

Ethnic Differences in Brain-derived Neurotrophic Factor Val66Met Polymorphism in Croatian and Korean Healthy Participants

Aim To compare the frequency of alleles and genotypes in brain-derived neurotrophic factor (BDNF) *val⁶⁶met* polymorphism in ethnically homogenous Caucasian (from Croatia) and ethnically homogenous Asian (from South Korea) healthy participants, as inter-population differences in BDNF *val⁶⁶met* may be responsible for the divergent findings in genetic and association studies.

Methods BDNF *val⁶⁶met* was genotyped in 800 (556 Croatian and 244 Korean) healthy participants. Frequencies of alleles and genotypes were evaluated using a χ^2 test.

Results The frequencies for genotypes ($\chi^2_2 = 114.69$; $P < 0.001$) and alleles ($\chi^2_1 = 120.07$; $P < 0.001$) between Korean and Croatian individuals differed significantly, due to significantly lower (46.3% and 19.5%, $P < 0.001$) frequency of "Met" allele and significantly higher (53.7% and 80.5%, $P < 0.001$) frequency of "Val" allele in Croatian than in Korean participants.

Conclusion The study found significant ethnic differences in BDNF *val⁶⁶met* polymorphism. The most frequent genotype among Korean participants was "Met/Val" and they had similar distribution of "Met" and "Val" alleles. In contrast, the most frequent genotype among Caucasian participants was "Val/Val" and they had different distribution of "Met" and "Val" alleles. These ethnic differences require matching participants for ethnicity in pharmacogenetic studies and in the studies investigating genetic variations in neuropsychiatric disorders.

Nela Pivac^{1*}, Byungsu Kim^{2*},
Gordana Nedić¹, Yeon
Ho Joo², Dragica Kozarić-
Kovačić³, Jin Pyo Hong²,
Dorotea Muck-Seler¹

¹Laboratory for Molecular Neuropsychiatry, Division of Molecular Medicine, Ruđer Bošković Institute, Zagreb, Croatia

²Department of Psychiatry, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea

³Department of Psychiatry, Referral Centre for the Stress-related Disorders of the Ministry of Health of the Republic of Croatia, University Hospital Dubrava, Zagreb, Croatia

*Nela Pivac and Byungsu Kim equally contributed to this work.

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Correspondence to:

Nela Pivac
Laboratory for Molecular
Neuropsychiatry
Division of Molecular Medicine
Rudjer Boskovic Institute
POBox 180
10002 Zagreb, Croatia
npivac@irb.hr

Brain-derived neurotrophic factor (BDNF) is a member of neurotrophin family of growth factors (1). BDNF is located in the hippocampus, neocortex, amygdala, cerebellum, and hypothalamus, and it regulates memory formation and processing, learning, locomotion, food intake, nociception, mood, cognition, behavior, and stress response (1). It affects cholinergic, dopaminergic, and serotonergic neurotransmitter systems, which are altered in many neurodegenerative and psychiatric disorders (2). BDNF modulates neural proliferation, survival, repair, neurodegeneration (reductions in hippocampal neuronal plasticity and resiliency), and is associated with Alzheimer disease, major depression, posttraumatic stress disorder, schizophrenia, and bipolar affective disorder (1).

A single nucleotide polymorphism (SNP) of the BDNF gene (*val⁶⁶met*) exists in the 5' prodomain region of the BDNF gene. This polymorphism includes the substitution of valine ("Val") into methionine ("Met") in codon 66 (BDNF *val⁶⁶met*). Although this polymorphism does not affect the structure of the mature BDNF protein (3), the mechanism by which this polymorphism initiates the changes in human memory and hippocampal function is related to alterations in intracellular trafficking and secretion of BDNF (4). BDNF *val⁶⁶met* is a functional polymorphism (G196A, NCBI database dbSNP rs6265), reported to be associated with alterations in hippocampal volume, emotional reactivity, and anxiety and depression-related personality traits (5). It is also related to poorer episodic memory performance, lower depolarization-induced production of BDNF, lower hippocampal measure of neuronal integrity and synaptic abundance, n-acetyl aspartate content, and lower levels of hippocampal activation during memory processing (3,6-8).

There are divergent findings of the positive or negative associations between BDNF *val⁶⁶met* polymorphism and schizophrenia, unipolar depression and bipolar disorder, eating disorders, alcoholism, substance abuse, and anxiety and depression-related personality traits in Caucasian and Asian participants (2). These differences might be induced by ethnic differences in Caucasian and Asian participants (2,6,8-12). However, the cited studies frequently included psychiatric patients and healthy participants, who were not homogenous within the particular ethnic groups. The hypothesis of our study was that BDNF *val⁶⁶met* polymorphism differed between ethnically homogenous Caucasian and ethnically homogenous Asian healthy individuals. The aim of the study was to compare the frequency of "Met/Met," "Met/Val," and "Val/Val" genotypes and

"Met" and "Val" alleles in healthy male and female participants of Caucasian (from Croatia) and Asian (from South Korea) origin.

PARTICIPANTS AND METHODS

Participants

This comparative study included 800 unrelated healthy volunteers from two centers: 556 Caucasian healthy participants of Croatian origin from Zagreb, Croatia (349 men and 207 women; mean age (\pm standard deviation) 33.8 ± 9.05 years) and 244 Asian healthy participants of Korean origin from Seoul, South Korea (129 men and 115 women; mean age 32.5 ± 8.4 years). Caucasian participants were recruited from October 2001 to March 2006 from the University Hospital Dubrava, Zagreb, Croatia. Asian participants were recruited from May 2000 to October 2005 from the Asan Medical Center, University of Ulsan College of Medicine, Seoul, South Korea. Inclusion criteria were not taking medications; no previous or current psychiatric disorders; no alcohol abuse or suicidal attempts; no family history of psychiatric disorders (this was determined according to the answers of participants about the mental health status of their parents, grandparents, and close relatives); not being mutually related; and belonging to the native ethnic group of the regions studied. Written informed consent was obtained from all participants, after the aims and procedures of the study had been explained. The study was performed in accordance with the ethical standards of the Declaration of Helsinki and approved by the Ethics Committees of the University Hospital Dubrava, Zagreb, Croatia or Asan Medical Center, University of Ulsan College of Medicine, Seoul, South Korea.

Genotyping of BDNF Val/Met

Genomic DNA was isolated from peripheral blood leukocytes according to standard procedures of proteinase-K-RNase digestion followed by phenol-chloroform extraction in Korean participants (13) or by a salting out method in Croatian participants (14). In Korean participants, BDNF *val⁶⁶met* polymorphism (rs6265) was genotyped using a single-base primer extension assay (ABI PRISM SNaPshot Multiplex kit; ABI, Foster City, CA, USA) according to the manufacturer's recommendations. Genomic DNA flanking the SNP (rs6265) was amplified by polymerase chain reaction (PCR) using the primers: 5'-TGATGACCATCCTTTTCCTT-3' (forward) and 5'-CACTGGGAGTTCCAATGC-3' (reverse); each PCR reaction utilized 10 ng genomic DNA, 0.5 pM of each oligonucleotide primer, 1 μ L 10X PCR Gold buffer, 250 μ M dNTP, 3 mM MgCl₂, and 0.25 unit i-StarTaq DNA Poly-

merase (iNtRON Biotechnology, Sungnam, Kyungki-Do, Korea) in a total reaction volume of 10 μ L. The amplification protocol consisted of one cycle of denaturation at 95°C for 10 minutes, 30 cycles of initial denaturation at 95°C for 30 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute, followed by a final extension at 72°C for 7 minutes. The PCR products were treated with 1 unit of shrimp alkaline phosphatase (SAP) (Roche, Mannheim, Germany) and 1 unit of exonuclease I (USB Corporation, Cleveland, OH, USA) for 60 minutes at 37°C followed by 15 minutes at 72°C to purify the amplified products. A 1 μ L aliquot of each purified amplification product was added to a SNaPshot Multiplex Ready reaction mixture containing 0.15 pmol genotyping primer for the primer extension reaction, which consisted of 25 cycles at 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 30 seconds. These reaction products were treated with 1 unit of SAP for 1 hour at 37°C, followed by 15 minutes at 72°C to remove excess fluorescent dye terminators. A 1 μ L aliquot of each sample was added to 9 μ L of Hi-Di formamide (ABI) and incubated at 95°C for 5 minutes, followed by 5 minutes on ice. The reaction products were analyzed by electrophoresis using an ABI Prism 3730xl DNA analyzer, with the results interpreted using GeneScan analysis software (ABI).

In Croatian participants, BDNF *val⁶⁶met* polymorphisms was genotyped in ABI Prism 7000 Sequencing Detection System apparatus (ABI) using Taqman-based allele-specific polymerase chain reaction assay, according to the procedure described by the Applied Biosystems (Applied Biosystems, Foster City, CA, USA). The primers and probes were purchased from Applied Biosystems.

Statistical analysis

The genotype frequency was assessed for Hardy-Weinberg equilibrium with a χ^2 test. Statistical differences in genotype and allele frequencies between Korean and Croatian healthy participants were evaluated by a χ^2 test. Age of the

participants (expressed in years as means \pm standard deviation) was evaluated using one-way analysis of variance (ANOVA). Bonferroni correction was used for multiple testing, using the total number of pair comparison of ethnic groups ($N=2$) as a correction factor and the level of significance was set to $\alpha=0.025$. All statistical analyses were performed with SigmaStat 3.1 (Jandell Scientific Corp., San Raphael, CA, USA).

RESULTS

South Korean (age 32.5 ± 8.4 years) and Croatian (age 33.8 ± 9.05 years) participants were matched for age and one way ANOVA showed no significant differences in age ($F=2.73$; $df=1623$; $P=0.099$).

Genotype and allele frequencies of the BDNF *val⁶⁶met* polymorphism were determined in 800 healthy participants: 556 Croatian participants and 244 Korean participants (Table 1). The observed genotype distribution in Korean ($\chi^2_1=1.444$; $P=0.229$) or Croatian ($\chi^2_1=0.406$; $P=0.524$) samples did not differ significantly from the expected Hardy-Weinberg equilibrium. In Koreans, the genotype frequency "Met/Met" was found in 57 participants (23.4%), "Met/Val" in 112 participants (45.9%), and "Val/Val" in 75 participants (30.7%). In Croats, "Met/Met" was found in only 19 participants (3.4%), "Met/Val" in 180 participants (32.4%), and "Val/Val" in 357 participants (64.2%). There were significant differences in the frequency of "Met/Met," "Met/Val," and "Val/Val" genotypes between all Korean and Croatian participants ($\chi^2_2=114.68$; $P<0.001$), between male Korean and male Croatian participants ($\chi^2_2=65.25$; $P<0.001$), and between female Korean and female Croatian participants ($\chi^2_2=49.06$; $P<0.001$). The most frequent genotype among Korean participants (either in both sexes, or separately in men or women) was "Met/Val," while the most frequent genotype among Croatian participants (either in both sexes, or separately in men or women) was

TABLE 1. Genotype and allele frequencies of the brain-derived neurotrophic factor *val⁶⁶met* polymorphism in Asian (Korean) and Caucasian (Croatian) participants

Participants	Genotype (No, %)			Allele (No, %)	
	AA (Met/Met)	AG (Met/Val)	GG (Val/Val)	A (Met)	G (Val)
Korean (n = 244):	57 (23.4)	112 (45.9)	75 (30.7)	226 (46.3)	262 (53.7)
male (n = 129)	25 (19.4)	68 (52.7)	36 (27.9)	118 (45.7)	140 (54.3)
female (n = 115)	32 (27.8)	44 (38.3)	39 (33.9)	108 (47.0)	122 (53.0)
Croatian (n = 556):	19 (3.4)	180 (32.4)	357 (64.2)	217 (19.5)	894 (80.5)
male (n = 349)	12 (3.4)	111 (31.8)	226 (64.8)	135 (19.3)	563 (80.7)
female (n = 207)	7 (3.4)	69 (33.3)	131 (63.3)	82 (19.9)	331 (80.1)

*Abbreviations: Met – methionine; Val – valine.

"Val/Val." To control for the possible sex-related differences in the BDNF *val⁶⁶met* polymorphism within the particular ethnic group, BDNF *val⁶⁶met* was compared separately in male and female Korean or male and female Croatian participants. No significant differences in the occurrence of "Met/Met," "Met/Val," and "Val/Val" genotypes were found for Korean ($\chi^2_2=5.34$; $P=0.069$) or Croatian ($\chi^2_2=0.139$; $P=0.933$) men and women (Table 1).

The frequency of the "Met" and "Val" alleles was significantly different (Table 1) in Asian and Croatian participants ($\chi^2_1=120.07$; $P<0.001$), Korean and Croatian men ($\chi^2_1=66.09$; $P<0.001$), and Korean and Croatian women ($\chi^2_1=51.64$; $P<0.001$). All Korean participants, Korean men, and Korean women had similar distribution of "Met" and "Val" alleles (46.3%, 45.7%, and 47.0% and 53.7%, 54.3%, and 53.0%, respectively). All Croatian participants, Croatian men, and Croatian women had significantly ($P<0.001$) lower frequency of "Met" allele (19.5%, 9.3% and 19.9%) and significantly ($P<0.001$) higher frequency of "Val" allele (80.5%, 80.7%, and 80.1%) than Korean participants. No significant differences were detected in the allele frequency between male and female Korean ($\chi^2_1=0.03$; $P=0.858$) or between male and female Croatian ($\chi^2_1=0.02$; $P=0.896$) participants (Table 1).

DISCUSSION

Our comparative multi-centric study demonstrated significant ethnic differences in the frequency of the BDNF *val⁶⁶met* alleles and genotypes in healthy male and female participants recruited from ethnically homogenous Caucasian (of Croatian origin) and Asian participants (of Korean origin). Besides a meta-analysis of the case-control studies (2) and a comparison of the data with the previous reports (11), this is the first direct comparison of the frequencies of the BDNF *val⁶⁶met* alleles and genotypes in large groups of Caucasian and Asian healthy participants. In line with previous findings (2,6,8-12), our results showed the opposite genotype frequencies in the two ethnic populations: the "Met/Val" genotype was most frequent in Asian participants, while "Val/Val" genotype was most frequent in Caucasian participants. In our study, the distribution of the "Met" and "Val" alleles was almost equal in Asian population, a result confirmed by the reported frequency of 43-58% (15) or 45.9-41.7% (2) for "Met" allele in different Asian populations. On the other hand, the majority of our Caucasian individuals were carriers of the "Val" allele. This finding agrees with the reported frequency of 84-78% (15) or 80.1-81.5% (2) for "Val" allele in various Caucasian populations.

Ethnic differences between Asian or Caucasian participants in our study were not due to different sex of the participants, which is in agreement with previous data on BDNF (2,9,16,17). Absence of sex-related differences was also reported for BDNF concentration in serum or plasma (18-20)

Ethnic differences in BDNF *val⁶⁶met* might be associated with smoking status (9) and different anxiety-like personality traits (17). It has been reported that Caucasian participants (of German origin), carriers of the "Met" alleles, were more vulnerable to initiate and/or maintain smoking (9). Since Croatian (Caucasian) and Korean (Asian) healthy individuals were not controlled for smoking habits or anxiety-like personality traits, the possible association of smoking and/or anxiety-like personality traits and BDNF *val⁶⁶met* cannot be excluded.

BDNF regulates food intake, eating behavior, and energy expenditure (1). In healthy Caucasian individuals, "Met/Met" genotype was connected with the lower body mass index (BMI) (21). Hence, ethnic differences in BDNF *val⁶⁶met* might be associated with different BMI, dietary habits, and obesity status in Croatian (Caucasian) and Korean (Asian) participants. Epidemiological data (22,23) suggest that Croatian participants are overweight or obese, while Koreans still have lower BMI than most of the examinees in the Western countries (24). Although we did not determine BMI, eating patterns, caloric intake, daily exercise, and physical activity in our Caucasian and Asian groups, we might presume that these and other unknown factors might have accounted for the ethnic differences in BDNF *val⁶⁶met* in Asian compared with Caucasian healthy individuals.

Significant ethnic differences in the BDNF *val⁶⁶met* polymorphism may arise from a natural selection of a particular allele or from the influence of various environmental factors, or might be induced by a fixation of allele frequency through a founder effect (2). These differences, other genetic and environmental factors, and factors like population stratification, inadequate sample sizes, different diagnostic instruments, may contribute to the divergent findings in pharmacogenetic studies, and in the studies investigating genetic variations in neuropsychiatric disorders among different ethnic groups (2,4,15). Caucasian and Asian participants with homozygosity for the "Met" allele have an increased risk for schizophrenia and carriers of the "Val/Met" and the "Met/Met" genotypes have an increased risk for eating disorders, while Caucasian and Asian carriers of "Val" allele have an increased risk for substance-re-

lated disorders (2). Caucasian carriers with "Val/Val" genotype have higher anxiety scores than carriers of "Met/Met" or "Val/Met" genotype (17). These data suggest an involvement of BDNF *val⁶⁶met* polymorphism in the etiology of substance-related disorders, eating disorders, schizophrenia (2,15), anxiety related personality traits (17), and neuroticism (10).

The limitation of the study was that we did not control for the smoking status, anxiety-like personality traits, eating patterns, body mass index, caloric intake, daily exercise, and physical activity in our participants. The advantages of the study were its size and homogeneity, as well as the evaluation of BDNF *val⁶⁶met* in all participants and separately in men and women, and conservative Bonferroni correction used for multiple testing.

In conclusion, our study showed significant ethnic differences in the BDNF *val⁶⁶met* polymorphism in Korean and Croatian healthy participants, with significantly lower frequency of "Met" allele and significantly higher frequency of "Val" allele in Croatian than in Korean participants. Ethnic differences in BDNF *val⁶⁶met* polymorphism found in the present and previous (2,11,15) studies, with other multiple genetic and environmental factors, might be responsible for the diversity of the reported positive and negative associations between this polymorphism and various neuropsychiatric disorders. Our results suggest that a careful matching of the participants for ethnicity, among other factors, should be done in pharmacogenetic studies, or in the studies investigating complex molecular pathways involved in the pathogenesis of psychiatric and neurodegenerative disorders.

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