Association studies of WD repeat domain 3 and chitobiosyl diphosphodolichol beta-mannosyltransferase genes with schizophrenia in a Japanese population

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Abstract

Schizophrenia and schizophrenia-like symptoms induced by the dopamine agonists and N-methyl-D-aspartate type glutamate receptor antagonists occur only after the adolescent period. Similarly, animal models of schizophrenia by these drugs are also induced after the critical period around postnatal week three. Based upon the development-dependent onsets of these psychotomimetic effects, by using a DNA microarray technique, we identified the WD repeat domain 3 (WDR3) and chitobiosyl diphosphodolichol beta-mannosyltransferase (ALG1) genes as novel candidates for schizophrenia-related molecules, whose mRNAs were up-regulated in the adult (postnatal week seven), but not in the infant (postnatal week one) rats by an indirect dopamine agonist, and phencyclidine, an antagonist of the NMDA receptor. WDR3 and other related proteins are the nuclear proteins presumably involved in various cellular activities, such as cell cycle progression, signal transduction, apoptosis, and gene regulation. ALG1 is presumed to be involved in the regulation of the protein N-glycosylation. To further elucidate the molecular pathophysiology of schizophrenia, we have evaluated the genetic association of WDR3 and ALG1 in schizophrenia. We examined 21 single nucleotide polymorphisms [SNPs; W1 (rs1812607)-W16 (rs6656360), A1 (rs8053916)-A10 (rs9673733)] from these genes using the Japanese case-control sample (1,808 schizophrenics and 2,170 matched controls). No significant genetic associations of these SNPs were identified. However, we detected a significant association of W4 (rs319471) in the female schizophrenics (allelic P = 0.003, genotypic P = 0.008). Based on a haplotype analysis, the observed haplotypes consisting of W4 (rs319471)–W5 (rs379058) also displayed a significant association in the female schizophrenics (P = 0.016). Even after correction for multiple testing, these associations remained significant. Our findings suggest that the WDR3 gene may likely be a sensitive factor in female patients with schizophrenia, and that
modification of the WDR3 signaling pathway warrants further investigation as to the pathophysicsology of schizophrenia.

Introduction

Schizophrenia typically develops after adolescence [1]. Methamphetamine, an indirect dopamine agonist, and phencyclidine (PCP) and ketamine, antagonists of the N-methyl-D-aspartate (NMDA) type glutamate receptor, are known to cause schizophrenia-like symptoms only after the adolescent period [2–4]. Similarly, in experimental animals, the psychotomimetic effects of these drugs have also been observed after the critical period around postnatal week three [5–7]. These observations suggest that the neuron circuits and molecules in the brain related to schizophrenia might show an age-related response to these psychotomimetics. In support of this assumption, we have found that methamphetamine and PCP elicit developmental changes in the c-Fos protein expression pattern, which reflects activity modification of the cell activities in the nervous systems, in the rat neocortex across the critical period [8, 9]. Consequently, we have explored the gene transcripts that are developmentally regulated after methamphetamine and PCP administrations in the rat cerebral neocortex. Based on this series of experiments using a DNA microarray technique, we detected as candidates for this type of novel schizophrenia-related genes the WD repeat domain 3 (WDR3) and chitobiosyldiphosphodolichol beta-mannosyltransferase (ALG1), whose mRNAs were up-regulated greater in the adult (postnatal days 50) than in the infant (postnatal days 8) rats by these psychotomimetics. Furthermore, these genes are located in linkage regions with schizophrenia [10, 11]. WDR3, also known as DIP2 and UTP12, is broadly expressed including in the brain [12]. This protein is contained in the nuclear, nucleolus and the main component of the small 40S ribosome subunit [12–14]. It also plays an essential role in the processing of 18S rRNA [14]. However, the specific function in the brain is unexplained. On the one hand, ALG1 is presumed to be involved in the regulation of the protein N-glycosylation, having the activity to add the first mannose residue to the lipid-linked oligosaccharides [15]. Abnormal glycosylation of the glutamate transporter, which may also be regulated by N-glycosylation, was reported in the post-mortem study of schizophrenia [16]. Therefore, WDR3 and ALG1 may also be associated with the susceptibility and/or pathogenesis of schizophrenia.

In the present study, we used single nucleotide polymorphisms (SNPs) in the Japanese case-control sample to implement a genetic association study of the WDR3 and ALG1 genes in schizophrenia.

Materials and methods

Subjects

We analyzed 1,808 schizophrenics (male N = 992; mean age 48.9 ± 13.7 years, female N = 816; mean age 50.9 ± 14.2 years) and 2,170 matched controls (male N = 889; mean age 39.2 ± 13.8 years, female N = 1,281; mean age 44.6 ± 14.1 years) from the Japanese population. All the case-control subjects were assembled from the Honshu area of Japan (the main island of the nation). The populations of Honshu are categorized as a single genetic cluster [17, 18]. For the same subset used in a previous study [18], the Pr (K = 1) value (specifically, number of population present in sample = 1 [19]) was greater than 0.99 [20, 21], and k (the genomic control factor [22]) was 1.074. These data showed a negligible population stratification effect in our Japanese samples [23]. All patients were diagnosed by well-trained psychiatrists based on the
Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV Criteria). The control subjects were assembled from hospital staff and volunteers. Expert psychiatrists checked whether or not they have a present or past history of psychosis and a family history of mental disorder within the second degree of relationships by brief interviews. The present study was approved by the ethics committees of the Tokyo Medical and Dental University and RIKEN Brain Science Institute. All participants gave informed and written consent to participate in the study.

Gene and SNP selection and genotyping

**Exploration of target genes for association analysis.** Before the gene and SNP selection and genotyping, we prepared the male Wistar rats (ST strain, Clea Japan, Japan) to explore of the novel candidate genes of schizophrenia. In this study, only male rats were used in the pharmacological experiment in order to avoid changes in behavior and biochemical response to various drugs due to the onset of the female menstrual cycle. The animals were bred under a 12 hour light / dark cycle (lights on 08:00 hours) at 24.0 ± 0.5 degrees (Celsius) and had free access to food and water. The animal experiments were approved by the ethics committee of animal experiment of the Tokyo Medical and Dental University, and were strictly performed following the guidelines of the university.

To explore the novel target genes for the present association analysis, we performed a DNA microarray analysis using the GeneChip® Rat Gene 1.0 ST Array (Affymetrix, Santa Clara, CA, USA) to find in the neocortex the developmentally regulated transcripts responsive to the psychotomimetic doses (adult period) of PCP and methamphetamine across the critical period around postnatal week three. The array system interrogates 27,342 well-annotated genes with 722,254 distinct probes. A detailed explanation can be found at [https://www.affymetrix.com/support/technical/datasheets/gene_1_0_st_datasheet.pdf](https://www.affymetrix.com/support/technical/datasheets/gene_1_0_st_datasheet.pdf). Data analyses have been achieved by the software, GeneSpring GX 11.0 (Agilent Technologies, Santa Clara, CA, USA).

For this screening stage, six experimental groups of rats were prepared; five saline-administered control rats at PD50; five PCP (7.5 mg/kg, s.c.)-injected rats at PD50; five methamphetamine (4.8 mg/kg, s.c.)-injected rats at PD50; five saline-administered control rats at PD8; five PCP (7.5 mg/kg, s.c.)-injected rats at PD8; and five methamphetamine (4.8 mg/kg, s.c.)-injected rats at PD8. Equal amounts of the total RNA individually isolated from the respective five animals per each treatment group were combined. The cDNA synthesis, cRNA labeling, hybridization and scanning were done according to the manufacturer’s instructions (Affymetrix).

Based upon the DNA microarray data, we finally chose WDR3 and ALG1 as the genes for the present human association study by screening the transcripts of their rat homologues that showed the development-dependent upregulation by PCP and methamphetamine injection with the log2 ratio for the PCP/control (saline) and methamphetamine/control of more than 0.263 (1.2 times the control value) at PD 50 and that less than 0.137 (1.1 times the control value) in the ratio at PD 8 [log2 ratio of the WDR3: PCP 0.595 at PD50 (151%), 0.058 at PD8 (104%), methamphetamine 0.571 at PD50 (149%), 0.009 at PD8 (101%); log2 ratio of the ALG1: PCP 0.273 at PD50 (121%), 0.128 at PD8 (109%), methamphetamine 0.265 at PD50 (120%), 0.047 at PD8 (103%)].

**Selection of SNPs and genotyping.** We first retrieved the region 10kb up- and downstream of these genes that provided the correlation coefficient of \( r^2 > 0.85 \) and minor allele frequency of MAF>0.10 from the public databases [dbSNP (build 149) of the National Center for Biotechnology (NCBI) [http://www.ncbi.nlm.nih.gov/projects/SNP/]]. We then used Carlson’s LD-Select algorithm to evaluate the selection of the SNPs [24]. Additionally, we added SNPs from the insulator regions (CTCF binding site) between the target and adjacent gene as an effective region using CTCFBSDB 2.0 [http://insulatordb.uthsc.edu/][25].
SNP genotyping was performed by TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA, USA). We used an ABI PRISM 7900HT (Applied Biosystems) or C1000 Touch Thermal Cycler with a 384-Well Reaction Module (BIO-RAD, Hercules, CA, USA) for the Polymerase Chain Reaction (PCR), and we analyzed the fluorescent signals using the 7900HT Sequence Detection System and SDS v2.3 software (Applied Biosystems).

Statistical analyses

Fisher’s exact test using the PLINK v1.07 program was used to calculate the Hardy-Weinberg equilibrium (HWE) and the count of the alleles and genotypes in the case-control samples for association (http://zzz.bwh.harvard.edu/plink/) [26]. We calculated the P-value of the false discovery rate (FDR) using the Benjamini-Hochberg procedure as a multiple testing for deriving the observed significance to correct.

For analysis of the linkage disequilibrium (LD) test to estimate the degree of LD, we used two LD parameters, i.e., the standardized disequilibrium coefficient ($D'$) and $r^2$, calculated by Haploview v4.2 (http://www.broad.mit.edu/mpg/haploview/) [27]. We computed the standardized disequilibrium coefficient based on $D'$ according to the method of Gabriel et al. (2002) [28]. We executed the haplotype correlated analysis for common haplotypes (frequency $\geq 0.05$), then we calculated the individual and global haplotypic P-values using UNPHASED 3.1.4 (http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/). The multiple testing was calculated by FDR.

Moreover, we undertook an association analysis between these genes and schizophrenia in a stratified manner according to gender and age-at-onset using Fisher’s exact test with the PLINK v1.07 program. In the age-at-onset analysis, we divided the group into two age-at-onset categories, a) $<18$ years old, or $\geq 18$ years old and greater, and b) $<16$ years old, $16-25$ years old, $26-35$ years old, or $36$ years old and greater. In schizophrenia, even if the disease has similar symptoms, the age-at-onset of the disease with different causes occasionally changes. Therefore, the latter analysis is important to eliminate the possibility of heterogeneity which is considered to be present in schizophrenia. Moreover, schizophrenia with an onset age below 18 is often classified as early-onset schizophrenia in biological and clinical studies [29].

Furthermore, we examined the interaction of these genes using the multifactor dimensionality reduction (MDR) analysis [30], available in the open-source software package (http://www.multifactordimensionalityreduction.org/). An MDR analysis was performed by the MDR 3.0.2 program, and the permutation analysis used MDRpt Version 1.0.2 beta 2 (1,000 runs) for the testing accuracy and cross-validation consistency [31]. We used the false discovery rate as a multiple testing for the chi-square P-value. Before the analysis, the specific SNPs were excluded to avoid any false evaluation, the SNP showed a low MAF ($<0.05$), and the SNPs displayed a high LD ($r^2>0.95$).

The statistical power was calculated by the genetic power calculator (http://zzz.bwh.harvard.edu/gpc/cc2.html). The assumptive parameter is as follows: An additive model with the genotypic relative risk = 1.3, prevalence of disease = 0.01, risk allele frequency = 0.2, type I error rate = 0.05 and 1-type II error rate (determine N) = 0.8. These tests were used to the level such that the statistical significance was set at $P<0.05$.

Results

Association result

In this study, we selected 26 SNPs (16 SNPs from WDR3 and 10 SNPs from ALG1). A schematic representation of the structures of the human WDR3 and ALG1 genes and location of the SNPs are shown in Fig 1 and Table 1. The LD block structures are shown in Fig 2. Five of the WDR3
SNPs were excluded from the subsequent analysis; two SNPs due to unclear clustering by the TaqMan Assay [W3 (rs1469919) and W9 (rs6696092)], one SNP due to monomorphism [W16 (rs6656360)], and two SNPs due to significant deviations from the HWE in the controls [W11 (rs2295629) and W14 (rs3753262)]. Therefore, we examined 11 SNPs of the human *WDR3* gene and 10 SNPs of the human *ALG1* gene as the genetic association study of schizophrenia.

The allelic frequency and genotypic distributions of all the experimentally genotyped SNPs are summarized in Table 2. Two *ALG1* SNPs and one *WDR3* SNP showed a tendency of association at the level of $P < 0.05$ [A9 (rs7195893) and A10 (rs9673733) in the allelic tests, W8 (rs1321663) in the genotypic test]. The block-based haplotype analysis is shown in Table 3. For the haplotype analysis, the *WDR3* block [W4 (rs319471)–W5 (rs379058)] and the *ALG1* block [A1 (rs8053916)–A2 (rs9924614)] showed a related trend at the Global and Individual $P$-value, respectively. However, these allelic, genotypic, and haplotypic associations did not remain after correction for multiple testing.

For the gender-stratification analysis, the allelic and genotypic distributions of each SNP in the schizophrenic patients and controls are shown in Table 4. Among the males, all the SNPs

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**Fig 1. Genomic structure of human *WDR3* and *ALG1*.** Genomic structures and positions of the SNPs in human *WDR3* (A) and *ALG1* (B). Exons are denoted by boxes with untranslated regions in gray, and translated regions in white. SNPs denoted in light blue are located in the CTCF binding site, and in green are the tag SNPs (correlation coefficient: $r^2 > 0.85$, minor allele frequency: MAF > 0.10).

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did not show deviations from the HWE. On the other hand, among the females, WDR3 SNP W8 (rs1321663) was omitted from the analysis due to a significant deviation from the HWE in the female controls. In the female schizophrenia patients, WDR3 SNP W12 (rs10802003), ALG1 SNP A4 (rs3760030) and A7 (rs8045294) showed significant deviations from the HWE (P = 0.023, 0.026 and 0.024, respectively). We carefully interpreted the results of these 3 SNPs in the females.

As shown in Table 4, among the females, WDR3 SNP W4 (rs319471) exhibited a significant allelic association in the female schizophrenic patients compared to the female controls [the C allele is overrepresented in the patients; P = 0.003; odds ratio (OR), 95% confidence interval (95% CI) = 1.38, 1.12–1.70]. This association remained even after correction for multiple testing (P = 0.033). WDR3 SNP W4 (rs319471), W12 (rs10802003) and ALG1 SNP A7 (rs8045294) also displayed a tendency to genotypic association in the female subjects with schizophrenia compared to the female controls, however, it was not significant after multiple testing (Table 4).

### Table 1. SNP information for WDR3 and ALG1 genes.

<table>
<thead>
<tr>
<th>SNP ID</th>
<th>rs number</th>
<th>Major/minor</th>
<th>Strand</th>
<th>Location</th>
<th>Function</th>
<th>MAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>W1</td>
<td>rs1812607</td>
<td>C/T</td>
<td>+</td>
<td>5' upstream region</td>
<td>INS</td>
<td>T = 0.1616/353</td>
</tr>
<tr>
<td>W2</td>
<td>rs965361</td>
<td>A/T</td>
<td>+</td>
<td>5' upstream region</td>
<td>INS</td>
<td>T = 0.1625/355</td>
</tr>
<tr>
<td>W3</td>
<td>rs1469919</td>
<td>C/T</td>
<td>-</td>
<td>5' upstream region</td>
<td>INS</td>
<td>T = 0.2798/611</td>
</tr>
<tr>
<td>W4</td>
<td>rs319471</td>
<td>C/T</td>
<td>-</td>
<td>5' upstream region</td>
<td>INS</td>
<td>T = 0.1529/334</td>
</tr>
<tr>
<td>W5</td>
<td>rs379058</td>
<td>T/A</td>
<td>+</td>
<td>5' upstream region</td>
<td>INS</td>
<td>A = 0.3608/788</td>
</tr>
<tr>
<td>W6</td>
<td>rs3754127</td>
<td>C/T</td>
<td>+</td>
<td>5' upstream region</td>
<td>tag</td>
<td>T = 0.2807/613</td>
</tr>
<tr>
<td>W7</td>
<td>rs17037749</td>
<td>A/C</td>
<td>+</td>
<td>5' upstream region</td>
<td>INS</td>
<td>C = 0.0412/900</td>
</tr>
<tr>
<td>W8</td>
<td>rs3121663</td>
<td>G/C</td>
<td>+</td>
<td>intron1</td>
<td>tag</td>
<td>C = 0.0971/212</td>
</tr>
<tr>
<td>W9</td>
<td>rs6696092</td>
<td>A/G</td>
<td>+</td>
<td>intron3</td>
<td>tag</td>
<td>G = 0.4318/943</td>
</tr>
<tr>
<td>W10</td>
<td>rs1321666</td>
<td>T/C</td>
<td>+</td>
<td>intron13</td>
<td>tag</td>
<td>C = 0.4881/1066</td>
</tr>
<tr>
<td>W11</td>
<td>rs2295629</td>
<td>G/A</td>
<td>+</td>
<td>intron14</td>
<td>tag</td>
<td>A = 0.1946/425</td>
</tr>
<tr>
<td>W12</td>
<td>rs10802003</td>
<td>G/C</td>
<td>+</td>
<td>3' downstream region</td>
<td>tag</td>
<td>C = 0.0536/117</td>
</tr>
<tr>
<td>W13</td>
<td>rs10754369</td>
<td>C/T</td>
<td>+</td>
<td>3' downstream region</td>
<td>tag</td>
<td>T = 0.0847/185</td>
</tr>
<tr>
<td>W14</td>
<td>rs3753262</td>
<td>A/T</td>
<td>-</td>
<td>3' downstream region</td>
<td>INS</td>
<td>A = 0.3571/780</td>
</tr>
<tr>
<td>W15</td>
<td>rs3753261</td>
<td>C/T</td>
<td>-</td>
<td>3' downstream region</td>
<td>INS</td>
<td>T = 0.0627/137</td>
</tr>
<tr>
<td>W16</td>
<td>rs6656360</td>
<td>G/A</td>
<td>+</td>
<td>3' downstream region</td>
<td>INS</td>
<td>A = 0.0394/86</td>
</tr>
</tbody>
</table>

**WDR3**

**ALG1**

INS: insulator, MAF: minor allele frequency.

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As displayed in Table 5, in the haplotype analysis, the block range from W4 (rs319471) to W5 (rs379058) showed a significant association in the female subjects with schizophrenia compared to the female controls (global haplotypic $P = 0.016$), even after correcting for the multiple testing; T [W4 (rs319471)]–T [W5 (rs379058)] is overrepresented in the controls ($P = 0.003$; OR, 95% CI = 0.731, 0.592–0.901). We did not observe such an association in the male schizophrenics compared to the male controls.

Based on the age-at-onset stratification analysis, three of the WDR3 SNPs [W4 (rs319471), W8 (rs1321663) and W12 (rs10802003)] and four of the ALG1 SNPs [A1 (rs8053916), A5 (rs3760029), A6 (rs3760027) and A9 (rs7195893)] displayed a tendency to correlation with the different onset age groups of schizophrenia, although it was not significant after multiple testing (S1 Table).

By classifying the onset age groups of the male and female (S2 Table), five of the WDR3 SNPs [W1 (rs1812607), W2 (rs965361), W4 (rs319471), W12 (rs10802003) and W13 (rs10754369)] exhibited a tendency to correlation in several of the onset age groups, although did not remain significant after multiple testing. In addition, these SNPs have a commonality that the onset-aged between the 26 and 35 year groups in the male and female schizophrenics. In the case sample, three of the WDR3 SNPs [W4 (rs319471), W7 (rs17037749) and W13 (rs10754369)] showed a slight deviation from the HWE in the specific onset age groups of the males and females (16–25 years old in the males: $P = 0.010$, over 36 years old in the females: $P = 0.003$, over 36 years old in the females: $P = 0.040$, over 36 years old in the females: $P = 0.022$, respectively). Three of the ALG1 SNPs [A1 (rs8053916), A4 (rs3760030) and A7 (rs8045294)], although not significant after multiple testing, showed a tendency to correlation. Moreover, in the case sample, A4 (rs3760030) showed a slight deviation from the HWE in specific groups (26–35 years old in the females; $P = 0.006$). We further cautiously interpreted the results of the SNPs deviating from the HWE in the males and females.

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Fig 2. LD block structure of WDR3 and ALG1 genes. (A) WDR3 gene consists of three, and (B) ALG1 gene consists of two haplotype blocks in schizophrenia. In the left panel, the number in the box represents $D' \times 100$, blank means $D' = 1$. In the right panel, the number in the box represents $r^2 \times 100$. 
Table 2. Genotyping and allele distribution of SNPs on WDR3 and ALG1 genes in schizophrenia and controls from the Japanese population.

**WDR3**

<table>
<thead>
<tr>
<th>SNP ID</th>
<th>Affection</th>
<th>N</th>
<th>HWE P</th>
<th>Allele count</th>
<th>MAF</th>
<th>Allelic P (FDR P)</th>
<th>OR (95% CI)</th>
<th>Genotypic count</th>
<th>Genotypic P (FDR P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1812607</td>
<td>SCZ</td>
<td>1,806</td>
<td>0.222</td>
<td>3,806 806 0.223</td>
<td>0.430  (0.701) 1.045 (0.932–1.62)</td>
<td>AA AT TT</td>
<td>1,320 762 86</td>
<td>0.080 (0.220)</td>
<td></td>
</tr>
<tr>
<td>W2</td>
<td>CON</td>
<td>2,168</td>
<td>0.066</td>
<td>3,402 810 0.223</td>
<td>0.074  (0.413) 0.877 (0.759–1.012)</td>
<td>CC CT TT</td>
<td>1,709 433 28</td>
<td>0.200 (0.367)</td>
<td></td>
</tr>
<tr>
<td>rs965361</td>
<td>SCZ</td>
<td>1,808</td>
<td>0.277</td>
<td>2,810 806 0.223</td>
<td>0.076  (0.220)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W4</td>
<td>CON</td>
<td>2,170</td>
<td>0.914</td>
<td>3,851 489 0.100</td>
<td>0.543  (0.710) 0.972 (0.890–1.062)</td>
<td>TT TA AA</td>
<td>534 1,121 513</td>
<td>0.681 (0.955)</td>
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<tr>
<td>W5</td>
<td>CON</td>
<td>2,168</td>
<td>0.122</td>
<td>2,189 2,147 0.495</td>
<td>0.581  (0.710) 1.033 (0.922–1.158)</td>
<td>CC CT TT</td>
<td>1,445 658 66</td>
<td>0.840 (0.955)</td>
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<td>rs319471</td>
<td>SCZ</td>
<td>1,807</td>
<td>0.794</td>
<td>3,252 362 0.100</td>
<td>0.080  (0.220)</td>
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<tr>
<td>W7</td>
<td>CON</td>
<td>2,169</td>
<td>0.530</td>
<td>4,180 158 0.036</td>
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<tr>
<td>rs379058</td>
<td>SCZ</td>
<td>1,807</td>
<td>0.541</td>
<td>1,851 1,765 0.488</td>
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<td></td>
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<td></td>
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<tr>
<td>W6</td>
<td>CON</td>
<td>2,169</td>
<td>0.428</td>
<td>3,548 790 0.187</td>
<td>0.904  (0.904) 0.979 (0.772–1.240)</td>
<td>AA AC CC</td>
<td>2,015 150 4</td>
<td>0.947 (0.955)</td>
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<tr>
<td>rs3754127</td>
<td>SCZ</td>
<td>1,807</td>
<td>0.354</td>
<td>2,938 676 0.187</td>
<td>0.161  (0.590) 1.086 (0.969–1.217)</td>
<td>GG GC CC</td>
<td>1,485 605 79</td>
<td>0.042 (0.220)</td>
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<tr>
<td>W8</td>
<td>CON</td>
<td>2,169</td>
<td>0.088</td>
<td>3,487 129 0.036</td>
<td>0.075  (0.413) 1.085 (0.993–1.185)</td>
<td>TT TC CC</td>
<td>630 1,068 469</td>
<td>0.182 (0.367)</td>
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<td>0.248</td>
<td>2,934 680 0.188</td>
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<tr>
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<td>0.697</td>
<td>3,684 654 0.158</td>
<td>0.350  (0.701) 1.061 (0.939–1.198)</td>
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<td>1,572 540 57</td>
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<td>3,043 573 0.158</td>
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<td>CC TC TT</td>
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<td>0.717 (0.955)</td>
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<td>0.112</td>
<td>1,865 1,743 0.483</td>
<td>0.431  (0.701) 1.047 (0.934–1.174)</td>
<td>CC CT TT</td>
<td>1,461 641 68</td>
<td>0.955 (0.955)</td>
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<td>W13</td>
<td>CON</td>
<td>2,170</td>
<td>0.884</td>
<td>3,563 777 0.179</td>
<td>0.858  (0.904) 1.013 (0.881–1.166)</td>
<td>CC CT TT</td>
<td>1,715 429 25</td>
<td>0.955 (0.955)</td>
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<td>rs10754369</td>
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<td>0.756</td>
<td>2,942 672 0.186</td>
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<td>0.635</td>
<td>3,212 404 0.112</td>
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**ALG1**

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<th>HWE P</th>
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<th>MAF</th>
<th>Allelic P (FDR P)</th>
<th>OR (95% CI)</th>
<th>Genotypic count</th>
<th>Genotypic P (FDR P)</th>
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<td>0.652</td>
<td>2,543 1,071 0.296</td>
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<td>AA AT TT</td>
<td>1,192 827 151</td>
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<td>CON</td>
<td>2,170</td>
<td>0.656</td>
<td>3,211 1,129 0.260</td>
<td>0.314  (0.449) 0.948 (0.857–1.049)</td>
<td>CC CT TT</td>
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<td>0.531</td>
<td>2,712 904 0.250</td>
<td>0.554  (0.612) 0.965 (0.859–1.084)</td>
<td>CC TC TT</td>
<td>1,460 630 71</td>
<td>0.319 (0.638)</td>
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<td>CC TC TT</td>
<td>1,230 514 55</td>
<td>0.319 (0.638)</td>
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(Continued)
Gene-gene interaction analysis

Based on the MDR analysis, five of the WDR3 SNPs were excluded for the same reasons as for the case-control Fisher’s exact test: W3 (rs1469919), W9 (rs6696092), W11 (rs2295629), W14 (rs3753262) and W16 (rs6656360). The LD block which consisted of W1 (rs1812607) - W2 (rs965361) showed a high LD ($r^2 > 0.95$). Therefore, we searched the tag SNP for avoid any false evaluation. Using the HaploView program to examine the tag SNP, W1 (rs1812607) was detected. Therefore, SNP W2 (rs965361) was omitted. For the sex stratified analysis, the same six SNPs were excluded due to same reasons in the males. In the females, WDR3 SNP W8 (rs1321663) was additionally excluded for the same reasons as for the case-control Fisher’s exact test. Therefore, 10 ALG1 SNPs and 10 WDR3 SNPs were analyzed in all the samples of the case-controls and male case-control samples. In the females, 10 ALG1 SNPs and 9 WDR3 SNPs were analyzed.

The testing accuracy (TA) represents the average value of the sensitivity and specificity. A TA of 0.55 and greater means that the MDR model is typically statistically significant. The best $P$-value was the combination of ALG1 SNP A9 (rs7195893) and WDR3 SNP W10 (rs1321666) in the female schizophrenia ($P = 0.047$), but the TA of this model was less than 0.55 (TA = 0.543). The chi-square $P$-value supported this result ($P = 0.208$). Therefore, it was not enough to indicate the interaction of these genes (Table 6).

Power estimation

The power analysis showed a 99.09% power in the genotypic test and a 99.64% power in the allelic test for the case-control statistics in our sample. Based on the stratified analysis
according to sex, the powers of the female and male groups were 85.48% and 82.51% in the genotypic test and 91.3% and 89.02% in the allelic test, respectively. The other stratified groups consisting of the classified age at onset are shown in Table 7.

**Discussion**

This is the first genetic study of the *WDR3* and *ALG1* genes in schizophrenia to the best of our knowledge. We detected related signals between the *WDR3* genes and female schizophrenic patients. In the allelic tests, W4 (rs319471) indicated a significant association with schizophrenia among the female schizophrenia patients. In our block-based haplotype analysis, the block range from W4 (rs319471) to W5 (rs379058) exhibited a significant association in the female schizophrenics. In these analyses, no association was detected in the male or the group of all subjects. Indeed, gender differences related to schizophrenia have been widely known [32]. For example, the clinical observations showed that male patients were inclined to have earlier onset and a more severe course than female patients. In addition, male schizophrenics have more negative symptoms and cognitive deficits, while female schizophrenics show more affective symptoms [33]. For the molecular biological approaches, several genes that have sex-
Table 4. Stratification analysis of sex on WDR3 and ALG1 gene in schizophrenia and controls from Japanese population.

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<thead>
<tr>
<th>SNP ID</th>
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<th>HWE P (FDR)</th>
<th>Genotypic count</th>
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<th>Female</th>
<th>Male</th>
<th>Female</th>
<th>Male</th>
<th>Female</th>
<th>Male</th>
<th>Female</th>
<th>Male</th>
<th>Female</th>
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<th>Male</th>
<th>Female</th>
<th>Male</th>
<th>Female</th>
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<td>rs1812607 SCZ 992 816</td>
<td>0.484</td>
<td>0.573</td>
<td>AG/AA</td>
<td>1,305 2</td>
<td>1,294</td>
<td>0.399 0.417</td>
<td>1,449</td>
<td>323</td>
<td>646</td>
<td>0.455</td>
<td>0.491</td>
<td>0.216</td>
<td>0.231</td>
<td>0.727</td>
<td>0.697</td>
<td>0.008</td>
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<tr>
<td>rs1812607 SCZ 992 816</td>
<td>0.484</td>
<td>0.573</td>
<td>GG/AA</td>
<td>1,305 2</td>
<td>1,294</td>
<td>0.399 0.417</td>
<td>1,449</td>
<td>323</td>
<td>646</td>
<td>0.455</td>
<td>0.491</td>
<td>0.216</td>
<td>0.231</td>
<td>0.727</td>
<td>0.697</td>
<td>0.008</td>
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<tr>
<td>rs1812607 SCZ 992 816</td>
<td>0.484</td>
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<td>AG/GG</td>
<td>1,305 2</td>
<td>1,294</td>
<td>0.399 0.417</td>
<td>1,449</td>
<td>323</td>
<td>646</td>
<td>0.455</td>
<td>0.491</td>
<td>0.216</td>
<td>0.231</td>
<td>0.727</td>
<td>0.697</td>
<td>0.008</td>
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<tr>
<td>rs1812607 SCZ 992 816</td>
<td>0.484</td>
<td>0.573</td>
<td>GG/AG</td>
<td>1,305 2</td>
<td>1,294</td>
<td>0.399 0.417</td>
<td>1,449</td>
<td>323</td>
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<td>0.455</td>
<td>0.491</td>
<td>0.216</td>
<td>0.231</td>
<td>0.727</td>
<td>0.697</td>
<td>0.008</td>
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Association study of WDR3 and ALG1 with schizophrenia

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<td>0.337</td>
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<td>0.967</td>
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<td>484</td>
<td>1.269</td>
<td>363</td>
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<td>0.222</td>
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<td>(0.752)</td>
<td>(0.926–1.252)</td>
<td>(0.833–1.122)</td>
<td>570</td>
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<td>1.000</td>
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<td>0.157</td>
<td>0.158</td>
<td>0.289</td>
<td>0.876</td>
<td>0.909</td>
<td>624</td>
</tr>
<tr>
<td>rs3760029</td>
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<td>814</td>
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<td>1,698</td>
<td>284</td>
<td>1,393</td>
<td>235</td>
<td>0.143</td>
<td>0.144</td>
<td>(0.495)</td>
<td>(0.650)</td>
<td>(0.733–1.047)</td>
<td>(0.763–1.082)</td>
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<td>1.011</td>
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<tr>
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<td>813</td>
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<td>0.137</td>
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<td>(1)</td>
<td>(0.917–1.321)</td>
<td>(0.831–1.195)</td>
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<tr>
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<td>773</td>
<td>1,026</td>
<td>602</td>
<td>0.391</td>
<td>0.370</td>
<td>(0.650)</td>
<td>(0.650)</td>
<td>(0.845–1.098)</td>
<td>(0.814–1.052)</td>
<td>368</td>
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<tr>
<td>A8</td>
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<td>908</td>
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<td>997</td>
<td>830</td>
<td>796</td>
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<td>0.490</td>
<td>(0.495)</td>
<td>(0.650)</td>
<td>(0.932–1.204)</td>
<td>(0.832–1.068)</td>
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<td>0.872</td>
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<td>814</td>
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<td>0.870</td>
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<td>233</td>
<td>1,432</td>
<td>196</td>
<td>0.118</td>
<td>0.120</td>
<td>(0.495)</td>
<td>(0.650)</td>
<td>(0.684–1.005)</td>
<td>(0.723–1.052)</td>
<td>769</td>
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<td>0.181</td>
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<td>330</td>
<td>1,370</td>
<td>262</td>
<td>0.166</td>
<td>0.161</td>
<td>(0.495)</td>
<td>(0.650)</td>
<td>(0.754–1.056)</td>
<td>(0.734–1.024)</td>
<td>693</td>
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N: number of subjects, HWE: Hardy-Weinberg equilibrium, MAF: minor allele frequency, FDR: the false discovery rate using the Benjamini-Hochberg procedure, OR: odds ratio, 95% CI: 95% confidence interval, CON: control, SCZ: schizophrenia
Table 5. Sex stratified block-based haplotype analysis of WDR3 and ALG1 genes.

(A) WDR3

<table>
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<th>Frequency</th>
<th>OR (95% CI)</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>SCZ</td>
<td>CON</td>
<td>Individual P</td>
</tr>
<tr>
<td>W1, W2</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C, A</td>
<td>0.783</td>
<td>0.774</td>
<td>1.053 (0.903–1.229)</td>
</tr>
<tr>
<td>T, T</td>
<td>0.216</td>
<td>0.225</td>
<td>0.949 (0.813–1.108)</td>
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<td>W4, W5</td>
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<tr>
<td>C, A</td>
<td>0.477</td>
<td>0.493</td>
<td>0.933 (0.821–1.061)</td>
</tr>
<tr>
<td>C, T</td>
<td>0.413</td>
<td>0.400</td>
<td>1.051 (0.923–1.198)</td>
</tr>
<tr>
<td>T, T</td>
<td>0.111</td>
<td>0.105</td>
<td>1.055 (0.858–1.298)</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Marker</th>
<th>Frequency</th>
<th>OR (95% CI)</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>SCZ</td>
<td>CON</td>
<td>Individual P</td>
</tr>
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</tr>
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<td>0.186</td>
<td>1.009 (0.855–1.189)</td>
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<tr>
<td>C, G, C</td>
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<td>0.099</td>
<td>1.069 (0.865–1.322)</td>
</tr>
<tr>
<td>C, G, T</td>
<td>0.521</td>
<td>0.533</td>
<td>0.953 (0.838–1.084)</td>
</tr>
<tr>
<td>T, G, C</td>
<td>0.187</td>
<td>0.183</td>
<td>1.031 (0.874–1.216)</td>
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</table>

(B) ALG1

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<th>Frequency</th>
<th>OR (95% CI)</th>
<th>P-values</th>
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<tbody>
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<td>Individual P</td>
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<tr>
<td>C, C</td>
<td>0.459</td>
<td>0.430</td>
<td>1.125 (0.989–1.280)</td>
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<tr>
<td>C, T</td>
<td>0.252</td>
<td>0.268</td>
<td>0.92 (0.795–1.065)</td>
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<td>G, C</td>
<td>0.290</td>
<td>0.303</td>
<td>0.939 (0.816–1.080)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Marker</th>
<th>Frequency</th>
<th>OR (95% CI)</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SCZ</td>
<td>CON</td>
<td>Individual P</td>
</tr>
<tr>
<td>C, C, C, C, G, C</td>
<td>0.114</td>
<td>0.121</td>
<td>0.924 (0.756–1.129)</td>
</tr>
<tr>
<td>C, C, C, G, G</td>
<td>0.489</td>
<td>0.477</td>
<td>1.044 (0.917–1.189)</td>
</tr>
<tr>
<td>C, T, C, C, C, C</td>
<td>0.145</td>
<td>0.159</td>
<td>0.89 (0.743–1.065)</td>
</tr>
<tr>
<td>T, C, C, C, C</td>
<td>0.087</td>
<td>0.088</td>
<td>0.992 (0.790–1.247)</td>
</tr>
<tr>
<td>T, C, T, C, C</td>
<td>0.150</td>
<td>0.136</td>
<td>1.114 (0.926–1.340)</td>
</tr>
</tbody>
</table>

Association study of WDR3 and ALG1 with schizophrenia

PLOS ONE | https://doi.org/10.1371/journal.pone.0190991 January 8, 2018 13 / 19
Specific genetic associations with schizophrenia were reported such as Disrupted in Schizophrenia 1 (DISC1), reelin (RELN), D-amino acid oxidase (DAO) and synapse-associated.

Table 5. (Continued)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>C/C</th>
<th>C/T</th>
<th>G/C</th>
<th>A4 A5 A6 A7 A8 A9</th>
</tr>
</thead>
<tbody>
<tr>
<td>OR</td>
<td>0.448</td>
<td>0.248</td>
<td>0.304</td>
<td>0.048 0.024 0.004</td>
</tr>
<tr>
<td>95% CI</td>
<td>1.085 (0.957–1.230)</td>
<td>0.963 (0.835–1.112)</td>
<td>0.941 (0.823–1.076)</td>
<td>0.375 0.437 0.720</td>
</tr>
</tbody>
</table>

OR: odds ratio, 95% CI: 95% confidence interval, CON: control, SCZ: schizophrenia, FDR: the false discovery rate using the Benjamini-Hochberg procedure.

https://doi.org/10.1371/journal.pone.0190991.t005

Table 6. The MDR analysis for the best determined model.

<table>
<thead>
<tr>
<th>Total model</th>
<th>TA</th>
<th>CVC</th>
<th>Permutation P</th>
<th>χ²</th>
<th>P</th>
<th>FDR P</th>
</tr>
</thead>
<tbody>
<tr>
<td>WD-08 (rs1321663)</td>
<td>0.499</td>
<td>4/10</td>
<td>0.868</td>
<td>0.947</td>
<td>0.001</td>
<td>0.978</td>
</tr>
<tr>
<td>AL-10 (rs9673733), WD-10 (rs1321666)</td>
<td>0.478</td>
<td>2/10</td>
<td>0.999–1.000</td>
<td>0.999–1.000</td>
<td>0.759</td>
<td>0.384</td>
</tr>
<tr>
<td>AL-01 (rs8053916), AL-08 (rs8045473), WD-08 (rs1321663)</td>
<td>0.506</td>
<td>4/10</td>
<td>0.665</td>
<td>0.947</td>
<td>0.062</td>
<td>0.803</td>
</tr>
<tr>
<td>AL-02 (rs9924614), AL-07 (rs8045294), AL-10 (rs9673733), WD-10 (rs1321666)</td>
<td>0.501</td>
<td>2/10</td>
<td>0.834</td>
<td>0.999–1.000</td>
<td>0.001</td>
<td>0.973</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Male model</th>
<th>TA</th>
<th>CVC</th>
<th>Permutation P</th>
<th>χ²</th>
<th>P</th>
<th>FDR P</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL-09 (rs7195893)</td>
<td>0.498</td>
<td>7/10</td>
<td>0.886</td>
<td>0.621</td>
<td>0.003</td>
<td>0.960</td>
</tr>
<tr>
<td>WD-04 (rs319471), WD-12 (rs10802003)</td>
<td>0.518</td>
<td>9/10</td>
<td>0.453</td>
<td>0.349</td>
<td>0.262</td>
<td>0.609</td>
</tr>
<tr>
<td>AL-01 (rs8053916), AL-09 (rs7195893), WD-13 (rs10754369)</td>
<td>0.490</td>
<td>2/10</td>
<td>0.964–0.965</td>
<td>0.999–1.000</td>
<td>0.077</td>
<td>0.781</td>
</tr>
<tr>
<td>AL-01 (rs8053916), AL-03 (rs9932909), AL-08 (rs8045473), WD-08 (rs1321663)</td>
<td>0.488</td>
<td>3/10</td>
<td>0.972–0.973</td>
<td>0.990</td>
<td>0.106</td>
<td>0.745</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Female model</th>
<th>TA</th>
<th>CVC</th>
<th>Permutation P</th>
<th>χ²</th>
<th>P</th>
<th>FDR P</th>
</tr>
</thead>
<tbody>
<tr>
<td>WD-04 (rs319471)</td>
<td>0.511</td>
<td>8/10</td>
<td>0.623</td>
<td>0.493</td>
<td>0.123</td>
<td>0.726</td>
</tr>
<tr>
<td>AL-09 (rs7195893), WD-10 (rs1321666)</td>
<td>0.543</td>
<td>10/10</td>
<td>0.047</td>
<td>0.212</td>
<td>1.584</td>
<td>0.208</td>
</tr>
<tr>
<td>AL-10 (rs9673733), WD-05 (rs379058), WD-10 (rs1321666)</td>
<td>0.516</td>
<td>2/10</td>
<td>0.516</td>
<td>0.999–1.000</td>
<td>0.215</td>
<td>0.643</td>
</tr>
<tr>
<td>AL-02 (rs9924614), AL-07 (rs8045294), WD-05 (rs379058), WD-10 (rs1321666)</td>
<td>0.516</td>
<td>4/10</td>
<td>0.519–0.520</td>
<td>0.962</td>
<td>0.207</td>
<td>0.649</td>
</tr>
</tbody>
</table>

TA: testing accuracy, CVC: cross-validation consistency, FDR: the false discovery rate using the Benjamini-Hochberg procedure.

https://doi.org/10.1371/journal.pone.0190991.t006
protein 97/discs, large homolog 1 of Drosophila (*DLG1*) in previous studies [34–37]. Therefore, the female specific association revealed in the WDR3 gene might be involved in the molecular basis of the schizophrenic pathology. WDR3 SNP W4 (rs319471), which is located in the CTCF binding site of the 5' upstream of the WDR3 gene, showed a significant association with female schizophrenics. This study focused on the CTCF binding site to select the SNPs as the gene expression control by the insulator function. This function is well known to enhancer-blocking activity and as a barrier to chromosomal position effects [38]. Consequently, the polymorphism of this site might be linked to the insulator function/dysfunction of the WDR3 and flanking cluster genes. We searched the sequence that contains 50 base pairs up- and downstream of W4 (rs319471) at CTCFBSDB 2.0; a database for CTCF binding sites and genome organization (http://insulatordb.uthsc.edu/) [25, 39]. As a result, only when the SNP consists of the C allele does the sequence (ATCACTGCC) closely conform to the CTCF consensus. It might influence the expression level. We searched the expression quantitative trait loci (eQTLs) using the Brain eQTL Almanac (http://www.braineac.org/) to investigate whether W4 (rs319471) affects the expression of WDR3 in the females. The change in the expression level was reported in a multitude of genes, however, there is no significant report on the expression level of WDR3 in the database. Although we have to carefully consider that the database does not categorize the data by sex. To estimate the difference in the expression level in the female schizophrenics, the data by sex are needed. If it formed a CTCF consensus, the possibility is considered that the minor allele frequency is lower than in the healthy control at the female W4 (rs319471, minor

<table>
<thead>
<tr>
<th>Total</th>
<th>N Genotypic Allelic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SCZ</td>
</tr>
<tr>
<td>Under 17</td>
<td>264</td>
</tr>
<tr>
<td>Over 18</td>
<td>1426</td>
</tr>
<tr>
<td>Under 15</td>
<td>107</td>
</tr>
<tr>
<td>16–25</td>
<td>918</td>
</tr>
<tr>
<td>26–35</td>
<td>461</td>
</tr>
<tr>
<td>Over 36</td>
<td>204</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Male</th>
<th>N Genotypic Allelic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SCZ</td>
</tr>
<tr>
<td>Under 15</td>
<td>50</td>
</tr>
<tr>
<td>16–25</td>
<td>519</td>
</tr>
<tr>
<td>26–35</td>
<td>252</td>
</tr>
<tr>
<td>Over 36</td>
<td>94</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Female</th>
<th>N Genotypic Allelic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SCZ</td>
</tr>
<tr>
<td>Under 15</td>
<td>57</td>
</tr>
<tr>
<td>16–25</td>
<td>399</td>
</tr>
<tr>
<td>26–35</td>
<td>209</td>
</tr>
<tr>
<td>Over 36</td>
<td>110</td>
</tr>
</tbody>
</table>

N: number of subjects, N (80%): Number that reaches 80% detection power, CON: control, SCZ: schizophrenia

https://doi.org/10.1371/journal.pone.0190991.t007
allele: T), thus it is possible that the CTCF-binding activity is higher in schizophrenia. Moreover, based on a block-based haplotype analysis, the block consisting of W4 (rs319471) showed a significant correlation in female schizophrenia. It may support the fact that W4 (rs319471) is located in the disease susceptibility region. Actually, the influence of the CTCF-binding activity in the brain was reported. The CTCF-deficient neuron showed defects in the dendritic arborization and spine density during brain development [40]. Additionally, a decline in the cohesin function in the brain leads to a defective synapse development and anxiety-related behavior [41]. This means that the CTCF-binding activity has relevance to functional neural development and neuronal diversity. Accordingly, W4 (rs319471) has the possibilities involved in the pathophysiology of schizophrenia via the chromatin conformational changes.

The ALG1 SNPs showed only a statistically-weak correlation, however, two SNPs [A4 (rs3760030) and A7 (rs8045294)] that showed a tendency of association with female schizophrenia were reported as the eQTLs [42, 43]. Furthermore, the chromosome 16p13 region, ALG1 located, was reported to have copy number variations associated with schizophrenia [44]. The ALG1 gene did not show a strong correlation with schizophrenia in this study, however, the SNPs that showed a trend associated with a specific onset-age groups were observed. This may suggest that the SNPs or there genomic region affects the onset age of schizophrenia.

As for the age-at-onset analysis, there was no statistically significant association. This may be explained by the low statistical power in our stratified age-at-onset groups (<80%). Therefore, a larger sample size group needs to be studied to use the age-at-onset analysis.

In conclusion, our present associations study demonstrated that the WDR3 gene is selectively related to female schizophrenia. These results indicated that the WDR3 gene may be a susceptibility factor in female subjects with schizophrenia, and that regulation of the WDR3 signaling pathway ensures further research from the aspect of the pathophysiology of schizophrenia.

Further study is required to elucidate the gender-dependent correlation between the WDR3 gene and schizophrenia using different ethnic populations and larger sample sizes.

Supporting information

S1 Table. Stratification analysis of onset-age groups on WDR3 and ALG1 genes in schizophrenia and controls from Japanese population. N: number of subjects, HWE: Hardy-Weinberg equilibrium, MAF: minor allele frequency, FDR: the false discovery rate using the Benjamini-Hochberg procedure, OR: odds ratio, 95% CI: 95% confidence interval, CON: control, SCZ: schizophrenia.

S2 Table. Stratification analysis of onset-age groups by sex on WDR3 and ALG1 genes in schizophrenia and controls from Japanese population. N: number of subjects, HWE: Hardy-Weinberg equilibrium, MAF: minor allele frequency, FDR: the false discovery rate using the Benjamini-Hochberg procedure, OR: odds ratio, 95% CI: 95% confidence interval, CON: control, SCZ: schizophrenia.

Acknowledgments

We are sincerely thankful to the patients and healthy volunteers who participated in this study.

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**Resources:** Yoshimi Iwayama, Tomoko Toyota, Katsuyaki Suzuki, Mitsuru Kikuchi, Tasuku Hashimoto, Nobuhisa Kanahara, Akeo Kurumaji, Takeo Yoshikawa, Toru Nishikawa.

**Supervision:** Naoki Yamamoto, Akeo Kurumaji, Takeo Yoshikawa, Toru Nishikawa.

**Validation:** Naoki Yamamoto, Tomoko Toyota, Takeo Yoshikawa, Toru Nishikawa.

**Writing – original draft:** Momoko Kobayashi, Daisuke Jitoku.

**Writing – review & editing:** Toru Nishikawa.

**References**


