主論文の要旨

Rare loss of function mutations in *N*-methyl-D-aspartate glutamate receptors and their contributions to schizophrenia susceptibility

（NMDA 型グルタミン酸受容体における稀な機能喪失型変異と統合失調症の発症脆弱性への関与）

名古屋大学大学院医学系研究科　総合医学専攻
脳神経病態制御学講座　精神医学分野
（指導：尾崎 紀夫　教授）
余 妍洁

【Introduction】

Schizophrenia (SCZ) is a common, devastating mental disorder that affects approximately 1% of the global population. Twin studies estimated its heritability to be up to 80%.

N-methyl-D-aspartate receptors (NMDARs) are the first class of glutamate receptors. The pathology of anti-NMDAR encephalitis implies that abnormalities in glutamatergic signaling can result in cognitive impairment, hallucination, mood changes, and impairment of behavior, which are the symptoms often observed in SCZ patients. NMDAR subunits are encoded by *GRIN1, GRIN2A, GRIN2B, GRIN2C, GRIN2D, GRIN3A, and GRIN3B*. Target sequencing studies have identified potentially damaging de novo mutations in *GRIN2A*, *GRIN2B* and truncating mutations in *GRIN2C*, *GRIN3A* and *GRIN3B* in SCZ patients. NMDAR genes knockout mice exhibit impairments in associative and executive learning and synaptic plasticity, which were suggested to be associated with the pathogenesis of SCZ. These findings suggested the importance of NMDAR genes in the pathogenesis of SCZ.

Recently, there have been increasing evidences supporting a role of rare mutations in causation of mental disorders. To assess the relationship between rare mutation in NMDAR genes and SCZ, we performed mutation screening for the NMDAR genes and assessed how a splice site mutation affects the splicing pattern using a plasmid minigene. Lastly, we set out to examine genetic association between rare loss of function (LoF) mutations in NMDAR genes and SCZ/ASD.

【Methods】

In this study, two independent sample sets were used: 1) the first set (370 SCZ and 192 autism spectrum disorder (ASD) patients) for mutation screening; 2) the second set (1877 SCZ patients, 382 ASD patients, and 2040 healthy controls) for association analysis of selected mutations identified in the first set. All participates in our study are ethnically Japanese. The Ethics Committees of the Nagoya University Graduate School of Medicine and co-institutes and co-hospitals approved this study.

In mutation screening of NMDAR genes, custom amplification primers were designed to cover coding regions of *GRIN1, GRIN2A, GRIN2C, GRIN2D, GRIN3A,* and *GRIN3B* by FastPCR. We then sequenced the PCR products using Ion Torrent PGM.

Among identified mutations in NMDAR genes, rare (minor allele frequency [MAF] <1%), nonsynonymous mutations were selected and validated by Sanger

sequencing. Then, we analyzed them with the following methods: 1) We explored whether they were registered in the NCBI dbSNP database, the 1000 Genomes Project, the Exome Aggregation Consortium, the Human Genetic Variation Database of Japanese genetic variation consortium, or the Integrative Japanese Genome Variation; 2) We examined the impact of these mutations on protein function using three *in silico* tools (PolyPhen-2, SIFT and Mutation Taster).

To predict splicing consequence of a splice site mutation, we performed three *in silico* analysis: SD–Score, human splice finder and MaxEntScan. Then we constructed the minigene in the pcDNA3.1(+) vector and transfected into HEK 293 cells to confirm the prediction results. Forty-eight hours after the transfection, cells were harvested for RT-PCR. Total RNA was extracted, cDNA was synthesized with an oligo-dT primer. Then, we performed agarose gel electrophoresis of PCR products. Bands of the correct size were excised from the gel and sequenced by the Sanger method to confirm the sequence of every band.

Finally, we examined the genetic association between rare LoF mutations in NMDAR genes and SCZ/ASD (i.e., nonsense, frameshift, splice site mutations).

【Results】

Resequencing the first simple set of the coding regions of *GRIN1, GRIN2A, GRIN2C, GRIN2D, GRIN3A,* and *GRIN3B* identified 102 mutations including 38 rare, missense mutations and two LOF mutations (one 4bp deletion frameshift mutation which creating a premature stop codon (p.P132FsX192 in *GRIN2C*) and one splice site mutation [c.1412G>A] in *GRIN2D*) in two SCZ patients, respectively(Figure 1). All 40 mutations were heterozygous and confirmed by Sanger sequencing. We searched five genetic databases and identified 8 mutations to be novel mutations including the two LoF mutations. Seven of the 38 rare missense mutations were predicted to be deleterious by three *in silico* tools.

The splice site mutation, c.1412G>A in *GRIN2D*, was predicted to affect splicing pattern by all the three *in silico* tools (Table 1). Consistent with these predictions, we confirmed the splicing pattern change (intron 5 retention) using the minigene assay (Figure 2).

Frameshift mutation p.P132Fs in *GRIN2C* and splice site mutation c.1412G>A in *GRIN2D* were selected for association analysis in the second sample set (1877 SCZ cases, 382 ASD cases, and 2040 controls). The result showed that no mutations were found in the sample set used for association analysis.

Importantly, both of them were singleton mutations in this study, and not registered in the public genome databases. Thus, we considered them as ultra-rare LOF mutations (Table 1).

【Discussion】

We performed mutation screening of NMDAR genes in SCZ and ASD, and detected 40 rare, nonsynonymous mutations in this study. Among them, two LoF mutations in two SCZ patients, respectively, were identified: one frameshift mutation (p.P132Fs in *GRIN2C*) and one splice site mutation (c.1412G>A in *GRIN2D*). p.P132Fs in *GRIN2C* is located in the beginning of the gene sequence, which considered to possibly create damaging NR2C protein. In addition, minigene assay confirmed that c.1412G>A in *GRIN2D* resulted in intron 5 retention which carries two stop codons, also possibly causing nonfunctional NR2D receptor to be created.

LoF mutations were often assumed to confer a greater susceptibility to neurodevelopmental disorders than other missense mutations. Some studies have identified genome wide significant association between rare LoF mutations and risk for SCZ.

Furthermore, we conducted association analysis for p.P132Fs in *GRIN2C* and c.1412G>A in *GRIN2D*. Both of the two mutations were singleton in our study and not registered in the public genome databases, which indicated them to be ultra-rare mutations (table 1). As ultra-rare LoF mutations are suggested to be more abundant among cases with SCZ than controls, the two mutations may confer a strong genetic influence on SCZ risk.

In conclusion, we identified one frameshift mutation and one splice site mutation in NMDAR genes in patients with SCZ. Our results might imply that these mutations increase susceptibility to SCZ.