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Research Article

A New Heterozygous Deletion Mutation of the *SYCP2* Gene Caused Male Infertility Due to Non-Obstructive Azoospermia

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ABSTRACT

Infertility affects 10%-20% of heterosexual couples worldwide and 50% are due to male factors. The most severe type is non-obstructive azoospermia, which is distinguished by a total lack of sperm in the seminal fluid. However, the genetic causes of non-obstructive azoospermia have been incompletely understood. Here, an investigation was conducted on a Chinese family with non-obstructive azoospermia to examine its pathogenesis. Using whole-exome sequencing, we identified a new heterozygous deletion mutation (c.1937_1942delTAAATA, p. Ile646_Asn647del) in exon 24 of *SYCP2* gene. SYCP2 is a synaptonemal complex protein that plays an essential role in meiosis. Conservation analysis indicated that amino acid at position 647 was highly conserved among different species. Moreover, there was a notable modification in the three-dimensional transformation of the mutant SYCP2 protein. In vitro functional experiments showed that the SYCP2 protein expression decreased in HEK293T cells transfected with plasmids containing *SYCE2* c.1937_1942delTAAATA. Immunofluorescence staining showed that the subcellular localization of mutant SYCP2 protein altered, which was present in both the cytoplasm and nucleus, whereas wide-type SYCP2 was only found in the nucleus. In conclusion, our research suggests that the heterozygous deletion variant *SYCP2* c.1937_1942delTAAATA causes non-obstructive azoospermia occurrence and ultimately result in male infertility. This study expands the variant spectrum of non-obstructive azoospermia-associated genes and highlights the essential role of *SYCP2* in spermatogenesis.

Keywords: Infertility; Non-obstructive azoospermia; Deletion mutation; *SYCP2*; Synaptonemal complex

INTRODUCTION

Approximately 10%-20% of heterosexual couples worldwide experience infertility [1], which is characterized by failure to conceive after engaging in regular unprotected sexual intercourse for 12 months. Approximately half of the infertility cases are believed to be caused by male factors [2]. Male infertility can be attributed to various factors, which can be categorized into four main causes: quantitative deficiencies in spermatogenesis, dysfunction or obstruction of the ductal system, disturbances in the hypothalamic-pituitary axis and qualitative deficiencies in spermatogenesis [3]. Among the male infertility community, azoospermia is considered the most clinically severe form. Azoospermia, which is defined as the total lack of sperm in the semen, is found in approximately 1% of males overall and in 10%-15% of males who are unable to conceive [4]. Azoospermia

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exhibits a wide range of phenotypic presentations, which can be categorized into Non-Obstructive Azoospermia (NOA) and Obstructive Azoospermia (OA) based on the underlying cause. OA is characterized by a lack of sperm in the ejaculate resulting from a blockage in sperm transportation, whereas NOA is caused by either primary or secondary testicular dysfunction [5]. NOA, an important type of azoospermia, represents >70% of all azoospermia cases [6].

Our current understanding shows that NOA appears to be strongly associated with genetic factors. Due to the significant advancements in high-throughput sequencing in recent years, Whole-Exome Sequencing (WES) has emerged as a valuable asset in identifying genetic variations linked to NOA. Recent extensive WES investigations concerning NOA have revealed that established genetic elements contribute to 23% of the etiology of this disorder [7], emphasizing the crucial involvement of genetics in NOA progression. Mutations in meiotic genes are primarily responsible for NOA occurrence. Many genes have been identified and their genetic variants may result in human NOA, including *C14orf39* [8], *DMC1* [9], *KASH5* [10], *MSH4* [11], *SHOC1* [12,13], *SPINK2* [14], *SYCP1*, *SYCP2*, *TEX11*, etc. Among them, Synaptonemal Complex Protein 2 (*SYCP2*), an essential molecule, plays an irreplaceable role during sperm occurrence and development.

SYCP2 is located on chromosome 20q13.33 and consists of 45 exons. In addition, it encodes an intracellular protein containing 1530 amino acids. Moreover, SYCP2 protein in the human body has tissue specificity since it is mainly expressed in testicular tissue. In previous studies, Offenberg et al., discovered for the first time that *Sycp2* was transcribed specifically in meiotic prophase cells in rat testes. It can bind to DNA and assemble into axial elements. Yang et al., conducted a series of mouse experiments to prove that SYCP2 plays a crucial role in the Synaptonemal Complex's (SC) assembly and synapsis of chromosomes during male meiosis. *Sycp2-/-* male mice appear sterile. By utilizing *sycp2* hypomorphic and knockout mutant zebrafish lines, Takemoto et al. demonstrated that Sycp2 plays a crucial role in SC formation, as well as in the early stages of homologous pairing and meiotic recombination in vertebrates. These results indicate that *SYCP2* mutation may influence male genital cell meiosis, which further causes male infertility [15].

To date, there have been only two documented instances where NOA is linked to *SYCP2* mutations. However, the causes of NOA have been incompletely understood. The current research focuses on examining a Chinese Han family affected by NOA. To investigate the etiology of NOA, we examined pathogenic mutations in this family by analyzing clinical phenotypes and functional aspects.

MATERIALS AND METHODS

Patient

A 24-year-old male patient who had previously undergone sperm testing at different hospitals was referred to the Department of Andrology at the Reproductive Medical Center, Women and Children's Health Hospital of Guangxi Zhuang Autonomous Region, for additional examination due to sperm loss. This study was approved by the Institutional Review Board and Ethics Committee of Guangxi Maternal and Child Health Hospital (GXMC20230310) and performed in accordance with the Declaration of Helsinki. All participants provided written consent before participating in the study [16].

WES and Sanger sequencing

WES was conducted on the blood samples from the proband. Azoospermia-associated gene mutations within exon 24 of *SYCP2* identified by WES were confirmed by Sanger sequencing. Blood samples from the proband's parents were subjected to Sanger sequencing.

Bioinformatics analyses

Iterative Threading ASSEmbly Refinement (I-TASSER) was utilized to forecast the three-dimensional (3D) protein structures of mutant (MUT) and wild-type (WT) SYCP2 proteins. The prediction can be found at the website (https://zhang group.org//I-TASSER/). The sequences of SYCP2 from various species were acquired from the UniProt database (https://www.uniprot.org/). We aligned SYCP2 sequences among different species on the T-coffee website (https://www.ebi.ac.uk/Tools/msa/tcoffee/) to analyze the conservation of the mutant sites [17].

Plasmids construction and cell transfection

The pEGFP-C1 vector was used to generate the WT plasmid by directly inserting the cDNA of human *SYCP2*. Using the WT plasmid as a template, the MUT plasmid was created and verified using Sanger sequencing. The analysis of function was conducted using HEK293T cells, derived from human embryonic kidney, that were cultured in complete medium prepared with 90% DMEM(Gibco, USA) and 10% fetal bovine serum (Gibco, USA) at a temperature of 37°C and with a CO_2 concentration of 5%. HEK293T cells were transfected with recombinant plasmids carrying either MUT or WT *SYCP2* genes at a ratio of 3:1 for PEI (1 μg/μL) to the plasmid [18].

RNA expression analysis

HEK293T cells were transfected with 1.5 μg/well of the recombinant plasmids in 12-well plates. Following a 24-h transfection period, the cell lysates were utilized to extract total RNA with Trizol reagent (Invitrogen, USA). Subsequently, the RNA was reverse-transcribed into cDNA using RevertAid Master Mix (Thermo Scientific, USA). PowerUp™ SYBR™ Green Master Mix (Thermo Scientific, USA) was used for quantitative real-time PCR (qPCR) to compare *SYCP2* mRNA expression levels between HEK293T cells carrying WT and MUT plasmids. The $2^{-\Delta\Delta CT}$ method was used to calculate results. QPCR primers for amplifying *SYCP2* were as follows: the forward primer sequence was AGATTCACAGGCAGCGGAAA and the reverse primer sequence was TACAGGTGTCCAACATGCCC. The forward primer for GAPDH amplification was ATCAGCAATGCCTCCTGCAC and the reverse primer was TGGCATGGACTGTGGTCATG.

Western blotting analysis

Cells transfected with recombinant plasmids harboring normal or altered *SYCP2* were collected and rinsed with chilled PBS (Gibco, USA). To lyse the cells and prevent protein degradation, RIPA lysis buffer (Beyotime, China) was utilized, along with adding 1% PMSF (Beyotime, China). Next, the protein lysates were separated using 7.5% SDS-polyacrylamide gel electrophoresis (EpiZyme, China) and transferred to PVDF membranes (Millipore, USA). Following a 1.5-h incubation with 5% nonfat milk at room temperature, the membranes were incubated with either SYCP2 rabbit polyclonal antibody (CN.A16098, ABclonal, China) or β-tubulin rabbit polyclonal antibody (CN.10094-1-AP, Proteintech, China) overnight at 4°C. After incubation with goat secondary antibodies (CN.CW0103S, CWBIO, China) at ambient temperature for 1 h, protein expression was detected using an enhanced chemiluminescent reagent (NCM Biotech, China) and digital chemiluminescence system (Tanon Science and Technology, China) [19].

Immunofluorescence staining

HEK293T cells were grown on confocal dishes and treated with 4% paraformaldehyde for 15 min after 48 h of transfection. After rinsing thrice with PBS, the samples were treated with 0.2% Triton X-100 (Thermo Fisher Scientific, USA) for 15 min to achieve permeabilization. Finally, the cells were washed thrice with PBS and stained with DAPI (Beyotime, China) for 10 min in the dark. A inverted laser confocal microscope (LSM 880; Carl Zeiss, Germany) was utilized to observe the fluorescence of HEK293T cells [20].

Statistical analysis

Statistical analyses were conducted using the GraphPad Prism 10.0 software. A minimum of three independent biological replicates were conducted for all experiments. The results are expressed as the mean ± standard deviation. The two independent sample t-test was employed to determine the statistical significance of the disparities between the two groups. We defined a P-value<0.05 as a statistically significant difference. Asterisks indicate significance in more detail, where *means $p<0.05$, **means $p<0.01$, ***means $p<0.001$ and ****means $p<0.0001$ [21].

RESULTS

Clinical phenotype

The proband had a history of male infertility for more than one year. He was married in 2021 and did not take any precautions after marriage. However, to date, he has been infertile. He had a normal sexual life and could ejaculate smoothly, with a moderate volume, without erectile dysfunction or premature ejaculation. Regarding medical history, no consanguineous marriage was observed in the Proband's family. Physical examination revealed that the proband had normal male secondary sexual characteristics and the volume of the bilateral testes was 12 mL. The texture of his bilateral epididymis was normal, without nodules, tenderness or varicoceles. The vas deferens of both sides can be palpated. Laboratory tests revealed that the serum FSH level was within the normal range (4.28 mIU/mL). The karyotype was 46, XY, del (10) (p13). Genetic testing for Y chromosome microdeletions yielded negative results. Nevertheless, semen analysis revealed a normal volume but an absolute absence of sperm (Table 1) [22].

Table 1: Semen analysis results of the proband.

Items	Results	Reference value
Liquefaction time	20 min	<60 min
Consistency	Medium	-
pH	7.5	≥ 7.2
Color	Gray-white	Gray-white, light yellow
Volume of sperm	3.4	≥ 1.5 mL
Concentration of sperm	0	≥ 15 × 10^6/mL
Percentage of the progressively motile	0	≥ 32%
Percentage of the non-progressively motile	0	-
Percentage of the immotile	0	<60%
Total count of sperm	0	-

Concentration of round cells	3.5 × 10^6/mL	<5 × 10^6/mL
Quantification of seminal plasma fructose	122.8	≥ 13 μmol/a ejaculation
Quantification of seminal plasma neutral α-Glucosidase	87.4	≥ 20 mU/a ejaculation

Mutation detection and bioinformatics analysis

To identify the cause of azoospermia, we conducted a genealogical analysis of this lineage. The lineage map was shown in Figure 1A. We collected the proband's peripheral blood for WES. The results indicated that the patient carried a novel heterozygous deletion variant, c.1937_1942delTAAATA, p. Ile646_Asn647del, located at exon 24 of *SYCP2* on chr20 (Figure 1B). SYCP2 participates in SC formation during meiosis. This deletion mutation may be a possible pathogenic mutation in azoospermia. Sanger sequencing confirmed the deletion mutation of *SYCP2* in the proband (Figure 1C). The mutation also existed in the proband's mother, indicating that it was inherited from her. To estimate amino acid conservation in SYCP2 p.I646 and p.A647, we compared sequences from various species. The results depicted that the amino acid at position 647 was highly conserved, whereas the amino acid at position 646 was not (Figure 2A). Furthermore, a notable disparity was observed in the protein's 3D structures between WT and MUT, as depicted in Figure 2B [23].

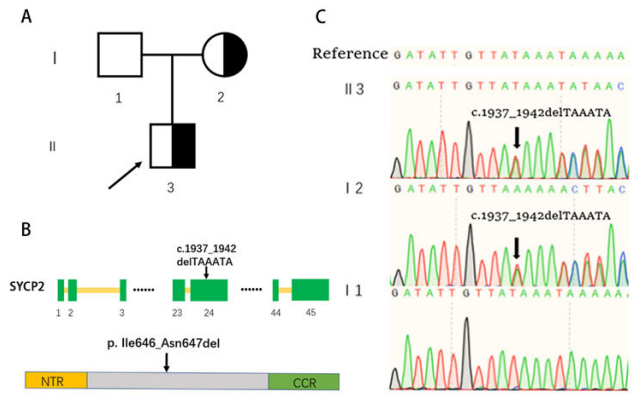

Figure 1: Identification of a novel deletion mutation c. 1937_1942delTAAATA in *SYCP2* gene. (A) The pedigree chart of the NOA family. Black arrow indicates the proband. (B) A schematic diagram of the *SYCP2* mutation c. 1937_1942delTAAATA, p. Ile646_Asn647del. Punctuation means ellipsis. NTR represents N-terminal region and CCR represents C-terminal coiled-coil domain. (C) Sanger sequencing confirmed the mutation in the proband and his parents.

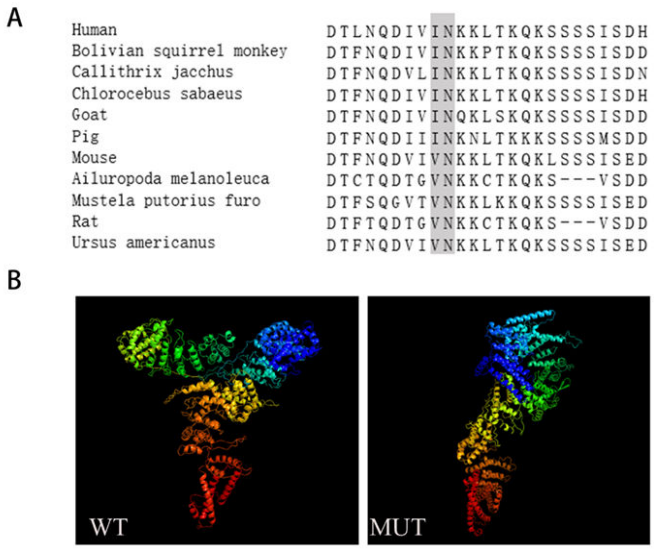

Figure 2: Biological analysis of the mutation. (A) The conservation analysis of the amino acid mutant sites. The Asn at 647 was highly conserved while the Ile at 646 was not. (B) The 3D structure visualization of the Wide-Type (WT) and mutant (MUT) protein. The mutant models of p. Ile646_Asn647del is significantly distinct compared with the wide-type.

Functional analysis after plasmid transfection

To clarify the impact of *SYCP2* p.Ile646_Asn647del on SYCP2 expression, we transfected MUT and WT plasmids into HEK293T cells and extracted RNA and protein. In the level of *SYCP2* mRNA expression, we didn't find any difference between WT and MUT groups (Figure 3A). At the protein level, the expression of the MUT SYCP2 protein was significantly decreased compared with that of the WT protein (P=0.0101, Figure 3B and 3C). Furthermore, immunofluorescence was conducted to determine the subcellular localization of MUT and WT proteins following transfection with *SYCP2* recombinant plasmids. It was observed that MUT SYCP2 was present in both the cytoplasm and nucleus, whereas WT SYCP2 was only found in the nucleus (Figure 4) [24].

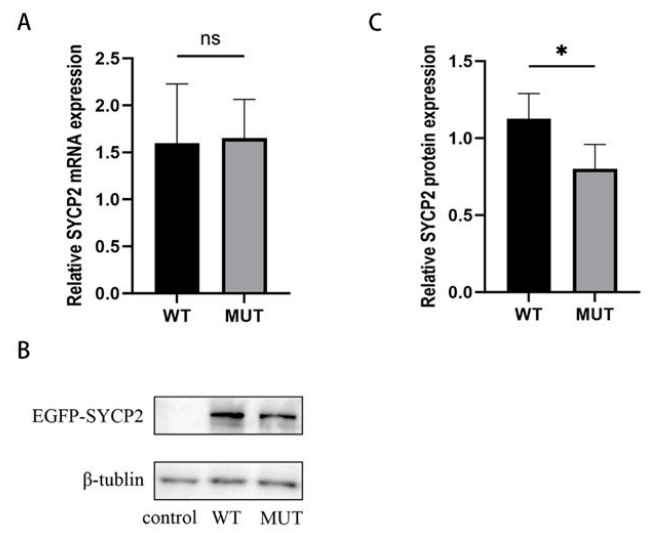

Figure 3: The effect of the mutation on SYCP2 expression. (A) Relative SYCP2 mRNA expression of HEK293T cells after transfected with wide-type (WT) and mutant (MUT) plasmids (ns, no significance). (B-C) Western blotting tested the SYCP2 protein expression of wild-type and mutant *SYCP2* gene in HEK293T cells (*P=0.0101). The control group is HEK293T cells transfected with blank plasmid.

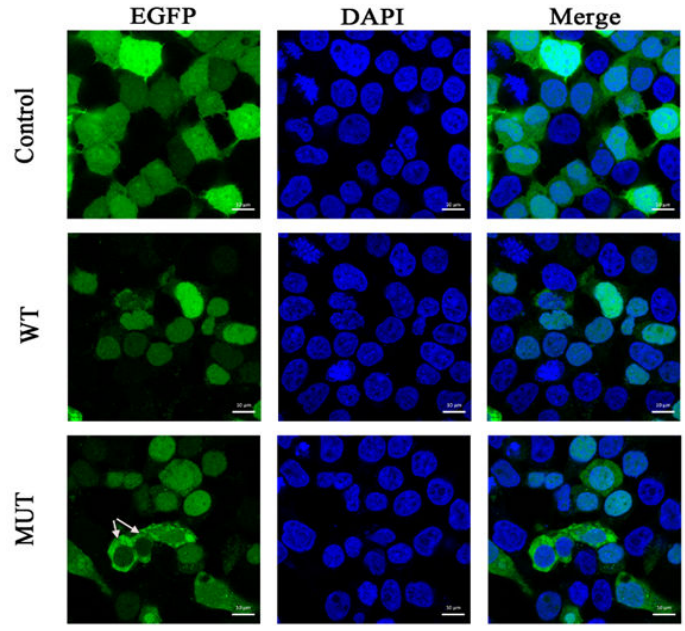

Figure 4: Immunofluorescence staining revealed the subcellular location of Wide-Type (WT) and mutant (MUT) SYCP2 in HEK293T cells. Wide-type SYCP2 was exclusively located in nucleus. Mutant SYCP2 not only located in nucleus but also in cytoplasm as red arrow indicated. Confocal images of EGFP (green), DAPI nuclear staining (blue) and merged signals.

DISCUSSION

In the current study, we discovered a new deletion mutation c. 1937_1942delTAAATA in *SYCP2* from a family with NOA affected by one person. It has been verified that this genetic alteration causes a reduction in the production of the SYCP2 protein, alteration in its distribution within the cell and consequently leads to sperm disorders and inability to conceive. The results of our study emphasize the crucial function of *SYCP2* in spermatogenesis and broaden the range of genetic variations associated with NOA. In addition, our findings offer fresh perspectives on NOA development [25].

The gold standard investigation for male infertility is a semen analysis conducted following the guidelines of the World Health Organization (World Health Organization, 2010). In this family, the proband was infertile for more than one year without taking precautions. Semen analysis displayed that the sperm concentration was zero, which conformed to clinical azoospermia. Using WES, we identified a heterozygous *SYCP2* frameshift variant (c.1937_1942delTAAATA) in the proband. Sanger sequencing verified his mother as a *SYCP2* (c. 1937_1942delTAAATA) heterozygote. The *SYCP2* mutation in the proband was derived from his mother. According to the lineage map, the inheritance mode was dominant. This is consistent with the inheritance mode of an azoospermic family reported by Schilit in 2019, in which the proband and his mother were *SYCP2* (c.2793_2797del) heterozygote. Because of the specificity of SYCP2 protein expression in the human body and the genital differences between males and females, *SYCP2* mutations cause male infertility while females are fertile. Consequently, it is plausible that most *SYCP2* mutations are inherited maternally. However, it is notable that Xu et al. reported a male infertile patient with a homozygous mutation in *SYCP2* (c.2689_2690insT) presented with NOA, whose parents were *SYCP2* (c.2689_2690insT) heterozygote. This finding contradicts the results of the current study. We believe that haploinsufficiency and incomplete dominance may be the reasons. As a result of different expressivity caused by *SYCP2* encoding errors and deletions, disease manifestations vary from oligozoospermia to azoospermia. The differences between the present study and animal experiments can also be explained by this notion. In a previous study, male *Sycp2-/-* mice were infertile, whereas *Sycp2+/-* mice were fertile. This may be due to human haploinsufficiency and/or mouse incomplete dominance. Furthermore, the interplay between genes and the environment is intricate and this collaboration may collectively govern the manifestation of various phenotypes. Moreover, there may be certain compensatory mechanisms in mice that are incompletely understood, but they still play an equal role in shaping the ultimate phenotype [26,27].

SYCP2 encodes synaptonemal complex protein 2, which participates in meiosis in sexually reproducing organisms. Meiosis is a unique cellular fission process that decreases chromosome count by half *via* two consecutive rounds of meiotic division, forming haploid gametes, SYCP2 facilitates the formation of pairs of homologous chromosomes by constructing SC during meiosis. The highly conserved protein structure known as SC is crucial in connecting homologous chromosomes in various species. The SC consists of three components: the Central Component (CC), Transverse Filaments (TF) and Lateral Elements (LE). Among them, the LE mainly comprises SYCP2 and SYCP3 proteins. The interaction between these two proteins forms a central core for the chromosome axis, providing

a framework for chromatin looping and assisting in the assembly of the chromosome axis [28,29].

To investigate the impact of c.1937_1942delTAAATA on the functionality of SYCP2, we constructed a MUT *SYCP2* vector specifically for conducting *in vitro* experiments. In our functional investigations, we found that MUT SYCP2 expression was decreased compared to the WT SYCP2 protein and the two independent samples t-test demonstrated that the difference was statistically significant. The reduction in SYCP2 protein may lead to incomplete SC, further influencing homologous chromosome synapsis. Additionally, we found significant differences between the MUT and WT SYCP2 proteins by analyzing the protein 3D structures. The C-terminal coiled-coil domain of SYCP2 can recruit SYCP3 and incorporate SYCP3 into the LE. SYCP3 effectively localizes to the SC in WT mice, but in *Sycp2-/-* mice, it forms aggregates within the nucleus instead of localizing to the LE. Moreover, the N-terminal region (NTR) of SYCP2 mediates the combination of the centromere with the SC. SYCP2NTR interacts with two centromere proteins, CENP J and CENP F. The significant change in the 3D structure of the MUT SYCP2 protein may cause SYCP3 to combine unsuccessfully and then fail to form a holonomic SC while also affecting the centromere combined with the SC. This study also observed that the novel *SYCP2* variant (c.1937_1942delTAAATA) led to abnormal subcellular localization. This may be because the novel mutation influences the nuclear localization signal. Abnormal nuclear localization of proteins may further aggravate the poor integrity of the SC during meiosis. However, the underlying molecular mechanisms must be explored in depth [30].

CONCLUSION

In summary, we detected a heterozygous deletion mutation (c. 1937_1942delTAAATA, p. Ile646_Asn647del) in *SYCP2* through WES in a NOA family. Functional *in vitro* studies revealed the influence of the new variant on SYCP2 protein localization and expression. These results suggest that the *SYCP2* novel mutation leads to abnormal meiosis by affecting SC formation, ultimately resulting in male NOA. This investigation is advantageous in enhancing our understanding of the molecular foundation of meiosis abnormality and the pathological mechanism of NOA.

DATA AVAILABILITY STATEMENT

The data underlying this article are available in the article and in its online supplementary material, further inquiries can be directed to the corresponding authors.

ETHICAL APPROVAL

This study was approved and performed in accordance with the Institutional Review Board and Ethics Committee of Guangxi Maternal and Child Health Hospital (GXMC20230310). Patients and relatives analyzed in the project signed informed consent prior to inclusion.

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AUTHORS' CONTRIBUTIONS

Lin Tan and Jianan Liu designed this study. Qi Yang recruited the patient and collected clinical datas. Lin Tan, Jianan Liu, Weijun Zhong, Zhongzhi Gan and Qian Liu performed functional experiments and data analysis. Lin Tan and Jianan Liu performed biological analysis. Lin Tan, Qi Yang and Jianan Liu drafted the manuscript. Fu Xiong and Xuedong Wu were responsible for the manuscript review. All authors contributed to acquisition of data and draft of the manuscript. All authors have read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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