A novel rare variant R292H in RTN4R affects growth cone formation and possibly contributes to schizophrenia susceptibility

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INTRODUCTION

Schizophrenia (SCZ) is a devastating psychiatric disorder that is characterized by hallucinations, delusions and cognitive deficits, and which causes tremendous societal burdens.¹ The lifetime prevalence of SCZ is ~1% in the general population. The heritability of SCZ is up to 80%, making this condition a target for human genetics research.²,³ Recent large-scale genome-wide association analysis,⁴ whole-exome sequencing⁵,⁶ and copy-number variant analysis⁷ with SCZ samples have revealed that deleterious rare gene variants such as single-nucleotide variants (SNVs) and copy-number variants exert significantly larger effects than common single-nucleotide polymorphisms (SNPs). Furthermore, rare SNVs, discovered from sequencing of susceptibility genes, may have large effect sizes and account for a part of the heritability of SCZ, and could contribute to an understanding of the etiopathophisiology of neurodevelopmental disease through further functional assays.⁸–¹¹ Recently, disturbances of neuronal connectivity in both local neural circuits and broad networks of interconnected brain areas have been implicated in SCZ.¹²,¹³ and white matter, myelin and oligodendrocyte have received increasing attention in terms of their potential role in the etiopathophysiolo of SCZ.¹⁴,¹⁵ Thus, the sequencing of myelin-related genes might be a hopeful method for uncovering the etiopathophysiology of SCZ.

The Reticulon 4 receptor (RTN4R) encodes the RTN4 receptor, which is a known receptor subunit for RTN4 that is one of the most potent myelin-associated inhibitor of axon regeneration and structural plasticity in the central nervous system.¹³,¹⁶ RTN4R is considered to be a promising candidate gene for SCZ and ASD because RTN4R is located on chromosome 22q11.2, a region that is known to be a hotspot for susceptibility mutations due to its high deletion rate in patients with SCZ and ASD.¹⁷,¹⁸ RTN4R is known to form a co-receptor complex with other proteins such as immunoglobulin domain-containing protein (LINGO1),¹⁹ p75, and tumor necrosis factor receptor orphan Y (TROY),²⁰ thereby activating the Rho homolog gene family, member A (RhoA).²¹ This activation initiates a cascade of intracellular molecular events that results in the destabilization of the actin cytoskeleton and leads to the collapse of growth cones, preventing further axonal growth and inhibiting myelination.²² Furthermore, during development, there is evidence that RTN4R is involved in limiting the critical period of experience-driven plasticity²³ and restricts synapse development in the hippocampus.²² Thus, RTN4-related signaling may act to stabilize brain wiring both in adulthood and during development and may relate to both learning and memory.²³ Moreover, mice lacking RTN4R exhibit reduced working memory function, consistent with an end phenotype of SCZ.²⁴ In addition, the SNP...

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(rs701428) in RTN4R is associated with the internal capsule of SCZ based on a diffusion tensor imaging study of the brain. Considering that RTN4R is strongly related to the neurodevelopment and is located at 2q11.2, we hypothesized that variants in RTN4R possibly contribute to ASD and SCZ susceptibility. Although there have been studies that have focused on SNVs of RTN4R, some have been no study that has detected SNVs with a large effect size on SCZ and that focused on SNVs in RTN4R in a Japanese SCZ population. Furthermore, it has reported that the common variants of the RTN4R had association with Japanese SCZ. Therefore, in this study, in order to discover novel rare RTN4R mutations with large effect size and to evaluate the pathogenesis of the discovered mutations, we sequenced the RTN4R coding exons using SCZ and ASD samples, and performed the association analysis and in vitro functional assays of the variants that could have large effects. Through this study, we elucidated that a novel rare variant R292H in RTN4R affects growth cone formation and possibly contributes to SCZ susceptibility.

MATERIALS AND METHODS

Participants

Two independent sample sets were used in this study. The first set, which comprised 370 SCZ patients (mean age = 49.7 ± 14.7 years; males = 52.9%) and 192 ASD patients (mean age = 16.3 ± 8.36 years; males = 77.6%), was sequenced for rare variants of RTN4R. The second, larger set, which comprised 16 patients (mean age = 47.6 ± 15.2 years; males = 52.5%), 382 ASD patients (mean age = 19.6 ± 10.7 years; males = 77.7%) and 4009 controls (mean age = 44.1 ± 14.2 years; males = 45.5%), was used for association analysis of selected variants detected in the first set. All of the cases were included if they met DSM-5 criteria for SCZ and ASD. In addition, the patient’s capacity to consent was confirmed by a family member when needed. Subjects with a legal measure of reduced capacity were excluded. Control subjects were healthy volunteers from the general public who had no history of mental disorders, based on questionnaire experiences or if they had received treatment for any psychotic disorders. This study protocol was approved by the Ethics Committees of the Nagoya University Graduate School of Medicine and other participating institutes and hospitals. The study was conducted in accordance with the established ethical standards of all institutions.

Mutation screening

Genomic DNA was extracted from whole blood or saliva using the Qiagen QIAamp DNA blood kit or tissue kit (Qiagen, Hilden, Germany). Custom amplification primers were designed to span coding exons and flanking intron regions of the selected genes (transcription ID of RTN4R: ENST00000344302 from ensemble database; human reference sequence NCBI built 37) using the Ion AmpliSeq Designer (Thermo Fisher Scientific, Waltham, MA, USA). Sample amplification and equalization were achieved using Ion AmpliSeq Library Kits 2.0 and the Ion Library Equalizer Kit, respectively (Thermo Fisher Scientific). Amplified sequences were ligated with Ion Xpress Barcode Adapters (Thermo Fisher Scientific). 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). The final product was then sequenced on the Ion PGM sequencing platform (Thermo Fisher Scientific). Raw data output from the sequencer with the default setting; call quality ≥ 20, read depth ≥ 10 was uploaded to the Torrent Server (Life Technologies, Carlsbad, CA, USA) for variant calling with NCBI GRCh37 as a reference. The resulting VCF files were analyzed by Ingenuity Variant Analysis (Qiagen) for annotation and visualization.

Prioritization

Nonsense mutations, missense mutations, small insertions/deletions and canonical splicing site variations with an allele frequency of < 1% were selected from the annotated data. The variants selected through the above-mentioned filtering were validated by Sanger sequencing. About the missense mutations, we chose the variants located in a functional domain or motif of the protein, according to the Human Protein Reference Database (http://www.hprd.org).

All mutations were evaluated in silico for possible structural and functional consequences using the following tools: (1) localization of a functional domain or motif of the protein was based on the Human Protein Reference Database (http://www.hprd.org/index.html) and existing literature; (2) evolutionary conservation was assessed with the Ewola var. 7.5 (http://www.h-invitational.jp/ewola/search.html); and (3) prediction of deleterious effects was performed by in silico analytic methods (Polyphen-2[ref. 28] [http://genetics.bwh.harvard.edu/pph2/) and SIFT [http://sift.jcvi.org/]). To investigate the association of discovered rare variants with susceptibility to neuropsychiatric disorders, we performed the association analysis with the following criteria: (1) novel, 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 the NHLBI GO Exome Sequencing Project (ESP6500078-V2) (http://exac.broadinstitute.org), the Human Genetic Variation Database (HGVD) of Japanese genetic variation consortium (http://www.genome.med.kyoto-u.ac.jp/SnpDB) or the Exome Aggregation Consortium (ExAC) (http://exac.broadinstitute.org).

Association analysis

Custom TaqMan SNP genotyping assays were designed and ordered from Applied Biosystems (Foster City, CA, USA). Allelic discrimination analysis was applied on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Differences in allele and genotype frequencies of the mutations were compared between SCZ patients/controls and ASD patients/controls using Fisher’s exact test (one-tailed), with a threshold of significance set at P < 0.05. Statistical calculations were performed using SPSS v21 (SPSS, Armonk, NY, USA). We performed power analysis using the Genetic Power Calculator (http://pngu.mgh.harvard.edu/purcell/gpc/) based on the multiplicative model.

Plasmid constructs and RTN4R mutagenesis

For expression cloning of RTN4R-EGFP, the Myc tag sequence of the pSecTag2-Hygro vector (Invitrogen, Carlsbad, CA, USA) was replaced with EGFP. The cDNA of human RTN4R was amplified from pENTR221-Hs RTN4R (IMAGE clone 100005521), and was inserted into the KpnI/XhoI site of the pSecTag2-Hygro vector by using the signal peptide of pSecTag2. All site-specific mutagenesis experiments were carried out using the KOD-Plus-Mutagenesis Kit (TOYOBO, Shiga, Japan), using the pSecTag2-Hygro-RTN4R vector as a template. The following primers were used for mutagenesis: R292H, 5′-CACCCTGGCTGGCGTACCTCAAC-3′; 5′-CAGGAGCTCCGACGCTCAGGG-3′; 5′-GACCCCTACGATAATGCCACGTC-3′; 5′-CAACGGCTCTCGGCTGACATTC-3′; 5′-GGACCTCACGGATAATGCCACGTC-3′; 5′-CACGGGCTCTCGGCTGACATTC-3′; 5′-CAACGGGCTCTCGGCTGACATTC-3′.

Gust binding assay

Semi-confluent HEK293FT cells (Thermo Fisher Scientific) were transfected with RTN4R-EGFP-related plasmids and GST-LINGO1 expressing plasmid using Lipofectamine 3000 (Thermo Fisher Scientific). After 48 h, the transfected cells were harvested and lysed in 1 ml lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1% Triton X-100 and 10% glycerol) on ice. Glutathione sepharose beads were incubated with the cell lysates for 1 h at 4°C and the beads were subsequently washed three times with lysis buffer. After washing, the beads were resuspended with sodium dodecyl sulfate polyacrylamide gel electrophoresis sample buffer, and the bound glutathione S-transferase (GST) and green fluorescent protein (GFP) fusion proteins were subjected to immunoblot analysis using the antibodies as indicated in Figure.
Figure 1. The results of mutation screening. (a) RTN4R gene structure based on ENST0000040608; the protein-coding exons sequenced in this study are indicated by yellow box. Untranslated regions (UTR) are indicated by gray box. Four rare missense mutations were discovered in exon 2. (b) RTN4R protein structure (473 amino acids). N-terminal (NT) leucine-rich repeat (LRR) domain (green box), eight LRR domains, CT-LRR domain, a stalk domain and GPI anchorage site (purple box) are contained in RTN4R. R68H, D259N and R292H are located in LRR domain. V363M is located in the Stalk domain. LRR domain is ligand-binding regions such as RTN4, MAG, OMGP and LGI1.35 Stalk domain is the interaction site for co-receptor p75NTR of TROY and is needed for RhoA activation.36 Variants discovered in this study are indicated in square box. Previously published missense mutations24,26,37 associated with schizophrenia (SCZ) are underlined. (c) RTN4R protein structure (473 amino acids). N-terminal (NT) leucine-rich repeat (LRR) domain (green box), eight LRR domains, CT-LRR domain, a stalk domain and GPI anchorage site (purple box) are contained in RTN4R. R68H, D259N and R292H are located in LRR domain. V363M is located in the Stalk domain. LRR domain is ligand-binding regions such as RTN4, MAG, OMGP and LGI1.35 Stalk domain is the interaction site for co-receptor p75NTR of TROY and is needed for RhoA activation.36 Variants discovered in this study are indicated in square box. Previously published missense mutations24,26,37 associated with schizophrenia (SCZ) are underlined. (c) RTN4R gene structure based on ENST00000040608; the protein-coding exons sequenced in this study are indicated by yellow box. Untranslated regions (UTR) are indicated by gray box. Four rare missense mutations were discovered in exon 2. (d) The results of evolutionary conservation analysis.

RESULTS

Mutation screening and prioritization

In order to elucidate the association of rare SNVs in RTN4R with the pathophysiology of SCZ and ASD, we sequenced RTN4R coding regions using 370 SCZ and 192 ASD. The resulting nucleotide sequence data have been deposited in the DNA Data Bank of Japan (DDBJ) databases (http://www.ddbj.nig.ac.jp) under the accession number DRA004490. Through this sequencing, we identified four rare missense mutations (MAF < 1%) that were validated by Sanger sequencing (Figures 1a–c). We discovered all of the missense mutations within exon 2 (Figures 1a and b). The R292H mutation was not documented in several databases (Table 1). Results obtained from evolutionary conservation analysis of the discovered rare missense mutations are shown in Figure 1d.

Association analysis

Among the four discovered mutations, R292H was assumed to have a high effect on SCZ based on the following findings: (1) two unrelated cases in the 370 SCZ samples had the R292H mutation, (2) this mutation was not registered in several public databases (Table 1), (3) R292H was located in the RTN4R ligand-binding domain (Figure 1b), which, following ligand binding initiates a cascade of intracellular molecular events that result in destabilization of the actin cytoskeleton and lead to the collapse of growth.
cones. We therefore performed genetic association analysis using RTN4-R292H. For our SCZ sample of cases ($N = 2086$), we computed a statistical power of $> 80\%$ based on the following parameters: disease prevalence of 0.01, observed rare allele frequency of 0.0027 (Table 1), odds ratio (OR) for dominant effect of $> 2.3$ and type I error rate of 0.05. The result of association analysis of RTN4-R292H is shown in Table 2. A marginally significant association between SCZ and R292H was observed (OR = 3.9, $P = 0.048$). The clinical features of the six SCZ cases with RTN4R-R292H are shown in Table 3.

Functional analysis of RTN4R-R292H

We considered that the RTN4R-R292H mutation might have strong relations to the pathogenesis of SCZ by affecting RTN4R function from the following findings: (1) R292H was marginally associated with SCZ, (2) according to a previous study, RTN4R-R119W, which is also in the RTN4R ligand-binding site, also significantly decreased RTN4-induced growth cone collapse compared with RTN4R-WT (Figure 2b). Consistent with a previous study, RTN4R-R119W, which is also in the RTN4 ligand-binding site, also significantly decreased RTN4-induced growth cone collapse compared with RTN4R-WT (Figure 2b).

Based on in silico 3D protein structure analysis, we predicted that R292 could be located at the site of interactions between RTN4R and LINGO1 (Figure 3a, Supplementary Methods, and Supplementary Figure S1), which is also an SCZ candidate gene and is known to build a receptor complex with RTN4R and regulate myelination.41 RTN4R-R292H is predicted to form a hydrogen bond with LINGO1-Q404 (Figure 3b). RTN4R-R292H is predicted to be located too far from LINGO1-Q404 for the formation of hydrogen bonds (Figure 3c). We discovered a reduced interaction of LINGO1 with RTN4R-R292H compared to WT (Figure 3d) by the GST-binding assay as our in silico structural analysis predicted.

### DISCUSSION

We performed mutation sequencing of RTN4R coding regions as a candidate gene for SCZ and ASD. Through this study, four missense mutations in RTN4R (Table 1a) were discovered. Among four discovered mutations, RTN4R-R292H was not found in several existing databases but detected in two SCZ cases after the mutations screening using 370 SCZ. Through association analysis, a marginally significant association (OR = 3.9, $P = 0.048$) was not found in several existing databases but detected in two SCZ cases after the mutations screening using 370 SCZ. Through association analysis, a marginally significant association (OR = 3.9, $P = 0.048$) was observed. Furthermore, we found a possible biological effect of RTN4R-R292H by investigating growth cone collapse of chick-dissociated...
Table 3. Clinical information of carriers of the RTN4R-R292H mutation

| Subject | Diagnosis       | Age (at participation), years | Sex | Family history (mental illness) | Education (years) | Occupation                  | Marriage | Clinical course after onset | Symptoms at onset | Physical illness | Cognitive test after onset | Clinical test after onset | Clinical course after | Physical illness | Neurobehavioral symptoms | Clinical course after onset | Symptoms at onset | Clinical course after onset |
|---------|-----------------|-------------------------------|-----|---------------------------------|-------------------|-----------------------------|----------|-----------------------------|-----------------|-----------------|-----------------------------|------------------------|------------------------|-----------------|----------------------------|-----------------------------|----------------|-----------------------------|
| 1       | Schizophrenia   | 55                            | Male| Schizophrenia (cousin)          | High school (12)  | Public servant              | ++       | Repeated hospitalization    | Delusion of possession, and auditory hallucinations, loose association, and auditory hallucination | Physical illness | Chronic bronchitis, lung cancer, schizophrenia | Diabetes mellitus | Apathy and mood disorder | Neurodevelopment | RhoA signaling should be investigated in future studies |
| 2       | Schizophrenia   | 59                            | Female| Schizophrenia (cousin), mental retardation (daughter) | Junior high school (9) | Restaurant staff for 10 years | ++       | Repeated hospitalization    | Persecutory delusion, and auditory hallucinations, loose association, delusion of observation, and auditory hallucination | Physical illness | Chronic bronchitis, lung cancer, schizophrenia | Diabetes mellitus | Apathy and mood disorder | Neurodevelopment | RhoA signaling should be investigated in future studies |
| 3       | Schizophrenia   | 68                            | Male| Schizophrenia (cousin)          | University (14)   | Clerical staff              | −        | Repeated hospitalization    | Delusion of possession, and auditory hallucinations, loose association, and auditory hallucination | Physical illness | Chronic bronchitis, lung cancer, schizophrenia | Diabetes mellitus | Apathy and mood disorder | Neurodevelopment | RhoA signaling should be investigated in future studies |
| 4       | Schizophrenia   | 40                            | Male| Depression (mother)            | High school (12)  | Part-time job               | ++       | Repeated hospitalization    | Delusion of possession, and auditory hallucinations, loose association, and auditory hallucination | Physical illness | Chronic bronchitis, lung cancer, schizophrenia | Diabetes mellitus | Apathy and mood disorder | Neurodevelopment | RhoA signaling should be investigated in future studies |
| 5       | Schizophrenia   | 20                            | Male| Mental retardation (mother)    | University (14)   | Welfare service worker      | ++       | Repeated hospitalization    | Delusion of possession, and auditory hallucinations, loose association, and auditory hallucination | Physical illness | Chronic bronchitis, lung cancer, schizophrenia | Diabetes mellitus | Apathy and mood disorder | Neurodevelopment | RhoA signaling should be investigated in future studies |
| 6       | Schizophrenia   | 28                            | Female| Mental retardation (father)    | University (14)   | Public servant              | ++       | Repeated hospitalization    | Delusion of possession, and auditory hallucinations, loose association, and auditory hallucination | Physical illness | Chronic bronchitis, lung cancer, schizophrenia | Diabetes mellitus | Apathy and mood disorder | Neurodevelopment | RhoA signaling should be investigated in future studies |

Abbreviations: −, absent; +, present; IQ, intelligence quotient; NA, not available; PIQ, performance IQ; TIQ, total IQ; VIQ, verbal IQ; WAIQ, Wechsler Adult Intelligence Scale.

Several limitations should be discussed when interpreting the results of this study. Firstly, our sequencing did not cover the several potentially informative regions of RTN4R, including untranslated regions and intronic regions. Indeed, it has recently been reported that de novo synonymous mutations in the gene SETD1A could contribute to the genetic etiology of SCZ. Secondly, due to limited access to the detailed clinical phenotypes of the subject and subjects’ family members and control subjects with RTN4R-R292H, and to lack of DNA samples from subjects’ family, we could not fully explore the impact of the novel variants segregation. Third, we only discovered a reduction of growth cone collapse by SNVs in RTN4R was related to SCZ susceptibility. The possible mechanisms by which the RTN4R-R292H mutation induced reduced growth cone collapse may involve changes in the following: (1) interaction of RTN4R with ligands including RTN4 that are required for myelin-mediated neural development; (2) RhoA signal transduction due to alteration of ligand-mediated conformational changes of the RTN4 receptor; and/or (3) co-receptor association with LINGO1, which is also an SCZ candidate gene and one of the components of RTN4R signaling complex and regulate myelination activating RhoA signaling, predicted that R292 could be located at the site of interactions between RTN4R and LINGO1 (Figure 3a, Supplementary Methods and Supplementary Figure S1), and which is also an SCZ candidate gene that is known to build a receptor complex with RTN4R and regulate myelination, based on in silico 3D protein structure analysis. We discovered a reduced interaction of LINGO1 with RTN4R-R292H compared to WT (Figure 3d) by the GST-binding analysis as our in silico structural analysis predicted. Through these genetic and functional studies, considering that RTN4R is located at chr22q11.2, which is a hotspot locus for SCZ and ASD, RTN4R-R292H could be related to the etiopathological role of SCZ, because the pathophysiology of SCZ is believed to be that of aberrant conditions of neurodevelopment.
CONFLICT OF INTEREST
The authors declare no conflict of interest.

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