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Original Article

Psychiatry/Mental Health Section

Study of NF-κB Gene Promoter Region {rs28362691 (-94 ins/del ATTG)} Polymorphism in Patients with Bipolar Disorder: A Case-control Study

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ABSTRACT

Introduction: Bipolar Disorder (BD), a multifactorial disorder, is associated with increased proinflammatory cytokines due to chronic low-grade inflammation. It affects a relatively younger population. A transcription factor, Nuclear Factor Kappa-B (NF-κB) has an essential role in regulating the cascade of inflammatory responses to psychological stress that occurs in BD. Polymorphism of its promoter region has been a matter of discussion in recent years in schizophrenia but rarely any study has been done in BD.

Aim: To evaluate the NF-κB promoter gene polymorphism in healthy subjects and in both types of BD patients, those with mania, and those with depression.

Materials and Methods: The current case-control study was conducted at the Department of Biochemistry, SCB Medical College, Cuttack, Orissa, India from March 2020 to February 2022. Individuals aged 18-30 years with BD were included in the study group and age-matched healthy individuals in the control group. A total of 100 cases of newly diagnosed BD (61 BD patients with depressive phase and 39 BD patients with manic phase) and 100 controls were included in the study. Demographic variables such as age and Body Mass Index (BMI) were recorded. A sample of blood was collected from all the participants for genotyping of BD patients. DNA was extracted from Ethylene Diamine Tetraacetic Acid (EDTA) blood and NF-κB gene polymorphism was seen by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method in gel electrophoresis. The quantitative statistical analysis was conducted using SPSS. A p-value of ≤0.05 was considered statistically significant.

Results: The common genotype found was del/del in patient groups. Among patients of depression, 32 out of 61 (52.46%) had del/del allele. Similarly in mania patients, 23 out of 39 (58.97%) had del/del allele. The odds of individuals developing BD with depression were the highest in the del/del {9.93 (4.36-22.4)} group. The odds of developing BD with mania were higher in the del/del group {12.94 (5.19-32.24)} than that in the ins/ins group {0.09 (0.04-0.23)}. The del/del type of genotype is higher in mild, moderate, and severe forms of depression as compared to ins/ins and ins/del.

Conclusion: The presence of deletion allele in both phases of BD could throw some light on its pathophysiology in neuroinflammation. The findings of the study could lend scope to early genetic screening based on factors including family history to predict the course of BD based on the polymorphisms. This will help strategise the management and genetic counselling approach.

Keywords: Alleles, Inflammation, Mutation, Nuclear factor kappa B1

INTRODUCTION

The BD is a chronic, complex disorder displaying episodes of mania, hypomania, or depression in symptomatic phases in the affected individuals leading to functional disability and deteriorated quality of life [1]. They are usually associated with a manic phase characterised by irritable, elated, and energised behaviour, and the depressive phase characterised by indifferent, sad, and hopeless periods [2].

In India, it is estimated that more than 7.6 million people suffer from BD [3], with the mean age of onset being early twenties and earlier onset have observed worse outcomes including high recurrence and resistance to treatment [3-6]. Three different conditions namely, bipolar I, bipolar II, and cyclothymic disorder are included in BD [7]. Scientific research has observed a common genetic risk between BD (especially bipolar I disorder), schizophrenia, and autism spectrum disorders [8,9]. Several studies exploring the underlying mechanism of BD development in traumatised children identified reduced limbic grey matter volume, elevations in the level of certain cytokines such as brain-derived neurotrophic factor, and Hypothalamic–Pituitary–Adrenal (HPA) axis alterations [10-12]. Multiple genetic loci associated with BD have been identified from genome-wide association studies and linkage studies have identified susceptible genes for BD on chromosomes 4,12,16,18 and in X-chromosome, suggesting polygenic risk [13,14].

At the molecular level, a cross-talk between BD and inflammation is observed due to the overlapping genetic polymorphisms, there seems to be a cross-talk between BD and inflammation process [15]. The aberrant NF-κB pathway is an integral component of dysregulation in the inflammatory cascade of psychiatric disorders [16]. Elevated NF-κB activity has also been observed among adult BD patients during depressed or euthymic states as compared to the healthy volunteers [17,18]. Further, phosphorylated p65 NF-κB protein levels were elevated by 7.2-fold among euthymic bipolar I patients compared to healthy volunteers [19]. The chromosome 4q24 contains the human NFκB1 gene which encodes for a 50 kDa DNA-binding protein (p50) [20]. NFκB1 gene shows few polymorphic variants in the promoter region, with rs28362691 (-94 ins/del ATTG) of NF-κB being a common variant associated with schizophrenia [21]. The -94 loci of the NF-kB gene promoter region have been observed to house four-base pair ATTG ins/del variation from previous studies. This encodes three genotypes: II genotypes (wild-type homozygous insertion i.e., ins/ins), DD genotype (variant homozygous deletion i.e., del/del),

and ID genotype (heterozygous i.e., ins/del). Despite their key role in the regulation of inflammatory cascade of BD, studies dedicated to exploring the upstream processes of signalling mechanisms of inflammation such as NF-κB are scant and studies on NF-κB gene polymorphism in BD patients have not been recorded in this part of the country [22,23].

The current study aims to find the NF-κB gene polymorphism types in BD patients and controls.

The objective of the study is to determine associations between different biochemical parameters in cases and controls and study specific polymorphism types in the manic and depressive phases of bipolar disease.

The null hypothesis is that there is no difference in the NF-κB gene polymorphisms associated with different stages of BD. The research sought to explore the frequency of different NF-κB gene polymorphisms in individuals with BD as compared to individuals with normal intelligence.

MATERIALS AND METHODS

The current case-control study was conducted among BD patients from the outpatient and in-patient departments of Department of Biochemistry, SCB Medical College, Cuttack, Orissa, India from March 2020 to February 2022. The study was approved by the institutional ethical committee (ECR/Inst/OR/2013/RR-20) according to the standards of the Declaration of Helsinki. The control group included age-matched healthy individuals aged 18-30 years of both sexes (not gender matched), individuals of normal intelligence (determined by the Wecshler Intelligence scale) [24], those who were physically stable with no disease, those with no history of head injury, those without any neurological disease, and those with no history of mental disorders in the family.

The exclusion criteria included patients with a history of substance abuse (cannabis, cocaine), drug dependence or alcohol intake; those with neurodevelopmental disorders; those with bulimia nervosa, anorexia nervosa, space-occupying lesion, or diabetes mellitus; pregnant patient, or those with any other psychiatry disorders other than BD.

The case group included patients of either sex between 18-30 years of age, diagnosed as BD, according to the International Classification of Diseases-Tenth Revision (ICD-10) criteria [25]. The case group followed the same exclusion criteria as the controls.

Based on a reference study [26], sample size was estimated to be around 200 participants equally distributed in both groups and the participants were recruited by continuous sampling.

The severity of the bipolar disease was assessed by the Hamilton Depression Score (HAM-D) [27] for symptoms of depression and similarly, Young Mania Rating Scale (YMRS) was used for the evaluation of mania severity [28]. After obtaining the written consent of the enrolled study participants, 5 mL of venous blood from the individuals and sputum samples from cases were collected. All the routine biochemical parameters such as Fasting Blood Sugar (FBS) were done in MISPA Clinia Autoanalyser and serum Liver Function Tests (LFT) like total bilirubin (mg/dL), direct bilirubin (mg/dL), Serum Glutamic-Oxaloacetic Transaminase (SGOT) or aspartate transaminase (mg/dL), Serum Glutamic Pyruvic Transaminase (SGPT) or alanine transaminase (mg/dL), serum lipid profile estimation like Low Density Lipoprotein (LDL), Very Low Density Lipoprotein (VLDL), High Density Lipoprotein (HDL), total cholesterol, and serum Triglycerides (TGs) were done in TOSHIBA Automated Biochemical Analyser Model TBA-120FR. Anthropometric measurements such as height, weight, and BMI were recorded are important in evaluating the degree of obesity [29].

For the study of NF-κB1 genotyping in BD patients, EDTA whole blood was collected, and extraction and purification of nucleic acids was done by spin column-based manual extraction kit. QIAGEN DNA Mini extraction kit was used for DNA extraction as per the manufacturer's instructions. DNA quality/quantity was checked using a ThermoFisher Scientific Nanodrop spectrophotometer. PCR was run for extracted DNA (GeNet Bio commercial kit). PCR run was conducted in an Applied Biosystems thermocycler. The primers for PCR amplification used were 5′-TGGGCACAAGTCGTTTATGA-3′ (forward) and 5′-CTGGAGCCGGTAGGGAAG-3′ (reverse). Electrophoresis of post-PCR products was run on an agarose gel stained with 2% ethidium bromide for 30 minutes at 90 V and visualised using the Bio-Rad Gel Documentation system. RFLP was employed to study the polymorphism, rs28362691 (-94 ATTG ins/del) associated with the promoter site of the NF-κB gene. For detection of the -94ins/del ATTG polymorphism in NF-κB1, a PCR product of 281 base pair deletion allele, 285 base pair insertion allele were digested with restriction enzyme PflMI (10 U/μL, BioLabs), where the recognition site resides. From gel electrophoresis analysis, different types of NF-κB genotypes were studied.

Two ATTG repeats are present in the NF-κB promoter region. Among them, one allele has an ATTG insertion. A unique recognition site at the ins allele is identified as 5′…. CCANNNNNTGG…. 3′. The insertion allele of 285 bp is cleaved into two fragments of 200 bp and 45 bp after restriction digestion. In the case of the deletion allele of 281 bp, only one ATTG remains at its promoter region and is hence not cleaved. Ideally, the heterozygotes allele (ins/del) must show three fragments, a 281 bp fragment, 240 bp, and 45 bp fragments (cleaved from 285 bp). Since the 45 bp is an exceedingly small fragment only two fragments were visualised. The primary outcome of the study is to perform genetic tests to identify genetic polymorphisms in BD patients and to evaluate the distribution patterns of polymorphism in disease severity in the manic and depressive phases of bipolar disease. Genetic polymorphism was ascertained by PCR and RFLP study of the promoter site of the NF-κB gene i.e., rs28362691 (-94 ATTG ins/del).

STATISTICAL ANALYSIS

The quantitative statistical analysis was conducted using SPSS (IBM Inc, Chicago, Illinois). Continuous variables were expressed as mean±standard deviation and continuous variables were expressed as percentages. One-way Analysis of Variance (ANOVA) with post-hoc Tukey test was applied to test for differences in the continuous variables. Odds ratio was calculated, with 95% Confidence Interval (CI). A p-value of ≤0.05 was considered statistically significant.

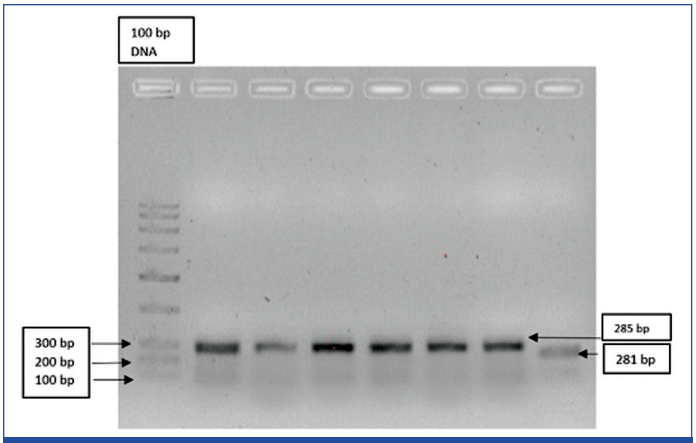
RESULTS

During the study period, we analysed 100 newly diagnosed cases of BD. The maximum number of cases belonged to the depressive phase group (61%; n=100) in comparison to the manic phase group (39%; n=100). [Table/Fig-1] shows the comparison of demographic variables including age, BMI, and biochemical parameters like FBS, liver and lipid profile among cases and controls. No significant differences in the age between the groups were noted as shown in [Table/Fig-1] BMI was significantly higher among BD patients with depression as compared to the control group (p=0.013*). A significant difference in serum total cholesterol and serum TG was observed among the three groups (BD depressive phase; BD manic phase and control). Serum total cholesterol (p=0.004*) and serum TG (p=0.003*). [Table/Fig-1] are significantly higher in individuals in the depressive phase and healthy individuals than in individuals in the manic phase.

[Table/Fig-2] shows the agarose gel electrophoresis image of the post-PCR products as DNA bands before restriction enzymes were used. Two alleles were identified i.e., ins allele of 285 bp bands in lanes 2 to 7 and in lane 8, the deletion allele of 281 bp band. LANE 1: 100 bp ladder, lane 2-7: 285 bp bands (insertion allele), LANE 8: 281 bp band (deletion allele).

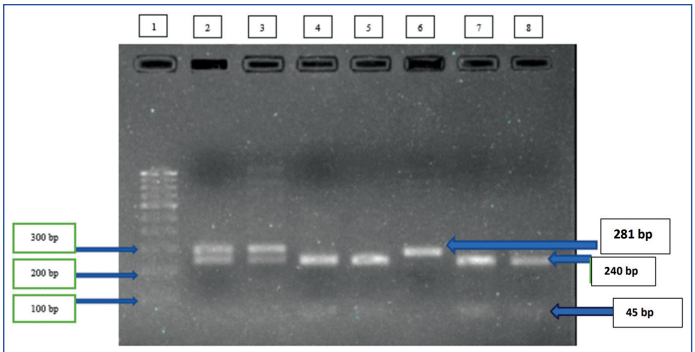
	Controls (n=100)	BD with depression (n=61)	BD with mania (n=39)	p-value
Age (years)	26.29±3.06	26.25±3.45	25.10±3.29	0.130
BMI (kg/m^2)	23.73±2.97	25.70±2.04	24.53±4.41	0.013*
FBS (mg/dL)	102.40±9.95	102.26±10.52	102.16±9.15	0.132
Total bilirubin (mg/dL)	0.66±0.27	0.64±0.37	0.65±0.14	0.082
Direct bilirubin (mg/dL)	0.25±0.13	0.24±0.16	0.24±0.32	0.131
SGOT (U/L)	21.14±5.9	22.17±4.9	22.11±4.2	0.136
SGPT (U/L)	19.91±4.54	20.52±4.7	20.12±4.6	0.156
Alkaline phosphatase (U/L)	59±47.03	60±45.12	159±46.16	0.329
Total cholesterol (mg/dL)	171.55±17.28	173.54±35.47	166.12±16.19	0.004*
TG (mg/dL)	121.25±15.09	123.49±16.19	114.35±27.13	0.003*
HDL (mg/dL)	58.02±11.65	61.0±31.74	59.70±28.85	0.112
LDL (mg/dL)	88.98±7.35	89.96±27.24	90.76±22.62	0.317
VLDL (mg/dL)	25.49±4.18	26.13±12.39	26.30±8.99	0.851

[Table/Fig-1]: Comparison of age, BMI, FBS, LFT and lipid profile among cases and controls.
BMI: Body mass index; FBS: Fasting blood sugar; SGOT: Serum glutamic-oxaloacetic transaminase; SGPT: Serum glutamic pyruvic transaminase; TG: Triglycerides; HDL: High density lipoprotein; LDL: Low density lipoprotein; VLDL: Very low density lipoprotein. One-way ANOVA was the statistics test employed. p-value <0.05 was considered to be statistically significant



[Table/Fig-2]: The agarose gel electrophoresis image of the post PCR products as DNA bands of NF-κB1 gene.

PflMI enzyme digestion band profile of the NF-κB1 gene on 2% agarose gel observed bands identifying polymorphisms. Lane 2, 3 shows ins/del polymorphism (ideally 3 fragments of 281 bp, 240 bp, and 45 bp should be seen). The 45 bp exceedingly small could not be visualised in gel doc. Lanes 4, 5, 7, and 8 show ins/ins polymorphism, and lane 6 shows del/del polymorphism. A 100 bp ladder was used in lane 1 [Table/Fig-3].



[Table/Fig-3]: PflMI (BioLabs) restriction digestion band profile of the NF-κB1 gene on 2% agarose gel. Lane 1 (Gene direx): 100 bp ladder, Lane 2, 3: ins/del polymorphism (ideally 3 fragments of 281 bp, 240 bp and 45 bp should be seen). Lane 4, 5, 7, 8: ins/ins polymorphism, Lane 6: del/del polymorphism. The 45 bp fragment is seen very faintly.

The odds of individuals developing BD with depression were higher in the del/del {9.93 (4.36-22.4)} and ins/del {7.40 (1.52-36.09)} alleles than in the ins/ins allele {0.07 (0.03-0.17)}. The odds of developing BD with mania was higher in the del/del group {12.94 (5.19-32.24)} than that in the ins/ins group {0.09 (0.04-0.23)}.

[Table/Fig-4] presents the summary of the different alleles present and various genotype frequencies of NF-κB1 gene polymorphism in case and control groups of the study population. The del/del genotype is present in equal frequency across all stages of BD. The ins/del is present only in the BD with depression group of the study population.

Genotype	Controls (n=100) n (%)	BD with depression (n=61) n (%)	Odds ratio (95% CI) compared to control	BD with mania (n=39) n (%)	Odds ratio (95% CI) compared to control
ins/ins	88 (88%)	21 (34.43%)	0.07 (0.03-0.17)	16 (41.03%)	0.09 (0.04-0.23)
del/del	10 (10%)	32 (52.46%)	9.93 (4.36-22.64)	23 (58.97%)	12.94 (5.19-32.24)
ins/del	2 (2%)	8 (13.11%)	7.40 (1.52-36.09)	0	-

[Table/Fig-4]: Distribution of the genotype frequencies of NF-κB1 gene polymorphism in controls and cases group.

[Table/Fig-5] shows NF-κB1 polymorphism with a degree of severity (HAM-D SCALE) in bipolar patients with a depressive phase. The del/del type of genotype is higher in mild, moderate, and severe forms of depression as compared to the ins/ins and ins/del genotypes.

	Allele distribution	
Severity	del/del (n=23), n (%)	ins/ins (n=16), n (%)
Mild	6 (26.1)	3 (18.75)
Moderate	14 (60.9)	10 (62.5)
Severe	3 (13.0)	3 (18.75)

[Table/Fig-5]: Distribution of NFκB1 polymorphism with degree of severity (YMRS) in bipolar patients with mania.

NF-κB1 polymorphism with respect to the del/del allele was seen in 14 (43.75%) individuals with moderate and severe disease and the ins/ins allele was seen in 12 (57.1%) individuals with moderate disease condition [Table/Fig-6].

Alleles	Mild disease	Moderate disease	Severe disease
del/del (n=32), n (%)	4 (12.5)	14 (43.75)	14 (43.75)
ins/del (n=8), n (%)	1 (12.5)	4 (50)	3 (37.5)
ins/ins (n=21), n (%)	1 (4.8)	12 (57.1)	8 (38.1)

[Table/Fig-6]: Distribution of NF-κB1 polymorphism with degree of severity (HAM-D SCALE) in bipolar patients with depressive phase.

DISCUSSION

In this study, 100 newly diagnosed cases of BD were analysed. BD is a debilitating lifelong neuropsychiatric disorder with unstable episodes of moods that extend between the extremes of mania and depression. The study explored to understand the underlying differences in the gene polymorphisms that could be associated with severity or different stages of the disease. In our study population, there were higher cases of BD patients with depressive phases (61%) as compared to mania (39%) Previous research by Tondo L et al., also observed higher prevalence of depressive phases of BD than manic or hypomanic phases of illness [30]. Consistent with previous studies, the current study also observed the age of onset to be between 20 and 30 years [5]. BD patients with depressive phase were observed to have higher BMI levels when compared to healthy volunteers and those with manic phase (p=0.013). A research observed higher levels of obesity among BD patients with depressive phase. As per the present study, lipid profile analysis also indicated higher levels of total cholesterol (p=0.004) and TG (p=0.003) among bipolar depressive patients than manic patients. A Scientific Statement from the American Heart Association published an article where BD and major depressive disorder have also

indicated higher predisposition of the affected youths to the rapid progression of early cardiovascular disease and atherosclerosis [31]. The increase in obesity and young age of onset could lead to a significant burden among BD patients. Despite the high prevalence of metabolic syndrome along with glucose intolerance among newly diagnosed with BD patients, the current study observed no significant differences in the plasma glucose levels between the study groups. No significant difference were observed between cases and controls for Total Bilirubin (TBIL), Direct Bilirubin (DBIL), SGOT, and Alkaline Phosphatase (ALKP) (p=0.152, 0.155, 0.182, 0.156, and 0.172, respectively). This could be attributed to the inclusion of newly diagnosed cases in the study group, having no history of intake of antipsychotic drugs.

Elevation of proinflammatory cytokine levels in BD patients have been well-established [32]. BD has been strongly associated with immune dysfunction. Further, high rates of inflammatory medical comorbidities among BD patients have been evidenced from epidemiological studies, including chronic infections, autoimmune disorders, metabolic disorders, and cardiovascular diseases. Associations between BD and chronic low-grade inflammation have been evidenced from cytokine studies, which increase pro-inflammatory cytokines [33]. A study was conducted to understand the role of increased NF-kB activity in the brain. The study compared mRNA and protein levels of neuroinflammatory and excitotoxicity markers in the postmortem frontal cortex of BD patients [34]. The NF-κB1 is one such gene with both proinflammatory and anti-inflammatory properties. The gene encoding for non-DNA binding 105 kD protein (p105) of NF-κB can undergo co-translational processing, which produces a 50-kD DNA-binding protein (p50) [35]. Alterations in NF-κB1 transcription can lead to a variation in the synthesis of p50 production. Given the role of NF-κB1 in inflammatory processes, polymorphisms in the promoter region of NF-κB1 could play key roles in modulating neuroinflammation in patients with BD.

The current study observed that in the control group comprising 100 healthy volunteers, 88 persons (88%) had insertion (ins/ins) allele whereas 10 had deletion (del/del) allele and only two had insertion/deletion (ins/del) allele. But the most common genotype found was del/del in the patient group. Among patients of depression, 32 out of 61 (52.46%) had del/del allele. Similarly, in mania patients, 23 out of 39 (58.97%) had del/del allele. This could be potentially indicative that presence of deletion allele in our candidate gene resulting from polymorphism in the promoter region of NF-κB1 could be contributing to the predisposition to symptoms of BD. The NF-κB signalling pathway is not studied much in BD patients, but its abnormal activity has been widely explored in related mental disorder, like schizophrenia [21].

To confirm the presence of polymorphism, this study used PCR-RFLP method after restriction digestion of the required DNA fragment of interest. [Table/Fig-2] shows the agarose gel electrophoresis image of the post-PCR products as DNA bands before restriction enzymes were used. When the gene contains an insertion allele, bands of 285 bp were obtained. Deletion allele when present should be 4 bp less than insertion allele i.e., of 281 bp band because-94 ins/del ATTG polymorphism has 4 bp change. When PflMI enzyme digestion performed band profile of the NF-κB1 gene on 2% agarose gel, we observed different DNA bands of NF-κB1 gene identifying polymorphisms [Table/Fig-3].

Further analysis conducted to understand the association between polymorphisms in NF-κB1 and severity of BD. Tuncay S et al., studied identification of NF-κB1and NF-κB1-alpha polymorphisms using PCR-RFLP assay in a Turkish population [36]. It is speculated that the -94 ins ATTG allele influences increased expression of NF-κB1 and in turn promoting inflammation. According to the YMRS scale in the present study, the del/del type of genotype is higher in mild, moderate, and severe forms of depression as compared to the ins/ins and ins/del genotypes. Distribution of NFκB1 polymorphism with degree of severity (HAM-D SCALE) in bipolar patients with depressive phase also found del/del genotype in moderate and severe cases of depression.

The chronic nature of BD and schizophrenia, another common neuropsychiatric disorder, has some studies to show possible inter-relationship between the immune and central nervous systems. Both diseases have some genetic risks, such as overlapping susceptibility loci in immune-related genes with markedly increased transcript levels of Interferon-Induced Transmembrane Proteins (IFITMs) and other immune markers [37]. As there are no studies in the literature till date, the rationale to conduct this novel study is to find out the association of NF-κB1gene polymorphism with BD needs further study and evaluation.

The results of the current study indicate that the presence of deletion allele in our candidate gene resulting from polymorphism in the promoter region of NFKB1 could be contributing to the predisposition to symptoms of BD. Future research on these aspects can help decipher the molecular underpinnings of BD and further large-scale studies can be conducted to elucidate on the importance of polymorphism patterns of NF-κB1 gene in BD. Genetic screening would be helpful in early identification of the susceptible population. The findings of the study could lend scope to early genetic screening based on factors including family history to predict the course of BD based on the polymorphisms. This will help to strategise the management and genetic counselling approach.

Limitation(s)

The sample size of the study was small. The genetic polymorphism was studied with the genomic DNA extracted from whole blood, but a better association could be made from studying the gene polymorphisms from brain tissues such as the cortex. Some of the confounding variables such as family history, detailed clinical presentations and allele-specific analysis have not been included in the study.

CONCLUSION(S)

In the current study, genotypes including del/del were more common among BD patients as opposed to the ins/ins genotype which is more in the control population. Among BD patients with mania, del/del genotype was more common among those with mild and moderate severity, while it was the most common among all severities among those with BD patients with depressive phases. Since an accurate diagnosis of BD in young people is challenging, genotyping may be suggested as a surrogate approach to predict disease severity.

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