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A novel *GATA3* frameshift mutation causes hypoparathyroidism, sensorineural deafness, and renal dysplasia syndrome

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ABSTRACT

Background: Hypoparathyroidism, sensorineural deafness, and renal dysplasia (HDR) syndrome (Barakat syndrome) is a rare autosomal dominant disorder caused by mutations in the gene encoding *GATA3* on chromosome 10p14.

Method: Informed consent was obtained from a 38-year-old female patient. 5 mL of venous blood was collected and sent for whole-exome sequencing. *GATA3* constructs of both wild-type and mutant were transfected into HEK-293 T cells. Three-dimensional modeling, luciferase-reporter gene test, western blotting and cellular immunofluorescence were used to evaluate the effect of the mutation.

Results: A novel frameshift mutation c. 677dup(p.Pro227AlafsTer77), named P227Afs, was found in *GATA3*. Three-dimensional modeling revealed that the mutation caused the loss of the dual zinc finger structures 1 and 2 (ZNF1 and ZNF2) of the synthesized protein. Expression of wild-type GATA3 produced a six-fold increase in luciferase activity when compared with pcDNA3.1 vector only ($P < 0.001$), whereas the P227Afs mutant showed no increase. The mutation significantly reduced the transcriptional activity of *GATA3*. Immunofluorescence and western blotting analyses demonstrated that the mutation changed the nuclear location of GATA3 and caused difficulty in nuclearization.

Conclusion: A novel heterozygous frameshift mutation in *GATA3* was identified and showed to result in difficult nuclearization, and a dominant-negative effect on the wild-type.

1. Introduction

Originally identified by *Barakat* et al. in 1977, hypoparathyroidism, sensorineural deafness, and renal dysplasia (HDR) syndrome (OMIM # 146255), also referred to as Barakat syndrome, is an uncommon autosomal dominant condition [1]. *Hasegawa* et al.[2]named this combination HDR. Literature-identified cases have varied components with or without *GATA3* deficiency, confusing the classification of the condition, although the trio of conditions defines the phenotype [3]. Patients with HDR syndrome have a wide range of clinical characteristics. The most prevalent clinical trait is sensorineural deafness (93%), with numerous expressions used to characterize hypoparathyroidism (87%), renal dysplasia (61%), and other conditions [4].

Genes causing HDR syndrome are found on the short arm of chromosome 10 (10 pter–10p13 area) [2]. Deletion-mapping studies conducted by *Van Esch* et al. confirmed that the gene causing the mutation in HDR syndrome is located on chromosome 10p15 in the GATA-binding protein 3 (GATA3) gene [5]. GATA3 is a zinc-finger transcription factor family member that binds to the GATA (A/G) consensus sequence (A/T]). As illustrated in Fig. 1, *GATA3* comprises two N-terminal transactivating domains (TA1 and TA2) and two C-terminal zinc-finger domains (ZNF1 and ZNF2). Currently, >90 *GATA3* mutations linked to both sporadic and familial HDR syndrome have been found [6]. *GATA3* haploinsufficiency has been proposed as an underlying mechanism [7].

Herein, we describe a novel heterozygous frameshift mutation of *GATA3* at exon 3(c. 677dup) that is linked to a Chinese family with HDR syndrome and that predates the appearance of the stop codon TAA, which produces a premature stop at amino acid residue 302, causing the loss of the zinc-finger structure of the protein. To understand the role of *GATA3*, we examined the three-dimensional (3D) structure, cellular location, and transcriptional activity of this mutant GATA3.

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2. Patient and methods

2.1. Patient and mutation analysis

Whole-exome gene sequencing (WES) was performed using venous blood of the proband by MyGenostics. Genomic DNA obtained from her parents and her daughter were also performed by Sanger sequencing (Forward primer: TCTCCAAGACGTCCATCCAC and Reverse primer: ATCTCAGCAGGCTTTGGGAC) by MyGenostics. Sequencing results were interpreted following the recommendations of the American College of Medical Genetics and Genomics (ACMG). The patient and her family provided written informed consent before the trial, which was approved by the Institutional Ethics Council of the Tianjin Medical University General Hospital.

2.2. Computer modeling of GATA3 ZNF1 structure

The *GATA3* sequence was obtained from the NCBI Genome database (https://www.ncbi.nlm.nih.gov/genome). Using Mutalyzer, the *GATA3* variation was aligned to NM_001002295.2 [9]. 3D protein structure modeling was performed using AlphaFold2 [10,11]. The predicted local distance difference test was used to select the model with the highest confidence level. Structures were visualized using PyMol 2.5.5 (https://pymol.org/2/).

2.3. Cell culture and transfection

HEK293T cells were grown in Dulbecco's modified Eagle medium with 1% penicillin–streptomycin solution (Solarbio, China) and 10% fetal bovine serum (VISTECH, New Zealand). EZ Cell Transfection Reagent (Life-iLab Biotech, China) was used to transfect cells after they were seeded into 24-well culture plates according to the manufacturer's instructions.

2.4. Expression vector

The entire human *GATA3* coding sequence was inserted into pcDNA3.1 using the *GATA3* wild-type plasmid (GATA-WT) with a FLAG tag and an MYC tag at the C-terminal. Nucleotide T was subsequently inserted at position 677 of the wild-type *GATA3* to create the Myc-Flag-tagged-P227Afs-*GATA3* (GATA-MU) plasmid. Both plasmids were produced by Tsingke Biological Technologies (China). Beyotime Biotechnology (China) provided the pGL6-basic plasmid, which was cloned to contain *GATA3*-response elements.

2.5. Luciferase assay

Approximately 500 ng of the reporter plasmid pGL6-*GATA3*, which contains the luciferase gene driven by the *GATA3* binding site sequence (Beyotime Biotechnology, China), and approximately 120 ng of human wild-type or mutant *GATA3* plasmids were cotransfected into HEK293T cells with 50 ng of the internal control pTK-Renilla (Tianjin Sheweisi Biotech Co., Ltd). The pcDNA3.1 expression vector (30–120 ng) with wild-type *GATA3* and/or mutant *GATA3* was cotransfected with the reporter plasmid pGL6-*GATA3* (500 ng) and 50 ng of internal control pTK-Renilla (Tianjin Sheweisi Biotech Co., Ltd). Following a 48-h transfection period, cells were lysed at room temperature, and the Dual-Luciferase Assay System (Promega, USA) was used to assess the luciferase activities, in accordance with a prior protocol [13]. Renilla luciferase, an internal control, was used to normalize the activity of firefly luciferase to fix transfection efficiency. Each cotransfection experiment was performed at least three times.

2.6. Subcellular localization

The next day, 500 ng of wild-type or mutant *GATA3* expression plasmids were transfected into HEK293T cells that had been plated on coverslips in 24-well plates. Cells were transfected for 24 h, fixed at room temperature, permeabilized, and blocked using 4% paraformaldehyde, 0.1% TritonX-100, and goat serum. Primary antibodies were rabbit monoclonal antibodies against MYC (Immunology Consultants Laboratory, USA), and secondary antibodies were goat antirabbit IgG Alexa Fluor 555 (Invitrogen, USA). 4′, 6-Diamidino-2-phenylindole Fluoromount (Southern Biotech, Birmingham, Alabama) was used as a nuclear stain. Finally, fluorescent images were collected using a fluorescence microscope with a × 20 objective (Axio Imager M2; Carl Zeiss, Oberkochen, Germany).

2.7. Western blotting analysis

After HEK293T cells were transfected with either the wild-type or *GATA3* mutant plasmid, total cell lysates were separated using nuclear and cytoplasmic protein extraction kits (Absin, Shanghai, China). Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Primary antibodies used included anti-FLAG (Immunology Consultants Laboratory, USA), anti-Lamin B1 (Boster, China), and mouse antibodies against HSP (Santa Cruz Biotechnology Inc., USA). Secondary antibodies used were goat antirabbit/mouse-HRP polyclonal antibodies (Invitrogen). *GATA3* expression was measured using an ECL kit (Millipore). Densitometric analysis was performed using the ImageJ software, and GATA3 band intensity was calibrated against HSP90 band intensity.

2.8. Statistical analysis

The mean ± standard deviation was used to represent the results. GraphPad Prism 8.0 (San Diego, CA, USA) was used to assess the one-way ANOVA test between groups. Significance was set at $P < 0.05$.

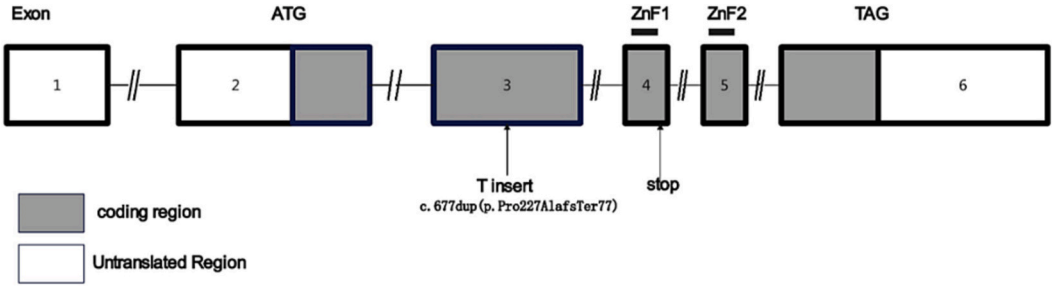

Fig. 1. A diagram of the genome structure of the *GATA3* gene. The *GATA3* consists of six exons spanning 20 kb of genomic DNA and encodes a transcription factor of 444 amino acids, as shown in the box. Coding exons are shown in gray. The *GATA3* encodes two transactivation domains (TA1 and TA2) and two zinc finger domains (ZNF1 and ZNF2). The coding regions of exon 1,2,3,4,5 and 6 are 188,610,537,146,126 and 806 bp, respectively, starting from ATG in exon 2 and stop codon in exon 6, tagged as a stop codon [8]. The arrow represents the mutation site in our patient.

3. Results

3.1. Clinical characteristics

A 38-year-old female had been suffering from hypocalcemia for almost a decade. Since childhood, the patient has experienced right-sided sensorineural deafness. At 27 years of age, the patient was diagnosed with bilateral hydronephrosis. The patient also has a history of Graves' disease and left lobe thyroid nodules. Her father had a history of hydronephrosis while both parents and her daughters had normal hearing. Laboratory examination identified low serum levels of intact parathyroid hormone (iPTH) (<0.32 pmol/L; normal range 1.1–7.3 pmol/L), high serum levels of phosphorus (2.02 mmol/L; normal range 0.80–1.45 mmol/L), severe hypocalcemia (1.8 mmol/L; normal range 2.10–2.55 mmol/L), and high levels of creatinine (94 mmol/L, normal range 41–73 mmol/L). Ultrasonography reveals hypertrophic kidneys with irregular contours. Thyroid ultrasound showed a diffuse thyroid lesion with enlargement and a medium to strong echogenic nodule in the left lobe of the thyroid (TI-RADS category 3). Detailed clinical characteristics of the proband are summarized in Table 1. Consequently, HDR syndrome was suspected. The patient was treated with calcium carbonate (1200 mg/day) and calcitriol (0.75 mg/day). The patient status is currently well controlled with a normal serum calcium level (2.27 mmol/L) (Table 1).

3.2. Mutation analysis and protein structure prediction

Whole-exome gene sequencing of the proband venous blood showed that exon 3 of *GATA3* (677dup, nucleotide number based on NCBI Reference Sequence: NC_000010.11) had a heterozygous base insertion. Consequently, the code-shift mutation changes the proline of 227 to alanine and alters the sequence of the next 77 amino acids, with the termination codon appearing earlier and protein translation being terminated earlier at amino acid 302. Significantly, this mutation was not present in the Human Gene Mutation Database (https://www.hgmd.cf.ac.uk/ac/index.php), Leiden Open Variation Database (https://www.lovd.nl/), or ClinVar database (https://www.ncbi.nlm.gov/clinvar), indicating that it is a novel mutation in *GATA3*. According to the ACMG, this variant was initially recognized as probably pathogenic (PVS1 + PM2_Supporting+PS2). Nevertheless, neither her parents nor daughter had a frameshift mutation(Fig. 2), and significant conformational changes were revealed by a 3D structure prediction study in mutant GATA3 protein, indicating premature translation termination (Fig. 3).

GATA3 P227Afs mutation lacks transactivation activity and exert dominant-negative effect on wild-type GATA3.

To understand the functional changes caused by this mutation, we evaluated the ability of mutant GATA3 to activate *GATA3*-promoter luciferase expression in HEK293 cells. When cotransfected with GATA3-WT, the luciferase activity increased by approximately six-fold relative to that in mock-transfected cells (Fig. 4). When GATA3 was introduced with a P227Afs mutation, the luciferase activity was slightly reduced compared with that in the mock-transfected cells (Fig. 4). The mutation decreased the transactivation of the gene. The control HDR-induced GATA3 mutant, P227Afs, involves deletion of ZNF1 and ZNF2, and has previously been shown to cause loss of DNA binding [12], revealing that this mutation produces complete loss of transactivation. Next, we tested whether the mutant GATA3 had any negative effects on wild-type

Table 1
Results of laboratory investigations in the proband before and after treatment.

Test	Values before treatment	Values after treatment	Reference range
Serum corrected calcium (mmol/L)	1.80	2.27	2.10–2.55
Serum phosphate (mmol/L)	2.14	1.76	0.80–1.45
Serum magnesium (mmol/L)	0.78		0.80–1.45
Parathyroid hormone(PTH) (pmol/L)	<0.32	0.46	1.1–7.3
24-h urinary calcium(mmol/day)	1.12 mmol	5	2.5–7.5
Total 25-hydroxy vitamin D(ng/mL)	20.67		>30
Serum creatinine (μmol/L)	85	100	41–73
Blood urea (mmol/L)	5.6		2.6–7.5
eGFR(mL/min/1.73 m2)	77.5	61.5	≥90
Serum potassium (mmol/L)	4.3		3.5–5.3
Serum sodium (mmol/L)	137		136–145
ALP(IU/L)	50		50–135
Urine full report and cytology	Normal No active sediment		
Renal sonography	The shape of both kidneys is irregular, the lower pole of both kidneys is smaller than the upper pole, and the parenchyma is thinner		

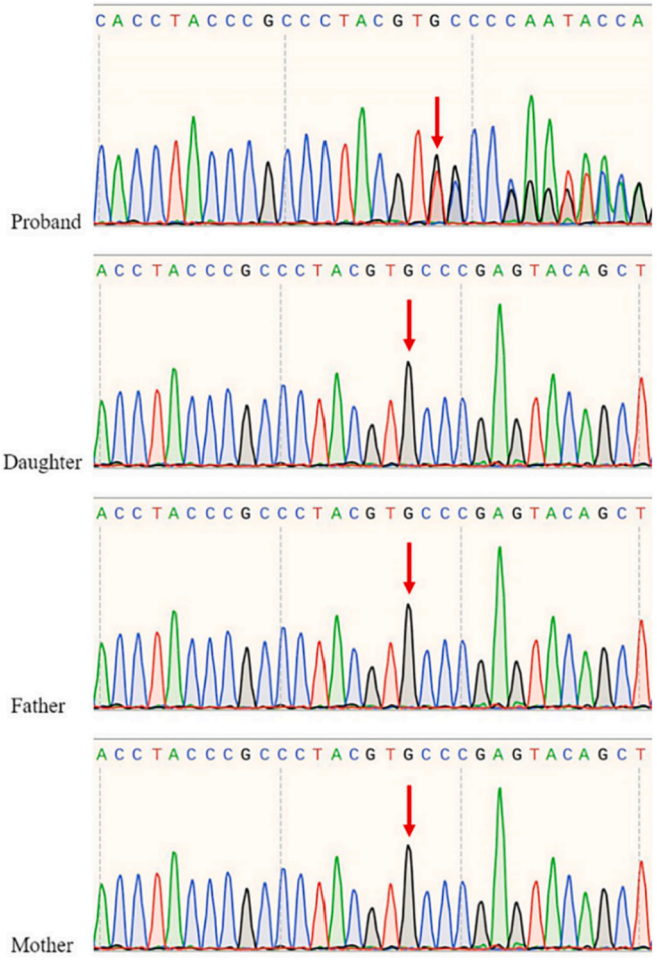

Fig. 2. Sequence analysis of the *GATA3* gene. Electrochromatograms show that the patient had a heterozygous c.677dup mutation. The patient's parent and her daughter did not have the mutation.

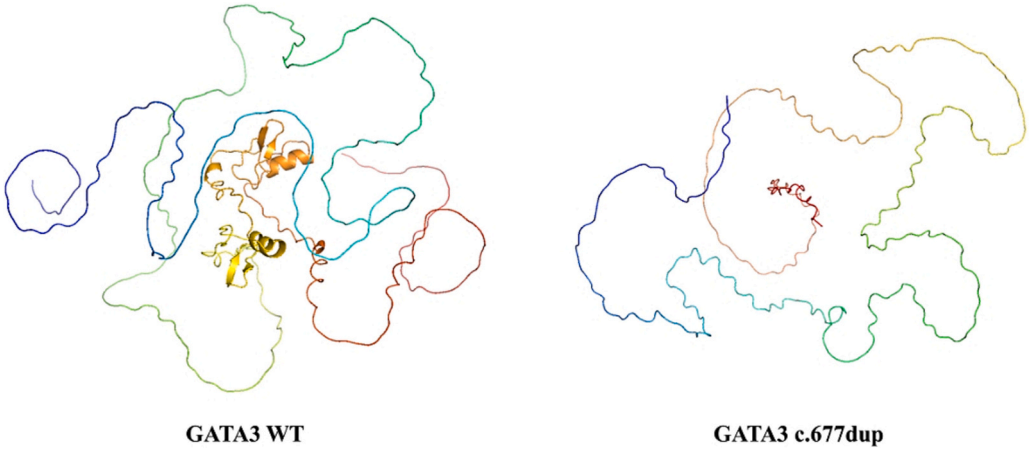

Fig. 3. Three-dimensional structure of the GATA3. Structural features of the GATA3 protein modeled by AlphaFold2 and displayed in the PyMol 2.5.5 software. The mutant GATA3 showed prematurely terminated translation with the loss of ZNF1 and ZNF2.

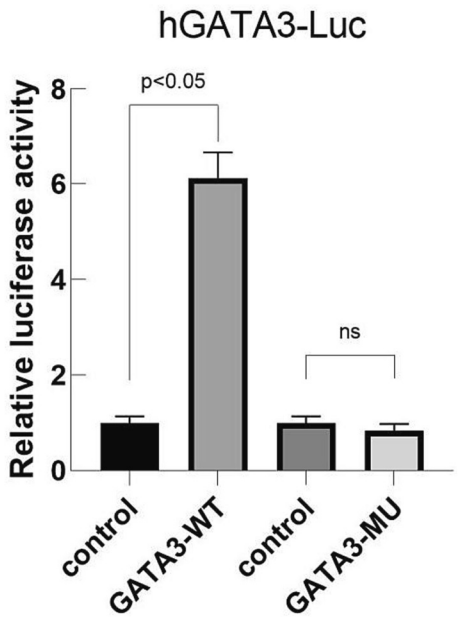

Fig. 4. When co-transfected with wild-type GATA3, luciferase activity increased by approximately six-fold relative to mock-transfected cells. While P227Afs-mutated GATA3 exhibits no transactivation of the *GATA3* promoter.

GATA3. When the wild-type *GATA3* expression vector was cotransfected with the 677dup-mutated *GATA3* vector, the luciferase activity of the wild-type GATA3 decreased with increased GATA3 expression (Fig. 5a). Therefore, the 677dup-mutated GATA3 had a dominant-negative effect on the transactivation activity of wild-type GATA3. Inhibition of luciferase activity by mutant GATA3 was attenuated in a dose-dependent manner when expression of wild-type GATA3 was progressively increased with fixed amounts of cotransfected 677dup-mutated GATA3; excess expression of wild-type GATA3 overcame the inhibition of luciferase activity (Fig. 5b).

3.3. Nuclear localization

To assess the functional significance of the *GATA3* c. 677dup (p. P227Afs) mutation, HEK293T cells were transfected with both wild-type and mutant GATA3 constructs. Immunofluorescence revealed that the GATA3 wild-type protein was localized in the nucleus, whereas the mutant protein was partially distributed outside the nucleus (Fig. 6a). These findings were confirmed by western blot analysis of subcellular components. This suggested that the size of the GATA3 wild-type and mutant P227Afs proteins was 59.5 and 48 kDa, respectively, and that the wild-type protein is mainly distributed in the nucleus, whereas the mutant protein is mainly distributed outside the nucleus (Fig. 6b). Quantitative analysis of western blot results showed that mutant nucleation decreased by approximately 35% compared with that of the wild type (Fig. 6b,c). Therefore, this mutation has a significant impact on nuclear localization.

4. Discussion

In the present case, we identified a novel heterozygous frameshift mutation in *GATA3* in a Chinese family with HDR. This P227Afs mutation induces the loss of both zinc-finger domains and abolishes the physiological function as a transcription factor. Our functional studies indicate that GATA3/P227Afs overexpression failed to increase the transcriptional activity of the GATA-responsive element, which may not only be attributed to the mutation distorting the nuclear distribution of the protein but also because GATA3/ P227Afs has a dominant-negative effect on the function of the wild-type protein.

GATA3 is located on chromosome 10p14 and comprises six exons encoding 444 amino acids [13]. GATA3 is a transcriptional regulator that belongs to a family of dual zinc-finger transcription factors and plays a role in the development and regulation of the immune system, embryonic development, and multiple cancer types [14,15]. It contains two N-terminal transactivating domains (TA1 and TA2) and two C-terminal zinc-finger domains (ZNF1 and ZNF2), as shown in Fig. 1.

In this study, the mutation is a frameshift variant that produces a highly conserved Pro227 mutation in ALA227. It also caused a 77-amino acid change, leading to premature termination of the polypeptide chain at codon 302 and loss of both the ZNF1 and ZNF2 domains (Fig. 1). Notably, the two zinc-finger domains have been described as necessary for the GATA3 protein to bind to DNA, as well as to stabilize the binding function [4]. ZNF2 specifically recognizes and binds to N-(A/T) GATA (t/g)-C sequences in DNA [16]. ZNF1 stabilizes the binding of ZNF2 to DNA and interacts with various zinc-finger proteins [17]. In our study, the insertion mutation disrupted the zinc-finger domain of GATA3. Thus, this mutation completely abolished the DNA-binding ability of GATA3.

To investigate whether the mutations have normal transcriptional activity, we performed luciferase-reporter gene experiments. It showed that the transcriptional activation of GATA3-MU was completely abolished compared with that of GATA3-WT. Mutant loss not only provided evidence of the pathogenicity of the mutation but also proved the importance of the double zinc-finger structure.

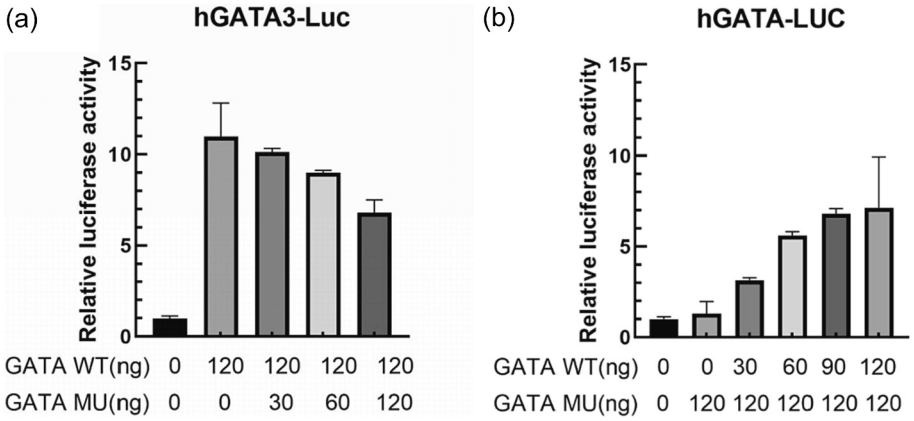

Fig. 5. a.P227Afs GATA3 exhibits no transactivation of the *GATA3* promoter and exerts a dominant-negative effect over wild-type GATA3 (luciferase assay). Normalized reporter gene activity of the control transfection (Mock) was arbitrarily set at 1. Representative data from repeated experiments are shown. b.The dominant-negative effect of the P227Afs GATA3 was relieved by increasing the amount of wild-type GATA3.

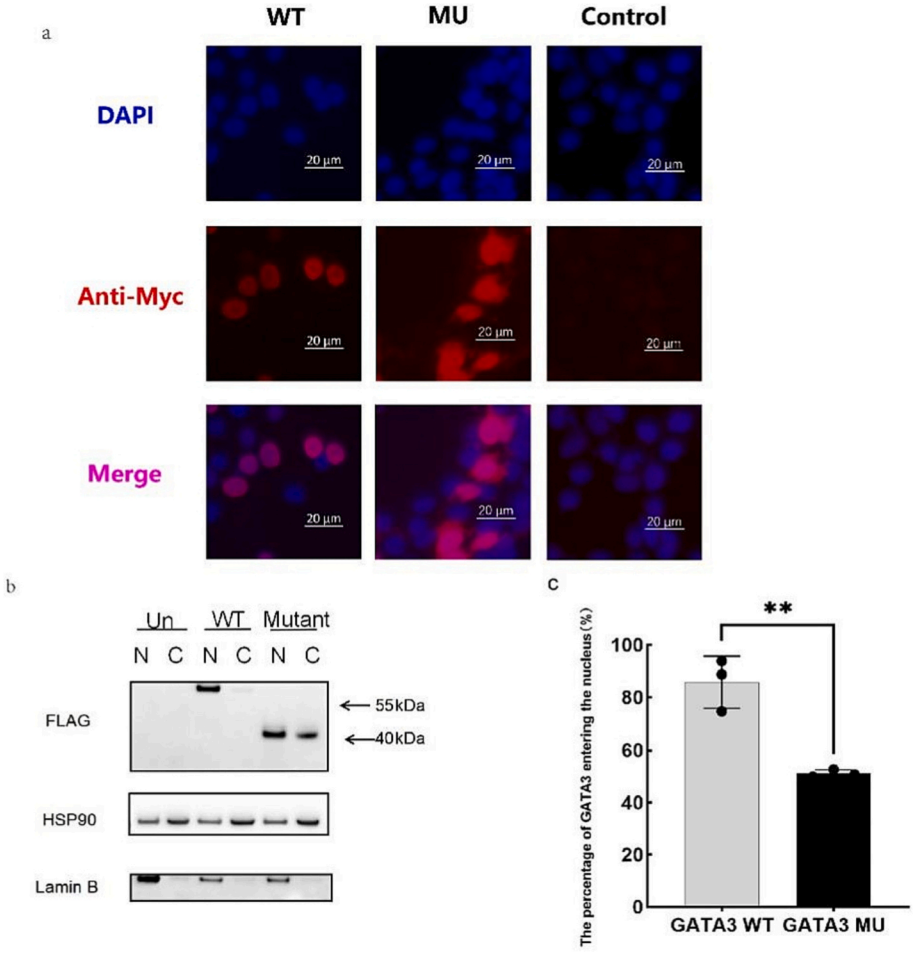

Fig. 6. a. Subcellular localization of GATA3 wild-type and mutant P227Afs. 293 T cells were transfected with GATA3 wild-type or mutant P227Afs constructs and visualized by immunofluorescence. The GATA3 wild-type accumulated in the nucleus. Interestingly, the GATA3 P227Afs mutant is around the **nucleus**, but not all of them go into the **nucleus**. Blue, Nuclear stain DAPI; red, GATA3 staining labeled by GATA3-myc antibody(Immunology Consultants Laboratory, Inc.). b.Western blot analysis of subcellular fractions (N-nuclear and C-cytoplasmic) from 293 T cells transiently transfected with wild-type (WT) and mutant(MU) P227Afs constructs. The GATA3-flag antibody detected the expected 58.5 kDa GATA3 wild-type and 42.5 kDa mutant P227Afs. The GATA3 wild-type was predominantly seen in the nuclear fraction, however, the mutant protein does not enter the nucleus as much as the wild-type protein, and remains more than wild-type protein in the cytoplasm. c. The percentage of GATA3 entering the nucleus represents that the ratio of protein only located in the nucleus and protein not only located in the nucleus but also in the cytoplasm.Three independent experiments were conducted on different days. Significance values compared with wild type from statistical tests: **, $P < 0.01$.

To explore why the loss of transcriptional activity occurred, we evaluated the subcellular localization of GATA3 mutants, considering that normal nuclear localization is important for GATA3 biological function, which is a prerequisite for regulating the transcriptional activity of downstream genes. Our results showed that the mutant protein did not fully enter the nucleus and was partially distributed around the nucleus compared with the distribution of the wild type. Thus, incomplete nuclearization may be partly responsible for decreased transcriptional activity, which is consistent with the results of two mutants (deletion of C in codon 201 and E228X) that also lack an N-terminal ZNF1 as described by Nesbit et al. [17] Moreover, our findings are consistent with the nuclear localization signal of GATA3, which is present within residues 249–311 of ZNF1 (263–287) [18].

In addition, we proved that GATA3 has a dominant-negative effect on the transactivation activity of wild-type GATA3, which can be rescued when the expression of wild-type GATA3 is progressively increased with fixed amounts of cotransfected mutant GATA3 (Fig. 5b). The mechanism whereby GATA3 overexpression and dominant-negative effects can cause HDR syndrome has been reported [3]. The Cys321Ser mutation found by *Ohta* et al. has a dominant-negative effect as this mutation lacks DNA-binding activity and is therefore unlikely to competitively inhibit the binding of wild-type GATA3 to the target DNA. However, it is more likely to inhibit wild-type GATA3 activity through competitive interactions with other proteins, such as FOG2, via ZNF1. Furthermore, GATA factors, including GATA3, can self-bind on DNA to form dimers [19], and this self-binding is mainly mediated by conserved motifs in the distal ZNF2.Therefore, the ability to dimerize with wild-type GATA3 is retained and interfere with the transactivation activity in a dominant-negative manner by sequestration. Interestingly, GATA3 with the P227Afs mutation also exerted a dominant-negative effect on the transcriptional activity of wild-type GATA3, as shown by the luciferase assay, although the P227Afs mutation resulted in a truncated GATA3 protein lacking both zinc-finger structures. Studies on somatic GATA3 truncating mutations in breast cancer have shown that the encoded proteins actively contribute to tumor growth, suggesting that the mutant proteins have a residual or novel function rather than simply resulting from null alleles [20,21]. This dominant-negative mechanism may be associated with the vital cofactors of wild-type GATA3. Thus, the results showed that the dominant-negative manner may be partly responsible for the decreased transcriptional activity.

HDR syndrome is characterized by parathyroid hormone deficiency, sensorineural hearing loss, and renal dysplasia [22]. Patients show considerable clinical heterogeneity, and the prevalence of each HDR defect outlier increases with age [1,17,23]. Triad HDR symptoms are present in 62.3% of patients; 28.6% of patients only show hypoparathyroidism and deafness, and 2.6% only show deafness and kidney disease [24]. *Mino* et al. [25] reported a Japanese family with HDR syndrome associated with a novel heterozygous mutation of *GATA3* at exon 3 (709 insC) that causes a premature stop at codon 302 with a loss of both zinc-finger domains, which is similar to the mutation reported here. The proband and her daughter harbored the same mutation. Similar to our patients, these patients also had a diffuse goiter. However, whether goiter is related to zinc-finger loss remains to be investigated. Our results expanded the spectrum of HDR-associated *GATA3* mutations.

In conclusion, we identified a novel mutation in *GATA3*, P227Afs, that results in a frameshifted and truncated mutant GATA3 whose abnormal nuclear localization and dominant-negative inhibition of wild-type GATA3 could partly explain mutation-induced functional abnormalities. This study provides key insights into the pathogenesis of this disease and lays a solid foundation for more accurate diagnosis and individualized treatment in the future.

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CRediT authorship contribution statement

Bo Huang: Methodology, Investigation. **Shiwei Li:** Data curation. **Yun Chai:** Formal analysis. **Yu Fan:** Project administration. **Xin Li:** Supervision. **Yue Liu:** Writing – original draft. **Yunhong Fu:** Writing – review & editing. **Xixi Song:** Visualization. **Jingqiu Cui:** Conceptualization.

Declaration of competing interest

None.

Data availability

No data was used for the research described in the article.

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