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Genome-Wide Association Identifies a Common Variant in the Reelin Gene That Increases the Risk of Schizophrenia Only in Women

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Sex differences in schizophrenia are well known, but their genetic basis has not been identified. We performed a genome-wide association scan for schizophrenia in an Ashkenazi Jewish population using DNA pooling. We found a female-specific association with rs7341475, a SNP in the fourth intron of the reelin (RELN) gene (p = 2.9 × 10^-5 in women), with a significant gene-sex effect (p = 1.8 × 10^-7). We studied rs7341475 in four additional populations, totaling 2,274 cases and 4,401 controls. A significant effect was observed only in women, replicating the initial result (p = 2.1 × 10^-3 in women; p = 4.2 × 10^-3 for gene-sex interaction). Based on all populations the estimated relative risk of women carrying the common genotype is 1.58 (p = 8.8 × 10^-7; p = 1.6 × 10^-5 for gene-sex interaction). The female-specific association between RELN and schizophrenia is one of the few examples of a replicated sex-specific genetic association in any disease.

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Introduction

Schizophrenia (181500) is a common psychiatric disorder of unknown aetiology. Individual twin studies and meta-analyses of twin studies [1] estimate that the heritability of schizophrenia is approximately 80%. Analysis of family, adoption and twin data indicate that inheritance acts in a complex fashion, in combination with the environment, to mediate the risk of developing the illness [2]. However, despite the relatively large heritability of schizophrenia, efforts to identify the molecular risk factors have so far yielded equivocal results (reviewed in [3]).

Sex differences in the risk of a disorder can provide clues about its pathogenesis. For schizophrenia, the age of onset, premorbid functioning, symptomatic characteristics, and course of illness differ significantly between men and women [4]. Two systematic reviews have demonstrated a sex difference in the risk of developing schizophrenia [5,6]; both studies report that the male to female risk ratio is 1.4. Sex-specific associations with schizophrenia have previously been reported for a number of loci [7–11], but the robustness of these claims is open to doubt; results have yet to be corroborated [8–10] or replication has not been found with the same single nucleotide polymorphism (SNP) in the same direction in the same sex (e.g. [7,11]). This difficulty has afflicted attempts to establish sex-specific association in other diseases. An empirical assessment of 432 published sex differences in genetic association studies for different conditions found a single valid interaction that was consistently replicated in at least two other studies [12].

In the present study, we carried out a genome-wide association study using DNA pools of cases and controls constructed separately for men and women to allow the identification of sex-specific effects. Several studies have shown that DNA pooling detects the most promising loci with considerable savings in time and costs [13–18]. We previously scanned a three Mb region spanning the 22q11 microdeletion for association with schizophrenia using DNA pools. Our previous study [7,19] showed that the pools are representative of the allele frequencies in the sample and are adequate for
DNA Pooling

detection of even modest association signals (odds ratios of about 1.3–1.5).

Results

DNA Pooling

Four separate pools of DNA were constructed as follows, 419 male cases, 241 female cases, 1,807 male controls and 964 female controls. Increasing the number of controls for a given number of cases significantly increases the power of the experiment. For example, our experiment with 660 cases and 2,771 controls is equivalent in power to about 1,100 cases and 1,100 controls [20]. Since estimating allele frequencies in pooled DNA samples introduces measurement errors, each of the four pools was independently analyzed in ten replicates with Affymetrix 500K SNP arrays. The results from the ten replicates were used to rank the SNPs (the 1,000 top ranked SNPs are listed in Table S1).

Our previously reported findings with several SNPs around both the COMT (116790) and DGCR2 (600594) genes in Chromosome 22 acted as a positive control for the performance of the current study. The association of multiple SNPs in the vicinity of both genes was clearly identified using our method (Figure 1). The best SNPs were selected for individual genotyping using an integrative approach taking into account their statistical significance, ranking and potential biological relevance (see Methods). As a result, a total of 194 SNPs were selected for individual genotyping.

Individual Genotyping

The 194 selected SNPs were individually genotyped. At this stage, we used a sample of 759 controls and 745 patients from the Ashkenazi Jewish population. We genotyped a subset of the control sample used in the pools and enlarged the sample of cases (compared to the sample used in the pools), in order to differentiate in a cost-effective way between SNPs with real differences in allele frequencies between cases and controls and SNPs showing spurious differences due to technical anomalies in the DNA pooling procedure. Fifty-two SNPs out of 167 SNPs that passed our quality control (including a manual examination of genotype clusters and test of Hardy Weinberg equilibrium) showed p-values below 0.05 at any of the tests (male, female and combined) and nine SNPs had p-values below 0.005 (specific results for all SNPs are presented in Table S2).

The lowest p-value was found for SNP rs7341475 (G→A), for women only. SNP rs7341475 is located in Chromosome 7 (bp position = 103192051, NCBI Build 36), in the fourth intron of the reelin gene (RELN (600514)). This particular SNP was prioritized for individual genotyping because it resides within a gene previously studied for association with schizophrenia, in addition to having a high rank in the pools result (top 99.98% in the female pools). The common genotype of this SNP (GG) has a higher frequency in women with schizophrenia (75.5%) relative to female controls (59.3%) with an odds ratio (ORGG) of 2.1 (pgenotype = 9.8 × 10−5), and a significant gene-sex interaction (pinteraction = 5.3 × 10−5). In men, however, no effect was observed (ORGG = 1.1, pgenotype = 0.47, GG frequency = 60.6%). Since the AA genotype is relatively rare, the genotype distribution in cases and controls was analyzed with the GA and AA genotypes grouped together. Similarly, the G allele was significantly overrepresented in women with schizophrenia (frequency = 86.6%) relative to female controls (frequency = 76.2%).

<table>
<thead>
<tr>
<th>SNP</th>
<th>Effect on Women</th>
<th>Effect on Men</th>
<th>p-genotype</th>
<th>p-interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs7341475</td>
<td>G→A</td>
<td></td>
<td>9.8 × 10−5</td>
<td>5.3 × 10−5</td>
</tr>
</tbody>
</table>

Figure 1. Association Signal on Chromosome 22 in a Sliding-window of Nine Consecutive SNPs

The −log10 values are the negative logarithm (base 10) of the sliding windows ranks divided by the number of windows on Chromosome 22. The combined rank across men and women is based on a silhouette statistic [36]. The inset shows a magnification of the region indicated by a vertical line and the position of DGCR2 and COMT genes within this interval.

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We considered whether the result could be due to
population stratification effect. We categorized parents of the Ashkenazi Jews individuals by their country of origin and compared the allele frequencies of rs7341475. We examined subjects whose parents were from the same country (77% of all individuals) and focused on those countries with more than 100 individuals (80% of individuals): Argentina, Germany, Russia, Poland, Ukraine, and USA (Figure S1). There was no significant difference in allele frequency between Ashkenazi Jewish individuals originated from different areas of the world ($p = 0.9405$).

The association results and the linkage disequilibrium (LD) structure of RELN are presented in Figure 2. Based on HapMap LD data (CEU), there are no other SNPs in high LD (maximum $r^2 = 0.4$) with rs7341475 on the 500K Affymetrix array. One other SNP (rs2106173) in the RELN gene was also tested by individual genotyping but no statistically significant difference was found. The association signals for other SNPs in RELN (based on DNA pools) were moderate (maximum $r^2 > 0.8$) are observed between rs7341475 and other SNPs distributed up to 39.8 kb upstream to rs7341475. The SNPs that are highly correlated with rs7341475 (rs10435342, rs6951931, rs17290575, rs6954479 and rs39327) are all located in the third or fourth intron of the gene. There is a substantial correlation ($r^2 > 0.5$) between rs7341475 and other SNPs in a 77.4 kb interval (103188857–103966305). There are no SNPs in neighboring genes in high correlation with rs7341475.

Replication Study
To confirm the female-specific association between rs7341475 and schizophrenia, we tested rs7341475 in four other sample sets, three of European ancestry (UK, Ireland and USA) and one Chinese. In this replication study we tested a total of 2,274 cases (768 women and 1,506 men) and 4,401 controls (2,194 women and 2,207 men). We separated all samples by sex and tested the association for male and female controls (2,194 women and 2,207 men). We separated all samples by sex and tested the association for male and female controls (2,194 women and 2,207 men). We separated all samples by sex and tested the association for male and female controls (2,194 women and 2,207 men).

Table 1. Frequency of GG Genotype (Sample Size)

<table>
<thead>
<tr>
<th>Population</th>
<th>Schizophrenia</th>
<th>Control</th>
<th>$P_{GG}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Men</td>
<td>Women</td>
<td></td>
</tr>
<tr>
<td>AJ</td>
<td>0.606 (470)</td>
<td>0.755 (265)</td>
<td>0.619 (1988)</td>
</tr>
<tr>
<td>UK</td>
<td>0.709 (320)</td>
<td>0.813 (155)</td>
<td>0.725 (1439)</td>
</tr>
<tr>
<td>Irish</td>
<td>0.750 (669)</td>
<td>0.762 (311)</td>
<td>0.733 (337)</td>
</tr>
<tr>
<td>USA</td>
<td>0.692 (295)</td>
<td>0.725 (109)</td>
<td>0.695 (202)</td>
</tr>
<tr>
<td>Chinese</td>
<td>0.806 (222)</td>
<td>0.845 (193)</td>
<td>0.830 (229)</td>
</tr>
</tbody>
</table>

AJ, Ashkenazi Jews; $P_{GG}$, significance level for differences in the frequency of the GG genotype between female cases and controls, calculated with a $\chi^2$ statistics. For men, $P_{GG}$ is not significant for all samples.

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Discussion
We have carried out a genome-wide association analysis of schizophrenia, using pooled DNA. We identified one SNP in the fourth intron of RELN that confers a sex-specific risk of schizophrenia. Although the significance of the association between rs7341475 and schizophrenia in the Ashkenazi Jewish sample would not withstand a Bonferroni correction for multiple testing, we were able to replicate the sex-specific association in other samples from different populations that
were tested for this specific SNP. The same allele and genotype are overrepresented in women, but not men, with schizophrenia in three different populations, Ashkenazi Jews, Europeans and Chinese (although the overrepresentation is not independently statistically significant in all populations tested). This observation, together with the fact that the association in the combined replication samples is significant and robust (even to the removal of the sample showing the most significant association) increases our confidence that we have found a genuine association.

The association observed in this study is unlikely to be the result of population stratification. The samples from the UK showing robust replication of the initial association were individually genotyped for SNPs across the whole genome. As such, this sample was rigorously evaluated for possible population stratification [21]. We also found no evidence for stratification in the Ashkenazi sample, indicating that the increase in allele frequency observed in women with schizophrenia cannot be caused by population structure in the Ashkenazi Jewish sample.

Genetic association studies have so far failed to report any consistent association between Reelin gene polymorphisms and schizophrenia [23–27]. However the gene has not so far been systematically screened. According to HapMap data, 183 tag SNPs would be needed to capture common variation at the gene (with $r^2 > 0.8$, MAF > 5%). While the SNPs on the Affymetrix 500K arrays capture 60% of SNPs. Most studies report data on a SNP in the promoter region, or on a CGG repeat polymorphism in the 5' untranslated region; none have tested rs7341475. The two previously reported polymorphisms are located in LD blocks that do not contain rs7341475.

Our finding is important on two counts: first, it supports the hypothesis of a neurodevelopmental origin for schizophrenia, assuming that the genetic association reflects variation in the function of the RELN gene. Reelin, the
protein product of RELN, is a serine protease [28] that acts via a number of receptor-mediated pathways on neurons [29]. It plays a key part in corticogenesis, as demonstrated by the cytoarchitectural abnormalities of the null mutant reeler (rl/rl) mouse [30]. RELN mutations in humans are associated with an autosomal recessive form of lissencephaly [31], a mental retardation syndrome that does not include psychosis. Second, while a sex difference in the risk for schizophrenia has been found, its molecular basis has so far been unclear. Here we establish a replicated sex-specific association. Intriguingly, there is prior evidence for sexual dimorphism at the reelin locus. Sex effect have been noted in one study reporting that RELN expression was higher in women compared to men (in layer I neurons) and a reduction in RELN expression observed only in men with schizophrenia (in the superficial interstitial white matter neurons). However, as the authors acknowledge, these have not been noted in other brain regions [32]. In mice, loss of Purkijne cell was observed in male mice with only one functional reelin gene (rl/−) but not in reelin-deficient female mice [33].

Finally we note that sex hormones are likely to mediate changes in RELN expression: for example, administration of testosterone decreases reelin expression in the brains of male European starlings [34]. Our result of a female-specific association of RELN with schizophrenia may suggest a possible pathway where sex hormones modulate gene expression, which by altering cortical structure, increases susceptibility to psychosis.

Materials and Methods

Ashkenazi Jewish sample. Samples from individuals diagnosed with schizophrenia were collected from hospitalized patients from seven medical centers in Israel (described in [7]). Schizophrenia was diagnosed by Diagnostic and Statistical Manual criteria (DSM-IV, American Psychiatric Association). Ashkenazi controls were collected from volunteers in blood banks. All four grandparents of each subject were of Ashkenazi Jewish origin, and all subjects (or the subject's legal representative) signed an informed-consent form.

Other studied samples. Chinese Han schizophrenia patients, their families and control subjects were recruited from Sichuan Province, SW China and consisted of 415 unrelated patients (222 males and 193 females) and 438 normal Han Chinese controls (229 males and 229 females). All patients were interviewed by an experienced psychiatrist using the SCID, and a diagnosis of schizophrenia was made according to DSM-III-R or DSM-IV criteria. Information was also collected from examination of medical records and all other available sources. Genomic DNA was extracted from peripheral blood according to standard phenol-chloroform methods. This study was approved by the South London and Maudsley Trust ethical committee and informed consent was obtained from all patients and control individuals. The Irish Case – Control Study of Schizophrenia (ICCSS) samples were collected in Northern Ireland and the Republic of Ireland. Individual genotypes were obtained for 980 affected cases (699 males and 311 females) and 582 controls (357 males, 245 females). The affected subjects were selected from in-patient and outpatient psychiatric facilities in the Republic of Ireland and Northern Ireland. Subjects were eligible for inclusion if they had a diagnosis of schizophrenia or poor-outcome schizoaffective disorder by DSM-III-R criteria, which were confirmed by a blind expert diagnostic review. Excluding the relatively small number of schizoaffective female patients (n = 36) did not significantly alter the female-specific association result. Controls, selected from several sources, including blood donation centers, were included if they denied a lifetime history of schizophrenia. Both cases and controls were included only if they reported all four grandparents as being born in Ireland or the United Kingdom.

The UK sample consisted of unrelated subjects with schizophrenia (320 males and 155 females). All were white and born in the British Isles. All patients had a consensus diagnosis of schizophrenia according to DSMIV criteria made by two independent raters following a semi-structured interview by trained psychiatrists or psychologists using the Present State Examination or the Schedules for Clinical Assessment in Neuropsychiatry (SCAN) interview and review of case records. Cases were screened to exclude substance-induced psychotic disorder or psychosis due to a general medical condition. The mean age at first psychiatric contact was 23.6 (SD 7.7) years and the mean at ascertainment was 41.7 (SD 14.6) years. Multicentre and Local Research Ethics Committee approval were obtained, and all subjects gave written informed consent to participate. The control sample used by the Wellcome Trust Case Control Consortium (WTCCC) is described in detail elsewhere [21]. Briefly, controls (n = 2938) came from two sources, the 1958 British Birth Cohort (58BC) and UK blood donors. At a genome wide level, the two groups do not significantly differ with respect to allele frequencies, justifying their use as a single control group. Individuals included in the study were living within England, Scotland and Wales. Individuals (n = 26) with non-Caucasian ancestry as determined by multidimensional scaling (MDS) were previously removed by the WTCCC from the sample. Controls were not screened for psychosis, but given the expected modest frequency in an unscreened sample, this has little effect on power. For the genome-wide association study from which these data were taken (manuscript submitted), \( \lambda \) was estimated at 1.06 genome-wide, which is at the lower end observed for other phenotypes when compared with the same controls. The call rate at this test locus for subjects in the genome-wide association study was >99.5% in each of cases and controls.

The USA sample consisted of unrelated subjects with schizophrenia (295 males and 105 females) and unrelated controls (202 males and 232 females) selected from a family-based sample that was
ascertained as part of the Clinical Brain Disorders Branch/National Institute of Mental Health Sibling Study. All subjects were diagnosed using the Structured Clinical Interview (SCID). Proband met DSM-IV criteria for broad schizophrenia diagnosis. Control individuals were ascertained from the National Institutes of Health Normal Volunteer Office and were screened by SCID diagnosis for psychiatric disorders and substance abuse. They had no relatives with schizophrenia spectrum diagnosis. All participants gave informed consent and self-identified as Caucasian of European ancestry. All subjects also underwent extensive physical and laboratory screening to rule out complicating medical conditions and substance abuse.

**DNA pooling.** The DNA pools were constructed using DNA samples from 690 schizophrenic patients (419 males and 241 females) and 2,771 controls (1,807 males and 964 females). Pools were created by using equal aliquots of each sample, as described in ref. [7, 19]. The pools were created from DNA samples from both sexes separately. Each pool (n = 4) was allelotyped ten times with the Affymetrix arrays sets as previously described [18].

**SNP selection for individual genotyping.** We selected 194 unique SNPs using three different ranking methods (Figure S2), in addition to prioritizing SNPs in genes previously studied for association with schizophrenia (schizophrenia candidate genes). We used the SchizophreniaGene database [35] to identify genes that were previously tested for association with schizophrenia (515 genes were listed at the time of access [1105057]). SNPs with minor allele frequencies below 5%, and SNPs within 100 bp of the common allele to HapMap (CEU) were excluded from further analysis due to the limited power to detect a significant association and the decrease in the accuracy of DNA pooling with rare variants.

Eighty SNPs were selected by the following criteria (some SNPs were selected by more than one method). We calculated for each SNP a modified chi-squared statistic, which includes the error variance introduced in DNA pooling. The SNP selection procedure [18, 19] SNPs were ranked based on logarithm (base 10) of the \( p \)-value \( (\log P) \). Sixty-first ranked SNPs (30 from each array type, Nsp & Sty) were selected for individual genotyping. Additional 20 SNPs with \( \log P > 3 \) were selected because they reside in candidate genes. Fifty-five SNPs were selected by the following criteria. We used the GenePool software with silhouette statistic and Manhattan distance as the distance measure [30]. The SNPs were ranked based on the silhouette statistic separately for males and females patients, ranging from 1 for the highest silhouette score to 510,552 for the lowest score. We generated a combined rank for all samples based on the average rank in males and females weighted for the different size of samples. The first 10 ranked SNPs with a minor allele frequency above 5% (based on HapMap, CEU) were selected for individual genotyping. This was done for each of three sets; male, female and combined. Twenty-five additional SNPs in candidate genes for schizophrenia were also selected for individual genotyping if they were also ranked in the first 1,000 SNPs in any of the sets.

Eighty-two SNPs were selected by the following criteria: first, we calculated rank score in a sliding window of non-consecutively neighboring SNPs. For each SNP we assigned a rank based on the mean score in the window showing the largest score out of the different possible windows. The sliding window method was used to identify regions where neighboring SNPs consistently show difference in allele frequency between cases and controls. This method minimizes spurious differences arising from technical anomalies in the analysis of pooled DNA [37], but could preferentially select SNPs in large blocks of LD. The analysis was carried out separately for male, females and the combined sets. SNPs in the first 1,000 ranked windows were selected based on the SNP’s individual rank score. The first 60 SNPs for the total sample and the 10 top-ranked SNPs for males and females samples were selected for individual genotyping. Two additional SNPs in candidate genes for schizophrenia that were also ranked in the first 1,000 SNPs in the combined analysis were also selected for individual genotyping.

**Individual genotyping.** DNA samples from the Ashkenazi Jewish and Chinese populations were genotyped using the Sequenom iPLEX system. LD in the Ashkenazi Jewish control sample was assessed using genotypes obtained from an Illumina HumanHap500 BeadChip in the course of another study [22]. Samples from the Irish Case – Control study of Schizophrenia and from the US were genotyped using a Taqman 5′-exonuclease allelic discrimination assay. Quality checks for the Sequenom system included concordance rate (>98%) for genotypes of 83 DNA samples from HapMap cell lines, call rate (>95%), Hardy-Weinberg equilibrium in control (\( p > 0.001 \)), manual checking for genotyping artifacts and genotype call rate (>98%) for re-genotyping. Genotypes for the UK sample were taken from a genome-wide association study performed in concert with the WTCCC study of common diseases [21] based upon the GeneChip 500K Mapping Array Set. Samples were genotyped at the Affymetrix service laboratory in San Francisco (USA) using the same pipeline as the WTCCC disease and controls samples.

**Statistical analysis.** All data analysis was performed using the R language and environment for statistical computing (http://www.r-project.org/). Single-SNP analysis for the individual genotyping data was carried out using a \( \chi^2 \) test on allele and genotype counts. For the replication samples a one-tailed test was employed, since we tested a specific hypothesis of an increase in GG frequency in female patients and a decrease in genotype frequency in male patients. We confirmed the rare homozygote genotype with the heterozygotes for the genotype association analysis. Hardy-Weinberg was assessed using the \( \chi^2 \) statistic with one degree of freedom. To test for a gene by sex interaction, a z-score was calculated using a ratio between the difference in the natural logarithm of the odds ratio between males and females and the square root of the variance of the difference. We used the Mantel-Haenszel method to combine the data of different populations with a fixed effect model and Cochran’s Q statistic to test for heterogeneity as implemented in the R package ‘meta’ (version 0.5). LD was calculated using the Haplovie software package. Population attributable risk of rs7341475 was calculated for women as \( (K - 1)/K \), where \( K = \sum g_i^2 \), \( g_i \) is the frequency of the \( i \) genotype, and \( g \) is the estimated genotype relative risk of the \( i \) genotype assuming multiplicative model.

**Supporting Information**

**Figure S1.** Allele Frequency of rs7341475 in Ashkenazi Jews from Different Origin

The height of each bar represents the frequency of the G allele of rs7341475 in Ashkenazi Jews originated from different countries. Error bars are 95% confidence interval for the frequency. The vertical solid line is the frequency of the G allele in schizophrenia female patients and the dashed lines are the 95% confidence interval for that frequency.

Found at doi:10.1371/journal.pgen.0040028.sg001 (42 KB DOC).

**Figure S2.** SNP Selection for Individual Genotyping

The diagram shows the method used to select SNPs for individual genotyping. SNPs were selected based on three ranking methods. SNPs were also prioritized in each ranking system if they reside within genes previously studied for association with schizophrenia (see text for more details).

Found at doi:10.1371/journal.pgen.0040028.sg002 (28 KB DOC).

**Table S1.** Results of DNA Pooling

The 1,000 top rank-ordered SNPs based on a silhouette statistic calculated using the GenePool software for men, women, and combined sample.

Found at doi:10.1371/journal.pgen.0040028.st001 (321 KB XLS).

**Table S2.** Results of Individual Genotyping

Found at doi:10.1371/journal.pgen.0040028.st002 (52 KB XLS).

**Accession Numbers**

The Online Mendelian Inheritance in Man (OMIM) (http://www.ncbi.nlm.nih.gov/omim/) accession numbers for disease and genes mentioned in the paper are Schizophrenia (181500), RELN (600514), COMT (116790), and DGCR2 (600594).

**Acknowledgments**

**Author contributions.** The study was conceived by AD and designed by SS. SS and MJ performed the genome-wide association experiment. SS analyzed the data. SXC, DAC, NJC, KSK, TL, MO, FAO, MJO, DW, DRW, and CS performed the replication experiments. MB, DAC, TL, JF, and AD contributed reagents/materials/analysis tools. SS, JF, and AD wrote the paper.

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**Competing interests.** The authors have declared that no competing interests exist.
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