Mutation Screening of the Neurexin 1 Gene in Thai Patients with Intellectual Disability and Autism Spectrum Disorder

Supaporn Yangngam,^{1,2} Oradawan Plong-On,³ Thanya Sripo,³ Rawiwan Roongpraiwan,⁴
Tippawan Hansakunachai,⁵ Juthamas Wirojanan,⁶ Tasnawat Sombuntham,⁴
Nichara Ruangdaraganon,⁴ and Pornprot Limprasert³

Aim: Neurexin 1 has two major protein isoforms using alternative promoters, coding for the alpha-neurexin 1 (α -NRXN1) and beta-neurexin 1 (β -NRXN1) genes. This study is to explore the possibility that variants of the NRXN1 gene predispose to intellectual disability (ID) and autism spectrum disorder (ASD). **Methods:** The coding regions in 24 exons and exon–intron boundaries of the NRXN1 gene were investigated in 115 Thai patients with ID and ASD by direct DNA sequencing. **Results:** Nine novel variants of the NRXN1 gene were identified. Four novel variants were found in the β -NRXN1 gene, one variant of six GGC repeats in exon 1, and three variants at the 5'UTR. Five novel variants were identified in the α -NRXN1 gene, four intronic variants and one missense variant in exon 14 (c.2713T>A or p.F905I). **Conclusion:** Mutation screening of the NRXN1 gene in patients with ID and ASD may be useful to identify potential variants predisposing to ID and ASD. However, further studies utilizing protein functional analysis of the novel variants are required for a more definite conclusion.

Introduction

AUTISM SPECTRUM DISORDER (ASD) is a complex developmental disorder that is characterized by deficits in social interaction and communication along with restricted and stereotyped patterns of behavior. According to the review by Miles *et al.* (2010), 50–70% of children with autism have been classified as having an intellectual disability (ID) by nonverbal IQ testing. The causes of ID and ASD are very complex, as they are influenced by either genetic or environmental factors or a combination of both. Although chromosomal microarray is currently the recommended first line of investigation for clinical genetic testing for ASD and ID (Miller *et al.*, 2010), this technique cannot be used for some research questions (i.e., identification of mutations or variants in genes).

Mutations in synaptic genes, particularly neuroligin and neurexin genes, have been reported in patients with ID and ASD. Two recent studies reported nonsense and missense mutations in two neuroligin genes, *NLGN3* and *NLGN4X*, in

ASD (Jamain *et al.*, 2003; Laumonnier *et al.*, 2004). In addition, evidence is available from several studies that rare variants in the neurexin 1 (*NRXN1*) gene are associated with ASD and ID (Feng *et al.*, 2006; Kim *et al.*, 2008; Yan *et al.*, 2008; Zweier *et al.*, 2009; Gauthier *et al.*, 2011; Camacho-Garcia *et al.*, 2012; Liu *et al.*, 2012). The *NRXN1* gene is located on chromosome 2p16.3. It contains 24 exons and encodes the two major isoforms of the neurexin 1 protein: alpha-neurexin 1 (α -NRXN1), a long isoform encoded by 23 exons (except exon 18), and beta-neurexin 1 (β -NRXN1), a short isoform encoded by 7 exons (exons 18–24) (Tabuchi and Sudhof, 2002). The structure of *NRXN1* gene makes it possible to generate many different protein isoforms due to the alternative splicing of RNA transcripts (Rowen *et al.*, 2002).

To explore the possibility that structural variants of the *NRXN1* gene predispose to ID and ASD, the coding regions in 24 exons and exon–intron boundaries of the *NRXN1* gene were investigated in 115 Thai patients with both features (ID and ASD) using direct DNA sequencing.

¹Graduate Program in Biomedical Sciences, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla, Thailand.

²Faculty of Medical Technology, Prince of Songkla University, Hat Yai, Songkhla, Thailand.

³Division of Human Genetics, Department of Pathology, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla, Thailand.

⁴Department of Pediatrics, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand.

⁵Department of Pediatrics, Faculty of Medicine, Thammasat University, Pathumthani, Thailand.

⁶Department of Pediatrics, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla, Thailand.

Materials and Methods

Subjects

The study patients were recruited from child developmental clinics at three university hospitals in Thailand: Songklanagarind, Thammasat, and Ramathibodi. The research protocol was approved by the ethics committees from the three participating institutes. A total of 203 Thai patients previously diagnosed with ASD were initially recruited for the study. The ASD diagnoses were based on the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) criteria for autistic disorder and pervasive developmental disorders—not otherwise specified (PDD-NOS). Informed consent forms were completed by the parents of each patient. After we excluded known medical causes and nongenetic risk factors of ASD (i.e., low birth weight, congenital malformations, perinatal asphyxia, and perinatal infection), 169 patients with ASD remained for further investigation. The patients were tested for common genetic causes of ID and ASD, which found normal standard karyotyping (all cases), normal CGG repeats and methylation of the *FMR1* gene (all cases), and normal exon 1–4 of the *MECP2* gene (female cases). One hundred thirty-four patients were available for nonverbal IQ testing using the Stanford-Binet Intelligence Scale: Fifth edition (Stanford-Binet-V). The inclusion criterion of the present study was ASD patients with nonverbal IQ scores less than 80, and 115 patients tested with the Stanford-Binet-V met this criterion, 92 with autistic disorder (76 males, 16 females) and 23 with PDD-NOS (19 males, 4 females). Their ages ranged from 17 months to 16 years and 7 months (average 52 months), with nonverbal IQ scores ranging from 41 to 79 (average 59). Of the 115 cases, whole exome sequencing of the *NLGN3* and *NLGN4X* genes was investigated in 92 cases (80%), none of which had pathologic variants as in a previous report (Mikhailov *et al.*, 2014).

The control samples were taken from blood donors at the Blood Bank units of Thammasat University Hospital and Songklanagarind Hospital, who were recruited voluntarily with consent. The controls were screened by questionnaires with exclusion criteria that comprised no current or past psychiatric or neurological disorders, no developmental delay, no learning disabilities or medical disorders with implications for the central nervous system, or not taking any regular medications.

Mutation analysis and DNA sequencing

Five milliliters of blood from cases and controls was collected. DNA was extracted using the standard phenol-chloroform method. To target the coding region, the 24 exons of the *NRXN1* gene, 27 primer pairs of exons, and the exon-intron boundaries were designed or modified from the previous report by Zweier *et al.* (2009). Polymerase chain reaction (PCR) and DNA sequencing methods are shown in the Supplementary Data S1 (Supplementary Data are available online at www.liebertpub.com/gtmb). All potential significant variants were confirmed by the opposite primers, and parental DNA was analyzed when available by direct DNA sequencing for the specific base changes identified in the probands.

Screening for novel and rare missense variants in the controls

A modified PCR-restriction fragment length polymorphism (RFLP) method was designed to look for the c.3715G>A (p.A1239T) variant in 310 controls and the c.4131G>C (p.E1377D) variant in 207 controls. GGC repeats were screened by direct DNA sequencing or fluorescent PCR in 122 controls. The PCR-RFLP and fluorescent PCR methods are described in Supplementary Data S1. Two variants of unknown significance, p.S14L and p.F905I, were screened by direct DNA sequencing in 310 controls. We selected fewer controls for the p.E1377D and GGC repeat studies because these are likely nonpathogenic variants.

Bioinformatic analysis

We used PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph/>) to predict the evolutionary amino acid conservation. The novel missense variants were investigated for possible functional consequences using PolyPhen-2 and DomPred (<http://bioinf.cs.ucl.ac.uk>).

Results

Twenty-four variants of the *NRXN1* gene were identified in the study (Table 1). Twelve SNPs were identified across the *NRXN1* gene, including 2 rare missense variants in both *NRXN1* proteins: p.A1239T and p.E1377D in the α -*NRXN1* protein and p.A164T and p.E272D in the β -*NRXN1* protein. The p.A1239T and p.E1377D have been previously reported and can be found in the SNP database with reference IDs rs201336161 and rs200935246, respectively. Four novel variants were exclusively identified in the α -*NRXN1* gene, three intronic variants (c.889+117T>C, c.930-56C>T, and c.2467+60G>A) and one missense variant in exon 14 (c.2713T>A or p.F905I, Fig. 1a). In the β -*NRXN1* gene, we found three novel variants at 5'UTR (c.3485-110728T>A, c.3485-110654G>T, and c.3485-110196G>C), three ins/del GGC repeat variants and one rare variant of unknown significance (c.41C>T or p.S14L). We converted the ins/del GGC variants as repeat numbers (shown in Table 1). One novel variant was found in both genes: intron 19 of the α -*NRXN1* gene (c.3667-38A>C) or intron 1 of the β -*NRXN1* gene (c.442-38A>C).

Four cases of three missense variants, p.S14L (one case), p.F905I (one case), and p.E1377D (two cases), were confirmed in the parental DNA, showing that all variants were transmitted from a parent (p.F905I, p.E1377D paternal transmission, and p.S14L, p.E1377D maternal transmission). Parental DNA from the patient with the p.A1239T variant was not available. The patient with p.F905I (T/A alleles) inherited the A allele from the heterozygous father. Although the father had no abnormal physical features, the complete physical examination report and psychological tests were not available. The proband with p.F905I was 4 years and 2 months of age and diagnosed with PDD-NOS and ID (nonverbal IQ = 52). He had normal coding regions of the *NLGN3* and *NLGN4X* genes (Mikhailov *et al.*, 2014). To exclude copy number variants (CNVs) at either of the *NRXN1* genes or other loci, whole genome analysis of 298,649 SNPs using the Illumina HumanCytoSNP-12v2.1 array was performed on DNA from the patient, which found no pathological CNVs (unpublished data). The p.F905I variant changes the nonpolar, hydrophobic

TABLE 1. VARIANTS IDENTIFIED IN THE *NRXN1* GENE IN THIS STUDY

Location	Nucleotide change (amino acid change)		Variant types	Patients (n = 115)/ (no. found in controls)
	α - <i>NRXN1</i> : NM_001135659.1 (exon 1–17, 19–24) (NP_001129131.1/ 1547 AA)	β - <i>NRXN1</i> : NM_138735.2 (exon 18–20, 22–24) (NP_620072.1/442 AA)		
Exon 1	c.511C>T (p.L171L)	NA	rs1045874	35/ND
Intron 3	c.889+117T>C ^a	NA	Novel intronic	1/ND
Intron 5	c.930-56C>T ^a	NA	Novel intronic	1/ND
Exon 6	c.999C>T (p.P333P)	NA	rs2303298	7/ND
Intron 8	c.1440+85C>T	NA	rs186874890	1/ND
Intron 11	c.2467+55_2467+56insT	NA	rs5831131	30/ND
Intron 11	c.2467+60G>A ^a	NA	Novel intronic	1/ND
Exon 14	c.2713T>A ^a (p.F905I)	NA	Novel missense	1/(0/310)
Intron 17	c.3484+20T>C	NA	rs3213756	41/ND
Intron 17	c.3485-110728T>A ^a	c.-749T>A (5'UTR β) ^b	Novel intronic	2/ND
Intron 17	c.3485-110654G>T ^a	c.-675G>T (5'UTR β) ^b	Novel intronic	1/ND
Intron 17	c.3485-110624G>A	c.-645G>A (5'UTR β) ^b	rs3732049	35/ND
Intron 17	c.3485-110377T>C	c.-398T>C (5'UTR β) ^b	rs13422484	65/ND
Intron 17	c.3485-110196G>C ^a	c.-217G>C (5'UTR β) ^b	Novel intronic	1/ND
Exon 18	c.3485-109939C>T	c.41C>T (p.S14L, exon 1 β) ^b	Known missense	1/(0/310)
Exon 18	c.3485-109930G>T	c.50G>T (p.G17V, exon 1 β) ^b	rs13413205	2/ND
Exon 18	c.3485-109916_3485-109902del5GGC	c.64-78del5GGC (p.22-26del5G, exon 1 β) ^b	5 GGC repeats ^c	1/(0/122)
Exon 18	c.3485-109913_3485-109902del4GGC ^a	c.67-78del4GGC (p.23-26del4G, exon 1 β) ^b	6 GGC repeats ^c	2/(0/122)
Exon 18	c.3485-109902insGGC	c.78insGGC (p.26insG, exon 1 β) ^b	11 GGC repeats ^c	1/(3/122)
Intron 19	c.3667-29C>G	c.442-29C>G	rs74387895	8/ND
Intron 19	c.3667-38A>C ^a	c.442-38A>C	Novel intronic	1/ND
Exon 20	c.3715G>A (p.A1239T)	c.490G>A (p.A164T)	rs201336161	1/(1/310)
Exon 22	c.4131G>C (p.E1377D)	c.816G>C (p.E272D)	rs200935246	2/(6/207)
Intron 22	c.4248+71C>T	c.933+71C>T	rs1363049	85/ND

^aNovel variants in this study.^bNucleotides found as part of the β -*NRXN1* gene.^cTen GGC repeats in the reference sequence (NM_138735.2) and the most common allele in our controls. α -*NRXN1*, alpha-neurexin 1; β -*NRXN1*, beta-neurexin 1; NA, not applicable; ND, not done.

amino acid, phenylalanine, to the same group amino acid, isoleucine, and it was not found in the 310 controls. The variant with phenylalanine at the 905 position is located in the evolutionary conserved amino acid region on the LNS(4) domain across different species (Fig. 1b). Two bioinformatics programs, DromPred and PolyPhen-2, have shown that the p.F905I variant changes the secondary structure of the α -*NRXN1* protein and damages the protein function.

The p.S14L variant in the β -*NRXN1* protein was found in one 3-year-old boy with autistic disorder and ID (nonverbal IQ=64), but was not found in 310 controls. This variant changes the polar uncharged amino acid, serine, to a nonpolar hydrophobic amino acid, leucine, in the signal peptide domain of β -*NRXN1*. The DomPred program predicted that the p.S14L variant should change the secondary structure of the amino acid positions 10–12 from a helix to a coil. However, the PolyPhen-2 prediction result was benign. A summary of the GGC repeat and rare missense variants within the *NRXN1* gene from previous reports and the present study are shown in Figure 2.

Discussion

This is the first study of the whole coding region of the *NRXN1* gene in individuals with the two clinical features of

ASD and ID. The most promising finding in this study was the identification of one novel missense variant, c.2713T>A (p.F905I), in the α -*NRXN1* gene according to NCBI access no. NM_001135659.1 and NP_001129131.1 (1547 amino acids). Due to alternative splicing, one other α -*NRXN1* transcription of 1477 amino acids has been reported (NM_004801.4/NP_004792.1), and therefore, we can also report this novel variant as c.2593C>T (p.F865I). Although the bioinformatic programs have predicted that the isoleucine variant would damage the α -*NRXN1* protein function, the variant did not show a deleterious effect on the father who carried the isoleucine variant. Previous reports have shown that ASD patients with rare variants of the *NRXN1* gene inherited the variants from either normal fathers or normal mothers (Feng *et al.*, 2006; Gauthier *et al.*, 2011; Camacho-Garcia *et al.*, 2012; Liu *et al.*, 2012). This phenomenon is not uncommon in ASD and ID because both disorders are known as having multifactorial inheritance involving more than one gene and environmental factors. However, there are some limitations to our study, in that we cannot exclude other causes as possibly connected. First, we did not look at the promoter and sizes of mRNA. Several deletions of the *NRXN1* gene have been reported in patients with a wide spectrum of neuropsychiatric disorders, including ASD, ID,

FIG. 1. (a) DNA sequencing shows the nucleotide change of the *NRXN1* gene position 2713 from thymine to adenine (c.2713T>A) resulting in alteration of the amino acid from phenylalanine to isoleucine (p.F905I). (b) Alignment of the amino acid at position 905 shows high conservation of the phenylalanine across different species using the Poly-Phen-2 program.

schizophrenia, epilepsy, and Pitt-Hopkins-like syndrome 2 (Zweier *et al.*, 2009; Schaaf *et al.*, 2012; Bena *et al.*, 2013; Dabell *et al.*, 2013). Second, other synaptic molecules, including the neurexin synaptic binding partners, *CNTNAP2* and *SHANK3*, have been shown to be associated with ASD

(Ye *et al.*, 2010). Recently, a model of oligogenic heterozygosity in ASD patients has been proposed (Schaaf *et al.*, 2011). For these reasons, we could not exclude other genetic mechanisms that could be affecting different phenotypes in the family members.

FIG. 2. Summary of the rare missense variants and GGC repeats in the alpha and beta isoforms of the neurexin 1 protein. Figure is not drawn to scale. References: 1, Feng *et al.* (2006); 2, Kim *et al.* (2008); 3, Yan *et al.* (2008); 4, Liu *et al.* (2012); 5, Gauthier *et al.* (2011); 6, Camacho-Garcia *et al.* (2012); *This study (see Supplementary Data S2, Table S2-2 for details). Protein access no: α-neurexin 1, NP_001129131.1; β-neurexin 1, NP_620072.1. SP, signal peptide domain; βN, short beta-neurexin-specific sequence; LNS, laminin/neurexin/sex-hormone-binding protein domain; EGF, epidermal growth-factor-like domain; CH, O-glycosylation sequence; TM, transmembrane domain; PDZ-BD, cytoplasmic domain.

The p.F905I variant located on the LNS(4) domain is found exclusively in the α -NRXN1 protein. There are six LNS domains in the α -NRXN1 protein, but only one in the β -NRXN1 protein (Fig. 2). The six LNS domains of the α -NRXN1 protein form an extracellular presynaptic configuration binding with other synaptic ligands, such as neuroligin, leucine-rich repeat transmembrane 2 (LRRTM2), neuroligin, and dystroglycan (Comoletti *et al.*, 2010; Miller *et al.*, 2011). Thus, alteration of the LNS(4) domain may affect protein folding resulting in disruption of the α -NRXN1 protein function.

The GGC indel variants are likely rare polymorphisms in the exon 1 of β -NRXN1 gene. The common allele is 10 GGC repeats and this allele frequency in the present study was 0.98. Although one study has shown that shorter GGC repeats could enhance protein production in the androgen receptor (*AR*) gene (Ding *et al.*, 2005), no study has been reported on the effect of GGC repeats in the β -NRXN1 gene expression. Further studies on the effect of GGC repeats on the expression of the β -NRXN1 gene would be interesting.

The p.S14L variant in the β -NRXN1 protein was identified in 5 of 607 ASD cases screened (3 White Americans, 1 German/French, and 1 Thai in the present study), but only 1 was found in 1429 combined controls, including this study (Feng *et al.*, 2006; Gauthier *et al.*, 2011; Camacho-Garcia *et al.*, 2012). This finding implies that the leucine may be a rare variant associated with ASD (p -value=0.0108, Fisher's exact test, see Supplementary Data S2, Table S2-1). Gauthier *et al.* (2011) reported that the p.S14L variant did not alter surface trafficking in COS cells. However, this variant might affect neuron-specific trafficking or interactions with neuroligin 1 partners. Thus, the effect of the p.S14L variant on the NRXN1 protein is still unclear. Likewise, the effect of the p.F905I variant on the α -NRXN1 protein function needs to be further studied to elucidate a more definite conclusion.

In conclusion, our study identified nine novel variants of the *NRXN1* gene in 115 Thai patients with ID and ASD. Therefore, mutation screening of the *NRXN1* gene may be useful to identify potential variants predisposing to ID and ASD. Further study of these novel variants found in the *NRXN1* gene using protein functional analysis is required.

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Author Disclosure Statement

The authors declare no conflict of interests.

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Address correspondence to:
Pornprot Limprasert, MD, PhD
Division of Human Genetics
Department of Pathology
Faculty of Medicine
Prince of Songkla University
Hat Yai, Songkhla 90110
Thailand

E-mail: lpornpro@yahoo.com