A Novel Dyskerin (*DKC1*) Mutation Is Associated With Familial Interstitial Pneumonia

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Short telomeres are frequently identified in patients with idiopathic pulmonary fibrosis (IPF) and its inherited form, familial interstitial pneumonia (FIP). We identified a kindred with FIP with short telomeres who did not carry a mutation in known FIP genes *TERT* or *hTR*. We performed targeted sequencing of other telomere-related genes to identify the genetic basis of FIP in this kindred. The proband was a 69-year-old man with dyspnea, restrictive pulmonary function test results, and reticular changes on high-resolution CT scan. An older male sibling had died from IPF. The proband had markedly shortened telomeres in peripheral blood and undetectably short telomeres in alveolar epithelial cells. Polymerase chain reaction-based sequencing of *NOP10*, *TINF2*, *NHP2*, and *DKC1*

revealed that both affected siblings shared a novel A to G 1213 transition in *DKC1* near the *hTR* binding domain that is predicted to encode a Thr405Ala amino acid substitution. *hTR* levels were decreased out of proportion to *DKC1* expression in the T405A *DKC1* proband, suggesting this mutation destabilizes *hTR* and impairs telomerase function. This *DKC1* variant represents the third telomere-related gene identified as a genetic cause of FIP. Further investigation into the mechanism by which dyskerin contributes to the development of lung fibrosis is warranted.

CHEST 2014; 146(1):e1–e7

Abbreviations: AEC = alveolar epithelial cell; DC = dyskeratosis congenita; FIP = familial interstitial pneumonia; HRCT = high-resolution CT; ILD = interstitial lung disease; IPF = idiopathic pulmonary fibrosis; PBMC = peripheral blood mononuclear cell; PCR = polymerase chain reaction; PFT = pulmonary function test; SIFT = sorting intolerant from tolerant; TRF = telomere restriction fragment

Idiopathic pulmonary fibrosis (IPF) is a progressive and frequently fatal lung disease with no known effective treatment. While early reports suggested inherited forms of IPF were rare, several more recent studies suggest $\geq 20\%$ of IPF is heritable^{1–3}; this syndrome has been termed familial interstitial pneumonia (FIP).⁴ To date, four genes have been linked to FIP: two components of the telomerase complex, *TERT* and *hTR*^{5,6}; the RNA component of telomerase; and two surfactant proteins (surfactant protein C^{7–9} and surfactant protein-A2).¹⁰ *TERT* mutations are the most common identified genetic cause of FIP, representing 10% to 15% of FIP families; nonetheless, in $>80\%$ of FIP kindreds, the gene responsible remains unknown.¹¹ Interestingly, up to one-third of patients with IPF have short telomeres in peripheral blood mononuclear cells (PBMCs) in the absence of a known mutation in *TERT* or *hTR*,^{12,13} suggesting other mechanisms may link telomerase function to the pathogenesis of IPF.

Mutations in *TERT*, *hTR*, and other components of the telomerase complex were first identified in families with dyskeratosis congenita (DC), a syndrome characterized by skin hyperpigmentation, nail dystrophy, oral leukoplakia, and aplastic anemia.¹⁴ Lung disease, cirrhosis, and other bone marrow dyscrasias (including macrocytic anemias, myelodysplastic syndrome, and acute myeloid leukemia) also occur in some kindreds with DC.¹⁵ We and others have recognized kindreds with FIP who share some features of DC but do not carry mutations in *TERT* or *hTR*. In this study, we screened affected family members from one such kindred with FIP for mutations in other telomere-related genes and identified a novel missense mutation in dyskerin (*DKC1*).

Manuscript received September 19, 2013; revision accepted January 2, 2014.

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Some of these results have been presented previously in abstract form (Kropski JA, Mitchell DB, Markin C, et al. *Am J Crit Care Med*. 2013;187:A1081).

Funding/Support: The study was supported by grants from the National Institutes of Health/National Heart, Lung, and Blood Institute [Grants HL92870, HL085317, and HL094296 to Dr Blackwell and Grant HL105479 to Dr Lawson] and the Department of Veterans Affairs (grants to Drs Lawson and Blackwell).

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DOI: 10.1378/chest.13-2224

FIP Kindred and Specimens

The Vanderbilt University institutional review board approved this investigation (#020343), and subjects or their surrogates provided written informed consent prior to enrollment in the study. The proband was identified from the Vanderbilt Familial Interstitial Pneumonia Registry and underwent a clinical evaluation including high-resolution CT (HRCT) scan, pulmonary function tests (PFTs), and bronchoscopy with transbronchial biopsies. A pedigree was constructed using Progeny (Progeny Software LLC). Clinical records of affected members were obtained and reviewed for research purposes. Hematoxylin and eosin-stained slides from paraffin-embedded lung tissue from biopsy specimens were reviewed by an expert lung pathologist.

DNA was isolated from blood and paraffin-embedded lung tissue using a PureGene Kit (Gentra Systems Inc). Control DNA was obtained from married-in subjects from other kindreds in the FIP registry. Lymphoblastoid cell lines were generated by Epstein-Barr virus transformation of lymphocytes from the proband, other members of the kindred, and healthy control subjects ($n = 5$), as previously described.¹⁶ RNA was isolated from lymphoblastoids using a Qiagen RNeasy Mini Kit (Qiagen NV).

DNA Sequencing

Modified Sanger sequencing of DNA from the proband was performed for *NOPI0*, *TINF2*, *NHP2*, and *DKC1* by a Clinical Laboratory Improvements Amendment-approved laboratory. Confirmatory sequencing of DNA samples for the *DKC1* Thr405Ala mutation was performed by polymerase chain reaction (PCR) amplification of the specific region of exon 12. The primer sequences used were forward 5'-TTCTGGACAAGCATGGGAAG-3' and reverse 5'-CAGCAAGTGTGCCGTCTCTA-3'. The PCR used Platinum TAQ polymerase (Thermo Fisher Scientific Inc) with cycling conditions of 94°C for 3 min for denaturation, followed by 60 cycles of 94°C for 30 s, 62°C for 30 s, and 68°C for 30 s, and a final extension of 68°C for 3 min. This yielded a 122 base pair product, which was visualized on a 2% agarose, ethidium bromide gel. Each amplification product was treated with ExoSAP-IT (USB Corp) prior to sequencing. Sequencing reactions were performed using the reverse primer listed and BigDye Terminator Version 3.1 Sequencing Kit (Thermo Fisher Scientific Inc) according to manufacturer's protocol. Products were analyzed by capillary electrophoresis using an ABI Prism 3100 Genetic Analyzer (Thermo Fisher Scientific Inc).

Telomere Restriction Fragment Analysis

Telomere restriction fragment (TRF) analysis was performed using the Southern blotting technique. Peripheral blood-derived genomic DNA (1 μ g) was processed according to the instructions found in the TeloTAGGG Telomere Length Assay Kit (Roche Diagnostics Corp). Blots were exposed to radiographic film and imaged using the AlphaImager FC (Cell Biosciences Inc) and quantified with AlphaVIEW SA software (Cell Biosciences Inc). TRF

length was calculated as described in the TeloTAGGG kit protocol.

Tissue Telomere Measurements

Telomere length in type 2 alveolar epithelial cells (AECs) was measured by fluorescence in situ hybridization using Cy3 tag as previously described.^{12,17} Sporadic IPF lung tissue was obtained from our tissue repository of well-phenotyped patients with IPF ($n = 7$). Normal control lung tissue was obtained from donor lungs rejected for transplant ($n = 8$). Identification of type 2 AECs was performed by costaining with rabbit antihuman prosurfactant protein C primary antibody (Merck KGaA) and fluorescein isothiocyanate anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories Inc). Ten images were performed for each slide with fixed digital camera settings using $\times 630$ magnification, and all fluorescein isothiocyanate-positive type 2 AECs were analyzed. Specifically, each nucleus was marked, and fluorescent intensities were measured for both Cy3 (telomere-specific signal) and 4',6-diamidino-2-phenylindole (general DNA content) separately using Image-ProPlus 7.0 software (Media Cybernetics Inc). Then, telomere length was calculated as a ratio of red fluorescent intensity to the blue fluorescent intensity.

Sorting Intolerant From Tolerant

Sorting intolerant from tolerant (SIFT) analysis,¹⁸ which uses changes in amino acid sequence to determine whether a mutation is likely to be deleterious, was performed. A SIFT score < 0.05 is considered likely to deleteriously affect function.

Quantitative PCR

DKC1 and *hTR* expression were measured by quantitative PCR (qPCR) using RNA isolated from lymphoblastoids. Data are expressed as copies relative to β -actin expression. The following primer sequences were used: *DKC1*: (F) 5'-CTCGGAAGTGGGGTTAGGT-3' (R) 5'-TCTTCTTTTCCTTCTTGATCAACTG-3'¹⁹; *hTR*: (F) 5'-GGAAGCTTGCTAGCGCACCGGGTTGCGGAGG-3' (R) 5'-GTTTGCTCTAGAATGAACGGTGAAG-3'; β -actin: (F) 5'-GAAGCATTTGCGGTGGACGAT-3' (R) 5'-CGAGGTCATCACCATTTGGCAA-3'.

Statistical Analysis

A linear regression analysis of TRF on age was performed using data from normal control subjects, and prediction lines for new data with several significant levels were obtained from the regression. The TRF for affected proband, carrier daughters, and unaffected maternal aunt were plotted against their age on top of the prediction lines to present their percentiles in the prediction. Differences among groups were assessed using one-way analysis of variance and between groups using a two-sample t test. Results are presented as mean $\pm$ SD. The programming language R version 2.15.2²⁰ (R Project for Statistical Computing) and GraphPad Prism,

FIGURE 1. Clinical features of affected subjects. A, Affected subjects manifested a hyperpigmented rash on the hands with dyskeratotic nail changes. B, Reticular changes and mild bronchiectasis on high-resolution CT scan. C, Hematoxylin and eosin (H&E)-stained transbronchial biopsy specimen from the proband demonstrates interstitial fibrosis (original magnification $\times 200$). D, H&E-stained surgical lung biopsy specimen from an affected relative demonstrates temporal heterogeneity and extensive interstitial fibrosis consistent with a usual interstitial pneumonia pattern (original magnification $\times 100$).

version 5.0 (GraphPad Software Inc) were used to make plots and to perform the statistical analyses.

RESULTS

Case History

A 69-year-old man with diabetes, hypertension, hyperlipidemia, and prior tobacco use presented with 2 years of progressive dyspnea and cough. He had worked as an electrician and had no notable exposure history. His brother had recently died of IPF. His physical examination was notable for an oxygen saturation of 91% on room air, bibasilar crackles, and a hyperpigmented rash on the trunk with an erythematous, dyskeratotic rash on the palmar and dorsal surfaces of both hands (Fig 1A). Laboratory studies were notable for a macrocytic anemia. Connective tissue disease serologies, including anti-Jo1 antibodies, were negative. PFTs revealed moderate restriction with a severely reduced diffusing capacity. A HRCT scan of the chest showed bilateral reticular changes with mild bronchiectasis and rare ground-glass opacities consistent with idiopathic interstitial lung disease (ILD) (Fig 1B). Transbronchial biopsy specimens demonstrated interstitial fibrosis (Fig 1C). Review of a surgical lung biopsy specimen of the affected brother confirmed changes consistent with usual interstitial pneumonia (Fig 1D). A pedigree for this kindred was constructed, which was consistent with an autosomal dominant or X-linked inheritance pattern (Fig 2).

Genetic Investigation

In this kindred, two brothers presented with ILD accompanied by skin rash, macrocytosis, and nail changes. Since some features of DC, including bone marrow abnormalities and skin changes, have been identified in individuals from FIP families possessing telomere-related mutations,

FIGURE 2. Four-generation pedigree of the affected kindred. Numbers below individual symbols are age at death or study. *DKC1* mutation status is also depicted below age in subjects for whom DNA was available for sequencing. Subject II-2 is an obligate carrier; however, no DNA was available for analysis. Insufficient historical information was available to clinically phenotype generation I.

FIGURE 3. Affected subjects have marked telomere shortening. **A**, TRF analysis of the proband, his daughters (obligate carriers), and his maternal aunt (age 87 y), in addition to a healthy control subject. Lane markers correspond to generation and number, as depicted in Figure 2. **B**, TRF percentile for age plot. Affected proband's TRF at age 69 y is shown in red, confirmed carrier daughters' TRFs in pink, and unaffected maternal aunt's in blue. **C**, Fluorescence in situ hybridization (FISH) showing relative telomere length in type 2 alveolar epithelial cells (AECs) from lungs of the affected proband. **D**, FISH showing relative telomere length in type 2 AECs from a normal control lung. Red = telomeres; green = prosurfactant protein C; blue = 4',6-diamidino-2-phenylindole (nuclear staining, original magnification $\times 640$). **E**, Quantification of AEC telomere length by FISH in normal lung ($n = 8$), sporadic idiopathic pulmonary fibrosis ($n = 7$), and affected siblings ($n = 2$). Telomere length is quantified by ratio of red to blue fluorescence. Data are presented as mean $\pm$ SD. $*P = .003$. IPF = idiopathic pulmonary fibrosis; TRF = telomere restriction fragment.

we evaluated telomere length in PBMCs from this kindred. Using PBMCs from the proband and multiple first-degree relatives, we measured telomere length and found that the proband had markedly shortened telomeres (below first percentile for age) (Figs 3A, 3B); two of three offspring of the proband also had short telomeres (below 10th percentile for age).

With the confirmation of FIP and short telomeres, we began to search for mutations in telomere-related genes. We first sequenced the proband for mutations in *TERT* and *hTR* but did not detect a mutation. We then performed targeted sequencing of additional telomerase related genes *TINF2*, *NHP2*, *NOP10*, and *DKC1*. No mutations were detected in *TINF2*, *NHP2*, or *NOP10*; however, a previously undescribed A-to-G 1213 transition that is predicted to encode a Thr405Ala amino acid substitution was identified in *DKC1* (Fig 4). SIFT analysis, which uses an algorithm using the amino acid sequence to predict whether a muta-

tion is likely to significantly affect function, predicted this to be a deleterious mutation (SIFT score, 0.02). Confirmatory sequencing of the affected sibling's DNA identified the same Thr405Ala mutation. As *DKC1* lies on the X chromosome, each of the female offspring is an obligate carrier of the variant. To prove transmission of this variant, we sequenced *DKC1* in each daughter and confirmed heterozygosity for the same Thr405Ala mutation; a maternal aunt with normal length telomeres did not carry this variant.

Functional Characterization

Having identified this novel Thr405Ala substitution in *DKC1* in the affected subjects, we next sought to characterize its functional consequences. Since the proband had markedly shortened telomeres in peripheral blood, we sought to determine if short telomeres were also found within the lung. We measured relative telomere length in type 2 AECs

FIGURE 4. Sanger sequencing confirms *DKC1* mutation in affected sibling and obligate carriers. An A-to-G transition was identified at position 1213 that is predicted to encode a Thr405Ala amino acid substitution in the mature peptide.

from lung biopsy specimens from the proband, affected sibling, normal control lungs, and sporadic IPF cases, using a dual-fluorescence approach with fluorescence in situ hybridization (for telomere length) and fluorescence immunostaining for prosurfactant protein C (to identify type 2 AECs). Sporadic IPF lungs had shorter AEC telomeres than control lungs; however, both the affected subjects had telomeres that were below the detection threshold in this assay (Figs 3C-E).

Next, we used an in vitro approach to analyze the effect of this Thr405Ala *DKC1* substitution on mRNA expression and *DKC1* function. We isolated RNA from lymphoblastoid cell lines generated from the proband and healthy control subjects. The *DKC1* transcript level was similar

between the proband and control subjects (Fig 5A), suggesting this variant does not significantly alter *DKC1* expression or transcript stability. Notably, Thr405Ala lies in the RNA-binding domain of *DKC1*, where *DKC1* binds *hTR*, which acts as the template for TERT in the active telomerase complex. Mutations in this region have been reported to decrease *hTR* stabilization in children with DC.²¹ We found that *hTR* level was markedly decreased in lymphoblastoid cells from the proband compared with control lymphoblastoid cells (Fig 5B). Additionally, whereas *DKC1* and *hTR* levels were strongly correlated among control subjects, *hTR* level was decreased out of proportion to *DKC1* in the proband (Fig 5C), suggesting impaired *hTR* stabilization caused by the Thr405Ala *DKC1* variant.

FIGURE 5. The Thr405Ala *DKC1* substitution is associated with disproportionate reduction of *hTR* levels compared with *DKC1* expression. A, Lymphoblastoid *DKC1* mRNA quantified by quantitative polymerase chain reaction (qPCR). B, *hTR* RNA expression quantified by qPCR. Results are expressed as copies relative to β -actin normalized to healthy control subjects ($n = 4$). C, *hTR* levels were plotted vs *DKC1* expression for the proband and control subjects. Sample size precluded formal statistical analysis between the groups.

DISCUSSION

In this report, we describe a kindred with FIP caused by a previously unreported Thr405Ala substitution in *DKC1*, a component of the telomerase complex. This mutation was associated with profound telomere shortening in peripheral blood and lung tissue from two affected brothers (Fig 2). Lymphoblastoid cells from the proband showed very low *hTR* levels, suggesting that the Ala405 mutation leads to impaired *hTR* stabilization, like previously reported *DKC1* RNA binding-motif mutations. These data suggest that Thr405Ala *DKC1* is a functionally deleterious mutation and, along with the observed X-linked mode of inheritance, short telomeres, and clinical findings, represents the likely genetic basis of FIP in this kindred. Identification of this mutation in a third telomere-related gene underscores the importance of better understanding the mechanisms linking telomere dysfunction to IPF/FIP. Given the frequency of telomere shortening in FIP, we expect that mutations in other telomere-related genes will be identified in FIP families in the future.

Mutations in *DKC1* have been recognized as a cause of X-linked DC²²; however, these mutations typically present with aplastic anemia early in childhood and those affected rarely survive to adulthood.²³ Most disease-causing *DKC1* mutations cluster near the N-terminus or near the *hTR* binding/pseudourine synthase domain; the Thr405Ala substitution that we describe maps to the latter. While pulmonary disease occurs in about 20% of childhood DC cases,¹⁵ we are not aware of studies reporting the prevalence of ILD specifically in adults carrying *DKC1* mutations. This is likely because severe mutations often lead to other life-limiting complications early in life. Safa et al²⁴ reported a pair of brothers aged 35 and 40 years who presented with mucocutaneous disease and who had restrictive PFTs and HRCT scan abnormalities, and Alder et al²⁵ reported a kindred in whom an individual had pulmonary fibrosis at age 46 years. This group has previously reported another FIP with short telomeres and reduced *DKC1* expression, but no coding mutations in *TERT*, *hTR*, or *DKC1* were detected.²⁶ We are not aware of other reports of a *DKC1* mutation presenting late in adulthood as IPF/FIP.

The extreme telomere shortening in the type 2 AECs we observed in *DKC1* mutation carriers was particularly striking; however, another important finding we observed was that all patients with IPF had short telomeres in AECs. This observation is similar to what was seen in one other report,¹² suggesting that telomere shortening specifically in AECs may play a mechanistic role in the pathogenesis of IPF/FIP; further study will be needed to better clarify the mechanisms and effects of AEC telomere shortening.

The principal limitation of this study is the relatively small size of this kindred and limited number of affected individuals. Given the small numbers of affected individuals, linkage analysis is significantly underpowered to confirm “causation” with this variant. However, this variant was not present in multiple single-nucleotide polymorphism databases (dbSNP [National Center for Biotechnology Information]²⁷ or 1000 Genomes [1000 Genomes Project]²⁸) nor in the Exome Sequencing Project database²⁹; thus, we are confident this represents a rare variant. Coupled with marked

telomere shortening and evidence of *hTR* destabilization, we believe there is compelling evidence to suggest this is a deleterious mutation that represents the genetic basis of FIP in this family. The clinical phenotype in this kindred is very different from that described in other *DKC1* mutations, namely that the age of presentation of any abnormalities was much later in life and with lung disease as the predominant feature. We suspect that while this variant impairs *DKC1* function, the degree of impairment is less than that seen in other mutations, resulting in more severe phenotypes such as seen in classic X-linked DC³⁰ and Hoyeraal-Hreidarsson syndrome.³¹

The mechanism through which *DKC1* and other telomere-related genes contribute to lung fibrosis remains incompletely understood and, for a variety of reasons, animal modeling of telomere deficiency has been challenging and is of uncertain relevance to human disease.^{11,32,33} As a group, mutations in telomere-related genes tend to decrease telomerase activity, lead to short telomeres, and affect DNA damage responses. These changes can result in cell-cycle arrest, senescence, and apoptosis.³⁴ How these events lead to lung fibrosis and whether other mechanisms may be involved remain areas of active investigation. Further study is required to clarify the mechanistic links between telomeres, telomerase function, and lung fibrosis.

CONCLUSIONS

Mutations in the telomerase pathway are the most frequently identified genetic cause of FIP. In this study, we describe a novel mutation in *DKC1* that represents the likely genetic basis of FIP in a kindred with short telomeres in PBMCs and type 2 AECs in the lungs. With the identification of a third telomere-related gene associated with FIP, further study into the mechanisms linking short telomeres, telomerase function, and lung fibrosis is warranted.

ACKNOWLEDGMENTS

Financial/nonfinancial disclosures: The authors have reported to *CHEST* that no potential conflicts of interest exist with any companies/organizations whose products or services may be discussed in this article.

Role of sponsors: The sponsors had no role in the design of the study, the collection of and analysis of the data, or in the preparation of the manuscript.

Other contributions: *CHEST* worked with the authors to ensure that the Journal policies on patient consent to report information were met.

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