Assessment of Brain-Derived Neurotrophic Gene and its Polymorphism Frequency in Patients With Bipolar Disorder in Hamadan

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Abstract

Background: Bipolar disorder is a biological brain disorder which is associated with debilitating fluctuation in mood and adverse effects on patients, their families and society. The importance of genetics and its role in bipolar disorder is a controversial issue to discuss. Evidence indicates a relation between the risk of bipolar disorder and specific genes. Amongst the genes whose role has been established in bipolar disorder, the most notable gene is BDNF (Brain-derived neurotrophic factor).

Methods: The study is based on a case-control methodology. During 18 months, the blood samples of patients diagnosed with bipolar mood disorder who were admitted to Farshchian hospital of Hamadan from March 2011 to September 2012 and for the control group, the blood samples of patients admitted to other parts of Farshchian hospital except psychiatric ward were taken and DNA extraction from white blood cells was performed. In general, 84 patients and 85 controls were examined in this study and an expert in vials containing EDTA anticoagulant collected 4ml of blood samples. These samples were sent to the molecular biology lab of Hamadan University of Medical Science to determine their genetic polymorphisms. Genomic DNA was extracted from peripheral blood cells using the real extraction DNA kit (DNP Tm kit, Cat# DN8115C, CinnaGen co., Iran). The allele specific polymerase chain reaction technique was used to determine the frequencies of listed genotype. Considering the different variations for each gene, primers design was carried out using the Allele ID software (Allele ID 6, premier Bio soft Int, USA). For this purpose, 401 nucleotide sequences of targeted gene polymorphisms was chosen as the control sequence and desired primers for this sequence was designed and ordered (Takapouzist Co., Iran). Finally, using the mentioned method the sequences were amplified and examined on 2% agarose gel during electrophoresis. The young mania rating scale (YMRS) was used to evaluate manic symptoms. A written consent was obtained from each individual patient during the study. In addition, all patients in this study were anonymous and ethical considerations were taken into account. Statistical data analysis was performed using SPSS Software and Chi-square test was used to analyze their significance.

Results: The results of this study, which was conducted on 84 patients in the case group and 85 patients in the control group indicated that the frequencies of evaluated alleles in the case and control groups for AA genotype were 4 and 4, for GA genotype were 23 and 28, and for GG genotype were 53 and 53, respectively.

Conclusions: According to the obtained data, there is no significant relationship between genetic and bipolar disorder. Some studies in this field have also confirmed this issue.

Keywords: Bipolar Disorder, BDNF, Val66Met, GG, GA and AA Genotype

1. Background

Bipolar mood disorder is a genetic disease, which has a 5 to 10 percent prevalence rate amongst parents, approximately 10% amongst two-egg twins, and more than 50% in single-egg twins. Furthermore, 60 to 80 percent of clinical differences in bipolar disorder is caused by genetic variants (1, 2).

Epidemiological studies suggest that biological and environmental factors play a crucial role in the pathophysiology of bipolar disorder. Among the biological factors, synaptic plasticity and survival of neurons can be noted as the most important factors (3). Discussing the genetic ba-

sis of bipolar disorder has created challenges for a better understanding of this disease (4, 5).

Nowadays, more than ever before, researchers have attempted to crack the etiologic code of mental disorders since the identification of these key genes ultimately allows a completely different approach to the diagnosis, prevention and treatment of mental disorders using gene targeted interventions and drug treatment measures. Moreover, it can be helpful in the etiology and differential diagnosis of this common and disabling psychiatric disorder (6-8).

The long-standing controversy in this field is whether multiple genes and polymorphisms in the etiology and sustainability of such disorder in the family plays a role or not (9). Studies conducted concerning twins and adoption showed the evidences of genetic factors in the etiology of this disorder (9, 10).

Amongst the candidate genes that may be linked with bipolar disorder, the most notable gene is BDNF that has been investigated in numerous studies (11, 12).

The BDNF gene is located on the short arm of chromosome 11 (11P13 substitution of valine acid with methionine acid at codon 66 or rs6265). G allele replacements with A allele at position 196 of exon 2 is a functional polymorphism of BDNF. According to the researches, this is the most remarkable polymorphism amongst all BDNF gene polymorphisms.

Polymorphism refers to a state in which a single nucleotide on a sequence or genome is different between two biological species of a chromosomes pair in humans. Common polymorphisms have two alleles (12).

The BDNF gene is located at chromosome 11p13, and contributes to the neurodevelopment, growth, migration, and survival of neurons and learning process (13).

Thus, it is assumed that the dysfunction of this protein is a risk factor for some neurological and psychiatric disorders.

The BDNF transcript in hippocampus in bipolar disease is reduced. This gene is expressed in hippocampus and neocortex and has different polymorphisms. One of them, which is called Val66Met results in the translocation of 2 amino acids and therefore affects its function in cellular models (14, 15).

Amongst the known polymorphisms of the BDNF gene, the most noticeable one is Val66Met polymorphism (11). In addition, in one study, the relation between 76 candidates genes and BPD was investigated through genotyping 90 SNPs. In a primary SNP analysis on BDNF and alpha subunit of voltage dependent on calcium canal genes, the obtained evidence showed relation with BPD. According to the results of most studies, only BDNF is considered as a potentially high-risk gene (4).

The ultimate goal of this study was to determine the genetic role in bipolar mood disorder based on BDNF analysis and assess the polymorphism frequency of this gene amongst bipolar disorder patients in Hamadan, Western province of Iran. For this purpose, we assessed the frequencies of G196G, G196A and A196A genotypes, determined the frequencies of G196, A196 alleles and specified the amount (frequencies) of the aforementioned genotypes based on gender, age, location and YRMS clinical diagnosis score.

2. Methods

2.1. Patient Selection

In this case-control study, 84 patients were submitted in the case group and 85 patients in the control group. The criteria for the case group included all the hospitalized patients in Farshchian Hospital of Hamadan who suffer from bipolar disorder or single-episode mania during the 18 of March 2011 to September 2012. All patients have given a signed consent to be a part of this study.

For the control group, the blood samples of patients admitted to other parts of Farshchian Hospital except psychiatric ward were used. Written consents were obtained from all patients during the study.

Excluding criteria for the case group were those who did not give consent or were not willing to participate in the study. Those patients who had simultaneous mood disorder or suffered from multiple episode mania were also excluded.

In regards to the control group, those who did not give consent or were not willing to participate in the study or had psychiatric history were excluded.

In both case and control groups, those with any major medical illness affecting brain function, neurological conditions and a history of head injury and substance-related disorders were excluded from the study.

2.2. Sampling Process

4 milliliters of blood was taken from each patient in the case group by an expert sampler and was collected in EDTA containing tubes. Each sample was transferred to the molecular lab of Hamadan University of Medical Science on ice. Then, DNA extraction was performed on 100 μl of each sample and the obtained sample was frozen in -80°C for further steps. 100 μL of blood samples were stored for test repetition cases if necessary.

2.3. YMRS (Young Mania Rating Scale)

YMRS is the most used scale to evaluate manic symptoms. This scale has 11 items, which have been set on the basis of the major symptoms of the disease according to

the patient's subjective history of clinical conditions over the past 48 hours (16). A small part of information is also obtained during the interview by the interviewer. For each treatment and control group patient, a questionnaire containing demographic information including gender, age, urban and rural and the Yang clinical diagnosis score was completed.

2.4. Ethical Considerations

The reason for sampling was explained to patients and patients were authorized to make decisions whether they wanted to participate in the research project or not. Consent forms, which were developed and approved by the medical ethics committee of the University of Medical Sciences were signed by the patients themselves or their parents. Patients' names and personal information during and after the study were kept private.

2.5. Equipment
2.5.1. Materials

Reverse (R) and Forward (F) primer sequences for the amplification of Val66Met DNA polymorphisms are as follows:

P1 (Forward): 5' CCTACAGTTCCACCAGGTGAGAAGAGTG 3'.

P2 (Reverse): 5' TCATGGACATGTTTGCAGCATCTAGGTA 3'.

P3 (G Allele specific): 5'CTGGTCCTCATCCAACAGCTCTTC-TATaAC 3'.

P4 (A Allele specific): 5' ATCATTGGCTGACACTTTCGAAC-cCA 3'.

Considering the different variations for each gene, primers design was carried out using Allele ID software (Allele ID 6, premier bio soft Int, USA). To work with the software, the number of target genes were extracted from the NCBI site. Then, by entering the gene number in this application, the overall profiles, including the name and desired sequence were automatically obtained from the NCBI database. With regards to the physical and chemical parameters such as the primer length, PCR product, melting temperature and also chemical component of primer structure the primer design was performed (Table 1).

Table 1. Primers' Characteristics

Primers	Melting Temperature	Concentration, μmol/L
P1	68	1.5
P2	68	1.25
P3	67	0.75
P4	70	1.25

2.5.2. PCR Mixture

The PCR mixture with a total volume of 25 μL contains 25 ng of genomic DNA and 4 above-mentioned primers. It also included 100 mmole per liter of dNTP, 3 mmole per liter of magnesium sulfate, 10X buffer and Taq DNA polymerase. Other materials used in this process including 50 and 100 bp DNA ladder and agarose gel. DNA Ladder, 6X Loading (cat nom: Ro611), Taq DNA polymerase (cat nom: EPO402) and MgCl$_2$ were purchased from Fermentas Company, Lithuania.

10X buffer, dNTP, and DNPTm DNA extraction kit were purchased from Cinnagen company. Syber safe (cat nom: S33102) was purchased from Invitrogen. 2% agarose was prepared in the laboratory.

2.5.3. DNA Extraction

Cinnagen DNA extraction kit (DNPTm kit, cat no: DN8115C) was used to extract genomic DNA.

2.5.4. Checking the Quality and Quantity of Extracted DNA

Two methods can be used to check the quality and quantity of extracted DNA: spectrophotometer and electrophoresis.

2.5.4.1. Determination of DNA Quantity (Concentration) by Spectrophotometry

DNA concentration was obtained using absorbance at 260 nm and the following relationship:

Concentration of DNA in solution in μg / mg = Dilution factor $\times$ 50 $\times$ absorbance at 260 nm

Equation 1. DNA Concentration

$$C\frac{\mu}{mL} = OD260 \times 50 \times Dilution \quad Factor \qquad (1)$$

OD260 = 1 is equivalent to 50 micrograms per milliliter of double-stranded DNA.

DNA quality can also be investigated using this method; it means that the amount of protein impurities can be measured by absorbance at 280nm and then using the following formula to obtain the DNA purity.

Equation 2. Absorption Ratio

$$Absoption \quad ratio = \frac{Absorbance \quad in \quad 260nm}{Absorbance \quad in \quad 280nm} \qquad (2)$$

The closer this ratio is to 1.8 - 2 the less DNA impurities are with proteins and RNA. Ratios more than 2 indicate that there is contamination with RNA and ratios less than 1.8 are indicative of the presence of protein impurities (Table 2).

Table 2. Purity Determination of Extracted DNA With Spectrophotometry Technique

Nucleic Acid	Protein, %	260/280 Ratio
100	0	2
95	5	1.99
90	10	1.98
70	30	1.64

2.5.4.2. Electrophoresis

Agarose gel electrophoresis is used for separation, identity determination and purification of DNA fragments. This is a simple and fast technique capable of separating fragments of DNA, which cannot be separated by other methods such as concentration gradient centrifugation.

2.5.4.3. Materials Required for Gel Preparation

1, TBE 10X (Tris base, Boric acid, EDTA), Tris Base 108 gr, Boric acid 55 gr, 20 mL of 0.5 M EDTA (pH 8).

In order to prepare TBE, the above-mentioned materials were mixed together and then the volume was brought to one liter. 1X TBE was used to provide agarose gel; 2, Agarose LE.

2.5.4.4. Agarose Gel Preparation

The concentration of gel was determined according to the size of the piece. The amount of required gel and buffer was calculated based on the electrophoresis tank. Agarose gel powder was weighed according to the percentage of gel and was heated in the TBE buffer to create a transparent liquid.

After the solution cooled off a little, a fluorescent dye such as Syber safe and safe stain were used. The amount of the used color is proportional to the gel volume according to the manufacturer's instructions. The comb was placed at one end of the pre-prepared tray and the cooled agar was poured. It was let to sit on a leveled surface for 20 - 30 minutes until it was completely solidified. The comb was gently removed by wiggling and pulling straight upward and the agarose gel was placed into the gel box (electrophoresis unit). The gel box was then filled with 1xTBE until the gel was covered. Samples were carefully loaded into the wells of the gel according to the following proportions:

2 microliter PCR product + 1 microliter 6X loading buffer + 4 microliter deionized water.

These were mixed on a parafilm and loaded into the wells. The DNA ladder (certain molecular weight marker) was used to separate PCR product sizes. After electrophoresis, DNA fragments were visualized using UV trans-illuminator (Figure 1)
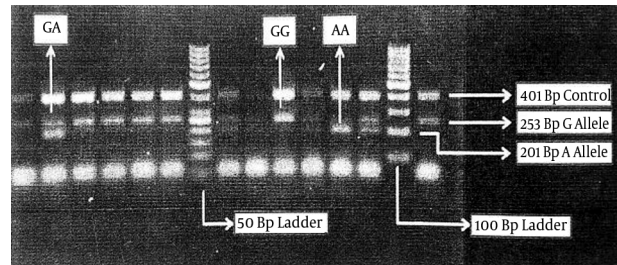
Figure 1. A and G Alleles on Gel Electrophoresis

2.5.5. DNA Amplification Using PCR

Each cycle of PCR contains these steps: Denaturation step, which typically occurs at 93 - 95°C for human genomic DNA; annealing step in which primers bind to desired sequences at 50 - 70°C; synthesis of DNA strand (extension step), which typically is conducted at approximately 70 - 75°C.

However, the main elements of PCR include Taq DNA polymerase, primers, dNTPs and magnesium ions.

In this study, the denaturation phase was performed at 94° C for 5 minutes. Then, 35 cycles of three phases including 45 seconds at 94°C, 60 seconds at 62.5°C and 60 seconds at 72°C were performed. Finally, another extension phase at 72° C for 5 minutes was conducted.

In this study, to amplify the BDNF gene polymorphism located at rs6265 genomics position the allele specific PCR method was used. For this purpose, 401 nucleotide sequences of targeted gene polymorphisms was chosen as the control sequence and the desired primers for this sequence was designed and ordered (Takapouzist Co., Iran).

Finally, using the mentioned method the sequences were amplified and examined on 2% agarose gel during electrophoresis (12).

2.5.6. Allele Specific PCR

Single nucleotide polymorphism genotyping requires a low-cost, accessible and fast technique. Allele specific PCR has overcome this need. This method allows effective separation of single nucleotide polymorphism by a single standard PCR reaction. In a typical PCR, primers are selected from a fixed part of the genome and lead to the amplification of polymorphic part, while in the allele specific PCR the opposite occurs. This means that at least one of the primers is selected from the polymorphism and has a mutation in the 3'.

In order to determine the presence or absence of polymorphisms, one reverse primer and two forward primers are sufficient. However, to determine homozygosity or heterozygosity, at least two reverse and two forward primers

are necessary ([12](#)).

3. Results

The results of this study show that the mean age in the case and control groups are 36.65 and 55.13 years, respectively. Amongst the case group, 61 patients were male (73%) and 23 patients were female (27%). In addition, in the control group, 48 patients were male (56%) and 37 patients were female (44%). A total of 109 male and 60 female patients were examined in this study ([Table 3](#)). Entirely, there is not any significant difference in the patients' gender and its association with bipolar disorder in the studied groups (P = 0.028).

The average scores of YRMS ([Table 4](#)) for the case and control groups were 30.17 and 2.02, respectively. The difference in YRMS scores between the two groups is statistically significant (P < 0.001). In this study, patients were also evaluated in terms of location.

The number of the case patients living in the city was approximately 56 people and rural residents were 28. Similarly, in the control group, 27 and 58 patients live in the city and rural areas, respectively. In other words, amongst the 169 studied patients in the case and control group, 114 patients were urban (67.5%) and 55 patients were rural patients (32.5%). According to the statistical analysis, the difference between the two groups in terms of location was not significant (P = 0.828).

In this study, the frequency of A196A, G196A and G196G genotypes in patients with bipolar disorder in Hamadan in both case and control groups was investigated ([Table 5](#)).

The results showed that the frequency of A196A genotype was 4 patients in the case group (4.7%) and 4 patients in the control group (4.7%). According to the chi-square test, the difference between presence and absence of A196A genotype in the two groups was not significant (P = 0.986).

G196A genotype frequencies in the case group were 24 patients (28.6%) and in the control group were 28 patients (33%). Based on the chi-square test, the difference between presence and absence of G196A genotypes in two groups was not significant (P = 0.431).

In the case group, 58 patients (69%) were reported to have G196G genotype and 26 patients (30%) did not have this genotype. Similarly, in the control group, 58 patients had this genotype (69%) and 32 patients did not have this genotype (38%) ([Table 5](#)). Chi-square test results did not indicate any significant differences between existence and non-existence of G196G genotype in the two groups (P = 0.921).

Another factor, which was examined in this study, was the determination of the frequency of each genotype based on gender. The results for A196A genotype in the case group based on the presence or absence of this genotype showed that 2 males (3%) and 2 females (9%) had this genotype and 59 males (97%) and 21 females (91%) did not have this genotype. On the other hand, in the control group, 3 males (6%) and 1 female (3%) had this genotype and 45 males (94%) and 36 females (97%) did not have this genotype ([Table 3](#)). According to the results of the chi-square test, the relationship in any of the groups was not significant (P = 0.229 and P = 0.444).

The results for G196A genotype in the case group based on the presence or absence of this genotype indicated that 19 males (31%) and 4 females (17%) had this genotype and 42 males (69%) and 19 females (83%) did not have this genotype. On the other hand, in the control group, 14 males (29%) and 14 females (38%) had this genotype and 34 males (71%) and 23 females (62%) did not have this genotype. According to the results of the chi-square test, the relationship in any of the groups was not significant (P = 0.210 and P = 0.399).

The results for G196G genotype in the case group based on the presence or absence of this genotype indicated that 38 males (62%) and 15 females (65%) had this genotype and 23 males (38%) and 8 females (35%) did not have this genotype. On the other hand, in the control group, 31 males (64.5%) and 22 females (59.5%) had this genotype and 17 males (35.5%) and 15 females (40.5%) did not have this genotype. According to the results of the chi-square test, the relationship in any of the groups was not significant (P = 0.805 and P = 0.629).

4. Discussion

In one study, the role of BDNF in the pathophysiology of BPD due to the decrease in BDNF serum levels in patients with this disorder has been confirmed ([17](#)). In two other studies, it was reported that BDNF levels are reduced in a manic phase ([10](#), [17](#)). Although there are theories indicating that this manic phase is due to the excessive activation of BDNF in the brain ([18](#)), the level of serum BDNF in patients with depression and bipolar disorder has shown a significant reduction compared to the control group ([10](#), [19](#)). Post-mortem brain autopsy in patients with bipolar disorder has shown a significant reduction in BDNF protein compared to control ([19](#)).

The use of anti-depression drugs, electroconvulsive therapy (ECT) and mood stabilizers such as lithium increase the transcription of the BDNF gene ([10](#), [19](#)).

Numerous studies conducted in North America and Europe have reported a significant relationship between val66met and BPD ([10](#)). Val66met polymorphism seems to change the volume of the hippocampus and cause disturbance of the hypothalamic-pituitary-adrenal axis. It is also

Table 3. Frequency of Genotype in two Groups Based on Sex

Group	Genotype	Sex		Total
		Female	Male	
Case	A/A			
	Yes	2	2	4
	No	21	59	80
	Total	23	61	84
Control	A/A			
	Yes	1	3	4
	No	36	45	81
	Total	37	48	85
Case	G/A			
	Yes	4	20	24
	No	19	41	60
	Total	23	61	84
Control	G/A			
	Yes	14	14	28
	No	23	34	57
	Total	34	48	85
Case	G/G			
	Yes	16	41	57
	No	7	20	27
	Total	23	61	84
Control	G/G			
	Yes	22	31	53
	No	15	17	32
	Total	37	48	85

Table 4. Mean of YMRS Score in Two Groups

Group			Mean	SD
YMRS	Case	84	30.17	4.279
	Control	85	2.02	3.761

associated with major depressive disorder and BPD. Egan in 2003 showed that hippocampal function and quality of memory are affected by this functional polymorphism (14). Since then several other researchers have investigated the relationship between this gene and both BPD disorder and schizophrenia. Enormous animal and pharmacological studies were even carried out to show a clear association between Val66Met with increased risk of psychotic diseases, but reports did not suggest a significant association between this polymorphism and any of the psychotic dis-

order (18, 20, 21).

Although these inconsistent results may be due to the causal heterogeneity, random errors, and methodological differences, the different subtypes of the disease is due to small sample size. However, this polymorphism has been investigated in several studies and the results are misleading (11). For example, two other studies showed that there is a significant correlation between the "val" allele of this polymorphism in the BDNF gene with BPD (19). In another study, a significant correlation between the Met allele of

Table 5. Frequency of Genotype in Two Groups

Genotype	Groups		Total
	Control	Case	
A/A			
Yes	4	4	8
No	81	80	161
G/A			
Yes	28	24	52
No	57	60	117
G/G			
Yes	53	58	111
No	32	26	58

this polymorphism and BPD was reported. In one study, it was shown that the distribution of met/met homozygous genotype in the patient group is more than the control group (10). In another study, it was reported that there was not a significant relationship between certain allele or genotype with BPD, but it was found that BDNF causes rapid cycling in this disorder (22).

In a meta-analysis study, there is strong evidence showing that the BDNF is associated with the etiology of BPD, but there was no significant difference in val66Met polymorphism amongst treated patients and control group (9).

Many methods can be used to identify the SNPs such as PCR restriction fragment length polymorphism (PCR-RFLP), high resolution melting (HRM), pyrosequencing and probe hybridization based techniques. PCR-RFLP has some disadvantages such as the requirement of an incubation period for enzymatic digestion by restriction endonuclease to apart the restriction fragments (23). The Allele Specific PCR method used in this study only requires basic equipment such as a conventional thermal cycler and a gel documentation system, which are available in most genetic laboratories.

4.1. Conclusion

Although the current study did not find any significant association between genetic role and bipolar disorder, based on the conflicting results obtained in various studies, it can be noted that considering bipolar disorder is a debilitating disorder with significant prevalence in society, it is better to examine genes involved in this disorder; and in the case of any connection, address tracking, treating and even preventing this disorder.

Since numerous studies were carried out in this field the results are contradictory. Due to the fact that there were no similar studies in the Middle East, it was necessary to conduct this.

The main limitation of this study was the low number of patients, which was due to the inaccessibility of more patients in the medical centers of Hamadan. Neurotrophins such as BDNF genes and molecules, and their role and importance in psychiatric disorders, such as bipolar disorder and schizophrenia and neurological disorders such as epilepsy, Alzheimer's and Axonal damage are the basis of future studies. Future work within the context of this study can be replicated in the framework of a similar study with a larger sample size in order to be able to discuss the results with more certainty.

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