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Genome wide significant associations in schizophrenia to *ITIH3/4, CACNA1C* and *SDCCAG8*, and extensive replication of associations reported by the Schizophrenia PGC

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Abstract

The Schizophrenia Psychiatric Genome-Wide Association Consortium (PGC) highlighted 81 single nucleotide polymorphisms (SNPs) with moderate evidence for association to schizophrenia. After follow up in independent samples, 7 loci attained genome wide significance (GWS), but multi-locus tests suggested some SNPs that did not do so represented true associations. We tested 78 of the 81 SNPs in 2640 individuals with a clinical diagnosis of schizophrenia attending a clozapine clinic (CLOZUK), 2504 cases with a research diagnosis of bipolar disorder, and 2878

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The members of WTCCC2 and WTCCC+ are given in supplementary material

Conflict of Interest

none

controls. In CLOZUK, we obtained significant replication to the PGC-associated allele for no fewer than 37 (47%) of the SNPs, including many prior GWS MHC SNPs as well as 3/6 non-MHC SNPs for which we had data that were reported as GWS by the PGC. After combining the new schizophrenia data with those of the PGC, variants at three loci (*ITIH3/4*, *CACNA1C* and *SDCCAG8*) that had not previously been GWS in schizophrenia attained that level of support. In bipolar disorder, we also obtained significant evidence for association for 21% of the alleles that had been associated with schizophrenia in the PGC. Our study independently confirms association to 3 loci previously reported to be GWS in schizophrenia and identifies the first GWS evidence in schizophrenia for a further 3 loci. Given the number of independent replications and the power of our sample, we estimate 98% (C.I. 78–100%) of the original set of 78 SNPs represent true associations. We also provide strong evidence for overlap in genetic risk between schizophrenia and bipolar disorder.

Keywords

association; psychosis; ITIH3/4; CACNA1C; SDCCAG8

Introduction

Epidemiological, pharmacological, and neurobiological studies have advanced general understanding of schizophrenia[1, 2] but the disorder is poorly understood at the molecular and cellular level. Genetic epidemiology has documented high heritability[3, 4]. Through the application of genome wide association technology, strongly implicated common risk variants with small effects have been identified,[5–10] as have low-frequency structural chromosomal abnormalities known as copy number variants (CNVs) which confer high risk of the disorder[11, 12]. The findings are consistent with a mixed model of schizophrenia.[13]

The schizophrenia group of the PGC reported[5] a mega-analysis of GWAS datasets comprising 9394 cases and 12462 controls of European ancestry (PGC Stage 1) with follow up of the top 81 SNPs (from regions where at least one SNP had $p<2\times10^{-5}$ in Stage 1) in up to 8442 independent cases and 21397 controls (PGC Stage 2). Meta-analysis of all individuals identified 7 loci where the association was genome-wide significant (GWS), 5 of which were novel. A sign test for consistency between Stages 1 and 2 was highly significant ($p<10^{-6}$), the same allele being over-represented in cases in both stages for 49 of 59 non-MHC alleles (the authors excluded MHC SNPs because the long range linkage disequilibrium (LD) in that region makes it difficult to be confident that multiple associated SNPs are independent). The result of the sign test implies the 81 SNPs from Stage 1 included true risk loci that the study was underpowered to identify at GWS. Thus, schizophrenia is like most complex disorders; extremely large samples are required to capture a substantial proportion of the common risk variants involved. However, unlike many other phenotypes, it has been customary in psychiatry that case inclusion be contingent upon diagnoses derived from lengthy research interviews, clinical note reviews and the application of consensus diagnoses based upon operationalized research diagnostic criteria. As the costs of genome analysis have decreased, the cost of this process has become a limiting step for exploiting GWAS technology.

The imposition of rigorous standards stems from concern that clinician diagnosed samples may be too heterogeneous. This view was supported by studies showing marked discrepancies in diagnostic practices between the US and Europe.[5] Nevertheless, perhaps as a consequence of the introduction of operationalized criteria, clinician diagnoses of schizophrenia has been demonstrated to have high specificity and positive predictive values when validated against research based approaches,[14–16] and clinical diagnosis has been used in a wide-variety of research settings requiring large samples including epidemiological studies. This suggests that for gene identification, it may be possible to exploit clinician diagnosis to rapidly access large samples, although more richly phenotyped samples will be required for probing the specificity of the identified risk with respect to well defined disorder categories.

Here, we exploit one such sample, a series of UK cases registered for clozapine treatment with a clinical diagnosis of schizophrenia. We call the sample CLOZUK. Our aims were to investigate in this sample the top 81 hits from the PGC to a) provide independent replication b) identify additional GWS associations through meta-analysis c) establish if subjects so ascertained are, with respect to genetic risk at common alleles, similar to rigorously ascertained and evaluated cases d) given the overlap in polygenic risk between schizophrenia and bipolar disorder, to test the hypothesis that schizophrenia and bipolar disorder are the same with respect to common alleles identified initially in a schizophrenia sample.

Materials and Methods

Details of samples, genotyping, quality control, and statistical analyses including meta-analysis with extant PGC data are given as supplementary information (SI).

Samples

CLOZUK—Patients taking clozapine provide blood samples to allow detection of adverse drug effects. Through collaboration with Novartis, the manufacturer of a proprietary form of clozapine (Clozaril), we acquired blood from people with treatment resistant schizophrenia according to the clozapine registration forms completed by treating psychiatrists. In the UK, treatment resistant schizophrenia implies a lack of satisfactory response to adequate trials of at least two other antipsychotics. The CLOZUK sample has not been used in published genetic studies, although it has been used by the WTCCC2 as a replication sample in a manuscript under review.[17]

Bipolar Samples—The bipolar sample is an extension of[18], collected in the UK.

Controls—Comprised 4539 individuals who were not screened for psychiatric illness.

All aspects of the study, including recruitment of anonymous samples via the clozapine monitoring service, were approved by relevant ethics panels and were in accordance with the UK Human Tissue Act.

Analyses

Samples were genotyped on the Immunochip at the Wellcome Trust Sanger Institute as part of the WTCCC+ (bipolar cases and controls) and WTCCC2 (CLOZUK) pipelines. Following quality control, we initially retained 2652 CLOZUK individuals, 2505 with bipolar disorder, and 4266 controls. The controls overlapped with those in a previous schizophrenia study[19] included in the PGC.[5] Similarly, many of the bipolar and control samples were included in WTCCC1.[18] Using the genotype data, we identified 2640 CLOZUK and 2878 controls independent of those used by the schizophrenia PGC. The bipolar sample contained 1332 from WTCCC1[18] and 1172 new subjects. Unless specified, all bipolar individuals were included.

Of the 81 targeted SNPs, we had genotypes for 68, good proxies (r^2>0.8 CEU 1000Genomes Pilot 1) for 10, and no good proxies for 3 (table S2).

Owing to concerns that the association signals at the MHC are not independent,[5] and because some of the findings at the MHC are discussed in a WTCCC2 paper[17] our primary analyses are for the loci outside the MHC. However, we also report the findings including the MHC to allow a complete picture of the extent of replication of the top PGC results.

We estimated power to replicate associations (one-sided p<0.05) based upon the direction of effect in PGC Stage 1 (see SI). Based upon these power estimates, if all PGC Stage 1 associations are true, we expect 21.9 (95% CI 15.5–28.4) replications. For non-MHC SNPs, power ranged between 1.1×10^{-4} and 0.72, (mean 0.22), and the number of expected replications if all PGC associations are true is 12.4 (95% CI 7.1–17.7).

Results

CLOZUK vs. controls

The results for all SNPs are fully documented in Table S3 and summary results for SNPs reported by the PGC as GWS, or which attained that in the present study, are given in Table 1. In CLOZUK, we replicated (p<0.05) association to 47% (37/78) markers; 35% (20/57) excluding the MHC. Both rates are greater (7–9 fold) than chance. The maximum likelihood estimates of the proportion of true associations among all 78 SNPs (given the estimated power of our sample) was 0.98 (95% C.I. 0.78–1); 0.85 (95% C.I. 0.51–1) for non-MHC SNPs. We found no two-tail significant associations to the unexpected allele, a finding incompatible with the high rates of significant associations being attributable to random uncontrolled stratification effects in either the original[5] or our own study.

Of alleles associated with schizophrenia in PGC Stage 1, 85% (66/78) showed the same direction of effect in CLOZUK, a highly significant excess (sign test $p=1.7\times10^{-10}$, table S4). A highly significant excess ($p=6.6\times10^{-6}$) was seen for the non-MHC SNPs (79%, 45/57), even after excluding loci reported as GWS by the PGC (40/51, $p=2.9\times10^{-5}$). In that set of 51 non-MHC SNPs which, in the PGC, had not attained GWS, the distribution of p-values in CLOZUK highly significantly differed from the null (Fishers test for combining p-values; $p= 6.3\times10^{-14}$), providing further evidence that true associations reside among these sub GWS SNPs.

To evaluate the utility of CLOZUK for genetic studies relative to samples based upon strict research diagnoses, we compared the distributions of the $\log_e$ ORs from CLOZUK and PGC Stage 2 samples (Figure S2 and Table S5). ORs were calculated for risk alleles defined by PGC Stage 1. On average, the effect sizes are larger in CLOZUK (p=0.0001). This was true for MHC (p=0.0062) and non-MHC loci (p=0.0072). The increased effect sizes might arise from either the phenotypic characteristics of the CLOZUK sample or from the use of an ethnically more homogenous sample. To examine this, we compared the distributions of effect sizes in CLOZUK with the Irish WTCCC2 sample from PGC stage 2. The advantages of that sample for comparison are that it is both the largest single country PGC stage 2 sample where diagnoses were based on standardized interviews, clinical note reviews, consensus diagnoses, and operationalized research diagnostic criteria, and it is also geographically the closest to the UK. The training of clinicians in Ireland and the UK is very similar. Effect sizes in CLOZUK were still larger (p=0.039) but not when restricted to non-MHC SNPs (p=0.31). Given that it is unclear to what extent the MHC SNPs, if at all, tag more than a single functional variant, we consider the latter test the more reliable. Thus, our data suggest the greater signal in our sample most likely relates to an as yet unknown aspect of homogeneity arising from ascertainment within a single country, rather than other properties of the CLOZUK sample.

Regarding single locus analysis, in CLOZUK, we obtained strong replications of the GWS associations reported by the PGC at the MHC region (reported in the WTCCC2 study[17]) with all 5 of the MHC SNPs that were GWS in the PGC (table S3) replicating in our sample (median p=0.0006, median OR=1.17). Combining CLOZUK with PGC Stages 1 and 2, the strongest PGC MHC association (rs2021722) improved by almost 2 orders of magnitude ($p=3.7\times10^{-14}$). Six novel MHC markers attained GWS.

Of the 7 GWS non-MHC markers reported by the PGC, (two were statistically independent but located within 130kb on chromosome 10), we obtained data for 6 (Table 1). Associations at *CCDC68, CNNM2 and NT5C2* replicated, while the *MIR137* locus just failed to do so (p=0.074). For these 4 SNPs, the combined evidence increased with the addition of our data above that from the PGC study alone. The evidence at *CSMD1* and *MMP16* dropped below GWS but given power considerations, it does not follow these were false associations.

As a result of significant associations in CLOZUK for *CACNA1C* (rs4765905, $p=3.6\times10^{-4}$) and the broad locus of *ITIH3/4* (rs2239547, $p=4.2\times10^{-4}$), when our data were combined with the PGC, both *CACNA1C* ($p=1.2\times10^{-8}$) and ITIH3/4 ($p=3.6\times10^{-10}$) surpassed GWS. We refer to the locus as *ITIH3/4*, although it is actually a region of extensive LD containing many genes. In the PGC[5], neither locus was GWS in schizophrenia, although in a combined analysis of schizophrenia and bipolar disorder, GWS was attained for both. In their joint schizophrenia-bipolar disorder analysis, a variant at *ANK3* was also identified at GWS. In CLOZUK, *ANK3* only showed a trend (rs16915157, p=0.11) and our combined CLOZUKPGC analysis (schizophrenia only) did not reach GWS. In the combined data, rs6703335, within an intron of *SDCCAG8* (*Serologically Defined Colon Cancer Antigen 8*) attained for the first time GWS.

One other locus is worthy of comment. *TCF4* has been strongly implicated before in schizophrenia[8, 20]. A SNP at this locus, rs17512836, was the strongest single finding from the stage 1 PGC analysis ($p=2.35\times10^{-8}$) that subsequently failed to attain GWS in the overall PGC study. In CLOZUK, we did replicate that association (p=0.012) and in the context of the findings from previous larger datasets[8, 20] with rs9646596, a marker at this locus in strong LD (D'=1) we consider this additional evidence that *TCF4* is likely a true susceptibility locus for schizophrenia.

Bipolar vs. controls

Excluding the MHC, alleles that were more frequent in PGC stage 1 schizophrenia cases than controls were also overrepresented in bipolar cases at 39 of 56 SNPs (p=0.0023). The number of SNPs showing this trend is not significantly different from that in the CLOZUK sample (p=0.28), although in the bipolar dataset, fewer alleles (15/78; excluding MHC, 7/57) achieved nominal levels of significance.

Although single locus analysis of the bipolar dataset is not the primary aim of this study, we note that of the GWS associations reported by the PGC, significant associations were observed for the schizophrenia risk allele (1-tailed) for two variants at the MHC including the most significant PGC SZ allele (rs2021722, p=0.024) as well as for *NT5C2* (rs11191580, p= 0.018) on chromosome 10. No studies of *NT5C2* and bipolar disorder have been reported but the finding at rs2021722 is consistent with a previous association between bipolar disorder and a schizophrenia risk allele at the MHC[21]. Although that analysis[21] contained some bipolar cases that overlap with those in the present study, association was significant at rs2021722 in bipolar disorder after those samples were excluded (retaining 1172 bipolar and 2878 controls, p=0.034). In bipolar disorder, we obtained strong evidence for association for two of the three loci which in the PGC study only obtained GWS when schizophrenia and bipolar disorder were combined. Excluding samples reported in that analysis[5], our bipolar sample was associated at *CACNA1C* ($p=2.3\times10^{-4}$) and *ITIH3/4* (p=0.0033) but not *ANK3*.

CLOZUK, bipolar and control comparisons

The overlap between schizophrenia and bipolar disorder for the set of 78 alleles is substantial, but it does not follow that the effects on disease risk are equivalent. To evaluate this, we directly compared allele frequencies in the CLOZUK with bipolar samples. Across the MHC, 20 out of 21 of the alleles that were more common in the PGC schizophrenia cases were more common in the CLOZUK than the bipolar cases (table S4). At non-MHC loci, this figure was 82% (47/57; $p=3.8\times10^{-7}$) and the mean effect size for non MHC SNPs was significantly different ($p=7.8\times10^{-5}$) in CLOZUK than bipolar disorder (OR respectively1.057 and 1.030). However, the finding of larger effect sizes in schizophrenia did not generalize. Thus the effect sizes for the non-MHC loci were not significantly different between our bipolar sample and either the complete PGC Stage 2 sample (table S5) or the WTCCC2 Irish sample (table S6).

Discussion

CLOZUK vs. controls

We provide extensive replication of the findings of the schizophrenia PGC and, in doing so, demonstrate the utility of the CLOZUK sample for analysis of common schizophrenia risk alleles. At nominal levels, we replicated association to 17/21 markers at the MHC region as well as 35% of all other markers. The latter is a more conservative estimate of the replication rate given extensive LD at the MHC. The rate is higher than the PGC obtained in their replication dataset (25%) despite the much larger replication sample available to the PGC. Moreover, while sign test analysis in the CLOZUK sample was comparable to that in the PGC replication dataset, an assessment of the average effect size significantly favoured CLOZUK. One possible explanation for this is that relative to other samples used in GWAS, those with a diagnosis of schizophrenia attending clozapine clinics by virtue of treatment resistance have a more severe form of disorder characterized by more core schizophrenia deficits and that they do so by virtue of a higher genetic loading. While, plausible, this is speculative, and our comparison of the CLOZUK sample with the WTCCC2 Irish sample suggests the larger effects may simply be due to increased homogeneity arising from the use of a sample from a single country. We aim to resolve this through future GWAS and CNV studies in larger samples of clozapine clinic attenders.

We additionally confirm the hypothesis of the PGC that a substantial number of the top associations from the Stage 1 mega-analysis are likely to be true associations. Given the estimated power of our study, assuming that all Stage 1 findings are true, we would expect 12 replications, with an the upper limit of 17.7 for the 95% confidence interval. Thus, our finding of 20 replications (excluding the MHC) is consistent with the hypothesis that many, or even all, the PGC Stage 1 associations are true. Also consistent with this is our maximum likelihood estimation that 85% (95% CI 50–100%) of the 78 tested alleles are true associations (given the estimated power of our sample).

While we show the top PGC hits are highly enriched for true associations, to implicate loci individually rather than *en masse* requires GWS support, and, ideally independent replication. Of the loci that were reported by the PGC as GWS, we obtained independent replication for *CCDC68, CNNM2, NT5C2* and the extended region of the MHC, while at the *MIR137* locus we just failed to do so. We also observed strong associations at *CACNA1C* and *ITIH3/4*, loci that in the PGC study had only showed GWS in a combined analysis of schizophrenia and bipolar disorder. Through joint analysis with the PGC schizophrenia data, we also established the first genome wide significance in schizophrenia alone for each of *CACNA1C* and *ITIH3/4* and *SDCCAG8*. With the exception of *SDCCAG8*, the potential relevance of the other loci to the pathophysiology of major mental disorders has been discussed.[5, 22–24] *SDCCAG8* encodes a centrosome-associated protein thought to play a role in cell division. Mutations in *SDCCAG8* are known cause of Nephronophthisis-related ciliopathies,[25] a group of disorders in which there is abnormal development and/or degeneration of kidney, retina, and brain. The mechanisms behind for this are not fully understood, but are thought to involve perturbed non-canonical Wnt signaling and dysregulated cell cycle control[26]. Interestingly, two other genes that are known to be causal

for this group of disorders have been associated with schizophrenia before, *RPGRIP1L* encoding RPGR-interacting protein 1-like protein[7] and *AHI1* encoding Abelson Helper Integration site 1,[27] although for neither of these genes is the evidence GWS.

CLOZUK, bipolar and control comparisons

Our study adds to previous findings that *en masse*[6, 28] alleles that confer risk of schizophrenia also confer risk of bipolar disorder. We additionally show that the proportion of the most strongly supported schizophrenia risk alleles from the PGC study that are more common in independent samples is similar for schizophrenia and bipolar disorder. While the effect sizes at these loci were significantly greater in the CLOZUK than the BD samples, that finding of a significant difference did not generalize to comparison between the BD sample and either the complete PGC Stage 2 replication sample or the WTCCC2 Irish subset. Given that these alleles were selected on the basis of association to schizophrenia, this is a somewhat surprising observation. One possible explanation is that risk alleles most easily detected in large heterogeneous samples are enriched for those that are relatively nonspecific for broad diagnostic category or to postulated genetic subtypes of the disorder(s). Another is that common alleles in general are non-specific with respect to the two major diagnostic groups. This study cannot distinguish between these possibilities.

In summary, using an independent sample of clozapine clinic attenders with a clinician reported diagnosis of treatment resistant schizophrenia, we independently confirm association to 3 loci previously reported to be GWS, identify GWS evidence for three loci, two of which were only previously GWS in a combined schizophrenia and bipolar analysis, and provide independent support for an additional 15 loci that had not previously attained GWS. We provide further evidence for overlap in genetic risk between schizophrenia and bipolar disorder. Given the number of replications and the power of our sample, we estimate a large proportion, possibly all, of the tested SNPs represent true schizophrenia associations. Taken together, the findings suggest some samples based upon clinician reported diagnosis may make an important contribution to the large samples required for genetic studies of schizophrenia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

SNPs reported as genome-wide significant for schizophrenia.

					PGC		CLOZUK		PGC + CLOZUK	
SNP	**chromosome**	**position (Mb)**	**nearby gene**	**alleles**	**OR**	**p-value**	**OR**	**p-value***	**OR**	**p-value**
rs1625579	1	98.3	MIR137	TG	1.13	1.59×10^{-11}	1.08	7.4×10^{-2}	1.12	7.76×10^{-12}
rs6703335	1	241.7	SDCCAG8	GA	1.10	5.47×10^{-8}	1.05	1.1×10^{-1}	1.09	4.22×10^{-8}
rs17662626	2	193.7	PCGEM1	AG	1.20	4.65×10^{-8}				
rs2239547	3	52.8	ITIH3/4	TC	1.11	6.02×10^{-8}	1.17	4.2×10^{-4}	1.11	3.62×10^{-10}
rs2021722	6	30.3	TRIM26	CT	1.16	2.18×10^{-12}	1.17	5.8×10^{-4}	1.16	1.05×10^{-14}
rs10503253	8	4.2	CSMD1	AC	1.12	4.14×10^{-8}	1.03	3.2×10^{-1}	1.10	1.35×10^{-7}
rs7004633	8	89.8	MMP16	GA	1.11	2.75×10^{-8}	0.99	5.4×10^{-1}	1.10	1.84×10^{-7}
rs7914558	10	104.8	CNNM2	GA	1.11	1.82×10^{-9}	1.09	1.4×10^{-2}	1.10	1.55×10^{-10}
rs11191580	10	104.9	NT5C2	TC	1.15	1.11×10^{-8}	1.19	1.0×10^{-2}	1.16	7.48×10^{-10}
rs4765905	12	2.2	CACNA1C	CG	1.09	1.87×10^{-6}	1.15	3.6×10^{-4}	1.09	1.23×10^{-8}
rs12966547	18	50.9	CCDC68	GA	1.10	2.60×10^{-10}	1.10	1.1×10^{-2}	1.10	1.74×10^{-11}
rs17512836	18	51.3	TCF4	CT	1.23	1.05×10^{-6}	1.32	1.2×10^{-2}	1.24	8.89×10^{-8}

Included SNPs were either reported by the PGC[5] as statistically independent genome-wide significant (GWS) schizophrenia associations (Stage 1 and 2 combined) and/or meet that criterion in the present manuscript. For the MHC region, only one SNP (rs2021722) of the many attaining GWS is listed for clarity. rs17662626 on chromosome 2 was not available in our sample. Alleles are listed with the PGC associated allele first. OR for CLOZUK and PGC+CLOZUK are odds ratio for associated allele in PGC.

*One-tailed p-values.