Here we report the association of the rs694539 variant of nicotinamide-N-methyltransferase gene with bipolar disorder in a case–control study of 95 bipolar disorder patients and 201 healthy controls ($\chi^2 = 13.382, P = 0.001$). With the polymerase chain reaction restriction fragment length polymorphism method we developed we were able to show the association for the first time. This new finding may provide evidence to understand the mechanism of the disease.

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1. Introduction

Bipolar disorder is a complex disorder involving an interaction of multiple susceptibility genes with environment. Environmental factors may be mediated through epigenetic mechanisms involving DNA methylation. These epigenetic factors may modulate gene expression by interfering with DNA structure and binding of transcription factors (Comb and Goodman, 1990). DNA methylation is coupled with one-carbon metabolism involving inter-conversion between homocysteine and methionine (Friso and Choi, 2002).

One of the factors contributing neurocognitive deficiency in bipolar disorders may be hyperhomocysteinemia (Dettmann et al., 2007, 2008). Greater functional deterioration was observed in euthymic bipolar patients with hyperhomocysteinemia (Dettmann et al., 2008; Osher et al., 2004). Another study reported higher levels of homocysteine in euthymic bipolar patients associated with cognitive impairment (Dettmann et al., 2007, 2008). More recently, worse performances on task of executive function (WCST) were found to be associated with elevated homocysteine levels in patients with bipolar disorders (Osher et al., 2008). In light of these observations, one can put forward the hypothesis that elevated levels of homocysteine may play a role in the pathophysiology of cognitive deficits and executive dysfunction in bipolar disorders. Neurocognitive improvement was obtained in patients with schizophrenia after the reduction of homocysteine levels (Levine et al., 2005). Homocysteine levels were associated with white matter lesions and silent brain infarcts (Dufouil et al., 2003). In addition, oxidized forms of homocysteine, homocysteic acid, are potent neurotoxic agents leading to apoptosis and leukoaraiosis (Prins et al., 2002; Sachdev et al., 2002). Homocysteine also elicits a DNA damage response in neurons leading to apoptosis and hypersensitivity to excitotoxicity (Krum et al., 2000).

To examine whether the rs694539 variant of NNMT gene was associated with bipolar disorders, we analyzed the allele and genotype frequencies of NNMT gene rs694539 variant in 95 bipolar disorder patients and 201 healthy controls.

2. Materials and methods

2.1. Genotyping

Genomic DNA was isolated from subjects with a conventional salting-out method (Miller et al., 1988). Genotypes of the subjects were determined by the PCR-RFLP method developed in our laboratory (Fig. 1). The PCR cycling conditions for rs694539 variant were as follows: briefly genomic DNA was denatured at 95 °C for 5 min followed by 35 cycles at 95 °C for 1 min, 55 °C for 30 s, 72 °C for 1 min and a final extension step of 72 °C for 10 min. The digestion of the amplified 187 bp fragment with the NlaIII restriction endonuclease was carried out at 37 °C overnight. Subsequently the digested fragment was run on an 8% PAGE for 30 min at 20 W, followed by silver staining and scanning (Fig. 1) (Sazci et al., 2013).
2.2. Subjects

All subjects were recruited from the University of Kocaeli Hospital, Psychiatry clinic and diagnosis of the bipolar patients was based on DMS-IV criteria. The subjects were 95 bipolar disorder patients and 201 healthy controls. The mean age of the patients was 38.35 ± 11.690 years, with an age range of 21–75 years. All patients were diagnosed by an experienced psychiatrist using the DSM-IV criteria. Informed consent was obtained from all subjects and the institutional review board approved the study. The control group was also from the city of Kocaeli, Turkey with an average age of 36.42 ± 11.514, ranging from 17 to 72 years of age.

2.3. Statistical analysis

The Hardy–Weinberg equilibrium was verified for both groups. Allele and genotype frequencies were compared using the \( \chi^2 \) test. The relative risk as odds ratio (OR) was determined using 2 × 2 cross-tabulation and a binary logistic regression model for age and gender. Differences between groups were examined using \( \chi^2 \) test and Student’s \( t \)-test. All statistical analyses were performed using SPSS software package version 21.0. The P-value < 0.05 was considered statistically significant.

3. Results and discussion

Here we report for the first time that the rs694539 variant of NNMT gene is associated with bipolar disorders (\( \chi^2 = 13.382; P = 0.001 \)). The statistical power for overall controls is 0.84 and for overall cases is 0.53. Although the individuals with GG genotype showed protection towards bipolar disorders (\( \chi^2 = 11.981, P = 0.001, \text{OR} = 0.414, \text{95\% CI} = 0.250–0.686 \)), the individuals with GA genotype had a 2.141-fold increased risk for bipolar disorders (\( \chi^2 = 8.777, P = 0.003, \text{OR} = 2.141, \text{95\% CI} = 1.288–3.559 \)). Moreover the A allele showed statistically significant association with bipolar disorders (\( \chi^2 = 11.981, P = 0.001, \text{OR} = 2.414, \text{95\% CI} = 1.457–4.000 \)). After the stratification analysis according to gender, we found that there seemed only to be association in female bipolar patients (\( \chi^2 = 15.582; P = 0.000 \)) in which the individuals with GG genotype showed protection against bipolar disorders (\( \chi^2 = 15.296, P = 0.000, \text{OR} = 0.270, \text{95\% CI} = 0.138–0.528 \)). On the contrary, the individuals with GA genotype showed an increased risk for bipolar disorders (\( \chi^2 = 13.077, P = 0.000, \text{OR} = 3.373, \text{95\% CI} = 1.721–6.610 \)). The A allele also showed statistically significant association with bipolar disorders (\( \chi^2 = 15.296, P = 0.000, \text{OR} = 3.708, \text{95\% CI} = 1.893–7.262 \)). In terms of female gender analysis, the statistical power for female controls is 0.99 and for cases 0.82. However there was no association between male patients and bipolar disorders (\( \chi^2 = 2.118, P = 0.347 \)) probably due to the lack of statistical power. With regard to male gender analysis, the statistical powers for male controls and cases are 0.24 and 0.14 respectively. The statistical powers for the male controls and cases are too small to offer a conclusive suggestion. All controls and cases were in Hardy–Weinberg equilibrium (Table 1).

Although the global leukocyte DNA methylation is not altered in euthyrmic bipolar patients (Bromberg et al., 2009), elevated homocysteine levels were reported to be associated with cognitive impairment (Dittmann et al., 2008; Osher et al., 2004).

Homocysteine is formed after transmethylation of methionine into S-adenosylmethionine (SAM), and subsequently S-adenosylhomocysteine (SAH) which is later hydrolyzed to homocysteine and adenosine under physiological conditions. Hyperhomocysteinemia is a complex trait determined by multiple genetic and environmental factors (Carr et al., 2009; Malinowska and Chmurzynska, 2009). Methylene tetrahydrofolate reductase (MTHFR) is one of the key enzymes of one-carbon metabolism. The MTHFR 677T and 1298C alleles associated with elevated plasma homocysteine levels (Frosst et al., 1995) were also found to be associated with neuropsychiatric disorders including schizophrenia and bipolar disorders (Ezzaher et al., 2011; Kempisty et al., 2006; Ozbek et al., 2008; Peerbooms et al., 2011; Reif et al., 2005; Sazci et al., 2003, 2005). Another enzyme involved in one-carbon metabolism is

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Fig. 1. A schematic illustration of the NNMT gene showing the location of NlaIII recognition sequences (5′–CATG–3′) relative to the primers' annealing sites. The forward and reverse primers depicted on the gene after amplification can create a fragment of 187 bp of which there is always a NlaIII site at position of 114133419. Below is the polyacrylamide gel electrophoresis (PAGE) of NNMT gene. The amplified 187 bp fragment was cut with the restriction endonuclease and run on an 8% PAGE at 20 W for 30 min followed by silver staining. Lane M showing the marker (M), lane GG showing the GG genotype with one fragment of 187 bp, lane GA showing the GA genotype with three fragments of 187, 106 and 81 bp, lane AA showing the AA genotype with two fragments of 106 and 81 bp.
nicotinamide-N-methyltransferase (NNMT) methylating nicotinamide and other pyridine compounds during which homocysteine is produced as an end product (Aksoy et al., 1994).

The NNMT gene, located on chromosome 11q23.1, is 16,703 bp in length and has three exons and two introns. The rs694539 variant of NNMT gene located at the 114133419th bp (G→A transition; dbSNP) is shown to be significantly associated with elevated homocysteine levels (Souto et al., 2005). This particular variant was found to be associated with schizophrenia in an Israeli population (Bromberg et al., 2012). The hypothesis is that whether the rs694539 variant of NNMT gene is associated with bipolar disorders as well. Hence we analyzed the allele and genotype frequencies of NNMT gene rs694539 variant in 95 bipolar disorder patients and 201 healthy controls which were recruited from the University of Kocaeli, Hospital Psychiatry Clinic.

In human, the NNMT gene is highly polymorphic. Most of the single nucleotide polymorphisms (SNPs) are within the noncoding regions. The rs694539 variant of NNMT gene in the noncoding region affects the regulation of transcription. This may also alter the cellular pathways, thus causing genetic risk for bipolar disorders (Saito et al., 2001).

To summarize, this report shows that the rs694539 variant of NNMT gene is significantly associated with bipolar disorders. The association was seen in overall bipolar patients and female bipolar patients in a gender specific manner. There was genotypic and allelic association. The role of this variant of NNMT gene in bipolar disorders is unclear, though dysregulation of epigenetics and/or elevated homocysteine and/or dysregulation of the nicotinamide levels may be one of the causes of the disease. Finally our findings suggest that the rs694539 variant of NNMT gene is a genetic risk factor for bipolar disorder in the Turkish population studied.

Disclosure statement

The authors declare that they have no conflicts of interest. We also declare that all authors were fully involved in the study and preparation of the manuscript.

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