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Research article

# Novel heterozygous variant of *ADPRHL2* causes pathogenic variation in CONDSIAS

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ABSTRACT

Adprhl2 (OMIM: 610624) mutation associated stress-induced childhood-onset neurodegeneration with variable ataxia and seizures (CONDSIAS, OMIM: 618170) is a sporadic neurodegenerative disease with poor prognosis. *ADPRHL2* encodes ADP-ribosylhydrolase 3 (ARH3), which participates in ADP-ribosylation to remove poly-ADP ribose (PAR). We found a new compound heterozygous mutation in the *ADPRHL2* gene c.580C > T (p.Gln194Ter) and c.803-1G > A in a 30-month-old boy, who showed gait instability, abnormal EEG, and developmental delay after respiratory infection. He died of convulsions 4 months after onset. By constructing a mutant plasmid and using Western blot to detect the expression of ARH3 and PAR, it was demonstrated that the *ADPRHL2* gene c.580C > T (p.Gln194Ter) and c.803-1G > A is pathogenic according to ACMG guidelines.

## 1. Introduction

CONDSIAS is caused by a mutation in the *ADPRHL2* gene (OMIM: 6106240). The main clinical manifestations include gait abnormalities, ataxia, epilepsy, and neurological degeneration [1]. The *ADPRHL2* gene is located on chromosome 1p34.3 and encodes ADP-ribosylated hydrolase protein 3 (ARH3). ARH3 plays a role in DNA damage repair by primarily engaging in ADP-ribosylation, a reversible post-translational modification of proteins crucial for preserving genome integrity and facilitating the repair of DNA damage in cells [2–5]. Poly ADP ribose polymerase 1 (PARP1) modifies proteins by chains of repeating ADP-ribose units that are referred to as poly(ADP-ribose) (PAR). PARP1 enzymatic activity is activated explicitly by binding to DNA breaks, which promotes timely DNA repair [6]. However, the excessive migration of PAR to the cytoplasm triggers mitochondrial-dependent PAR binding to the apoptosis-inducing factor (AIF) PAR binding site, releasing AIF from mitochondria into the nucleus, leading to DNA breakage and cell death [7,8]. In mammals, two enzymes can reverse PARylation, namely ARH3 and poly ADP ribose glycohydrolase (PARG). PARG has both exonuclease and endonuclease activities, acting on the terminal and internal sites of PAR, respectively, and can hydrolyze ADP ribose long chains. ARH3 has the crucial function of removing mono-ADP ribose units from proteins at the serine site, which PARG

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does not have [9]. ARH3 knockout cells survived well under non-stress conditions. However, after DNA damage induced by hydrogen peroxide ($H_2O_2$), these cells exhibited an accumulation of PAR. Therefore, ARH3 is a critical enzyme in DNA damage repair, which controls the PAR content and determines the cell fate during the reaction [7,10].

Our previous study first reported CONDSIAS induced by compound heterozygous variants in the ADPRS gene. A two-and-a-half-year-old male patient exhibited gait instability after a respiratory tract infection, rendering him incapable of autonomous ambulation. Neurological assessment unveiled acute cerebellar ataxia, aberrant EEG readings, and a marginally expanded cerebellar fissure on cerebral MRI imaging. Notably, the cerebrospinal fluid glucose test yielded a positive result. Laboratory analysis indicated diminished levels of thyroid-stimulating hormone alongside elevated plasma lactate and serum creatine kinase concentrations. Tragically, four months post-onset, the boy succumbed to sudden seizures. Through comprehensive next-generation sequencing, we discerned two novel compound heterozygous ADPRS variants: c.580C > T (p.Gln194Ter) and c.803-1G > A (referred to as 580 and 803 hereafter). RNA-seq implicated the 580 mutation in potential nonsense-mediated mRNA decay. Conversely, the 803 variant emerged as a splice site mutation, precipitating intron 5 retention. This investigation aims to delve deeper into the functional ramifications of these two variants.

## 2. Methods

### 2.1. Western blot analysis

Mutant sites were introduced into the pcDNA3.1 plasmid via homologous recombination, amplified, and subsequently transfected into HEK293 cells for expression. Cell lysates from HEK293 cells were subjected to SDS-PAGE analysis. The separated peptides were transferred onto a PVDF membrane (Millipore, USA), followed by Western blotting using a standard protocol. Immunoreactive proteins were visualized using an enhanced chemiluminescence (ECL) reagent kit (Powerful Biology, China).

### 2.2. Statistical analysis

Grayscale values of protein bands were quantified using ImageJ software. Each protein band recorded the values of three sites. Statistical analyses were performed using SPSS version 23.0. Group comparisons were conducted using t-tests, with significance set at $p < 0.05$.

## 3. Results

### 3.1. Expression of ARH3 with different mutations in ADPRHL2

To investigate the effect of 580 and 803 mutations in *ADPRHL2* on ARH3 expression, we constructed 580 mutant (580), 803 mutant (803), 580 + 803 mutant (580 + 803) and wild-type (WT) plasmids were transfected into 293T cells. After 48h by transient transfection, total cellular proteins were extracted from cells cultured under conditions of 37 °C and 5 % CO2. The intracellular expression levels of mutant and wild-type ARH3 were detected by Western blotting(WB)(All antibodies were sourced from literature [11]). Since the *ADPRHL2* is expressed in multiple cells throughout the body [10], a group of 293T cells was not transfected with the plasmid (control). The WT was transfected with *ADPRHL2* plasmid expression, which was equivalent to overexpression of ARH3. The control group still had a trace expression of ARH3, while the WT group overexpressed ARH3 protein, and the expression level was very significant. The expression levels of ARH3 in 580 and 803 decreased, while the expression levels of concurrent mutations (580 + 803) were almost similar to 580. A significant difference was observed in the total protein expression of ARH3 between the WT and ADPRHL2 mutant (580, 803) groups ($p < 0.01$). There was also a significant difference in the total protein expression of ARH3 between 580 and 803 ($p < 0.01$). The *ADPRHL2* gene 580, 803 mutation affected the expression of ARH3 in 293T cells (Fig. 1).

### 3.2. Expression of ARH3 with different mutations in ADPRHL2 under H2O2 stress

Furthermore, to investigate the function of ARH3 under exogenous stress conditions, we treated cells with 2 mM $H_2O_2$, a known

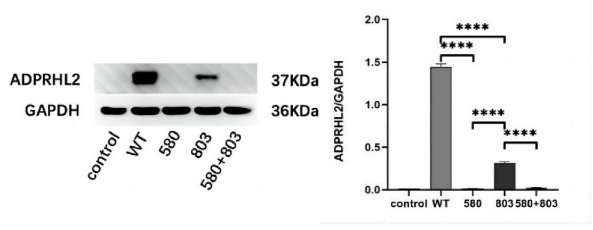


**Fig. 1.** WB results of wild type and mutant ARH3 protein(****$p < 0.01$). The expression of ADPRHL2 in control, wild type (WT), 580, 803 and double variant (580 + 803).

inducer of oxidative stress, for 10 min. Total ARH3 expression showed a significant difference between the WT + $H_2O_2$ group and the ADPRHL2 mutant (580+ $H_2O_2$, 803+ $H_2O_2$) group ($p < 0.01$), as well as between the 580 and 803 groups ($p < 0.01$). Compared to the groups without $H_2O_2$, ARH3 protein expression was increased in the control (control + $H_2O_2$), wild type (WT + $H_2O_2$) and mutant (580+ $H_2O_2$, 803+ $H_2O_2$) groups under a short duration oxidative stress condition of 10 min (Fig. 2).

We further investigate the effect of 580 and 803 mutations in *ADPRHL2* on PAR expression, with cells treated with DMSO for 8 days. The 580 expressed increased amounts of PAR compared to the WT. However, The intracellular expression levels of total PAR in the WT and 580 groups showed no significant difference ($p > 0.05$), and the interference of other hydrolytic enzymes could not be excluded. The 803 expressed a reduced amount of PAR compared to the WT, with a significant difference in PAR expression between WT and 803 ($p < 0.01$). The 803 variant did not affect the ARH3 structure and could still partially express ARH3 hydrolyzed PAR; normally, the expression was decreased. Additionally, a significant difference in total PAR expression was observed between 580 and 803 ($p < 0.01$).The *ADPRHL2* gene 580, 803 mutations affected PAR expression in 293T cells expression (Fig. 3).

Humans have two genes that encode specific PAR-degrading enzymes: ADPRHL2 and PARG [10,12]. While PARG exhibits higher hydrolytic activity against long PAR chains in vitro, ARH3 is also capable of removing PAR, albeit with lower efficiency [9]. To investigate whether these two enzymes collaborate in regulating cellular PARylation levels, all groups of cells were treated with the PARG inhibitor (PARGi). The cells were either treated with DMSO for 8 days or with 25 mM PARGi for the specified duration [13]. After the addition of PARGi, the expression levels of PAR in all groups decreased compared to Fig. 2, but the overall expression trend remained the same. It can be considered that ARH3 showed more significant activity in hydrolyzing PAR than PARG. The degree of functional differentiation or fusion between the ARH3 protein encoded by the *ADPRHL2* gene and the PARG protein encoded by the PARG gene is still unclear [7]. A significant difference was observed in the total protein expression of PAR between WT + PARGi and 803+PARGi ($p < 0.01$), as well as between WT + PARGi and 580+PARGi. Additionally, there was a significant difference in the total protein expression of PAR between 580+PARGi and 803+PARGi ($p < 0.01$) (Fig. 4).

## 4. Discussion

The disease associated with the *ADPRHL2* gene is a newly discovered neurodegenerative disorder in recent years, characterized by variable ataxia and seizures [1]. In the report, the majority of patients come from families married to close relatives. CONDSIAS patients are born normally and develop symptoms after infection, mainly in infancy and adolescence, with a rapidly progressing course that worsens with each infection or stress. The mortality rate is high, and deaths are often caused by respiratory failure after infection, sudden death during sleep, and asphyxia caused by seizures. At present, the reported clinical phenotypes include motor and language lag or regression, gait abnormalities, ataxia, seizures, organ abnormalities (such as head, spine, eyes, heart, etc.), gastrointestinal reactions, etc [14]. In terms of auxiliary examination, the more characteristic changes are brain MRI and neuroelectrophysiological examination. Brain MRI often indicates cerebellar atrophy, and a few cases of combined cerebral cortex or spinal cord atrophy exist. Electromyography or muscle histology examination shows damage to the myelin sheath and/or axon of peripheral nerve sensory and motor fibers. The current reported genotypes are all homozygous mutations. The patient has a typical clinical phenotype of CONDSIAS, which is consistent with the gene mutation. Due to the acute course of the disease after onset and seeking medical attention, cerebellar atrophy and peripheral nerve damage have not been found yet. Durmus et al. [15] proposed a novel biallelic mutation in the *ADPRHL2*, c.838G > A (p.Ala280Thr), causing ataxia, motor neuropathy with pyramidal signs, Episodic psychosis, with a focus on episodic psychobehavioral abnormalities that need to be distinguished from this. Other articles have reported mutations in the *ADPRHL2* leading to CONDSIAS, but there are no reports of 580 and 803 mutation sites [16–19].

In previous reports, both 580 and 803 were confirmed to be pathogenic mutations through RNA-seq [20]. In this experiment, the aim was to verify the pathogenicity of mutations at protein expression levels. This study transfected 293T cells with plasmids containing 580 mutant, 803 mutant, and 580 + 803 mutant, respectively, to compare the expression levels of ARH3. Due to the expression of ARH3 in various human cells, overexpression of ARH3 serves as a wild-type control. The results showed that the expression levels of 580 mutant and 803 mutant were lower than those of the wild-type, with statistical differences. There was also a statistical difference in ARH3 expression between the 580 mutant and the 803 mutant, suggesting that the 580 mutation disrupts the protein results and is more pathogenic than the 803 (Fig. 1). Due to the unreported heterozygous mutation in the patient, a plasmid transfection expression was constructed with simultaneous mutations at two sites. The results showed that the expression level of ARH3 at both sites was almost the same as that at 580. Since the 580 mutation caused glutamine to become a termination codon, transcription was terminated

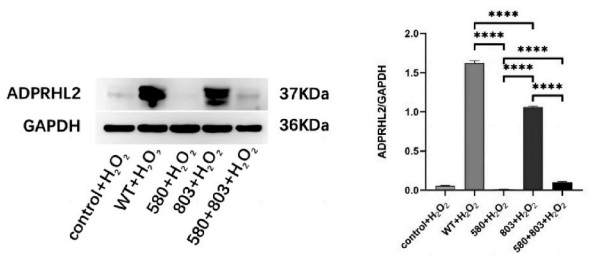


**Fig. 2.** WB results of wild type and mutant ARH3 protein (post $H_2O_2$ stress) (****$p < 0.01$). After exposure to H2O2 stress, the expression of ADPRHL2 in control, wild type (WT), 580, 803, and double variant (580 + 803).

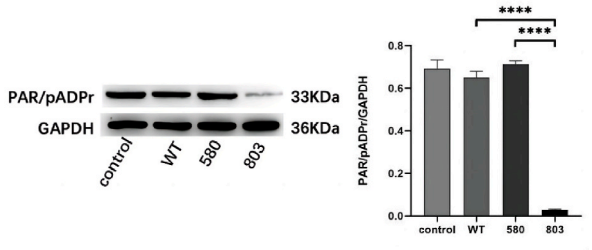


**Fig. 3.** WB results of wild type and mutant PAR protein(****$p < 0.01$). The expression of PAR in control, wild type (WT), 580, and 803.

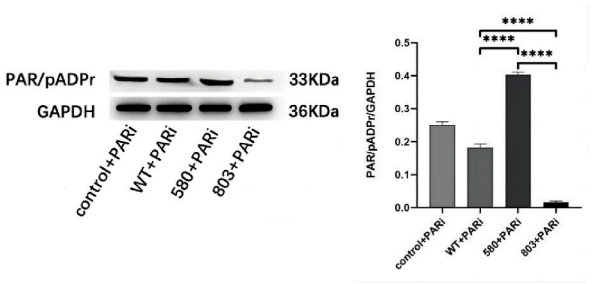


**Fig. 4.** WB results of wild type and mutant PAR protein (add PARG inhibitor) (****$p < 0.01$). After treatment with PARGi, the expression of ADPRHL2 in control, wild type (WT), 580, and 803.

prematurely, and expression could not proceed to position 803. CONDSIAS often develops under conditions such as infection or stress. Therefore, we cultured well-expressed cells in 2 mM $H_2O_2$ for 10 min. The results showed that the expression levels in the 803 mutant group and the wild-type group increased compared to before. Considering that ARH3 reactivity may increase after experiencing short periods of stress, 580 affects the ARH3 structure and cannot continue to express (Fig. 2). In previous studies, it has been demonstrated that ARH3 exhibits a protective effect against $H_2O_2$-induced cell death. This could explain the observed increase in ARH3 protein expression following $H_2O_2$ treatment [21,22].

ARH3 is an ADP ribohydrolase that hydrolyzes PAR and completes DNA repair. If ARH3 expression decreases, the PAR expression will increase. Therefore, the expression levels of PAR were compared between 580 mutant, 803 mutant, and wild-type. The results indicated an increase in the expression level of the 580 mutant and a decrease in the expression level of the 803 mutant. However, there was statistical significance between the expression level and the wild-type. c. 803-1G > A did not affect the structure of ARH3 and can still partially express ARH3 hydrolyzed PAR normally, resulting in a decrease in expression level. After experiencing oxidative stress, PAR cannot be hydrolyzed and accumulates, resulting in cell death. After culturing in 2 mM $H_2O_2$ for 10 min, the expression of PAR in 293T cells as a control group increased, while wild-type and c.580C > T mutant decreased in response to increased ARH3 reactivity (Figs. 3 and 4).

The functional divergence or convergence between ADPRHL2 and PARG is not well understood, partly due to a lack of detailed comparative expression analysis and biochemical function studies. Both ARH3 and PARG play a critical role in safeguarding the cell from excessive PARylation, with ARH3 responsible for removing initial serine-ADPr attachments and PARG for hydrolyzing their elongation products [11]. In other experiments, PARG has shown a more pronounced specific activity than ARH3 in removing PAR from proteins [10], and the loss of PARG in mice results in embryonic lethality [23]. However, in our experiments, PAR expression decreased in all groups after the addition of PARGi inhibitors compared to the previous ones, and it could be assumed that ARH3 exhibits more significant activity compared to PARG in hydrolyzing PAR.

Our research has identified that the mutation 803 may lead to two abnormal splicing events, resulting in a 1-bp deletion in exon 6 and 321-bp retention in intron 5, ultimately causing premature termination of the protein due to frameshift. The proteins resulting from these two abnormal splicing events were not detected in our analysis of the mutated protein, possibly due to our gel electrophoresis resolution not being sufficient to distinguish proteins of similar sizes.

This study demonstrates that mutations such as 580 (p.Gln194Ter) and 803 have an impact on *ADPRHL2* signal transduction and the expression of ARH3 and PAR, thus proving to be pathogenic mutations in this child. However, due to time and technical reasons, the failure to construct ARH3 knockout 293T cells may have an impact on the experimental results. This study is only conducted at the cellular level, and animal models can also be established for experimental verification of new genes or novel mutations in genes.

## Ethics Statement

The research involving human participants underwent a comprehensive review and received approval from the Ethics Committee of Wuhan Children's Hospital (2021R185-E01). Written consent for participation was obtained from the legal guardians or relatives of

the participants.

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## Data availability statement

Due to the anonymity of the participants/patients, the dataset of this article is not publicly available. Requests to access the dataset should be directed to the corresponding author.

## CRediT authorship contribution statement

**Shuang Yan:** Writing – original draft, Formal analysis. **Jie Ren:** Writing – review & editing, Data curation. **Hongting Su:** Writing – original draft, Investigation. **Jiehui Ma:** Project administration, Conceptualization. **Weijie He:** Writing – review & editing, Methodology. **Xiaofang Cai:** Supervision, Resources, Conceptualization. **Dan Sun:** Funding acquisition.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Dan Sun reports financial support was provided by Wuhan Knowledge innovation special project. Dan Sun reports financial support was provided by the Key research and development program of Hubei Province. Dan Sun reports financial support was provided by Construction Project of Hubei Provincial Clinical Medical Research Center for Childhood Neurodevelopmental Disorders. Dan Sun reports financial support was provided by Nature Science Foundation of Hubei Province. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e32945.

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