ORIGINAL RESEARCH

A Novel Variant and a Missense Variant Identified in the *DKC1* Gene in Three Chinese Families with Dyskeratosis Congenita

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Purpose: Dyskeratosis congenita (DC) is an inherited telomere biology disorder characterized clinically by mucocutaneous triad of reticulate hyperpigmentation, nail changes and oral leukoplakia. Bone marrow failure, pulmonary fibrosis and malignancies are the mainly life-threatening causes. There are X-linked recessive, autosomal dominant and autosomal recessive patterns of DC. *DKC1* is the most common pathogenic mutation gene responsible for X-linked DC, and it encodes a protein, dyskerin, which is a component of telomerase holoenzyme complex essential for telomere maintenance. Patients with DC have very short telomeres, but the precise pathogenic mechanism remains unclear. This study aimed to identify the causative mutations in the *DKC1* gene in three Chinese families with the X-linked form of DC.

Patients and Methods: Three Chinese families with DC were included in this study. Whole exome sequencing and Sanger sequencing were performed to clarify the mutation of *DKC1* gene. Measurement of relative telomere length through qPCR. Predictions of protein structure and function were performed using bioinformatics tools, including I-TASSER, Polyphen-2 and SIFT.

Results: There were four males with DC and a female carrier in three Chinese pedigrees. The novel mutation c.92A>C (p. Q31P) and the missense mutation c.1058C>T (p. A353V) in *DKC1* were identified. Both mutations locally changed the structure of dyskerin. Variant Q31P and A353V were predicted to have "deleterious" and "natural" effects on the function of dyskerin, respectively.

Conclusion: The novel variant and missense variant detected in the *DKC1* gene improve our understanding of DC and broaden the mutation spectrum of the *DKC1* gene.

Keywords: dyskeratosis congenita, mucocutaneous triad, *DKC1* gene, mutation

Introduction

Dyskeratosis congenita (DC) is a rare inherited disease, also known as Zinsser–Cole–Engman syndrome, with an estimated prevalence of 1/1,000,000. Three hereditary patterns have been recognized: X-linked recessive (XLR, OMIM #305000) (accounting for about 30% of the cases), autosomal dominant (AD, OMIM #127550) and autosomal recessive (AR, OMIM #224230).[1] It occurs predominantly in males and clinically presents with a mucocutaneous triad of reticulate hyperpigmentation, nail changes and oral leukoplakia.[2,3] In addition, hair and teeth involvements, palm-plantar hyperkeratosis and hyperhidrosis and other cutaneous manifestations are reported in DC.[2] A variety of other abnormalities of digestive system, cardiovascular system and bones and so on are also

described in DC patients.[2,3] The cardinal and life-threatening extracutaneous manifestations are bone marrow failure, pulmonary disorders and malignancies, accounting for 60–70%, 15–18% and 8–10% of the deaths in DC, respectively.[2] It is clear that a patient with DC has a high risk of cancers, such as squamous cell carcinoma and lymphoma.[4] In general, the prognosis of DC is poor and the mean age of death is the third to fifth decade of life.[2,4] Actually, the phenotype is variable where some patients with severe multi-system involvement in early life and others with mild presentations.[4]

DC is now primarily regarded as a telomere biology disorder of defective telomere maintenance.[4] There are 19 mutated genes reported in association with DC and they shorten the length of telomere directly or indirectly.[2] *DKC1* (OMIM #300126) is the most common pathogenic mutation gene where X-linked recessive pattern is noticeable, maps to Xq28 and encodes a highly conserved protein called dyskerin.[1,5] It participates in premessenger RNA splicing events and pseudouridylation of ribosomal RNA and spliceosomal small nuclear RNAs.[2] Furthermore, as a part of telomerase holoenzyme complex, dyskerin also interacts with a particular H/ACA RNA, the telomerase RNA component (TERC, or hTR in humans), regulating the accumulation of hTR. Consequently, dyskerin is crucial to the activity of telomerase in elongation of telomeres.[1,2,4] Likewise, mutations in *TERC* (OMIM #602322) and telomerase reverse transcriptase, *TERT* (OMIM #613989) have been found in AD-DC patients.[1,5] It is also been identified that AR-DC is a result of mutations in *NHP2, NOP10, TERT, USB1, TCAB1, CTC1,* and *RTEL1.*[1,2,5]

Collectively, DC has a wide spectrum of clinical characteristics and genetic heterogeneity. Here, we reported three Chinese DC families associated with *DKC1* gene mutations, and identified a novel mutation c.92A>C (p. Q31P) and a missense mutation c.1058C>T (p. A353V) in the *DKC1* gene.

Patients and Methods

Patients

Family 1 was a three-generation Chinese DC pedigree with two affected males (Figure 1A). The proband (III2) was a 23-year-old man presented with skin pigmentation for more than 10 years. Physical examination showed mottled and reticulate hyperpigmentation of the face, neck, trunk and upper arms (Figure 1B–D), leukoplakia on the dorsum of the tongue (Figure 1E) and mild fingernail changes (Figure 1F) without systemic involvement. His elder brother in this family had similar clinical features.
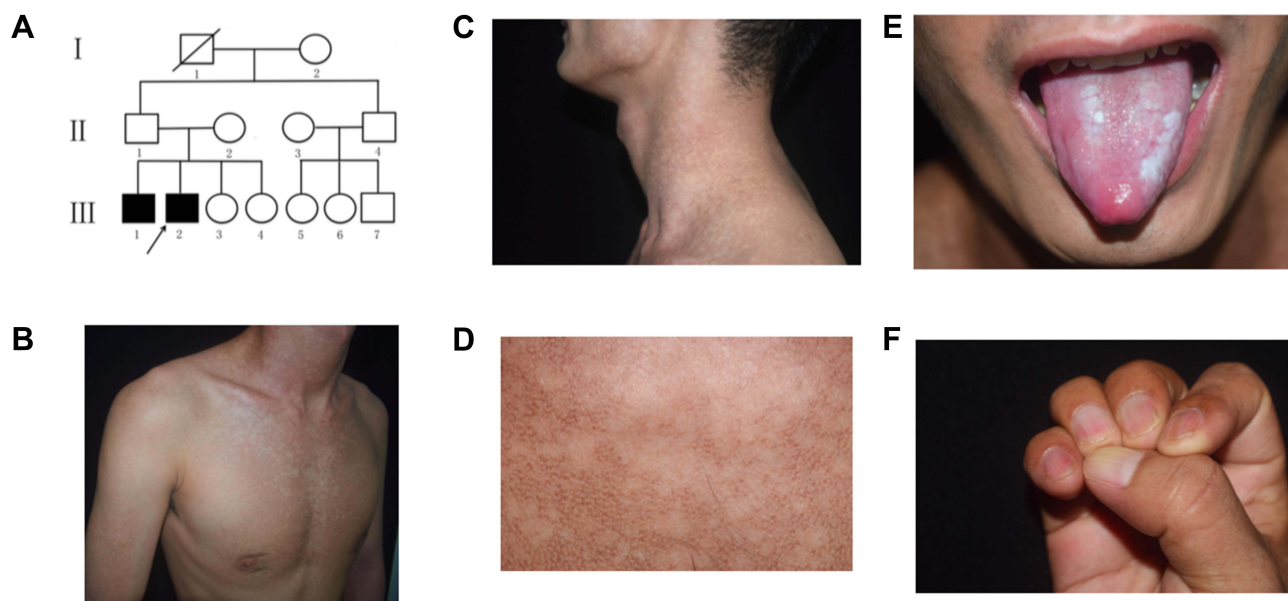

Figure 1 Pedigree and clinical features of the proband in family 1. (**A**) The pedigree of the family. (**B** and **C**) The mottled and reticulate pigmentation on the chest and neck. (**D**) Detail of reticulate hyperpigmentation of the neck. (**E**) Oral leukoplakia. (**F**) Mild longitudinal ridges, onychoschizia and brown lines of fingernails. The black arrow indicated the proband.
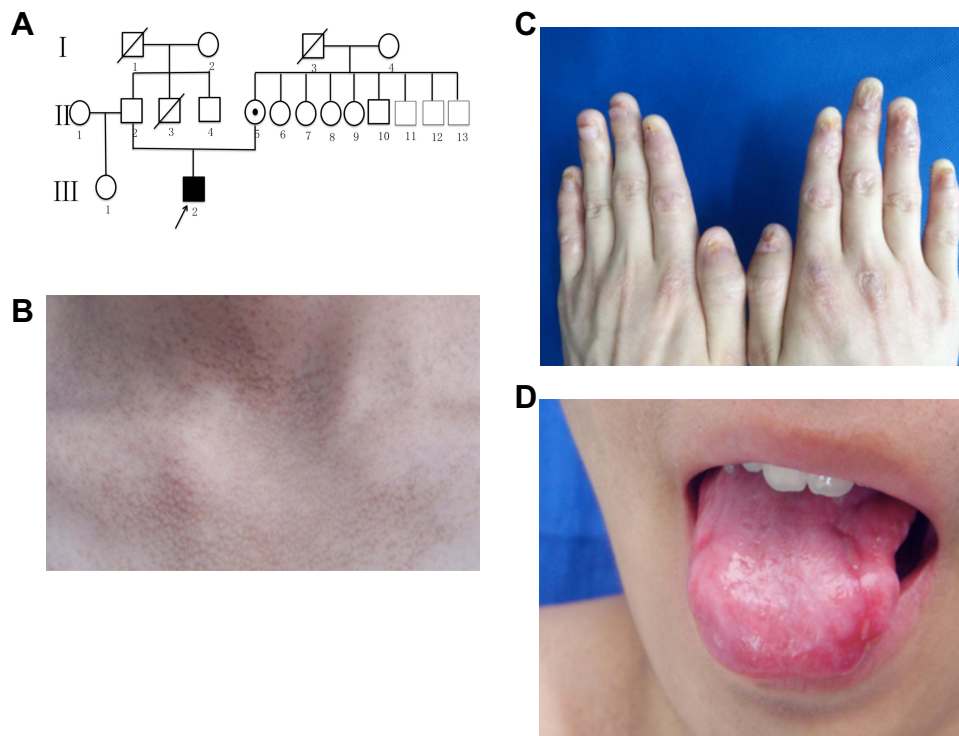
Figure 2 Pedigree and clinical features of the proband in the family 2. (**A**) The pedigree of the family. (**B**) The reticulate hyperpigmentation on the neck. (**C**) Fingernails atrophy. (**D**) White spots and ulcers on the tongue.

Family 2 was a three-generation Chinese DC lineage with one affected male and one carrier (Figure 2A). The proband (III2) was a 17-year-old boy with the complaint of skin hyperpigmentation and nail changes for about 10 years. The reticulate hyperpigmentation was initially from the ears and then gradually spread to face, neck and limbs (Figure 2B). White nails, atrophy and longitudinal ridges were found in 20 nails (Figure 2C). He developed oral disorder at 14 years of age and oral examination showed leukoplakia and ulcer on the tongue (Figure 2D). His mother (II5) was a carrier with normal clinical manifestations as same as unaffected person in this family and none of them had systemic disorders. Other relatives were clinically normal.

The last case was a 22-year-old man who developed hyperpigmentation on the face, neck and upper trunk since childhood, and his parents were clinically normal (Figure 3A). At the age of 10, he was found to have abnormal features of nails and tongue. Moreover, he suffered from trachoma, pulmonary fungal infection and abnormal liver function and macrocytic anemia. His complete blood count included a red-blood-cell count (RBC) of 3.29×10^{12}/L, hemoglobin level of 113 g/L, mean corpuscular volume of 105.3fL, and platelet count of 166×10^{9}/L. Physical examination revealed a poor health, reticulate hyperpigmented macules of the face, neck and upper trunk (Figure 3B), atrophic hypopigmented spots on the back of hands (Figure 3C), nail dystrophy (Figure 3C and D) and partial loss of the tip of the tongue (Figure 3E). Other routine laboratory examinations as well as lung function were within normal limits. Other relatives were clinically normal.

Whole Exome Sequencing and Data Analysis

The process of whole exome sequencing was done in Shanghai We-Health Biomedical Technology Co., Ltd. All genomic DNA samples were extracted from peripheral blood using commercial kit (TIANGEN, China). Onedrop OD1000 spectrophotometer and agarose gel electrophoresis were used to assess the quantity/quality of DNA. Exome capture was performed with xGen Exome Research Panel v1.0 (Integrated DNA Technologies, Inc., Iowa, United States) and 150 base pair paired end sequencing was executed using the Illumina HiSeq4000 platform (San Diego, CA). Raw reads were aligned using the Burrows–Wheeler Aligner (BWA) and SAMtools. After removing duplicates from sorted alignment using Picard, mutations were recognized through using the Genome Analysis Toolkit (GATK v3.70). Next, the *DKC1* gene mutation was confirmed by Sanger sequencing.
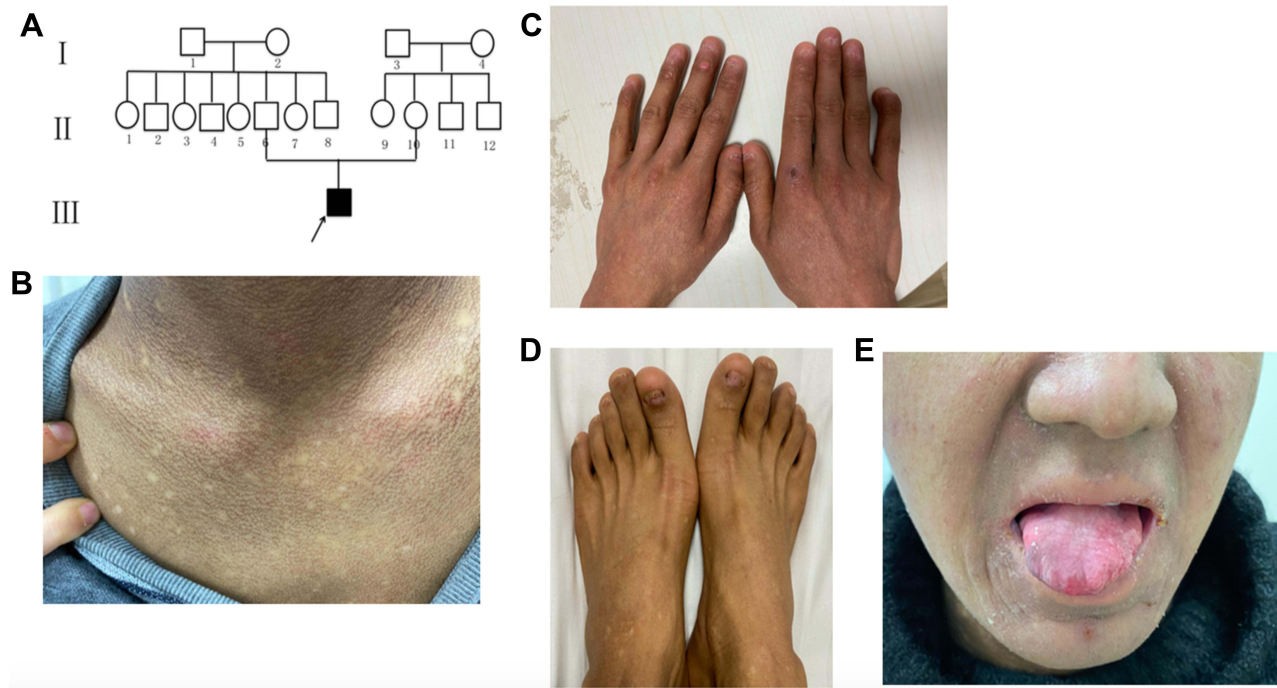
Figure 3 Pedigree and clinical features of the proband in family 3. (**A**) The pedigree of the family. (**B**) Reticulate scattered hyperpigmentation with hypopigmented macules on the neck. (**C** and **D**) Nails atrophy and dystrophy. (**E**) Oral leukoplakia and partial loss of the tongue tip.

Sanger Sequencing

Genomic DNA was extracted from blood samples using TIANamp Blood DNA Kit (Tiangen Biotech, Beijing, China). Primers were designed with the Primer3Plus software (http://www.primer3plus.com/cgibin/dev/primer3plus.cgi). Polymerase chain reaction (PCR) amplification was performed as described previously.[6] The PCR products were analyzed by 2% agarose gel electrophoresis. Purified PCR products were sequenced directly using an ABI3730xl DNA Analyzer (AppliedBiosystems, FosterCity, California, USA).

Measurement of Telomere Length

The relative telomere length was calculated by quantitative PCR (qPCR). The normalizing control gene was HBG. According to the manufacturer's instruction, targeted DNA was added to a reaction containing the pair of primers (telomere or HBG) and SYBR $^®$ Premix Ex Taq (MEIXUAN Biotech, Shanghai, China), in a total reaction volume of 20ul. Then, qPCR was performed on Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System and StepOnePlusTMReal-Time PCR System. The standard process was 1 cycle of 95°C for 5 min followed by 40 cycles of 95°C for 15 sec, 58°C for 30 sec and 72°C for 45 sec. Data collection and analysis used 7500 Fast Real-Time PCR software v2.0. Data were collected from repeated reactions for each sample. The standard deviation of Ct value was lower than 0.5, then the repeated values were received. The relative telomere length of normal population was regarded as 1. The final results were shown as $2^{-\Delta\Delta Ct}$, which represented the relative telomere length of DC patients in comparison with the normal population of certain age.

Primer sequences for telomere and HBG were as follows:

TEL - forward: 5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3'.

TEL - reverse: 5'-GGCTTGCCTACCCTTACCCTTACCCTTACCCTTACCCT-3'.

HBG - forward: 5'-GCTTCTGACACAACTGTGTTCACTAGC-3'.

HBG - reverse: 5'-CACCAACTTCATCCACGTTCACC-3'.

DKC1 Protein Structure Visualization and Function Prediction

Three-dimensional structures of DKC1 protein encoded by the wild-type and mutated *DKC1* gene (NM_001363.5) were predicted using I-TASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER-MR/). Furthermore, we visualized the architectures using PyMOL (PyMOL Molecular Graphics System, Version 2.0, Schrödinger, LLC). Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.jcvi.org/) were applied to predict the possible impact of an amino acid substitution on the structure and function of the DKC1 protein.

Results

Clinical Features

All affected individuals were males and had classical mucocutaneous features (Figures 1–3). Skin pigmentation and nails changes developed in about the first decade of life.

The Mutations of *DKC1* Gene

A novel missense variant c.92A>C (p. Q31P) in exon 3 of the *DKC1* gene (NM_001363.5) was identified in affected members (III1, 2) in family 1 (Figure 4A), thus confirming the diagnosis of DC in the proband and X-linked recessive inherited pattern. Other relatives were clinically normal and unable to go to hospital for sequencing. In the second pedigree, the proband (III2) was identified to have a transition mutation of C to T in the coding region at nucleotide position 1058 (Figure 4B) which caused a replacement of the alanine at amino acid residue 353 by valine in dyskerin. His mother (II5) carried a heterozygous mutation of 1058C to T (Figure 4C). No such mutation was found in his father, and other family members had no genetic examination. Similarly, the diagnosis of DC was definite and X-linked recessive inheritance was clear. As the only one affected individual of family 3, the proband (III1) showed the same variant c.1058C>T in exon 11 of the *DKC1* gene (Figure 4D) resulting in an amino acid change of A353V in dyskerin, however, his mother did not have sequencing because of death. His father was genetically normal.

The Telomere Length

Four DC patients in three families conducted the telomere length testing. The values of $2^{-\Delta\Delta Ct}$ were 0.831, 0.803, 0.887 and 0.815, respectively, which indicated that the telomere lengths of our DC patients were shorten.

The Prediction of Potential Effects of Mutations

The variant c.92A>C (p. Q31P) was predicted to be "possibly damaging" and "deleterious" with Polyphen-2 and SIFT, respectively. The variant c.1058C>T (p. A353V) was predicted to be "benign" and "natural" with Polyphen-2 and SIFT, respectively.

The mutation c.92A>C caused the replacement of a polarly amino acid glutamine by a non-polarly proline. In a three-dimensional structure, mutated PRO-31 could form a hydrogen bond with VAL-27 at the distance of 3.1 Å (Figure 5A). Wild-type GLN-31 could form a hydrogen bond with VAL-27 and the distance was 3.3Å, GLN-31 also could form a hydrogen bond with SER-280 at the distance of 3.5 Å (Figure 5B). Variant c.1058C>T (p. A353V) could not change the polarity of amino acids. Mutated VAL-353 could form a hydrogen bond with THR-351, THR-357, and SER-356 at the distance of 2.9 Å, 3.3 Å and 2.4 Å, 2.6 Å and 2.4 Å, respectively (Figure 5C). Wild-type ALA-353 could form a hydrogen bond with THR-352, THR-357 and SER-356; the distance was 2.7Å, 3.4 Å and 2.4 Å, 2.0 Å and 2.5 Å, respectively (Figure 5D). Both mutations locally affected the protein structure.

Discussion

According to diagnostic criteria,[1] our four patients in three families with mucocutaneous triad (reticulate pigmentation, nail dystrophy, and oral leukoplakia) were considered to have classic DC. A research about DC patients in Chinese mainland shows that 87% of the patients have mucocutaneous symptoms and 13% of the patients with two or three symptoms of the classical triad.[3] Abnormal skin pigmentation usually appears within 10 years of age.[2] Reticulate hyperpigmentation is a typical feature, but hypopigmentation is also been reported.[7] Lesions tend to occur on sun-exposed areas such as the neck,
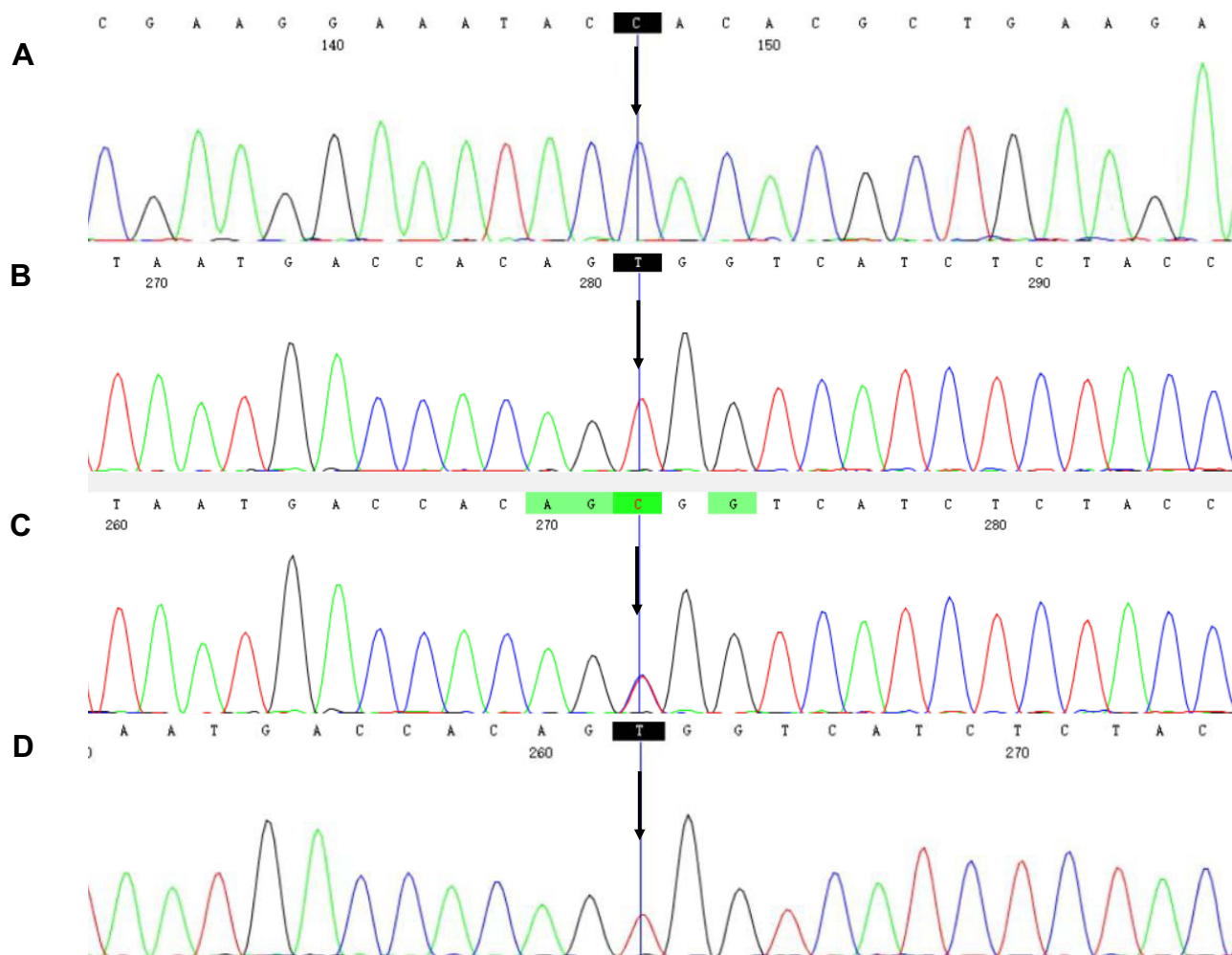
Figure 4 *DKC1* gene mutations in three DC families. (**A**) A hemizygous mutation c.92A>C was identified in III1 and III2 of family 1. (**B**) Mutation c.1058C>T was identified in the proband (III2) in family 2. (**C**) A heterozygous mutation c.1058C>T was detected in II5 in family 2. (**D**) A hemizygous mutation c.1058C>T was identified in the proband in family 3. The black arrow indicated mutated bases.

trunk and limbs.[7–9] Commonly reported nail changes are atrophy and longitudinal ridges, and involvement of the fingernails is more severe than toenails.[2,10] As the most common dental finding, oral leukoplakia is associated with an increased risk of head and neck squamous cell carcinoma (HNSCC) (primarily tongue) and approximately 30% of oral leukoplakia can be transformed to carcinoma.[11,12] It is investigated that erosive oral leukoplakia and carcinoma develop about 20–30 years of age.[12] In general, DC-affected individuals have 100-fold higher risk for developing HNSCC and anogenital SCC compared with general population.[13] Significant cancers in DC are HNSCC, acute myeloid leukemia, non-Hodgkin lymphoma and anogenital SCC.[13]

Other mucocutaneous features include hair loss, early graying and palmoplantar hyperkeratosis and so on. A study shows that the severity of mucocutaneous phenotypes has a positive relation with higher-risk genotypes and poorer prognosis.[14] Therefore, careful examination of mucocutaneous features is important for diagnosis and prediction of outcomes. Moreover, close monitoring should be suggested once patients are diagnosed with DC for preventing malignancies. In patients with classic mucocutaneous features, the diagnosis of DC is apparent. However, some patients present with aplastic anemia lacking mucocutaneous features and the diagnosis is challenging.

The deficiency of telomere biology resulting in short telomeres and limitation of cellular replication can affect multiple organs, especially those undergoing rapid replication.[4] Our four DC patients were confirmed to have shorter telomere lengths than normal people of the same age. Moreover, the proband of family 3 had infections, liver complication and
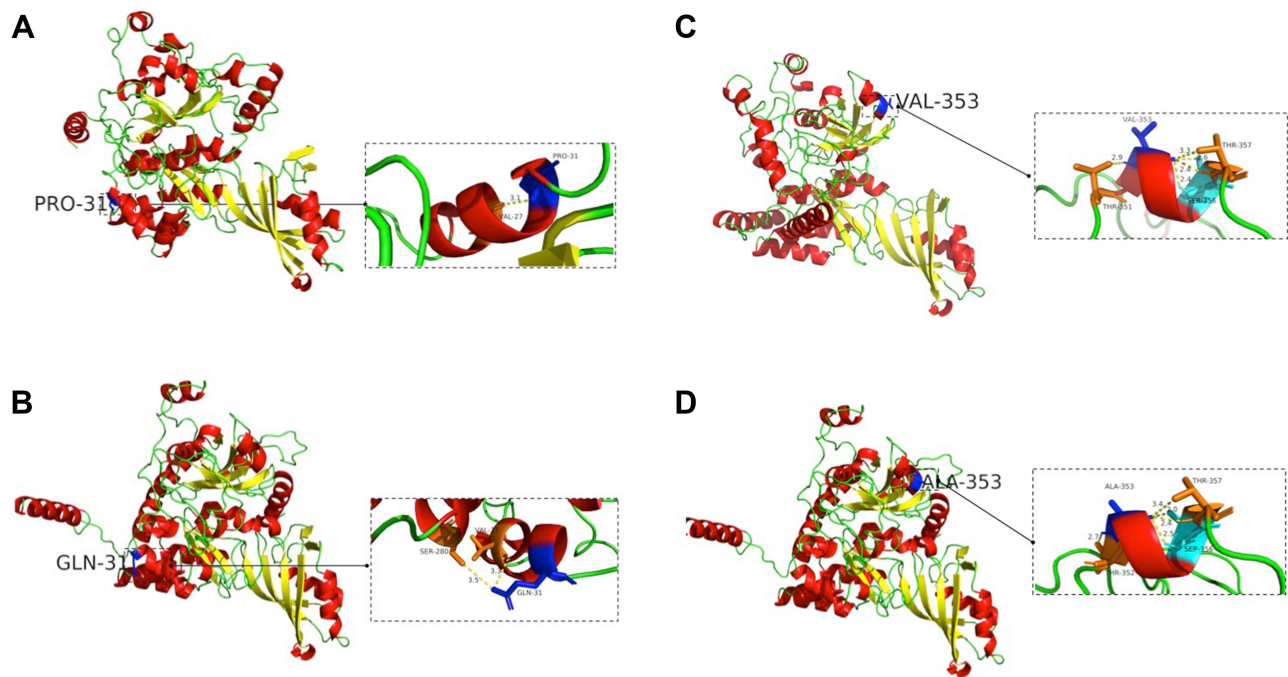
Figure 5 Three-dimensional structure of dyskerin. (**A**) PRO replaced GLN at position 31, and the hydrogen bond between amino acids changed. (**B**) Three-dimensional structure of wild-type with GLN 31. (**C**) ALA was replaced by VAL at position 353, and the hydrogen bond between amino acids changed. (**D**) Three-dimensional structure of wild-type with ALA 353. The left picture showed overall map of protein three-dimensional structure, and the right small picture showed partial map of protein three-dimensional structure.

anemia. The clinical complications include bone marrow failure, pulmonary fibrosis, liver disease and immunologic abnormalities and other medical disorders.[4] Bone marrow failure is the principal cause of premature mortality. It is estimated that 80% of the patients with classic DC develop at least one cell lineage cytopenia by 30 years of age.[15] Then it progresses into pancytopenia and eventually evolves into myelodysplastic syndromes (MDS).[13] Because of low numbers of B-lymphocytes and dysfunction of T-lymphocytes, DC-affected individuals suffer from recurrent infections including virus, fungi and bacteria.[16] Pulmonary complications are reported in more than 20% of the DC patients, and pulmonary fibrosis is the most frequent pulmonary manifestation.[17] The mutation of *DKC1, TERC* and *PARN* genes is related to pulmonary fibrosis.[17,18] The clinical management of DC is complex, and allogeneic hematopoietic stem cell transplant (allo-HSCT) is the only curative therapy for bone marrow failure.[1]

In our study, all of the patients were males and their parents and sisters presented with normal phenotype, suggesting the X-linked recessive inheritance in these three pedigrees. The substitution of nucleotide 92A with C in exon 3 of the *DKC1* gene leading to a Q31P mutation in family 1 is not reported before, and a mutation of 1058C to T in exon 11 of the *DKC1* gene leading to an A353V mutation in the last two families has been reported several times.

The *DKC1* gene mutation can be found in XLR-DC and Hoyeraal-Hreidarsson syndrome (HHS).[4] HHS is considered as a severe variant form of DC, and can be diagnosed by cerebellar hypoplasia combined with DC-related features or four of the six common HHS features: intrauterine growth retardation, developmental delay, microcephaly, cerebellar hypoplasia, immunodeficiency, or aplastic anemia.[4,15] As reported, the mutation of c.1156G>A in the *DKC1* gene is found both in a DC patient and an HHS patient in one family.[19] Since A353V mutation is also found in an HHS case, showing the same mutation has widely various phenotypes.[20] DC-affected males owing to *DKC1* mutations often present with classic mucocutaneous triad and early onset bone marrow failure.[20] Female carriers with *DKC1* pathogenic mutations occasionally show subtle DC-related phenotypes because of skewed X-chromosome inactivation and epigenetics and other mechanisms.[21]

To our knowledge, *DKC1* gene contains 15 exons that are translated into a ~58kDa L-shaped protein, dyskerin, which primarily presents with five domains: pseudouridine synthase domain (TRUB), pseudouridine synthase and archaeosine

transglycosylase domain (PUA), dyskerin-like domain (DKCLD), nuclear and nucleolar localization signals (NLS/ NoLS), amino-terminal extension (NTE), and carboxy-terminal extension (CTE).[22,23] As previously noted, mutations found in *DKC1* mainly cause amino acid substitutions. Mutation sites are primarily distributed in exons 2–6 and 9–12, and c.1058C>T (p. A353V) in exon 11 is described as the most frequent site which is known to occur de novo in many cases.[6,24,25] The cause of this hotspot site may be that CpG-to-TpG mutation is relatively common in human DNA.[26] A353V mutation localizes to the PUA domain, the RNA-binding motif in dyskerin, and the transformation of amino acid affects the interaction between dyskerin and H/ACA RNAs.[27] A study reports that A353V mutation has the most severe effect on the telomere maintenance. The mechanism of DC may be related to WNT signaling.[28] The mutation of c.92A>C in exon 3 causes replacement of a polarly amino acid glutamine by a non-polarly proline. Exons 3–5 encode the DKCLD domain, in which mutations may affect the interaction within domains.[23]

As known using PolyPhen-2 and SIFT softwares, Q31P mutation may have a damaging impact on the function of dyskerin. However, the specific mechanism is unclear. We suspect this mutation leads to malfunction of dyskerin resulting in phenotypes of DC. Further studies are necessary to understand the role of mutation in the pathogenesis of DC.

Conclusion

In summary, a novel mutation c.92A>C (p. Q31P) in the *DKC1* gene was detected in family 1, and a missense mutation c.1058C>T (p. A353V) in the *DKC1* gene was detected in the last two families. The substitution of amino acid Q31P was predicted to locally change the structure of dyskerin and to be deleterious. Similarly, the substitution of amino acid A353V was predicted to locally change the structure of dyskerin and to be natural. These results improve our understanding of DC and broaden the mutation spectrum of the *DKC1* gene.

Abbreviations

DC, dyskeratosis congenita; HNSCC, head and neck squamous cell carcinoma; MDS, myelodysplastic syndromes; allo-HSCT, allogeneic hematopoietic stem cell transplant; HHS, Hoyeraal–Hreidarsson syndrome; PUA, pseudouridine synthase and archaeosine transglycosylase domain; DKCLD, dyskerin-like domain.

Ethics Statement

Ethics Committee at the Suzhou Dushu Lake Hospital approved the study. This study was complied with the Declaration of Helsinki. The participants provided their written informed consent to participate in this study and allowed to publish images and clinical details.

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Disclosure

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