Autism and Nonsyndromic Mental Retardation Associated with a De Novo Mutation in the *NLGN4X* Gene Promoter Causing an Increased Expression Level

Hussein Daoud, Frédérique Bonnet-Brilhault, Sylviane Védrine, Marie-Véronique Demattéi, Patrick Vourc'h, Nadia Bayou, Christian R. Andres, Catherine Barthélémy, Frédéric Laumonnier, and Sylvain Briault

Background: Pathogenic mutations in the X-linked *Neuroligin 4* gene (*NLGN4X*) in autism spectrum disorders (ASDs) and/or mental retardation (MR) are rare. However, nothing is known regarding a possible altered expression level of *NLGN4X* that would be caused by mutations in regulatory sequences. We investigated this issue by analyzing these regions in patients with ASDs and no mutation in the *NLGN4X* coding sequence.

Methods: We studied 96 patients who met all DSM-IV criteria for autism. The entire coding sequence and the regulatory sequences of the *NLGN4X* gene were analyzed by polymerase chain reaction and direct sequencing.

Results: We identified a de novo 1 base pair (−335G>A) substitution located in the promoter region in a patient with autism and nonsyndromic profound MR. Interestingly, this variation is associated with an increased level of the *NLGN4X* transcript in the patient compared with male control subjects as well as his father. Further in vitro luciferase reporter and electrophoretic mobility shift assays confirmed, respectively, that this mutation increases gene expression and is probably caused by altered binding of transcription factors in the mutated promoter sequence.

Conclusions: This result brings further insight about the phenotypic spectrum of *NLGN4X* mutations and suggests that the analysis of the expression level of *NLGN4X* might detect new cases.

Key Words: Autism, mental retardation, *NLGN4X*, overexpression, regulatory sequences

Autism (MIM 209850) is a complex neurodevelopmental disorder characterized by impaired reciprocal social interaction and communication and markedly restricted repertoire of activities and interests (1). Twin and family studies suggested the existence of a strong genetic background for this condition (1). The high frequency of affected male subjects in autism would suggest the involvement of X-linked genes (2). This hypothesis has been reinforced by several observations in which truncating mutations in the *Neuroligin 4X* gene (*NLGN4X*) (MIM 300427) were associated with autism spectrum disorders (ASDs) and/or mild mental retardation (MR) (3,4). These findings indicate that some autism cases might result from mutations with a Mendelian transmission and that similar truncating *NLGN4X* mutations result in a wide variety of phenotypes. Subsequent mutational analyses in more than 400 patients showed that mutations in the coding sequence of the *NLGN4X* gene seem to be rare in the autistic population (5–9).

From the Institut National de la Santé et de la Recherche Médicale (INSERM U930) (HD, FB-B, SV, PV, CRA, CB, FL, SB); Université François-Rabelais (HD, FB-B, SV, M-VD, PV, CRA, CB, FL, SB); Centre Hospitalier universitaire de Tours (FB-B, PV, CRA, CB); CNRS (M-VD), Tours; Centre Hospitalier Régional (S), Laboratoire de Génétique Chromosomique, Orléans, France; and the Service des maladies congénitales et héréditaires (NB), Hôpital Charles Nicole, Tunis, Tunisia.

Address correspondence to Hussein Daoud, Ph.D., INSERM U930, Université François-Rabelais, Faculté de Médecine, 10 Bd Tonnellé, 37032 Tours Cedex, France; E-mail: daoud_h@med.univ-tours.fr.

Received May 12, 2008; revised May 6, 2009; accepted May 8, 2009.

Recently, the phenotypes spectrum associated with *NLGN4X* mutations has been enlarged with the description of a family with autism, severe MR, and Tourette syndrome caused by an interstitial gene deletion probably leading to a truncated protein (10).

To further investigate the potential contribution of *NLGN4X* gene mutations in ASDs, we have screened by direct sequencing the entire coding sequence as well as promoter and 3′ untranslated (3′ UTR) regions in 96 individuals diagnosed with ASDs.

Methods and Materials

Subjects

Autistic patients (80 male subjects and 16 female subjects) were recruited through the Child Psychiatry Center at the University Hospital of Tours (France) by a multidisciplinary team. All patients were Caucasians. Written informed consent was obtained from parents. Exclusion criteria included: severe sensory problems (e.g., visual impairment or hearing loss); significant motor impairments (e.g., failure to sit by 12 months or walk by 24 months); and identified metabolic, genetic, or progressive neurological disorders. Genetic disorders have been excluded by clinics and cytogenetics. Patients with FRAXA mutation were excluded.

Mutation Analysis of *NLGN4X* Gene

Genomic DNA was extracted from lymphoblastoid cell lines (LCLs) with the Gentra Puregene Blood Kit (Qiagen, Hilden, Germany) as per manufacturer's instructions. The entire coding sequence as well as 1 kilobase pair of the promoter and 3′ untranslated regions of the *NLGN4X* gene were analyzed (primer sequences and amplification conditions are available upon request). Polymerase chain reactions (PCRs) were performed with the GoTaq Flexi DNA Polymerase (Promega, Madison, Wisconsin). The PCR products were verified by agarose gel electrophoresis. Amplifications abnormalities were confirmed in a sec-

0006-3223/09/$36.00
doi:10.1016/j.biopsych.2009.05.008

ond independent PCR. Purified products were run on an automated sequencer (XL3130 BioAnalyzer; Applied Biosystems, Foster City, California).

Real-Time Reverse Transcription PCR

Total RNA was extracted from LCLs with Trizol reagent (Invitrogen, Carlsbad, California). The RNA concentrations were determined by ultraviolet spectrophotometry, and integrity was verified by agarose gel electrophoresis. One microgram of DNAse-treated RNA from each sample was used to generate complementary DNA (cDNA) with the Superscript II Reverse transcriptase (Invitrogen) according to manufacturer's instructions. Primers were designed to amplify a 214-base pair (bp) fragment of the *NLGN4X* cDNA with a forward primer in exon 4 and a reverse primer in exon 5. The *Glyceraldehyde-3-Phosphate Dehydrogenase* (*GAPDH*) cDNA real-time amplification was used for normalization, and the relative expression levels were calculated with the δ-δ Ct method (11).

Luciferase Assays

To assess the consequence of the 1-bp substitution on gene expression, 715 bp from wild-type and mutated promoter region were subcloned into the HindIII/NheI sites of the pGL4.10(*luc2*) promoter-less vector (Promega), which contains the firefly luciferase gene (F-luc). Human embryonic kidney (HEK) 293T cells plated in 96-well format (2.10^4 cells/well) were transiently transfected with Exgen500 (Euromedex, Souffelweyersheim, France) with 80 ng of pGL4.10(*luc2*) and 40 ng of pGL4.74(*hRluc*/TK) Renilla vector (containing the Renilla luciferase [R-luc]) to serve as an internal control for transfection efficiency. Luciferase assays using the Dual-glo luciferase assay system (Promega) were performed 24 hours after transfection. Sixty microliters of Dual-Glo Luciferase reagent were added to the 60 μL of culture medium and incubated for 30 min. The F-luc luminescence was measured. Before measuring of R-luc, 60 μL of the Stop-and-Glo reagent were added to each well to quench the F-luc reaction. The R-luc luminescence was measured after an incubation of 30 min. Results were expressed as F-luc/R-luc means ± SEM.

Electrophoretic Mobility Shift Assay (EMSA)

All reactions included double-stranded, ^{32}P-labeled, oligonucleotide probes corresponding to wild-type or mutated *NLGN4X* promoter nucleotides −357 to −30. Electrophoretic mobility shift assay (EMSA) was performed on human adult brain nuclear proteins (Biochain, Hayward, California). Nuclear protein extract (8 μg) was incubated at room temperature with or without 100× molar excess unlabeled competitor probe and bovine serum albumin for 30 min before addition of ^{32}P-labeled probe, then incubated an additional 20 min at room temperature before loading on a 6% nondenaturing acrylamide gel. After migration, the gel was exposed for 10 hours and revealed with the Storm gel imaging system (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom).

Results

We did not detect any mutation or polymorphism in the *NLGN4X* coding sequence of all patients. However, we identified a 1-bp substitution G>A located in the promoter region sequence 335 bp upstream from the transcription initiation site in a boy with autism and profound MR (Figure 1). The sequence variation has been confirmed in a second independent PCR with genomic DNA extracted from peripheral blood. This variation occurred de novo, and a paternity test using different independent and informative markers excluded false paternity (data not shown). Lastly, this variation was absent in 450 normal X chromosomes as well as in the expressed sequence tags or single nucleotide polymorphisms databases.

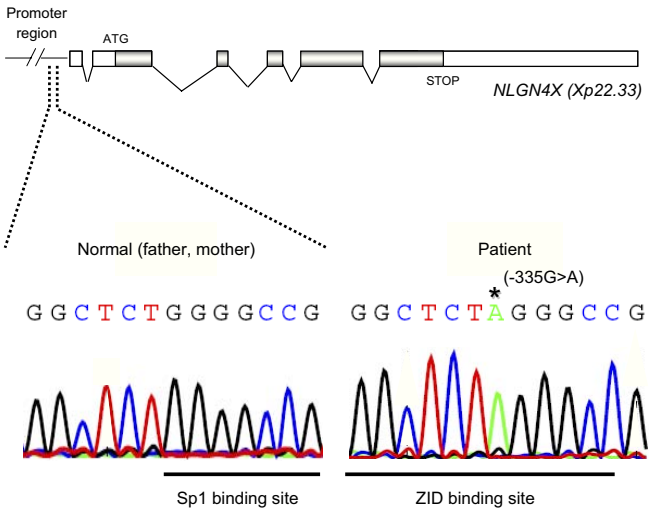

Figure 1. Sequencing analysis of the *NLGN4X* gene promoter region in the patient. Representation of the normal and variant nucleotide sequences between the bases −341 and −329 upstream from the transcription start site of the *NLGN4X* gene (NM_020742). Modifications involving transcription factors binding sites on the promoter region with MatInspector and Match prediction software are indicated (horizontal bars). The −335G>A substitution is predicted to suppress an SP1 binding site (ggGGCCGtgt) and to create a Zing finger protein with Interaction Domain (ZID) binding site (cGGCTCtagggcc). *Identification of a de novo 1-base pair substitution (−335G>A) in the patient; this variation was not found in his parents or 450 control X chromosomes.

This patient has no particular familial history of pervasive developmental disorders or psychiatric history. He had a history of a preterm birth at 36 weeks of amenorrhea with birth weight at 2350 g, height at 47.5 cm, and head circumference at 32 cm (−2 SD). There was an accelerated increase in head circumference with 44 cm at 7 months (+1 SD), 46.5 cm at 1 year (+1 SD), and 50 cm at 23 months (+2 SD). He had normal developmental milestones in his first 2 years with sitting at 6 months and walking at 10 months. Language development and efficient communication and socialization were observed. The first words appeared at 16 months with progression to 2 years. At 2 years old, parents worried about poor eye contact and language stagnation. At 2.5 years old, language regression, absence of communication, restrictive interests, sensory particularities, and behavior disorders happened. Stereotypic movements appeared at 8 years old. Clinical evaluation was conducted at 12 years old. His figures were 40 kg, 152 cm, and 54 cm for head circumference (+.5 SD). He met DSM-IV criteria for autistic disorder. His Autism Diagnostic Interview scores (based on "ever" scores) were 27 in the social domain (cutoff = 10), 14 in the communication (non verbal) domain (cutoff = 7), and 7 in the repetitive/restrictive behaviors domain (cutoff = 3). Developmental abnormalities were identified before 36 months. Developmental ages (Vineland Adaptive Behavior Scale) were 12 months for communication, 24 months for daily living skills, 10 months for socialization, and 24 months for motricity. Childhood autism rating score was 40 (cutoff = 37.5 for severe autism). His intellectual quotient was 11, leading to an associated diagnosis of profound MR. Physical examination revealed normal growth parameters with no dis-