Supplementary Methods

Method for detecting variants using WES

The WES data analysis pipeline was designed based on the best practices workflow recommended by the Broad Institute's Genome Analysis Tool Kit (GATK) instructions (McKenna et al., 2010). Genomic DNA was extracted from the peripheral blood of probands and their parents. Genomic DNA was randomly fragmented into 180-280 bp segments using a Covaris sonicator. The fragmented DNA underwent end repair and A-tailing, followed by the ligation of sequencing adapters to both ends to construct the DNA library. Libraries with specific indices were pooled and hybridized with biotin-labeled probes in a liquid-phase hybridization. Streptavidin-coated magnetic beads were then used to capture the exonic regions. After PCR amplification, the libraries were quality-checked, and those that passed the quality control were sequenced. After library construction, initial quantification was performed using Qubit 2.0. The insert size of the libraries was then assessed using NGS3K/Caliper. Once the insert size met the expected criteria, the effective concentration of the libraries (3 nM) was accurately quantified using qPCR to ensure library quality. Libraries that passed quality control were subjected to high-throughput paired-end sequencing, with 150 bp reads generated from each end. Exome capture was performed using the Agilent SureSelect Human All Exon V6 Kit (Agilent Technologies, Santa Clara, CA, USA). WES was conducted on the Illumina Novaseq 6000 platform (Illumina Inc., San Diego, CA, USA), generating 150-bp paired-end reads with a minimum coverage of 10× (average coverage of 100×), covering approximately 99% of the genome. Reads were aligned to the GRCh37/hg19 reference genome using the Burrow-Wheeler aligner (BWA). Variant calling was performed using HaplotypeCaller by GATK in GVCF mode. Variant annotation was performed using ANNOVAR, which includes annotation information from the dbSNP database, the 1000 Genomes Project, and other existing databases (Wang et al., 2010). Annotations included the location information, variant type, minor allele frequencies from public data sets, deleteriousness, and conservation scores enabling further filtering and assessment of the variant's

pathogenicity.

Screening of deleterious genetic variants

Filtering of deleterious variants was performed as follows: (1) Filter out common variants with minor allele frequency (MAF) more than 0.01 in 1000 Genomes Project (1000g_all), Exome Sequencing Project (esp6500iv_all), and Genome Aggregation Database (gnomAD_ALL and gnomAD_EAS). (2) Only variants located in exons and splice site regions (splicing junction 10 bp) were retained. (3) Synonymous variants not located in highly conserved areas and not predicted to affect splicing are removed. (4) Small fragment non-frameshift InDels (<10 bp) located in repeat regions are excluded. (5) Variants are retained if they are predicted to be deleterious by at least half of the following tools: SIFT (Vaser et al., 2016), PolyPhen-2 HumVar (Adzhubei et al., 2010), PolyPhen-2 HumDiv (Adzhubei et al., 2010), MutationTaster (Schwarz et al., 2010) and CADD (Kircher et al., 2014). (6) Variants (>2bp) predicted not to affect alternative splicing were removed.

Genotyping

Peripheral blood samples were collected from participants, and genomic DNA was extracted using the Magnetic Universal Genomic DNA Kit (DP705, Tiangen Biochemical Technology Co., Ltd., Beijing, China). Primers were designed using Primer Premier 5.0 and synthesized by Shanghai MAP Biotech Co., Ltd. The PCR amplification was performed in a total reaction volume of 25 μL, consisting of 20 μL of PCR Master Mix, 1.5 μL of forward primer (10 μM), 1.5 μL of reverse primer (10 μM), and 1.5 μL of genomic DNA template. The PCR procedure was carried out with an initial denaturation at 98°C for 2 min, followed by 35 cycles consisting of denaturation at 98°C for 10 seconds, annealing at 60°C for 15 seconds, and extension at 72°C for 20 seconds per kilobase. A final extension was performed at 72°C for 5 minutes, and the reaction was held at 4°C. The PCR products were purified using the E.Z.N.A. Gel Extraction Kit (D2500-01, Omega Bio-Tek, USA) according to the manufacturer's instructions. Sequencing was conducted on an ABI 3730XL sequencer using fluorescently labeled dideoxynucleotides to generate chromatograms, which were analyzed to determine the genotype.

Supplementary Table 1 Primer sequences and PCR product lengths for genotyping of folate metabolism gene polymorphism

Gene	Locus position	Forward primer	Forward sequence	Reverse primer	Reverse sequence	Product length (bp)
MTHFR	MTHFR-c677	MTHFR-c677-F	TGACTGTCATCCCTATTGGCAG	MTHFR-c677-R	AAGAACTCAGCGAACTCAGCA	301
MTRR	MTRR-c66	MTRR-c66-F	TCATATTATGTGTGGGTATTGTTGC	MTRR-c66-R	ATTCTTCAAAGCACAAAACGGTA	313
BHMT	BHMT-c716	BHMT-c716-F	CCACTCACAGGAGCATCCATCA	BHMT-c716-R	AATACGAAGGGGCTTTTGGCTG	337

Supplementary Table 2 Summary of whole-exome sequencing coverage and quality metrics

Metric	Mean Value
Raw data (G)	15.78 G
Mapping rate (%)	99.90%
Sequencing depth (×)	181.09×
Coverage (%）	99.70%
Proportion of target regions with > 20× coverage (%)	96.8
Proportion of target regions with > 10× coverage (%)	98.6
Proportion of target regions with > 4× coverage (%)	99.4

Note: Average sequencing depth refers to the total amount of sequencing data mapped to target regions divided by the total length of target regions. Coverage refers to the proportion of the reference genome bases covered by sequencing reads.

Supplementary Table 3 Enrichment of deleterious variants in folate metabolism–related KEGG pathways among ASD patients

KEGG Pathway	Number of Enriched Patients	Patient ID	Genes Involved
Folate-mediated one-carbon metabolism (hsa00670)	45	F1, F3_1, F4, F5, F6, F7_1, F7_2, F8, F9, F10_2, F11, F12, F14, F15, F16, F17, F18, F19_1, F20, F21_1, F21_2, F22, F23, F24, F25, F26, F28, F30, F32, F36, F40_1, F40_2, F43, F44, F46, F47, F48, F49, F50_1, F50_2, F52, F53, F57, F58, F59	DHFR2, MTHFD2, MTHFR, SHMT1, SHMT2, MTR, MTRR, SLC19A1, FOLR1, BHMT, MAT2B, CBS, CTH
Folate transport and metabolism (hsa04981)	39	F1, F3_3, F5, F6, F7_1, F8, F9, F10_1, F10_2, F11, F12, F15, F17, F18, F19_1, F21_1, F22, F23, F26, F30, F31, F32, F36, F39, F44, F45_1, F45_2, F46, F47, F48, F49, F50_1, F50_2, F52, F53, F55, F57, F58, F59	DHFR2, MTHFD2, MTHFR, SHMT1, SHMT2, MTR, MTRR, SLC19A1, FOLR1
Cysteine and methionine metabolism (hsa00270)	22	F3_3, F7_1, F7_2, F14, F16, F17, F18, F22, F24, F27, F29, F33_1, F33_2, F34, F36, F46, F47, F49, F50_1, F53, F58, F59	MTR, MTRR, MAT, BHMT, SAHH, CBS, CTH

Supplementary Table 4 Deleterious folate metabolism gene polymorphisms detected by whole exome sequencing

Gene	Chromosome	ID	Nucleotide changes	Amino acid changes	Transcript	Mutation region	Mutation type
DHFR2	3q11.1	rs17855824	c.496G>A	p.V166I	NM_176815	Exon	Missense
MTHFD2	2p13.1	rs199529089	c.1A>G	p.M1V	NM_006636	Exon	Missense
MTHFR	1p36.22	rs1801131	c.1286A>C	p.E429A	NM_005957	Exon	Missense
	1p36.22	rs1801133	c.677C>T	p.A222V	NM_005957	Exon	Missense
	1p36.22	rs13306558	c.6C>A	p.D2E	NM_001330358	Exon	Missense
SHMT1	17p11.2	rs78909145	c.647A>G	p.K216R	NM_004169	Exon	Missense
SHMT2	12q13.3	rs376369904	c.206G>A	p.R69H	NM_001166356	Exon	Missense
	12q13.3	rs375584473	c.361C>T	p.R121C	NM_001166356	Exon	Missense
	12q13.3	rs11557166	c.537C>T	p.D179D	NM_001166356	Exon	Nonsense
	12q13.3	rs189439132	c.1280G>A	p.R427H	NM_001166356	Exon	Missense
MTR	1q43	rs142648132	c.742G>C	p.V248L	NM_000254	Exon	Missense
	1q43	rs1805087	c.2756	p.D919	NM_000254	Exon	Missense

			A>G	G			
MTRR	5p15.31	rs148267849	c.-1580G>T	/	NM_002454	UTR5	/
	5p15.31	rs1801394	c.66A>G	p.I22M	NM_002454	Exon	Missense
	5p15.31	rs374239028	c.208C>T	p.R70C	NM_002454	Exon	Missense
	5p15.31	rs2287780	c.1243C>T	p.R415C	NM_002454	Exon	Missense
	5p15.31	rs16879334	c.1349C>G	p.P450R	NM_002454	Exon	Missense
SLC19A1	21q22.3	rs543796693	c.1760A>C	p.Q587P	NM_194255	Exon	Missense
	21q22.3	rs549433809	c.421T>A	p.F141I	NM_194255	Exon	Missense
FOLR1	11q13.4	rs76191655	c.292C>T	p.R98W	NM_000802	Exon	Missense
BHMT	5q14.1	rs56709544	c.46C>T	p.R16C	NM_001713	Exon	Missense
	5q14.1	rs752243322	c.700G>T	p.G234C	NM_001713	Exon	Missense
	5q14.1	rs3733890	c.716G>A	p.R239Q	NM_001713	Exon	Missense
MAT2B	5q34	rs80145956	c.905C>T	p.T302I	NM_013283	Exon	Missense
CBS	21q22.3	rs765134080	c.1380G>A	p.T460T	NM_000071	Exon	Nonsense
	21q22.3	rs760214620	c.346G>A	p.G116R	NM_000071	Exon	Missense

	21q22.3	rs201827340	c.52C>T	p.R18C	NM_000071	Exon	Missense
CTH	1p31.1	rs775272253	c.556G>A	p.V186M	NM_001902	Exon	Missense

Supplementary Table 5 Mutation frequency and harmfulness score of folate metabolism gene polymorphism

Gene	ID	Nucleotide changes	Wild type	Heterozygous	Homozygous	Mutation frequency	CADD
DHFR2	rs17855824	c.496G>A	65	5	0	0.071	15.92
MTHFD2	rs199529089	c.1A>G	68	2	0	0.029	20.6
MTHFR	rs1801131	c.1286A>C	49	18	3	0.3	19.89
MTHFR	**rs1801133**	**c.677C>T**	**36**	**28**	**6**	**0.486**	**25**
MTHFR	rs13306558	c.6C>A	68	2	0	0.029	19.17
SHMT1	rs78909145	c.647A>G	69	1	0	0.014	22.5
SHMT2	rs376369904	c.206G>A	69	1	0	0.014	23
SHMT2	rs375584473	c.361C>T	69	1	0	0.014	26.7
SHMT2	rs11557166	c.537C>T	58	10	2	0.171	17.72
SHMT2	rs189439132	c.1280G>A	66	4	0	0.057	23.7
MTR	rs142648132	c.742G>C	67	3	0	0.043	/
MTR	rs1805087	c.2756A>G	62	8	0	0.114	23.5
MTRR	rs148267849	c.-1580G>T	69	1	0	0.014	/
MTRR	**rs1801394**	**c.66A>G**	**36**	**27**	**7**	**0.486**	**23.3**
MTRR	rs374239028	c.208C>T	69	1	0	0.014	23.7
MTRR	rs2287780	c.1243C>T	44	23	3	0.371	23.4
MTRR	rs16879334	c.1349 C>G	48	19	3	0.314	29.6
SLC19A1	rs543796693	c.1760A>C	69	1	0	0.014	21.9
SLC19A1	rs549433809	c.421T>A	69	1	0	0.014	24.5
FOLR1	rs76191655	c.292C>T	69	1	0	0.014	25.8
BHMT	rs56709544	c.46C>T	68	2	0	0.029	35
BHMT	rs752243322	c.700G>T	69	1	0	0.014	27.6
BHMT	**rs3733890**	**c.716G>A**	**32**	**31**	**7**	**0.543**	**21.8**

MAT2B	rs80145956	c.905C>T	68	2	0	0.029	18.57
CBS	rs765134080	c.1380G>A	69	1	0	0.014	19.19
CBS	rs760214620	c.346G>A	69	1	0	0.014	35
CBS	rs201827340	c.52C>T	69	1	0	0.014	15.96
CTH	rs775272253	c.556G>A	69	1	0	0.014	31

Supplementary Table 6 Hardy-Weinberg equilibrium test for folate metabolism gene polymorphisms

ID	Gene polymorphism	Wild type	Heterozygous	Homozygous	*HWE P-value**
rs1801133	MTHFR C677T	178	129	33	0.218
rs1801394	MTRR A66G	180	135	25	0.942
rs3733890	BHMT G716A	151	160	29	0.167

Note: ID refers to the standardized identifiers in the dbSNP database.

**HWE P-value* were calculated based on the Hardy-Weinberg equilibrium test.

Supplementary Table 7 Comparison of genotype frequencies of folate metabolism gene polymorphisms between the ASD and the control group

Genotype	Total	ASD group	Control group	*P*
	（n = 340）	(n = 170)	(n = 170)	
MTHFR C677T				**0.009**
CC	178	75 (44.1%)	103 (60.6%)	
CT	129	77 (45.3%)	52 (30.6%)	
TT	33	18 (10.6%)	15 (8.8%)	
MTRR A66G				**0.018**
AA	180	77 (45.3%)	103 (60.6%)	
AG	135	79 (46.5%)	56 (32.9%)	
GG	25	14 (8.2%)	11 (6.5%)	
BHMT G716A				0.809
GG	151	78 (45.9%)	73 (42.9%)	
GA	160	77 (45.3%)	83 (48.8%)	
AA	29	15 (8.8%)	14 (8.2%)	

Data are presented as frequency (percentage).