Resequencing and Association Analysis of Six PSD-95-Related Genes as Possible Susceptibility Genes for Schizophrenia and Autism Spectrum Disorders

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PSD-95 associated PSD proteins play a critical role in regulating the density and activity of glutamate receptors. Numerous previous studies have shown an association between the genes that encode these proteins and schizophrenia (SZ) and autism spectrum disorders (ASD), which share a substantial portion of genetic risks. We sequenced the protein-encoding regions of DLG1, DLG2, DLG4, DLGAP1, DLGAP2, and SynGAP in 562 cases (370 SZ and 192 ASD patients) on the Ion PGM platform. We detected 26 rare (minor allele frequency <1%), non-synonymous mutations, and conducted silico functional analysis and pedigree analysis when possible. Three variants, G344R in DLG1, G241S in DLG4, and R604C in DLGAP2, were selected for association analysis in an independent sample set of 1315 SZ patients, 382 ASD patients, and 1793 healthy controls. Neither DLG4-G241S nor DLGAP2-R604C was detected in any samples in case or control sets, whereas one additional SZ patient was found that carried DLG1-G344R. Our results suggest that rare missense mutations in the candidate PSD genes may increase susceptibility to SZ and/or ASD. These findings may strengthen the theory that rare, non-synonymous variants confer substantial genetic risks for these disorders.

Schizophrenia (SZ) is a debilitating disorder that affects approximately 1% of the population. A large portion of its heritability, which is estimated at 80%¹, remains to be explained. Results from multiple large-scale genome-wide association studies as well as whole-genome/whole-exome sequencing support a polygenic model for explaining the susceptibility to the disorder. In this model, deleterious rare variants exert significantly larger effects than common single nucleotide polymorphisms (SNPs)²–⁴.

The postsynaptic density (PSD) is a protein complex localized at the postsynaptic plasma membrane of excitatory synapses. The PSD is essential for protein trafficking in neurons and synaptic plasticity⁵, processes commonly associated with the pathogenesis of SZ⁶. Scaffolding proteins, the primary components of the PSD structure, interact closely with glutamate receptors and play a major role in the dynamic regulation of their signaling activities⁷. PSD-95, which is a key protein in this subgroup, is a member of the membrane-associated guanylate kinase (MAGUK) family and is encoded by the disks large homolog 4 (DLG4) gene. Its linkage to SZ has been well established through both variant association⁸ and expression studies⁹–¹⁴. DLG1 and DLG2, which encode two other MAGUK family proteins, have been similarly linked to SZ¹⁵–²⁰. The products of DLGAP1 and DLGAP2 are guanylate kinase-associated protein (GKAP) family proteins that bind to the MAGUKs, mediating their interaction with other components of the PSD complex²¹,²². Resequencing studies have implicated these genes as susceptibility genes for SZ²³,²⁴. SynGAP1, another major scaffolding protein, has multiple protein-protein interacting motifs.
that enable it to act as a structural and regulatory anchor in synaptic homeostasis. Its association with SZ has been shown in human expression studies and animal models. Recently, many whole-genome/whole-exome sequencing studies focusing on deleterious rare mutations, including copy number variants (CNVs), have frequently identified the PSD gene group, especially the PSD-95-related subgroup. In a study conducted by Purcell et al. who analyzed the exome sequences of 2536 SZ cases and 2543 controls for the burden of rare, disruptive mutations, the PSD, activity-regulated cytoskeleton-associated scaffolding protein, and PSD-95 gene sets were associated with SZ (p = 0.0808, p = 0.0016, p = 0.0017, for singletons, respectively). Multiple de novo CNVs spanning the coding regions of DLG1, DLG2, and DLGAP1 have been discovered in European and Asian SZ patients. A Swedish study of 4719 SZ cases and 5917 controls found a significantly increased burden of large CNVs (>500 kb) in genes present in the PSD, especially in the 3p29/DLG1 locus, which has been implicated in previously conducted genome-wide association studies.

Autism spectrum disorders (ASD) are a range of conditions characterized by persistent deficits in social communication and interaction, as well as restricted, repetitive patterns of behavior, interests, or activities. Both ASD and SZ belong to a group of distinct clinical entities known as neurodevelopmental disorders, as defined in DSM-V. It has been indicated by clinical and epidemiologic studies that neurodevelopmental disorders have a high comorbidity rate, overlapping signs and symptoms, and significant similarities in genetic background. Furthermore, various previous researches provide strong evidences of common underlying molecular pathways and shared genetic causes between ASD and SZ. A recent review of targeted large-scale resequencing studies has pointed out that genetic evidence converges on three functional pathways, one of which is synaptic function. This review also predicted that PSD genes such as DLG4 and SynGAP1 will be identified as key nodes in the connected network. A similar study utilizing the network-based analysis of genetic associations system identified a large biological network of genes that are affected by rare de novo CNVs in autism, with DLG4, DLG1, and DLG2 as important nodes in the cluster. Individually, DLGAP2 and SynGAP1 are established risk genes for ASD, and DLG1, DLG2, and DLG4 have also been implicated in various studies. Based on the results of these studies, we selected six candidate genes with the most evidence implicating an association with SZ and ASD: DLG1, DLG2, DLG4, DLGAP1, DLGAP2, and SynGAP1. The exonic regions of these genes were sequenced to look for rare, protein-altering point mutations.

Materials and Methods

Participants. Two independent sample sets were used in this study (Table 1). The first set, comprising 370 SZ patients (mean age = 49.73 ± 14.75 years; males = 52.97%) and 192 ASD patients (mean age = 16.34 ± 8.36 years; males = 77.60%), was sequenced for rare point mutations. The second, larger set, comprising 1315 SZ patients (mean age = 47.41 ± 15.35 years; males = 53.92%), 382 ASD patients (mean age = 19.61 ± 10.71 years; males = 77.75%), and 1793 controls (mean age = 45.11 ± 14.61 years; males = 51.25%), was used for association analysis of selected variants detected in the first set.

All participants in this study were recruited in the Nagoya University Hospital and its associated Institutes. Patients were included in the study if they (1) met DSM-5 criteria for SZ or ASD and (2) were physically healthy. Controls were selected from the general population and had no personal or family history of psychiatric disorders (first-degree relatives only based on the subject's interview). The selection was based on the following: (1) questionnaire responses from the subjects themselves during the sample inclusion step; or (2) unstructured diagnostic interview conducted by an experienced psychiatrist during the blood collection step. All subjects were unrelated, lived in the central area of the Honshu island of Japan, and self-identified as members of the Japanese population. The Ethics Committees of the Nagoya University Graduate School of Medicine approved this study. All experiments were performed in accordance with the Committee’s guidelines and regulations. Written informed consent was obtained from all participants. In addition, each patient's capacity to provide consent was confirmed by a family member when needed. Individuals with a legal measure of reduced capacity were excluded.

Resequencing and Data Analysis. Genomic DNA was extracted from whole blood or saliva using the QIAGEN QIAamp DNA blood kit or tissue kit (QIAGEN Ltd., Germany). Custom amplification primers were designed to cover coding exons and flanking intron regions of the selected genes with Ion AmpliSeq Designer (Thermo Fisher Scientific, USA). Sample amplification and equalization were achieved using Ion AmpliSeq Library Kits 2.0 and the Ion Library Equalizer Kit, respectively (Thermo Fisher Scientific, USA). Amplified sequences were ligated with Ion Xpress Barcode Adapters (Thermo Fisher Scientific, USA). Emulsion PCR and subsequent enrichment were performed using the Ion OneTouch Template Kit v2.0 on Ion OneTouch 2 and Ion OneTouch ES, respectively (Thermo Fisher Scientific, USA). The final product was then sequenced on the Ion PGM sequencing platform (Thermo Fisher Scientific, USA). Raw data output from the sequencer was deposited in the DNA Data Bank of Japan (DDBJ) (http://www.ddbj.nig.ac.jp) under the accession number DRA004490,
and uploaded to the Torrent Server (Life Technologies, USA) for variant calling, with NCBI GRCh37 as a reference. The resulting VCF files were analyzed by Ingenuity Variant Analysis (QIAGEN Ltd., Germany) for annotation and visualization.

Association Analysis. Missense mutations, small insertions/deletions, and splicing site variations with a minor allele frequency <1% were selected from the annotated data. The mutation calls were then validated for confidence by Sanger sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Genotyping prioritization was based on whether the mutation was 1) located in a functional domain or motif of the protein, according to the Human Protein Reference Database (http://www.hprd.org), Pfam (http://pfam.xfam.org/), and existing literature [21,50–59, 2] functionally important, such as causing a frame shift, stop gain, or cysteine gain/loss, 3) novel, as in not documented in the NCBI dbSNP database (Build 137) (http://www.ncbi.nlm.nih.gov/SNP/), the 1000 Genomes Project (http://www.1000genomes.org/), the Exome Variant Server of NHLBI GO Exome Sequencing Project (ESP6500SI-V2) (http://evs.gs.washington.edu/EVS/), or the Human Genetic Variation Database of Japanese genetic variation consortium (http://www.genome.med.kyoto-u.ac.jp/SnpDB), and 4) predicted to be deleterious by in silico analytic methods. In addition to PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.jcvi.org/) that were originally incorporated in the Ingenuity Variant Caller, we also employed PROVEAN (http://provean.jcvi.org/index.php), PMut (http://www.ngrl.org.uk/Manchester/page/pmut), Mutation Taster (http://www.mutationtaster.org/), and PANTHER (http://pantherdb.org/) for enhanced prediction of the consequences of protein alterations.

Custom TaqMan SNP genotyping assays were designed and ordered from Applied Biosystems. Allelic discrimination analysis was performed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, USA). Differences in allele and genotype frequencies of the mutations were compared between SZ patients/controls and ASD patients/controls using Fisher’s exact test (two-tailed), with a threshold of significance set at p < 0.05.

Additional Analysis for Amino Acid Changes. Conservation status of genotyping candidates in 11 common species was investigated using HomoloGene (http://www.ncbi.nlm.nih.gov/homologene). Possible 3D changes caused by mutations in the protein structure were predicted and modeled with I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) and UCSF Chimera (http://www.cgl.ucsf.edu/chimera/).

Results

Resequencing and Genetic Association Analyses. Thirty-seven rare, non-synonymous mutations were called by Ingenuity Variant Analysis during resequencing. Among them, 26 were validated via the Sanger method (Table 2). All variants were heterozygous. The carriers of four variants had pedigree DNA available. Sanger sequencing revealed that all four were inherited. Based on the selection criteria mentioned in Materials and Methods, G344R in DLG1, G241S in DLG4, and R604C in DLGAP2 were selected for association analysis (Fig. 1). The DLG4-G241S and DLGAP2-R604C variants were not found in any of the samples used for genotyping, whereas an additional DLG1-G344R variant carrier was detected in the SZ sample group (Table 3).

Protein 3D Structure Analysis. 3D modeling of the wild-type and mutated protein sequences indicated that for the DLGAP2-R604C variant, the additional cysteine gained from the mutation significantly changes the secondary and tertiary structures by adding a local β strand (Fig. 2).

Evolutionary Conservation Analysis. Results obtained from HomoloGene showed that the amino acids corresponding to the three mutations in DLG1, DLG4, and DLGAP2 were highly conserved among different species (Table S1).

Clinical Information of Mutation Carriers. Detailed descriptions of the clinical information can be found in the Supplement. Interestingly, the variant DLGAP2-R604C in one ASD patient was inherited from a parent who is also affected with ASD.

Discussion

Both SZ and ASD are disorders involving polygenic inheritance, with rare variants having a much higher impact on susceptibility than common variants. Recent large-scale genetic studies have reported that ultra-rare and private non-synonymous mutations are highly enriched in patient populations, especially in sets of genes with functions closely involved in neurodevelopment [2,60–63]. DLG4-G241S and DLGAP2-R604C were only present in single cases among a collective sample size of 562 patients during resequencing as well as in 1697 patients and 1793 controls, and DLG1-G344R was present in two SZ cases from the same sample sets. Therefore, they may confer a much higher risk than regular rare mutations discovered with the criterion of a minor allele frequency of <1%.

The second PDZ domain (PDZ2) of SAP-97, where DLG1-G344R is located, folds into a compact globular domain comprising six β-strands and two α-helices, which is a typical architecture for PDZ domains. During synaptic transmission, SAP-97 interacts with key protein partners such as ligand-binding units in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) [53,64] and N-methyl-D-aspartate receptor (NMDA) [57] through the PDZ2 domain to regulate glutamate signaling and neuronal growth, which are major factors in the pathogenesis of SZ and ASD. The same domain also functions as a binding site for receptors for the neurotrophic growth factors corticotropin-releasing hormone (CRF) [65] and epidermal growth factor receptor (ErbB1) [66], which are linked to SZ.

The first PDZ domain (PDZ1) of PSD-95, where DLG4-G241S is located, similarly binds to NMDAR [57], PDZ1 is also the site at which PSD-95 interacts with other PSD proteins such as SynGAP [57]. Mutated PDZ domains have been linked to defective PSD clustering and dendrite spine morphology in cultured cells [67], as well as disrupted glutamate signaling and learning ability in animal models [68]. Interestingly, one study showed an association of this
Table 2. Rare, non-synonymous mutations identified during the resequencing stage. 1. Based on NCBI Build GRCh37/hg19. 2. Positions of allele/amino acid changes are determined with reference to the following RefSeq accessions: DLG1: NM_004087.2; NP_004078.2 DLGAP2: NM_004745.3; NP_004736.2 DLG2: NM_001142699.1; NP_001136171.1 DLG4: NM_001365.3; NP_001356.1 DLGAP1: NM_004746.2; NP_004737.2. 3. GK: guanylate kinase-like domain; SH3: SRC homology 3 domain; PDZ: PSD95-Dlg1-zo1 domain; GKAP: guanylate kinase-associated protein domain.

| Genomic Position | Gene Symbol | Transcripts | Protein Variant | Case Samples With Variant | SIFT Prediction | PolyPhen-2 Prediction | Mutation Taster Prediction | PROVEAN Prediction | PAN-THER Prediction | dbSNP ID | 1000 Genomes Frequency | HGVD Frequency | Domain | Pedigree Analysis |
|------------------|-------------|-------------|----------------|--------------------------|----------------|-----------------------|--------------------------|------------------|-----------------|----------|---------------------|----------------|--------|---------------------|
| 3:19678777 | DLG1 | c.2186A>T | p.K655I | 1 Damaging | Benign | Disease Causing | Deleterious | Neutral | Neutral | 0.001 | GK |
| 3:196812488 | DLG1 | c.1552G>C | p.E643Q | 1 Damaging | Probably Damaging | Disease Causing | Neutral | Neutral | Neutral | 0.008 | SH3 |
| 3:196812570 | DLG1 | c.1470C>G | p.N606K | 1 Damaging | Benign | Disease Causing | Neutral | Neutral | Neutral | SH3 |
| 3:196857519 | DLG1 | c.1143A>C | p.E381D | 1 Tolerated | Benign | Disease Causing | Neutral | Neutral | Neutral | 0.001 | PDZ |
| 3:196863502 | DLG1 | c.1030G>C | p.G344R | 1 Damaging | Probably Damaging | Disease Causing | Deleterious | Neutral | Neutral | PDZ |
| 3:197009653 | DLG1 | c.215C>T | p.P72L | 1 Damaging | Benign | Disease Causing | Neutral | Neutral | Neutral | 0.001 | Inherited |
| 8:1496995 | DLGAP2 | c.136G>A | p.D46N | 1 Damaging | Possibly Damaging | Disease Causing | Neutral | Neutral | 58497511 |
| 8:1497230 | DLGAP2 | c.371G>T | p.R124L | 1 Damaging | Probably Damaging | Disease Causing | Deleterious | Neutral | Neutral | Inherited |
| 8:1497379 | DLGAP2 | c.520G>A | p.A174T | 1 Tolerated | Benign | Polymorphism | Neutral | Neutral | Neutral | |
| 8:1574928 | DLGAP2 | c.1255A>G | p.S409G | 1 Tolerated | Benign | Polymorphism | Neutral | Neutral | 0.001 |
| 8:1574992 | DLGAP2 | c.1289C>T | p.S430F | 1 Damaging | Probably Damaging | Disease Causing | Deleterious | 201068222 | 0.02 |
| 8:1616734 | DLGAP2 | c.1810C>T | p.R604C | 1 Damaging | Probably Damaging | Disease Causing | Neutral | Neutral | Neutral | |
| 8:1624733 | DLGAP2 | c.1997G>A | p.R652H | 1 Damaging | Possibly Damaging | Polymorphism | Neutral | Neutral | 375426065 | 0.04 | 0.002 | GKAP |
| 8:1626417 | DLGAP2 | c.2044G>A | p.A696T | 1 Damaging | Probably Damaging | Polymorphism | Neutral | Neutral | 0.003 | GKAP |
| 8:1626550 | DLGAP2 | c.2219C>A | p.T740N | 1 Damaging | Probably Damaging | Disease Causing | Deleterious | Deleterious | GKAP |
| 8:1626657 | DLGAP2 | c.2284G>A | p.V776I | 1 Tolerated | Probably Damaging | Disease Causing | Neutral | Neutral | Neutral | 0.001 | GKAP |
| 11:83984282 | DLG1 | c.177T>A | p.V61D | 1 Tolerated | Benign | Polymorphism | Neutral | Neutral | Neutral | |
| 11:84822760 | DLG2 | c.302C>T | p.P101L | 1 Tolerated | Probably Damaging | Disease Causing | Neutral | Neutral | Neutral | |
| 17:7100164 | DLG4 | c.1124A>G | p.D375G | 1 Tolerated | Probably Damaging | Disease Causing | Deleterious | Deleterious | PDZ |
| 17:7106562 | DLG4 | c.583G>A | p.G241S | 1 Damaging | Possibly Damaging | Disease Causing | Deleterious | Deleterious | PDZ | Inherited |
| 18:3534411 | DLGAP1 | c.2260G>A | p.D754N | 1 Tolerated | Possibly | Disease Causing | Neutral | Neutral | 376569562 | GKAP |
| 18:3534564 | DLGAP1 | c.1273G>A | p.D703N | 1 Damaging | Possibly | Disease Causing | Deleterious | Deleterious | GKAP |
| 18:3742510 | DLGAP1 | c.1173T>C | p.I392T | 2 Tolerated | Benign | Disease Causing | Neutral | Neutral | 0.002 |
| 18:3879572 | DLGAP1 | c.497G>A | p.G166D | 3 Tolerated | Benign | Disease Causing | Neutral | Deleterious | |
| 18:3879854 | DLGAP1 | c.215G>A | p.R72H | 1 Damaging | Probably | Disease Causing | Deleterious | Deleterious | | |
| 18:3880047 | DLGAP1 | c.22C>A | p.R88 | 1 Damaging | Probably | Disease Causing | Deleterious | Deleterious | 0.001 |

domain with Angelman Syndrome, a genetic disorder exhibiting a high occurrence rate in patients with autism, due to its functional relevance in the TrkB-PSD-95 signaling pathway. Cysteine is a 'special' amino acid that forms disulfide bonds between cysteine residues. These bonds are the basis of secondary and quaternary structures and are critical for the stabilization of tertiary structures of a...
The presence of DLGAP2-R604C introduces a new cysteine to the protein sequence and is highly likely to cause the formation or breaking of a disulfide bond that in turn disrupts the normal folding of DLGAP2.

The Exome Aggregation Consortium (ExAC, http://exac.broadinstitute.org/) integrates the exome sequencing data from 60,706 unrelated individuals from various studies and populations, which was reprocessed through the same pipeline, and jointly variant-called. While individuals in this dataset aren't necessarily healthy controls since they only removed subjects with pediatric diseases, it is a useful reference set of allele frequencies due to its

**Table 3. Association analysis results of three rare missense mutations**

| Variant      | Genotype counts (resequencing)* | Genotype counts (association) | P value |
|--------------|---------------------------------|-------------------------------|---------|
|              | SZ     | ASD    | SZ     | ASD    | Control | SZ     | ASD    |
| DLG1-G344R   | 0/1/739 | 0/0/384 | 0/1/2629 | 0/0/764 | 0/0/3586 | 0.4231 |        |
| DLG4-G241S   | 0/0/740 | 0/1/383 | 0/0/2630 | 0/0/764 | 0/0/3586 |        |        |
| DLGAP2-R604C | 0/0/740 | 0/1/383 | 0/0/2630 | 0/0/764 | 0/0/3586 |        |        |

*a: Homozygote of minor allele/heterozygote/homozygote of major allele.

**Figure 2. Predicted protein structure of mutated DLGAP2 protein with the R604C variant compared to the wildtype.** α-helices are marked in red and β-strands in purple.

**Figure 1. Locations of amino acid changes caused by detected mutations in the DLG1, DLG4 and DLGAP2 genes.** 1. Protein sequence and domain data was obtained from Human Protein Reference Database. 2. Mutations validated in association analysis are marked in red.
scale and data consistency. We searched ExAC for the frequencies of variants we detected in our study (Table S2). It should be noted that DLG1-G344R and DLG4-G241S did not exist in the database, while DLGAP2-R604C was found twice in European (non-Finnish) subjects.

Several limitations should be considered when interpreting the results of our study. First, our relatively small sample set did not have sufficient power to detect statistical significance in an association analysis. Second, we did not conduct molecular biological analysis of the detected mutations. The in vitro and in vivo impacts of these mutations on the pathophysiology of the disorders need to be examined in future research. In addition, our stringent criteria for selection of variants for further analyses may have left out potentially interesting targets, such as DLG4-D375G, which is located in the PDZ domain of the encoded protein and was predicted by four in silico tools to be pathological. In addition, R72H and D703N in DLGAP2 are not present in a known functional domain but were predicted to be pathological by all five tools. These variants may be good candidates for a follow-up study (Fig. 1). Finally, our sequencing did not cover the promoter, untranslated regions, or intronic regions of the target genes, which may contain important mutations at regulatory sites.

Conclusion
In this study, we sequenced the exonic regions of PSD-95 and related genes in SZ and ASD patients using the Ion PGM platform and discovered 26 rare, non-synonymous variants. We then conducted an association analysis in a much larger sample set for three of these variants to investigate their relationship with SZ and/or ASD. Although statistical significance was not obtained, the observation that these mutations were only detected in cases, together with the structural relevance and in silico prediction results, indicates that they may impact the susceptibility of carriers to these disorders.

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