Brief Report

DOI: 10.5582/irdr.2025.01041

Identification of a novel *de novo AFF4* variant (c.778A>G) associated with CHOPS syndrome

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SUMMARY: CHOPS (cognitive impairment, coarse facies, heart defects, obesity, pulmonary involvement, short stature, and skeletal dysplasia) syndrome is an extremely rare disorder with multiple congenital anomalies caused by missense variants in the ALF transcription elongation factor 4 gene (*AFF4*). This study aimed to identify causative variants in a Chinese family with CHOPS syndrome. A Chinese girl with short stature, obesity, and developmental delay underwent comprehensive clinical and genetic evaluations, including karyotyping analysis, multiple ligation-dependent probe amplification, detection of aberrant methylation, whole exome sequencing, Sanger sequencing, and copy number variation analysis, followed by *in silico* analyses. Reverse transcription, polymerase chain reaction, and Sanger sequencing were performed to evaluate the gene expression levels. The patient exhibited cognitive impairment, coarse facial appearance, obesity, short stature, skeletal involvement, and ophthalmic abnormalities. Genetic analyses identified a *de novo* heterozygous c.778A>G (p.Met260Val) variant in *AFF4* in the proband, absent in parents and little sister, with no other remarkable results. This novel variant was classified as pathogenic, without apparent effect on relative gene expression. The identification of this *de novo* missense variant as the genetic cause of CHOPS syndrome in this Chinese family broadens the genetic and phenotypic spectrum of the disorder.

Keywords: CHOPS syndrome, *AFF4* gene, novel variant, *de novo* variant, whole exome sequencing

1. Introduction

CHOPS syndrome (OMIM 616368) is an extremely infrequent genetic disease with multiple congenital anomalies caused by missense variants in the ALF transcription elongation factor 4 gene (*AFF4*, OMIM 604417) (*1,2*). Since three originally reported individuals with *AFF4* variants shared highly similar phenotypes, including cognitive impairment, coarse facies, heart defects, obesity, pulmonary involvement, short stature, and skeletal dysplasia, the acronym "CHOPS" has been given to this specific clinical entity (*2*). The clinical features of CHOPS patients, such as short stature, intellectual disability, and craniofacial features, resemble those of Cornelia de Lange syndrome (CdLS), a multisystem developmental disorder sharing common molecular pathogenesis caused by transcriptional elongation abnormalities, but the recognizable symptoms and distinct molecular etiologies suggest they are unique clinically diagnostic entities (*1-3*). Currently, the diagnosis of rare pediatric disorders relies heavily on genetic diagnostics, and the use of genomic technologies to identify novel variants is essential for deepening the understanding of rare diseases and ultimately improving patient outcomes (*4*).

The *AFF4* gene encodes the AFF4 protein, a member of the AF4/FMR2 family, which serves as a central scaffold protein of the super elongation complex (SEC) involved in the regulation of transcription elongation (*5*). The SEC encompasses eleven-nineteen Lys-rich leukemia (ELL) family members ELL1/ELL2/ELL3, ALL1-fused gene from chromosome 9 (AF9)/eleven-nineteen leukemia (ENL), AF4/FMR2 family member 1 (AFF1, also termed AF-4)/AFF4, and

positive transcription elongation factor b (P-TEFb) (*5-7*). The AFF4 protein, located downstream of the transcription start sites, plays a pivotal role in regulating RNA polymerase II (RNAP2) transcription (*5*). This protein is also important in tumorigenesis, osteogenesis, odontogenesis, and adipogenic differentiation (*6*). Gain-of-function variants in *AFF4* lead to resistance to proteasomal degradation and resultant transcriptional activation, which underlies CHOPS syndrome (*1,2*). Hitherto, only 16 cases of CHOPS syndrome have been reported worldwide, and they all carried heterozygous *AFF4* missense variants enriched in the highly conserved ALF (AF4-LAF4-FMR2) domain of AFF4 (*1-3,8-10*).

In this study, we identified a *de novo* heterozygous *AFF4* missense variant in a Chinese family with CHOPS syndrome through whole exome sequencing (WES) combined with Sanger sequencing. The heterozygous variant in *AFF4* (NM_014423.3), c.778A>G (p.Met260Val), was novel and classified as "pathogenic" following the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) guidelines. Herein, we also present the clinical and genetic findings of the patient and summarize the phenotypic and molecular characteristics of previously reported CHOPS syndrome cases.

2. Patient and Methods

2.1. Subject and clinical evaluation

A 9-year-old Chinese girl suspected of CHOPS syndrome, along with her available family members, was recruited from the Third Xiangya Hospital, Central South University, Changsha, China (Figure 1A). Detailed physical, neurocognitive, auditory, and ophthalmic examinations, laboratory tests, and radiographic assessments were performed by professionals to evaluate her growth pattern, craniofacial issues, intellectual disability, hearing loss, ocular signs, and anomalies in cardiac, pulmonary, skeletal, and other systems. Clinical data and peripheral blood samples were collected from the proband (II:1) and the available unaffected family members (I:1, I:2, and II:2). The study was conducted according to the tenets of the Declaration of Helsinki and under approval of the Institutional Review Board of the Third Xiangya Hospital. Written informed consent was obtained from all participants or their guardians.

2.2. G-banding karyotyping analysis

Conventional G-banding karyotyping analysis was performed at the Prenatal Diagnosis Center, Guizhou Provincial People's Hospital, Guiyang, China. In brief, a peripheral blood sample was taken from the patient for cell culture, colchicine treatment, and chromosome binding. After chromosome banding through trypsin using Giemsa staining, karyotyping analysis was carried out under a microscope (CytoVision®, Leica Biosystems, Germany).

2.3. Multiple ligation-dependent probe amplification (MLPA) and detection of aberrant methylation

Genomic DNA (gDNA) was extracted from the blood sample of the proband to perform MLPA and detection of aberrant methylation on the 15q11 region for Prader Willi Syndrome (PWS) or Angelman syndrome (AS) by BGI-Shenzhen (Shenzhen, China). Qualified DNA was denatured at 98°C for 5 min and hybridized with probes at 54°C for 15 min. Amplified probes were sequenced on Applied Biosystems 3730xl DNA analyzer (Thermo Fisher Scientific, Waltham, MA, USA) and data were analyzed using Coffalyser.Net software (MRC Holland, Amsterdam, Netherlands).

2.4. WES

WES was performed on the patient by BGI-Shenzhen. The gDNA was isolated from blood sample of the patient

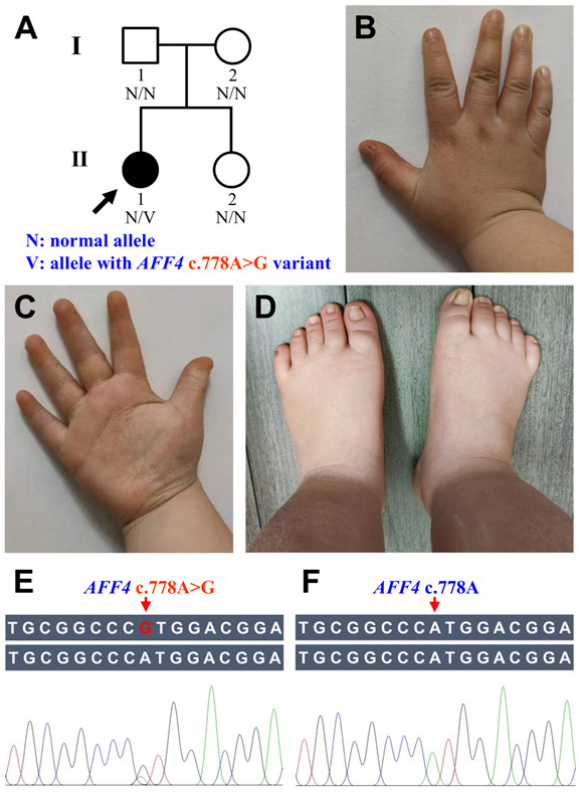

Figure 1. Pedigree, clinical, and molecular characterization of a Chinese proband with CHOPS syndrome. (A) Pedigree of a Chinese patient with CHOPS syndrome. Square represents male and circle represents female. Full black symbol and white symbol represent patient with CHOPS syndrome and unaffected individual, respectively. An arrow indicates the proband. **(B-D)** Hands and feet of CHOPS syndrome. **(E, F)** Sequencing of the *AFF4* c.778A>G variant in the proband and the wild-type c.778A in proband's unaffected father. *AFF4*, the ALF transcription elongation factor 4 gene.

using magnetic silica-coated sub-microspheres, and 50 ng of gDNA was fragmented randomly. Generated fragments of 100–500 bp were processed for end-repair, A-tailing, and adaptor ligation. Polymerase chain reaction (PCR) amplification and purification were conducted for further DNA nanoball preparation. Exome capture and enrichment were performed with KAPA HyperExome Probes (Roche, Basel, Switzerland). A DNA library was constructed utilizing combinatorial probe-anchor synthesis technology and DNA nanoballs, and sequencing was conducted on MGISEQ-2000 (MGI, Shenzhen, China).

2.5. Variant analysis and Sanger sequencing

After strictly filtering of the raw data obtained from the sequencing platform, the generated clean data was aligned with the human genome GRCh37/UCSC hg19 utilizing Burrows-Wheeler Aligner software. Genome Analysis Toolkit tools were used for base quality score recalibration and detection of single nucleotide variants (SNVs) and insertions/deletions (indels). High-confident SNVs and indels were further filtered and annotated against multiple databases of the human genome, including the Single Nucleotide Polymorphism database (dbSNP, build 156), Exome Sequencing Project 6500 (ESP6500, V2), the 1000 Genomes Project (1000G, Phase3), the Genome Aggregation Database (gnomAD, v4.1.0), the Exome Aggregation Consortium (ExAC, r0.3.1), the BGI-Phoenix genetic database (BPGD, V3.1), the BGI in-house variant database secondaryFinding Var (V1.1_2020.3), dbscSNV (v1.1), SpliceAI (v1.3), dbNSFP (v2.9.1), ClinVar, and the Human Gene Mutation Database (HGMD), as well as databases of mitochondrial genome containing Human Mitochondrial Genome Database (MtDB, *http://www.mtdb.igp.uu.se/*), MITOMAP (*https://www.mitomap.org/*), gnomAD (v.3.1), MitImpact_db (v3.0.6), APOGEE (v.1.0), MitoTIP (3.0.6), and HmtVAR (*https://www.hmtvar.uniba.it*). Variant pathogenicity was predicted based on multiple tools: MutationTaster (*http://www.mutationtaster.org/*), Sorting Intolerant from Tolerant (SIFT, *http://provean.jcvi.org/*), Protein Variation Effect Analyzer (PROVEAN, *http://provean.jcvi.org/*), Polymorphism Phenotyping version 2 (Polyphen-2, *http://genetics.bwh.harvard.edu/pph2/*), LoGoFunc predictor (*https://itanlab.shinyapps.io/goflof/*), PhyloP, and Genomic Evolutionary Rate Profiling (GERP) (*11-18*). Sanger sequencing was used to validate the potential variants identified by WES in the patient and her parents, using the following five pairs of primer sequences:

5'-ATCAACGCTCCAAATCACCTCG-3'
and 5'-ACAGATCTACGCATCCACTTGG-3',
5'-TCCCAACAATTCTGCAGTGA-3'
and 5'-GCTAAGCTTTCTATTTGGGCATA-3',
5'-TCTTCACGGTGCTGTAGAGTTT-3'
and 5'-CCATGTCCTCATCCACAATTTC-3',
5'-TGAGCCAAAGATGGATAACTGC-3'
and 5'-GACACTACTATCCACAGGTTCT-3',
5'-ATCCTATCCCAGTGCTGGTTCG-3'
and 5'-CACGTTAACTCCGCTCAACACC-3'.

For the second verification and analysis, Sanger sequencing for the candidate pathogenic variant was performed on family members with the following primers:

5'-TGGGCAGCACTCAACTCAAT-3'
and 5'-TGGTGAGATGTGCTTTGCTG-3'.

2.6. Copy number variation (CNV) analysis

The WES data were used to detect exome CNVs with ExomeDepth (BGI-Shenzhen). Sequence alignment, deduplication, GC bias correction, and CNV analysis algorithm setting were conducted. Following quality control, variation analysis results were filtered against databases including the Online Mendelian Inheritance in Man (OMIM), HGMD, and the Database of Genomic Variants, producing semiautomatic interpretation and classification.

2.7. Conservation analysis and variant evaluation

Conservation of protein sequences was analyzed by the Basic Local Alignment Search Tool (BLAST, *https://blast.ncbi.nlm.nih.gov/BlastAlign.cgi*). Three-dimensional structures of the wild-type and mutant AFF4 proteins were predicted using SWISS-MODEL tool (*http://www.swissmodel.expasy.org*), based on the model retrieved from the AlphaFold Protein Structure Database (AF-Q9UHB7-F1, *https://alphafold.com/*). PyMOL software (version 2.5.8, Schrödinger, LLC, Portland, USA) was used to visualize protein structures. Identified variant was classified following the ACMG/AMP variant classification guidelines (*19*).

2.8. RNA isolation, PCR, and gene expression analysis

Total RNA was isolated from the patient's leukocytes using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the extracted RNA was converted to complementary DNA (cDNA) using the First Strand cDNA Synthesis Kit (Toyobo, Japan). PCR amplification was conducted using the following primers:

5'-GGGCAGCACTCAACTCAAT-3'
and 5'-AGGTTCTGTTTTGCATGGTGT-3'.

PCR amplification products were checked by agarose gel electrophoresis and subsequently sequenced on Applied Biosystems 3730xl DNA analyzer (Thermo Fisher Scientific). Gene expression analysis was

conducted by Sanger sequencing of *AFF4* cDNA and comparison of the areas under peaks of wild-type and mutant alleles, which were quantified and calculated using ImageJ software (v1.54d, National Institutes of Health, USA). Statistical analysis was performed using Student's *t*-test *via* GraphPad Prism (v8.2.1, GraphPad Software, LLC, Boston, MA, USA) and $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. Clinical findings

The patient, a 9-year-old Chinese girl with non-consanguineous parents, was born at full term *via* vaginal delivery without postnatal problems. She showed a round face with a coarse appearance, facial fullness, arched eyebrows, and long eyelashes, along with brachydactyly (Figure 1B-D). At the age of 6 years, she was noted to have short stature. Her height was 101.8 cm, and her weight was 23.5 kg, indicating obesity. The ultrasound scan revealed no abnormalities of the uterus or its appendages. Based on extensive radiological screening, including skeletal maturation assessment, full-spine radiographs in frontal and lateral views, chest X-ray, and dedicated magnetic resonance imaging of the pituitary, and auxological and biochemical investigations, she was diagnosed with growth hormone deficiency, short stature, and vitamin D deficiency. Recombinant human growth hormone medication was started. Her height and weight began to increase over time, and after six months of treatment, ophthalmic examination showed increased intraocular pressure associated with the therapy, as well as amblyopia, myopia, and astigmatism.

At her latest clinic visit at 9 years old, her height was 116.5 cm (3rd percentile), her weight was 33.0 kg (75th–90th percentile), and her BMI was 24.31 kg/m^2. Her full-scale intelligence quotient was 72 based on the Wechsler Intelligence Scale for Children, indicating a marginal defect. Ultrasonic measurement of bone mineral density showed relatively low bone mineral density. Cardiac ultrasonography showed no significant cardiac abnormalities, except for mild pulmonary valve regurgitation. The results of auditory evaluations, including pure tone audiometry, otoacoustic emission, tympanometry, and acoustic reflex testing, were normal. Fiberoptic nasopharyngoscopy indicated adenoid hypertrophy (Table 1).

We reviewed the literature and summarized 17 patients with CHOPS syndrome worldwide, relating to 8 heterozygous *AFF4* variants, as shown in Table 1 (*1-3,8-10*). The patients usually exhibited cognitive impairment and characteristic facial features (100%, 17/17), short stature and skeletal involvement (100%, 17/17), obesity (94%, 16/17), heart defects (76%, 13/17), pulmonary involvement (76%, 13/17), and other features including hearing loss (59%, 10/17), ocular abnormalities (59%, 10/17), genitourinary abnormalities (47%, 8/17), and gastrointestinal issues (47%, 8/17). Our patient exhibited symptoms similar to those observed in previous CHOPS sufferers, including characteristic facial features, intellectual disability, obesity, short stature, and brachydactyly, as well as ocular abnormalities and adenoid hypertrophy. Intriguingly, a previously reported 7-year-old boy carrying a different substitution (c.779T>C, p.Met260Thr) occurring at the same position as our variant exhibited similar manifestations, including cognitive impairment, coarse facial features, obesity, short stature, brachydactyly, and ophthalmic abnormalities (*1*). However, our patient showed growth hormone deficiency, vitamin D deficiency, and adenoid hypertrophy, which were not observed in that boy, who exhibited additional symptoms including aspiration history, mixed hearing loss, and skin changes absent in our patient (*1*). Characteristic facial features, obesity, intellectual disability, and short stature also occur in other rare genetic disorders, such as PWS and AS (*20,21*). The obesity observed in CHOPS syndrome and PWS may result from excessive food-seeking behaviors or reduced resting energy expenditure due to impaired hypothalamic function (*1,2,20*).

3.2. Exclusion of chromosomal and other genomic disorders

Considering the overlap of clinical manifestations with other genetic disorders, karyotyping analysis, MLPA, DNA methylation analysis, and CNV analysis were performed. Karyotyping analysis of the patient's peripheral blood revealed a chromosome karyotype of 46, XX, without chimerism. MLPA and methylation detection showed unremarkable results and lack of large segment variation or abnormal DNA methylation on chromosome 15q11 region, excluding diagnosis of PWS or AS. No clinically significant chromosomal microdeletions or microduplications (over 100 kb) were found in this sample through CNV analysis. These results ruled out PWS, AS, and other significant genomic disorders.

3.3. WES and variant analysis

WES of the proband (II:1) produced a total of 29,462.20 Mb of raw data. The target sequence covered 99.88% and 99.80% of bases at >10× and >20×, respectively, with a mean sequencing depth across the target region of 405.47×. Five potential phenotype-related heterozygous variants were identified in the proband through WES and validated by Sanger sequencing, c.778A>G in *AFF4*, c.247dup in the component of oligomeric golgi complex 6 gene (*COG6*), c.129dup in the cereblon gene (*CRBN*), c.8612A>C in the lysine methyltransferase 2A gene (*KMT2A*), and c.1282A>C in the oligophrenin 1 gene (*OPHN1*). Sanger sequencing confirmed that the

Table 1. Clinical and genetic characteristics of CHOPS syndrome patients with *AFF4* variants

Case no.	Variant	Age (y)/ Sex	Cognitive impairment and coarse facies	Heart defects	Obesity	Pulmonary involvement	Short stature and skeletal dysplasia	Hearing loss	Ocular abnl	GU	GI	Additional features (*Ref.*)
1	c.758C>G, p.Pro253Arg	9/F	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Otorrhea, chronic bilateral otitis media, polycythemia, steroid-induced diabetes mellitus, iatrogenic adrenal insufficiency, and prematurity (*1*)
2	c.758C>T, p.Pro253Leu	9/F	Yes	Yes	Yes	Yes	Yes	NR	NR	NR	NR	Sinusitis, recurrent otitis media with effusion, and Moyamoya-like vasculopathy (*3*)
3	c.760A>G, p.Thr254Ala	17/M	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	NR (*1,2*)
4	c.760A>G, p.Thr254Ala	2/M	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	NR	NR (*9*)
5	c.761C>G, p.Thr254Ser	20/F	Yes	Yes	Yes	Yes	Yes	NR	NR	Yes	Yes	Coarse hair (*1,2*)
6	c.763G>A, p.Ala255Thr	11/F	Yes	Yes	Yes	Yes	Yes	NR	NR	NR	NR	Hypothyroidism (*1*)
7	c.772C>T, p.Arg258Trp	12/F	Yes	Yes	Yes	Yes	Yes	NR	Yes	Yes	Yes	Eczema (*1,2*)
8	c.772C>T, p.Arg258Trp	23/M	Yes	NR	Yes	NR	Yes	Yes	Yes	Yes	NR	Gynecomastia, acanthosis nigricans, and hirsutism (*1*)
9	c.772C>T, p.Arg258Trp	2/F	Yes	Yes	Yes	Yes	Yes	Yes	NR	NR	Yes	Obstruction of nasolacrimal duct, chronic bilateral otitis media, sacral mass (removed), and jaundice (*1*)
10	c.772C>T, p.Arg258Trp	6/M	Yes	Yes	Yes	Yes	Yes	Yes	Yes	NR	Yes	Growth hormone deficiency (*1*)
11	c.772C>T, p.Arg258Trp	7/F	Yes	Yes	Yes	NR	Yes	Yes	Yes	NR	Yes	Low lying conus with lipoma of the filum terminale (*1*)
12	c.772C>T, p.Arg258Trp	1/M	Yes	NR	Yes	NR	Yes	Yes	NR	Yes	NR	NR (*1*)
13	c.772C>T, p.Arg258Trp	17.5/F	Yes	Yes	Yes	Yes	Yes	NR	NR	NR	NR	NR (*8*)
14	c.772C>T, p.Arg258Trp	13/M	Yes	Yes	Yes	Yes	Yes	Yes	Yes	NR	NR	NR (*9*)
15	c.772C>T, p.Arg258Trp	0/M	Yes	Yes	NR	Yes	Yes	NR	NR	Yes	Yes	Congenital hypothyroidism and nervous system involvement (*10*)
16	c.778A>G, p.Met260Val	9/F	Yes	No	Yes	No	Yes	No	Yes	No	No	Adenoid hypertrophy, growth hormone deficiency, and vitamin D deficiency (*This study*)
17	c.779T>C, p.Met260Thr	7/M	Yes	NR	Yes	Yes	Yes	Yes	Yes	NR	NR	Sun-exposed erythema and mottling (*1*)

AFF4: the ALF transcription elongation factor 4 gene, y: years, F: female, M: male, Ocular abnl: ocular abnormalities, GU: genitourinary abnormalities, GI: gastrointestinal abnormalities, NR: data not reported in the original publication.

asymptomatic father (I:1) carried variants in *COG6*, *CRBN*, and *OPHN1*, and the asymptomatic mother (I:2) carried the *KMT2A* variant, while the *AFF4* variant was absent in all tested asymptomatic family members (I:1, I:2, and II:2), suggesting that the candidate pathogenic *AFF4* variant was *de novo* with paternity and maternity confirmed (ACMG/AMP: PS2, Figure 1E, F). Based on clinical features, auxiliary examinations, Sanger sequencing, and co-segregation analysis, the *de novo AFF4* variant was proposed as the genetic cause of the family, while another four variants were excluded. The heterozygous c.778A>G (p.Met260Val) variant in *AFF4*, a CHOPS syndrome-associated gene, was predicted to be deleterious by bioinformatics tools (ACMG/AMP: PP3), *via* a gain-of-function mechanism (Table 2). It was absent in multiple databases including ESP6500, 1000G, gnomAD, and ExAC, and has not been reported in the literature (ACMG/AMP: PM2, Table 2). Conservation analysis showed residue p.Met260 in the AFF4 protein is highly evolutionarily conserved from zebrafish to human (Figure 2A). The residue is located within a mutational hotspot area, in which all previously identified missense variants are reported to be pathogenic, including different amino acid substitutions at the same position, moreover, the two variants altered the significantly conserved amino acid residue and the resulting conformational changes are shown in Figure 2B (ACMG/AMP: PM1 and PM5) (*1*). cDNA sequencing confirmed the presence of the mutant allele c.778A>G, and comparative analysis of electropherograms from the patient showed no statistically significant difference between the wild-type and mutant alleles (p = 0.2151, Figure 2C), indicating that the mutant allele was not degraded and that the c.778A>G variant did not affect the relative gene expression. According to ACMG/AMP guidelines, the *AFF4* c.778A>G (p.Met260Val) variant was classified as "pathogenic" (PS2+PM1+PM2+PM5+PP3).

The *AFF4* gene, located on chromosome 5q31, is a fusion partner of the *KMT2A* gene (*22*). *AFF4* comprises 21 exons and encodes a 1,163-amino-acid protein AFF4 that serves as a scaffold for the SEC complex assembly (*22*). The AFF4 protein plays a monitoring role in transcription elongation, competing with AFF1 for binding to other SEC components (*16*). The SEC includes P-TEFb, a C-terminal domain kinase composed of cyclin-dependent kinase 9 (Cdk9) and cyclin T1/T2, contributing to the regulation of transcription elongation by the release of paused RNAP2 (*7,23*). In cells, the majority of P-TEFb is found reserved in the inactive 7SK small nuclear ribonucleoprotein particle complex, and upon signal stimulation, it is released and recruited by Brd4 and SEC, forming active complexes to stimulate transcription of inducible genes (*24*). The N-terminus of AFF4 (residues 1–300) can facilitate the autophosphorylation of Cdk9 at residue p.Thr186 by binding the Cdk9 T-loop to either Brd4 or AFF1/AFF4, which is essential for reconstituting P-TEFb and promoting the release of paused RNAP2 or restarting global gene transcription (*24*).

AFF4 includes an N-terminal intrinsically disordered region that interacts with other SEC subunits and a C-terminal homology domain (CHD) conserved among AFF1–AFF4 (*25*). The CHD of AFF4 can fold into a domain encompassing eight helices and mediates AFF4 homodimerization or heterodimerization with AFF1 (*25*). The domain contains a surface loop region that serves as a substrate for the P-TEFb Cdk9, promoting release of promoter-proximal paused RNAP2 (*25*). All identified *AFF4* variants associated with CHOPS syndrome are *de novo* missense variants clustered in a 13-amino-acid region (residues 251–263) located on conserved ALF homology domain of the AFF4 protein (Figure 2D), and all were predicted to be deleterious by *in silico* tools (*1,26*). The ALF homology domain is a 175-amino-acid motif with an average sequence identity of 82% among the AFF1, AF4/FMR2 family member 2 (AFF2, also termed LAF-4), and AF4/FMR2 family member 3 (AFF3, also termed FMR-2) proteins (*27*). The *de novo* c.772C>T (p.Arg258Trp) variant is the most common variant, accounting for over half of CHOPS syndrome cases, indicating that nucleotide 772 is a mutational hotspot. A likely explanation is that the C>T transition is the most common genetic change, resulting from replication of the U:G mispair after spontaneous hydrolytic deamination of cytosine and producing an aberrant uracil (*28*). The *AFF4* variants p.Pro253Arg, p.Thr254Ala, p.Thr254Ser, p.Ala255Thr, and p.Arg258Trp showed gain-of-function effects, leading to resistance to proteasomal degradation by SIAH1 and resulting in excessive accumulation of mutant AFF4. This accumulation causes alterations in RNAP2 distribution and transcriptional elongation abnormalities, which underlie CHOPS syndrome (*1,2*). The novel heterozygous *de novo AFF4* c.778A>G (p.Met260Val) variant identified in this study was absent from multiple databases. *In silico* analyses and ACMG/AMP criteria evaluation suggested that it was a "pathogenic" variant *via* a gain-of-function mechanism. Since p.Met260Val also locates in the critical ALF homology domain, a similar gain-of-function pathogenic mechanism may underpin development of CHOPS syndrome. Further cellular and animal model experiments are needed to thoroughly elucidate the detailed mechanisms by which abnormally elevated AFF4 proteins cause transcriptional abnormalities and affect downstream signaling pathways, which would deepen the understanding of their role in the overlapping clinical manifestations observed in other rare disorders, such as CdLS, PWS, and AS.

In conclusion, we identified a novel *de novo AFF4* variant, c.778A>G (p.Met260Val), as the genetic cause of CHOPS syndrome in a Chinese family. This discovery broadens the phenotypic and genetic spectrum of CHOPS syndrome and may enhance our understanding of its clinical and genetic features.

Table 2. Summary of *AFF4* variants in patients with CHOPS syndrome

Items	Variant 1	Variant 2	Variant 3	Variant 4	Variant 5	Variant 6	Variant 7	Variant 8
Exon	3	3	3	3	3	3	3	3
Nucleotide change	c.758C>G	c.758C>T	c.760A>G	c.761C>G	c.763G>A	c.772C>T	c.778A>G	c.779T>C
Amino acid change	p.Pro253Arg	p.Pro253Leu	p.Thr254Ala	p.Thr254Ser	p.Ala255Thr	p.Arg258Trp	p.Met260Val	p.Met260Thr
dbSNP rs number	No	No	rs786205233	rs786205679	No	rs786205680	rs2150098305	rs1761368316
1000G	No	No	No	No	No	No	No	No
ESP6500	No	No	No	No	No	No	No	No
ExAC	No	No	No	No	No	No	No	No
gnomAD	No	No	No	No	No	No	No	No
ClinVar	No	Pathogenic/ Likely pathogenic	Pathogenic	Pathogenic/ Likely pathogenic	No	Pathogenic/ Likely pathogenic	Likely pathogenic	Uncertain significance
HGMD accession number	CM199228	No	CM152549	CM152550	CM199227	CM152551	No	CM199229
MutationTaster	Disease causing	Disease causing	Disease causing	Disease causing	Disease causing	Disease causing	Disease causing	Disease causing
PolyPhen-2	Probably damagin g	Probably damaging	Probably damaging	Probably damaging	Probably damaging	Probably damaging	Possibly damaging	Possibly damaging
PROVEAN	Deleterious	Deleterious	Deleterious	Deleterious	Deleterious	Deleterious	Deleterious	Deleterious
SIFT	Damaging	Damaging	Damaging	Damaging	Damaging	Damaging	Damaging	Damaging
LoGoFunc	Gain of function	Gain of function	Gain of function	Gain of function	Gain of function	Gain of function	Gain of function	Gain of function
ACMG/AMP classification	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Pathogenic
ACMG/AMP criteria	PS2+PS3+PM2+ PP3+PP4	PS2+PM2+PM5+ PP3+PP4	PS2+PS3+PM2+ PP3+PP4	PS2+PS3+PM2+ PP3+PP4	PS2+PS3+PM2+ PP3+PP4	PS2+PS3+PM2+ PP3+PP4	PS2+PM2+PM5+ PP3+PP4	PS2+PM2+PM5+ PP3+PP4

AFF4: the ALF transcription elongation factor 4 gene, dbSNP: Single Nucleotide Polymorphism database, rs: Reference SNP, 1000G: 1000 Genomes Project, ESP6500: Exome Sequencing Project 6500, ExAC: Exome Aggregation Consortium, gnomAD: Genome Aggregation Database, HGMD: Human Gene Mutation Database, PolyPhen-2: Polymorphism Phenotyping version 2, PROVEAN: Protein Variation Effect Analyzer, SIFT: Sorting Intolerant from Tolerant, ACMG/AMP: American College of Medical Genetics and Genomics and the Association for Molecular Pathology, PS: pathogenic strong, PM: pathogenic moderate, PP: pathogenic supporting.

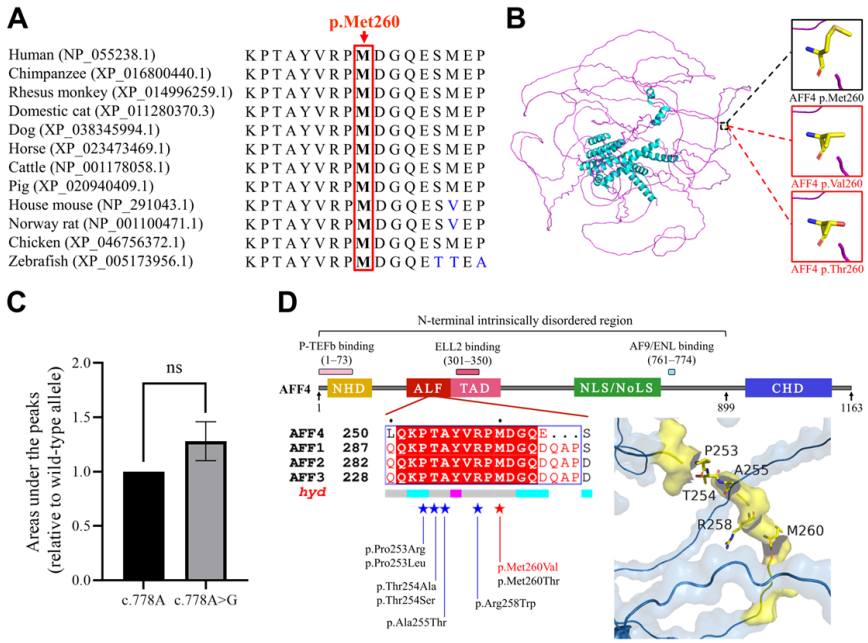

Figure 2. Effect of the *AFF4* c.778A>G (p.Met260Val) variant and schematic representation of the AFF4 protein illustrating the identified variants in CHOPS syndrome. (A) Conservation analysis of the AFF4 p.Met260 residue. **(B)** Cartoon model of the AFF4 protein structure by PyMOL based on the SWISS-MODEL: the methionine and the altered valine and threonine at position 260 are shown as stick models. **(C)** The areas under the peaks of wild-type and c.778A>G mutant alleles by sequencing analysis of *AFF4* complementary DNA from the proband. **(D)** Scheme of AFF4 protein demonstrating the location of the *de novo* missense variants identified in the patients with CHOPS syndrome. All reported *AFF4* variants are located within the highly conserved ALF homology domain of AFF4. The interaction regions of AFF4 with P-TEFb, ELL2, and AF9/ENL are shown as rectangles on top of the human AFF4 scheme. Sequence alignments of human AFF1–AFF4 proteins suggest conservation of the 13-amino-acid region in ALF homology domain. Sequences used (proteins and UniProtKB accession numbers): AFF1 (P51825), AFF2 (P51816), AFF3 (P51826), and AFF4 (Q9UHB7). The hydropathy is rendered by a bar below, where pink indicates hydrophobic, grey indicates neutral, and cyan indicates hydrophilic. Sequence alignments and hydropathic properties were visualized using the ESPript 3.0 web server *(26)*. Highly conserved 13-amino-acid residues are highlighted with red background. Mutated residues reported in previous studies and in this study are indicated by stars under the sequences. Three-dimensional structure model of wild-type AFF4 protein (residues 250–265) visualized by PyMOL is shown at the bottom right corner. The 13-amino-acid region is marked in yellow as surface and the corresponding mutant residues were labeled and depicted as sticks. AF9/ENL, ALL1-fused gene from chromosome 9/eleven-nineteen leukemia; AFF1–AFF4, AF4/FMR2 family member 1–4; *AFF4*, the ALF transcription elongation factor 4 gene; ALF, AF4-LAF4-FMR2; CHD, C-terminal homology domain; ELL2, RNA polymerase II elongation factor ELL2; *hyd*, hydropathy; NHD, N-terminal homology domain; NLS, nuclear localization signal; NoLS, nucleolar localization signal; ns, not significant; P-TEFb, positive transcription elongation factor b; TAD, transactivation domain..

Acknowledgements

We acknowledge and thank the patient and her family members.

Funding: This work was supported by Natural Science Foundation of Hunan Province (2023JJ30715), Scientific Research Project of Hunan Provincial Health Commission (A202303018385), Health Research Project of Hunan Provincial Health Commission (W20243024), Distinguished Professor of the Lotus Scholars Award Program of Hunan Province, Sublimation Scholars Project of Central South University, Wisdom Accumulation and Talent Cultivation Project of the Third Xiangya Hospital of Central South University (YX202109), and College Students' Innovative Training Plan Program (S202510533147).

Conflict of Interest: The authors have no conflicts of interest to disclose.

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Received July 11, 2025; Revised August 2, 2025; Accepted August 10, 2025.

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Released online in J-STAGE as advance publication August 15, 2025.