymorphisms (SNPs) G-308A (rs1800629) and T-1031C (rs1799964) in 83 Polish patients with depression by restriction fragment length polymorphism analysis. The results were compared with the frequencies of genotypes in a geographically- and ethnically-matched group of individuals without depression. No statistically significant difference in genotype/allele frequency was observed for either SNP between the two groups. No association was found between the particular genotypes and selected demographic/clinical features, including sex, age at diagnosis or severity of depressive symptoms before/after pharmacotherapy. Thus, there does not appear to be any connection between the studied SNPs and the development and progression of depression; however, further studies are required with larger cohorts to better understand this aspect of depression.

Introduction

One of the most common mental health diseases is depression. The World Health Organization (WHO) predicts that depression will be the most widespread worldwide health

problem by 2030. However, the causes of depression remain incompletely understood (1).

It has been repeatedly confirmed that patients with diagnosed depression present with altered levels of inflammatory markers characteristic of systemic inflammation in both the blood and the cerebrospinal fluid (2). Pro-inflammatory cytokines, such as tumor necrosis factor α (TNF- α) are able to penetrate the blood-brain barrier from the peripheral circulation; this results in microglial activation, which initiates a cascade of various cytokines and other factors, leading to a loss of synaptic plasticity, disturbances in neuronal transmission and ultimately neurodegeneration, which is also characteristic of the aging brain (3).

TNF- α is a pro-inflammatory molecule known to be involved in neuroinflammation. This inflammatory process forms the base of Alzheimer's disease (4), vascular dementia (5) and Parkinson's disease (6). It is not necessary for the blood brain barrier (BBB) to be broken for information about ongoing inflammation to be received by the brain cells; however, when the BBB is damaged, this allows migration of inflammatory cytokines/other substances between the peripheral tissues and brain tissue. The incomplete activation of microglia occurring during chronic and slowly progressive trauma initiates the process of neurodegeneration, which is exacerbated by peripheral or cerebral inflammatory stimulus. Such chronic neurological disorders may be provoked by BBB impairment, as well as by bacterial and/or viral infections (5).

In addition to cytokines, noncoding RNAs such as microRNAs (miRNAs), which regulate gene expression through a number of different mechanisms, can also migrate between the peripheral system and the CNS. These RNA fragments are found in the majority of organs and tissues, and are highly expressed in brain tissues (7). It has been found that amongst individuals infected with HIV, circulating miRNA levels differ between those with high and low neuropsychological performance. In addition, several miRNAs are involved in inflammatory pathways, suggesting that miRNAs may play a role in modulation of microglial-mediated immune response during chronic HIV infection (8).

Conversely, a number of polymorphisms in the *TNFA* gene promoter are associated with complex human diseases (9-11),

Correspondence to: Dr Marta Żebrowska-Nawrocka, Laboratory of Molecular Diagnostics and Pharmacogenomics, Department of Pharmaceutical Biochemistry and Molecular Diagnostics, Medical University of Łódź, Muszynskiego 1, 90-151 Łódź, Łódź Voivodeship, Poland
E-mail: marta.zebrowska@umed.lodz.pl

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of which, two were selected for the present study. The first, single nucleotide polymorphisms (SNP), G-308A (rs1800629), is associated with increased TNF- α production compared to the wild-type allele. The second, SNP T-1031C (rs1799964), co-creates a haplotype associated with an increased risk of different diseases (12). The aim of this preliminary study was to determine the frequency of the G-308A and T-1031C SNPs in a group of patients with depression in Poland, and identify any potential association between their presence, the course of disease and its treatment.

Materials and methods

Patients. Blood samples were taken from 83 patients (58 women; 25 men; median age 51; age range 17-62 years) suffering from recurrent depressive disorder (rDD). All participants were hospitalized in the J. Babinski Memorial Psychiatric Hospital in Łódź (Łódź, Poland). The involvement of patients in this study was based on the ICD-10 criteria [categories of mood (affective) disorders: F32.0-F32.2, F33.0-F33.8] (13). For all study participants, the standardized Composite International Diagnostic Interview (CIDI) (14) was used to obtain a case history. The investigation was performed in accordance with the principles of the Declaration of Helsinki (15) and was approved by the Ethical Committee of Medical University of Łódź (approval no. RNN/566/08/KB; KE/126/20). All participants included in the study or their parents provided verbal informed consent to take part. The median age at diagnosis of the first depressive episode in this group was 44 years and the age range of first diagnosis was 14-58 years old. The severity of depression was evaluated amongst the enrolled individuals based on the 17-item version of the Hamilton Depression Rating Scale (HDRS). The severity of disease symptoms before treatment (HDRS I) ranged from 10-37 (median, 22). During hospitalization, all the patients received pharmacotherapy based on a range of medicines (Selective Serotonin Receptor Inhibitors, Serotonin Norepinephrine Reuptake Inhibitors, agomelatine) or a combination thereof. The severity of depressive symptoms measured after treatment (HDRS II) ranged from 2-35 (median, 16). The effect of therapy was calculated based on two parameters. The first was *HDRS change*, determined by HDRS I - HDRS II (median, 16), and the second was *%HDRS change*, determined by (HDRS I - HDRS II/HDRS I) x 100% (median, 0.73).

The control group consisted of 248 healthy individuals from the Blood Transfusion Center; the participants were matched geographically and ethnically to the group of patients with depression (16).

PCR. DNA samples were isolated using the Blood Mini kit according to the manufacturer's protocol (A&A Biotechnology S.C.). The purity and concentration of DNA samples were assessed nanospectrophotometrically (Implen GmbH) and then stored at -20°C, until required. Genotyping was performed by restriction fragment length polymorphism, in which the products of PCR are digested by a specific restriction enzyme. PCR for SNPs were conducted according to the Jump Start™ AccuTaq™ LA DNA Polymerase kit protocol (Sigma-Aldrich; Merck KGaA). The PCR mixture consisted of a DNA template

(50 ng), primers (0.5 μ M each forward and reverse), enzyme (0.5 U) and nucleotide free water to a final volume of 20 μ l. Every experiment included a blank sample. The digestion reaction was performed according to the manufacturer's protocols (Thermo Fisher Scientific, Inc.). Genotypes were identified by electrophoresis in 2% agarose gel. The primer sequences, PCR conditions and details of the digestion reaction for the investigated polymorphisms are given in Table I. The number of PCR cycles was 35. The PCR was performed using a Bio-Rad Laboratories thermal cycler (Bio-Rad Laboratories, Inc.).

DNA structural analysis. DNA secondary structures were predicted using the RNAstructure software package version 6.3 (released April 5, 2021; rna.urmc.rochester.edu). All parameters were set to default values.

Databases. The Genome Browser database (genome-euro.ucsc.edu) was used for characterization of the *TNFA* promoter region.

Statistical analysis. All statistical analyses were performed using STATISTICA version 13.3 (StatSoft Europe). Continuous variables were analyzed using a Student's t-test or Mann-Whitney U test, and categorical variables using the χ^2 test. The genotypes for G-308A and T-1031C were in agreement with the Hardy-Weinberg equilibrium in both groups. Deviations of allelic frequencies from Hardy-Weinberg equilibrium were verified a χ^2 test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

The first stage of the analysis was to compare the genotype and allele frequencies for both selected SNPs between the patient and control groups. No statistically significant difference was observed (Table II).

The next stage of the analysis examined the association between the particular genotypes of investigated polymorphisms (G-308A and T-1031C) and some selected demographic or clinical features of rDD patients. The initial P-value concerns the G-308A polymorphism, and the second the T-1031C polymorphism. No significant relationships were found with regard to sex ($P=0.1977$, $P=0.3065$), age at diagnosis ($P=0.1554$, $P=0.2282$), severity of depressive symptoms before pharmacotherapy ($P=0.1920$, $P=0.3628$), change in severity of treatment according to the Hamilton scale ($P=0.1090$, $P=0.1361$; Student's t-test) or percentage change in HDRS ($P=0.2221$, $P=0.0948$). The influence of investigated *TNFA* gene polymorphisms on the treatment response is presented in Table III.

Regarding the influence of the tested SNPs on DNA secondary structure, the free energy change (ΔG) value of the most stable structure was compared between wild-type or allelic variants. Regarding the variants, the presence of the -308A allele increased ΔG from -212.9 to -209.3 and the -1031C allele from -212.9 to -211.4. The structure with both -308A and -1031C, had a ΔG of -207.8. Decreases in the ΔG value suggests that the DNA structure is less stable, and this may influence the function of transcription factors leading to changes in the mRNA level.

Table I. PCR-restriction fragment length polymorphism conditions.

Single nucleotide polymorphism	Primer sequence, 5'-3'	Product size, bp	Annealing temperature, °C	Restriction enzyme	Digestion conditions, °C/h	Digested fragment size, bp
-308 <i>TNFA</i>		147	59	<i>NcoI</i>	37/16	WT: 126,21 M: 147
Forward	GAGGCAATAGGTTTTGAGGGCCAT					
Reverse	GGGACACACAAGCATCAAG					
-1031 <i>TNFA</i>		264	59	<i>BpI</i>	37/16	WT: 249, 15 M:180, 69, 15
Forward	TATGTGATGGACTCACCAGGT					
Reverse	CCTCTACATGGCCCTGTCTT					

WT, wild-type; M, mutant.

Table II. Genotype and allele frequencies of *TNFA* single nucleotide polymorphisms in patients with depression and healthy individuals.

Genotype	Patients, n (%)	Healthy individuals, n (%)	P-value, χ^2 test
<i>TNFA</i> -308			
GG	58 (69.9)	172 (69.4)	0.5050
GA	25 (30.1)	72 (29.0)	
AA	0 (0.0)	4 (1.6)	
G	141 (84.9)	416 (83.9)	0.7442
A	25 (15.1)	80 (16.1)	
HWE P	0.3040	0.5878	-
<i>TNFA</i> -1031			
TT	55 (66.3)	167 (67.3)	0.7141
TC	25 (30.1)	76 (30.6)	
CC	3 (3.6)	5 (2.1)	
T	135 (81.3)	410 (82.7)	0.6961
C	31 (18.7)	86 (17.3)	
HWE P	1.0000	0.6228	-

HWE P, Hardy-Weinberg equilibrium P-value.

Discussion

Several patients with depression demonstrate increased TNF- α levels in the peripheral blood. As such, it was proposed that a genetic predisposition to elevated pro-inflammatory cytokine synthesis may play a role in the pathogenesis of depression (17).

Both of the polymorphisms in the *TNFA* promoter region chosen for the present study are located in a region that is known to enhance gene expression (H3K27Ac) in the GM12878 lymphoblastoid cell line. Additionally, chromatin state segmentation evaluation in K562 cells found the G-308A polymorphism region to have the features of a weak enhancer, whereas the T-1031C region was weakly transcribed. Theoretical models of *TNFA* promoter secondary structure, predicted by RNAstructure software, also indicate that the

evaluated SNPs may play important role in alterations to physiological processes that occur in this gene fragment; however, this hypothesis requires experimental confirmation.

The increase in ΔG resulting from the presence of the -1031C and -308A allelic variant (data not presented) may affect the thermodynamic stability of the structure, which may influence gene expression. It was found that G-308A and T-1031C SNPs only appear to have a small influence on the ΔG ; however, it should be noted that even minor changes can be significant in a process as complex as gene transcription.

According to previous a case-control study, allele A may be associated with a greater bioactivity of TNF- α . Initial reports based on an Asian population indicated that the -308A allele and the AA genotype were significantly more common in the depression group than the control group (18). Later studies

Table III. G-308A and T-1031C *TNFA* genotype frequencies in relation to effectiveness of therapy.

A, HDRS change						
Genotype	n	Mean	Minimum	Maximum	Standard deviation	P-value
<i>TNFA</i> G-308A						0.109
GG	53	16.89	2	35	7.02	
GA	25	14.32	4	25	5.3	
<i>TNFA</i> T-1031C						0.1361
TT	53	16.83	4	35	6.6	
CT or CC	25	14.44	2	26	6.4	
B, % of HDRS change						
Genotype	n	Median	Minimum	Maximum	Interquartile range	P-value
<i>TNFA</i> G-308A						0.2221
GG	53	0.76	0.13	1	0.58-0.88	
GA	25	0.67	0.2	0.95	0.62-0.86	
<i>TNFA</i> T-1031C						0.0948
TT	53	0.75	0.2	1	0.62-0.89	
CT or CC	25	0.64	0.13	0.95	0.56-0.79	

HDRS, Hamilton Depression Rating Scale.

yielded conflicting results; all of which have been reviewed elsewhere (19).

The first SNP chosen for analysis in the present study was G-308A. Neither genotype nor allele appear to be disease risk factors; in addition, neither are clinicopathological features of depression. This observation is consistent with the results of other studies performed on Caucasian populations (20,21). Other authors also emphasize the lack or low frequency of the AA genotype in patients (20), which is consistent with the present findings. A recent study suggested that the GG genotype may be a depression risk variant (22); however, this previous study was based on a population of relatively young individuals, which may suggest that features other than age should be included in further genetic analyses of depression. Higher numbers of studies report the results of observations made in Asian populations. Due to this diversity of research findings, a number of meta-analyses have been performed; their findings indicate that *TNFA* SNP G-308A is not associated with susceptibility to depression (23,24). These findings are consistent with the results obtained amongst patients with chronic hepatitis C. No differences in -308 *TNFA* genotype frequency between healthy controls and HCV-infected patients was found (25). Therefore, this polymorphism apparently does not influence mood disorders or infectious diseases development, in a direct way.

The second SNP of the *TNFA* gene chosen for study was T-1031C. Few studies to date have examined the influence of this SNP on the risk of major depressive disorder (MDD). The results of the present study indicated that the *TNFA* gene T-1031C was not associated with an increased incidence of depression, nor did it affect the severity of symptoms. These

findings are in line with previous studies, as no involvement was found for the T-1031C SNP in patients with a single depressive episode with or without stressful life events prior to MDD (26). Similar findings were noted by Misener *et al* (27) who found the presence of this SNP to have no significant association with childhood depression in a study of 384 families, primarily of Caucasian ethnicity. However, a recent publication by Bialek *et al* (28) indicated that the TT genotype and the T allele were associated with a low effectiveness of pharmacotherapy, and the CT genotype and C allele with positive response to the treatment of patients with MDD (28). No such observations were made in the present study.

The present study is intended as only a preliminary study, and is hence not without limitations. The first is the lack of data on age and sex differentiation in the control group. These data were not provided in the original study by Bednarczuk *et al* (16) from which the genotyping results were obtained. A second limitation is the small sample size, which could be attributed to the relatively low frequency of depression without any comorbid medical conditions or addictions that can trigger a depressive episode. In addition, it is possible that the patient data may be subject to potential biases stemming from the desire of a patient to take part in the study; this may have been influenced by factors associated with psychiatric status. Furthermore, this research does not include information about the percentage of rDD patients who did not meet the inclusion criteria, or why some participants did not complete the study. In addition, no data was collected on occupation, home-income, tobacco smoking, diet, physical activity, sleep and other lifestyle information; this information may affect the interpretation of the findings. Therefore, our results

should be treated as preliminary, and final conclusions should only be drawn tentatively. Further trials in this field are highly desirable. Such studies should involve larger groups of patients and healthy controls. In addition, as the promoter region of the *TNFA* gene is rich in other polymorphisms that may affect gene expression, these studies should examine the effects of SNPs G-238GA, C-857T and C-863A on the mRNA and protein levels. Finally, similar studies of other cytokines (including IL-1 β , IL-10 and IL-8) amongst patients with depression are needed to conclude whether increased/decreased production of pro-/anti-inflammatory cytokines is substantially controlled by genes.

In conclusion, there no association between the G-308A and T-1031C polymorphisms of the *TNFA* gene and the development and progression of depression were identified; however, further studies to assess the potential influence of the selected SNPs (calculated during bioinformatics analysis) on gene promoter stability are required.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

EB and AJ conceptualized the study. AJ performed the analysis. MZN, DSK and KM performed the investigation. PG obtained the clinical data. AJ and DSK wrote the manuscript. EB, AJ, MZN and DSK reviewed and edited the manuscript. All authors have read and approved the final manuscript. EB and AJ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was conducted according to the criteria described in the Declaration of Helsinki and was approved by the Ethical Committee of Medical University of Łódź (Łódź, Poland; approval no. RNN/566/08/KB; KE/126/20). All participants included in the study provided verbal informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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