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Trio-WES with CNV-seq for Genetic Diagnosis of DD/ID in Children: A Single-Center Study in China

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Abstract

Background: Developmental delay/intellectual disability (DD/ID) is characterized by genetic heterogeneity. Early and accurate diagnosis that identifies pathogens is crucial for providing targeted clinical treatment.

Methods: To evaluate the utility of gene sequencing and explore the potential molecular pathogenesis of DD/ID, we retrospectively analyzed the gene sequencing results of 106 patients who underwent trio whole-exome sequencing (trio-WES) at our hospital. We also conducted bioinformatics analysis to elucidate the pathogenesis of DD/ID and identify therapeutic targets.

Results: We found that the positive detection rate of trio-WES combined with copy number variation sequencing (CNV-seq) was nearly 10% greater than that of trio-WES alone (61.3% vs. 50.0%), and 36 previously unreported variants associated with DD/ID were reported. Additionally, bioinformatics analysis indicated that the cellular response to nitrogen compounds and the metabolism of sulfur compounds in cells play significant roles in the pathogenesis of DD/ID.

Conclusions: In conclusion, the results of this study suggest that trio-WES combined with CNV-seq has significant clinical application value in the etiological diagnosis and clinical management of DD/ID in children. We discovered new associations between gene variations and clinical phenotypes, expanding the mutation spectrum of DD/ID. Our results also suggest that the levels of nitrogen and sulfur compounds may be associated with DD/ID, which might open new directions for DD/ID research.

Introduction

Neurodevelopmental disorders (NDDs) are chronic conditions that affect the functioning of the central nervous system (CNS) during the development of motor skills, cognition, communication and behavioral domains; these conditions may involve a range of dysfunctions, such as developmental delay and regression, ID, autism and attention-deficit/hyperactivity disorder (ADHD) [1], and their total prevalence is up to 3% of the general population [2]. The most important NDD is DD/ID, whose etiology is highly heterogeneous; gene mutations and chromosomal variants are among the main pathogenic mechanisms that cause DD/ID [3–5].

Owing to the genetic heterogeneity of DD/ID, clinical diagnosis of its etiology is relatively difficult. The 2010 guidelines of the American College of Medical Genetics and Genomics (ACMG) recommended chromosome microarray (CMA) and fragile X chromosome (FXS) testing as the primary tests for individuals with unexplained GDD/ID and/or ASD [6]. However, the molecular diagnostic yield of CMA in individuals with DD/ID, ASD, and/or multiple congenital anomalies (MCAs) has been reported to be only approximately 15–20% [4, 7]. Owing to the rapid development of next-generation sequencing technologies, particularly the popularity of trio-based (parent–offspring model) whole-exome sequencing (trio-WES) technology, comprehensive genetic analyses capable of detecting causative single-nucleotide variants (SNVs) and CNVs are being performed more frequently than before in individuals with unexplained or idiopathic DD/ID. In addition, CNV-seq, a chromosomal microdeletion/microduplication assay based on WES or WGS data, has an advantage over CMA testing in that it not only detects mutations found in known regions but also may identify new disease-associated causative sites. Trio-WES in combination with CNV-seq can increase the diagnostic rate of DD/ID by 10–40% [8–10]. Despite significant advancements in genetic testing and biomedical technology, the complex heterogeneity and diagnostic challenges associated with DD/ID persist.

Although there have been investigations into the diagnostic yield of combined trio-WES and CNV analysis versus either test alone in DD/ID cohorts, as mentioned above, studies report conflicting findings regarding the extent of the additional diagnostic gain. In addition, progress in the etiological study of DD/ID has been relatively slow, with most attention focused on analyzing the pathogenicity of individual genes, and there is little in-depth research based on analyzing clinical phenotypes. The aim of this study was to evaluate the utility of trio-WES in combination with CNV-seq in pediatric DD/ID patients and to explore the underlying molecular pathogenesis of DD/ID as well as targets for early intervention.

Methods

Case collection

A total of 106 unexplained familial cases of DD/ID in patients attending the First Affiliated Hospital of Zhengzhou University between January 2017 and June 2024 were included. The diagnostic criteria for DD were defined using the Diagnostic and Statistical Manual of Mental Disorders (5th edition). The Gesell Developmental Schedules (for children aged < 5 years) and the Wechsler Intelligence Scale for Children (for children aged ≥ 5 years) were used to diagnose DD/ID. Autism spectrum disorder (ASD) and attention-deficit/hyperactivity disorder (ADHD) were clinically diagnosed according to the DSM-5 diagnostic criteria for ASD and ADHD, along with other clinical assessments such as the Autism Behavior Checklist, Childhood Autism Rating Scale, or Modified Checklist for Autism in Toddlers. The International League Against Epilepsy criteria were used to diagnose epilepsy (EP), epilepsy syndromes, and epileptic encephalopathy. (2) Written informed consent was obtained from the legal guardians of the children for data collection and trio-WES or trio-WES combined with CNV-seq sequencing. The exclusion criteria eliminated the following groups: (1) children with DD/ID caused by conditions such as cerebral palsy; (2) children with acquired neurological injury from definite etiologies such as encephalitis, traumatic brain injury, or cerebral hemorrhage; (3) children with incomplete clinical data; and (4) children with low-quality blood samples or substandard next-generation sequencing data. This study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (2024-KY-1873-001), and written informed consent was obtained from each participant or their guardian(s). All families were got appropriate genetic counseling in our outpatient, and some families went to the genetic specialist outpatient department of our hospital for further detailed genetic counseling.

Trio-WES sequencing

Two milliliters of peripheral venous blood was collected from the probands and their parents. Exons were enriched using the SeqCap EZ MedExome Kit (Roche NimbleGen) and SureSelect Human All Exon V6 (Agilent, USA), after which they were sequenced on the Illumina X10 platform (2×150 nucleotide paired-end reads) by Berry Genomics and Chigene Company. Reads were aligned to the UCSC human reference genome (hg19) using the Burrows–Wheeler Aligner (BWA). Variants were called with GATK HaplotypeCaller v3.7 and annotated using the Variant Effect Predictor (VEP). For splice variants, two prediction tools—Human Splice Finder (HSF, http://www.umd.be/HSF3/) and Splice Site Prediction (SSP, http://www.fruitfly.org/seq_tools/splice.html)—were applied to assess the impact of the variants on the transcripts. For each patient, variants were filtered and prioritized according to an internal bioinformatics workflow. Variant classification followed the guidelines recommended by the American College of Medical Genetics and Genomics (ACMG). All candidate variants were validated by Sanger sequencing.

CNV-seq

For CNV-seq, genomic DNA was fragmented into 200–300 bp fragments using the TruSeq Library Preparation Kit. Sequencing was performed on the Illumina HiSeq 2500 platform (Illumina, San Diego, United States), and the raw data were processed by the base-calling analysis software fastq v0.18.1. The sequencing results were subsequently aligned to the human reference genome sequence (hg19). Potential copy number variations (CNVs) identified by WES and CNV-seq were annotated by comparing the CNVs and associated genes against databases including DECIPHER, ClinVar, ClinGen, and OMIM. Candidate CNVs were filtered using normal population frequency databases (DGV and ISCA). CNVs were interpreted on the basis of annotation information and frequency databases according to previously reported standards and guidelines from the American College of Medical Genetics and Genomics (ACMG). Pathogenicity screening was conducted using databases such as OMIM, DECIPHER, and Orphanet.

Statistical analysis

SPSS 26.0 statistical software was used for data processing. The Kolmogorov–Smirnov test was used to test whether the data were normally distributed. Measurement data conforming to a normal distribution are expressed as ± s, and comparisons between groups were made via the t test for two independent samples; nonnormally distributed measurement data are expressed as M (Q 1, Q 3), and intergroup comparisons were made via the Mann–Whitney U test. Count data are expressed as cases (%), and the χ^2 test or two-tailed Fisher's exact test was applied to the clinical data of patients with and without positive exome results. The ratios of categorical variables (ORs) were calculated. A two-sided $P < 0.05$ was considered to indicate statistical significance.

Results

General information

A total of 106 families with DD/ID probands were included in this study, all included families strictly adhered to the defined inclusion and exclusion criteria. The workflow of the study is shown in Fig. 1. A total of 58.5% (n = 62) of the patients were males, with a male/female ratio of 1.41 (62/44), and all the children were born to parents who were not related. The clinical characteristics of the probands were defined by the Human Phenotype Ontology (HPO: http://www.human-phenotype-ontology.org) and ChinaHPO (http://www.chinahpo.org). Epilepsy (50/106) was the most common symptom co-occurring with developmental delay. In addition to an affected nervous system, other frequently observed systemic abnormalities (found in ≥ 3 affected individuals) included craniofacial abnormalities (12), organ abnormalities (11), muscular hypotonia/hypertonia (9), abnormal hormone levels (9), eye movement abnormalities (6), malnutrition (6), dysaudia (4) and skin abnormalities (4).

Among the 106 probands included, 74 children who received trio-WES, 1 child who received trio-WES combined with *MECP2* MLPA and 31 children who received trio-WES combined with CNV-seq analysis were included. There were 57 families with positive gene test results, with a total positive detection rate of 53.7%. In the trio-WES pedigrees, 37 patients had positive results, with a positive detection rate of 50.0%. In the analysis group that underwent trio-WES combined with CNV-seq, 19 patients had positive results, with a diagnostic detection rate of 61.3%. Across all the positive families, there were 31 male and 25 female probands.

Correlation of clinical phenotypes with genotypes

We investigated the associations between clinical features and identified genetic variants to determine whether some clinical phenotypes were more likely to suggest a genetic origin. Unfortunately, we did not find significant associations with sex, age, family history, skin abnormalities, hearing disorders, malnutrition, oculomotor abnormalities, hypotonia, hypertonia, craniofacial abnormalities, organ abnormalities, autism spectrum disorders, or epilepsy in pre-diagnostic patients (Table 1). Notably, DD in patients younger than 12 months (19 positive and 10 negative patients vs. 37 positive and 40 negative patients; $P = 0.130$) was associated with an increased risk of harboring genetic variants.

Table 1
Comparison of genetic testing results across various clinical phenotypes.

Characteristic	No. of test results		*P* value((Fisher exact test))
	Positive	Negative	
Sex			
Male	31	31	
Female	25	19	0.556
Age group, years			
0<Age ≤ 1	19	10	
1<Age ≤ 2	4	7	
2<Age ≤ 5	18	23	
5<Age ≤ 12	13	8	
12<Age ≤ 18	2	2	0.219
Family history of DD/ID			
Yes	8	7	
No	48	43	>0.999
Brain MRI abnormalities			
Yes	7	9	
No	49	41	0.588
Epilepsy			
Yes	25	25	
No	31	25	0.697
ASD			
Yes	4	5	
No	52	45	0.732
Eye movement abnormalities			
Yes	4	2	
No	52	48	0.682
Skin abnormalities			
Yes	2	2	
No	54	48	>0.999
Craniofacial			
Yes	5	7	
No	51	43	0.542
Muscular hypotonia/hypertonia			
Yes	6	3	
No	50	47	0.495
Organ abnormalities			
Yes	6	5	
No	50	45	>0.999
Dysaudia			
Yes	2	2	
No	55	47	>0.999
Dyshormonia			

Characteristic	No. of test results		*P* value((Fisher exact test))
	Positive	Negative	
Yes	7	2	
No	49	48	0.167
Malnutrition			
Yes	4	2	
No	52	48	0.681

Positive results indicate the detection of clinically relevant variants by trio whole-exome sequencing or copy number variation sequencing. In contrast, negative results from trio whole-exome sequencing and copy number variation sequencing indicate the absence of clinically relevant variants.

Genetic spectrum of diagnostic variants

Ten chromosomal microdeletions (10/56) were detected across 56 positive families (Table 2). A total of 10 CNVs, including 1 duplication and 9 heterozygous deletions, were identified. These 10 CNVs ranged in size from 0.32 Mb to 38.6 Mb, of which 2 (20%) CNVs were smaller than 1 Mb and 8 (80%) CNVs were larger than 1 Mb (Table 2). All of the diagnostic CNVs were validated via CNV-seq or RT–qPCR. CNVs located on chromosomes 4, 5, 11, 14, 15, 16, 17, 19, and 21 were identified in 1, 1, 1, 1, 1, 1, 2, 1, and 1 patient, respectively. The 10 CNVs were all classified as pathogenic on the basis of the ACMG guidelines and the DECIPHER database. Among these 10 CNVs, 9 (90%) were de novo, and 1 (10%) was inherited from an unaffected father along with Prader–Willi syndrome (OMIM:176270).

Table 2
Clinical and mutational data of 10 microdeletions/microduplications identified in the cohort.

Patients ID	Sex	Age	Phenotypes	Family history	Position (hg19)	Size	Inheritance /zygosity	ACMG
1	F	10m22d	Developmental delay, hypotonia, congenital heart disease and craniofacial abnormalities	Negative	17p12-p11.2 del	4.64Mb	De novo	P
2	F	1y	Developmental delay, hypotonia and epilepsy	Negative	16p11.2 del	0.64Mb	De novo	P
3	F	7y	Global developmental delay, congenital pyloric stenosis, visual impairment, severe pneumonia	Negative	11q24.1-q25 del	13.68Mb	De novo	P
4	F	1y2m	Developmental delay and epilepsy	Negative	14q12 del	2.35Mb	De novo	P
5	M	5y	Developmental delay	Negative	4p15.33-p15.1 del	19.55Mb	De novo	P
6	M	3y6m	Developmental delay and funnel chest	Negative	17p11.2 del	3.46Mb	De novo	P
7	F	6y6m	Developmental delay, deafness and craniofacial abnormalities	Negative	19p13.12 del	0.32Mb	De novo	P
8	F	4y	Developmental delay and fat	Negative	15q11.2-q13 del	5.20Mb	Paternal	P
9	F	7 m	Developmental delay and epilepsy	Negative	5q14.3 del	2.72Mb	De novo	P
10	F	6 m	Global developmental delay, severe pneumonia and congenital heart disease.	Negative	21p11.2-q22.3 dup	38.6Mb	De novo	P

F: female; M: male; y: years old; m: months; P: pathogenic; del: deletions; dup: duplications.

Five of the remaining 46 prevalent cases carried two disease-causing gene variants each (Table 3), involving a total of 68 variants corresponding to 46 Mendelian diseases identified and verified by Sanger sequencing. The recurrent genes included *MMACHC* (2 patients), *GABRB3* (3 patients), *SCN2A* (3 patients), the *SCN1A* gene (2 patients), *CACNA1A* (2 patients), *SCN8A* (2 patients), *KCNQ2* (2 patients) and *SLC6A8* (2 patients) (Fig. 2). Among the 65 DD/ID-associated variants (except for 3 variants of *KIF2A1*, *SMN1* and *CHRNE*, these genes have not been previously reported to be related to DD/ID), 44.6% (29/65) of the 65 DD/ID-associated variants have already been reported, and 55.4% (36/65) of the variants have not been previously reported (Table 4). A total of 47.7% (31/65) of the variants were de novo mutations. Disorders with autosomal dominant (AD), autosomal recessive (AR), X-linked dominant (XLD), and X-linked recessive (XLR) inheritance were diagnosed in 25, 14, 1, and 6 patients, respectively, and in 1 patient with mitochondrial inheritance. Among these 65 variants, 26 (40.0%) were de novo AD or XLD variants, 38 (58.5%) had biparental or maternal inheritance with XLR or AR inheritance, and 1 was a mitochondrial mutation (*ND5* gene). Among patients with these variants, 5 probands carried 2 pathogenic mutated genes each.

Table 3
Clinical and mutational data of 5 patients carrying 2 disease-causing mutated genes

Patient ID	Sex	Age	Phenotypes	Family history	Mutated gene 1	Variants 1	Mode	Inheritance/zygosity	Mutated gene 2	Variants 2
13	M	3 m	Developmental delay, hypothyroidism, congenital heart defects, hearing impairment, and enlarged liver.	Negative	GPC3	c.1358del: p.K453Rfs*14	XLR	Maternal/ Hemizygous	SMN1	Exon7 del
23	M	1y1m	Global developmental delay, pneumonia and nephritis	His uncle has nephritis.	HERC2	c.8537C > T (p.P2846L)/ c.13963C > T (p.R4655C)	AR	Paternal/Maternal Compound heterozygous	GRIN1	c.1805C > T (p.T602I)/ c.1468_1489d (p. V490Sfs*5
26	M	2y5m	Global developmental delay and squint	Negative	NAA15	c.244 + 47C > G	AD	De novo Heterozygous	KIF2A1	c.3152T > C (p.V1051A)
32	M	8y6m	Global developmental delay	His brother also has developmental delay	THOC2	c.836T > C (p.I279T)	XLR	Maternal/ Hemizygous	SHANK2	c.2414G > A (p.C805Y)
33	M	7m22d	Developmental delay, malnutrition, epilepsy and hypotonia	Negative	MVK	c.197delG (p.R66Sfs*26)/ c.655G > A (p.G219R)	AR	Paternal/Maternal Compound heterozygous	CHRNE	Exon7-8 del

F: female; M: male; y: years old; m: months; d: days; AR: autosomal recessive inheritance; AD: autosomal dominant inheritance; XLR: X-linked recessive inheritance; XLD: X-linked dominant inheritance.

Table 4
Summary of clinical and mutation data for 19 proband families with 36 novel DD/ID-related va

Patient ID	Sex	Age	Phenotypes	Family history	Mutated gene 1	Variants 1	Mod
11	F	2y	Episodic limb dysfunction, and DD	Negative	DNM1L	c.2161C > T (p.Q721*)	AD
12	M	2y	DD, epilepsy	Negative	GLB1	c.1769G > A (p.R590H) c.734-8A > G	AR
13	M	3 m	DD, hypothyroidism, precardiosis, deafness, liver enlargement	Negative	GPC3	c.1358del (p.K453Rfs*14)	XLR
14	F	11y	ID, epilepsy	Negative	SCN2A	c.180del (p.K60Nfs*33)	AD
15	M	13y	ID, epilepsy	Negative	SLC6A8	c.1136_1137del (p.E379Vfs*85)	XLR
16	M	13y	ID, left strabismus	Negative	SOX5	c.1586del (p.G529Efs*28)	AD
17	M	7y	ID	Negative	TSC2	c.3718C > T (p.R1240C)	AD
18	F	3m13d	DD, pneumonia, ventricular septal defect, malnutrition, nystagmus.	Mother has ID and nystagmus	ARID1B	c.2371 + 1G > C	AD
19	F	5 m	DD, feeding difficulties, pneumonia	Negative	ASXL3	c.1494del (p.L499Wfs*6)	AD
20	M	3y9m	DD, epilepsy	Negative	CACNA1A	c.5408A > G (p.N1803S)	AD
21	F	7y10m	ID, epilepsy	Negative	GRIN2D	c.2511C > G (p.S837R)	AD
22	M	1y1m	DD, pneumonia, nephritis	Negative	HERC2	c.8537C > T (p.P2846L) c.13963C > T (p.R4655C)	AR
22	M	1y1m	DD, pneumonia, nephritis	Negative	GRIN1	c.1805C > T(p.T602I) c.1468_1489del:(p.V490Sfs*5)	AR
23	M	7y5m	ID, ASD	Elder sister has ID	HGSNAT	c.819T > G (N273K) c.1277G > A (G426E)	AR
24	M	7m17d	DD, epilepsy	Negative	ITPR1	c.1655A > G (p.Y552C)	AD

Patient ID	Sex	Age	Phenotypes	Family history	Mutated gene 1	Variants 1	Mod
25	M	2y5m	DD, strabismus	Negative	NAA15	c.244 + 47C > G	AD
26	F	1y2m	DD, ASD, epilepsy	Negative	SCN2A	c.3084del (p.F1029Lfs*8)	AD
27	M	4 m	DD, epilepsy	Negative	SCN8A	c.3946G > A (p.V1316M)	AD
28	M	6y	ID, epilepsy	Negative	SLC6A8	c.1082_1088dup (p.G364Hfs*103)	XLR
29	M	2y8m	DD	Elder brother has DD	UPF3B	c.1121del (p.R374Lfs*20)	XLR
30	M	7 m	DD, pneumonia, microcephaly	Negative	VPS13B	c.6940 + 1G > T c.6780del (p.E2260Dfs*57)	AR
31	M	8y6m	ID	Younger brother has DD, anut and grandmother has insanity	THOC2	c.836T > C (p.I279T)	XLR
31	M	8y6m	ID	Younger brother has DD, anut and grandmother has insanity	SHANK2	c.2414G > A (p.C805Y)	AD
32	M	1 m	DD, epilepsy, malnutrition, hypotonia	Negative	MVK	c.197delG (p.R66Sfs*26) c.655G > A (p.G219R)	AR
33	M	3m15d	DD, epilepsy, coma, poor spontaneous breathing	Negative	HTRA2	c.844_c.845delGC (p.A282Qfs*16) c.510C > G (p.H170Q) c.754C > T (p.R252W)	AR
34	□	4y	DD, epilepsy	Negative	GABRB3	c.323T > G (p.L108R)	AD
35	□	9 m	DD, epilepsy	Negative	PCLO	c. 10348G > A(p.E3450K) c.1124_c.1125delAGinsCTCTTGGTCCTGCTAAGCCTCCAGCTCAGCAC	AR
36	□	4 m	DD, epilepsy, malnutrition,	Negative	SCN1A	c.796_c.797delCTinsG (p.L266Gfs*8)	AD
37	M	6y7m	ID, epilepsy, craniofacial abnormalities	Negative	CSNK2A1	c.28A > T(p.R10*)	AD
38	F	2y8m	DD	Negative	MMACHC	c.445_c.446delTG(p.C149fs*32) c.80A > G(p.Q27R)	AR

Patient ID	Sex	Age	Phenotypes	Family history	Mutated gene 1	Variants 1	Mod
39	M	3 m	DD, epilepsy	Negative	GABRB3	c.757C > T (Pro253Ser)	AD

F: female; M: male; y: years old; m: months; d: days; AR: autosomal recessive inheritance; AD: autosomal dominant inheritance; XLR: X-linked recessive inheritance; XLD: X-linked dominant inheritance. DD: developmental delay; ID: intellectual disability.

Associations among Genetic Factors and Clinical Phenotypes

We performed enrichment analysis of the positive genetic test results in the DD/ID cohort, and the GO enrichment results were significant at an adjusted $P < 0.01$ (Fig. 3). In this analysis, the genes involved in the synaptic signaling pathway had the greatest importance, with genes such as *CACNA1A*, *GABRB3*, *GRIN1*, *GRIN2D*, *ITPR1*, *KCNQ2*, *MECP2*, *STXBP1*, *PCLO*, and *PRRT2* possibly being involved. Other involved functions were related to ion channel transport, brain development and the cell response to nitrides. Among these functions, the cellular response to nitrogen compounds and sulfur compound metabolism may play important roles in developmental disorders of the nervous system and the pathogenesis and progression of DD/ID.

Discussion

Genetic factors play important roles in the pathogenesis of DD/ID[11, 21]. At present, analyses and precise treatments for DD/ID biomarkers are lacking; therefore, appropriate genetic testing and comprehensive assessment are essential for understanding the etiology of DD/ID and developing intervention strategies. The main genetic causes of DD/ID are associated with single-nucleotide variations (SNVs), insertions/deletions, and CNVs[12]. Advances in next-generation sequencing technologies have demonstrated the ability to detect genetic material across the entire genome, reducing diagnostic errors and improving diagnosis rates for patients with DD/ID. However, as mentioned above, various genetic testing methods still differ in their detection rates of genetic causes of DD/ID[8–10]. Compared with proband-only WES, trio-WES enhances the identification of de novo variations and structural rearrangements in the DNA coding region and results in a 12% increase in diagnostic rates[13, 14]. In addition, CNV-seq surpasses the limitations of traditional chromosomal microarrays in detecting CNVs across the genome and identifying new CNVs. Despite these advances, relatively few studies have reported the use of trio-WES combined with CNV-seq for the diagnosis of DD/ID in children.

The integration of genetic testing has significantly improved diagnostic rates, reduced missed diagnoses, and clarified indications for testing[15, 21]. In this study, trio-WES combined with CNV-seq in children with DD/ID yielded a positive detection rate of 61.3%. This combined approach was a significant improvement over trio-WES alone, increasing the positive detection rate by nearly 10%. Although the increased diagnostic rate in this study is similar to that reported in previous literature, the high diagnostic rate of trio-WES in this study can be attributed to the small size of our cohort and the strictly selected DD/ID patients. This approach not only saves families time but also reduces the financial costs that might arise from trying different testing methods. Owing to technical limitations, trio-WES may miss 55% of CNVs, while the missed detection rate of CNV-seq is only 3% [16]. CNV-seq cannot detect rare balanced translocations, cannot determine the exact mechanism of a gain or loss, and cannot detect single-gene-level deletions or duplications. The continuous development of whole-exome sequencing and genomic sequencing analysis, as well as the identification of copy number variants from these data via new algorithms, may overcome these limitations [17]. In summary, the combination of trio-WES and CNV-seq detection addresses the limitations of each method, thereby achieving a more comprehensive genetic analysis and reducing the rate of missed detection. Finally, the phenotype of DD/ID is clinically heterogeneous, and identifying the cause is relatively difficult. In addition, with the development of the social economy and genetics, understanding of DD/ID genetic factors continues to improve, and patient demand for genetic testing is constantly expanding to new disease risk factors. We believe that the indications for genetic testing should be appropriately expanded. Previous studies have shown correlations between the rates of positive genetic tests and factors such as comorbid ASD, positive family history, complex phenotypes, moderate- or high-severity developmental disorders, and craniofacial abnormalities [18–22]. In this study, we found that there was no clear indicator of an increase in the positive rate in general pediatric patients without considering the overall degree of development. Additionally, our study revealed a trend suggesting that infants younger than 12 months of age have a higher prevalence of positive genetic test results (19 positive cases vs. 10 negative cases in this age group, compared to 37 positive cases vs. 39 negative cases in older cohorts; $P = 0.130$). Although this finding was not statistically significant, it aligns with emerging evidence implying that early-onset developmental disruptions may be disproportionately influenced by de novo genetic variants, potentially because of heightened genetic vulnerability during rapid synaptogenesis in infancy[23, 24]. The P value (0.130) likely stems from insufficient sample size within the younger subgroup (n = 29 in the cohort under < 12 months of age) or heterogeneity in age classifications, limiting statistical power. This observation underscores the importance of age as a potential modifier of the diagnostic yield of DD/ID, particularly given the predominance of de novo variants in our cohort (e.g., 47.7% of variants were *de novo* mutations) and the critical role of synaptic pathways identified in our gene ontology analysis. Future studies should prioritize larger, prospective cohorts with age stratification to elucidate this association and explore whether early genetic diagnosis is correlated with improved intervention efficacy, addressing the current limitations in long-term follow-up.

Understanding the complex links among genetic variation, brain development, and the phenotype of DD/ID is critical to enhancing our understanding of the underlying mechanisms of DD/ID. The exact mechanisms through which the genome influences cognitive function and brain development are still being studied[25–27]. In previous studies, 36 cases of DD/ID-related gene mutations were first found, which not only broadened the gene mutation spectrum of the disease but also provided an important reference for research on the pathogenesis of DD/ID. Furthermore, we identified five probands carrying mutations in two distinct pathogenic genes, which suggests that clinicians should opt for broad-spectrum sequencing protocols even when a specific genetic disorder is highly suspected, as targeted approaches may carry the risk of missed diagnoses.

The etiological mechanism of DD/ID is still unclear, and some studies in model systems suggest that synaptic development may be involved in its pathogenesis and progression[26]. Some studies suggest that changes in the functional state of nerve cells may be affected by environmental factors, which may lead to abnormal synaptic development. While these insights are derived mainly from studies of specific diseases, such as Down syndrome, fragile X syndrome, and Rett syndrome, with their general applicability to all conditions remaining uncertain, this study revealed that synaptic dysplasia still plays an important role in the pathogenesis of DD/ID. Notably, we also found that the cellular response to nitrogen compounds and the metabolic process of sulfur compounds may play a significant role in the pathogenesis of DD/ID. Nitrogen compounds in the human body can affect the level of oxidative stress in cells. A variety of studies have shown that reactive nitrogen species (RNS) are involved in neurodegenerative diseases, and only a few reports have suggested that RNS might be involved in the progression of DD/ID through activation of the AMPK pathway [28, 29]. Recent studies have shown that the aberrant transfer of nitric oxide (NO) can trigger neuronal bioenergetic compromise and microglial phagocytosis and may contribute to the synapse loss that underlies cognitive decline, which provides an important reference for research on nitrogen compounds in brain development[30]. As for sulfur compounds, some studies have shown that hydrogen sulfide may be an important factor in neuroinflammation [31] and that low and physiological levels of H_2S may improve the clinical phenotype of neurodegenerative diseases by exerting anti-inflammatory and antiapoptotic effects [32]. In addition, H_2S has been shown to protect neurons from secondary neuronal damage by acting as an antioxidant, anti-inflammatory, and antiapoptotic mediator [33–35]. However, some reports indicate that H_2S increases the neuronal Ca^{2+} concentration and may lead to calcium overload in secondary neuronal injury [36–38]. H_2S also triggers calcium waves in primary cultures of astrocytes and may mediate signaling between neurons and glial cells [39]. Recent research has shown that mitochondrial dysfunction in the anterior cingulate cortex (ACC) brain region in a mouse model of autism can cause an increase in H_2S in brain tissue, drive synaptic dysfunction, and induce social disorders, further revealing the impact of sulfur compounds on neuronal function[40]. However, few studies have investigated the occurrence and development of DD/ID. Based on the clinical sequencing results, we hypothesize that intracellular nitride and sulfide levels may be associated with developmental delay/intellectual disability (DD/ID) and aim to provide a potential research direction for future precision medicine targeting ID/DD. However, the accuracy of these findings still requires further validation through basic and clinical experimental studies..

Limitations of the study

This study has several limitations that warrant careful consideration. First, the findings are derived exclusively from a single-center cohort of children with unexplained DD/ID, which may introduce selection bias and limit the generalizability of our results to broader populations, particularly those with diverse demographic or comorbid conditions. Second, the lack of long-term follow-up data represents a critical gap. Without longitudinal monitoring (e.g., tracking neurodevelopmental trajectories or response to intervention), we cannot assess the clinical utility of our diagnostic strategy over time or its impact on patient outcomes, weakening the translational relevance of our high diagnostic yield. Third, we observed a potential signal suggesting that children aged < 12 months had a heightened rate of positive genetic findings (P = 0.130), but this finding lacked statistical significance, likely because of our limited sample size —a condition that restricts our ability to draw definitive conclusions about age as a predictive factor. Fourth, we identified an absence of functional validation of pathogenic variants. We did not perform in vitro or in vivo functional assays. This omission raises the possibility that some putative pathogenic variants lack direct causative links to DD/ID or that their mechanisms remain unverified, particularly for novel gene variants (36 unreported mutations). Future work should prioritize multicenter collaborations and longitudinal cohorts to address these concerns.

Conclusions

In conclusion, this study supports the clinical utility of trio-WES combined with CNV-seq in advancing the etiological diagnosis and clinical management of DD/ID in children. In addition, we identified associations between genetic variation and clinical phenotypes, increasing the mutation spectrum associated with DD/ID. Our results also suggest a possible correlation between the levels of nitrogen and sulfur compounds and DD/ID, which may be a new target for DD/ID research, although further comprehensive research is needed to confirm the efficacy of this approach.

Declarations

Author contributions

Conceptualization, AJC and HLW; resources, PNJ, JDW, ZC, WM, EHW and ZHZ; data curation, YZ, YW and JCN; writing—original draft preparation, AJC and PNJ; review and editing, WW, ZHZ and HLW; funding acquisition, AJC, ZHZ and HLW.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethical approval

Informed consent was obtained from all the patients and parents. Ethical approval was obtained from the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (2024-KY-1873-001).

Consent for publication and consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

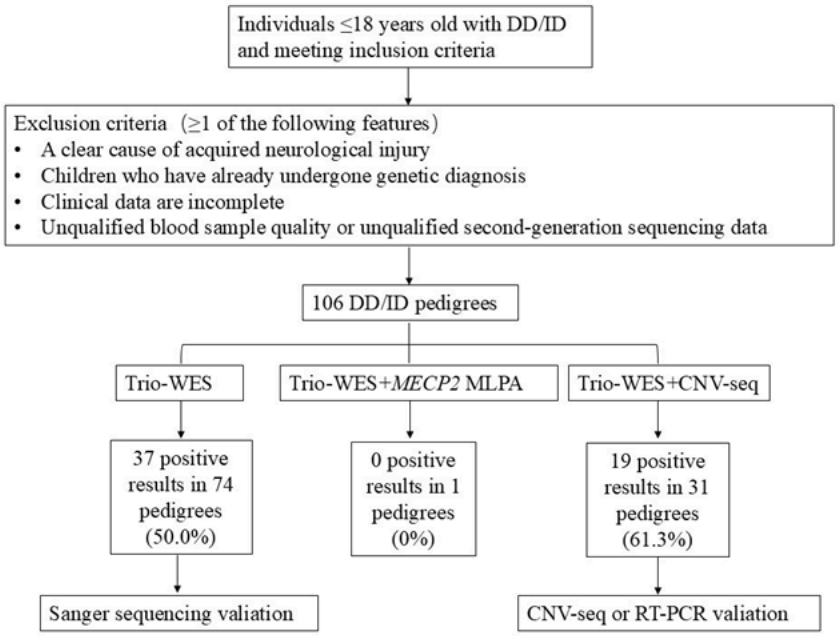


Figure 1

Flow diagram of the study.

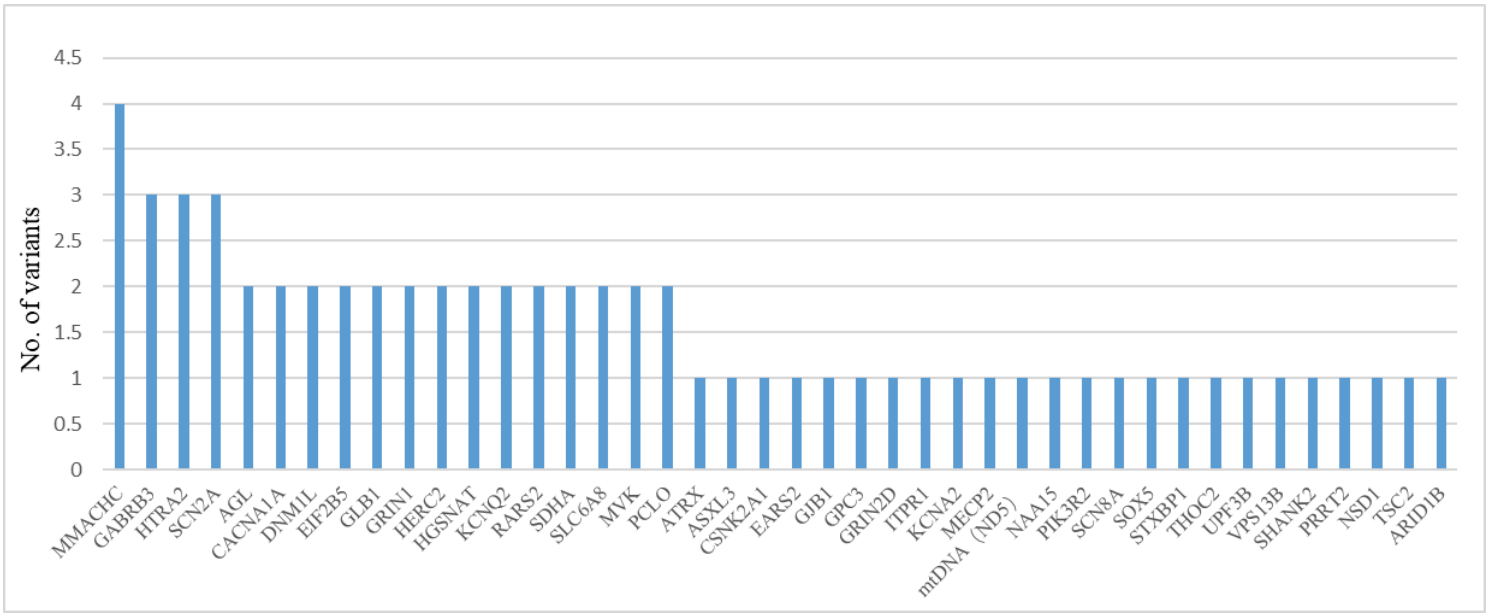


Figure 2

Diagnostic mutations and their variants identified in the cohort.

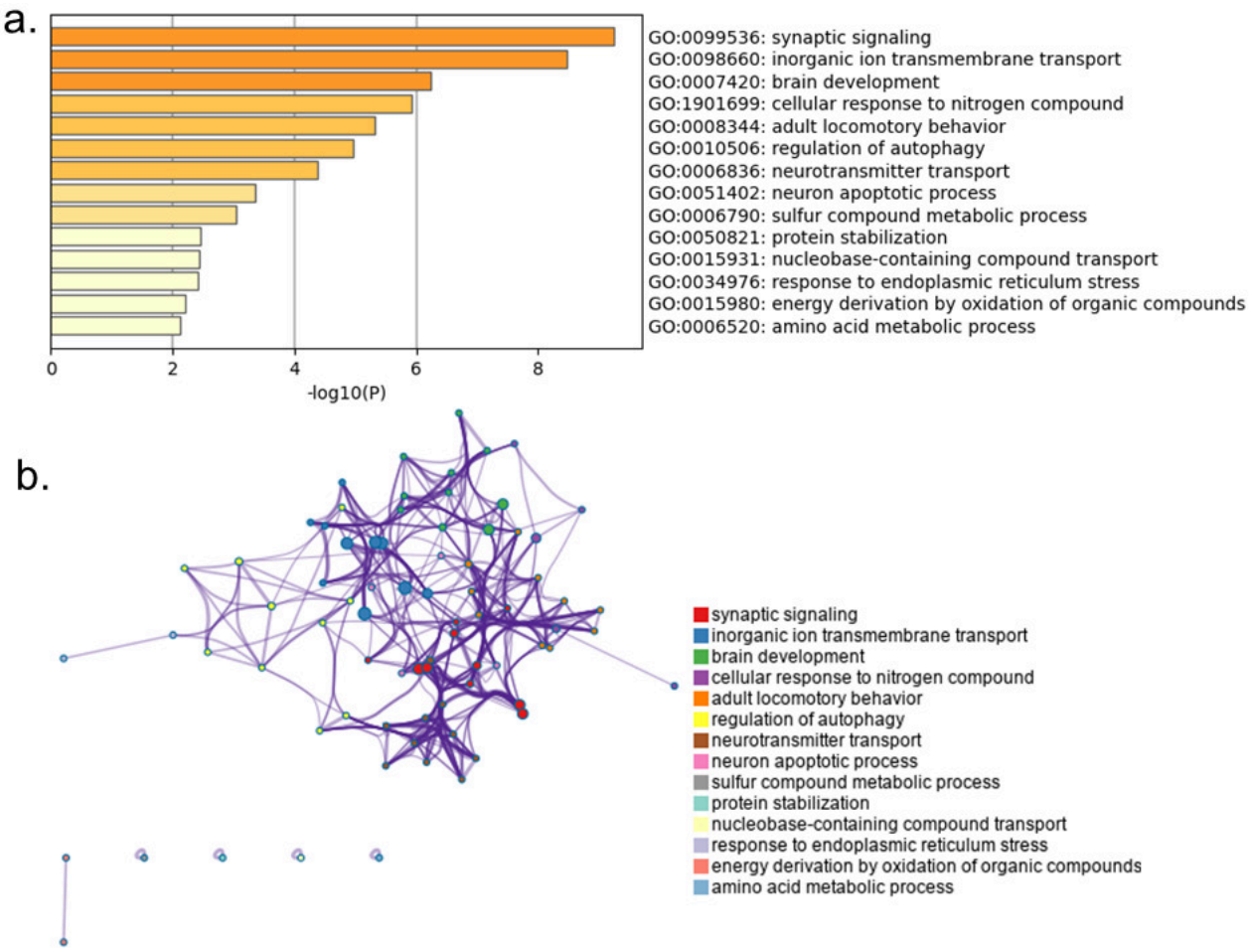


Figure 3

The analysis revealed a diverse and intricate network of pathways associated with the genes in the cohort. The pathways are arranged in descending order of significance, with the *P* value gradually increasing. a. Bar chart of terms enriched in the gene list, colored by p value. b. Nodes colored by cluster ID, where those that share the same cluster ID are usually close to each other.