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ORIGINAL ARTICLE

Intron retention by a novel intronic mutation in *DKC1* gene caused recurrent still birth and early death in a Chinese family

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Abstract

Background: *DKC1*, the dyskerin encoding gene, functions in telomerase activity and telomere maintenance. *DKC1* mutations cause a multisystem disease, dyskeratosis congenita (DC), which is associated with immunodeficiency and bone marrow failure.

Methods: In this research, we reported a novel intronic mutation of *DKC1* causing dyskerin functional loss in a Chinese family. Whole exome sequence (WES) of the proband and validation by sanger sequencing help us identify a pathogenic *DKC1* mutation. Minigene splicing assays were performed to evaluate functional change of *DKC1*.

Results: A pathogenic *DKC1* intronic mutation(c.84 + 7A > G) was identified in the proband, which was inherited from heterozygous mother and not reported before. We detected the novel transcript with a 7 bp intron retention through minigene splicing assay. The newly spliced transcript is so short that would be degraded by nonsense-mediated mRNA decay in vitro and we infer that the novel DKC1 mutation would influences normal physiological function of dyskerin.

Conclusions: Our study identified a novel intronic mutation, which expands the spectrum of pathogenic *DKC1* gene mutations and can be used in molecular diagnosis. The mutant allele was transmitted to the next generation with high frequency in the family and causes still birth or early death.

Qiufang Guo and Ping Zhang contributed equally to this work.

KEYWORDS
DKC1, intron retention, intronic mutation, minigene splicing assay

1 | INTRODUCTION

DKC1 (OMIM 300126), located at chromosome Xq28, is the dyskerin encoding gene. Dyskerin is multifunctional and highly conserved among species and has been reported to be associated with telomerase activity and involved in telomere maintenance (Vulliamy et al., 2006). Dyskerin is associated with H/ACA box snoRNAs in small nucleolar ribonucleprotein particles which can modify uridine residues of rRNA and is essential for ribosome biogenesis (Filipowicz & Pogačić, 2002; Kiss, 2002). Dyskerin is also a component of telomerase complex and responsible for telomere maintenance (Mason & Bessler, 2011; Mitchell et al., 1999). Mutations in *DKC1* gene might disturb both the two processes and can lead to ribosomopathies and telomeropathies. Dyskeratosis congenita (DC) is a rare inherited multisystem disorder that clinically defined by abnormal skin pigmentation, nail dystrophy, and leukoplakia of the oral mucosa (Nelson & Bertuch, 2012). Its characteristics include short telomeres, immunodeficiency and bone marrow failure (Dokal et al., 2011). Several genes have been identified that might cause DC: *DKC1, TERT, TERC, TIN2, NHP2, NOP10, TCAB1, RTEL1, TINF2, ACD, PARN, NAF1, WRAP53*, and *CTC1* (Ratnasamy et al., 2018; Walne & Dokal, 2009). Among these, *DKC1* mutations related X-linked DC (OMIM 305000) is the most common one in DC (Mason & Bessler, 2011).

Mechanisms behind *DKC1* mutations contributing to DC is not completely understood. Donaires et al. described telomeres eroded immediately after reprogramming in patient-derived *DKC1* p.A353V iPSCs but stabilized in late passages (Donaires et al., 2019). Their findings suggest telomeres were maintained by a telomerase-dependent mechanism, even in cells with *DKC1* mutation. Richards et al. revealed that upregulation of *DKC1* bolsterd telomerase activity and promoted telomere lengthening in normal human erythroblasts (Richards et al., 2020). Gu et al. made a mouse model of X-linked DC, with a deletion of *DKC1* exon 15. They found growth disadvantage in cells expressing mutant dyskerin and the growth disadvantage was associated with an enhanced DNA damage response, which was dependent on telomerase (Gu et al., 2009). Recent researches show that depletion of *DKC1* induces oxidative stress and disrupts ribosomal biogenesis via NPM1 and p53 activation regardless of telomere shorting (Ibáñez-Cabellos et al., 2020). But these studies still cannot fully explain why mutations of *DKC1* cause DC. More studies were needed to illustrate roles of *DKC1* mutation in DC.

With the development of whole genome sequencing (WGS) and whole exome sequencing (WES), more and more mutations of disease-causing genes were identified, especially mutations outside of coding exons. Intronic mutations usually disturb normal alternative splicing events and result in novel transcripts. Identification of new alternatively spliced transcripts is essential to decipher its biological roles and processes that regulate gene functions (Mollet et al., 2010). Intron retention has been considered as a specific alternatively splicing event in primate genomes. 3 new transcripts due to intron retention of *DKC1* gene has been illustrated (Turano et al., 2013). In this study, we described a novel intronic mutation of *DKC1* gene in a Chinese pedigree with X-linked DC. The mutation was predicated to influence alternative splicing of *DKC1* transcript by providing a splicing donor site.

2 | MATERIALS AND METHODS

2.1 | WES sequencing

The peripheral blood samples from the proband and his parents were collected. Trio whole-exome sequencing was performed. DNA samples were extracted using a QIAamp DNA Blood Mini kit (Qiagen, Germany) following the manufacturer's protocol. The quality and quantity of the DNA samples were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). DNA fragments were enriched for exome sequences using the xGen Exome Research Panel v2 (Integrated DNA Technologies, Coralville, IA, USA). Sequencing was performed on NovaSeq 6000 (Illumina, San Diego, USA).

2.2 | Read mapping, variant calling and interpretation

WES data was analyzed as described in our previous study (Zhang et al., 2021). Reads were mapped to human reference genome hg19 with Burrows-Wheeler Aligner. Variants were called using Sentieon. The numbers of reads covering each exon of the captured region were calculated from the BAM file using BedTools with default parameters. Further analysis of SNV and small indels followed an in-house pipeline, an average of ~200 variants were kept in each sample. The variant filtering process was combined with a phenotype-scoring algorithm named

PhenoPro (Li et al., 2019). The interpretation of sequence variants followed the published standards and guidelines (Kleinberger et al., 2016).

2.3 | Mutation validation of genomic DNA

Sanger sequencing for confirmation of *DKC1* gene (NM_001363.5) variant was performed on an Applied Biosystems ABI3730XL genetic analyzer. Paired primers were designed by Primer3 website: primer-F (5'-GGGGAAAATTCCCAAACCT-3′) and primer-R (5'-GCCCATTCCTTGGAAACCT-3′). Sequencing data was analyzed using Mutation Surveyor v.4.0.9 software.

2.4 | Cell experiment

HEK 293T cells were cultured in DMEM supplemented with 10% FBS and 1% P/S at 37°C in a humidified atmosphere containing 5% CO_2. Transfection was performed according to the manufacturer's instructions (Promega, E4981), then transfected cells were incubated for 24 or 48 hr. To block nonsense-mediated mRNA decay (NMD), cells were treated with puromycin(50 ug/ml) before harvested.

2.5 | RNA analysis

Total mRNA was extracted from HEK 293T cells using Qiagen RNeasy Mini kit (Qiagen, 74,104). Concentrations were determined by NanoDrop 2000. Complementary DNA was synthesized from mRNA using the PrimeScript RT reagent kit (Takara, RR037A) according to the manufacturer's protocol. Real-time quantitative PCR was performed using TB Green Premix Ex Taq (Takara, RR420Q) on the manufacturer's specifications. All reactions were triplicated and data were analyzed according to the ΔΔCt method. TA clone was performed using Mighty TA-cloning Kit (Takara, 6028). Primer sequences for RT-PCR and qRT-PCR are primer-F (5′-ATGGCGGATGCGGAAGTAAT-3′) and primer-R (5′-CCACTGAGACGTGTCCAACT -3′).

3 | RESULTS

3.1 | Clinical features and family history

The proband was a 2-year and 1-month-old Chinese boy who had suffered from consecutive disease episodes. He is a child of physically healthy and non-consanguineous parents and was born at 32 weeks with a birth weight of 1400 g by Cesarean section delivery due to oligohydramnios and fetal intrauterine distress. The boy presented with thrombocytopenia in neonatal period, and he had suffered from recurrent infections and fever eversince 6 months old. At the age of 2 years, he was referred to our hospital because of infective fever, pancytopenia, cytomegalovirus infection, and liver dysfunction, for which he was hospitalized three times at local hospital during the past four months. During disease progression, laboratory analyses showed decreased white blood count (0.9–3.28 × 10^9/L, normal:4–10 × 10^9/L), platelet (16–45 × 10^9/L, normal:100–300 × 10^9/L) and hemoglobin (49.2-90 g/L, normal:110-140 g/L), while hepatic transaminases were elevated (ALT, 135-424 IU/L; AST, 129-775 IU/L, normal: 0-40 IU/L). Bone marrow biopsies revealed significantly increased histocytes and occasional haemophagocytosis. Several kinds of viruses were detected in fluid, including cytomegalovirus (CMV), epstein–barr virus (EBV), and parainfluenza virus 3 (PIV-3). A physical examination revealed low weight (8.5 Kg, −3.16 SD), short stature (79 cm, −2.72 SD) and microcephaly (head circumference: 42 cm, −4.61 SD). Meanwhile, the boy showed delayed development milestones. He could raise his head at 5 months old and sit at 9 months old, but still could not walk at 2 years old. Unfortunately, the boy died one month later due to multiple organ dysfunction syndrome (MODS), severe pneumonia, septic shock, hemophagocytic syndrome (HPS) and immunodeficiency disease.

The mother had four miscarriages, including three times before the boy was born and one time after the boy was born (Figure 1a). The first, second, and fourth miscarriages were all male fetus at 32 weeks for intrauterine fetal death due to oligohydramnios and fetal intrauterine distress. The third miscarriage was at 8 weeks for embryo damage. Unfortunately, the samples of the first three fetuses were unavailable. The mother had three sisters (Figure 1a II-1, II-2 and II-6) and two brothers (Figure 1a II-3 and II-4). Two brothers were died early, but clinical information was unclear.

3.2 | Gene mutation analysis

The average on-target sequencing depth for WES was 120×. WES data analysis revealed a previously unreported, likely pathogenic mutation (NM_001363.5: c.84 + 7A > G) in *DKC1* gene (Figure 1b). The novel variant is located at intron 2. It was not recorded in gnomAD database, 1000 genome database, Exome Aggregation Consortium (ExAC), Human Genome Mutation Database (HGMD), or Pubmed. For the validation of

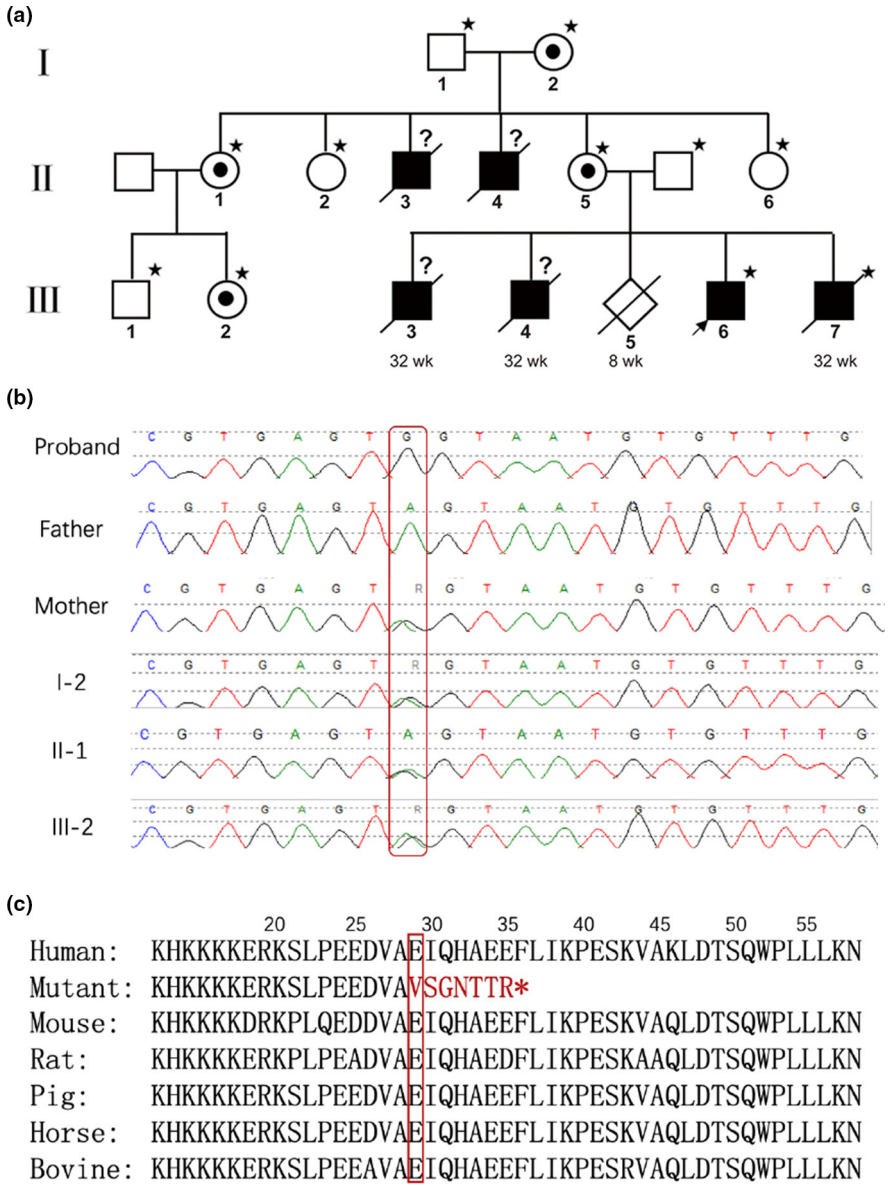

FIGURE 1 *DKC1* mutation analysis of the family. (a) The pedigree of the family. Arrow indicates the proband (III-6); black pots indicate carriers; asterisks indicate samples performed sanger sequence; wk numbers below symbols mean gestational weeks of unborn fetuses. (b) Validation of the c.84 + 7A > G mutation identified in WES by sanger sequencing. Samples were labeled consistent with Figure 1a. (c) Conservation analysis of amino acid sequence near mutant site among 6 species. The novel truncated dyskerin peptide was labeled as mutant

the novel *DKC1* variant, genomic DNA of the proband and his parents were amplified by PCR. Sanger sequencing results showed the variant was inherited from his mother, who was a carrier (Figure 1b, II-5). We also performed Sanger sequencing of other family members and found that the maternal grandmother, one of the proband's aunts and one cousin were variant carriers (Figure 1b, I-2, II-1 and III-2). X-linked recessive inheritance was clearly apparent in the family according to the pedigree (Figure 1a). This novel mutation was predicated as probably affecting alternative splicing by generating a splicing donor site (GTGAGTG) using SpliceAI and SOPHiA. The splicing donor site would result in incorporation of part of intron 2 into the *DKC1* mRNA. The reading frame would be disrupted by a premature stop codon because of the 7 bp intron retention. The predicated peptide would be composed of 35 amino acid according to the open reading frame. The disrupted amino acid region is highly conserved among species, including human, mouse, rat, pig, horse, and bovine (Figure 1c).

3.3 | Splicing analysis by minigene assay

To validate the novel transcript of mutant *DKC1* gene, we cloned a minigene to the pcDNA3.1 vector (Figure 2a). Exon 1, Intron 1, exon 2, intron 2, and exon 3 were included in the minigene. Wild type and mutant *DKC1* minigene plasmids were transfected to HEK 293 T cells. Considering the novel transcripts were only 108 bp, the NMD surveillance mechanism was inferred to commit them to a rapid degradation (Gardner, 2010). To detect whether novel *DKC1* transcripts could be degraded by NMD, cells were treated or untreated with puromycin, an inhibitor of protein synthesis that could inactivate translationally active

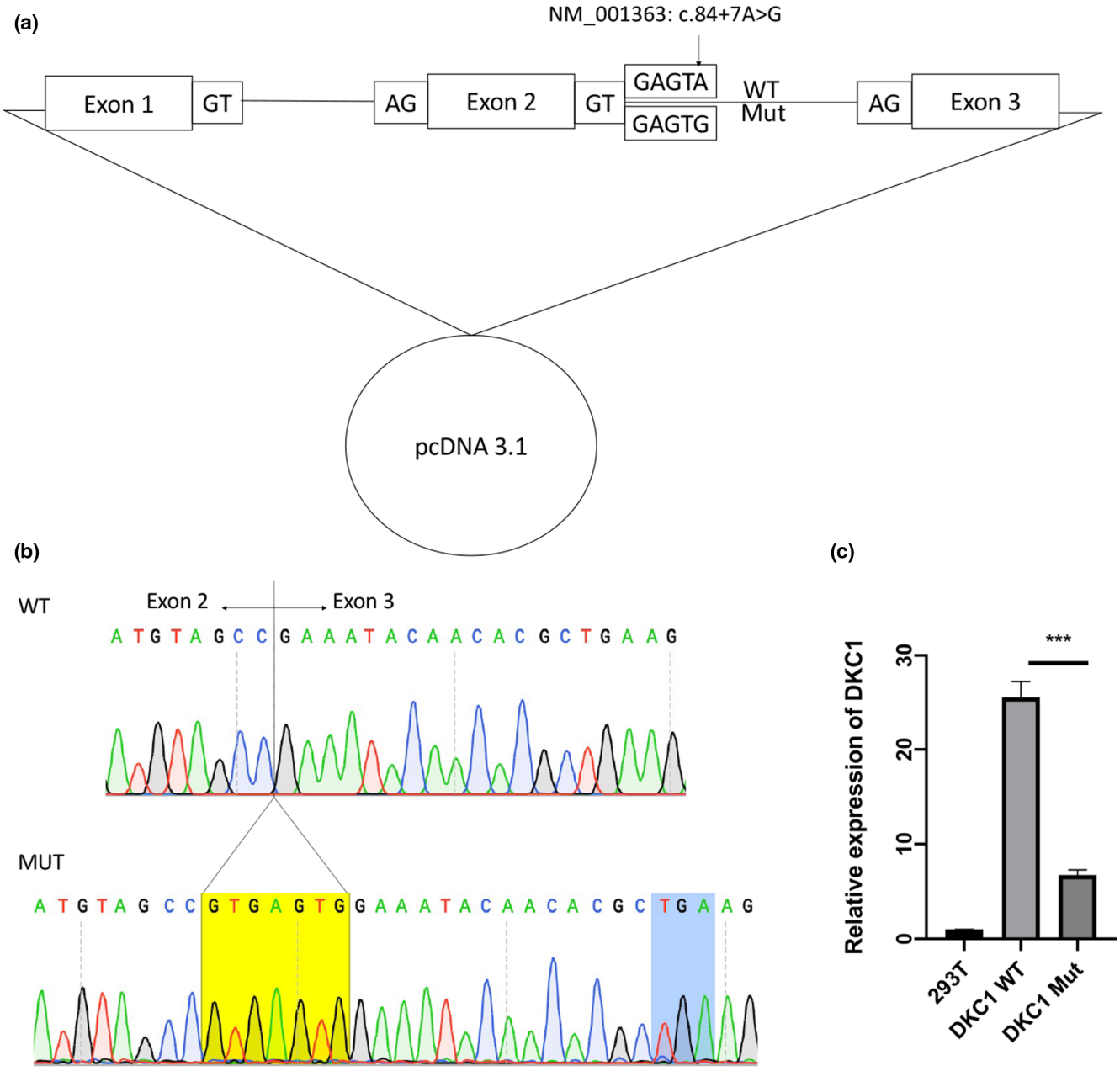

FIGURE 2 Functional change studies of the *DKC1* c.84 + 7A > G mutation by minigene splicing assay. (a) Schematic diagram of minigene expression plasmid construction. The arrow indicates the mutation site. (b) Nucleotide sequences of wild type and mutant *DKC1* TA clones after RT-PCR and sanger sequence. Yellow area indicates the 7 bp intron retention sequences. Blue area indicates the premature stop codon. (c) Relative expression of *DKC1* in wild type and mutant minigene group

ribosomes and represses NMD, for 4 hrs before harvested. RT-PCR and TA-clone were performed to detect the predicated transcripts. Sanger sequencing results showed incorporation of a portion of intron 2(7 bp, GTGAGTG) into the transcript in the mutant minigene group as predicated (Figure 2b). We also analyzed *DKC1* mRNA through RT-qPCR. Compared with wild type *DKC1* group, cells transfected with mutant *DKC1* has much lower expression level even after puromycin treatment (Figure 2c). It indicates that the novel *DKC1* mutation affect the dyskerin protein level and the decrease in the amount of the normal protein may cause DC. Therefore, there would be nearly null functional dyskerin protein and the mutation would lead to severe DC symptoms in the proband.

4 | DISCUSSION

X-linked recessive DC has severe clinical symptoms and often caused by mutations within the *DKC1* gene.

According to the HGMD website (http://www.hgmd.cf.ac.uk/ac/gene.php?gene=DKC1), 78 pathogenic mutations in *DKC1* gene have been identified up to now, including 59 missense/nonsense mutations, 6 splicing mutations, 2 regulatory mutations, and 4 small deletions, 1 small insertion, 4 small indels, and 1 cross deletion. Most mutations are located at N terminal region and PUA domain of dyskerin protein. The novel DKC1 mutation c.84 + 7A > G found in our study has not been reported in literatures before and belongs to splicing mutations. *DKC1* c.85-15T > A was the first reported splicing mutation in 1999 (S. W. Knight et al., 1999). Researchers detected 10 missense mutations and 1 splicing mutation in 21 of 37 families with dyskeratosis congenita by SSCP analysis. *DKC1* c.16 + 592C > G, c.1447-2A > G, c.1259 + 1G > A, c.85-15T > C, and c.942G > A were identified one after another (Kirwan & Dokal, 2007; S. Knight et al., 2001; Pearson et al., 2008; Vulliamy et al., 2006). All the six *DKC1* splicing mutations were predicated to influence normal alternative splicing. *DKC1* c.16 + 592C > G and c.1259 + 1G > A mutations have been verified and analyzed respectively in DC patients.

Several studies have described the mechanisms of failure to maintain telomere integrity in DC, but it still remains unclear. It is important to work out precisely how dyskerin interact with other components of telomerase and ribosomal biogenesis to control homeostasis. Allogeneic hematopoietic stem cell transplantation (HSCT) has been attempted to salvage a HHS child with *DKC1* c.1058C > T who is suffering from immunodeficiency and bone marrow failure. This result indicates that while HSCT can correct bone marrow failure and immunodeficiency, it may fail due to other fatal processes, such as portal hypertension and pulmonary arteriovenous shunting (Chen et al., 2019).

The *DKC1* c.84 + 7A > G mutation in our study causes a short transcript and disrupted protein expression because of 7 bp intron retention and a premature stop codon. We also performed RNA-seq of peripheral blood cells of the carrier mother, but we did not detect any read with the c.84 + 7A > G mutation (data not shown). It indicates the novel transcript would be degraded under the condition of normal NMD mechanism in blood. We performed minigene splicing assay to validate our predication due to the unavailability of samples of the proband. Through NMD block by puromycin, we detected the novel transcript with incorporation of part of intron 2 into the dyskerin mRNA in HEK293T cells after transfected with mutant *DKC1* minigene. In vivo transcripts that contain a premature stop codon are often degraded and the decrease in dyskerin mRNA would result in reduced normal protein level. Because *DKC1* gene is located at chromosome X, so there would be reduced dyskerin protein in female carriers and nearly null functional dyskerin protein in mutant male patients. Considering the important functions of dyskerin in telomerase activity and telomere maintenance, reduced expression of dyskerin would resulted in severe functional loss in the proband, who died because of multiple organ dysfunction syndrome and immunodeficiency disease.

DKC1 gene is essential for telomere maintenance and patients with DC have very short telomeres, which usually slightly increasing with age (Alter et al., 2007). We measured relative telomere length (RTL) of frozen blood cells of the carrier mother (II-5), proband (III-6), and aborted fetus (III-7) using a quantitative PCR–based assay developed by Cawthon (Raschenberger et al., 2013). The RTL data of 33–37 years old female comes from Shanghai Biowing Applied Biotechnology. Because we could not find RTL data of 0-5 years old, so we measured RTL of another 5 healthy donors under the age of 5 years old. Results showed that RTL of the proband, III-6, is shorter than all the 5 healthy boys; however, RTL of III-7 is on average of the 5 healthy donors (data not shown). RTL of the carrier mother, II-5, is in the 0th percentile of the 33–37 years old group and 4th percentile of the 31–40 years old group of the same sex, which means that the telomere length of II 5 is quite short.

After death of the proband, the couple got pregnant again. WES analysis of early trimester villi DNA showed the fetus also carried the same *DKC1* mutation but the couple insisted on continuing the pregnancy and the fetus died at 32 weeks due to poor circulation. It's unfortunate that the allele carrying this mutation was transmitted to the offspring with such a high efficiency in the family. Finally, we recommend the couple to perform assisted reproduction with preimplantation genetics test. The carrier mother is pregnant again with the help of the third generation IVF technology and the fetus does not carry this lethal genetic mutation. The mother is 33 weeks pregnant now and prenatal tests showed no abnormalities, which means the mother has passed the danger at 32 weeks when previous miscarriages happened. We are looking forward a healthy baby, which will further strengthen pathogenicity of the intronic DKC1 mutation.

In summary, we reported a novel intronic mutation in *DKC1* gene, validated the existence of novel transcript due to intron retention using a minigene splicing assay and inferred reduced expression of dyskerin protein in the proband. Our research expands the spectrum of pathogenic mutations in the *DKC1* gene. More importantly, it reminds the importance of prenatal consultation and genetic consultation.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

AUTHOR CONTRIBUTIONS

Bingbing-Wu and Jinqiao-Sun conceived the project. QG and PZ drafted the manuscript. JS and WY collected Peripheral blood. QG carried out minigene splicing assay. GL performed the sequencing analysis and performed the validation experiments. PZ, HW, and BW reviewed the genetic testing results and clinical diagnosis. All authors read and approved the final manuscript.

ETHICAL COMPLIANCE

Pre-test counseling was performed by physicians, and appropriate informed consent was signed by the proband's mother. The criteria for genetic testing were approved by the ethics committees of Children's Hospital, Fudan University (2015–130).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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