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Association of *CACNA1C* with bipolar disorder among the Pakistani population

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Abstract

Many single nucleotide polymorphisms (SNPs) have been identified for Bipolar Disorder (BD), but association between SNPs and BD can vary depending on the population tested. SNPs rs10994336 and rs9804190 in *ANKK1* and rs1006737 in *CACNA1C* have emerged as the most highly replicated SNPs significantly associated with BD. The aim of the present study was to assess the association of these SNPs with BD in the Pakistani population, which has never before been examined. A total of 120 BD and 120 control individuals from Pakistan were examined in this analysis. Genotyping results indicated that rs1006737 in *CACNA1C* was significantly associated with BD, while rs10994336 or rs9804190 in *ANKK1* was not significant when examined individually. However, risk score assessment found that the presence of two or more risk alleles was significantly associated with disease, indicating that risk alleles from *ANKK1* and *CACNA1C* may additively contribute to BD. A protein-protein interaction network was generated using STRING to probe the relationship between ANK3 and CACNA1C interactors and their associations with BD. While none of the interactors are directly linked to BD, they play a role in

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Conflict of Interest Statement

All authors report no conflicts of interest.

Author Contributions

M.K., G.K.R., and J.L. designed experiments. M.K., A.R., M.S., P.A.S., M.H., A.N. and G.K.R. collected DNA samples and handled clinical information for the study population. M.K., T.M.D., and L.T. conducted genotyping experiments. T.M.D., J.S.L., and A.T.D. performed all statistical analyses. M.K., T.M.D., J.S.L., L.T., A.T.D., G.K.R., and J.L. contributed to manuscript preparation.

pathways linked to BD, including oxytocin and dopamine signaling pathways. Collectively, these results reveal a significant association of *CACNA1C* with BD among the Pakistani population, extending results from other ethnic groups to the Pakistani population for the first time.

Keywords

CACNA1C; *ANKK1*; Bipolar Disorder; Pakistan

1. Introduction

Bipolar disorder (BD) is a neuropsychiatric disorder characterized by severe mood shifts alternating between episodes of mania and depression (Craddock and Sklar, 2013). Manic symptoms include high-risk behavior, decreased sleep, and impulsivity, whereas depressive episodes are associated with suicidality, impaired cognition, and anhedonia (Barnett and Smoller, 2009). Among adults, the estimated lifetime prevalence of BD is 1 to 3 percent worldwide (Pedersen et al., 2014). Previous studies indicate that susceptibility to BD is influenced by several genetic risk factors, each with small to moderate individual effects. Twin and familial studies have shown that BD has a high heritability rate of 89% to 93% in twin pairs, and there is an increased risk of disease among first degree relatives of probands relative to the general population (McGuffin et al., 2003; Kieseppa et al., 2004; Lichtenstein et al., 2009). Despite a genetic contribution underlying BD, individual-specific and family-specific environmental factors may play roles as well (Craddock and Sklar, 2013).

A large number of candidate gene association studies, family-based studies, linkage studies, genome-wide association studies (GWAS), and meta-analyses have been conducted to explore genetic markers linked to BD (Barnett and Smoller, 2009; Craddock and Sklar, 2013). From these studies, 681 putative associations have been reported in the National Human Genome Research Institute (NHGRI) Catalog of Published Genome Wide Association Studies (accessed 20 June 2017) from 69 BD and/or Schizophrenia (SZ) studies, with a total of 186 SNPs associations identified in GWAS. The advantage of using GWAS to interrogate patient genomes is that it offers an unbiased, high-throughput approach to search for disease-causing variants, and this approach is useful in the study of complex disorders. However, GWAS results often have minimal overlap between genetic risk variants, and have historically sampled from individuals from European descent (Rosenberg et al., 2010).

Variations in GWAS results have been found in different ethnic populations, indicating that studying disease variants in a diverse set of human populations is required to more thoroughly understand disease etiology (Adeyemo and Rotimi, 2010). Previous gene association studies and GWAS on BD have been conducted on European, East Asian, Japanese, and American patient cohorts; however, to our knowledge, no study has examined the genetic variants underlying BD in the Pakistani population. Examining the genetic variants in Pakistani individuals has previously posed a challenge, as individuals with psychiatric disorders often do not report their disease due to the possibility of social stigmatization (Cinnirella and Loewenthal, 1999). This has resulted in an under-reporting of

the number of individuals in Pakistan with mental illness, and has prevented patients from receiving care by trained professionals (Khalily, 2011).

The present study addresses this gap in our knowledge by examining three common BD SNPs among a Pakistani cohort for the first time, two of which reside in the intronic region of *ANKK3* and one in the intronic region of *CACNA1C*. A meta-analysis consisting of several GWAS provides strong evidence of an association of *ANKK3* SNP rs10994336 and *CACNA1C* SNP rs1006737 with BD (Ferreira et al., 2008). Furthermore, an additional SNP, rs9804190, in *ANKK3* was one of 88 SNPs that met criteria for replication across GWAS (Baum et al., 2008). *ANKK3* encodes a large protein Ankyrin G, whose neural-specific isoforms are found at the nodes of Ranvier and axonal initial segments (Kordeli et al., 1995). Ankyrin G is involved in the normal clustering of voltage-gated sodium channels within the nervous system, thus enabling the propagation of action potentials in myelinated neurons (Zhou et al., 1998). *CACNA1C* codes for the major L-type voltage-dependent calcium channel, CaV1.2 (alpha-1C subunit), which regulates the activity-dependent influx of calcium. Using allele-specific PCR, we probed the association of these SNPs in a cohort of Pakistani patients with BD and unaffected individuals.

2. Methods

2.1. Procedure and participants

Ethical approval for this research was obtained from the Ethics committee of Pir Mehr Ali Shah Arid Agriculture University Rawalpindi and the Pakistan Institute of Medical Sciences Hospital Islamabad. The present study was performed with 120 BD patients and 120 healthy controls. Informed consent and a complete medical history were obtained from all patients and controls who participated in this research. A comprehensive semi-structured interview based assessment was made by experienced psychiatrists, and a detailed history of manic and depressive episodes was collected for each patient. The consensus diagnosis was made by experienced psychiatrists and was based on the Diagnostic and Statistical Manual of Mental Disorders (American Psychiatric Association, 1994). Of the 120 BD patients, 111 patients met the lifetime criteria for bipolar I disorder based on DSM-IV classification. This episode included three or more manic symptoms listed in our Supplementary Table 3, and were severe enough to impair occupational or social functioning. Nine patients did not meet the DSM-IV classification of bipolar I disorder, in that they did not meet diagnostic criteria for three manic symptoms. However, based on the severity of manic and depressive symptoms and the mixed or rapid cycling between symptoms, including during clinical evaluation and self-reporting of past patient history, clinicians assigned the diagnosis of bipolar I disorder, and thus, the nine patients were included in the present study. Although each patient presented with manic symptoms at the time of the clinical evaluation, manic and depressive symptoms were identified based on a combination of clinical evaluation and self-report and past patient history. Patient clinical symptoms associated with BD were characterized before, or during, blood sample collection.

To identify correlations between BD clinical symptoms and SNPs, the clinical symptoms were categorized into two groups. Clinical symptoms that were severe enough to affect occupational and social functioning that occurred in the context of, and met the duration

criteria for a diagnosis of, a DSM-IV manic and major depressive episode respectively were listed as “yes”. Manic and major depressive episode symptoms that did not meet DSM-IV criteria in regards to their severity and longevity were listed as “no”. Clinical symptoms that were not presented by the patients were also listed as “no”. The clinical variables surveyed are commonly used in the diagnosis of manic and depressive episodes in BD as reported in the DSM-IV, and include changes in sleeping behavior, mood, cognitive function, and weight (American Psychiatric Association, 1994).

Healthy controls were recruited from local communities with a simple non-structured interview performed by psychiatrists. Control subjects with a history of mental health or neurological diseases, or first-degree relatives suffering from mental health or neurological diseases, were excluded from the present study. Healthy controls were drawn from the same geographical areas as patients and matched to the patient group based on ethnicity. All participants were unrelated Pakistani nationals born and residing in different areas of Pakistan.

2.2. Genomic DNA extraction and Quantification

Venous blood collected in EDTA vacutainers from patients and healthy controls was processed for genomic DNA extraction using the standard phenol chloroform method (Sambrook and Russell, 2001). Briefly, whole blood was diluted in a 1:1 ratio with red blood cell (RBC) lysis solution (0.32M Sucrose, 10mM Tris-HCl pH 7.5, 5mM MgCl₂, 0.01% Triton-X) and kept at room temperature for 5mins before centrifugation at 12000rpm for 2mins. RBC lysis solution was again added to the pelleted cells until a clear white blood cell (WBC) pellet was obtained. To the pelleted cells, 450µl of WBC lysis solution (10mM Tris-HCl pH 7.5, 400mM NaCl, 2mM EDTA pH 8.0), 10µl of 20% SDS solution, and 10µl of Proteinase K (20mg/µl) was added and incubated overnight at 37°C. Following incubation, 300µl of Chloroform Isoamylalcohol solution (24:1) and 300µl of Phenol (pH 7.8) were added to the lysed WBCs and centrifuged for 10mins at 12000rpm. The upper aqueous layer was collected and 55µl of 3M sodium acetate solution and 800µl of chilled Isopropanol were added to precipitate genomic DNA. At this stage, fine threads of DNA can be seen by inverting the tube gently. Following centrifugation for 10mins at 12000rpm, the supernatant was discarded and the pelleted DNA was washed with 250µl of 70% Ethanol. Samples were then centrifuged again and ethanol was removed and pellets allowed to dry. The DNA pellet was then dissolved in 100µl of TE (100mM Tris-HCl pH 7.5, 10mM EDTA pH 8.0) and stored at -20°C prior to genotyping. DNA quantification was done using a Thermo Scientific Nanodrop Spectrophotometer 2000. Each sample was diluted to a final concentration of 10ng/µl before genotyping.

2.3. Genotyping

Genotyping was conducted on SNPs in the genes *ANK3* and *CACNA1C* (Supplementary Table 1). Allele-specific primers and Sanger sequencing primers were designed for each SNP using BatchPrimer3 (Supplementary Table 2). PCR amplification was performed using 10× PCR buffer (Invitrogen, Catalog # 10342020), 1.5nM of MgCl₂, 0.2mM dNTPs, 0.5µM primer, 1 unit of Taq DNA polymerase (Invitrogen, Catalog # 10342020) and 20ng genomic DNA. Sequence amplification was performed with a Bio-Rad DNA Engine Peltier Thermal

Cycler with the following cycling conditions: initial denaturation at 95°C for 5mins followed by 30 cycles of 95°C for 30sec, 58°C for 30sec, and 72°C for 45sec, with a final elongation of 72°C for 5min. The PCR products were run on a 1.5% agarose gel, and randomly selected samples were sequenced to confirm the specificity of allele specific primers using allele flanking primers (Supplementary Table 2, Figure 1). For sequencing, bands were cut from the gel and DNA was extracted using the QIAGEN MinElute gel extraction kit (Cat. No. 28604). Samples underwent Sanger sequencing at the Keck DNA Sequencing Lab at Yale University, and electropherograms were visualized using 4Peaks (Figure 1).

2.4. Data Analysis

Differences within the age and gender distribution for the control and BD cases were calculated using an independent *t-test* and a *Chi-squared test*, respectively. Hardy Weinberg equilibrium and linkage disequilibrium were calculated using the R package “HardyWeinberg” and “genetics”, respectively, in R v3.3.2. To account for multiple testing of three different SNPs among the same samples, a Bonferroni corrected p-value threshold was applied to the Hardy Weinberg p-values and all subsequent statistical tests. This methodology has been employed for GWAS and genetic case-control studies, and has been described in greater detail in previously published protocol papers (Nyholt, 2001; Anderson et al., 2010; Clarke et al., 2011). The association between SNP genotypes and disease was determined using contingency tables and multivariate logistic regression modeling in SAS v9.4. Unadjusted and Bonferroni-corrected p-values, odds ratios (ORs), and 95% confidence intervals (95% CI) were calculated for each SNP. Adjusted models included age and gender as covariates. In order to determine whether gender stratification was necessary, multivariate logistic regression was performed to examine if there was a significant interaction between gender and SNP genotypes with disease risk. The genetic risk score was calculated to identify an additive effect of risk alleles from multiple SNPs that increase the risk of BD. The association of clinical variables with SNP genotypes were calculated using a *Chi-squared test* in Prism 7.

2.5. Protein-protein interaction network

The interaction between the two candidate proteins, ANK3 and CACNA1C, and other proteins related to BD were probed using STRING version 10.5 (Szklarczyk et al., 2015). Only high confidence protein-protein interactions obtained from experimental, co-expression, co-occurrence, or database sources were used in this analysis. In addition to the two candidate proteins, 20 primary and 20 secondary interactors were included in the analysis. Pathway analysis was conducted using ANK3, CACNA1C, and the 40 additional proteins to identify enriched KEGG pathways that may be linked to BD.

3. Results

In this study, we compared the distribution of SNPs in *ANK3* and *CACNA1C* in 120 patients with BD and 120 control individuals. Table 1 illustrates the average age and gender of patients and controls. There was a significant difference between the control and BD groups in age and gender ($p=4.19\times10^{-6}$ and $p=1.25\times10^{-5}$, respectively). As a result of this limitation, these variables were considered as covariates in subsequent analyses. All samples

passed linkage disequilibrium testing, indicating there was no random association between *ANK3* SNPs due to SNP proximity, or from selection bias within the sample population ($p=0.841$, $X^2 = 0.040$). Control samples passed Hardy Weinberg equilibrium for each SNP when accounting for multiple testing (*ANK3* rs10994336 $p=0.395$, $X^2=1.222$; *ANK3* rs980419 $p=0.0342$, $X^2 = 5.147$; *CACNA1C* rs1006737 $p=0.0255$, $X^2=5.094$).

Previous studies have found the T allele at rs10994336 in *ANK3* is found more frequently among BD patients compared to controls (Ferreira et al., 2008). Among the Pakistani population sampled in our analysis, 18.3% of controls and 30.8% of patients were heterozygous for the risk allele T ($p=0.026$, $OR=1.98$) (Table 2). There was a marginal change in OR after adjusting for age and gender (adjusted $p=0.041$, $OR=1.99$) (Table 2). Using a Bonferroni p -value cutoff of 0.0167, these results were non-significant, and indicate that there is no association between SNP rs10994336 in *ANK3* with BD. No individual carried two rs10994336 risk alleles in *ANK3*. A second *ANK3* SNP, rs9804190, did not yield a difference in genotypic frequency between cases and controls (adjusted $p=0.687$, $OR=1.15$) (Table 2).

For SNP rs1006737 of *CACNA1C*, there was a significant association for a heterozygous genotype with BD, with 34.2% of control individuals and 50.8% of cases having a heterozygous genotype ($p=0.009$, $OR=1.99$) (Table 2). The adjusted p -value accounting for gender and age was also significant for this SNP (adjusted $p=0.014$, $OR=2.05$). These results surpass the Bonferroni-corrected p -value threshold, indicating that the association between SNP rs1006737 and BD is significant after accounting for multiple-testing correction. Like *ANK3* rs10994336, no individual was homozygous for the risk allele.

Given that 80% of BD patients sampled for this analysis were male, the interaction between SNPs and gender was examined. There was no interaction between gender and the three SNPs tested (*ANK3* rs10994336 $p=0.965$; *ANK3* rs9804190 $p=0.828$; *CACNA1C* rs1006737 $p=0.326$). This indicates that the uneven distribution of gender in this analysis had little effect on the association between disease state and genotype.

Seventeen manic and depressive clinical symptoms used in the diagnosis of DSM-IV manic and major depressive episodes were documented for each patient to further examine the association of candidate SNPs with behavioral hallmarks of BD (Supplementary Table 3). These clinical symptoms were classified into two groups. If clinical symptoms met the duration and severity criteria for DSM-IV classified manic and depressive episodes, patients were considered positive for the symptom, and recorded “yes”. For clinical symptoms that occurred sporadically throughout the month, did not last the entire day, and did not sufficiently meet the diagnostic criteria for manic and major depressive episodes, patients were considered negative for the symptom, and recorded “no”. Based on this classification, two clinical symptoms, loss of energy (fatigue) and flight of ideas (disorganized thought), were nominally associated with *ANK3* rs9804190 and *CACNA1C* rs1006737, respectively ($p=0.014$, $p=0.023$) (Supplementary Table 3). However, only the association between loss of energy (fatigue) and *ANK3* rs9804190 were significant after accounting for multiple-testing (Supplementary Table 3).

Due to the polygenic nature of BD, we assessed how the number of risk alleles affected the risk of BD (Table 3). Genotype data from all three SNPs in *ANKK3* and *CACNA1C* were included in this analysis. There was a significant association between BD and the presence of 1 risk allele ($p=0.003$, $OR=2.46$), and this remained significant after adjusting for gender and age (adjusted $p=0.004$, $OR=2.57$) (Table 3). There was also a significant association between BD and the presence of 2 or more risk alleles (adjusted $p=0.004$, $OR=3.35$) (Table 3). A separate analysis assessing if a significant relationship persisted when accounting for 3 or more risk alleles was also conducted, however, those results were non-significant (data not shown). To parse out which genotypic combinations may be driving the significant correlation between BD and 2 or more risk alleles, each genotypic combination was analyzed separately. Using the presence of 0 risk alleles as a reference, there was a significant association with BD when accounting for the presence of 1 risk allele in *ANKK3* rs10994336 and 1 risk allele in *CACNA1C* rs1006737 ($p=0.005$, $OR=5.01$) (Supplementary Table 4). This significant association persisted after adjusting for age and gender (adjusted $p=0.01$, $OR=5.06$) (Supplementary Table 4). However, this genotypic combination analysis should be considered as a preliminary analysis due to the small number of individuals with this genotype (Supplementary Table 4). Collectively, this indicates that while rs10994336 in *ANKK3* may not be significantly associated with BD by itself, it may contribute to BD in conjunction with SNP rs1006737 in *CACNA1C*.

Previous studies have shown rs9804190 in *ANKK3* and rs1006737 in *CACNA1C* are associated with altered expression of *ANKK3* and *CACNA1C* mRNA levels (Roussos et al., 2012; Eckart et al., 2016). Since altered expression of these genes may affect *ANKK3* and *CACNA1C* native protein-protein (or genetic) interactions, we utilized STRING to visualize *ANKK3* and *CACNA1C* primary and secondary interacting partners. *ANKK3* and *CACNA1C* do not physically interact, and do not form any disease triplets, where *ANKK3* and *CACNA1C* are linked by a common protein interactor (*ANKK3*-interactor-*CACNA1C*) (Figure 2A; Supplementary Table 5). Disease quadruplets (*ANKK3*-Interactor1-Interactor2-*CACNA1C*) were identified using STRING, and include proteins associated with calcium ion binding (*CALM1*, *CALM2*), voltage-gated sodium channel subunits (*SCN1A*, *SCN10A*, *SCN11A*), and G-protein subunits (*GNAI2*, *GNAI3*). (Figure 2A; Supplementary Table 5). Enrichment analysis for the 40 proteins, in addition to *ANKK3* and *CACNA1C*, queried in STRING identified KEGG pathways associated with oxytocin signaling, dopaminergic synapses, and calcium signaling that are overrepresented in the protein list (Figure 2B).

4. Discussion

Previous GWAS and candidate gene association studies have focused on identifying risk variants for BD among predominantly European and East Asian patient cohorts. The complexity of the SNPs underlying neuropsychiatric disorders has been compounded by the realization that GWAS between ethnic populations are not always consistent, thereby necessitating the evaluation of risk variants in other populations. The purpose of the present study was to investigate three SNPs highly associated with BD among European and East Asian populations in a Pakistani BD patient cohort, a population that has never before been examined. We report that the A allele of SNP rs1006737 in *CACNA1C* is significantly associated with Pakistani BD (Table 2). These results are consistent with previous findings,

indicating that SNPs identified in other populations also occur in the Pakistani BD population (Ferreira et al., 2008). In contrast, the SNPs rs100994336 and rs9804190 in *ANK3* were not significantly associated with Pakistani BD (Table 2). This result suggests that SNPs associated with BD may vary based on ethnic background as has been shown in previous studies, or it may be in part due to the smaller sample size utilized in this study (Takata et al., 2011; Gonzalez et al., 2013).

We found that individuals with heterozygous genotype for *CACNA1C* rs1006737 have 2.05 times the odds of developing BD compared to individuals with a control genotype, adjusting for age and gender (Table 2). Although the association of *ANK3* rs10994336 and BD risk, alone, was not found to be significant, it displayed a significant association in combination with a SNP in *CACNA1C*. Individuals with a heterozygous genotype for *ANK3* rs10994336 and *CACNA1C* rs1006737 have 5.06 times the odds of developing BD compared to individuals with a control genotype for all SNPs after adjusting for age and gender (Supplementary Table 4). Though a limitation of this genotypic analysis is the small number of individuals used to arrive at the odds ratio (9 cases and 8 controls relative to 54 controls and 28 cases), the observation that an additive effect of SNPs contributing to BD was supported by risk score analysis. We found that individuals with 2 or more risk alleles from all SNPs tested have 3.35 times the odds of developing BD compared to individuals with 0 risk alleles, adjusting for age and gender (Table 3).

Previous studies have shown that these SNPs are associated with altered affective behaviors in cases and controls. The rs1006737 mutant A allele in *CACNA1C* is associated with paranoid ideation, lower extraversion, trait anxiety, and higher harm avoidance, while the rs10994336 T allele in *ANK3* is associated with decreased novelty seeking, lower behavioral activation scores, altered set-shifting, and decision-making (Roussos et al., 2011; Linke et al., 2012). Both alleles are nominally associated with high startle reactivity (Roussos et al., 2011). rs1006737 in *CACNA1C*, in conjunction with the underlying neural systems, influences language production on a semantic level (Krug et al., 2010). Cognitive abnormalities are also linked to allelic variation in *ANK3*, and this may impact visual signal detection in patients, increasing the risk for BD (Ruberto et al., 2011). The rs10994336 in *ANK3* is reported to be linked with facial affect processing disruption in patients with BD (Zhao et al., 2016). We found that rs1006737 in *CACNA1C* is nominally associated with flight of ideas (disorganized thought), and rs9804190 in *ANK3* is associated with loss of energy (fatigue). The two clinical symptoms are associated with DSM-IV classified manic and major depressive episodes, respectively (Supplementary Table 3). Our preliminary results are consistent with previous reports, indicating that these SNPs may play a role in altering affective behaviors. However, current assessment of the 17 clinical symptoms associated with manic and major depressive episodes comes with certain limitations, which include a lack of clear quantifiable and objective classifications. This analysis was instead used to generate hypothetical links between the SNPs and their potential roles in BD clinical symptoms. In the future, a more in-depth measurement of patient symptoms in relation to SNPs is necessary.

Mutations in *ANK3* and *CACNA1C* are linked to changes in brain volume in cases and controls. The rs10994336 T allele in *ANK3* is associated with reduced white matter integrity

in the anterior limb of the internal capsule (Linke et al., 2012). The association of risk variants of *CACNA1C* and alterations in brain volume is still under debate. Some reports indicate this SNP is associated with increased subcortical volume, grey matter density, and brainstem alterations (Franke et al., 2010; Perrier et al., 2011). There is an increased grey matter density in the right amygdala and right hypothalamus in carriers of this SNP (Perrier et al., 2011). A smaller left putamen was observed in BD patients carrying this risk allele compared to healthy controls, indicating that rs1006737 polymorphism may influence anatomical variation within subcortical regions involved in emotional processing (Perrier et al., 2011). In the corticolimbic frontotemporal neural system, mutant polymorphism at rs1006737 significantly increased gray matter volume and reduced functional connectivity (Wang et al., 2011). This reflects the influence of *CACNA1C* genetic variation on structural and functional aspects of the corticolimbic system, which may be a mechanism contributing to the neural circuitry of BD (Wang et al., 2011).

Brain regional activation has also been studied in patients with SNPs in *CACNA1C*. Regardless of BD status, carriers of the rs1006737 SNP displayed significantly reduced bilateral hippocampal activation during episodic memory recall (Erk et al., 2010). In the same study, the risk variant's role in disrupting the functional coupling between left and right hippocampal regions was validated (Erk et al., 2010). Another brain region that has been linked to the SNP is the subgenual anterior cingulate cortex. Carriers of the risk allele exhibit diminished activation of the region, which is known for regulating adaptive stress-related responses, and highly associated with affective disorders (Erk et al., 2010). The same genetic variant has been associated with changes in brain circuitries, including increased hippocampal and prefrontal activity during emotional processing and executive cognitive function, respectively (Bigos et al., 2010). Previous brain imaging studies have also highlighted the effect of rs1006737 genotype in brain function, such as increased amygdala activity during emotional processing, which can affect facial emotion recognition in the risk allele carriers (Wessa et al., 2010; Jogia et al., 2011; Nieratschker et al., 2015).

In the present study, no individual homozygous for the mutant allele of rs10994336 (T) in *ANK3* or rs1006737 (A) in *CACNA1C*, respectively, were identified, indicating an allelic expression imbalance also observed previously for rs10994336 of *ANK3* (Wober-Bingol et al., 2011). Allelic expression imbalance was also observed for rs3750800 in *ANK3*, which was not examined in the present study, with more than half of all heterozygous samples showing allelic imbalance and a small number of samples showing near mono-allelic expression (Quinn et al., 2010). Allelic expression imbalance for an *ANK3* SNP was also found in an American cohort with Northern and Western European ancestry (https://www.ncbi.nlm.nih.gov/SNP/snp_viewTable.cgi?pop=1409).

Expression levels of *ANK3* and *CACNA1C* transcripts are altered by rs9804190 in *ANK3* and rs1006737 in *CACNA1C* (Roussos, et al. 2012; Eckart, et al. 2016). As a result, this may lead to altered protein levels, thus affecting ANK3 and CACNA1C protein-protein interactions. We used STRING to determine the possible protein-protein (and genetic) interactions that may be adversely affected by changes in ANK3 and CACNA1C protein levels (Figure 2, Supplementary Table 5). Based on a literature search, none of the predicted proteins that interact with ANK3 and CACNA1C are associated with BD or other

neuropsychiatric disorders, although some are linked to epilepsy (Wallace et al., 1998; Liao et al., 2010).

However, in concordance with our pathway analysis, other groups have also found enrichment for similar biological pathways when assessing SNPs associated with BD. Importantly, these common biological pathway themes were found across platforms and methodologies, suggesting these pathways may be highly relevant to BD. One such study queried genes located near BD-linked SNPs and found that dopaminergic signaling regulation was significantly enriched (Torkamani et al., 2008). Utilizing four separate GWAS studies conducted for BD, a study found enrichment for glutamate signaling (Nurnberger et al., 2014). Due to the high degree of overlap between BD-linked SNPs and other psychiatric disorders, research on common pathways across disorders has also been conducted. Over-representation for genes linked to long term potentiation, glutamate receptor signaling, dopamine-DARPP32 feedback in cAMP signaling, and calcium signaling has been identified when assessing SNPs associated with BD and SZ (Forstner et al., 2017). Circadian entrainment pathway enrichment among 23 SNPs commonly associated with BD, SZ, and Autism has also been identified (Khanzada et al., 2017). While the genes utilized in these previously published pathway analyses were not identical to ours, similar pathway enrichment was found in our preliminary enrichment analysis (Figure 2). This suggests that common biological and molecular themes are affected in BD, though the genes driving those pathways may differ across studies.

Alterations in oxytocin levels have been studied across several neuropsychiatric disorders, including major depression and BD (Cochran et al., 2013). Previous studies have found that serum oxytocin levels are significantly higher in patients during a manic episode of BD compared to healthy controls, as well as BD patients going through depressive episodes (Turan et al., 2013). An additional study has shown that oxytocin levels are significantly reduced in a small mixed cohort of female individuals with depressive disorder or bipolar affective disorder depressive episode compared to healthy controls, while the same effect was not found between male patients and controls (Ozsoy et al., 2009). The evidence for dopamine signaling in BD is conflicting. Dopamine transporter up-regulation and down-regulation have been reported in patients with euthymic bipolar and bipolar depression, respectively (Chang et al., 2010; Anand et al., 2011). Whether these changes in the dopamine transporter availability can be correlated with disease severity or phase has yet to be confirmed. Alterations in the binding potentials of D₁ dopamine receptors in the frontal cortex of bipolar patients has also been observed (Suhara et al., 1992). While the role of oxytocin and dopamine is still being investigated in BD, our analysis suggests that ANK3 and CACNA1C may interact with proteins that play important roles in the etiology of BD, and alterations in ANK3 and CACNA1C protein expression may be detrimental to the endogenous function of these pathways.

Important caveats of the present study include the small sample size, as well as the significant difference between age and gender between cases and controls (Table 1). Misconceptions about mental health disorders among the Pakistani population can make it difficult to correctly diagnose and collect DNA samples for genetic testing. In recognition of

these limitations, appropriate statistical analyses accounting for gender and age as covariates was conducted to ensure there was no effect on statistical results.

Despite this, the conclusions from the present study will set the foundation for future studies utilizing larger samples from the Pakistani population.

5. Conclusion

We found that SNP rs1006737 of *CACNA1C* is significantly associated with BD among the Pakistani population, while rs10994336 and rs9804190 of *ANKK3* are not significant in our genetic case control study. However, risk score assessment found that the presence of 2 or more risk alleles from all three SNPs tested were significantly associated with disease. To determine which genotypic combinations contribute to the significant correlation between BD and 2 or more risk alleles, we assessed genotypic combinations and found that individuals with SNPs in *ANKK3* rs10994336 and in *CACNA1C* rs1006737 have 5.06 times the odds of developing BD than control individuals. To our knowledge, this study is the first conducted among the Pakistani population. Future studies utilizing a larger sample population will allow for a more thorough interrogation of the genetic variants underlying BD in Pakistani individuals. Nevertheless, this study provides a critical starting point in evaluating a population previously unstudied in the field of BD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BD	Bipolar disorder
SNPs	Single nucleotide polymorphisms
ORs	Odds ratios
GWAS	Genome-wide association studies
NHGRI	National human genome research institute
ANKK3	Ankyrin G
CACNA1C	L-type voltage-dependent calcium channel
PCR	Polymerase chain reaction
DSM-IV	Diagnostic and Statistical Manual-IV

RBCs	Red blood cells
WBCs	White blood cells
CALM1	Calmodulin 1
CALM2	Calmodulin 2
SCN1A	Sodium voltage gated alpha subunit 1
SCN10A	Sodium voltage gated alpha subunit 10
SCN11A	Sodium voltage gated alpha subunit 11
GNAI2	G protein subunit alpha I2
GNAI3	G protein subunit alpha I3
KEGG	Kyoto encyclopedia of genes and genomes

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Figure 1. Sanger sequencing of selected samples showing all observed genotypes
Electropherograms of individuals with *ANK3* rs10994336 (A), rs9804190 (B), and *CACNA1C* rs1006737 (C). The SNPs are outlined with the black boxes. Normal genotypes with no risk alleles are shown as reference for each SNP.

Figure 2. Protein or genetic interactions and enrichment analysis for ANK3 and CACNA1C interacting partners

(A) Forty proteins that interact with ANK3 and CACNA1C via primary or secondary interactions were plotted using STRING. High confidence interactions limited to experimental evidence and databases were used in this figure. The width of the edges connecting proteins correlates with the confidence level of the interactions. (B) Enrichment analysis for KEGG pathways among the forty proteins that interact with ANK3 and CACNA1C. X-axis is the enrichment score, which is the $-\log$ FDR adjusted p-value. The black line at 1.3 represents an FDR adjusted p-value of 0.05. Any pathways that surpass 1.3 are significantly over-represented among our proteins of interest.

Table 1

Mean age and gender frequency among control and BD patients.

	Controls	Cases	p-value
Total Samples	120	120	
Mean Age $\pm$ SD	41.6 $\pm$ 17.8	32.8 $\pm$ 9.7	<0.001 * ^a
Total Number (Percentage) Males	63 (52.5%)	96 (80.0%)	<0.001 * ^b

Comparison of mean age and gender across cases and controls.

* p-values that pass the Bonferroni p-value threshold of 0.0167 are highlighted.

^a Age differences was calculated using an independent *t-test*.

^b Gender differences was calculated using a *Chi-square test*.

Table 2

Genotypic frequencies of studied SNPs in control and BD patients.

Genotype	Controls (n=120)	Cases (n=120)	OR (95% CI); p-value	Adjusted OR (95% CI); p-value
<i>ANK3/rs10994336 (C/T)</i>				
CC	98 (81.7%)	83 (69.2%)	Reference	Reference
CT	22 (18.3%)	37 (30.8%)	1.98 (1.08, 3.63); 0.026	1.99 (1.03, 3.86); 0.041
<i>ANK3/rs9804190 (C/T)</i>				
CC	97 (80.8%)	93 (77.5%)	Reference	Reference
CT	19 (15.8%)	22 (18.3%)	1.22 (0.66, 2.29); 0.526	1.15 (0.58, 2.29); 0.687
TT	4 (3.3%)	5 (4.2%)		
<i>CACNA1C/rs1006737 (G/A)</i>				
GG	79 (65.8%)	59 (49.2%)	Reference	Reference
GA	41 (34.2%)	61 (50.8%)	1.99 (1.18, 3.35); 0.009 [*]	2.05 (1.15, 3.65); 0.014 [*]

Genotypic frequencies in cases and controls. The Adjusted OR, 95% CI, and p-value were calculated after adjusting for gender and age as covariates. The disease association of *ANK3/rs9804190* was calculated using a dominant regression model.

^{*} Odds ratios and p-values that surpass the Bonferroni corrected p-value threshold are highlighted

Table 3

Polygenic risk score between BD cases and controls.

Number of Risk Alleles	Controls (n=120)	Cases (n=120)	OR (95% CI); p-value	Adjusted OR (95% CI); p-value
0	54 (45.0%)	28 (23.3%)	Reference	Reference
1	47 (39.2%)	60 (50.0%)	2.46 (1.36, 4.46); 0.003 *	2.57 (1.34, 4.93); 0.004 *
2+	19 (15.8%)	32 (26.7%)	3.25 (1.57, 6.73); 0.0015 *	3.35 (1.49, 7.55); 0.004 *

Risk score OR, 95% CI and p-values for cases and controls.

* Odds ratios and p-values that pass the Bonferroni p-value threshold of 0.0167 were highlighted.