Genetic and phenotypic analysis of 101 patients with developmental delay or intellectual disability using whole-exome sequencing

Original Article

Genetic and phenotypic analysis of 101 patients with developmental delay or intellectual disability using whole-exome sequencing

Running title: Trio-WES for DD/ID

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Abstract

Whole-exome sequencing (WES) enables identification of pathogenic variants, including copy number variants (CNVs). In this study, we performed WES in 101 Japanese patients with unexplained developmental delay (DD) or intellectual disability (ID) (63 males and 38 females), 98 of them with trio-WES. Pathogenic variants were identified in 54 cases (53.5%), including four cases with pathogenic CNVs. In one case, a pathogenic variant was identified by reanalysis of exome data; and in two cases, two molecular diagnoses were identified. Among 58 pathogenic variants, 49 variants occurred *de novo* in 48 patients, including two somatic variants. The accompanying autism spectrum disorder and external ear anomalies were associated with detection of pathogenic variants with odds ratios of 11.88 (95% confidence interval (CI) 2.52 – 56.00) and 3.46 (95% CI 1.23 – 9.73), respectively. These findings revealed the importance of reanalysis of WES data and detection of CNVs and somatic variants in increasing the diagnostic yield for unexplained DD/ID. In addition, genetic testing is recommended when patients suffer from the autism spectrum disorder or external ear anomalies, which potentially suggests the involvement of genetic factors associated with gene expression regulation.

Keywords: Whole-exome sequencing, Intellectual disability, Developmental delay, Autism spectrum disorder, External ear anomalies

Introduction

Whole-exome sequencing (WES) is a comprehensive genetic test that enables the detection of single nucleotide variants, insertion/deletions, and copy number variants (CNVs)[1]. In addition, offspring-parental trio analysis can systemically identify both germline and somatic *de novo* variants, which have been reported to play important roles in neurodevelopmental disorders (NDDs)[2]. NDDs comprise intellectual disability (ID), the autism spectrum disorder (ASD), attention-deficit/hyperactivity disorder, neurodevelopmental motor disorders, and specific learning disorders. Among these, developmental delay (DD) or ID is a major condition of NDDs[3]. DD/ID has high clinical and genetic heterogeneities, and the genetic diagnosis of DD/ID is challenging[2]. The diagnostic rate in patients with DD/ID, using WES, has been recently reported to be 27% to 39%[4-6]. Genetic diagnosis helps facilitate family planning and the coping process for disease course, including fewer repeat investigations, discontinuation of unnecessary laboratory tests, and further assessment of related medical conditions.

Patients with DD/ID may show various clinical symptoms, such as ASD, seizures, hypotonia, facial dysmorphism, and neuroimaging abnormalities. It is important to identify the key clinical features that allow the identification of patients with a high probability of harboring relevant genetic variants. A recent large cohort study clarified factors that influence the diagnostic yield of *de novo* variants in NDDs. These included sex, abnormal cranial magnetic resonance imaging (MRI) findings, speech and walking delay, and paternal age[7]. Further analysis with detailed clinical features may help to elucidate the clinical features related to the diagnostic yield of DD/ID in clinical settings.

In this study, we performed WES for 101 Japanese patients with unexplained DD/ID, 98 of them with trio-WES. A variety of genetic causes, including CNVs and somatic variants, were identified. Phenotypic analysis revealed that the presence of ASD and external ear anomalies with DD/ID potentially suggests the involvement of genetic factors associated with the regulation of gene expression, leading to a higher diagnostic yield.

Methods

Patients

This study included 101 unexplained Japanese DD/ID patients (63 males, 38 females) without any identifiable non-genetic factors responsible for DD/ID, including congenital infections, hypoxia, trauma, central nervous system infection or malignancy. G-banded karyotyping was normal in all patients. They underwent WES at the Hamamatsu University School of Medicine between June 2016 and April 2020. Among them, trio-WES was performed in 98 patients, but parents' samples were not available in 3 cases (#402, #2059, #2686). These patients had been recruited for suspected genetic diseases, in pediatric care, including patients with long-term follow-up beyond childhood. The criteria for patients with DD/ID enrolled in this WES study were to have at least one of additional neurological, systemic, or other clinically characteristic features. We collected clinical information for each patient by retrospectively reviewing their medical records, and investigated the correlation between clinical information and diagnostic yield. Diagnosis of ASD is based on impaired social interaction and restricted and stereotyped behaviors by the DSM-V method[8]. We classified 84 patients into the following four

categories based on developmental quotient (DQ), intelligence quotient (IQ), or clinical evaluation by the attending physician if these scores were not available: profound (DQ/IQ, < 21), severe (DQ/IQ, 21–34), moderate (DQ/IQ, 35−49), and mild (DQ/IQ, 50–70). Seventeen cases were designated as "unclassified" because only the diagnosis of DD/ID without detailed information was available. For analysis of each clinical finding, we only included patients whose corresponding information was described in the medical record. Thus, the number of assessed patients varied from 61 to 101 (Table 1).

Genetic analysis

This study was approved by the Institutional Review Board Committee of the Hamamatsu University School of Medicine. After receiving written informed consent, genomic DNA of the patients and their parents were extracted from blood leukocytes or saliva. Forty-four patients previously underwent array comparative genomic hybridization (aCGH) with negative findings[9]. WES was performed using the SureSelectXT Human All Exon v6 (Agilent Technologies, Santa Clara, CA) with 150-base paired-end reads using Illumina HiSeq2500 or NextSeq500 (Illumina, San Diego, CA). Reads were aligned to the reference genome (GRCh37) using BWA with default parameters. Duplicate reads were removed using the Picard tool. Variants were identified using the GATK HaplotypeCaller. Raw variants were filtered out when their parameters met any of the following criteria: QD < 2.0, MQ < 40.0, FS > 60.0, MQRankSum < −12.5, and ReadPosRankSum < −8.0 for single nucleotide variants; QD < 2.0, ReadPosRankSum < −20.0, and FS > 200.0 for insertion/deletions. Mosaic variants were detected using Mutect2[10]. The final variants were annotated using Annovar[11] to predict

the functional impact of the coding variants. We focused on rare variants with minor allele frequencies <1% in an in-house exome database, Human Genetic Variation Database (http://www.hgvd.genome.med.kyoto-u.ac.jp/)[12], 1KJPN, 2KJPN, or 3.5KJPN (https://ijgvd.megabank.tohoku.ac.jp/)[13], and the gnomAD database (https://gnomad.broadinstitute.org/)[14]. Variant pathogenicity was predicted by SIFT (http://sift.jcvi.org/), Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), MutationTaster (http://www.mutationtaster.org/)[15], CADD (http://cadd.gs.washington.edu/score)[16] and M-CAP (http://bejerano.stanford.edu/mcap/index.html)[17]. We also examined possible pathogenic CNVs using WES data with the eXome Hidden Markov Model (XHMM)[18] and previously developed methods[19]. The identified single nucleotide variants and insertions/deletions were confirmed by Sanger sequencing. CNVs were validated by aCGH or real-time quantitative PCR. "Pathogenic" or "Likely pathogenic" variants, according to the American College of Medical Genetics and Genomics classification[20,21], were defined as the disease-causing pathogenic variants. In addition, we included three variants that we reported as novel genetic causes[22-24].

Statistical analyses

All statistical analyses were performed using SPSS version 26.0 (SPSS Inc., Chicago, IL). Clinical data of patients with or without exome positive results were tested using the two-tailed Fisher's exact test, and the odds ratio (OR) for categorical variables was calculated. The Mann-Whitney U test was used to compare medians. Continuous

variables were compared using the Student's t-test. The significance level was set at a P-value < 0.05.

Results

Identification of pathogenic variants by WES

The average read depth of the protein-coding regions of RefSeq genes was 99.29 (range across all patients, 70.41–144.10), such that 99.2% of the targeted coding sequences were covered by 10 reads or more (Supplemental Table S1). We found a total of 58 pathogenic variants in 54 cases (53.5%, Table 1). These included three variants that we previously reported as novel genetic causes[22-24]. Autosomal dominant (AD) disorders accounted for 48 cases. In these, 50 pathogenic variants were identified, including four CNVs and two somatic variants, and two molecular diagnoses were identified in two patients (#1376 and #2386). There were three cases of autosomal recessive disorders (five variants), two cases with X-linked recessive disorders (two variants), and one case with X-linked dominant (XLD) disorder (one variant) (Tables 2 and 3). The details of six cases have already been reported[22-27]. In AD and XLD disorders, 49 of 51 variants occurred *de novo*, further indicating the importance of *de novo* variants in DD/ID. In one case (#1108), the pathogenic variant was identified by reanalysis of exome data. One other patient (#1376) had a variant that was considered as a secondary finding (see Discussion). In one individual (#2386), two pathogenic variants in two genes (*CDKN1C* and *DNM1L*) were detected. These caused Beckwith-Wiedemann syndrome (OMIM# 130650) and encephalopathy due to defective mitochondrial and peroxisomal fission 1 (OMIM# 614388), respectively (Table 2). Patient #2386 displayed severe ID that could not be explained by the *CDKN1C* variant inherited from her affected mother. Thus, the *DNM1L*

variant was also considered to be involved in her clinical symptoms. The details of this patient will be published elsewhere. The candidate variants detected by WES in the unsolved cases are summarized in Supplemental Table S2.

We found four CNVs ranging from 1.3 Mb to 4.4 Mb using WES data. The number of protein-coding genes involved in the CNVs ranged from 15 to 42 (Table 3). All identified CNVs overlapped with previously described CNVs in patients with NDDs and were interpreted as pathogenic[21]. WES is useful for the identification of pathogenic CNVs in DD/ID patients[28].

Somatic variants were identified in two patients (#760 and #1465) with megalencephaly-capillary malformation syndrome (MCAP). Several cases of MCAP have been reported to harbor somatic variants in *PIK3CA*[29]. However, our standard WES using GATK HaplotypeCaller failed to identify the causative pathogenic variants due to the low variant allele fractions. With a suspicion of the involvement of somatic variants, we used the MuTect2 for variant calling and found the previously reported *PIK3CA* variants[10,29] (Table 2). The variant allele fraction of the two variants was 11.7% (12/103 reads) in #760 and 7.7% (4/52 reads) in #1465. Sanger sequencing (#760) and digital PCR (#1465) confirmed the somatic variant in *PIK3CA*[30] (Supplementary Figure S1 and S2). Because WES achieves relatively high depth compared with that of whole-genome sequencing, efforts should be made to identify somatic variants with WES data.

Correlation between clinical features and detection rates of pathogenic variants

Our DD/ID cohort was characterized by the presence of additional associated clinical findings in all patients. These included facial dysmorphism (n = 62/95, 65.3%), hypotonia

(n = 43/79, 54.4%), short stature (n = 44/87, 50.6%), and seizures (n = 37/84, 44.0%). ASD was noted in 20 of 72 individuals (25.6%), and MRI abnormalities were observed in 51 of 92 patients (55.4%). The clinical descriptions of the patients are summarized in Table 1 and Supplemental Table S1.

In our study, previously reported key clinical findings such as sex, postnatal feeding problems, abnormal cranial MRI, developmental milestone like first words and independent walking, and paternal age were not significantly associated with identification of pathogenic variants. Instead, ASD was significantly associated with a positive WES result (OR, 11.88; 95% confidence interval [CI], 2.52 – 56.00) (Table 1). This is consistent with previous reports describing a high diagnostic rate among ASD patients suffering from DD/ID[31,32]. Although there was no difference in WES results with or without facial dysmorphism (P = 0.133), the presence of external ear anomalies such as low set ear, macrotia, and cryptotia significantly increased the odds of having a positive WES result (OR 3.46, 95% CI 1.23 – 9.73) (Table 1, Supplemental Table S1). Abnormal eyebrow morphology (P = 0.052) and birth at term (P = 0.080) tended to affect the results of WES, but there were no statistically significant differences. We investigated the relationship between the positive WES results and the degree of DD/ID. No correlation was found between DQ/IQ value or the degree of DD/ID by dividing it into four groups and positive results of WES (Table 1).

Discussion

The overall diagnostic yield was 53.5% in the WES study. It was high compared to those of previous WES studies for DD/ID (27% to 39%)[4-6]. The yield of WES is reportedly higher in patients with NDDs along with any additional associated conditions

than in patients with NDDs alone (53% vs. 31%)[33]. A previous WES study of ID patients with additional clinical features in 31 of the 33 cases reported a relatively high diagnostic yield (57%)[34]. All DD/ID cases in our study had some associated conditions (Supplemental Table S1). Therefore, the present findings suggest that the presence of multiple phenotypic features in addition to DD/ID enriches the diagnostic yield in the context of comprehensive WES analysis, including CNVs and somatic variants.

The most strongly associated clinical feature with positive WES results was ASD. The data suggest that DD/ID and ASD share genetic backgrounds, which have strong effects, leading to higher diagnostic yields. ASD is associated with a variety of factors, including genetics and prenatal and postnatal environment[35]. In SFARI (Simons Foundation Autism Research Initiative) database (https://gene.sfari.org/; accessed February 2021), there are listed 1003 genes implicated in ASD susceptibility. The major functional categories of ASD genes are regulation of gene expression, such as chromatin regulators and transcription factors, and neuronal communication, such as synaptic function[36]. Alterations in the functions of gene expression regulation are known to be genetically associated with ID[37]. In patients with DD/ID and ASD, we observed that many of the genes with pathogenic variants were involved in gene expression regulation (10/14) (Supplemental Table S3).

We also found that external ear anomalies were weakly associated with positive WES results. In patients with DD/ID and external ear anomalies, 9 of 14 genes with pathogenic variants were associated with gene expression regulation in this study (Supplemental Table S4). In the development of the craniofacial region including external ear, reciprocal signaling between neural crest cells and the craniofacial ectoderm plays an important role

in driving craniofacial patterning and morphogenesis[38]. The regulation of neural crest development is mediated by epigenetic modifications such as DNA methylation, histone modification and chromatin remodelers[39]. An alteration in these signals of gene expression regulation results in a disruption of neural crest, and leads to a set of syndromes and diseases affecting broad spectrum of congenital malformations and DD/ID[40]. Among the 54 patients with molecular diagnosis, 18 had ASD, 18 had external ear anomalies, and seven had both ASD and external ear anomalies, indicating that a substantial proportion of patients showed both complications in this heterogeneous DD/ID population. In the study of Coffin-Siris Syndrome and Kabuki syndrome showing DD/ID, ASD and external ear anomalies, the dysfunction of neural crest cells reported to be caused by the mutations of genes associated with chromatin regulators[41,42]. Therefore, mutations in genes involved in gene expression regulation possibly cause ASD and/or external ear anomalies in addition to DD/ID. Our data suggest that the presence of three clinical features (DD/ID, ASD, and external ear anomalies) might indicate abnormalities in gene expression regulation.

We identified pathogenic variants by reanalysis in one case with negative exome results in the original WES (#1108). In this individual, the gene–disease association was unknown at the time of the first analysis, but the same *de novo* variant in *DHX30* was reported as pathogenic one year later[43]. Previous studies have reported that reanalysis of WES data may offer an additional diagnostic yield of 10-15%[44]. Systematic reassessment of exome data was recommended to be analyzed at a 2- to 3-year interval as knowledge related to gene–disease associations improved[45]. It is important to reanalyze WES data before additional testing, such as whole-genome sequencing or RNA sequencing.

In WES analysis, secondary findings that refer to genetic variants that are unrelated to the primary medical reason for testing but may have future medical significance remain concerns. In this study, one patient (#1376) had two pathogenic *de novo* variants, including *TCF4* causing Pitt-Hopkins syndrome (OMIM # 610954) and *LZTR1* causing Noonan syndrome 10 (OMIM # 616564) or Schwannomatosis-2, susceptibility to (OMIM # 615670). The *de novo* nonsense p.(Arg576*) variant in *TCF4* was considered to cause profound DD in this patient. On the other hand, *LZTR1* variants associated with Noonan syndrome were reported to be localized in the kelch (KT) domains[46], suggesting that the identified missense p.(Arg688Cys) variant in the BTB (bric-a-brac, tramtrack, broad complex) domain is less likely to cause Noonan syndrome. Instead, an identical variant has been reported to cause schwannomatosis[47]. Therefore, this patient is likely to develop multiple schwannomas in various areas of the body during adulthood. The American College of Medical Genetics and Genomics guidelines for reporting secondary findings included a list of 59 medically actionable genes recommended for clinical genomic sequencing[48]. *LZTR1* was not included in this list. However, we proposed regular imaging for this patient because the *LZTR1* variants are reportedly at risk for intracranial or spinal cord schwannomas in younger patients. Appropriate genetic counseling is needed prior to WES analysis, as identification of such secondary findings is expected in trio-WES.

In this study, we were unable to find a molecular diagnosis in almost half of the patients. WES has technological limitations, including the inability to detect noncoding variants, structural variants except for CNVs, epigenetic changes, and trinucleotide repeat expansion[49]. The screening of fragile X syndrome, a typical trinucleotide repeat disorder, has not been performed in most of our patients, and should be considered for patients with

DD/ID or ASD even if they do not present any characteristic clinical features for fragile X syndrome[50]. In addition, some studies suggested that CNV calling from WES data had limitations due to high false-positive rates and low sensitivity[51,52]. Therefore, it is necessary to consider the possibility of pathogenic CNV in undiagnosed patients. Whole-genome sequencing enable the accurate and comprehensive structural variant detection, and has become widely used because of its low costs, which continue to decrease[53]. However, many potential variants of unknown significance have been detected, making it difficult to evaluate variants. Recently, several studies used transcriptome sequencing to identify disease-causing variants[54]. Further research is needed to develop strategies for investigating pathogenic variants in cases that are unresolved by WES.

In conclusion, WES, as a first-line genetic analysis tool, is useful for detecting the genetic causes in patients with unexplained DD/ID. This benefits patients and their families in terms of cost-effectiveness, family planning, and patient management. Our study also suggests that the presence of ASD and external ear anomalies with DD/ID potentially indicates the involvement of genetic factors associated with gene expression regulation, thereby leading to a higher diagnostic yield.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Table 1. Clinical findings of the patients.

Clinical findings	Assessed cases	Number	%	WES positive	WES negative	p-Value	OR (95% CI)
Gender	101			54	47		
Male		63	62.4	32	31	0.541	
Female		38	37.6	22	16	0.541	
Examined age (median [range]) (years)	101	4.0 [0 – 42]		4.0 [0 – 32]	3.0 [0 – 42]	0.157†	
Paternal age at birth (mean [SD]) (years)	61	34 [± 5.4]		35 [± 5.4]	33 [± 5.3]	0.147‡	
Maternal age at birth (mean [SD]) (years)	61	31 [± 4.9]		31 [± 4.8]	31 [± 5.0]	0.740‡	
Family history	98	9	9.2	5	4	1.000	
Assisted reproductive technology	93	3	3.2	1	2	0.601	
Birth at term	95	81	85.3	46	35	0.080	3.29 (0.95–11.36)
Small for gestational age (10th percentile)	95	15	15.8	5	10	0.098	
Neonatal intensive care	94	33	35.1	16	17	0.524	
DD/ID	101	101		54	47		
DQ/IQ (median [range])	55	42 [7 – 70]		39 [7 – 69]	43 [14 – 70]	0.618†	
Profound (DQ/IQ <21)	101	26	25.7	17	9	0.178	
Severe (DQ/IQ, 21–34)	101	19	18.8	9	10	0.368	
Moderate (DQ/IQ, 35–49)	101	14	13.9	8	6	1.000	
Mild (DQ/IQ, 50–70)	101	25	24.8	13	12	1.000	
Unclassified	101	17	16.8	7	10	0.297	
Speech delay	88	84	95.5	48	36	0.318	
No single word	85	49	57.6	28	21	0.826	
Motor delay	91	82	90.1	45	37	1.000	
Unable to walk without support	85	27	31.8	15	12	1.000	
Stereotypies	76	4	5.3	4	0	0.123	
Dysphagia	82	23	28.0	11	12	0.623	
Deafness	84	6	7.1	3	3	1.000	
Sleep disorder	79	10	12.7	5	5	1.000	
Autism spectrum disorder	78	20	25.6	18	2	**0***	11.88 (2.52–6.00)
Seizure	84	37	44.0	19	18	0.661	
Short stature (<-2.0SD)	87	44	50.6	24	20	0.830	
Tall stature (>2.0SD)	87	2	2.3	2	0	0.502	
Macrocephaly	82	10	12.2	7	3	0.504	
Microcephaly	82	29	35.4	19	10	0.351	
Hypotonia	79	43	54.4	23	20	1.000	

Spasticity	79	7	8.9	3	4	0.696	
Strabismus	76	16	21.1	10	6	0.575	
Facial dysmorphism	95	62	65.3	37	25	0.133	
Hypertelorism	95	8	8.4	3	5	0.465	
Abnormal palpebral fissure	95	13	13.7	8	5	0.766	
Abnormal eyebrow morphology	95	22	23.2	16	6	0.052	2.90 (1.02–8.23)
External ear anomaly	95	24	25.3	18	6	**0.019***	3.46 (1.23–9.73)
Cleft palate	95	7	7.4	5	2	0.445	
Abnormal lips	95	12	12.6	8	4	0.373	
Nasal anomaly	95	22	23.2	15	7	0.147	
Dental crowding	95	9	9.5	6	3	0.498	
Micrognathia	95	14	14.7	9	5	0.563	
Microphthalmus	95	3	3.2	2	1	1.000	
Congenital heart defect	85	18	21.2	11	7	0.596	
Renal anomaly	83	3	3.6	3	0	0.246	
Limb anomaly	95	37	38.9	23	14	0.211	
Hand anomaly	95	30	31.6	19	11	0.269	
Foot deformity	95	15	15.8	10	5	0.398	
Ambiguous genitalia	95	12	12.6	4	8	0.215	
Dermatopathy	95	8	8.4	5	3	0.721	
MRI abnormalities	92	51	55.4	23	28	0.095	

Statistical Analysis: †, Mann-Whitney U test; ‡, Student's t-test; No symbols, Fisher’s exact test; *, significant difference.

Abbreviation: CI, Confidence interval; DD, developmental delay; DQ, developmental quotient; ID, intellectual disability; IQ, intelligence quotient; MRI, magnetic resonance imaging; OR, odds ratio; SD, standard deviation; WES, whole-exome sequencing.

Table 2. Summary of pathogenic variants identified by whole-exome sequencing.

Subject	DD/ID (DQ/IQ)	Gene (Transcript)	Variant	Inheritance	Known or Novel	Class	OMIM Phenotype (MIM#)	Ref
N3	Profound	*FOXG1* (NM_005249.5)	c.506del, p.(Gly169Alafs*23)	AD/*de novo*	Known	Pathogenic	Rett syndrome, congenital variant (613454)	
294	Mild (59)	*ZBTB7A* (NM_015898.4)	c.1152C>G p.(Cys384Trp)	AD/*de novo*	Novel	NA	NA	23
329	Profound	*CSNK2B* (NM_001320.7)	c.533_534insGT p.(Pro179Tyrfs*49)	AD/*de novo*	Novel	Pathogenic	Poirier-Bienvenu neurodevelopmental syndrome (618732)	26
454	Moderate (41)	*ANKRD11* (NM_001256182.2)	c.1909A>T p.(Lys637*)	AD/*de novo*	Novel	Pathogenic	KBG syndrome (148050)	
556	Severe (26)	*SATB2* (NM_001172509.2)	c.868C>T p.(Gln290*)	AD/*de novo*	Known	Pathogenic	Glass syndrome (612313)	
595	Profound	*CTCF* (NM_006565.4)	c.1102C>T p.(Arg368Cys)	AD/*de novo*	Novel	Pathogenic	Mental retardation, autosomal dominant (615502)	
748	Moderate (42)	*KMT2A* (NM_001197104.2)	c.2530delC p.(Gln844Argfs*105)	AD/*de novo*	Novel	Pathogenic	Wiedemann-Steiner syndrome (605130)	
757	Profound (10)	*SETD1B* (NM_001353345.1)	c.5704C>T p.(Arg1902Cys)	AD/*de novo*	Novel	Likely Pathogenic	Intellectual developmental disorder with seizures and language delay (619000)	25
760	Mild (68)	*PIK3CA* (NM_006218.4)	c.3104C>T p.(Ala1035Val)	AD/*de novo*, somatic	Known	Pathogenic	Megalencephaly-capillary malformation-polymicrogyria syndrome, somatic (602501)	
774	Mild (69)	*PIK3R2* (NM_005027.4)	c.1117G>A p.(Gly373Arg)	AD/*de novo*	Known	Pathogenic	Megalencephaly-polymicrogyria-polydactyly-hydrocephalus syndrome (603387)	
792	Mild (63)	*BRAF* (NM_004333.6)	c.722C>T p.(Thr241Met)	AD/*de novo*	Known	Pathogenic	Noonan syndrome (613706)	
832	Profound (7)	*KMT2A* (NM_001197104.2)	c.2565dup p.(Glu856Argfs*10)	AD/*de novo*	Novel	Pathogenic	Wiedemann-Steiner syndrome (605130)	
975	Mild (56)	*DYRK1A* (NM_001396.4)	c.1046G>A p.(Cys349Tyr)	AD/*de novo*	Novel	Likely Pathogenic	Mental retardation, autosomal dominant (614104)	
1108	Profound	*DHX30* (NM_138615.2)	c.2353C>T p.(Arg785Cys)	AD/*de novo*	Known	Pathogenic	Neurodevelopmental disorder with severe motor impairment and absent language (617804)	

1241	Mild (50)	*TBR1* (NM_006593.4)	c.443_444del p.(His148Profs*92)	AD/*de novo*	Novel	Pathogenic	Intellectual developmental disorder with autism and speech delay (606053)	
1376	Profound (18)	*TCF4* (NM_001083962.2)	c.1726C>T p.(Arg576*)	AD/*de novo*	Known	Pathogenic	Pitt-Hopkins syndrome (610954)	
		LZTR1 (NM_006767.4)	c.2062C>T p.(Arg688Cys)	AD/*de novo*	Known	Pathogenic	Schwannomatosis-2, susceptibility to (615670)	
1386	Profound (17)	*ARID1B* (NM_017519.3)	c.5616del p.(Ser1873Leufs*48)	AD/*de novo*	Novel	Pathogenic	Coffin-Siris syndrome (135900)	
1411	Profound	*EEF1A2* (NM_001958.5)	c.271G>A p.(Asp91Asn)	AD/*de novo*	Known	Pathogenic	Epileptic encephalopathy, early infantile (616409)	
1460	Moderate	*GABRG2* (NM_198904.3)	c.964G>A p.(Ala322Thr)	AD/paternal	Novel	Likely Pathogenic	Epilepsy, generalized, with febrile seizures plus (607681)	
1465	Severe (31)	*PIK3CA* (NM_006218.4)	c.2176G>A p.(Glu726Lys)	AD/*de novo*, somatic	Known	Pathogenic	Megalencephaly-capillary malformation-polymicrogyria syndrome, somatic (602501)	
1481	Profound	*COASY* (NM_025233.7)	c.112dupT p.(Tyr38Leufs*26)	AR/maternal	Novel	Pathogenic	Neurodegeneration with brain iron accumulation (615643)	
			c.1495C>T p.(Arg499Cys)	AR/paternal	Known	Pathogenic		
1493	Mild (59)	*SMARCA4* (NM_001128849.3)	c.1537C>T p.(Arg513Trp)	AD/*de novo*	Novel	Likely Pathogenic	Coffin-Siris syndrome (614609)	
1530	Moderate (49)	*GNB2* (NM_005273.3)	c.229G>A p.(Gly77Arg)	AD/*de novo*	Novel	NA	NA	21
1539	Moderate (48)	*ARID1B* (NM_017519.3)	c.2986+1G>C	AD/*de novo*	Novel	Pathogenic	Coffin-Siris syndrome (135900)	
1573	Profound	*TUBB4A* (NM_001289123.1)	c.916G>A p.(Val306Ile)	AD/*de novo*	Known	Likely Pathogenic	Leukodystrophy, hypomyelinating (612438)	
1615	Severe (30)	*GATAD2B* (NM_020699.4)	c.143_144insA p.(Leu49Alafs*10)	AD/*de novo*	Novel	Pathogenic	GAND syndrome (615074)	
1618	Profound	*AP1S2* (NM_001272071.2)	c.179+1G>A	XLR /maternal	Novel	Pathogenic	Mental retardation, X-linked syndromic (304340)	
1625	Severe (32)	*HECW2* (NM_020760.4)	c.4690G>A p.(Glu1564Lys)	AD/*de novo*	Novel	Likely Pathogenic	Neurodevelopmental disorder with hypotonia, seizures, and absent language (617268)	

1693	Severe (38)	*HDAC8* (NM_018486.3)	c.675C>G p.(Tyr225*)	XLD /*de novo*	Novel	Pathogenic	Cornelia de Lange syndrome (300882)	
1730	Severe (27)	*RAB11B* (NM_004218.4)	c.202G>A p.(Ala68Thr)	AD/*de novo*	Known	Pathogenic	Neurodevelopmental disorder with ataxic gait, absent speech, and decreased cortical white matter (617807)	
1775	Unclassified	*KMT2D* (NM_003482.4)	c.4262G>T p.(Gly1421Val)	AD/*de novo*	Novel	Likely Pathogenic	Kabuki syndrome (147920)	
1785	Mild	*EP300* (NM_001429.4)	c.3857A>G p.(Asn1286Ser)	AD/*de novo*	Known	Likely Pathogenic	Rubinstein-Taybi syndrome (613684)	
1788	Mild (68)	*CTCF* (NM_006565.4)	c.1699C>T p.(Arg567Trp)	AD/*de novo*	Known	Pathogenic	Mental retardation, autosomal dominant (615502)	
1799	Moderate	*SPTBN2* (NM_006946.4)	c.541G>C p.(Ala181Pro)	AD/*de novo*	Novel	Likely Pathogenic	Spinocerebellar ataxia (600224)	
1928	Severe	*BRAF* (NM_004333.6)	c.1593G>T p.(Trp531Cys)	AD/*de novo*	Known	Pathogenic	Noonan syndrome (613706)	
2017	Mild (68)	*KMT2D* (NM_003482.4)	c.12448C>T p.(Gln4150*)	AD/*de novo*	Novel	Pathogenic	Kabuki syndrome (147920)	
2040	Unclassified	*SYNGAP1* (NM_006772.3)	c.403C>T p.(Arg135*)	AD/*de novo*	Known	Pathogenic	Mental retardation, autosomal dominant (612621)	
2043	Moderate	*POLR3A* (NM_007055.4)	c.1771-6C>G p.(Pro591Metfs*9)	AR/maternal	Known	Pathogenic	Leukodystrophy, hypomyelinating, 7, with or without oligodontia and/or hypogonadotropic hypogonadism (607694)	24
			c.791C>T p.(Pro264Leu)	AR/paternal	Known	Likely Pathogenic		
2120	Mild (54)	*NSD1* (NM_172349.2)	c.4340-2A>G	AD/*de novo*	Novel	Pathogenic	Sotos syndrome (117550)	
2123	Profound (20)	*PPP2R5D* (NM_006245.4)	c.592G>A p.(Glu198Lys)	AD/*de novo*	Known	Pathogenic	Mental retardation, autosomal dominant (616355)	
2177	Unclassified	*ARID1B* (NM_017519.3)	c.2528C>A p.(Ser843*)	AD/*de novo*	Novel	Pathogenic	Coffin-Siris syndrome (135900)	
2181	Profound (19)	*AP4S1* (NM_007077.4)	c.289C>T p.(Arg97*)	AR/paternal, maternal	Known	Pathogenic	Spastic paraplegia (614067)	
2224	Unclassified	*STXBP1* (NM_003165.6)	c.1439C>T p.(Pro480Leu)	AD/*de novo*	Known	Pathogenic	Epileptic encephalopathy, early infantile (612164)	

2235	Profound	*TOP2B* (NM_001330700.1)	c.187C>T p.(His63Tyr)	AD/*de novo*	Novel	NA	NA	22
2306	Unclassified	*SMARCA2* (NM_003070.5)	c.3610T>G p.(Phe1204Val)	AD/*de novo*	Novel	Likely Pathogenic	Nicolaides-Baraitser syndrome (601358)	
2386	Profound (17)	*CDKN1C* (NM_000076.2)	c.209C>T p.(Pro70Leu)	AD/maternal	Known	Likely Pathogenic	Beckwith-Wiedemann syndrome (130650)	
		DNM1L (NM_012062.5)	c.2072A>G p.(Tyr691Cys)	AD/*de novo*	Known	Likely Pathogenic	Encephalopathy, lethal, due to defective mitochondrial peroxisomal fission (614388)	
2409	Unclassified	*RBMX* (NM_002139.4)	c.1063dup p.(Arg355Lysfs*8)	XLR /maternal	Novel	Pathogenic	Mental retardation, X-linked, syndromic 11, Shashi type (300238)	
2539	Unclassified	*ACTA2* (NM_001141945.2)	c.536G>A p.(Arg179His)	AD/*de novo*	Known	Pathogenic	Multisystemic smooth muscle dysfunction syndrome (613834)	
2658	Profound	*ASXL1* (NM_015338.6)	c.2415dup p.(Thr806Hisfs*16)	AD/*de novo*	Novel	Pathogenic	Bohring-Opitz syndrome (605039)	
2691	Mild (50)	*CSNK2B* (NM_001320.7)	c.94G>A p.(Asp32Asn)	AD/*de novo*	Novel	Likely Pathogenic	Poirier-Bienvenu neurodevelopmental syndrome (618732)	

AD, autosomal dominant; AR, autosomal recessive; Class, American College of Medical Genetics and Genomics classification[20]; C Het, compound heterozygous; DD, developmental delay; DQ, developmental quotient; Hemi, hemizygous; Hom, homozygous; ID, intellectual disability; IQ, intelligence quotient; NA, not assessed or not available; XLD, X-linked dominant; XLR, X-linked recessive.

Table 3. Summary of copy number variants identified by whole-exome sequencing.

Subject	DD/ID (DQ/IQ)	Chromosomal location	Type	Start	End	Length (bp)	Protein coding genes	Inheritance	Class	OMIM Phenotype (MIM#)
1512	Severe (27)	6q22.1-q22.31	deletion	117,046,930	119,346,836	2,299,906	15	*de novo*	Pathogenic	
1665	Moderate (39)	10p12.1	deletion	25,010,650	28,824,690	3,814,040	25	*de novo*	Pathogenic	Chromosome 10p12-p11 deletion syndrome (61670)
2117	Moderate (45)	12p13.33-p13.32	deletion	208,283	4,645,313	4,437,030	42	*de novo*	Pathogenic	
2707	Severe (31)	17p11.2	deletion	16,960,879	18,315,050	1,354,171	24	*de novo*	Pathogenic	Smith-Magenis syndrome (18229)

Class, American College of Medical Genetics and Genomics classification[21]; DD, developmental delay; DQ, developmental quotient; ID, intellectual disability; IQ, intelligence quotient.

Table S4. Causative genes identified in cases with external ear anomalies.

Subject	Gene	Gene Description	Function	Functional group
294†	*ZBTB7A*	Zinc finger and BTB domain containing 7A	Transcriptional repressor	GER
454	*ANKRD11*	Ankyrin repeat domain 11	Inhibiting ligand-dependent activation of transcriptio	GER
748†	*KMT2A*	Lysine (K)-specific methyltransferase 2A	Responsible for H3K4 methyltransferase activity	GER
792†	*BRAF*	v-raf murine sarcoma viral oncogene homolog B1	Regulating the MAP kinase/ERK signaling pathway,	NC
832	*KMT2A*	Lysine (K)-specific methyltransferase 2A	Responsible for H3K4 methyltransferase activity	GER
975	*DYRK1A*	Dual specificity tyrosine-phosphorylation-regulated k	Signaling protein	NC
1108	*DHX30*	DExH-box helicase 30	RNA-dependent RNA helicase	other
1386†	*ARID1B*	AT rich interactive domain 1B	Histone demethylase that demethylates Lys4 of histone H3	GER
1411†	*EEF1A2*	Eukaryotic translation elongation factor 1 alpha 2	Responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome.	other
1665†	10p12.1 deletion			
1775	*KMT2D*	Lysine methyltransferase 2D	Histone methyltransferase that methylates the H3K4	GER
1785	*EP300*	E1A binding protein p300	Histone acetyltransferase that regulates transcription	GER
2017	*KMT2D*	Lysine methyltransferase 2D	Histone methyltransferase that methylates the H3K4	GER
2306	*SMARCA2*	SWI/SNF related, matrix associated, actin dependent	Part of the large ATP-dependent chromatin remodelir	GER
2409	*RBMX*	RNA binding motif protein X-linked	Splicing control, transcription and genome integrity	GER
2658	*ASXL1*	ASXL transcriptional regulator 1	Chromatin-binding protein required for normal deterr	GER
2691	*CSNK2B*	Casein kinase 2 beta	Serine threonine kinase ubiquitously expressed in eukaryotic cells and involved in various cellular processes	other
2707†	17p11.2 del			

GER, Gene expression regulation (including chromatin regulators and transcription factors); NC, neuronal communication (synaptic function).

†, Cases with findings of both autism spectrum disorder and external ear anomalies.