Whole-exome sequencing and neurite outgrowth analysis in autism spectrum disorder

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Autism spectrum disorder (ASD) is a complex group of clinically heterogeneous neurodevelopmental disorders with unclear etiology and pathogenesis. Genetic studies have identified numerous candidate genetic variants, including de novo mutated ASD-associated genes; however, the function of these de novo mutated genes remains unclear despite extensive bioinformatics resources. Accordingly, it is not easy to assign priorities to numerous candidate ASD-associated genes for further biological analysis. Here we developed a convenient system for identifying an experimental evidence-based annotation of candidate ASD-associated genes. We performed trio-based whole-exome sequencing in 30 sporadic cases of ASD and identified 37 genes with de novo single-nucleotide variations (SNVs). Among them, 5 of those 37 genes, POGZ, PLEKHA4, PCNX, PRKD2 and HERC1, have been previously reported as genes with de novo SNVs in ASD; and consultation with in silico databases showed that only HERC1 might be involved in neural function. To examine whether the identified gene products are involved in neural functions, we performed small hairpin RNA-based assays using neuroblastoma cell lines to assess neurite development. Knockdown of 8 out of the 14 examined genes significantly decreased neurite development (P < 0.05, one-way analysis of variance), which was significantly higher than the number expected from gene ontology databases (P = 0.010, Fisher’s exact test). Our screening system may be valuable for identifying the neural functions of candidate ASD-associated genes for further analysis and a substantial portion of these genes with de novo SNVs might have roles in neuronal systems, although further detailed analysis might eliminate false positive genes from identified candidate ASD genes.

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INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by impairments in social interactions, reduced verbal communication abilities, stereotyped repetitive behaviors and restricted interests. The complex etiology of ASD includes genetic and environmental factors; the high heritability of this disorder supports the presence of a significant genetic contribution. Recent studies have shown that de novo single-nucleotide variations (SNVs) greatly contribute to the risk of several brain disorders, including ASD, schizophrenia and intellectual disability.1–9 In particular, numerous de novo SNVs were identified using whole-exome sequencing (WES) of individuals with sporadic ASD.1–3,6–9 Among them, several disruptive mutations associated with sporadic ASD were recurrently found in CHD8, SCN2A, GRIN2B, DYRK1 and SYNGAP1 (for review, see Krumm, N. et al4, Ronemus, M. et al10), which have led to a convergence on several functional pathways such as chromatin remodeling, the Wnt signaling pathway and synaptic function; however, the genetic mutations in these genes account for only a small proportion of all cases. To make matters worse, the functions of most of candidate ASD-associated genes, as well as the biological significance of the identified mutations in the central nervous system largely remain elusive despite extensive bioinformatics resources. Accordingly, although the precise functional analysis of each disease-associated gene and its mutation is important for understanding the etiology of ASDs, it is not easy to assign priorities to numerous candidate ASD-associated genes for further detailed biological analysis.

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To circumvent these problems, developing a rapid and convenient system for identifying an experimental evidence-based annotation of the numerous candidate ASD-associated genes is imperative.

In this study, we performed trio-based WES in ASD patients and identified 37 genes with de novo SNVs. Then, we developed a neuroblastoma cell-based rapid and convenient screening system for elucidating the functions of these gene products in the central nervous system. Of the 14 genes examined with this screening system, 8 were identified as regulators of neurite outgrowth, which is a significantly higher proportion than expected from simply using the gene ontology databases, although further detailed analysis might eliminate false positive genes from identified candidate ASD genes.

**MATERIALS AND METHODS**

Following a description of the study, written informed consent was obtained from each subject. This study was carried out in accordance with the World Medical Association’s Declaration of Helsinki and was approved by the Research Ethics Committee in Osaka University. All recombinant DNA experiments were reviewed and approved by the Gene Modification Experiments Safety Committee in Osaka University.

**Subjects**
The cohort consisted of 30 patients with ASD (21 males and 9 females; mean age ± s.d., 22.1 ± 7.8 years) and their parents (mean age ± s.d. at sampling; father: 54.5 ± 6.9 years, mother: 52.3 ± 7.7 years; mean age ± s.d. at conception of proband; father: 31.9 ± 3.9 years, mother: 28.9 ± 3.5 years; for details, see Supplementary Materials and Methods). All participants are Japanese and were recruited from either outpatient or inpatient services at the Osaka University Hospital. We obtained participant data from the research bio-resource of the Human Brain Phenotype Consortium in Japan (http://www.sp-web.sakura.ne.jp/consortium.html). Each ASD patient was diagnosed by at least two trained child psychologists according to the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5). The patients were assessed through unstructured or semi-structured behavioral observation and interviews with the patients and their parents or caregivers as previously described. In addition, the Autism Diagnostic Interview-Revised, the Pervasive Developmental Disorders Autism Society Japan Rating Scale and the Japanese version of Asperger’s Questionnaire were used to assist in the evaluation of ASD-specific behaviors and symptoms. Intelligence quotient (IQ) data for the subjects were collected using the Japanese version of the full-scale Wechsler Adult intelligence Scale-III (n = 23), the full-scale Wechsler Intelligence Scale for Children-Third Edition (n = 5), or the Japanese version of the NART 50, which can measure estimated IQ scores (n = 2). Among the patients with ASD, 2 patients had low IQ scores (≤ 69), 2 patients had borderline IQ scores (70–79), 2 patients had normal IQ scores (80–119) and 4 patients had superior IQ scores (≥ 120) (for detail, Supplementary Materials and Methods). The mean full-scale IQ ± s.d. of all patients with ASD was 100.9 ± 19.8.

**DNA preparation and WES**

Peripheral blood samples were collected from the subjects. Genomic DNA was extracted from whole blood using a QIAamp DNA Blood Maxi Kit (Qiagen, Hilden, Germany). Parentage was confirmed using nine polymorphic micro-satellite markers (D1S50, D1S260, D5S560, D5S410, D9S285, D9S1776, D13S217, D14S276 and D14S985). The amplicons were separated on a Genetic Analyzer 3500 (Life Technologies Inc., Carlsbad, CA, USA), and analyzed using GeneMapper software version 4.1 (Life Technologies Inc.). Trio-based WES was performed in every family. Genomic DNA was captured with the SureSelect Human All Exon v.4 Kit (Agilent Technologies, Santa Clara, CA, USA) and sequenced with four samples per lane on an Illumina HiSeq 2000 (Illumina, San Diego, CA, USA) with 101-bp paired-end reads. Image analysis and base calling were performed by Sequencing Control Software with Real-Time Analysis and CASAVA software v.1.8 (Illumina). Exome data processing, variant calling and variant annotation were performed as previously described. Mapping to human genome hg19 was performed using Novoalign (Novocraft Technologies, Selangor, Malaysia). Aligned reads were processed by Picard to remove PCR duplicates. Variants were called using the Genome Analysis Toolkits v.1.6-5 (GATK). Called SNVs, insertions and deletions (indels) were annotated using ANNOVAR. From the total variants within ± 10 bp of intronic regions from exon–intron boundaries, those registered in dbSNP135, ESP 5400 (http://evs.gs.washington.edu/ESVS/), 1000 Genomes (http://www.1000genomes.org/), the Human Genetic Variation Database as a reference database of genetic variations in 1208 Japanese controls (http://www.genome.med.kyoto-u.ac.jp/SnpDB) or our in-house (exome data from 408 individuals) databases at the Yokohama City University School of Medicine and those located with segmental duplications were removed. SNVs detected by WES were confirmed by Sanger sequencing. Primer sequences are available in Supplementary Table S1. The details of the PCR conditions are available on request. Our human genome researches and gene analysis researches were all performed under the Ethical Guidelines for Human Genome/Gene analysis Research, Ministries of Education, Culture, Sports, Science, and Technology, Health, Labor and Welfare, and Economy, Trade and Industry. According to the Guidelines, the research institution and its joint research institution approved by the ethics review committee but not third-party institutions can only deal with any potential personally identifiable information. In conformity to the Guidelines, we did not obtain the subjects’ consent for the disclosure of any potential personally identifiable information. Accordingly, we could not disclose any potential personally identifiable information such as whole-exome sequence data of each subject to third-party institutions or public databases.

**Cells**

Mouse neuroblastoma Neuro2a cells were cultured in Advanced Dulbecco’s Modified Eagle Medium (DMEM/F12 (Life Technologies) supplemented with 10% fetal bovine serum, Glutamax (Life Technologies), and non-essential amino acid solution (Life Technologies). Neuro2a cells were infected with recombinant lentivirus expressing small hairpin (shRNA) at a multiplicity of infection of ~ 40. Two days after infection, the cells were treated with 30 μM retinoic acid in Advanced DMEM/F12 supplemented with 0.2% fetal bovine serum to induce neuronal differentiation associated with neurite outgrowth. After 24 h, cell morphology was observed (Supplementary Figure S1). Lenti-X 293T cells (Clontech, Mountain View, CA, USA) were maintained in DMEM containing 10% fetal bovine serum for lentivirus production.

**Preparation of recombinant lentivirus**

Recombinant lentivirus was prepared essentially as described previously with some modifications. Briefly, 5.2 μg of pHPH-VESVG, 0.43 μg of pCEP4-tat and 2.6 μg of MISSON shRNA construct (Supplementary Table S2, Sigma-Aldrich, St Louis, MO, USA) were transfected into Lenti-X 293T cells (Clontech) in one 10-cm dish using polyethyleneimine (Polyethyleneimine ‘Max’, MW 40 000; Polysciences, Warrington, PA, USA). After 16–18 h of incubation, the culture supernatant was replaced with fresh media. After 24 h of incubation, the culture supernatant was collected, filtered through a syringe filter with a polycyovinylidene fluoride membrane (0.45-μm pore size, Millex-HV, Merck Millipore, Billerica, MA, USA) and stored in aliquots at −80 °C. Titration of recombinant lentiviruses was performed with Lenti-X qRT-PCR titration kit (Clontech). An shRNA sequence that does not target any mouse genes (SCEH002, Sigma-Aldrich) was used as a control.

**Quantification of neurite length in Neuro2a cells**

Before the experiment, we confirmed that almost all cells were positive for green fluorescent protein expression with lentivirus expressing green fluorescent protein at multiplicity of infection of ~ 40 (data not shown). Thus, lentiviruses expressing shRNA against candidate ASD-associated genes were used at multiplicity of infection of ~ 40. Neuro2a cells were fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilized with Triton X-100 and incubated with Hoechst 33258 dye (Calbiochem, San Diego, CA, USA) to identify nuclei and Alexa Fluor 546-phallolidin (Molecular Probes, Eugene, OR, USA) to identify cell bodies and neurites. For automated image analysis, images of Neuro2a cells were acquired using a ToxInsight automated microscope (Thermo Scientific, Waltham, MA, USA). Neurite number per cell, total neurite length per cell, neurite length per neurite and the longest neurite...
length were automatically measured. Approximately 9000–15 000 cells were observed for each construct (see Figure legends). The data presented are from three independent experiments performed in triplicate.

### Rescue experiments for the neurite growth assay

Human PRKD2 and PPCS complementary DNAs, purchased from Origene (Rockville, MD, USA), were amplified via PCR and subcloned into each MISSION shRNA construct. Recombinant lentiviruses expressing shRNA-resistant human PRKD2 and shRNA against PRKD2 or expressing shRNA-resistant human PPCS and shRNA against PPCS were prepared as described above. Quantification of neurite length in Neuro2a cells expressing these recombinant lentiviruses were performed as described above.

### Reverse transcription-PCR analysis

Total RNA was isolated from Neuro2a cells and reverse transcribed with Superscript III (Life Technologies). Real-time PCR was performed with SYBR premix EX Taq (Takara Bio Inc., Shiga, Japan) on a CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) according to the supplier’s protocol. Intron-spanning primer sets were used (primer sequences are available on request). The expression levels for each gene were normalized to that of GAPDH and were determined according to the 2^-ΔΔCt method.

### Gene annotation

Gene annotations were interrogated using AmiGO2 (http://amigo2.berkeleybop.org/amigo). The GO annotation terms related to the central
increased sensitivity.7,8 Because the possible functional impact of one trio, which is similar to that observed in previous studies indicating observed point-mutation rate in coding sequences was ~ 1.2 events per 4 synonymous and 3 possible splice-site mutations (Table 1). The SNVs included 3 nonsense, 1 frameshift, 27 non-synonymous, and one small deletion (Supplementary Materials and Methods).

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Table 2 Possible function of identified genes in the central nervous system

| Gene       | (Possible) function | Neuronal function | Description |
|------------|--------------------|-------------------|-------------|
| ABCA3      | ABC transporter    | –                 | Unknown     |
| ARHGAP19   | Rho GTPase-activating protein | –                 | Unknown     |
| CAMSAP3    | Microtubule organization | –                 | Unknown     |
| DDX50      | RNA helicase       | –                 | Unknown     |
| ECSIT      | Adapter protein    | –                 | Unknown     |
| ENO3       | Enolase            | –                 | Unknown     |
| FAHD1      | Hydrolase          | –                 | Unknown     |
| FRRS1      | Ferric-chelate reductase | –                 | Unknown     |
| HER1       | E3 ubiquitin ligase | +                 | Neuron projection development (experimental evidence) G0:0031175 |
| ITGA10     | Integrin alpha-10  | +                 | Neuron projection morphogenesis (sequence similarity) G0:0048812 |
| LARP1      | RNA metabolism     | –                 | Unknown     |
| NERAP2     | Endocytosis regulator | –                 | Unknown     |
| NUP205     | Nucleoporin        | –                 | Unknown     |
| OBSCN      | Rho guanine nucleotide exchange factor | –                 | Unknown     |
| OR6P1      | Olfactory receptor | –                 | Unknown     |
| OSMR       | IL-31 receptor     | –                 | Unknown     |
| PCNX       | Pecaxin-like protein | –               | Unknown     |
| PEAK1      | Tyrosine kinase    | –                 | Unknown     |
| PLEKHA4    | PH domain-containing | –               | Unknown     |
| PP1       | Zinc finger protein | –                 | Unknown     |
| POLR1E     | RNA polymerase     | –                 | Unknown     |
| PPCS       | PPC synthetase     | –                 | Unknown     |
| PRKD2      | Serine/threonine kinase | –               | Unknown     |
| RPA40      | Ribonuclease P     | –                 | Unknown     |
| SERPINB7  | Serpin peptidase inhibitor | –            | Unknown     |
| SLC4A12    | Magnesium transporter | –               | Unknown     |
| SMO        | GPCR/hedgehog signaling | +              | Axon extension/neuron differentiation (experimental evidence) G0:0048846 |
| WDR20      | WD repeat containing protein | –          | Unknown     |
| WNK2       | Serine/threonine kinase | –            | Unknown     |
| YAF2       | YY1-associated factor | +               | Forebrain development (zebrafish) (experimental evidence) G0:0030900 |
| ZFHX3      | Transcription factor | +               | Brain development (sequence similarity) G0:007420 |
| ZFPM2      | Transcription factor | –               | Unknown     |

Table 2 Possible function of identified genes in the central nervous system

**RESULTS**

WES of 30 sporadic autism trios

We selected 30 sporadic autism trios from human brain phenotype consortium in Japan (Supplementary Materials and Methods). We performed trio-based WES and validated 38 de novo events, 37 SNVs and one small deletion (Supplementary Materials and Methods). These SNVs included 3 nonsense, 1 frameshift, 27 non-synonymous, 4 synonymous and 3 possible splice-site mutations (Table 1). The observed point-mutation rate in coding sequences was ~ 1.2 events per trio, which is similar to that observed in previous studies indicating increased sensitivity.7,8 Because the possible functional impact of one frameshift SNV in a retrovirus gene and four synonymous SNVs is low, we selected 33 SNVs (3 nonsense, 27 non-synonymous and 3 possible splice-sites) in 32 genes for further analysis (Table 1). The 33 variants were confirmed by Sanger sequencing (Supplementary Table S1 and data not shown). The possible function, as well as neuronal function of these 32 gene products is shown in Table 2. Five of those 32 genes, POGZ, PLEKHA4, PCNX, PRKD2 and HERC1, have been previously reported as genes with de novo SNVs in ASD.1–3,6–9

**The strategy for gene knockdown-based assignment of priorities to the identified disease-related genes for further detailed analysis**

Recent genetic analyses have generally identified many disease-related genes, most of which have functions that are largely unknown, making further molecular etiological analysis difficult. Thus, it is important to develop a screening system with which we can rapidly and conveniently assign priorities to the number of candidate genes for further detailed analysis. One possible criterion is that autism-associated gene should be expressed in the brain; however, almost identified 32 genes except for SERPINB7 are expressed in the brain (GTEx Project (http://www.gtexnportal.org/home/)). Another possible criterion is that autism-associated gene products should have neuronal functions. To examine whether the identified genes with de novo SNV in autism are involved in neural functions, we focused on neurite development in neuronal cells. Neurite development is commonly used for monitoring early neuronal differentiation and is one of the most important events in the formation of neural circuits.29 In this study, we used mouse neural crest-derived Neuro2a neuroblastoma cells, which have been

nervous system function, including neurite outgrowth were manually searched for each gene.

**Statistical analyses**

Statistical analysis was performed using one-way analysis of variance, or Fisher's exact test, as indicated. All P-values reported are two-tailed. The level of significance was set at P<0.05.

**Neurite development**

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extensively used to study neuronal differentiation and its associated signaling pathways. A useful characteristic of Neuro2a cells is their ability to develop relatively simple neurites within a few days, which serves as an established model system for the study of neurite development of central nervous system neurons. Using the simple system, we easily determine that the candidate ASD-associated gene products can possibly be involved in neurite development, one of the important aspects of the central nervous system development and function. The system focuses only in neurite development, which gives us fragmentary information of the possible neural function of each gene product, however, the information is enough to drive us to further analyze the function of the gene product in detail. The strategy for gene knockdown-based assignment of priorities of the identified autism-related genes is summarized in Supplementary Figure S2. We searched a commercial shRNA library (MISSION TRC shRNA library SP1, Sigma-Aldrich) for the 32 target genes and found that the library offered 20 validated shRNAs for 15 of those genes (Supplementary Table S2). To maintain the rapid and convenient screening, the remaining 17 genes, for which validated shRNA constructs were not available from the library, were not subjected to further study.

Identification of neural functions of the autism-related genes in mouse neuroblastoma Neuro2a cells

We first tested the knockdown efficiency of the 20 commercially available validated shRNA constructs for the 15 genes and found that the validated shRNA constructs effectively decreased their target RNAs, except for that targeting ZFPM2, which was not detected in Neuro2a cells. For FRRS1, POGZ, POLRE1, PPCS, and PRKD2, we examined two validated shRNA constructs for knockdown efficiency and chose the more effective one for further morphological studies (data not shown). We then performed shRNA-based assays using Neuro2a cells, which are well known to undergo neural differentiation and extend bidirectional and multidirectional process outgrowth on retinoic acid treatment. Before our experiments, we confirmed that retinoic acid treatment increased the number of neurites and the total length of neurites in Neuro2a cells (Supplementary Figure S1). We compared cells infected with lentiviruses expressing validated shRNA vs cells infected with lentiviruses expressing control shRNA under retinoic acid-differentiated
conditions for a variety of morphological changes. The shRNA-mediated knockdown of these genes did not affect cell viability (data not shown). We analyzed ~9000–15 000 cells for each construct and found that knockdown of 8 out of 14 genes significantly decreased at least 1 parameter: retinoic acid-induced neurite outgrowth was decreased (Figure 1b and Supplementary Table S3). Among these genes, knockdown of PRKD2 or PPCS markedly impaired neurite outgrowth (Figure 2). In addition, knockdown of the other six genes did not significantly affect neurite outgrowth (Supplementary Table S3). These results suggest that the identified eight gene products, PRKD2, PPCS, POGZ, FRRS1, YAF2, ENO3, POLR1E and FAHD1, may be candidate regulators of neurite development in neurons in the central nervous system. We then performed a rescue experiment of PRKD2 and PPCS, disruption of which markedly impaired neurite outgrowth (Figure 2). We found that overexpression of PRKD2 but not PPCS rescued the impaired neurite outgrowth (Figure 3 and data not shown). These results suggest either that the impaired neurite outgrowth by shRNA-mediated knockdown of PPCS may be a false positive result or that overexpressed PPCS cannot substitute the endogenous function of PPCS.

**DISCUSSION**

For the comprehensive analysis of a large number of candidate genes identified by genetic studies, it is necessary to make biological sense of these genes in the central nervous system and to identify convergent signaling pathways in which multiple candidate genes are involved; however, molecular analysis of the function of each individual gene product in the central nervous system generally requires substantial effort. To circumvent this problem, we developed a high-throughput post-genomics screening method with which we can easily predict the neuronal function of the candidate gene products and assign priorities to the number of candidate genes for detailed analysis. We interrogated the annotations of the identified 32 disease susceptibility genes with a Gene Ontology browser (AmiGO2) and found that five of those genes, HERC1, ITGA10, SMO, YAF2 and ZFHX3, are involved in neuronal functions such as neurite outgrowth (Table 2). Our current screening system identified 7 (in the case of excluding PPCS) to 8 (including PPCS) out of the 14 genes examined (50 ~ 57.1%) as regulators of neurite development, which is 3.2 ~ 3.7-fold higher than that expected from all of AmiGO2 (5 out of 32 genes, 15.6%; \( P = 0.010, \) Fisher’s exact test). The system focuses only in neurite development in Neuro2a cells, which gives us fragmentary information of the possible neural function of each gene product, however, the information is enough to drive us to further analyze the function of the gene product in detail. Thus, the Neuro2a cell-based rapid and convenient screening of ASD genes can be valuable for identifying the ASD-related neural functions of enormous pools of candidate genes identified by genetic analyses. Further detailed analysis might eliminate false positive genes from identified candidate ASD genes in our current screening.

In this study, we performed WES of 30 individuals with sporadic ASD and their parents, identified 37 genes with 38 de novo SNVs, and selected 33 SNVs (3 nonsense, 27 non-synonymous and 3 possible splice site) in 32 genes for analysis. Five of those 32 genes, POGZ, PLEKHA4, PCNX, PRKD2 and HERC1, have been previously reported as genes with de novo SNVs in ASD.1–3,6–9 A previous study has shown that HERC1 regulates Purkinje cell physiology in the cerebellum;30 however, the functions of most of these gene products in the brain had remained unclear despite extensive bioinformatics resources. Using our system, we found that POGZ and PRKD2 regulate neural functions, such as neurite development. Further comprehensive analysis of these recurrently mutated gene products in neural development will unravel the molecular etiology of ASD. In addition to the recurrently mutated genes identified in our study, there are several recurrent disruptive mutated genes, such as CHD8, SCN2A, and DYRK1.4,10 It will also be interesting to examine the functions of these gene products in neurite development using our system.

Proper development of axons and dendrites is critical for the anatomical connectivity of brain regions and leads to functional neural circuit formation; precise axon and dendrite development is a biological basis for higher brain function. The elaboration of axons and dendrites is a highly dynamic process that is regulated by the combination of many genes.31 ASDs are often associated with altered synaptic connectivity and plasticity, resulting in marked disruptions in information processing and cognition.32–34 Several ASD-associated gene products such as EPAC2, TAOK2 and PTEN are involved in the maintenance of axonal and dendritic growth.34–38 We found that shRNA-mediated knockdown of PRKD2 or PPCS drastically impaired neurite growth in Neuro2a cells, suggesting that these gene products.
have important roles in dendrite development in neurons. Although the effects were not as marked, the shRNA-mediated knockdown of the remaining six genes also impaired neurite development. A more comprehensive analysis of the molecular mechanisms of axon and dendrite development is important for understanding the etiology of ASDs.

The current study has several limitations as follows:

1. The screening method lacks the positive controls (for example, robust ASD-associated genes such as CHD8 and MEPC2) and negative controls (for example, genes with de novo loss-of-function mutations in healthy siblings in previously published studies). Because of the lack of these important factors, we cannot definitively claim whether the screening system is valid or not.

2. The identified mutations may cause a variety of effects on protein functions, such as loss of function, gain of function, dominant negative suppression of the wild-type protein, or acquisition of a new function. Therefore, the identified mutations cannot be mimicked by simple knockdown experiments.

3. It is important to perform the knockdown experiments with ~50% knockdown efficiency for examining the putative loss-of-function mutations. Furthermore, the screening method lacks the rescue experiments.

4. In the screening method, we selected the candidate ASD genes for the neurite outgrowth assay in a nonrandom manner.

Thus, the data on sensitivity and specificity seems lacking; further detailed analysis might eliminate false positive genes from the candidate ASD genes, resulting in identifying causative ASD genes.

Given that recent studies argue that true ASD-associated exome SNVs make up ~3–5% of ASD (for review, see Krumm, N. et al., Malhotra, D. et al., Ronemus, M. et al.), the number of causative ASD genes among the 30 trios will be only one to two. Although the number of causative ASD genes is expected to be low, the other identified de novo SNVs may also be associated with risk for ASD.

Our current neurobiological screening system using neuroblastoma cell lines focuses only in neurite development, which gives us fragmentary information of the possible neural function of each gene product. It is also important to examine the precise role of the identified candidate gene products in various neural functions, including synapse development and function as well as neurite development. Furthermore, although our system can biologically select genes involving neural function from a vast number of candidate ASD-associated genes for further detailed analysis, the limitation of this study is that we are unable to identify the biological significance of the SNVs using our current screening system. It is important to develop a new system in which we can rapidly examine the biological significance of the SNVs identified by WES of patients with ASD in the near future.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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