Genome-Wide Association Study in Bipolar Patients Stratified by Co-Morbidity

Berit Kerner[1]*, Christophe G. Lambert[2], Bengt O. Muthén[3]

1 Department of Psychiatry, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, California, United States of America, 2 Golden Helix Incorporated, Bozeman, Montana, United States of America, 3 Graduate School of Education and Information Studies, Social Research Methodology Division, University of California Los Angeles, Los Angeles, California, United States of America

Abstract

Background: Bipolar disorder is a severe psychiatric disorder with high heritability. Co-morbid conditions are common and might define latent subgroups of patients that are more homogeneous with respect to genetic risk factors.

Methodology: In the Caucasian GAIN bipolar disorder sample of 1000 cases and 1034 controls, we tested the association of single nucleotide polymorphisms with patient subgroups defined by co-morbidity.

Results: Bipolar disorder with psychosis and/or substance abuse in the absence of alcohol dependence was associated with the rare variant rs1039002 in the vicinity of the gene phosphodiesterase 10A (*PDE10A*) on chromosome 6q27 ($p = 1.7 \times 10^{-8}$). *PDE10A* has been implicated in the pathophysiology of psychosis. Antagonists to the encoded protein are currently in clinical testing. Another rare variant, rs12563333 ($p = 5.9 \times 10^{-8}$) on chromosome 1q41 close to the MAP/microtubule affinity-regulating kinase 1 (*MARK1*) gene, approached the genome-wide level of significance in this subgroup. Homozygotes for the minor allele were present in cases and absent in controls. Bipolar disorder with alcohol dependence and other co-morbidities was associated with SNP rs2727943 ($p = 3.3 \times 10^{-8}$) on chromosome 3p26.3 located between the genes contactin-4 precursor (*BIG-2*) and contactin 6 (*CNTN6*). All three associations were found under the recessive genetic model. Bipolar disorder with low probability of co-morbid conditions did not show significant associations.

Conclusion: Conceptualizing bipolar disorder as a heterogeneous disorder with regard to co-morbid conditions might facilitate the identification of genetic risk alleles. Rare variants might contribute to the susceptibility to bipolar disorder.

Introduction

Bipolar disorder (BPD) is a mental disorder with dramatic and unpredictable mood swings between mania and depression. It affects approximately 5.7 million American adults or 2.6 percent of the U.S. population aged 18 and older in a given year (http://www.nimh.nih.gov/health/publications/the-numbers-count-mental-disorders-in-america/index.shtml). The symptoms of BPD vary considerably. Therefore, this disorder has also been conceptualized as a group of related mood disorders referred to as bipolar spectrum disorders. It remains unclear if these conditions share a common pathophysiology or common risk factors. Recently, co-morbid conditions in BPD have drawn attention as potential indicators of pathologically distinct subtypes [1].

Co-morbid conditions are common in BPD. They are often related to the severity of the disorder and may aggregate in families. Familial aggregation has been shown for co-morbid attention deficit hyperactivity disorder (ADHD) [2], alcohol use disorders, and panic disorder [3], as well as psychosis [4]. Familial aggregation of co-morbid conditions can be caused by shared environment, shared genetic risk factors or a combination of both. Therefore, co-occurrence in families alone cannot clearly differentiate between these possible causes. Twin studies comparing the occurrence rate in monozygotic twins versus dizygotic twins revealed that the susceptibility to BPD, substance abuse, as well as alcohol dependence could be influenced by genetic factors [5–9]. Shared pathophysiology might explain the common and characteristic co-occurrence of these disorders [10,11]. Exposure to substances of abuse at certain vulnerable times during development might in itself increase risk for psychotic disorders in individuals with certain genetic susceptibilities [12,13]. The identification of genetic risk alleles associated with BPD, substance

abuse, or a combination of both might assist in disentangling these complex and interrelated factors.

The search for genetic risk factors in BPD has recently drawn attention to some genomic variants that might play a role in BPD. In genome-wide association studies, several common variants have been found to be significantly associated with BPD. Among those are the single nucleotide polymorphism (SNP) rs1012053 in the first intron of the gene diacylglycerol kinase eta (*DGKH*) (HGNC:2854) on chromosome 13q14.1 [14], variants in the gene voltage-dependent L-type calcium channel subunit alpha-1C (*CACNA1C*) (HGNC:1390) on chromosome 12p13 [15] and the intronic SNP rs10994336 in the Ankyrin-3 (*ANK3*) (HGNC:494) gene on chromosome 10q21 [16,17]. In addition, rare structural variants as well as common variants in gene deserts have been suggested. However, replication of these findings has been challenging. A previous analysis of the data set used in our analysis failed to report genome-wide significant associations [18].

BPD is likely to be a heterogeneous disorder. Samples consisting of unidentified subgroups that behave differently with regard to the problem at hand introduce noise to the data and might lead to increased type 1 and type 2 errors [19]. In order to explore phenotype heterogeneity in the Genetic Association Information Network (GAIN) Bipolar Disorder sample, we used a multivariate latent class analysis (LCA) to define subgroups of BPD patients based on profiles of psychiatric co-morbid conditions. Using this approach, BPD patients could be assigned to their most likely latent class. We then tested the relationship of SNPs from a genome-wide scan genotyped on the Affymetrix 6.0 array for association with the latent classes. Our approach identified several highly significant associations with rare, as well as common, genomic variants.

Subgroups of patients defined by co-morbid conditions might be more homogeneous with respect to underlying genetic risk factors. Therefore, sub-grouping of BPD patients according to co-morbid conditions might identify additional genomic variants that are associated with a particular sub-phenotype.

Materials and Methods

Ethics statement

All participants gave informed consent for the inclusion in genetic studies on BPD. Written consent was given by the patients for their information to be stored in the National Institute of Heath database and used for research. No identifiable data were used in this study. Only completely de-identified data had been made available to the researchers involved in this study. The study had been exempt from institutional review by the Institutional Review Board at the University of California, Los Angeles, based on the fact that only preexisting and completely de-identified data were analyzed.

Sample

The BPD sample consisted of 1041 unrelated individuals from the Foundation for the National Institutes of Health Genetic Association Information Network (GAIN) Initiative (http://www.genome.gov/19518664) [20]. Individuals were of European descent according to self-reported heritage. The sample has been used in several previously published studies [15,18,21]. Best-estimate diagnosis procedures had been used to diagnose mood disorder, as well as co-morbid conditions according to the Diagnostic and Statistical Manual of Mental Disorder Version III Revised (DSM-III-R) and DSM-IV diagnostic criteria [22].

Table 1. Demographic description of the bipolar disorder sample and controls.

	Controls	BPD	Latent Class 1	Latent Class 2	Latent Class 3
	N = 1034	N = 1000	N = 258	N = 249	N = 493
	100%	100%	26%	25%	49%
AGE (mean)	52 (SD = 17.6)	42[#] (SD = 12.6)	41 (SD = 12.6)	41 (SD = 10.8)	44* (SD = 13.8)
MAX	90	88	64	80	88
MIN	18	17	18	17	18
Female	502 (49%)	501 (50%)	160 (62%)*	96 (39%)*	240 (49%)*
Male	532 (51%)	499 (50%)	98 (38%)	153 (61%)	248 (51%)
BPD	0 (0%)	1000 (100%)	258 (100%)	249 (100%)	493 (100%)
BPD+SUB	0	336 (34%)	111 (43%)	200 (80%)	25 (5%)
BPD+OCD	0	82 (8%)	50 (19%)	24 (10%)	8 (2%)
BPD+PD	0	236 (24%)	97 (38%)	77 (31%)	62 (13%)
BPD+SP	0	161 (16%)	64 (25%)	57 (23%)	40 (8%)
BPD+ED	0	70 (7%)	37 (14%)	25 (10%)	8 (2%)
BPD+ADHD	0	97 (10%)	43 (17%)	50 (20%)	4 (1%)
BPD+ALCAB	0	134 (13%)	133 (52%)	1 (0.4%)	0 (0%)
BPD+ALCDEP	0	332 (33%)	0 (0%)	249 (100%)	83 (17%)
BPD+NIC	0	241 (24%)	90 (35%)	125 (50%)	26 (5%)
BPD+PSYCH	0	252 (25%)	105 (41%)	68 (27%)	79 (16%)

[#]Age was missing in 8 individuals, 5 males and 3 females, all of whom clustered into Latent Class 3.
*Significant at the 0.05 level in the Wald Chi-Square test when testing the equality of means across the latent classes.
SUBA, substance abuse; OCD, obsessive compulsive disorder; PD, panic disorder; SP, social and specific phobia; ED, eating disorder; ADHD, attention-deficit hyperactivity disorder; ALCAB, alcohol abuse; ALCDEP, alcohol dependence; NIC, nicotine dependence; PSYCH, psychotic symptoms (presence of hallucinations and/or delusions).
doi:10.1371/journal.pone.0028477.t001

Information on mood symptoms, psychotic symptoms and co-morbid conditions was obtained based on the Diagnostic Interview for Genetic Studies (DIGS) Version 3 and 4 (http://www.nimhgenetics.org) [23], family information and medical records. Multiple diagnoses per individual were allowed if diagnostic criteria were fulfilled for each disorder independently. Information about lifetime symptoms of hallucinations and/or delusions was obtained through the K section of the DIGS. Based on these information sources forty-one subjects did not meet full DSM-IV criteria for BPD or schizoaffective disorder and therefore, they were subsequently excluded from the analysis. The remaining 1000 individuals, all of whom were diagnosed with BPD type I, were included in the latent class analysis and the genetic association study. The cases consisted of 499 males and 501 females. The age at interview ranged from 17 to 88 years. The mean age was 40.1 years, with a standard deviation (SD) of 12.6 years (Table 1). Age was missing in 8 individuals, 5 males and 3 females.
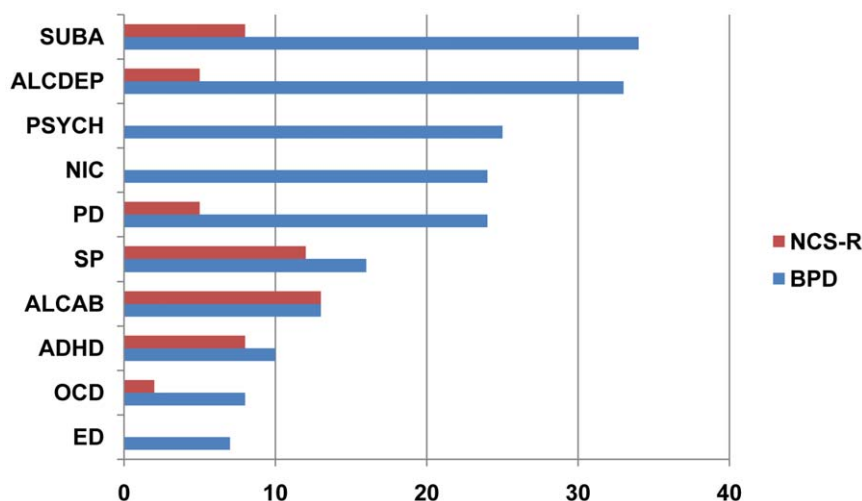
Co-morbid conditions were prevalent in the BPD patients (Table 1, Figure 1). Conditions present in at least 5% of individuals with BPD were used as indicators of the latent subclasses. Ten variables fulfilled this criterion: substance abuse or dependence (SUBA) (336 individuals), alcohol dependence (ALCDEP) (332 individuals), nicotine dependence (NIC) (241 individuals), panic disorder (PD) (236 individuals), social phobia and specific phobia (SP) (161 individuals). Alcohol abuse (ALCAB) (134 individuals), attention deficit hyperactivity disorder (ADHD) (97 individuals), obsessive compulsive disorder (OCD) (82 individuals), and eating disorder (ED) (70 individuals) were only slightly more frequent than in the general population [24]. Presence of psychotic symptoms, such as hallucination and/or delusions (PSYCH) (252 individuals) were also evaluated and included in the analysis, even though we did not consider them as a co-morbid disorder, but rather a part of

the bipolar phenotype. Prior evidence suggested that these symptoms might indicate a particular subtype of BPD [25].

The controls in the GAIN study had been ascertained independently through an NIMH funded initiative. Originally, a total of 4,586 subjects across the U.S. had been asked to complete a psychiatric and medical questionnaire. Based on the response, only individuals who did not fulfill diagnostic criteria for major depression, psychosis or BPD were included as controls. Out of this pool, one thousand and thirty-four control samples matched for gender and ethnicity (532 males and 502 females) were selected as controls in the GAIN sample. The age at interview ranged from 18 years to 90 years with a mean age of 52.5 years and a SD of 17.6 years. Since individual-level information on psychiatric conditions and symptoms was not available for the controls, this information could not be included in the latent class model. Our analysis is therefore limited to the evaluation of co-morbid conditions in the context of BPD only. The majority of cases and controls were older than the average age of onset for BPD or the co-morbid conditions, but since information on age was not available on the individual level, uncertainties in some controls could not be completely excluded. Since this study is a reanalysis of publicly available data, these design issues were beyond our control.

Latent class analysis

The LCA was performed in the statistical software program Mplus [26]. The latent class membership was estimated by maximum likelihood using the estimation maximization (EM) algorithm [27]. We used 1000 random sets of starting values to avoid local maxima in the log-likelihood. Missing data was handled by including all available observations in line with the assumption of "missing at random" (MAR) [28]. This assumption allows for missingness to be a function of covariates in contrast to

Figure 1. Life-time prevalence of co-morbid conditions and psychotic symptoms in bipolar patients of the GAIN study compared to lifetime prevalence in the National Comorbidity Survey Replication Study [51]. Co-morbid conditions and psychotic symptoms are common in bipolar disorder. In the GAIN sample of 1000 bipolar patients, substance abuse/dependence was the most prevalent co-morbid condition, followed by alcohol dependence. Nicotine dependence was present in about 25% of individuals. The prevalence of panic disorder was consistent with other reports in the literature [23]. Attention deficit hyperactivity disorder, obsessive-compulsive disorder and eating disorder were generally rare, present in less than 7% of individuals. As a comparison we used lifetime prevalence rates of psychiatric disorders from the National Comorbidity Survey Replication Study (NCS-R). In this study the evaluation of ADHD was limited to a subsample of individuals age 44 and younger, which might explain the high prevalence rate. Psychotic symptoms, nicotine dependence and eating disorders had not been evaluated in this study. SUBA, substance abuse; ALCDEP, alcohol dependence; PSYCH, psychotic symptoms (presence of hallucinations and/or delusions); NIC, nicotine dependence; PD, panic disorder; SP, social and specific phobia; ALCAB, alcohol abuse; ADHD, attention-deficit hyperactivity disorder; OCD, obsessive compulsive disorder; ED, eating disorder.
doi:10.1371/journal.pone.0028477.g001

the assumption "missing completely at random". The only variable with missing data was age at interview. The Bayesian information criterion (BIC) was calculated for the different class solutions, where the model with the smallest BIC was selected as the best [29].

In this latent class framework, C denotes a latent variable, and U stands for the binary, categorical or count observed indicator variables. Let C denote a latent categorical variable with K classes, $C_i = (c_{i1}, c_{i2}, ..., c_{iK})$, where $c_{ik} = 1$ if individual i belongs to class k and zero otherwise. For U, conditional independence is assumed given c_i,

$$P(u_{i1}, u_{i2}, ..., u_{i\tau}|c_i) = P(u_{i1}|c_i)P(u_{i2}|c_i)P(u_{ui\tau}|c_i) \qquad (1)$$

As a first step we conducted the latent class analysis without any covariates in the model in order to understand the substantive interpretation of the latent classes. Then we included sex and age as auxiliary variables in the model. Conditional class specific means were evaluated for each auxiliary variable based on the estimated latent class model (option e in Mplus). We then tested the equality of means across the latent classes using the Wald Chi-Square test based on draws from the posterior probabilities. The magnitude of the mean differences between the classes was interpreted as an indicator for the strength of the prediction that the auxiliary variable influenced the class membership [30–32].

Genotyping and quality control

Genotyping was carried out by the Broad Institute Center for Genotyping and Analysis using the Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA, USA) separately for cases and controls to facilitate sharing of controls with the Genome-Wide Association Study of Schizophrenia (dbGaP; Study Accession: phs000021.v2.p1). The identity of the samples including gender identity had been checked at Rutgers University by genotyping a 24-SNP panel on the Sequenom iPLEX platform. A detailed description of the extensive quality control of the data before public release can be found elsewhere [20]. Even after those quality control measures, significant batch effects due to non-randomization of cases and controls were present in the publicly available data.

Therefore, in this analysis, we recalled the genotypes on the raw signal intensity measurements of the data using the corrected robust linear model with the maximum likelihood distance (CRLMM) algorithm and the BEAGLECALL algorithm [33,34]. CRLMM used the Oligo 1.6.0 package from Bioconductor 2.3. (http://www.bioconductor.org /download/oldrelease/BioC2.3/). All samples were run together in a single computational batch using default parameters. Recently the BEAGLECALL methodology was introduced to improve the accuracy of genotype calls through the use of haplotype phase information and linkage disequilibrium (LD) structure [35]. We used BEAGLECALL version 0.9.4, which invokes the haplotype phasing methods of BEAGLE version 3.1. [36]. BEAGLECALL requires a matrix of genotype probabilities for the 3 genotypes as well as normalized A and B allele intensities. CRLMM calls were used as a starting point for BEAGLECALL – all calls were made regardless of confidence, and the initial probabilities for each call were set to one, with the other two genotype probabilities set to zero. Autosomal allele intensities were extracted from the raw CEL files and quantile normalized [37] using Golden Helix SNP & Variation Suite (SVS) software (http://www.goldenhelix.com). Three iterations of BEAGLECALL were run with the recommended call thresholds of 0.8 for the first iteration, 0.96 for the second and 0.97 for the third. We used the default BEAGLECALL

Hardy-Weinberg quality control threshold of 10^{-6}. Genotype calls with confidence less than 0.97 were set as missing. Our analysis was limited to autosomal chromosomes only, since the BEAGLECALL software currently does not support genotype calls for X and Y chromosomal markers in male individuals. Starting with 868,157 autosomal calls from CRLMM, BEAGLECALL provided 796,664 high quality autosomal SNP calls. After eliminating SNPs with minor allele frequency (MAF)<0.001, the final dataset consisted of 728,331 autosomal SNPs. When association studies were performed across all combined samples with the case/control status, the Q-Q plots and Manhattan plots indicated little evidence of spurious associations due to batch effects (File S1). A total of 1,000 cases and 1,034 controls had genotypes and phenotypes that met quality control standards and those were included in the genetic analysis using the latent class membership probability as phenotype. We tested for association under the dominant, additive and recessive model. Association analyses were performed with the SVS software from Golden Helix. Correction for population stratification and outliers in the genotype data was performed with principal component analysis [38]. Q-Q plots were examined for each analysis (File S1) and cluster plots were manually examined for all significant findings. Genome-wide significant results were retested with 1,000 permutations. In addition, we re-genotyped all genome-wide significant SNPs with the TaqMan® SNP Genotyping Assay from Applied Biosystems run on the ABI 7900 Fast Real-Time PCR System (Life Technologies Corporation, Carlsbad, CA, USA) according to the published protocol (http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_042998.pdf). The genome browser used for the bioinformatics analysis was the Ensemble database, assembly GRCh37.p2, Feb 2009, Version 60.37e.

Results

Prevalence of co-morbid conditions

In the GAIN sample ascertained for genetic studies on bipolar disorder, co-morbid conditions were prevalent. The frequency of alcohol dependence, substance abuse and panic disorder in the GAIN sample reflected the well-known and characteristic comorbidity in bipolar disorder that has emerged in recent studies in the U.S., as well as worldwide. In this regard, this relatively small sample was very similar to larger population samples of bipolar disorder published in recent years [24,39]. However, the sample differed from published data in the frequency of specific and social phobias, ADHD and OCD, which were only slightly higher than in the general population. The prevalence of alcohol abuse was comparable to the prevalence in the National Comorbidity Survey Replication Study (NCS-R) (). In order to evaluate possible clustering of co-morbid conditions and unobserved subgroups of individuals characterized by co-morbidity profiles, we performed a latent class cluster analysis.

Latent class analysis

In the latent class analysis, subgroups of patients with significant differences in co-morbidity profiles became apparent. A three-class solution was selected as indicated by the Bayesian information criterion (BIC) (Figure 2, Table 1). The entropy of the 3-class solution was 0.694, indicating a fair separation of the latent classes. Class 1 (26% of the sample) was BPD patients, who had a high probability of endorsing substance abuse and psychotic symptoms. Alcohol dependence was absent in this group (Table 2, Table 3). Overall, 91% of individuals had received life-time diagnoses for two or more co-morbid conditions in addition to BPD, 56% were diagnosed with three or more additional co-morbid conditions, and 24% carried four or more co-morbid diagnoses in addition to

BPD (Table 4). In this class, 62% were females and 38% were males. The mean age was 41 years with a standard deviation of 12.6. Class 2 (25% of the sample) was BPD with alcohol dependence and substance abuse/dependence. In this group, forty-seven percent had been diagnosed with four or more co-morbid conditions and seventy-five percent of individuals carried three or more diagnoses (Table 4). This class consisted of slightly more males (61%) than females (39%). Regarding obsessive compulsive disorder (OCD), panic disorder (PD), social phobia (SP), eating disorder (ED), and attention-deficit hyperactivity disorder (ADHD), Class 1 and Class 2 were not clearly differentiated (Table 1, Table 2, Table 3). The probability of endorsing psychotic symptoms (PSYCH) was higher in Latent Class 1 (41%) compared to Latent Class 2 (27%). Individuals in Class 3 (49% of the sample) were diagnosed with BPD, but their probability of endorsing any co-morbid condition was very low. Six percent of individuals were diagnosed with two co-morbid conditions; no individual carried more than two co-morbid diagnoses in addition to BPD. In 54% of individuals BPD was the only psychiatric diagnosis (Table 4). This class consisted of equal numbers of males and females and the mean age was the highest of all classes. In the Wald test significant differences in age and sex were present in all between-class comparisons, with the exception of the comparison between Class 1 and Class 2 on age. Since the assumption of heterogeneity in the sample with respect to co-morbidity was supported by the data, we were interested in testing if bipolar disorder with alcohol dependence might have different genetic risk factors than bipolar disorder without alcohol dependence. Taking heterogeneity with regard to co-morbid conditions into account

would facilitate the identification of common genomic variants associated with specific sub-phenotypes.

Genome-wide association analysis

The genome-wide association analyses with the most likely latent class membership as phenotype revealed three SNPs that reached or approached the level of genome-wide significance, two SNPs were associated with Latent Class 1 and one was associated with Latent Class 2 (Figure 3, Figure 4, Table 5, Table 6). In patients with co-morbidity in the absence of alcohol dependence (Latent Class 1) highly significant associations under the recessive model were found with SNP rs1039002 ($p = 1.7 \times 10^{-8}$, Bonf. P = 0.017, Perm. P = 0.023) and SNP rs12563333 ($p = 5.9 \times 10^{-8}$, Bonf. P = 0.04, Perm. P = 0.001). Rs1039002 is located on chromosome 6q27, 5′ of the processed transcript *RP11-252P19-001* (OTTHUMG00000015988) and close to the gene encoding phosphodiesterase 10A (*PDE10A*). Rs12563333 is located within the transcribed gene sequence *RP11-410C4.4* (OTTHUMG00000037350) on chromosome 1q41 immediately upstream of the gene encoding the MAP/microtubule affinity-regulating kinase 1 (*MARK1*) (HGNC:6896). Homozygotes for the minor allele of these SNPs were present only in Class 1 and absent in all other classes or the controls (Table 6). Two SNPs came close to the level of genome-wide significance. SNP rs9493867 on chromosome 6q23.2 within the gene encoding serine/threonine-protein kinase (*Sgk1*) (HGNC:10810) was associated with Latent Class 1 under the recessive model ($p = 1.0 \times 10^{-7}$). Homozygotes for SNP rs9493867 are absent in the HapMap sample. SNP rs13220542 located on chromosome 6q15 was associated under the dominant model ($p = 9.0 \times 10^{-8}$). This common variant is located 3′ to the gene
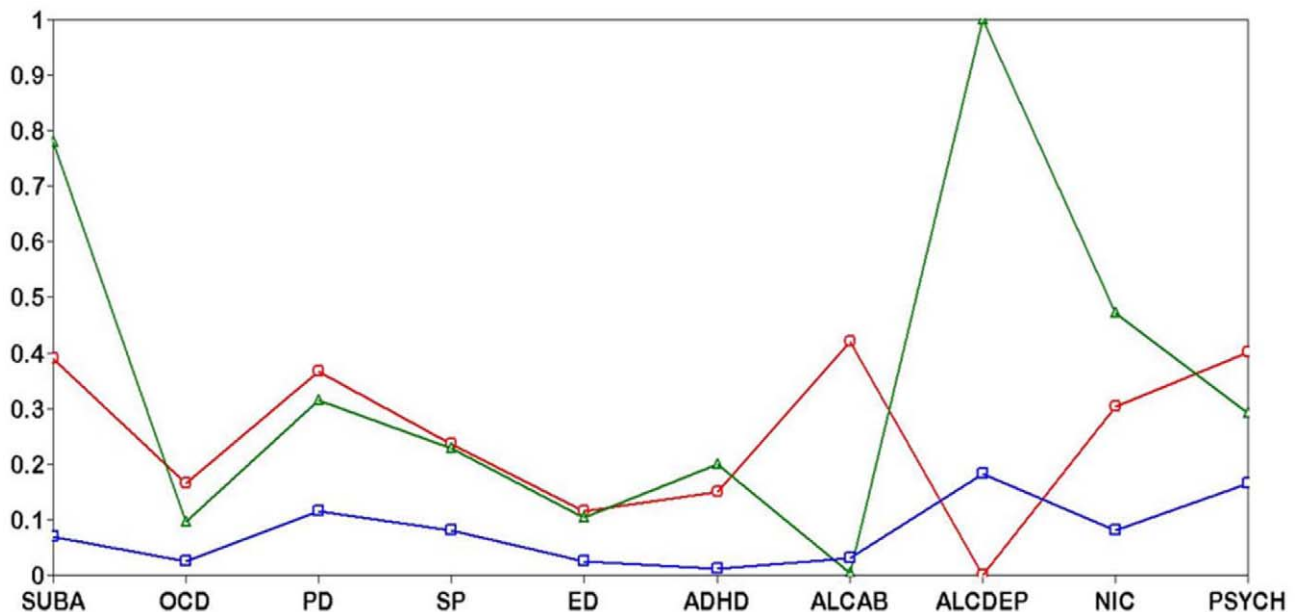
Figure 2. Latent class solution of co-morbid conditions in bipolar disorder (BPD). Co-morbid conditions might indicate heterogeneity in BPD. Latent class mixture modeling is a multivariate statistical approach to heterogeneity in data. The figure shows the co-morbidity profile of the three latent classes of BPD patients. Latent Class 1 (red line and ○ symbol) consisted of BPD patients with substance abuse and/or psychosis and a low probability of endorsing alcohol dependence. Class 2 (green line and Δ symbol) was BPD patients with a high probability of endorsing substance abuse and alcohol dependence. The probability of endorsing nicotine dependence in this class was the highest of all latent classes. Regarding obsessive compulsive disorder, panic disorder, social phobia, eating disorder, and attention-deficit hyperactivity disorder, Class 1 and Class 2 were not clearly differentiated. Individuals in Class 3 (blue line and □ symbol) were diagnosed with BPD, but their probability of endorsing any co-morbid condition was very low. The x-axis indicates the co-morbid conditions included in the LCA. The y-axis represents the probability of endorsing co-morbid conditions scaled from 0% to 100%. SUBA, substance abuse; OCD, obsessive compulsive disorder; PD, panic disorder; SP, social and specific phobia; ED, eating disorder; ADHD, attention-deficit hyperactivity disorder; ALCAB, alcohol abuse; ALCDEP, alcohol dependence; NIC, nicotine dependence; PSYCH, psychotic symptoms (presence of hallucinations and/or delusions).
doi:10.1371/journal.pone.0028477.g002

Table 2. Probability of endorsing a co-morbid condition by latent class.

	Latent Class 1	Latent Class 2	Latent Class 3
SUBA	0.39	0.78	0.07
OCD	0.17	0.1	0.03
PD	0.37	0.31	0.12
SP	0.24	0.23	0.08
ED	0.12	0.10	0.03
ADHD	0.15	0.2	0.01
ALCAB	0.42	0	0.03
ALCDEP	0	1	0.18
NIC	0.30	0.47	0.08
PSYCH	0.40	0.29	0.17

All estimates were significant with two-tailed P-values<0.001 comparing the probability of endorsing an item versus not endorsing an item. SUBA, substance abuse; OCD, obsessive compulsive disorder; PD, panic disorder; SP, social and specific phobia; ED, eating disorder; ADHD, attention-deficit hyperactivity disorder; ALCAB, alcohol abuse; ALCDEP, alcohol dependence; NIC, nicotine dependence; PSYCH, psychotic symptoms (presence of hallucinations and/or delusions).
doi:10.1371/journal.pone.0028477.t002

Discussion

In recent years, there has been increasing recognition of co-morbid conditions in mental disorders. A deeper understanding of this phenomenon and its risk factors could improve treatment and prevention. Our results indicate heterogeneity in bipolar disorder patients with regard to co-morbidity consistent with emerging evidence from other studies. In this study we have explored possible genetic risk factors that might shed some light on underlying patho-mechanisms. Latent class analysis indicated the existence of three distinct subgroups of patients characterized by co-morbidity profiles, one group with predominantly substance abuse and/or psychosis, one group in which alcohol dependence prevailed, and one group with very low probability for any co-morbid conditions. Addressing this heterogeneity led to the identification of several highly significant associations with SNPs in genome-wide association analyses. Our results suggest that phenotype heterogeneity in BPD might indicate genetic heterogeneity. However, the interpretation of our findings poses several problems. Since genome-wide association analyses can, by design, only point to regions of the genome associated with a disease phenotype, the actual functional variants often remain elusive. Genome-wide association studies are also underpowered to replicate association with rare variants. Therefore, it is now commonly agreed upon that re-sequencing approaches are necessary to follow-up on genome-wide association studies and to identify the underlying causal variants.

The associations found in this study point to several interesting genes and chromosomal regions that might justify re-sequencing approaches in order to find the underlying functional variants. The genomic variant rs1039002, which was associated with a subgroup of bipolar patients with substance abuse and/or psychotic symptoms, is located close to a transcribed genomic sequence with unknown function. The product of the closest gene sequence with known function is phosphodiesterase 10A (*PDE10A*), a protein involved in the elimination of the intracellular signaling molecules cAMP and cGMP. The highest expression levels of this gene are found in heart, brain, kidney and testes. In the brain, expression is particularly high in the medium spiny neurons of the striatum. Inhibitors of this phosphodiesterase have shown therapeutic potential for the treatment of psychotic symptoms in schizophrenia, as well as the treatment of Parkinson's disease, Huntington's disease,

encoding mitogen-activated protein kinase kinase kinase 7 (*MAP3K7*) (HGNC:6859). Both SNPs are located within gene regions.

BPD with high co-morbidity, substance abuse and alcohol dependence (Latent Class 2) was associated with SNP rs2727943 on chromosome 3p26.3 under the recessive model ($p = 3.3 \times 10^{-8}$, Bonf. P = 0.02, Perm. P = 0.03). SNP rs2727943 is a relatively common SNP (homozygotes for the minor allele were found in 3% of the HapMap samples) and it is not located in a gene region (Table 5, Table 6). The odds ratio of being a bipolar case for homozygote carriers of this SNP were 1.94; however, the odds ratio for being in Latent Class 2 compared to Latent Class 1, Latent Class 3 or controls was 4.9. No significant associations were found with Latent Class 3 or when all BPD cases combined were compared to the controls.

Table 3. Comparison of latent class profiles using odds ratios.

	LC1 compared to LC2		LC1 compared to LC3		LC2 compared to LC3	
	Estimate (S.E.)	P value[*]	Estimate (S.E.)	P value[*]	Estimate (S.E.)	P value[*]
SUBA	0.2 (0.1)	0.004	8.4 (3.9)	0.03	46.9 (19.9)	0.02
OCD	1.9 (0.6)	0.002	7.8 (6.8)	0.25	4.2 (3.4)	0.22
PD	1.3 (0.3)	0.000	4.4 (1.9)	0.02	3.5 (1.3)	0.006
SP	1.0 (0.3)	0.000	3.6 (1.7)	0.04	3.4 (1.4)	0.01
ED	1.1 (0.4)	0.001	5.1 (3.4)	0.14	4.5 (3.0)	0.14
ADHD	0.7 (0.2)	0.001	14.0 (13.5)	0.3	19.9 (18.7)	0.29
ALCAB	233 (340)	0.49	23.2 (25.0)	0.35	0.1 (0.2)	0.62
ALCDEP	N/A	N/A	N/A	N/A	N/A	N/A
NIC	0.5 (0.1)	0.000	4.9 (1.9)	0.01	10.2 (3.1)	0.001
PSYCH	1.6 (0.4)	0.000	3.4 (0.9)	0.000	2.1 (0.6)	0.000

*Two-tailed; LC, Latent Class; SUBA, substance abuse; OCD, obsessive compulsive disorder; PD, panic disorder; SP, social and specific phobia; ED, eating disorder; ADHD, attention-deficit hyperactivity disorder; ALCAB, alcohol abuse; ALCDEP, alcohol dependence; NIC, nicotine dependence; PSYCH, psychotic symptoms (presence of hallucinations and/or delusions).
doi:10.1371/journal.pone.0028477.t003

**Table 4.** Co-occurrence of co-morbid conditions.

| Diagnoses per individual in addition to BPD	Latent Class 1	Latent Class 2	Latent Class 3
Four or more disorders	24%	47%	0%
Three or more disorders	56%	75%	0%
Two or more disorders	91%	100%	6%

doi:10.1371/journal.pone.0028477.t004

addiction, and obsessive compulsive disorder. PDE10A inhibitors are now being tested in clinical trials [40,41].

RP11-410C4.4 is the closest expressed sequence to the SNP rs12563333, which is also associated with Latent Class 1 membership. This gene is located immediately 5′ to the serine/threonine-protein kinase gene *MARK1*. *MARK1* is highly expressed in brain and testes, with highest levels of expression found in the hippocampus. MARK1 phosphorylates microtubule-associated proteins and is involved in synaptic plasticity and dendritic trafficking [42].

Evidence for the involvement of this gene in autism has come from gene expression studies in postmortem brains, as well as genome-wide association studies [43]. Comparison of human *MARK1* with the mouse sequence showed significant differences and lack of conservation. It has therefore been hypothesized that *MARK1* could be involved in the development of higher cognitive functions that separate humans from mice [44]. However, it remains to be determined if rs12563333 is in linkage disequilibrium with any functional variants in *MARK1*.
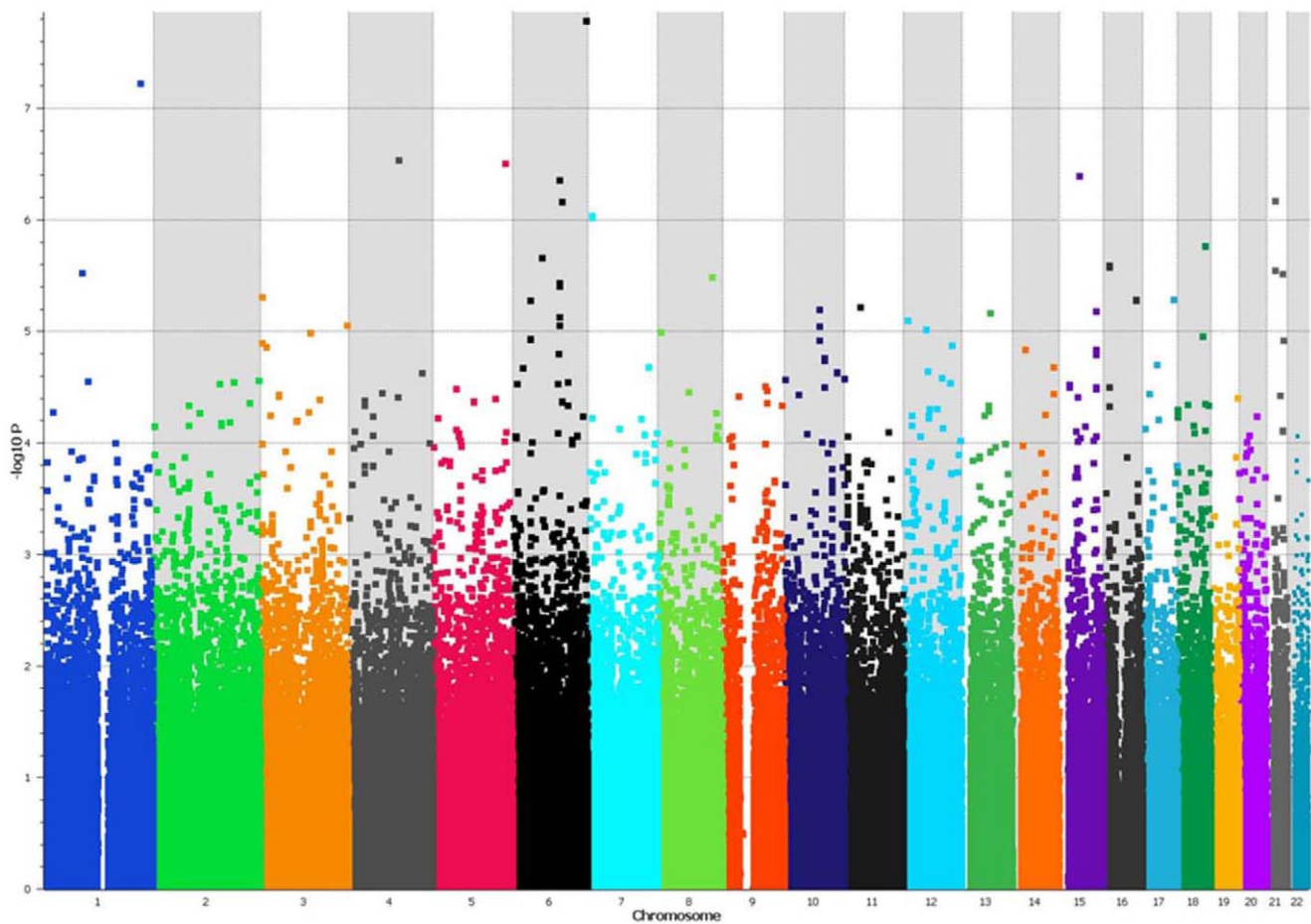
Figure 3. Genome-wide association analysis for Latent Class 1 under the recessive model. The Manhattan plot demonstrates the results of the genome-wide association analysis using the Latent Class 1 membership probability as phenotype. This subgroup of bipolar disorder patients was characterized by co-morbidity with substance abuse in 43% of individuals. In this group, 24% of individuals had been diagnosed with four or more co-morbid conditions in their lifetime. Alcohol abuse was prevalent in this patient group (52%), followed by substance abuse. Alcohol dependence was absent. Psychotic symptoms were found in 41% of individuals; about half of those had also used illegal substances. The x-axis depicts the position of the SNPs on the chromosomes with each chromosome shaded in a different color. The y-axis shows the −log10 p-value of the correlation trend test. The most significant finding was with the rare SNP rs1039002 on chromosome 6q27 (correlation trend test p = 1.7×10^{-8}), followed by rs12563333 on chromosome 1q41 (correlation trend test p = 5.9×10^{-8}). Within the chromosomal region indicated by SNP rs1039002 is the gene phosphodiesterase 10A. This gene has been implicated in the patho-physiology of psychosis in animal models.
doi:10.1371/journal.pone.0028477.g003
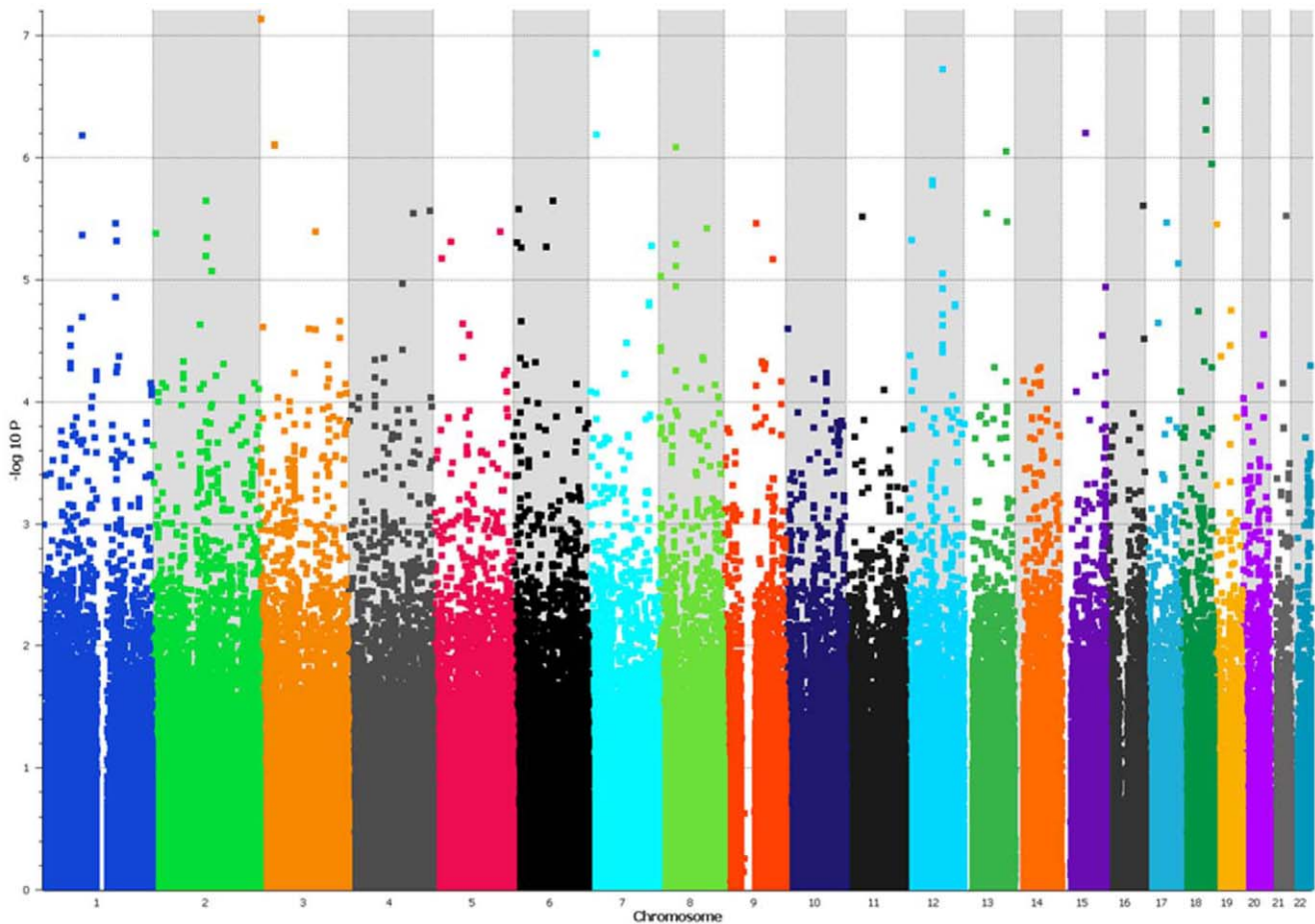
Figure 4. Genome-wide association analysis for Latent Class 2 under the recessive model. The Manhattan plot demonstrates the results of the genome-wide association analysis using the Latent Class 2 membership probability as phenotype. This subgroup of bipolar disorder patients was characterized by co-morbidity with alcohol dependence (100%) and substance abuse (80%). Forty-seven percent of individuals in this subgroup carried lifetime diagnoses of four or more co-morbid conditions. The x-axis depicts the position of the SNPs on the chromosomes, each chromosome shaded in a different color. The y-axis shows the −log10 p-value of the correlation trend test. The most significant association with Latent Class 2 membership probability was with SNP rs2727943 on chromosome 3p26.3 (correlation/trend test p = 3.0×10^{-8}). This common intergenic variant is located between the genes contactin-4 precursor (*BIG-2*) and contactin 6 (*CNTN6*). Deletions in this chromosomal region have been previously associated with autism spectrum disorders.
doi:10.1371/journal.pone.0028477.g004

Two SNPs that approached genome-wide significance are located in genes that are involved in response to environmental stresses, and therefore, might warrant further exploration. SNP

Table 5. Results of genome-wide case control association analyses using latent class membership as phenotype.

Class	SNP	Chr.	Position	Gene	Corr. P	Bonf. P
LC1	rs1039002	6q27	166155457	RP11-252P19.1	1.7×10^{-8}	0.01
	rs12563333	1q41	218724857	MARK1	5.9×10^{-8}	0.04
LC2	rs2727943	3p26.3	1897973	intergenic	3.0×10^{-8}	0.02

This table gives the SNP phenotype associations that reached or approached the genome-wide level of significance (after correction for multiple testing using the Bonferroni method). The position of the SNPs on the chromosomes is given in base pairs according to the Ensemble database, assembly GRCh37.p2, Feb 2009, Version 60.37e. SNP, single nucleotide polymorphism; LC1, Latent Class 1; LC2, Latent Class 2; Chr., chromosome; Corr P, p-value of the correlation-trend test, Bonf. P, Bonferroni corrected p-value.
doi:10.1371/journal.pone.0028477.t005

rs9493867 on chromosome 6q23.2 is located within the gene serum/glucocorticoid regulated kinase 1 (*Sgk1*). This gene encodes a serine/threonine-protein kinase that is involved in the activation of potassium, sodium and chloride channels [45]. In rats it appears to be involved in memory consolidation, spatial learning, and cellular stress response through negative regulation of the SK1-JNK1-MEKK1 pathway [46,47]. The second SNP is located in the 3′ region of the gene mitogen-activated protein kinase kinase kinase 7 (*MAP3K7*) on chromosome 6q15. This serine/threonine protein kinase is also involved in cell response to environmental stresses in related pathways through activation of MAPK8/JNK and the MAP2K4/MKK4 protein complex [48].

The most significant association with Latent Class 2 was within a region that has been found to be deleted in individuals with autistic features. SNP rs2727943 is located on chromosome 3p26.3 between the genes contactin-4 precursor (*BIG-2*) and the gene encoding the neural adhesion molecule contactin 6 (*CNTN6*). The protein products of these genes might play a role in the formation of axon connections in the developing brain [49,50].

A limitation of our study is, foremost, the small sample size. Re-sequencing of the most significant regions in a larger sample of

Table 6. Genotype counts for SNPs with genome-wide significant associations.

SNP	MAF	Depend. Average DD	Depend. Average Dd	Depend. Average dd	Genotype Count DD	Genotype Count Dd	Genotype Count dd
rs1039002	0.06	0.95	0.14	0.14	4	222	1801
rs12563333	0.03	0.92	0.17	0.14	4	109	1921
rs2727943	0.15	0.36	0.12	0.12	46	498	1485

This table shows the minor allele frequencies for each SNP, as well as the dependent average latent class membership probability for each genotype. SNP, single nucleotide polymorphism; MAF, minor allele frequency; DD, homozygotes for the minor allele; Dd, heterozygotes for the minor allele; dd, homozygotes for the major allele.

doi:10.1371/journal.pone.0028477.t006

bipolar patients would be desirable in order to evaluate all variants in the identified genomic regions and to determine their functional consequences. The high genotype quality of the re-called and re-genotyped significant SNPs, the fact that the identified rare variants were present only in cases and not in controls and strong evidence for the therapeutic potential of PDE10A inhibitors might justify further follow-up studies. Our analysis indicates three major points. 1. Rare variants might be important pathogenic factors in BPD. Rare variants in gene regions were the most significant signals in our analysis, and these variants were exclusively present in cases and not in controls. In our small sample and without replication it is difficult to claim certainty about the disease association; however, cumulative evidence for the implication of the identified genomic regions could justify further investigation. 2. Since the associated variants were very rare, a genome-wide association design might not be the most appropriate approach for replication. Future studies could focus on re-sequencing of the chromosomal regions in a sample of BPD patients and controls in order to identify all rare and possibly coding variants in the region that might play a role in BPD pathophysiology. The fact that all of our associations were found in or near genes that have been implicated in psychiatric disorders and even in psychosis underlines the importance of these significant associations. 3. Taking the heterogeneity of the phenotype into account when performing disease association studies might increase the power of detecting significant associations.

In summary, our paper describes a novel multivariate approach to the phenotype of BPD. We identified rare variants in regions of interest that might warrant further studies. However, we were unable to find common variants with functional consequences associated with BPD. Focused or genome-wide re-sequencing might be important for the identification of genetic risk factors in bipolar disorder.

Supporting Information

File S1 The supporting information provides the counts and proportions of the latent class solutions and the Bayesian information criteria, on which the latent class solution was selected. The QQ plot for the genome-wide association analysis under the recessive model is provided, followed by scatter plots for the genome-wide significant genotypes, first after the original genotyping, then after re-genotyping with the TagMan assay.
(DOC)

Author Contributions

Conceived and designed the experiments: BK CGL BOM. Performed the experiments: BK CGL. Analyzed the data: BK CGL. Contributed reagents/materials/analysis tools: BK CGL BOM. Wrote the paper: BK CGL BOM. Designed the software used in the analysis: CGL BOM.

References

1. Potash JB, Toolan J, Steele J, Miller EB, Pearl J, et al. (2007) The bipolar disorder phenome database: a resource for genetic studies. Am J Psychiatry 164: 1229–1237. Erratum in: Am J Psychiatry 164: 1616.
2. Faraone SV, Glatt SJ, Tsuang MT (2003) The genetics of pediatric onset bipolar disorder. Biol Psychiatry 53: 970–977.
3. Saunders EH, Scott LJ, McInnis MG, Burmeister M (2008) Familiality and diagnostic patterns of subphenotypes in the National Institutes of Mental Health bipolar sample. Am J Med Genet 147B: 18–26.
4. Potash JB, Chiu YF, MacKinnon DF, Miller EB, Simpson SG, et al. (2003) Familial aggregation of psychotic symptoms in a replication set of 69 bipolar disorder pedigrees. Am J Med Genet 116B: 90–97.
5. Kendler KS, Pedersen NL, Neale MC, Mathé A (1995) A pilot Swedish twin study of affective illness including hospital- and population ascertained subsamples: results of model fitting. Behav Genet 25: 217–232.
6. Kendler KS, Karkowski LM, Neale MC, Prescott CA (2000) Illicit psychoactive substance use, heavy use, abuse, and dependence in a US population-based sample of male twins. Arch Gen Psychiatry 57: 261–269.
7. McGuffin P, Rijsdijk F, Andrew M, Sham P, Katz R, et al. (2003) The heritability of bipolar affective disorder and the genetic relationship to unipolar depression. Arch Gen Psychiatry 60: 497–502.
8. Kieseppa T, Partonen T, Haukka J, Kaprio J, Lonnqvist J (2004) High concordance of bipolar I disorder in a nationwide sample of twins. Am J Psychiatry 161: 1814–1821.

9. Kendler KS, Aggen SH, Tambs K, Reichborn-Kjennerud T (2006) Illicit psychoactive substance use, abuse and dependence in a population-based sample of Norwegian twins. Psychol Med 36: 955–962.
10. Regier DA, Farmer ME, Rae DS, Locke BZ, Keith SJ, et al. (1990) Comorbidity of mental disorders with alcohol and other drug abuse. Results from the Epidemiologic Catchment Area (ECA) Study. JAMA 264: 2511–2518.
11. Barnett JH, Werners U, Secher SM, Hill KE, Brazil R, et al. (2007) Substance use in a population-based clinic sample of people with first-episode psychosis. Br J Psychiatry 190: 515–520.
12. Arseneault L, Cannon M, Witton J, Murray RM (2004) Causal association between cannabis and psychosis: examination of the evidence. Br J Psychiatry 184: 110–117.
13. Henquet C, Krabbendam L, de Graaf R, ten Have M, van Os J (2006) Cannabis use and expression of mania in the general population. J Affect Disord 95: 103–110.
14. Pickard BS, Knight HM, Hamilton RS, Soares DC, Walker R, et al. (2008) A common variant in the 3′UTR of the GRIK4 glutamate receptor gene affects transcript abundance and protects against bipolar disorder. Proc Natl Acad Sci USA 105: 14940–14945.
15. Baum AE, Akula N, Cabanero M, Cardona I, Corona W, et al. (2008) A genome-wide association study implicates diacylglycerol kinase eta (DGKH) and several other genes in the etiology of bipolar disorder. Mol Psychiatry 13: 197–207.

16. Sklar P, Smoller JW, Fan J, Ferreira MA, Perlis RH, et al. (2008) Whole-genome association study of bipolar disorder. Mol Psychiatry 13: 558–569.

17. Ferreira MA, O'Donovan MC, Meng YA, Jones IR, Ruderfer DM, et al. (2008) Collaborative genome-wide association analysis of 10,596 individuals supports a role for Ankyrin-G (ANK3) and the alpha-1C subunit of the L-type voltage-gated calcium channel (CACNA1C) in bipolar disorder. Nat Genet 40: 1056–1058.

18. Smith EN, Bloss CS, Badner JA, Barrett T, Belmonte PL, et al. (2009) Genome-wide association study of bipolar disorder in European American and African American individuals. Mol Psychiatry 14: 755–763.

19. Goodman LA (2002) Latent class analysis. In: Hagenaars JA, McCutcheon AL, eds. Applied latent class analysis. Cambridge: Cambridge University Press. pp 3–55.

20. GAIN Collaborative Research Group, Manolio TA, Rodriguez LL, Brooks L, Abecasis G, et al. (2007) New models of collaboration in genome-wide association studies: the Genetic Association Information Network. Nat Genet 39: 1045–1051.

21. Scott LJ, Muglia P, Kong XQ, Guan W, Flickinger M, et al. (2009) Genome-wide association and meta-analysis of bipolar disorder in individuals of European ancestry. Proc Natl Acad Sci USA 106: 7501–7506.

22. American Psychiatric Association (2000) Diagnostic and statistical manual of mental disorders. 4th edition. Washington D.C.: American Psychiatric Association.

23. Nurnberger JI, Jr., Blehar MC, Kaufmann CA, York-Cooler C, Simpson SG, et al. (1994) Diagnostic interview for genetic studies. Rationale, unique features, and training. NIMH Genetics Initiative. Arch Gen Psychiatry 51: 849–859.

24. Merikangas KR, Akiskal HS, Angst J, Greenberg PE, Hirschfeld RM, et al. (2007) Lifetime and 12-month prevalence of bipolar spectrum disorder in the National Comorbidity Survey Replication. Arch Gen Psychiatry 64: 543–552. Erratum in: Arch Gen Psychiatry 64: 1039.

25. Kerner B, Brugman DL, Freimer NB (2007) Evidence of linkage to psychosis on chromosome 5q33-34 in pedigrees ascertained for bipolar disorder. Am J Med Genet B Neuropsychiatr Genet 144B: 74–78.

26. Muthen LK, Muthen BO (1998–2009) Mplus User's Guide. Third Edition. Los Angeles: Muthen & Muthen.

27. Muthén B, Shedden K (1999) Finite mixture modeling with mixture outcomes using the EM algorithm. Biometrics 55: 463–469.

28. Little RJ, Rubin DB (2002) Statistical analysis with missing data. Second edition. New York: John Wiley & Sons.

29. Nylund KL, Asparouhov T, Muthen B (2007) Deciding on the number of classes in latent class analysis and growth mixture modeling. A Monte Carlo simulation study. Structural Equation Modeling 14: 535–569.

30. Asparouhouv T, Muthén BO (2007) Wald test of mean equality for potential latent class predictors in mixture modeling. From: http://www.statmodel.com/download/MeanTest1.pdf.

31. Wang CP, Brown CH, Bandeen-Roche K (2005) Residual diagnostics for growth mixture models: Examining the impact of preventive intervention on multiple trajectories of aggressive behavior. J Am Stat Association 100: 1054–1076.

32. Bandeen-Roche K, Miglioretti DL, Zeger SL, Rathouz PJ (1997) Latent variable regression for multiple discrete outcomes. J Am Stat Association 92: 1375–1386.

33. Carvalho B, Bengtsson H, Speed TP, Irizarry RA (2007) Exploration, normalization, and genotype calls of high-density oligonucleotide SNP array data. Biostatistics 8: 485–499.

34. Lin S, Carvalho B, Cutler DJ, Arking DE, Chakravarti A, et al. (2008) Validation and extension of an empirical Bayes method for SNP calling on Affymetrix microarrays. Genome Biology 9: R63.

35. Browning B, Yu Z (2009) Simultaneous genotype calling and haplotype phase inference improves genotype accuracy and reduces false positive associations for genome-wide association studies. Am J Hum Genet 85: 847–861.

36. Browning SR, Browning BL (2007) Rapid and accurate haplotype phasing and missing data inference for whole genome association studies using localized haplotype clustering. Am J Hum Genet 81: 1084–1097.

37. Bolstad BM, Irisarry RA, Astrand M, Speed TP (2003) A Comparison of Normalization Methods for High Density Oligonucleotide Array Data based on Variance and Bias. Bioinformatics 19: 185–193.

38. Price AL, Alkes L, Patterson NJ, Plenge RM, Weinblatt ME, et al. (2006) Principal components analysis corrects for stratification in genome-wide association studies. Nat Genet 38: 904–909.

39. Merikangas KR, Jin R, He JP, Kessler RC, Lee S, et al. (2011) Prevalence and correlates of bipolar spectrum disorder in the world mental health survey initiative. Arch Gen Psychiatry 68: 241–251.

40. Siuciak JA, Chapin DS, Harms JF, Lebel LA, McCarthy SA, et al. (2006) Inhibition of the striatum-enriched phosphodiesterase PDE10A: a novel approach to the treatment of psychosis. Neuropharmacology 51: 386–396.

41. Schmidt CJ, Chapin DS, Cianfrogna J, Corman ML, Hajos M, et al. (2008) Preclinical characterization of selective phosphodiesterase 10A inhibitors: a new therapeutic approach to the treatment of schizophrenia. J Pharmacol Exp Ther 325: 681–690.

42. Drewes G, Ebneth A, Preuss U, Mandelkow EM, Mandelkow E (1997) MARK, a novel family of protein kinases that phosphorylate microtubule-associated proteins and trigger microtubule disruption. Cell 89: 297–308.

43. Maussion G, Carayol J, Lepagnol-Bestel AM, Tores F, Loe-Mie Y, et al. (2008) Convergent evidence identifying MAP/microtubule affinity-regulating kinase 1 (MARK1) as a susceptibility gene for autism. Hum Mol Genet 17: 2541–2551.

44. Caenepeel S, Charydczak G, Sudarsanam S, Hunter T, Manning G (2004) The mouse kinome: discovery and comparative genomics of all mouse protein kinases. Proc Nat Acad Sci 101: 11707–11712.

45. Rajamanickam J, Palmada M, Lang F, Boehmer C (2007) EAAT4 phosphorylation at the SGK1 consensus site is required for transport modulation by the kinase. J Neurochem 102: 858–866.

46. Tsai KJ, Chen SK, Ma YL, Hsu WL, Lee EHY (2002) Sgk, a primary glucocorticoid-induced gene, facilitates memory consolidation of spatial learning in rats. Proc Nat Acad Sci 99: 3990–3995.

47. Kim MJ, Chae JS, Kim KJ, Hwang SG, Yoon KW, et al. (2007) Negative regulation of SEK1 signaling by serum- and glucocorticoid-inducible protein kinase 1. EMBO J 26: 3075–3085.

48. Chen ZJ (2005) Ubiquitin signalling in the NF-kappaB pathway. Nat Cell Biol 7: 758–765.

49. Fernandez T, Morgan T, Davis N, Klin A, Morris A, et al. (2004) Disruption of contactin 4 (CNTN4) results in developmental delay and other features of 3p deletion syndrome. Am J Hum Genet 74: 1286–1293.

50. Roohi J, Montagna C, Tegay DH, Palmer LE, DeVincent C, et al. (2009) Disruption of contactin 4 in three subjects with autism spectrum disorder. J Med Genet 46: 176–182.

51. Kessler RC, Berglund P, Demler O, Jin R, Merikangas KR, et al. (2005) Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. Arch Gen Psychiatry 62: 593–602. Erratum in: Arch Gen Psychiatry 62: 768. Merikangas, Kathleen R [added].