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Molecular genetic testing and cohort analysis of 32 twin pairs with neurodevelopmental disorders-Reporting a novel de novo variant of *TET3*

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Abstract

Neurodevelopmental disorders (NDDs) pose significant challenges due to their impact on cognitive, social and motor abilities, often rooted in genetic factors such as copy number variations (CNVs) and single nucleotide variantions (SNVs). Molecular genetic testing, advanced due to sequencing technologies, is instrumental in diagnosing NDDs, with twins offering unique perspectives in detecting novel de novo CNVs and SNVs. The study enrolled 32 pairs of twins that underwent molecular genetic testing and comprehensive clinical data collection. Additionally, we analyzed the potential deleterious effects of a novel de novo TET methylcytosine dioxygenase 3 (*TET3*) variant (c.4927G > A) using western blotting, immunofluorescence assay and enzymatic activity assay. Analyzing simultaneously, the overall detection yield of molecular genetic testing was 17.2% (11/64). Children with disease-related genetic variants had lower total developmental quotients (DQ) than children without disease-related genetic variants. One pair of monozygotic twins carried a novel de novo *TET3* variant. Immunostaining assay revealed that while the wildtype TET3 protein was evenly distributed in the nucleus, the variant was concentrated around the nucleus. Anenzymatic assay using corresponding TET2 mutants suggested that the variant has a significantly reduced activity. Taken together, our study elaborated molecular genetic testing results of 32 pairs of twins and found that children with lower developmental levels are prone to possessing identifiable genetic variants. We reported the clinical phenotype of a pair of monozygotic twins carrying a novel de novo *TET3* variant and confirmed the detrimental effects of this variant in vitro.

Keywords Neurodevelopmental disorders, Twin pairs, Molecular genetic testing, Developmental level, *TET3*

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Introduction

Neurodevelopmental disorders (NDDs), such as autism spectrum disorder (ASD), developmental delay (DD) and intellectual developmental disorder (IDD), are a group of early onset disorders impairing cognitive, social and motor abilities [1]. The incidence of NDDs, notably ASD affecting approximately 2.76% and IDD 1% of the population, underscores their significant societal impact [2]. The latest epidemiological research reports that the incidence of ASD is 2.3% and the incidence of IDD is 1.2% in Chinese children and adolescents [3]. Despite their prevalence, the etiology of NDDs remains complex, with genetic factors playing a prominent role. The common genetic causes of NDDs include chromosomal aberrations, copy number variations (CNVs), and single nucleotide variations (SNVs), however, currently chromosomal aberrations can mostly be detected by prenatal diagnosis. Therefore, the use of molecular genetic testing to detect CNVs and SNVs in patients with NDDs is particularly important for the etiological diagnosis [4, 5]. Recent advancements in sequencing technologies have facilitated the identification of various gene variants associated with NDDs, particularly ASD [6, 7]. Research reports on ASD twins began in 1977, and for the first time identified the important role of genetic factors in the pathogenesis of ASD twins [8]. ASD twins have since been extensively studied to dissect the contributions of genetic or environment factors to ASD. However, few studies have comprehensively evaluated the role of molecular genetic testing in detecting CNVs or SNVs in twins with NDDs while detailed analyses of genetic diagnoses, genetic risk, genotype, and genotype-phenotype association within twin cohorts. In this study, we elaborated on the demographics of 32 pairs of twins referred to the Children's Hospital of Fudan University for clinical genetics evaluation of ASD, DD/IDD. We reported the yield and specific findings of molecular genetic testing in the twin pairs. Besides, we analyzed the effects of genetic diagnoses and genetic risk on the development quotient (DQ) of the Griffiths Mental Development Scales (GMDS), as well as genotype and genotype-clinical phenotype association of the cohort. Importantly, we reported a novel de novo variant (c.4927G > A) of Tet methylcytosine dioxygenase 3 (*TET3*) in one pair of twins and confirmed the detrimental effects of this variant in vitro.

Materials and methods

Study subjects

The study included 32 twin pairs diagnosed with ASD/DD/IDD at the Department of Child Healthcare, Children's Hospital of Fudan University, from March 2016 to July 2022. Inclusion criteria for the twins were as follows: at least one of the twins has ASD/DD/IDD. Diagnosis of ASD/DD/IDD was conducted by experienced pediatricians according to the Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-5). Patients did not present congenital limb malformation and other serious chronic diseases as well as secondary epilepsy caused by intracranial infection, intracranial tumor and craniocerebral injury. Informed consent was obtained from their guardians for molecular genetic testing and further clinical evaluation.

Molecular genetic testing

All methods were conducted in accordance with relevant guidelines and regulations. Genomic DNA was extracted from peripheral blood samples in EDTA-coated Vacutainers. Blood samples from 16 pairs of twins were detected by Whole exome sequencing (WES) and Chromosomal microarray analysis (CMA)/Array-based comparative genomic hybridization (Array-CGH). Only WES/ Clinical targeted panel sequencing (CTPS) was performed on 14 pairs of twin blood samples and Array-CGH were performed on 2 pairs of twin blood samples. The DNA fragments were enriched using the Agilent SureSelect XT Human All Exon V5 kit and sequencing was performed on an Illumina HiSeq X10 platform. WES and bioinformatic analysis were performed in patients and their families as previously described [9]. And CTPS was used the Agilent ClearSeq Inherited Disease panel kit (Santa Clara, CA, USA) for enrichment based on Nest generation sequencing (NGS) [10, 11]. The CTPS included 2742 genes. The details about CTPS were described previously [12]. The filtering process began by removing variants with suboptimal quality scores. Remaining variants were then computationally compared with reported mutations in the Human Gene Mutation Database. Variants present in this database with a minor allele frequency (MAF) of less than 5%, based on the 1000 Genomes Project or ESP5400 data from the National Heart, Lung, and Blood Institute GO Exome Sequencing Project, were retained. For variants not listed in the Human Gene Mutation Database, synonymous variants, intronic variants located more than 20 bp from exon boundaries (as they are unlikely to affect mRNA splicing), and common variants with a MAF greater than 1% were discarded [13]. Variants were interpreted according to American college of medical genetics and genomics (ACMG) guidelines and were classified as pathogenic (P), likely pathogenic (LP), variants of unknown significance (VUS), likely benign (LB) or benign (B) [14]. Cases with disease-related variants were deemed positive, where "P/LP" variants that could explain the etiology were considered genetic diagnoses and "P/LP/VUS" variants were classified as genetic risk. Conversely, cases without any disease-related variants were considered negative. Monozygotic twins and dizygotic twins were identified by sequencing analysis. The kinship coefficient was defined as the probability

that two alleles sampled at random from two individuals are identical by descent. We calculated the kinship coefficient between twins based on the method from Manichaikul's study [15]. Twins with the kinship coefficient between 0.49 and 0.5 were defined as monozygotic twins, 0.25 and 0.4 were defined as dizygotic twins.

Clinical evaluations

Subjects were recommended to complete the Autism Diagnostic Observation Schedule, second edition (ADOS-2) and the GMDS. The ADOS included two subdomains: social affect (SA) and restricted and repetitive behavior (RRB). The total raw score was converted into the ADOS calibrated severity score, from 1 to 10 (none to severe). The GMDS included five subscales: Locomotor (Lm), Personal and Social (P/S), Hearing and Speech (H/Sp), Eye and Hand (E/Hd), and Performance (Pf). The raw score of each subscale was transformed into DQ. A DQ lower than 70 was considered indicative delay. Other additional medical information was also recorded from patients' medical history.

Crystal structure modeling

The PDB file, which included the crystal structure information of the human TET3 protein as predicted by the AlphaFold Monomer v2.0 pipeline, was obtained from the open-source UniProt database (Q9H2P0). The 3D structure of the human TET3 protein was visualized using ChimeraX v1.5, and its functionally conserved domains and variant identified in our study mapped onto it.

Plasmid construction

A full-length human TET3 (NM_001287491.2) expression plasmid with both Flag and HA tags at the C-terminal was constructed. A prokaryotic expression plasmid expressing the active domain of TET2 (hTET2-CS, residues 1129–1936Δ1481–1843) was constructed in pET28-b [16]. The variant was introduced into the plasmids using the KOD-Plus-Mutagenesis Kit (TOYOBO, SMK-101). All constructs were validated by sequencing.

Cell culture

Human embryonic kidney 293T cells (HEK293T cells) were cultured in DMEM (Gibco, C11995500BT) supplemented with 10% fetal bovine serum (ExCell Biotech, FSP500). For transfection, 1 μg of plasmids was introduced into HEK293T cells using Highgene transfection reagent (Abclonal, RM09014). The cells were cultured for 48 h before immunofluorescence staining, and for 72 h before Western blotting analysis.

Western blotting

HEK293T cells were harvested and lysed in lysis buffer (4% SDS, 20% glycerol, 0.125 M Tris HCl, pH6.8) supplemented with 1× protease inhibitor (Beyotime, P1005). The lysates were quantified using the Omni-Easy™ Instant BCA Protein Assay kit (Epizyme Biotech, ZJ102). Equal amounts of each sample were loaded for blotting. The primary antibodies used were anti-Flag (Smart lifesciences, SLAB01, 1:2000) and anti-GAPDH (Beijing Ray Antibody Biotech, RM2002, 1:5000). Cell lysate and protein marker (Epizyme, WJ103) were electrophoresed on a 10% SDS-PAGE and transferred to a PVDF membrane. After blocking with 5% nonfat milk for 30 min at room temperature, the membrane was incubated overnight at 4 °C with the primary antibodies. After washing with TBST buffer (Tris-buffered saline with 0.1% Tween 20) three times, the membrane was incubated with either an HRP-conjugated goat anti-rabbit (Proteintech, SA00001-2, 1:10000) or an HRP-conjugated mouse anti-Goat (Proteintech, SA00001-1, 1:10000) antibody for 1 h at room temperature. The signal was developed using an ECL luminescent solution (Tanon, 180–5001) and imaged using a digital chemiluminescence imager.

Immunofluorescence assay

HEK293T cells were cultured on coverslips and fixed with 4% paraformaldehyde for 30 min at room temperature. After washing with PBST (phosphate-buffered saline, 0.1% Triton X-100) three times, the cells were incubated in 1×PBS with 5% normal goat serum and 0.1% Triton X-100 for 30 min at room temperature. The cells were then incubated overnight at 4 °C with primary antibody: anti-Flag (Proteintech, SA00001-2, 1:2000). After washing with 1×PBS three times, the cells were incubated with secondary antibody: goat anti-rabbit-Fluor 488 (Jackson, 111-545-003, 1:1000) at room temperature for 1 h in dark room. The nuclei were counterstained with DAPI (Thermo, TC2546141, 1:10000). Following three washes with 1×PBS, the coverslips were mounted on slides using an anti-fade mounting solution (Beyotime, P0126). The images were captured using a Nikon fluorescent microscope.

Protein purification

The prokaryotic expression plasmids of WT and variant hTET2-CS were transformed into Escherichia coli strain Rosetta (DE3). The transformants were grown at 37 °C to an OD600 of 0.8 and induced by adding Isopropyl-beta-D-thiogalactopyranoside (IPTG) to 0.1 mM. After further 16 h incubation at 16 °C, cells expressing TET2 proteins were lysed and the supernatant was purified by Ni-NTA affinity purification. After on-column digestion at 4 °C overnight, the TET2 proteins were eluted out and further purified by an ion exchange and gel filtration

Table 1 Demographics and molecular genetic test results of twin pairs

Items	MZ	DZ	Total	*P*-value
N (Pairs, %)	22 (68.8)	10 (31.2)	32 (100.0)	-
Visiting ages (Months)	[b]39.0 (28.0, 47.0)	[b]32.5 (30.0, 41.0)	[b]36.5 (28.0, 46.0)	-
Gender (Male: Female)	10:1	3:1	55: 9	-
Birth weight (Kg)	[b]2.5 (2.2, 2.8)	[a]2.8 ± 0.3	[b]2.6 (2.4, 2.8)	-
Gestational week (Week)	[b]36.7 (34.0, 37.6)	[a]36.8 ± 1.6	[b]36.7 (35.0, 37.1)	-
Premature infant (Pairs, %)	11 (50.0)	5 (50.0)	16 (50.0)	-
Test-tube baby (Pairs, %)	0 (0.0)	3 (30.0)	3 (9.4)	-
Maternal age (Year)	[b]29.0 (25.0, 30.0)	[a]29.0 ± 4.7	[b]29.0 (25.0, 32.0)	-
Paternal age (Year)	[b]31.0 (27.0, 34.0)	[a]31.0 ± 4.7	[b]31.0 (27.5, 33.5)	-
Identical phenotype (Pairs, %)	21 (95.5)	7 (70.0)	28 (87.5)	[c]0.044
Non-identical phenotype (Pairs, %)	1 (4.5)	3 (30.0)	4 (12.5)	
Etiology related "P/LP" cases (Number, %)	10 (22.7)	1 (5.0)	11 (17.2)	[d]0.166
Rest of cases (Number, %)	34 (77.3)	19 (95.0)	53 (82.8)	

MZ, Monozygotic Twin; DZ, Dizygotic Twin; P, pathogenic; LP, likely pathogenic.

[a]The normally distributed continuous variables are shown as means ± SD.

[b]The skewed distributed continuous variables are shown as medians and IQR.

[c]Pearson Chi-square test.

[d]Calibration Chi-square test.

chromatography. The proteins were concentrated to 1 mg/ml and used for in vitro assays.

In vitro enzymatic assays and liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis

500 ng of 490 bp double-stranded DNA substrate (5mC was incorporated by PCR) was incubated with 1 μg hTET2-CS or its mutants in 50 μl of buffer containing 50 mM HEPES (pH 8.0), 100 mM NaCl, 100 μM Fe (NH4)2(SO4)2, 2 mM ascorbate, 1 mM DTT, 1 mM ATP, and 1 mM 2-KG. The samples were incubated for 3 h at 37 °C. The reactions were quenched by addition of 3 volumes of Buffer DP (Magen). Reaction products were purified using the Magen HiPure PCR Pure Micro Kit. The reaction products were digested by NP1 (NEB) at 55 °C for 4 h and CIAP (Takara) at 37 °C for 2 h. The reaction products were ultrafiltrated at 10,000 g for 30 min, then analyzed by LC-MS/MS.

Statistical analysis

Data analysis was performed using SPSS 27. Data were presented as the means ± standard deviation (SD) or medians and interquartile ranges (IQR) for continuous variables according to whether the data were normally distributed. Data were presented as percentages for categorical variables. We used unpaired t-test or Mann–Whitney U test depending on normality for the comparisons of DQ of the GMDS in subgroups. For categorical variables, the phenotypic concordance rate and detection yield of molecular genetic testing between monozygotic and dizygotic twins were compared by chi-squared tests.

Results

Demographics and molecular genetic test results

A total of 32 pairs of twins were included in the cohort, comprising 22 pairs of monozygotic twins and 10 pairs of dizygotic twins. The median age of the 64 subjects was 36.5 months. Among them, 55 were male and 9 were female. Based on the classification of NDD and non-NDD, both twins exhibited NDD as identical phenotype. In one pair, one twin exhibited NDD and the other exhibited non-NDD, defined as non-identical phenotype. Monozygotic twins had a higher concordance rate than dizygotic twins, and the difference was statistically significant ($P<0.05$). Other demographic data of the cohort are shown in Table 1. CMA or array-CGH was performed on 18 pair of twins and a total of 4 CNVs were found in two pairs of monozygotic twins (4/36). One pair of monozygotic twins (2 cases) carried the "P" CNV on chromosome 7 that could explain the etiology of William's syndrome (Table 2), and one pair of monozygotic twins carried the CNVs of unknown significance on chromosome 17 (Table S1). WES or CTPS was performed on 30 pairs of twins, with WES performed on 24 twin pairs and CTPS on 6 twin pairs. A total of 69 SNVs were found in 40 cases (40/60). In terms of ACMG classification, of the 69 SNVs, 1 was "P" SNV, 10 "LP" SNV and 58 "VUS" SNV. In a pair of dizygotic twins, one of the patients carried

Table 2 Pathogenic CNVs identified from molecular genetic testing

ID	Age (Months)	Gender	Genetic testing	Chromosome location	Position	Size	Deletion/duplication	ACMG classification
MZ-1-E	80	M	Array-CGH	7q11.23	Chr7:72645834–74,172,862	1527 kb	Deletion	P
MZ-1-Y	80	M	Array-CGH	7q11.23	Chr7:72645834–74,172,862	1527 kb	Deletion	P

CNVs, copy number variations; ACMG, American college of medical genetics and genomics; MZ, Monozygotic Twin; E, Elder one of twins; Y, Younger one of twins; M, male; Array-CGH, array-based comparative genomic hybridization; P, pathogenic.

a "P" SNV and a "LP" SNV that could explain his NDD phenotype, and the other one who exhibited non-NDD did not carry any disease-related SNVs (Table 3). Five pairs of monozygotic twins carried identical "LP" SNV involving loci of genes such as *POFUT1*, *SMAD6*, *TBCD*, *TET3* and *TMEM260* (Table 3). Notably, only four of the five pairs of monozygotic twins (8 cases) had the "LP" variants that could explain the phenotype of NDD in the patients. Other SNVs of unknown significance are shown in Table S2. In terms of type classification, among the 69 SNVs, 49 were missense variants, 6 were frameshift variants, 4 were nonsense variants, 4 were splicing variants, 6 were intronic variants. 24 pairs of twins underwent parental testing (both father and mother). Only 2 variants of one pair of twins were of maternal origin and 6 variants of one pair of twins were of paternal origin. Other twin pairs carried de novo variants. Analyzing etiology related pathogenic or likely pathogenic SNVs and CNVs simultaneously, the overall detection yield of molecular genetic testing was 17.2% (11/64) and detection yield of monozygotic twin and dizygotic twin was 22.7% (10/44) and 5.0% (1/20), respectively.

Analysis the effects of genetic diagnoses and genetic risk on developmental level

38 patients were diagnosed with ASD, 32 of whom completed the ADOS-2 and the median calibrated severity score was 7. Among the 60 children diagnosed with NDDs, 41 assessed using the GMDS and the average value of total DQ was 59.6. The distribution of ADOS and GMDS in the twin cohort is depicted in Fig. S1. To investigate the effects of genetic diagnoses on developmental level, we Compared of the DQ of GMDS between NDDs children with etiology related "P/LP" variants and those without disease-related genetic variants, revealing no statistical difference (Table 4). To explore the effects of genetic risk on developmental level, we compared the DQ of GMDS between NDDs children with "P/LP/VUS" variants and those without disease-related genetic variants. We found that children with genetic variants (P/LP/VUS) had significantly lower total DQ than those without disease-related genetic variants. Specifically, differences between the two subgroups were primarily observed in personal and social ability ($p<0.05$), performed ability ($p<0.01$) (Table 5). A more visually informative distribution of DQ on GDMS between these two groups is shown in Fig. 1B.

Analysis of genotype and genotype-clinical phenotype association

Analyzing the genotype of the twin cohort, we found that the "P/LP" variants were all identical among monozygotic twins and the "P/LP" variants were non-identical between dizygotic twins. Furthermore, upon analyzing of

Table 3 Pathogenic and likely pathogenic SNVs identified from molecular genetic testing

Pairs	Age (Month)	Gender	Genetic testing	Gene	Position	Variant	Type	Inheritance	Pattern	Zygosity	ACMG	Clinvar
*DZ-1-Y	73	M	WES	*RELN*	chr7:103322700	NM_005045.4:exon11:c.1152T>A:p.C384X	Missense	de novo	AD/AR	Het	P	-
				CIC	chr19:42777442	NM_001304815.1:exon2:c.1507C>T:p.R503C	Missense	de novo	AD	Het	LP	-
MZ-2-E	25	M	WES	*POFUT1*	Chr20:30804437	NM_015352.2:exon4:c.455G>A:p.W152X	Missense	de novo	AD	Het	LP	-
MZ-2-Y	25	M	WES	*POFUT1*	Chr20:30804437	NM_015352.2:exon4:c.455G>A:p.W152X	Missense	de novo	AD	Het	LP	-
MZ-3-E	37	M	WES	*SMAD6*	Chr15:66995941	NM_005585.5:exon1:c.345G>A:p.W115*	Nonsense	de novo	AD	Het	LP	p
MZ-3-Y	37	M	WES	*SMAD6*	Chr15:66995941	NM_005585.5:exon1:c.345G>A:p.W115*	Nonsense	de novo	AD	Het	LP	P
MZ-4-E	45	M	WES	*TBCD*	Chr17:80772743	NM_005993.5:exon13:c.1252_1318del:p.G418fs	Frameshift	de novo	AR	Het	LP	-
MZ-4-Y	45	M	WES	*TBCD*	Chr17:80772743	NM_005993.5:exon13:c.1252_1318del:p.G418fs	Frameshift	de novo	AR	Het	LP	-
MZ-5-E	50	M	WES	*TET3*	Chr2:74328842	NM_001287491.2:exon12:c.4927G>A:p.E1643K	Missense	de novo	AD/AR	Het	LP	-
MZ-5-Y	50	M	WES	*TET3*	Chr2:74328842	NM_001287491.2:exon12:c.4927G>A:p.E1643K	Missense	de novo	AD/AR	Het	LP	-
MZ-6-E	28	M	WES	*TMEM260*	Chr14:57085385	NM_017799.4:exon10:c.1131dup:p.E378fs	Frameshift	de novo	AR	Het	LP	-
MZ-6-Y	28	M	WES	*TMEM260*	Chr14:57085385	NM_017799.4:exon10:c.1131dup:p.E378fs	Frameshift	de novo	AR	Het	LP	-

SNVs, single nucleotide variations; ACMG, American college of medical genetics and genomics; HGMD, Human Gene Mutation Database; MZ, Monozygotic Twin; DZ, Dizygotic Twin; E, Elder one of twins; Y, Younger one of twins; M, male; F, female; WES, whole exon sequencing; AD, autosomal dominant; AR, autosomal recessive; Het, heterozygous; P, pathogenic; LP, likely pathogenic. * In one pair, one twin exhibited NDD that carried disease-related genetic variants and the other exhibited non-NDD that did not carry

Table 4 Comparison of the DQ of GMDS between NDDs children with etiology related "P/LP" variants and those without disease-related genetic variants

	Genetic findings		*P*-value
	Etiology related "P/LP" ($n=8$)	Negative ($n=16$)	
GMDS			
[a]DQ of Lm	76.5 ± 10.6	74.6 ± 18.0	0.7889
[a]DQ of P/S	51.6 ± 13.4	63.8 ± 19.2	0.1254
[a]DQ of H/Sp	41.6 ± 15.1	49.4 ± 19.4	0.3351
[a]DQ of E/Hd	60.5 ± 7.2	64.6 ± 17.0	0.5276
[a]DQ of Pf	69.3 ± 6.6	74.1 ± 14.2	0.3693
[b]Total DQ	56.9 (54.0, 74.2)	68.7 (55.0, 78.4)	0.2144

GMDS, griffiths mental development scales; DQ, developmental quotient; Lm, Locomotor; P/S, personal and social; H/Sp, Hearing and Speech; E/Hd, Eye and Hand; Pf, Performance.

[a]The differences of normally distributed continuous variables (shown as means ± SD) are tested by unpaired t tests.

[b]The differences of skewed distributed continuous variables (shown as medians and IQR) are tested by the Mann-Whitney U tests.

Table 5 Comparison of the DQ of GMDS between NDDs children with "P/LP/VUS" variants and without disease-related genetic variants

	Genetic findings		*P*-value
	"P/LP/VUS" ($n=25$)	Negative($n=16$)	
GMDS			
[a]DQ of Lm	71.7 ± 12.3	74.6 ± 18.0	0.5367
[b]DQ of P/S	47.0 (39.0, 60.5)	61.0 (48.0, 84.0)	[c]0.0283
[b]DQ of H/Sp	38.0 (28.5, 58.5)	52.0 (30.0, 62.0)	0.3289
[a]DQ of E/Hd	55.2 ± 17.3	64.6 ± 17.0	0.0965
[a]DQ of Pf	60.6 ± 14.2	74.1 ± 14.2	[d]0.0049
[a]Total DQ	56.2 ± 12.4	65.3 ± 15.2	[c]0.0432

GMDS, griffiths mental development scales; DQ, developmental quotient; Lm, Locomotor; P/S, personal and social; H/Sp, Hearing and Speech; E/Hd, Eye and Hand; Pf, Performance.

[a]The differences of normally distributed continuous variables (shown as means ± SD) are tested by unpaired t tests.

[b]The differences of skewed distributed continuous variables (shown as medians and IQR) are tested by the Mann-Whitney U tests.

[c]$p<0.05$.

[d]$p<0.01$.

genotype-clinical phenotype association, we discovered it interesting that a pair of monozygotic twins have identical "P/LP" variants and exhibited different clinical phenotype, with one diagnosed with ASD and the other as normal (Table 6).

Reporting a novel de novo *TET3* variant in a pair of monozygotic twins

In our study, 24 pairs of twins underwent parental testing, of which 22 pair of twins carried de novo variants. This suggests that twins may have an advantageous in identifying de novo variants. We focused on a novel de novo missense variant (c.4927G > A) of *TET3* that one pair of monozygotic twins carried in our twin cohort. The human *TET3* gene is composed of eleven exons. It encoded a full-length protein of 1776 amino acids, encompassing multiple functional domains, including the CXXC DNA binding domain, the Cys-rich insert domain and the double-stranded b helix (DSBH) domain. The DSBH domain is split in two by a low-complexity insert. This novel variant of *TET3* resulted in point mutation in the coding region (Fig. 2A). We subsequently visualized the predicted 3D structure of the human TET3 protein using ChimeraX v1.5, and revealed that the the Cys-rich insert domain and the DSBH domain tend to cluster in the center. Our variant (c.4927G > A) was in the core of the 3D organization of the TET3 protein, where the DSBH domain congregate (Fig. 2B). Reviewing the clinical data of the twins, their visiting age was 50 months. The elder brother's total DQ of GMDS was 74.2 (Lm 99, P/S 66, H/Sp 59, E/Hd 71, Pf 76). The younger brother' total DQ of GMDS was 54.0 (Lm 80, P/S 34, H/Sp 29, E/H d59, Pf 68). In neurological aspect, they had no previous seizures, hypotonia, or hypertonia and their electroencephalogram (EEG) and magnetic resonance imaging (MRI) were both normal. Physical examination showed that they had craniofacial dysmorphisms, including a broad forehead and protruding ears, but not long face, short nose, long philtrum, hypotonic face and highly arched palate (Fig. 2C). Besides, their weight, height and head circumference were all within the normal range and there were no other comorbidities of ophthalmological problems, cardiovascular anomalies, musculoskeletal problems, and gastrointestinal manifestations. Tracing the birth and developmental history, the gestational week of the twins was 39 weeks, and their birth weights were 3.15 kg (elder brother) and 3.90 kg (younger brother). They both began walking independently at 1 year and 4 months of age.

Verification of the cell function and enzymatic activity of the *TET3* variant in vitro

To study the potential deleterious effects on cells of the *TET3* variant (c.4927G > A), we constructed expression plasmids of the full-length human *TET3* cDNA (*hTET3*) encoding both Flag and HA tags at the C-terminal. We also constructed the variant plasmids (Fig. 2D). Then, we transfected both the *hTET3* and variant plasmids into human HEK293T cells, performed Western blotting (WB) and immunofluorescence (IF) staining using anti-Flag antibody. In WB analysis, an expressed band was observed in the *hTET3* group, detectable by anti-Flag antibody. A similar signal was detected in the group with *TET3* variant, but not in the sham (no transfection) group (Fig. 2E). This observation suggested this variant did not affect expression of TET3 in HEK-293T cells. For IF staining, signals were detected in both the *hTET3* and *TET3* variant groups, but not in the sham group when using the anti-Flag antibody. Interestingly, in the *hTET3*

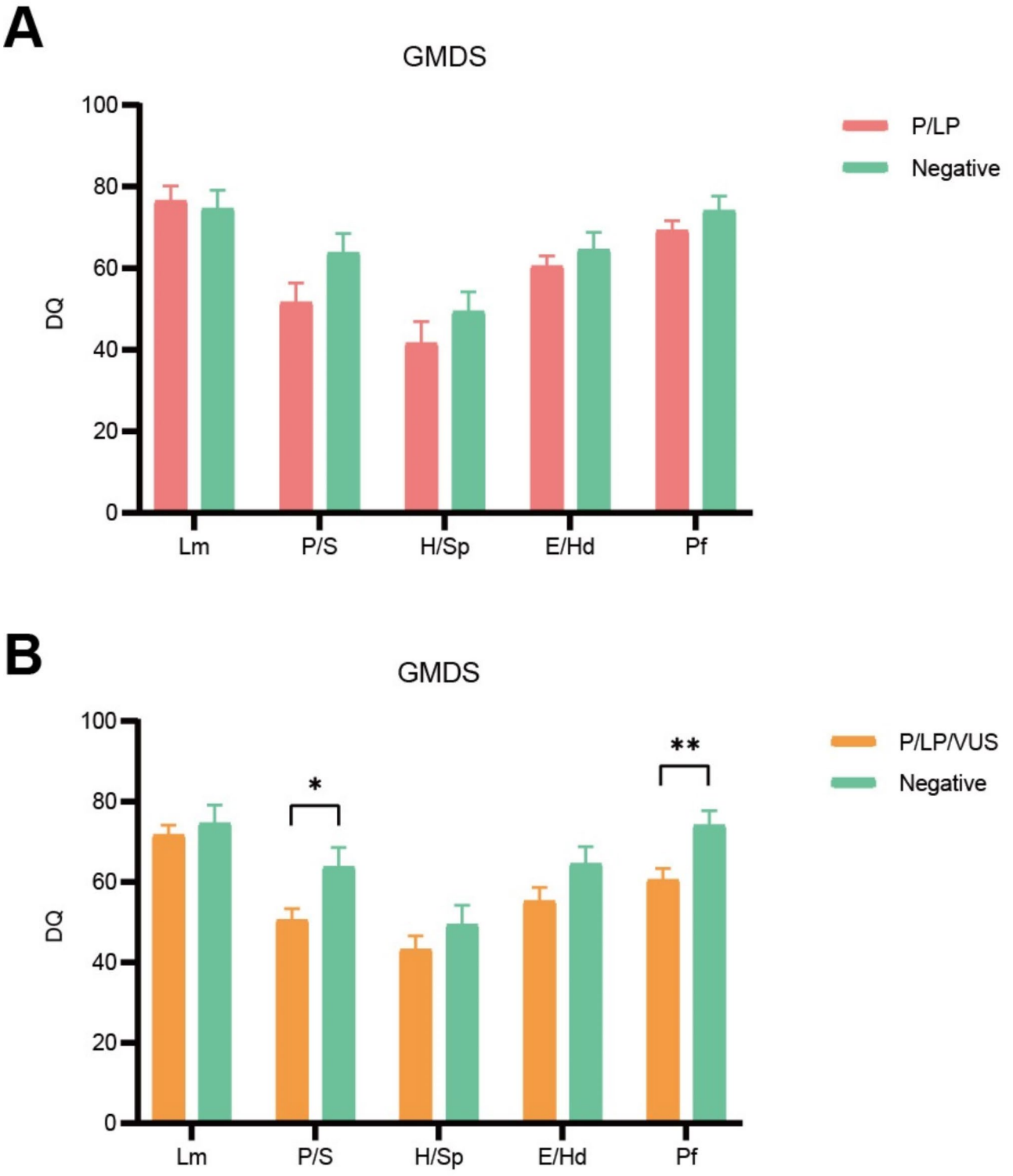

Fig. 1 The DQ distribution map of GDMS. **A** The DQ distribution map of GDMS between NDDs children with etiology related "P/LP" variants and those without disease-related genetic variants. **B** The DQ distribution map of GDMS between NDDs children with "P/LP/VUS" variants and those without disease-related genetic variants

Table 6 Analysis of genotype and genotype-phenotype association in twin pairs

Items (pairs)	MZ (22)	DZ (10)
Identical "P/LP" (pairs, %)	6 (27.3)	0 (0.0)
Non-identical "P/LP" (pairs, %)	0 (0.0)	1 (10.0)
Identical "P/LP" and identical phenotype (pairs, %)	5 (22.7)	0 (0.0)
Identical "P/LP" and different phenotype (pairs, %)	1 (4.5)	0 (0.0)
Non-identical "P/LP" and different phenotype (pairs, %)	0 (0.0)	1 (10.0)

MZ, Monozygotic Twin; DZ, Dizygotic Twin; P, pathogenic; LP, likely pathogenic.

group, the TET3 protein was evenly distributed in the nucleus, whereas in the *TET3* variant group, the TET3 protein was concentrated around the nucleus (Fig. 2F), indicating that the subcellular localization of the *TET3* variant in human cell had changed. TET3 is a classic methylcytosine dioxygenase. To assess the potential deleterious effects on enzymatic activity of the *TET3* variant, we used sequence alignment to identify the homologous mutation site of *TET3* (E1643) in *TET2* (E1851) (Fig. 2G) [17]. We constructed a *hTET2-CS* expression plasmid for prokaryotic purification and purified both the wild-type and variant hTET2-CS from Escherichia coli (Fig. 2H). The variant significantly reduced the enzyme activity of hTET2-CS (Fig. 2I). The result indicated that the *TET3* variant has significantly reduced enzymatic activity.

Discussion

In this study, we conducted an in-depth examination of the demographic characteristics of 32 pairs of twins. Consistent with findings from previous studies focus on twins, we observed a higher concordance rate among monozygotic twins compared to dizygotic twins. We investigated the detection yield and novel variants through CMA/Array-CGH and WES/CTPS in 32 twin pairs with NDDs. Analyzing etiology related pathogenic or likely pathogenic SNVs and CNVs simultaneously, the overall detection yield of molecular genetic testing was 17.2% (11/64). Previously, many articles have studied the detection yield of WES or panel in patients with ASD. Ghralaigh's study reported a diagnostic yield in ASD was 31% using WES [18], while Hu's investigation into the detection yield of CTPS in Children with ASD in China demonstrated an overall diagnostic yield of 19.16% (109/569) when combining SNVs and CNVs simultaneously [12]. However, scant literature exists concerning molecular genetic testing and analysis in twins diagnosed with NDDs. In a study by Sofia et al., CNVs analysis was conducted on 100 twin pairs, focusing on individuals predisposed to NDDs, revealing that postzygotic de novo CNVs events are typically infrequent [19]. Nevertheless, in our study, we observed a relatively high detection yield of de novo SNVs and CNVs. Our findings underscore the considerable diagnostic advantages offered by molecular genetic testing in identifying de novo pathogenic or likely pathogenic CNVs and SNVs in patients with NDDs, thereby facilitating the identification of causative and risk genes. Consequently, we advocate for the inclusion of molecular genetic testing such as WES, CTPS, CMA and Array-CGH, in the diagnostic evaluation of NDDs among twin pairs and their parents. Leveraging genetic information may elucidate the etiology of NDDs, leading to more targeted and effective interventions.

The DQ value across various subscales of GMDS can reflect the development level of children's gross motor skills, personal and social abilities, language, fine motor skills and performed capabilities. Sanders' investigation highlighted that individuals with lower cognitive abilities exhibit a higher likelihood of harboring identifiable genetic risk variants than those with higher IQ levels [20]. In our cohort study, we analyzed the impact of CNVs and SNVs on DQ scores, examining two distinct perspectives: genetic diagnoses and genetic risk. Cases characterized by etiology related "P/LP" variants were classified under genetic diagnoses, while those encompassing "P/LP/VUS" variants were categorized under genetic risk. Our analysis concerning genetic diagnoses revealed no statistically significant differences in GMDS DQ scores between NDDs children with etiology related "P/LP" and without disease-related genetic variants. Conversely, with regards to genetic risk, children carrying "P/LP/VUS" variants exhibited significantly lower total DQ scores compared to children without disease-related genetic variants. Upon disaggregating the GMDS subscales, the dissimilarities between the two groups predominantly manifested in personal and social abilities, as well as performance capabilities. Our findings underscore that children with lower developmental levels are prone to possessing identifiable genetic variants (P/LP/VUS), implying an association between diminished developmental proficiency and heightened genetic susceptibility.

Furthermore, we analyzed on the genotype-phenotype association within our twin cohort. An interesting finding emerged wherein a pair of monozygotic twins exhibited identical "P/LP" variants alongside differing clinical phenotype-one twin diagnosed with ASD and the other presented as neurotypical. This discrepancy in clinical presentation within monozygotic twins suggests that the "P/LP" variants could not explain the etiology of ASD patient. It is well known that monozygotic twins theoretically share identical DNA, only a minority of these twins exhibit genetic variations [21]. Therefore, this finding also suggests that the association between pathogenic or likely pathogenic variants and etiology in twins could also be corroborated by the clinical phenotype of twins.

Within our cohort, several de novo variants were identified, including a novel de novo missense variant (c.4927G > A) in the *TET3* gene, detected in a pair of

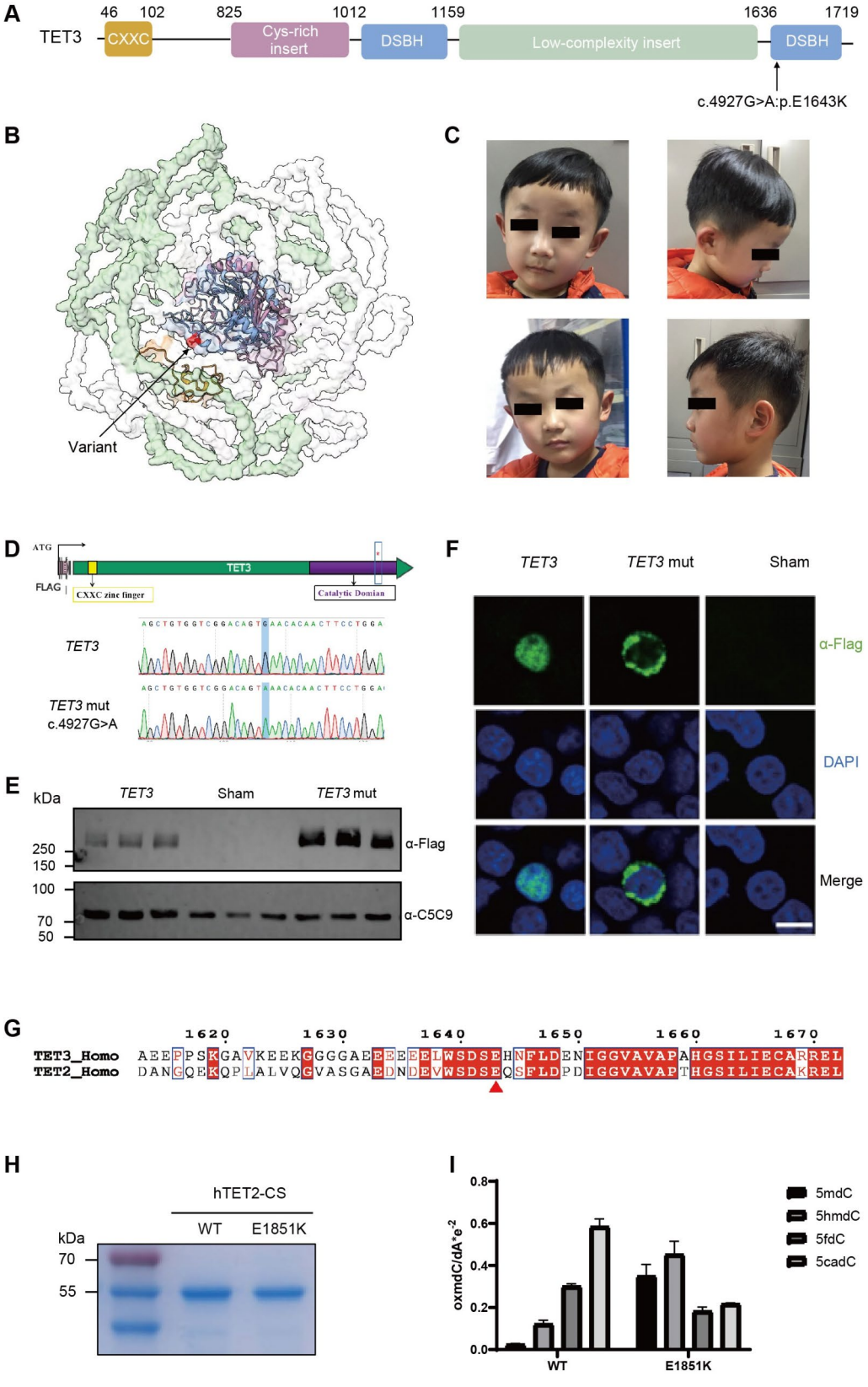

Fig. 2 (See legend on next page.)

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Fig. 2 A novel de novo *TET3* variant in a pair of monozygotic twins and verification of the cell function and enzymatic activity of the *TET3* variant in vitro. **A** Schematic depiction of TET3 showing domain structure with the Cys-rich insert in pink (aa 825–1012) and the double-stranded b helix domain in blue (DSBH; aa 1012–1159; aa 1636–1719). The DSBH domain is split in two by a low-complexity insert (aa 1159–1636). The N-terminal CXXC DNA binding domain is shown in orange (aa 46–102). **B** Predicted 3D structure of human TET3 with the functional domains highlighted in the same color of A and variant highlighted in red. **C** The front and side photos of the twins. **D** The schematic diagram of *TET3* plasmid construction. **E** Western blotting, *hTET3* group and *TET3* variant group successfully expressed 250KDa band by using anti-Flag antibody, but not in the sham group. **F** IF signals were detected in *hTET3* and *TET3* variant group except for the sham group when anti-Flag was used. The TET3 protein in *hTET3* group was evenly distributed in the nucleus and the TET3 protein in *TET3* variant group was concentrated around the nucleus. **G** Structure-based sequence alignment of TET3 and TET2 proteins. Identical and highly conserved residues are highlighted in red and conserved residues in white. The mutation site was identical in TET3 (E1643) and TET2 (E1851) proteins as shown above the red triangle. **H** The wild-type and variant hTET2-CS purified from *E. coli*. **I** The hTET2-CS variant showed a reduced enzyme activity of 5mdC

monozygotic twins. Variants in the *TET3* gene associated with Beck-Fahrner syndrome, categorized as one of the mendelian disorders affecting the epigenetic machinery, commonly characterized by motor and language impairments. Clinical manifestations typical include mild to severe DD/IDD with approximately one third of affected individuals exhibiting epilepsy. Additional neurobehavioral features may encompass ASD, attention-deficit/hyperactivity disorder and anxiety. Craniofacial dysmorphisms such as tall or broad forehead, long face, protruding ears, short nose, long philtrum, hypotonic face and highly arched palate, have been reported. Growth abnormalities, including macrocephaly, are also prevalent among affected individuals [22]. 28 patients have been documented with pathogenic variants of *TET3* [23–27]. Notably, our patients exhibited some overlapping clinical features described in previous literature, such as delayed language abilities, mild to moderate developmental delay and facial features. Through molecular genetic testing, the twins were diagnosed with Beck-Fahrner syndrome. In fact, most Beck-Fahrner syndrome rely on molecular confirmation.

Studies have shown that *Tet3* is crucial for cortical development in mice, and its knockout leads to neuronal differentiation defects and synaptic dysfunction [28, 29]. As a key regulator of DNA demethylation, TET3 influences gene expression, and its deficiency has been linked to developmental delays and neurobehavioral abnormalities, consistent with the clinical features of Beck-Fahrner syndrome [30, 31]. Furthermore, *Tet3* mutant mouse models exhibit impaired motor coordination, increased anxiety-like behaviors, and deficits in learning and memory, closely resembling the cognitive and motor impairments observed in affected individuals [32–35]. These findings highlight the pathogenic mechanisms underlying *TET3* mutations and reinforce the importance of this gene in neurodevelopment.

To elucidate the putative deleterious effects of the *TET3* variant (c.4927G > A) on cellular function, we conducted in vitro verification of its impact. The TET3 protein in the *hTET3* group exhibited nuclear distribution, consistent with previous study [26]. Conversely, in the *TET3* variant group, in the TET3 protein displayed concentration around the nucleus, suggestive of altered subcellular localization induced by the *TET3* variant (c.4927G > A), potentially leading to cell dysfunction. Within the TET family, TET2 exhibits markedly higher variant frequencies across various cancer types, particularly gliomas, lymphoid and myeloid malignancies, including acute myeloid leukemia (AML) [36–38]. To delineate the ramifications of this variant on TET family proteins, we used sequence alignment to identify the homologous variant site of *TET3* (E1643) in *TET2* (E1851). Previous studies have delineated the minimal regions requisite for TET2 catalytic activity (hTET2-CS, residues 1129–1936 Δ1481–1843) [16]. We constructed a *hTET2-CS* expression plasmid for prokaryotic purification and then purified both the wild-type and variant hTET2-CS from Escherichia coli. In vitro enzymatic assays indicated a significantly reduction in enzyme activity attributable to the variant, thereby indicating the criticality of variant site for TET family protein function.

There are some limitations in our study. Firstly, we recruited a small sample of twin pairs with NDD from the Department of Child Health Care of Children's Hospital of Fudan University in Shanghai, China. The sample size is not large and the data of our single center may be biased. Secondly, we only counted part of the 32 twin pairs in the statistics of clinical evaluations due to incomplete data. Additionally, we lacked in vivo evidence to further indicate the functional effects of the identified *TET3* variant.

Abbreviations

ACMG	American college of medical genetics and genomics guidelines
AD	Autosomal dominant
ADOS-2	The Autism Diagnostic Observation Schedule, second edition
AML	Acute myeloid leukemia
AR	Autosomal recessive
Array-CGH	Array-based comparative genomic hybridization
ASD	Autism spectrum disorder
B	Benign
CMA	Chromosomal microarray analysis
CNVs	Copy number variations
CTPS	Clinical targeted panel sequencing
DD	Developmental delay
DQ	Developmental quotients
DSBH	Double-stranded b helix

DSM-5	Diagnostic and statistical manual of mental disorders, fifth edition
DZ	Dizygotic Twin
EEG	Electroencephalogram
E/Hd	Eye and Hand
GMDS	The Griffiths mental development scales
HEK293T cells	Human embryonic kidney 293T cells
Het	Heterozygous
H/Sp	Hearing and Speech
hTET3	Human *TET3*
IDD	Intellectual developmental disorder
IF	Immunofuorescence staining
IQR	Interquartile ranges
LB	Likely benign
LC-MS	Liquid chromatography tandem mass spectrometry
Lm	Locomotor
LP	Likely pathogenic
MAF	Minor allele frequency
MRI	Magnetic resonance imaging
MZ	Monozygotic Twin
NDDs	Neurodevelopmental disorders
NGS	Nest generation sequencing
P	Pathogenic
Pf	Performances
P/S	Personal and social
RRB	Restricted and repetitive behavior
SA	Social affect
SD	Standard deviation
SNVs	Single nucleotide variations
TET3	TET methylcytosine dioxygenase 3
VUS	Variants of unknown significance
WB	Western blotting
WES	Whole exome sequencing

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions
Qiong Xu and Guoliang Xu designed the study. Chunchun Hu, Dongyun Li, Yiting Zhu, Huiping Li, Xiu Xu and Qiong Xu collected clinical data. Guangbo Jin did the experiment of enzymatic activity in vitro. Chuanhui Ge did the experiment of cell function in vitro. Wenzhu Peng did the Crystal structure modeling. Lianni Mei analyzed the data and wrote the manuscript. Yan Jiang helped to revise the manuscript. All authors read and approved the final manuscript.

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Data availability
The full uncropped Gels and Blots image is provided within the supplementary information files. The ethics approval file and patients's consents are provided within the related files. The sequencing data in this paper includes WES, CTPS, CMA, Array-CGH data, it can not be shared due to privacy.

Declarations

Competing interests
The authors declare no competing interests.

Ethics approval and consent to participate
The studies involving human participants were reviewed and approved by the Ethics Committee of the Children's Hospital of Fudan University (Approval number: 2017-178, approval date: 27 September 2017).

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