Research Article

Identification of Four Novel Synonymous Substitutions in the X-Linked Genes Neuroligin 3 and Neuroligin 4X in Japanese Patients with Autistic Spectrum Disorder

Kumiko Yanagi,1 Tadashi Kaname,1 Keiko Wakui,2 Ohiko Hashimoto,3 Yoshimitsu Fukushima,2 and Kenji Naritomi1

1 Department of Medical Genetics, Graduate School of Medicine, University of The Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215, Japan
2 Department of Medical Genetics, School of Medicine, Shinshu University, 3-1-1 Asahi, Matsumoto 390-8621, Japan
3 Faculty of Nursing and Rehabilitation, Aino University, 4-5-4 Higashi-Ohta, Ibaraki 367-0012, Japan

Correspondence should be addressed to Tadashi Kaname, tkaname@med.u-ryukyu.ac.jp

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Mutations in the X-linked genes neuroligin 3 (NLGN3) and neuroligin 4X (NLGN4X) were first implicated in the pathogenesis of X-linked autism in Swedish families. However, reports of mutations in these genes in autism spectrum disorder (ASD) patients from various ethnic backgrounds present conflicting results regarding the etiology of ASD, possibly because of genetic heterogeneity and/or differences in their ethnic background. Additional mutation screening study on another ethnic background could help to clarify the relevance of the genes to ASD. We scanned the entire coding regions of NLGN3 and NLGN4X in 62 Japanese patients with ASD by polymerase chain reaction-high-resolution melting curve and direct sequencing analyses. Four synonymous substitutions, one in NLGN3 and three in NLGN4X, were identified in four of the 62 patients. These substitutions were not present in 278 control X-chromosomes from unrelated Japanese individuals and were not registered in the database of Single Nucleotide Polymorphisms build 132 or in the Japanese Single Nucleotide Polymorphisms database, indicating that they were novel and specific to ASD. Though further analysis is necessary to determine the physiological and clinical importance of such substitutions, the possibility of the relevance of both synonymous and nonsynonymous substitutions with the etiology of ASD should be considered.

1. Introduction

Neuroligin 3 (NLGN3) and Neuroligin 4X (NLGN4X) are members of neuroligins expressed in the postsynaptic neurons and mediate transsynaptic signaling by interacting their ligand, neurexins [1]. Mutations in the X-linked genes NLGN3 and NLGN4X (GenBank accession numbers NM_181303.1 and NM_020742.2, resp.) were first reported as being involved in X-linked autistic spectrum disorder (ASD; OMIM#300425 and OMIM#300495) in Swedish families [2]. Some reports have also indicated that NLGN3 and NLGN4X are responsible for ASD. Mutations in these genes including 5 missense mutations [2–5], two small in-del mutations, which lead to a premature stop codon in the transcript [2, 6], an exon skipping mutation [7], and a large deletion [8], have been identified in ASD patients (Table 1). In vitro experiments using NLGN3 and NLGN4X proteins carrying amino acid changes that were identified in ASD patients indicated that the gene mutations could cause ASD by a loss-of-function mechanism [5, 9]. Neuroligin 3 p.R451C knock-in mice and neuroligin 4X-deficient mice exhibited autism-related behaviors [10, 11]. In addition, recent mutation screening studies revealed that the p.K378R substitution in the NLGN4X gene was common in individuals of different ethnicities, such as Portuguese and Greek [3, 4]. A recent association study regarding rare variants in NLGN3 and NLGN4X also supported the relationship between a specific NLGN3 haplotype and the etiology of ASD in Chinese Han male population [12]. Moreover, two synonymous variants are specifically found in ASD patients [13, 14].
Table 1: Previously identified sequence variations in coding regions in ASD patients.

| Gene    | NT1 change | A-A2 change | Mutation type | Ethnic background | Ref.3 | NCBI      |
|---------|------------|-------------|---------------|-------------------|-------|-----------|
| NLGN3   | c.222C>T   | p.Y74Y      | Synonymous    | Finnish           | [13]  | NM_181303.1|
|         | c.1351C>T  | p.R451C     | Missense      | Swedish           | [2]   | NM_018977.3|
|         | c.259C>T   | p.R87W      | Missense      | Irish and Scottish| [5]   | NM_020742.2|
|         | c.759G>A   | p.G99S      | Missense      | Portuguese        | [3]   | NM_020742.2|
|         | c.1186 insT| p.D396X     | Frameshift    | Swedish           | [2]   | NM_020742.2|
|         | c.1253delAG| p.D429X     | Frameshift    | French            | [6]   | NM_020742.2|
| NLGN4X  | c.1597A>G  | p.K378R     | Missense      | Portuguese and Greek| [3, 4]| NM_020742.2|
|         | Not described | p.A558A | Synonymous     | German            | [14]  | NM_020742.2|
|         | c.2574C>T  | p.R704C     | Missense      | Portuguese        | [3]   | NM_020742.2|
|         | del exon 4 | In-frame1   | Skipping      | AGRE6             | [7]   | NM_020742.2|
|         | del exon 4–6| Truncated7  | Large deletion| Irish and English | [8]   | NM_020742.2|

1NT: nucleotide.
2A-A: amino acid.
3Ref.: reference number.
4The number of substituted nucleotides was not mentioned in the reference.
5Exon 4 skipping mutation was predicted to result in an in-frame exclusion of 62 amino acids.
6Autism genetics resource exchange.
7The translated protein was predicted to be entirely truncated between exon 3 and exon 6.

In contrast, some data indicate that these two genes do not have a significant effect on ASD development. The frequency of mutation in the coding region was not high (<2%) among ASD patients [2–6]. It has been reported that mutations in these genes were not observed in ASD patients in the United Kingdom, Canada, the Autism Genetic Resource Exchange (AGRE) and the International Molecular Genetic Study of Autism Consortium (IMGSAC) [15–17].

Such contradictory results could possibly be due to genetic heterogeneity, regional characteristics, or differences in the ethnic background of ASD patients. It is, therefore, important to investigate the NLGN3 and NLGN4X sequences in ASD patients from various ethnic backgrounds.

Molecular screening of NLGN3 and NLGN4X in Japanese patients with ASD has not yet been reported. We, therefore, performed a mutation screening study of these genes in 62 unrelated Japanese patients with ASD and 278 control X chromosomes using polymerase-chain-reaction (PCR) high-resolution melting (HRM) and direct sequencing analyses.

2. Materials and Methods

2.1. Patients. The Japanese ASD patients analyzed in this study were described previously [18]. All the patients were diagnosed with ASD according to the Diagnostic and Statistical Manual of Mental Disorders (Fourth Edition criteria), the Autism Diagnostic Interview-Revised, and the Childhood Autism Rating Scale by at least two trained psychiatrists. No chromosomal aberration was found in all patients. The study was approved by the ethics committee of the University of the Ryukyus, and written informed consent was obtained from all subjects.

2.2. Source of Genomic DNA. Genomic DNA in patients (51 males, 11 females) and control Japanese individuals (30 males, 30 females) was isolated from successfully transformed B cells by Epstein-Barr virus using a standard protocol involving proteinase K digestion. In addition, control genomic DNA was isolated from blood of 198 healthy Japanese individuals (118 males, 80 females) using a QIAamp column (Qiagen, Hiden, Germany).

2.3. Mutation Screening. Initially, mutation screening was performed in the 62 ASD subjects by PCR-HRM analysis. HRM analysis is used to scan gene variations in a PCR amplification, for example, mutations and single-nucleotide polymorphisms (SNPs), prior to sequencing by detecting differences in the thermal denaturation of double-stranded DNA [19, 20]. We set up optimal conditions for PCR-HRM analysis of the entire coding regions of NLGN3 and NLGN4X. Primers for all exons were carefully designed in flanking introns of the genes taking into account the high sequence homology between neureligins (Table 2). Exons 2, 7, and 8 of NLGN3 and exons 2, 5, and 6 of NLGN4X were amplified in two or three segments to obtain a fine resolution of the HRM curves. Only an upstream part of exon 2 (exon 2.1 in Table 2) of NLGN4X was sequenced in all ASD patients because we failed to distinguish between the complicated SNPs located in the region.

2.4. Condition of PCR-HRM Analysis. PCR was performed under optimized conditions using Ex-Taq DNA polymerase (TAKARA Bio Inc., Japan) as follows: 20 μL reaction mixture containing Mg2+-free 1 × PCR buffer (TAKARA Bio Inc., Japan), 0.25 mM dNTPs (TAKARA Bio Inc., Japan), 0.25 units Ex-Taq DNA polymerase, 1 μM of each primer, and the optimal concentration of MgCl2 was loaded per well in a 96-well PCR plate (Roche, Basel, Switzerland). The amount of genomic DNA used in each reaction was 40 ng for males and 20 ng for females. Fluorescent DNA
Table 2: Primer sets used to screen for variants by PCR-HRM analysis.

| Gene     | Exon | Forward | Reverse               |
|----------|------|---------|-----------------------|
| NLGN3    | 2.1  | GCTCAGTTTTTGAGTTCAAGTC | TCACCTGCGGTGATGTCAG   |
|          | 2.2  | CACAGTCACACTCACTTGGG  | GATGTGAAGCAATTCAG     |
|          | 3    | GCCAGACTCTCGTTGATT    | CAAATCCCCCGACAAGGCA   |
|          | 4    | TGGCTTGCTGGCGCACTTG   | GCAAGAAGCAGATGAAAGCC  |
|          | 5    | AAGGTGAGCAACACATGTAG  | GGCGCAGGAGTAAACACATT  |
|          | 6    | CATCCTCTGCTCTCATGTC   | TAGAAGAGAGCCTGGCGATTC |
|          | 7.1  | CAGCTCTAGTGACAAAAAGAT | CAGGGTGCTCTTACCCCGAT  |
|          | 7.2  | GTAAGGACACCTGCGGAG    | GGGGCTCTCAAAAGGAAAGA  |
|          | 8.1  | GTGACCCCCATTTCCATGT   | GCCGCAAGAGTTAGGAGACA  |
|          | 8.2  | ATCACCGCAGGCCAATGG    | CCTCACACTCTGTTGGTG    |
|          | 8.3  | GAGAGAGCTGGCGACATAC   | CTGGAGATTGGTGCTGTCT   |
|          | 2.1  | AAAGGCACTATCTCTTGAGG  | TGAGTGAATTTCCGGATGCCA |
|          | 2.2  | AGAAGACCTGGTACACAAAGAG | GAGACATTAAAAACCTCTTAG |
|          | 3    | TTAGCATGTTGACAGTGCTGT | CCGTCAAAACAGAAGGTGACT |
|          | 4    | CTTTTCTTTATTTGGCCACCA | TTCTGGTTACAGGTAATTTGC |
|          | 5.1  | AGCTGCAATTCTTGCTGT    | TCTCCGCGCAAGATGTCTTC  |
|          | 5.2  | CCAACCCTGTGACACCTT    | ACCCAAACAGAAAGATGAC   |
|          | 6.1  | CAGTCACATGTTGGAAGATG  | GACGGCAATGGTGACACTTA  |
|          | 6.2  | TCCCTATTGAAACGAAAG    | AACATTCCCCTGGTCGAGAC  |

dye, SYTO 9 (0.5 μM; Life Technologies, Carlsbad, CA), was added before amplification. Genotype analysis of each sample was performed in duplicate. The melting curves were sequentially analyzed using LightCycler 480 Gene Scanning Software (Roche, Basel, Switzerland) to detect sequence variants. Evaluation of the HRM curves was sequentially confirmed by direct sequencing (Life Technologies, Carlsbad, CA).

2.5. Screening in Controls. Variants identified in ASD patients were then investigated for detecting the common variants by searching the database of Single Nucleotide Polymorphisms (dbSNP) build 132 and the Japanese Single Nucleotide Polymorphism database (JSNP). All variants found in ASD patients were scanned in genomic DNAs isolated from EBV transformed cells or blood cells in control Japanese individuals.

2.6. Assessment of the Substitutions. The effect of the substitutions was evaluated using Mutation Taster software (http://www.mutationtaster.org/).

3. Results

We identified four variants, a synonymous substitution and three intronic substitutions, in NLGN3 in the 62 ASD patients. The synonymous substitution (c.1698G>A, p.K566K) was observed in a male ASD patient. We also identified four variants, comprising three synonymous substitutions and a substitution in the 5’ UTR region of NLGN4X. One synonymous substitution, c.297C>T (p.G99G), was observed in a female patient, and two substitutions, c.516C>T (p.I172I) and c.1590C>T (p.F530F), were observed in two different male patients. The variants identified in this study are summarized in Table 3. Nonsynonymous substitutions, including seven substitutions reported previously, were not identified in the Japanese patients by PCR-HRM analysis (Tables 1 and 3). The four synonymous and an intronic substitution were not found in the control X-chromosomes from unrelated healthy Japanese individuals, without any neuropsychiatric disorder (Table 3).

4. Discussion

By using PCR-HRM analysis we identified an intronic and four exonic substitutions only in 62 Japanese patients with ASD, two out of 62 patients (3.2%) in NLGN3 and three out of 62 patients (4.8%) in NLGN4X. The exonic substitutions comprised one synonymous substitution in NLGN3 and three synonymous substitutions in NLGN4X (Table 3). The PCR-HRM analysis could detect 90% of the sequence variations with 100% accuracy [19, 20]; therefore; we were able to identify almost all the changes in NLGN3 and NLGN4X in these patients.

In this study, we analyzed genomic DNAs from EBV-transformed cells in patients with ASD. The source of genomic DNA, especially extracted from EBV-transformed cells, should be considerable in each experiment, because there is a possibility that unexpected substitutions occur during the transformation [21, 22]. Analyses in control genomic DNA showed that there was no sequence alteration or substitution bias in the exons of NLGN3 and NLGN4X between genomic DNA isolated from blood and EBV-transformed cells in this study (Table 3).

Our mutation screening study in Japanese ASD patients failed to detect novel nonsynonymous mutations and seven
known nonsynonymous mutations that were identified in previous studies [2–5] as well as a study in a Chinese Han population [12]. Considering the low frequency of nonsynonymous substitutions in these genes seen in previous reports [15–17], a larger number of ASD patients should be sampled. Nevertheless, our results suggest that nonsynonymous substitutions in NLGN3 and NLGN4X may account for only a small proportion of Japanese patients with ASD. In addition to ASD, there are some reports indicating that NLGN3 and NLGN4X are also relevant to Asperger syndrome [2], X-linked mental retardation [6], and Tourette syndrome [8]. Considering the function of NLGN3 and NLGN4X [1], additional mutation screening studies in such neurobehavioral disorders should be needed in near future.

Experimental evidence is increasing that synonymous substitutions could affect the protein function through transcription or translation impairment [23–25]. While the clinical and physiological importance of the four synonymous substitutions and an intronic substitution are not clear at this moment, Mutation Taster software [26] evaluated that the substitutions might affect protein structure by altering splice site. According to previous reports of mutation screening in NLGN3 and NLGN4X, two synonymous mutations, p.Y74Y in NLGN3 and p.A558A in NLGN4X (Table 1), were also observed in ASD patients but not in healthy controls [13, 14]. A recent study in a Chinese Han population has indicated that a common intrinsic variation in NLGN3 may influence the susceptibility of males to ASD [12]. Although further analysis is necessary to demonstrate the biological effects of synonymous and intronic substitutions on ASD, these substitutions as well as nonsynonymous substitutions should be taken into account.

5. Conclusion

We identified four substitutions, one in NLGN3 and three in NLGN4X, specific to Japanese patients with ASD. They were synonymous but the possibility of the association of both synonymous and nonsynonymous substitutions with the etiology of ASD should be considered.

### Table 3: Sequence variants identified in Japanese patients with autistic spectrum disorder.

| Gene   | Exon | SNP ID | Location | Translation | Male | Female | EBV cell line | Blood |
|--------|------|--------|----------|-------------|------|--------|---------------|-------|
| NLGN3  | 4    | —      | c.567+22C>T | Intrinsic   | 0/51 | 1/11   | 0/90          | 3/278 |
| NLGN3  | 5    | —      | c.727+47G>C | Intrinsic   | 1/51 | 0/11   | 0/90          | 0/278 |
| NLGN3  | 7    | —      | c.1698G>A   | p.K566K     | 1/51 | 0/11   | 0/90          | 0/278 |
| NLGN4X | 2    | —      | c.305-86T>G  | 5′UTR       | 0/51 | 1/11   | 1/90          | 4/278 |
| NLGN4X | 3    | —      | c.297C>T     | p.G99G      | 0/51 | 1/11   | 0/90          | 0/278 |
| NLGN4X | 5    | —      | c.516C>T     | p.I172I     | 1/51 | 0/11   | 0/90          | 0/278 |

1. Reference number of the variant documented in dbSNP build 132 or JSNP. (—) indicates that the variant does not have a reference number.
2. Number of ASD patients (50 males, 11 females) with the variant.
3. Number of control chromosomes with the variant in EBV transformed cell line (30 males, 30 females) and blood (118 males, 80 females).

### Conflict of Interests

The authors declare that they have no competing interests.

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