Genetic variation in the miR-708 gene and its binding targets in bipolar disorder

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Objective: rs12576775 was found to be associated with bipolar disorder (BD) in a genome-wide association study (GWAS). The GWAS signal implicates genes for the microRNAs miR-708 and miR-5579 and the first exon of the Odd Oz/ten-m homolog 4 gene (ODZ4). In the present study, miR-708, its surrounding region, and its targets were analyzed for potential BD-associated functional variants.

Methods: The miR-708 gene and surrounding regions were screened for variation using high-resolution melting (HRM) analysis in 1099 cases of BD, followed by genotyping of rare variants in an enlarged sample of 2078 subjects with BD, 1303 subjects with schizophrenia, and 1355 healthy controls. Whole-genome sequencing data from 99 subjects with BD were analyzed for variation in potential miR-708 binding sites. The minor allele frequencies (MAFs) of these variants were compared with those reported in reference individuals.

Results: Three variants detected by HRM were selected to be genotyped. rs754333774 was detected in three cases of BD, two cases of schizophrenia, and no controls. This variant is located 260 base pairs upstream from miR-708 and may play a role in controlling the expression of the miR. Four variants were identified in miR-708 targets binding sites. The MAFs of each of these variants were similar in BD and reference samples.

Conclusions: We report a single recurrent variant located near the miR-708 gene that may have a role in BD and schizophrenia susceptibility. These findings await replication in independent cohorts, as do functional analyses of the potential consequences of this variant.

KEYWORDS
bipolar disorder, microRNA, miR-708, sequencing, susceptibility, variation

Bipolar disorder (BD) is a common disease with a worldwide average population prevalence of 1.4%, which rises to 2.4% if bipolar spectrum disorders are included.1 BD is strongly familial, with a 10-fold increase in risk to the relatives of BD probands.2 Estimates of the heritability of BD range from 79% to 93%.3-6 Genome-wide association studies (GWAS) have identified a number of common polymorphisms which are convincingly associated with BD.7,8 The Psychiatric GWAS Consortium Bipolar Disorder (PGC-BD) working group performed a combined analysis of GWAS data from 7481 individuals with BD and 9250 controls. The same group also tested 34 single-nucleotide polymorphisms (SNPs), that were associated with P-values of <5 × 10−5 in the discovery sample, in an independent replication cohort of 4496 cases with BD and 42 422 controls. The combined analysis of the discovery and replication samples confirmed genome-wide significant (GWS) evidence of an association between BD and the calcium voltage-gated channel subunit alpha1C gene (CACNA1C), and identified a GWS intrinsic variant in the Odd Oz/ten-m homolog 4 gene (ODZ4; also known as tenascin-M4 [TENM4]), rs12576775...
Fiorentino et al. showed further evidence for an association between BD and the same SNP \((P=6.20 \times 10^{-7}\text{ and } 4.46 \times 10^{-7}, \text{ respectively})\). A large intergenic region distal to rs12576775 and a proximal genomic region with a high recombination rate in intron 1 of ODZ4 effectively restrict the BD association region to the genomic interval that includes the first exon of ODZ4 and the microRNA (miRNA) genes miR-708 and miR-5579. Of the two miRNA genes, only miR-708 has been experimentally validated and it is known to be expressed in the human nervous system.

The role of miRNAs in psychiatric disorders has recently become prominent as a result of findings in schizophrenia (SCZ). The PGC-SCZ working group reported GWS with a SNP association in the third intron of the primary transcript of miR-137. There is evidence that genetic variation in this gene influences the negative symptoms of SCZ. In addition to the findings with miR-137, several other microRNAs have also been implicated in susceptibility to SCZ, including an association with miR-206 \((r=17578796)\) in a Scandinavian sample and with miR-30e \((rs112439044)\) in a Han Chinese sample. Ultra-rare variants in the precursor or mature microRNA sequences have been reported in American males suffering from SCZ. An enrichment of rare copy number variations (CNVs) overlapping with microRNAs has also been reported in SCZ, with 25 microRNAs impacted by rare CNVs in two or more unrelated subjects. Several studies have investigated polymorphic miRNA binding sites in target genes related to SCZ. These include rs3822674, which is located in the 3’ untranslated region (UTR) of the gene encoding complexin II (CPLX2) and is predicted to interfere with repression of CPLX2 expression by miR-49R16, rs1130354 in the 3’ UTR of dopamine receptor D2 gene (DRD2) was reported to interfere with miR-326-mediated repression of DRD2 expression and rs11122396 in the 3’ UTR of the disrupted-in-schizophrenia-1 (DISC1) gene disrupted miR-135b-5p-mediated control of DISC1 expression.

The evidence that implicates miRNAs in the etiology of BD is not as strong as it is for SCZ. However, recent data have provided increasing support for the hypothesis that miRNAs also play a role in the etiology of BD. The largest GWAS of BD to date, a SNP in an intergenic region flanking ODZ4 and the microRNA (miRNA) genes miR-708 and miR-5579. Of the two miRNA genes, only miR-708 has been experimentally validated and it is known to be expressed in the human nervous system.

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1 | MATERIALS AND METHODS

1.1 | Subjects

The study included 2078 research subjects with bipolar I (BD-I) or bipolar II disorder and 1303 research subjects with SCZ. The University College London (UCL) control sample included 1355 subjects, comprising 875 screened subjects who had no first-degree family or personal history of psychiatric illness, and an additional 480 unscreened normal British subjects obtained from the European Collection of Cell Cultures. National Health Service (NHS) multicentre research ethics approval was obtained. All participants provided informed written consent. Ancestry screening was used as a selection criterion for the inclusion of cases. Samples were included if at least three out of four grandparents were English, Irish, Scottish, or Welsh, and if the fourth grandparent was non-Jewish European, before the EU enlargement in 2004. Participants with BD were interviewed using the lifetime version of the Schizophrenia and Affective Disorder Schedule. Participants fulfilling the research diagnostic criteria for BD were included. An analogous protocol was used to recruit participants with SCZ. Blood or saliva samples were collected for all participants. DNA from blood samples was extracted using a standard phenol-chloroform method and from saliva samples using the Oragene protocol for DNA extraction (DNA Genotek, Ottawa, ON, Canada).

1.2 | Detection and evaluation of new variants

High-resolution melting (HRM) variant screening was used to identify BD susceptibility variants 300 base pairs (bp) upstream and
downstream from the mir-708 gene (chr11:79112766-79113453, GRCh37/hg19).

This method of variant analysis allows cost-efficient detection of rare variations in large numbers of samples. HRM is particularly amenable to regions not efficiently targeted by established next generation sequencing selection panels such as introns and UTRs.

HRM was performed using three primer pairs in 1099 cases with BD. Reactions were carried out on a LightCycler 480 (Roche, Burgess Hill, UK). Primer sequences and reagents are shown in Table S1.

Samples with abnormal HRM curves were then sequenced using the BigDye terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Warrington, UK) on an ABI 3730xL DNA Analyzer (Applied Biosystems). Sequencing data were analyzed using the Staden Package (http://staden.sourceforge.net/). The reference minor allele frequency (MAF) for the general population was evaluated in the data from the 1000 Genomes (1000G) project, in the Exome Aggregation Consortium (ExAC), Cambridge, MA, USA (http://exac.broadinstitute.org [accessed January 2016]), from the Scripps Wellderly study (n = 534) and from two population cohorts in the UK10K study (UK10K ALSPAC [Avon Longitudinal Study of Parents and Children] and UK10K TWINS [TwinsUK]; MAF, minor allele frequency; nd, not detected.

For the ExAC data, it is important to take into consideration the presence of BD and SCZ diagnoses in the database. This did not affect our study because the reported MAFs of the miR-708 variants selected did not exceed our exclusion threshold for genotyping. Bioinformatic analysis to determine the potential functional effect of SNPs was carried out using the University of California, Santa Cruz genome browser (http://genome.ucsc.edu/), Alibaba 2 (http://www.gene-regulation.com/pub/programs/alibaba2/index.html) and PROMO using version 8.3 of TRANSFAC (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3).

Only variants with a MAF lower than 0.01 in the general population were considered for further analysis.

### 1.3 Genotyping

Genotyping of the selected SNPs was performed in an enlarged sample of 2078 cases with BD and 1355 ancestrally matched controls. It was performed in-house using the allele-specific polymerase chain reaction (PCR), using KASPar reagents (LGC Genomics, Hoddesdon, UK) on a LightCycler 480 real-time PCR machine. Allele-specific primers were designed for each of the SNPs using Primer Picker (KBiosciences, LGC Genomics, Hoddesdon, UK; primer sequences are listed in Table S2). The variants were also genotyped in 1305 SCZ samples. For all SNPs, 97% of samples were successfully genotyped. Genotyping for each heterozygote sample was repeated at least twice. All of these data were analyzed to confirm Hardy-Weinberg equilibrium. Allelic associations for SNPs were performed using Fisher’s exact test. Significance values shown for all analyses are uncorrected for multiple testing, and a cut-off significance value of P<.05 was used.

### TABLE 1

| SNP         | Position in chr11 | Position compared to miR-708 | 1000G MAF | Eur 1000G MAF | Exac MAF | Eur Exac MAF | Wellderly MAF | UK10K MAF | Position compared to miR-708 |
|-------------|-------------------|-----------------------------|-----------|---------------|-----------|-------------|---------------|------------|-----------------------------|
| rs754333774 | 79113407          | Upstream                    | G > A     | nd            | nd        | nd          | 0.000 94      | nd         | Upstream                    |
| rs768049399 | 79113040          | Downstream                  | A > C     | 0.082         | 0.082     | nd          | 0.069 29      | 0.063 54   | Downstream                  |

### TABLE 2

| Position on ch11 | Position compared with miR-708 | Change | N      | Genotype counts | MAF   |
|------------------|-------------------------------|--------|--------|-----------------|-------|
| rs754333774      | 79113407                      | Upstream | G > A | 2041 | BD | 0/3/2038 | 0.0007 |
|                  |                               |         |       | 1261 | SCZ | 0/2/1259 | 0.0008 |
|                  |                               |         |       | 3302 | BD + SCZ | 0/5/3297 | 0.0007 |
|                  |                               |         |       | 1310 | CTRL | 0/0/1310 | 0 |
| rs768049399      | 79113040                      | Downstream | A > C | 2001 | BD | 0/1/2000 | 0.0002 |
|                  |                               |         |       | 1274 | SZ | 0/0/1274 | 0 |
|                  |                               |         |       | 3275 | BD + SCZ | 0/1/3274 | 0.0001 |
|                  |                               |         |       | 1308 | CTRL | 0/1/1307 | 0.0004 |

BD, bipolar disease; CTRL, control; MAF, minor allele frequency; N, total number; SCZ, schizophrenia. The genomic reference sequence used is GRCh37/hg19; change: the nucleotide change indicated is on the negative strand; genotype count: number of homozygotes for the minor allele/heterozygotes/homozygotes for the major allele.
Detection and evaluation of variants in the has-miR-708 binding sites

Whole-genome sequencing (WGS) was performed on 99 of the subjects with BD-I selected from our BD cohort first on the basis of individuals with a strong positive family history of BD or bipolar spectrum disorder. Where the strength of the family history was tied, individuals with the earliest age at onset were selected. The mean age of onset for the cases selected for sequencing was 21.55 (standard deviation [SD] 8.90), and this was significantly lower than the total cohort (P = .0124). The genomic DNA was sequenced using 100 bp paired-end reads on a Hi-Seq 1000 (Illumina Inc., San Diego, CA, USA). Sequence data alignment to the National Center for Biotechnology Information human reference genome 37.1 (hg19) and variant calling was performed using the CASAVA 1.8.2 pipeline at Illumina (http://support.illumina.com/sequencing/sequencing_software/casava.html). The sequence data from these individuals was further analyzed and annotated using iGAP (Knome Inc., Boston, MA, USA). The BD WGS data was screened for variants in miR-708 binding sites (15 bp upstream and 1 bp downstream the microRNA seed) predicted by Targetscan 6.2: June 2012 (http://www.targetscan.org/).

1.4 | Detection and evaluation of variants in the has-miR-708 binding sites

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1.5 | Imputation and analysis of BD GWAS data

UCL BD and control GWAS data were included in the PGC-BD dataset. These data have been subjected to a standardized quality control and imputation pipeline (https://sites.google.com/a/broadinstitute.org/ricopili/). Association testing was performed using PLINK2.

2 | RESULTS

2.1 | Variant selection and genotyping

Three single nucleotide variants were detected by HRM analysis across the region selected to be analyzed: rs56158925, rs754333774, and rs768049399 (Table 1). rs56158925 is located 200 bp downstream from mir-708. This variant has an overall MAF of 0.082 in the general population of the 1000G Project, and the same frequency in the data from the European subpopulation of the project and with a slightly lower frequency in the Wellderly and UK10K cohorts data (Table 1). The other two variants, rs754333774 and rs768049399, are located 260 bp upstream and 27 bp downstream, respectively, of the mir-708 gene. rs768049399 was annotated in the ExAC database, with an overall MAF of 6.4 × 10−5 and 9.7 × 10−5 in the European subpopulation of the project but was not reported in data from the 1000G project, Wellderly subjects or in the UK10K cohorts. rs754333774 was also not reported in the 1000G project or in the UK10K cohorts however it was detected in one of 534 individuals from the Wellderly sample (Table 1). The estimated MAF of this variant in these two datasets is 0.0017.

Genotyping assays were designed for the two SNPs with MAFs lower than 0.01 in the general population. Genotyping was conducted in the complete UCL case–control sample, including cases with BD and SCZ. The rs768049399 variant allele was detected in one case with BD and in one control (Table 2). The rs754333774 variant allele
was detected in three cases with BD, two cases with SCZ, and no controls (Table 2).

Bioinformatic transcription factor binding analysis of the effect of rs75433774 did not identify consistent predictions for altered binding. By contrast, the variant allele of rs768049399 was predicted to destroy a binding site for the CCAAT/enhancer-binding protein beta transcription factor and to create a binding site for five transcriptional factors: hepatocyte nuclear factor (HNF)-3a; TATA box-binding protein; homeobox protein D8; HNF-1C; and HNF1-B.

2.2 | Variants in miR-708 binding sites

Targetscan 6.2 predicted 4377 transcripts with miR-708 binding sites (including many transcripts with overlapping 3’ UTRs), with a total of 381 conserved sites and 4999 poorly conserved sites (including many overlapping sites). These binding sites were analyzed in WGS from 99 subjects with BD for possible variants. Only four nonreference (GRCh37/hg19) allelic variants were identified. The variants were located in the genes encoding the following proteins: apolipoprotein C-III (APOC3); polypeptide N-acetylgalactosaminyltrnasferase 13 (GALNT13); ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminidase alpha-2,6-sialyltransferase 4 (ST6GALNAC4); and solute carrier family 22 member 23 (SLC22A3) (Table 3). All four variants are known common variants that have been annotated on The Single Nucleotide Polymorphism Database (dbSNP). rs5128 in APOC3 is 1 bp upstream from the miR-708 seed sequence, while the other three variants rs707082, rs1043026, rs5873874, located in GALNT13, ST6GALNAC4 and SLC22A3, respectively, are within the seed sequence of the miR-708 binding sites.

The allele frequencies of the four variants in the BD WGS samples were similar to those reported in both the entire sample and the European-only sample from the 1000G project (Table 3). Data were available only for rs5128 in the ExAC database, and these were similar to those of the BD WGS sample. The 3’ UTRs of the remaining genes were not included in the exome sequencing data in the ExAC database. Association testing of GWAS data for the first 491 UCL BD cases and 495 UCL controls, imputed using the 1000G project data as a reference panel, did not show evidence for involvement of rs5128 or rs707082 with BD (P=.5214 and .4286, respectively); rs1043026 and rs5873874 were not reliably imputed (Table S3).

3 | DISCUSSION

The potential role of miRNAs in psychiatric disorders has recently been highlighted by GWS association findings with SNPs within miR-137 and SCZ.13 Several reports have implicated microRNAs in BD, both genetically and biologically.11,23,25,26,39,40 The PGC-BD group performed a combined analysis of GWAS data that identified a GWS intronic variant in ODZ4 (TENM4), rs12576775.9 This association has been confirmed by two other independent studies.10,11 The two variants in miR-708 with a MAF lower than 0.01 (rs768049399 and rs75433774) were genotyped in our BD and control cohorts. The strong prior findings with miRNA genes in SCZ led us to genotype the selected variants in our SCZ cohort, in addition to the subjects with BD and control subjects.

The rare allele of rs75433774 was found only in individuals with BD or SCZ; in our own data and, this finding was not statistically significant. This variant was detected in a single individual from the Wellderly sample but was absent in a substantially larger number of individuals from the UK10K cohorts. Together the frequency of this variant in the combined UK10K cohorts and Wellderly sample was lower than that in our BD and SCZ case cohorts. This finding is intriguing but requires validation in large independent samples of subjects with BD and or SCZ and healthy controls. No support for a role for rs768049399 in BD (and/or SCZ) was found.

In order for microRNAs to regulate gene expression, they need to bind to a target region, normally in the 3’ UTR of a gene. It is important that nucleotides 2–8 of the miRNA have a perfect match with their target, and this region is defined as a seed region. The miRNA binding site and, more specifically, the seed regions are well conserved; they are more likely to reside within those targets in the transcriptome with lower variant densities, especially target regions in which nucleotides have low mutation frequencies.41 Analysis of enrichment of GWS signals for miRNA genes and their putative target regions implicated miR-137 and its pathway in SCZ in different populations.42,43 With the advent of WGS, it is therefore possible to implicate not only regions, but also specific variants, in disease. WGS data for 99 BD subjects were analyzed for possible variants in the miR-708 binding sites predicted by Targetscan. This analysis identified four miR-708 binding site variants, each of which was located in brain-expressed genes, including APOC3 and SLC22A23. Both of these genes have been reported to be associated with the response to antipsychotic drug treatment. Variants in APOC3 have been implicated in variations of cholesterol and triglyceride levels in SCZ patients treated with clozapine and olanzapine;44 variants in SLC22A23 have been reported to be associated with QT prolongation in a GWAS of subjects with SCZ treated with quetiapine.45 However, comparison of the allele frequency data of the four miR-708 binding site variants between the 99 WGS subjects with BD and those in reference databases suggested that none of the miR708 3’UTR binding site variants were likely to be etiological. Indeed, analysis of imputed data from our own BD case–control sample did not show evidence for an association for the two variants that were reliably imputed. One of the challenges of studying miRNAs is the complexity of miRNA-target gene networks. The study of these networks requires systematic computational prediction of miRNA-target gene interactions. However, there is a certain degree of imprecision in the predictions made by current miRNA target gene algorithms. This imprecision can be observed in the inconsistent prediction scores that are obtained from the different algorithms. Most algorithms do not consider whether miRNA genes and their potential target are co-expressed and this could be an effective way of improving predictions. Furthermore both miRNAs and their targets are located in areas of the genome that are not often covered by whole
exome sequencing. However, as larger scale WGS datasets become available, variation in 3'UTRs and intronic/intragenic regions will be better described, making it possible to begin to understand the functional role of these regions.

4 | CONCLUSIONS

In summary, we report a single recurrent variant located close to in the miR-708 gene that may have a role in susceptibility to BD and/or SCZ. This finding awaits replication in independent SCZ and BD cohorts, as does a functional analysis of the potential consequences of this variant.

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DISCLOSURES

The authors of this paper do not have any commercial associations that might pose a conflict of interest in connection with this manuscript.

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