ORIGINAL ARTICLE

Brain Derived Neurotrophic Factor (BDNF) Gene in Peripheral Blood Cells of Stable Schizophrenia

Tokhi E[a], Abd Rahim NEH[b], A. Talib N[c], Mohd NH[d], Ku Zaifah N[b]

[a]*Department of Paraclinic/Pharmacology, Faculty of Medicine, Herat University, Herat, Afghanistan*
[b]*Department of Basic Medical Sciences, Kulliyyah of Medicine, International Islamic University Malaysia, Kuantan, Pahang, Malaysia*
[c]*Department of Pathology and Laboratory Medicine, Kulliyyah of Medicine & Sultan Ahmad Shah Medical Centre @ IIUM, Kuantan, Pahang, Malaysia*
[d]*Psychological Medicine Unit, Medical Faculty, Universiti Sultan Zainal Abidin, Kuala Terengganu, Terengganu, Malaysia*

ABSTRACT

INTRODUCTION: DNA methylation is one of the epigenetic mechanisms regulating the gene functions without causing DNA sequence changes. Brain-derived neurotrophic factors (BDNF) contribute to the neurodevelopment hypothesis of schizophrenia. BDNF plays a role in facilitating neuronal signalling and survival. Epigenetics is the mutual interaction of genes and the environment. During the epigenetic event, BDNF DNA methylation may cause a reduction in BDNF transcription, thus contribute to the clinical presentation and treatment response in schizophrenia. Therefore, this study's objective was to evaluate the association of BDNF DNA methylation with schizophrenia and explore the relationship between BDNF DNA methylation and clinical presentation. **MATERIAL AND METHODS**: Overall, a total of 240 participants were recruited, which included 118 schizophrenia patients and 122 - healthy controls in the study, and methylation status was determined using the Methylight Taqman® assay. The psychopathological symptoms were assessed using the Positive and Negative Syndrome Scale (PANSS). **RESULTS**: There was no significant change in the DNA methylation status between schizophrenia patients and healthy controls (p=0.073). However, among males, the BDNF DNA methylation was significantly higher in schizophrenia patients than healthy controls. (p=0.016). Also, BDNF DNA methylation showed a significant positive correlation with the positive symptoms (r = 0.04, p=0.03) of schizophrenia. **CONCLUSION**: The BDNF DNA methylation could be affected by gender and has a clinical association.

Keywords
Epigenetics, DNA methylation, schizophrenia, BDNF

Corresponding Author
Asst. Prof. Dr. Nour El Huda Abd Rahim
Department of Basic Medical Sciences, Kulliyyah of Medicine, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia.
Tel. No: +609-5704000
Email: elhuda@iium.edu.my

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INTRODUCTION

Schizophrenia is a chronic and severe mental disorder characterized by delusions, hallucinations, impaired cognitive function, and displays incoherent behaviours.[1] The etiological cause of schizophrenia is multifactorial, an interplay of the environmental and genetic factors.[2-4] Despite extensive research, its pathogenesis and causes are unclear.[5]

Studies on twin families and adopted individuals suggested that genetic factors as one of the significant contributors. The genes and the environment mutually interact and contributes to the clinical presentation of schizophrenia.[6,7] The epigenetics studies have unveiled the association between DNA methylation and neurodevelopment of schizophrenia.[8] Genomic methylation profiling on brain tissues of schizophrenia discovered several methylation hotspots include those genes that contribute to neurodevelopment.[9] The affirmation on the roles of the methylated neurodevelopmental associated genes in schizophrenia requires more extensive data and additional analysis methods, which should be more sensitive and specific.[10]

Brain-derived neurotrophic factor (BDNF) facilitates the survival, maturation, and differentiation of the neurons during brain development.[11] *BDNF* also has significant

roles in regulating brain neurotransmitters serotonergic, glutamatergic, and dopaminergic neurotransmitter systems.[12] *BDNF* gene is located at chromosome 11, NC_000011.10 (27654893..27722030, complement). *BDNF* DNA methylation could affect its gene expression, whereby the hypomethylation in the regulatory region increases the synthesis of *BDNF* after depolarization.[13] Meanwhile, the association of *BDNF* DNA methylation and schizophrenia is still inconclusive. Although Ikegame et al.[14] stated significant hypermethylation of *BDNF* in schizophrenia, the later study by Copoglu et al.[15] had shown no significant relationship. The study findings' differences could be due to the lower sample size,[16] or the various methods used for DNA methylation quantitation.[17]

This present study investigated *BDNF* DNA methylation in stable schizophrenia patients who are outpatients with no relapse for at least 6 months and on stable antipsychotic therapy for at least 6 months prior to the study. The *BDNF* DNA methylation were then assessed for its relationship with the psycho-clinical presentation and the antipsychotic use.

MATERIALS AND METHODS

This study is a comparative case-control study that analyses *BDNF* DNA methylation in schizophrenia patients. The IIUM Research Ethics Committee (IREC) and Medical Research and Ethics Committee (MREC), Ministry of Health Malaysia, granted the research approval. Blood samples of schizophrenic patients from the Psychiatry clinic of Hospital Tengku Ampuan Afzan (HTAA) Kuantan, Pahang, Malaysia, and the healthy controls subjects were collected for further tests. Schizophrenic patients fulfilled the Diagnostic and Statistical Manual Five Edition (DSM-V) criteria with at least six months duration of symptoms. All subjects were older than 18 years old, interviewed, and signed informed consents were obtained before the blood taking. The demographic data came from the patients' records in the hospital or healthy control subjects. The samples were collected and managed as prior studies.[18]

Based on the estimation by Kordi et al. (2012)[19], to achieve a 95% power of study at a 95% two-sided confidence level, a minimum sample size of 80 was adequate to analyse *BDNF* DNA methylation. The study collected 118 schizophrenic patients and 122 healthy controls. Excluded cases were those mentally handicapped or suffered from drug-induced schizophrenia. The healthy controls were healthy adults who were age- and sex-matched. The clinical variables of all recruited subjects were evaluated by two trained psychiatrists using the Positive and Negative Syndrome Scale (PANSS subdomains: positive, negative, disorganization, excitement, depressed, and total) based on Kay et al. (1986)[20] and Van der Gaag et al. (2006).[21]

DNA Extraction and Bisulfite Modification

A total of 10 ml of the blood sample was withdrawn from the subjects and further divided into two separate EDTA container tubes; 5 ml for DNA purification and 5 ml for RNA purification. Two millilitres out of 5 ml for the DNA purification was used for the genomic DNA extraction, and 0.5 ml out of the 5 ml for RNA purification was mixed with 1.3 ml RNA*later* solution using RiboPure™-Blood Kit according to the manufacturer's as RNA stabilizer. (ThermoFisher Scientific, U.S) protocols. The RNA stabilized samples, were kept at -80°C until the RNA extraction steps for subsequent gene expression study. DNA extraction from blood samples was done using the GENTRA PUREGENE Blood Kit according to the supplier's (Qiagen, Germany) recommendations. A half microgram of genomic DNA was treated with sodium bisulfite, which converts unmethylated cytosine to uracil, while the methylated cytosine residues remain unmodified, using the EZ DNA Methylation Gold Kit according to the manufacturer's (Zymo Research, U.S) instructions. The universal methylated and universal non-methylated controls, the M. SssI Methyltransferase treated human cells DNA (Zymo Research, U.S.) were also bisulfite-treated. Elution was performed with 30 µl of elution buffer in the final elution step. The final concentration of the eluted bisulfite-treated DNA samples was 10 ng/ul.

Methylight Primer and Probe Sequences

This study was designed to target the methylated site of the *BDNF* gene specifically. The target gene, *BDNF* sequence, was identified through an online browser http://www.ensemble.org; (BDNF: EN SG00000176697) based on the protocol suggested by the following protocol Wojdacz et al. (2007).[22] The Methylight Primer Express v1.0 (Applied Biosystems, U.S) and Methprimer (http://www.urogene.org/methprimer/)[23] were used to design the methylated and unmethylated *BDNF* primer. The reference gene (*ACTB*) primer sequence was based on Eads et al. (2000).[24] The primer and probe sequences are shown in Table I. All primers and probes were bundled together as PrimeTime® qPCR primers (IDTDNA, U.S.) (https://www.idtdna.com/).

MethyLight Protocol

The *BDNF* methylation level quantitation was measured using the Methylight® Taqman probe SYBR green protocol. The serial dilution BDNF and ACTB standard curves were generated using the reaction mixture of the universal methylated and non-methylated DNA for the specificity and sensitivity assay. Seventy nanograms of the bisulfite-treated universal methylated DNA were added to the 140 ng universal non-methylated DNA and serially diluted into 1:3, 1:9, 1:27, 1:81, 1:243, 1:729, and 1:2187. For the polymerase chain reaction (PCR), 2 µl of each bisulfite-treated DNA was added to 1X PrimeTime® qPCR probe and primer mix (IDTDNA, U.S.) and 1X concentration of 10X SensiFAST™ Probe NO-ROX (Bioline, UK) and ran in triplicate. The reaction mixtures were pipetted in a clear 96-well plate and denatured at 95° C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds (BioRad CFX96™ Real-Time PCR (RT-PCR) System). The same PCR reaction mixture protocol was applied to all subjects' samples. The percentage methylation ratio (PMR) values were calculated using the mean Cq values (threshold) of the BDNF and ACTB genes according to this formula [24,25]:

$$\frac{(BDNF\ mean\ value)sample/(ACTB\ mean\ value)sample}{BDNF\ mean\ value\ (universal)/(ACTB\ mean\ value)universal} \times 100\%$$

Table I. Primer and Probe Sequence

Primer	Primer/probe sequence
ACTB	
Forward	5'- TGGTGATGGAGGAGGTTTAGTAAG T -3'
Reverse	5'- AACCAATAAAACCTACTCCTCCCTTAA -3'
Probe	5'-/6-FAM/ACCACCACC/Zen/ CAACACACAATAACAAACACA/3IABkFQ/-3'
BDNF	
Forward	5'- AGTTTGTCGGCGTTGATAAGTAA -3'
Reverse	5'- CCACTACGCAAATCACACCTAA -3'
Probe	5'-/6-FAM/ACTCCGACG/Zen/AAAC TAAATTCGACTCGAAA/3IABkFQ/-3'

Gene Expression

The *BDNF* mRNA gene expression was determined based on the five RNA samples of the highest *BDNF* DNA methylation status and five RNA samples of the lowest *BDNF* DNA methylation status of the schizophrenic patients. The blood in the RNA*later* underwent RNA purification using a Ribopure Blood Kit based on the manufacturer's (ThermoFisher Scientific, Waltham, MA, USA) protocol. The assessment of the RNA integrity of the samples was run on a denaturing agarose gel electrophoresis. Seventy-five nanograms of the RNA underwent reverse transcription into cDNA synthesis using iScript cDNA Synthesis (Bio-Rad). Two microlitres of each QuantiTect® Primer of the target (*BDNF*) and housekeeping genes (*GAPDH* and *ACTB*) were added to the 5 µL of the cDNA, 10 µL SsoFast™ EvaGreen® Supermix (Bio-Rad) and 3 µL of nuclease-free water. The reaction mixture was amplified in triplicate. The genes' standard curves were constructed by performing a serial dilution of 1:3, 1:9, 1:27, 1:81, 1:243, 1:729, 1:2187 of the mixtures. The real-time PCR program was performed using CFX96™ real-time PCR (Bio-Rad) with an initial incubation at 95°C for 30 seconds, followed by 45 cycles of denaturation at 95°C for 5 seconds, annealing at 60°C for 5 seconds and melt curve from 65°C to 95°C for 5 seconds per step. The data acquisition and analysis were performed on the Bio-Rad CFX Manager version 3.0 using the Cq value for the relative quantitation.

Statistical Analysis

The results are presented in mean and standard deviation (SD). The independent sample t-test was used to compare

the PMR between schizophrenic patients and healthy control; and the difference within each gender. A Pearson's correlation test was performed to compare the relationship between the *BDNF* DNA methylation and PANSS score. *P-value* < 0.05 was considered to be statistically significant.

RESULTS

The demography of the subjects is described in Table II. Of the 118 schizophrenic patients, 88 were male, and 30 were female. The healthy controls (n = 122) consisted of 92 males and 30 females. The mean age for the patients and healthy controls was 40 (8) and 38 (8) years.

Table II. Demographic data of patients with schizophrenia and healthy controls

Variables	Schizophrenia (n=118) n (%)	Healthy controls (n=122) n (%)	*p*-value
Gender[a]			
Male	88 (74.58)	92 (75.41)	0.881
Female	30 (25.42)	30 (24.50)	
Age (years)[b]	39.95 ± 8.266^	37.90 ± 8.186^	0.055
Male	39.74 ± 8.243^	38.34 ± 7.947^	0.247
Female	40.57± 8.443^	36.57 ± 8.889^	0.079

[a] Chi-square test
[b] Independent Sample t-test ^
^ Mean ± SD
**p*-value <0.05 is taken as statistically significant at 95% confidence interval

BDNF DNA Methylation in Schizophrenia

There is no significant difference in *BDNF* DNA methylation values between schizophrenics and healthy controls (Table III). However, further analysis for each gender, the *BDNF* DNA methylation was significantly different in male subjects between schizophrenic patients and healthy controls (p=0.016). There was no significant difference in the PMR of *BDNF* in female subjects (Table IV).

Relationship of BDNF DNA Methylation with Psychological Symptoms

There is a significant positive correlation between *BDNF* DNA methylation and the positive subdomain of PANSS as inferred by Pearson's correlation coefficient (r = 0.04, *p* -value=0.03) (Figure 1). The other subdomains of PANSS scores: negative (r=0.00, *p*=0.06), disorganized (r=0.01, *p*= 0.15), excited (r=0.01, *p*=0.21) and depressed (r=0.03, *p*=0.05) subdomains showed no significant correlations.

Table III. Comparison of *BDNF* PMR between schizophrenia patients and healthy controls groups

Variables	PMR Mean (SD)	Mean differences (95% CI)	*p*-value
Schizophrenia (n=118)	1.300 (0.039)	-0.009	0.073
Healthy controls (n=122)	1.293 (0.039)		
Males			
Schizophrenia (n=88)	1.301 (0.039)	-0.014	0.016*
Healthy controls (n=92)	1.287 (0.039)		
Females			
Schizophrenia (n=30)	1.304 (0.043)	-0.006	0.564
Healthy controls (n=30)	1.310 (0.035)		

Independent sample t-test
**p*-value <0.05 is statistically significant in a 95% confidence interval.

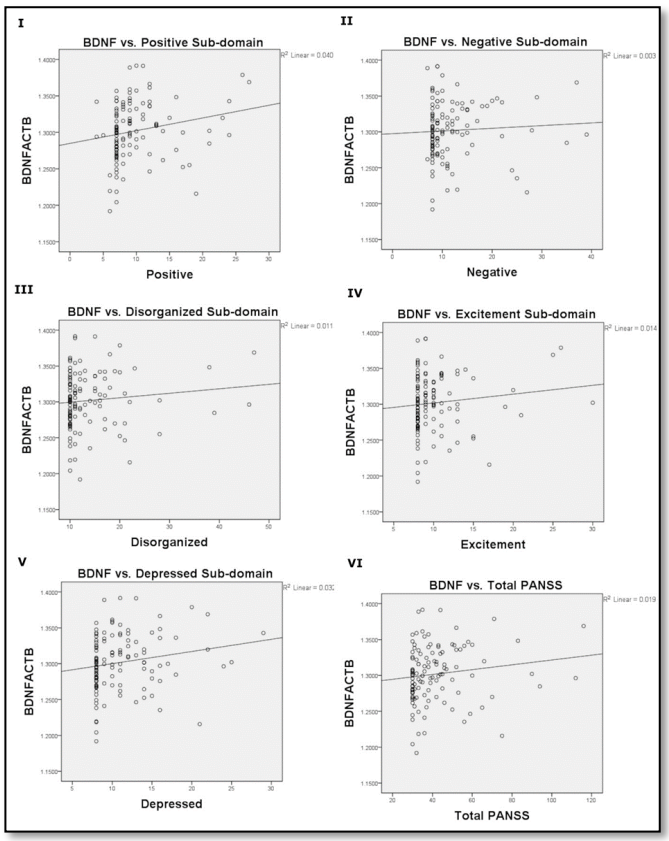

Figure 1. Correlation between PMR values of *BDNF* DNA methylation and PANSS: Positive (I), Negative (II), Disorganization (III), Excited (IV), and Depressed (V) subdomains, and Total PANSS score (VI). The graph shows significantly positive (*p*-value = 0.032) correlations between *BDNF* PMR and positive PANSS score.

BDNF Gene Expression in Schizophrenia

There was no significant *BDNF* gene expression between the high and low methylated groups in schizophrenia. The highest methylated group was less expressed than the lowest methylated group (*p*-value = 0.258) (Figure 2).

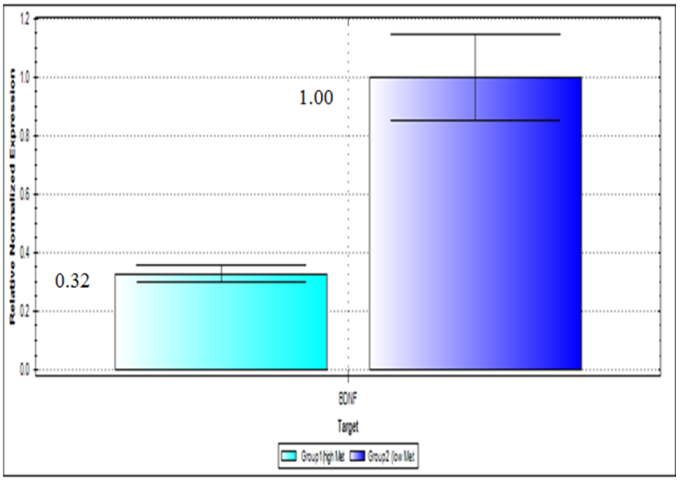

Figure 2. Results of BDNF gene expression. The higher methylated samples were 0.32 less expressed as compared to the lower methylated group (p-value = 0.258).

DISCUSSION

This study reports the Methylight assay's findings to measure DNA methylation analysis of *BDNF* methylation marker and the reference marker ACTB in peripheral blood mononuclear cells. *BDNF* has an essential role in neurodevelopment and the plasticity of the neurons.[26] Its genetic dysregulation may contribute to the pathophysiology of schizophrenia.[27] The analysis of *BDNF* DNA methylation showed some contradicting results. Many researchers found a high correlation of the *BDNF* DNA methylation between the brain and blood.[28,29] Indeed, DNA methylation in the blood mirrors the brain in several psychiatric disorders.[30] Thus, the peripheral blood have been used as a proxy for the brain tissues for epigenetic analysis of the *BDNF* gene because brain tissues are almost inaccessible in living patients. Kundovic et al. (2015) had suggested that the peripheral blood can be used as a predictor of epigenetic changes in the brain in an animal study.[31] The current study was performed to evaluate the *BDNF* DNA methylation in the peripheral blood of schizophrenic patients.

The majority of this study subjects were males (74.6% schizophrenic patients and 75.4% healthy controls). This finding corresponds with the data from the National Mental Health Registry of Malaysia (NMHR) that reported more than 60% of registered schizophrenic patients were males.[32] The age distribution and gender among cases and healthy controls were comparable, thus reduced biases and heterogeneity in the study populations.

There were no differences between the *BDNF* DNA methylation status between schizophrenic patients and healthy controls, similar to the previous study on epigenetic changes of the *BDNF* gene in peripheral blood.[15] This finding contradicts other studies that used brain tissues[33] or peripheral blood.[14,19] Differences in the method analysis of DNA methylation and sample size could contribute to the contradictory findings. The current study used real-time methylight assay, which is more sensitive and applicable for high throughput samples.[34] Nevertheless, a comparison of the *BDNF* DNA methylation in males found higher methylation in schizophrenic patients than healthy controls indicating the possibility of sex-specific DNA methylation susceptibility. Previous studies have shown a correlation of DNA methylation with age and sex,[35] and the methylation was also affected by medication.[36]

BDNF DNA methylation showed a significant positive correlation with the positive symptom of schizophrenia. This correlation suggested that the higher *BDNF* DNA methylation level is related to more substantial positive symptoms, thus supporting the evidence that *BDNF* DNA methylation plays a role in schizophrenia clinical presentation. *BDNF* controls the expression of the dopamine D3 receptor in the mesolimbic dopaminergic pathway[37] thus, may contribute to the positive symptoms of schizophrenia.[38]

Downregulation of the *BDNF* gene expression may alter neurodevelopment[39] and neurotransmission.[40] In the current study, though insignificant, the *BDNF* gene expression in schizophrenic patients showed a down-regulation trend in the *BDNF* highest methylated group as compared to the *BDNF* lowest methylated group. This

data support the previous findings of DNA methylation effects on gene silencing.[41] In conclusion, although there were no differences in the DNA methylation between schizophrenics and healthy controls, there is a sex-specific relationship between male and DNA methylation of *BDNF* in schizophrenia. The study showed significantly higher *BDNF* DNA methylation in male schizophrenic patients than the healthy controls. The positive symptoms of schizophrenia showed a significant correlation with the *BDNF* DNA methylation. Thus, this study showed the epigenetic aberration of *BDNF* is associated with the pathophysiology of schizophrenia.

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