Exome Sequencing Identifies Mutant *TINF2* in a Family With Pulmonary Fibrosis

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BACKGROUND: Short telomeres are a common defect in idiopathic pulmonary fibrosis, yet mutations in the telomerase genes account for only a subset of these cases.

METHODS: We identified a family with pulmonary fibrosis, idiopathic infertility, and short telomeres.

RESULTS: Exome sequencing of blood-derived DNA revealed two mutations in the telomere-binding protein *TINF2*. The first was a 15-base-pair deletion encompassing the exon 6 splice acceptor site, and the second was a missense mutation, Thr284Arg. Haplotype analysis indicated both variants fell on the same allele. However, lung-derived DNA showed predominantly the Thr284Arg allele, indicating that the deletion seen in the blood was acquired and may have a protective advantage because it diminished expression of the missense mutation. This mosaicism may represent functional reversion in telomere syndromes similar to that described for Fanconi anemia. No mutations were identified in over 40 uncharacterized pulmonary fibrosis probands suggesting that mutant *TINF2* accounts for a small subset of familial cases. However, similar to affected individuals in this family, we identified a history of male and female infertility preceding the onset of pulmonary fibrosis in 11% of *TERT* and *TR* mutation carriers (five of 45).

CONCLUSIONS: Our findings identify *TINF2* as a mutant telomere gene in familial pulmonary fibrosis and suggest that infertility may precede the presentation of pulmonary fibrosis in a small subset of adults with telomere syndromes.

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ABBREVIATIONS: FPF = familial pulmonary fibrosis; IPF = idiopathic pulmonary fibrosis; PCR = polymerase chain reaction

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The incidence of idiopathic pulmonary fibrosis (IPF) increases with age.¹ IPF also has a known genetic component, as evidenced by the fact that as many as one-fifth of affected individuals report having a family member with pulmonary fibrosis.² Familial pulmonary fibrosis (FPF) is inherited most frequently as an autosomal dominant trait with age-dependent penetrance, and mutations in the telomerase genes are its most commonly identifiable cause.¹ Loss-of-function mutations in the core telomerase genes, *TERT* and *TR*, can be identified in 8% to 18% of probands with FPF.¹ More rarely, FPF displays X-linked inheritance,³ and mutations in dyskerin, the X-linked telomerase holoenzyme component encoded by *DKC1*, have been linked to sporadic pulmonary fibrosis⁴ and FPF⁵ and account for about 1% of cases (M. Armanios, MD, unpublished data, July 2008). Despite these discoveries, extrapulmonary features of a telomere syndrome, such as liver

disease and bone marrow failure, are often detected in patients with pulmonary fibrosis who have short telomeres but do not have telomerase mutations, suggesting that the full complement of telomere genes in IPF is yet to be characterized.^{6,7}

Lung disease is the most common manifestation of germline defects in telomere maintenance in adults.^{1,8} In more severe forms, telomere-mediated disease manifests in children in the disorder dyskeratosis congenita, a mucocutaneous condition classically defined by abnormal findings in the skin, nails, and oral mucosa and marked by a predisposition to bone marrow failure.⁹ In children with dyskeratosis congenita, mutations in *DKC1* and in *TINF2* are the most common identified genetic causes.⁹ Here, we report identifying by exome sequencing a mutation in *TINF2* in a family with pulmonary fibrosis.

Materials and Methods

Human Subjects

Subjects were recruited to a Johns Hopkins study aimed at understanding the genetics and natural history of telomere-mediated disease.^{10,11} The study was approved by the Johns Hopkins Medicine Institutional Review Board, NA_33072, and all the participants gave written informed consent.

Exome Sequencing

Exome sequencing was performed using the SureSelect XT All Exome V4 kit and was sequenced on the Illumina HiSeq2500 platform as described.⁴ Variants were called using the Genome Analysis Tool Kit (GATK) and were annotated using ANNOVAR (<http://www.openbioinformatics.org/annovar/>).⁴ Unique variants in telomere genes that were not found in the 1000 Genome Project Database and the Exome Variant Server were prioritized for additional studies.

Telomere Length and DNA Sequencing

Telomere length was measured on peripheral blood mononuclear cells by flow cytometry and fluorescence in situ hybridization.¹² *TERT* and *TR* sequencing was performed as outlined previously.¹³ *TINF2* exon 6 was sequenced using the following primer sets: *TINF2.E6F* 5'-CCTGGAGACAATATGGTGTGG-3' and *TINF2.E6R* 5'-AGGCTGTGATCCAATCCTG-3' (834 bp product). Because DNA derived from formalin-fixed, paraffin-embedded tissues is fragmented, we used two primer sets to genotype the two variants: *TINF2.E6.1F* 5'-AGACCTTTTGAGGCAGTCCA-3' and *TINF2.E6.1R* 5'-CCTTGAAGATGGTCCCTGAGGAAG-3' for $\Delta 15$ (247 bp product), and *TINF2.E6.2F* 5'-CAGGGACCATCTTCAAGGAC-3' and *TINF2.E6.2R* 5'-TGGA-GGCTGCTCTGTGCCCATG-3' for the Thr284Arg missense (250 bp product). The proband had no deviations from the reference sequence at primer binding sites.

Haplotype and Clonality Studies

Genomic DNA was extracted from peripheral blood using a Puregene kit (QIAGEN) and from paraffin-embedded lung tissue using standard protocols. Variants were confirmed by Sanger sequencing, and the haplotype of adjacent variants was determined by thymidine and adenosine cloning polymerase chain reaction (PCR) products into a pCR4-TOPO vector according to the manufacturer's protocol (Life Technologies) and by the sequencing of individual clones.

TINF2 Expression Studies

We measured *TINF2* mRNA levels in lymphoblastoid lines generated from the proband as well as from healthy control subjects, as described previously,³ using the following quantitative real-time PCR primers: *TINF2.E3F*: GATTTTGGAGGCACAGGAAA and *TINF2.E5R*: CTGCATCCAACCTCAGCACAT. To test the in vivo stability of mutant *TINF2* proteins, we cloned the *TINF2* genomic locus (inclusive of introns and exons) into a pCDNA5/FRT/TO vector (Life Technologies). An N-terminal Myc tag was introduced immediately after the start codon to facilitate the detection of the exogenous *TINF2* protein. Site-directed mutagenesis was used to introduce mutations, and constructs were sequence verified. Constructs were then transfected into a HeLa Flp-In T-Rex system, alongside the pOG44 vector, to generate isogenic, tetracycline-inducible lines according to the manufacturer's recommendations (Life Technologies). Cells were treated with 1 μ g/mL doxycycline for 1 week prior to protein extraction. Total protein was extracted after cells were lysed in radioimmunoprecipitation assay buffer with protease inhibitors (Roche), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using standard procedures, followed by transfer to PVDF membranes. Anti-Myc (clone 4A6, Millipore) and Tubulin (ab6046, Abcam) antibodies were used for immunoblotting, and all membranes were scanned using an Odyssey Infrared Scanner (LI-COR).

Results

Exome Sequencing Identifies *TINF2* Mutations in a Proband With FPF

The proband presented with IPF at 49 years of age and died at age 50 (Figs 1A, 1B). She did not have prema-

ture graying, blood count abnormalities, or any mucocutaneous features of dyskeratosis congenita. Her brother died of IPF at age 44 years. Both the proband and her brother had a documented history of infertility that did not respond to reproductive assistance. The family history was notable for two maternal uncles

Figure 1 – Clinical features of family with pulmonary fibrosis. A, Pedigree of index case with summary of clinical history. The proband is delineated by the arrow; □ = male family members; ○ = female family members. The black shade refers to individuals with clear telomere phenotypes and gray indicates probable obligate carrier of a *TINF2* mutation. *TINF2* genotypes are denoted adjacent to the individuals sequenced. B, Lung window images from a chest CT scan from the proband showing peripheral honeycombing predominantly in the lung bases, typical of IPF. The UIP histology was subsequently confirmed on lung biopsy. C, Lymphocyte telomere length of affected and unaffected individuals from A are plotted relative to age-matched control subjects. D, Granulocyte telomere length relative to age-matched control subjects. Nomograms and percentiles were based on data from 192 control subjects. The pedigree identifiers refer to individuals in A. C/G = missense mutation; Δ15 = deletion at the intron 5-exon 6 junction; IPF = idiopathic pulmonary fibrosis; UIP = usual interstitial pneumonia; WT = wild-type reference sequence.

who died of cryptogenic liver disease in their 30s (Fig 1A). Lymphocyte telomere length in the proband was at the first age-adjusted percentile, and granulocyte length was below the 10th percentile (Figs 1C, 1D). There were no mutations detected in *TERT* or *TR* by Sanger sequencing; however, exome sequence analysis identified two mutations in *TINF2*. The first was dele-

tion IVS5-7-c.605-612, which spanned 15 base pairs across the intron 5-exon 6 boundary (hereon referred to as Δ15). This mutation deleted the splice acceptor site and thus predicted a functionally null *TIN2* protein (Figs 2A, 2B). In addition, there was a heterozygous substitution in exon 6, c.851 C to G, which predicted a missense variant p.Thr284Arg (Figs 2A, 2B).

Figure 2 – *TINF2* mutations studies in a proband with pulmonary fibrosis. A, Schema of *TINF2* genomic locus showing position of mutations detected by exome sequencing. The exon 6 hotspot is highlighted in red. B, Chromatogram traces of sequence data from the proband derived from blood and lung genomic DNA. C, Percentage of the polymerase chain reaction-amplified products derived from clonal analysis in the blood and lung.

The latter mutation had been described in an adult with short stature, dental loss, bone marrow failure, and lung disease¹⁴ and fell within the *TINF2* exon 6 hotspot within which the vast majority of mutations reported in children with dyskeratosis congenita have been identified¹⁵⁻¹⁷ (Fig 2A).

Haplotype Studies Suggest Acquired Mosaicism of the *TINF2* Genotype in the Blood

To determine the phase of the mutations, we amplified and cloned the *TINF2* region encompassing the variants and quantified the proportion of each clone by Sanger sequencing. Among the clones sequenced from blood-derived DNA (n = 56), the wild-type allele was in 34%. The $\Delta 15$ and Thr284Arg mutations were detected in cis in 45% of the clones. The remaining clones contained either the $\Delta 15$ (17%) or Thr284Arg (4%) alone. The clonal heterogeneity suggested two compatible possibil-

ities. The first was that there was mosaicism in the blood. The second was that there was low-grade polymerase-mediated template switching in the PCR amplification reaction that contributed to the allele heterogeneity we detected. We tested for template switching by mixing plasmids that carried the $\Delta 15$ and Thr284Arg separately and found rare PCR products that contained only wild-type *TINF2* or both products in cis. Template switching may, thus, explain the rare single variant clones detected in the blood. To test for mosaicism, we sequenced genomic DNA extracted from lung biopsy tissue and found that the Thr284Arg variant predominated (40% of clones [eight of 20 screened]). Only rarely, in 8% of clones (two of 26), was the deletion detected. These data were consistent with blood-derived contamination because the vast majority of blood-derived DNA clones contained the deletion (61%) (Fig 2C). The results indicated that the Thr284Arg was the germline

mutation and that the Δ15 represented a secondary acquired variant in the blood.

Segregation Studies Support Mutant *TINF2* Segregates With IPF Phenotype

To test for segregation in the family, we sequenced genomic DNA from the proband's father and sister, who had no telomere syndrome features and normal telomere lengths, and found no mutations (Figs 1A, 1C, 1D). These findings, along with the maternal history of liver disease, implicated the patient's mother as a likely obligate carrier of the Thr284Arg mutation (Fig 1A).

TINF2 Deletion Disrupts Protein Expression

We examined the functional consequences of the mutations on endogenous *TINF2* expression by measuring the mRNA levels in cells derived from the proband and found a reduction by quantitative real-time PCR (37% compared with 100 ± 21%, n = 4 control subjects). This was consistent with the Δ15-Thr284Arg mutation causing nonsense-mediated decay. To directly examine whether the Δ15 mutation affected the expression of the

missense mutation, we cloned the *TINF2* genomic sequence under the regulation of a tetracycline-inducible cytomegalovirus promoter in HeLa cells (Fig 3A). We compared the protein stability of *TIN2* encoded by wild-type, Thr284Arg, and Δ15-Thr284Arg. For additional comparison, we included *TIN2* Arg282His, the *TINF2* mutation that is the most commonly identified in severe pediatric telomere syndrome cases.¹⁵ Both missense *TINF2* mutations produced proteins that had comparable levels to wild-type by immunoblot (Fig 3B). However, the Δ15-Thr284Arg mutation abolished the long isoform of *TIN2* and produced lower levels of a degenerate product that was smaller than the *TIN2* short isoform consistent with the splice junction mutation disrupting protein expression (Fig 3B).

TINF2 Mutations Are a Rare Cause of FPF

We had previously screened 73 probands with FPF for *TINF2* mutations in exon 6.⁶ In this study, we screened 40 additional probands with FPF and found no mutations. These data suggest that *TINF2* mutations are a rare cause of FPF and explain the genetic risk in approximately 1% of cases.

Figure 3 – Consequences of mutations on *TIN2* protein stability and proposed model of functional consequences of *TINF2* mutations. A, Schema of *TINF2* at its genomic locus inserted in FRT sites in HeLa cells. B, Immunoblot of Myc-tagged *TIN2* expressed from a single promoter shows its two isoforms, *TIN2L* and *TIN2S* as described in Kaminker and Campisi Cell Cycle 2009. A third band, possibly representing a third *TIN2* isoform, is also seen at an intermediate size between *TIN2L* and *TIN2S*. In contrast to the wild-type, and the two missense variants, Δ15-Thr284Arg abolishes the expression of *TIN2L* and creates low levels of a smaller degenerate protein product below the expected size for *TIN2S*. C, Six known familial pulmonary fibrosis genes and their frequency estimates. D, Schema of model summarizing the putative functional consequences of the *TINF2* mutations within the context of genetic and functional studies delineated. CMV = cytomegalovirus; FRT = flippase recognition target; TetO = Tet operator; TetR = Tet repressor; *TIN2L* = long-form isoform; *TIN2S* = short-form isoform. See Figure 1 legend for expansion of other abbreviations.

A History of Infertility May Precede the Onset of IPF in Telomere Syndrome Cases

In both the proband and her brother, infertility preceded the onset of IPF, and we tested whether this pattern was recurrent in other telomere syndrome cases. We reviewed the history of 45 consecutively evaluated patients with pulmonary fibrosis with telomerase mutations in a Johns Hopkins-based study and documented a history of referral for reproductive assistance in five cases (11%, three male and two female, *TERT* $n = 3$, *TR* $n = 2$). In all these cases, the infertility evaluations preceded the diagnosis of pulmonary fibrosis. Among the total of the seven cases, including the two in this family, reproductive assistance led to a successful birth in three cases (43%, two male, one female).

Discussion

Here, we report mutations in *TINF2* in association with FPF. Our observations add to a growing body of literature underscoring the intimate connection between telomere dysfunction and IPF risk. The proband and her affected brother with IPF had no features of classic dyskeratosis congenita and no bone marrow failure, the predominant phenotypes heretofore linked to *TINF2* mutations that usually manifest before the age of 10 years.¹⁵⁻¹⁷ As such, our exome findings are notable and indicate that the spectrum of disease associated with *TINF2* mutations may at times, albeit rarely, include adult-onset disease in the absence of hematologic abnormalities. With the addition of *TINF2*, there are six known FPF genes.^{4,5,13,18-20} Four of these fall in the telomere pathway and together they explain approximately one-fifth of the familial clustering of pulmonary fibrosis (Fig 3C).

Telomere length is the primary determinant of disease severity in telomere syndromes.^{9,11} Severe telomere shortening usually manifests in children with aplastic anemia, whereas attenuated telomere defects are associated with adult-onset disease complicated by pulmonary fibrosis or emphysema.^{8,9,11} Pulmonary fibrosis has been reported in two adult cases with classic dyskeratosis congenita and aplastic anemia in association with *TINF2* mutations.^{14,21} These examples further underscore the rare nature of the isolated FPF presentation in the family we report here. The determinants of disease severity beyond telomere length in *TINF2* mutation carriers are not known, but it may be that some mutations are hypomorphic, such as in this case given the adult-onset clinical presentation, or that other genetic modifiers that are yet to be identified affect the severity of the telomere defect. It is also possible that the

acquired deletion in the blood may have had a protective effect against a bone marrow failure phenotype in the proband.

The mosaicism we report here points to functional reversion as a likely acquired event and, to our knowledge, this has not been reported in the telomere syndromes. Somatic reversion is known to occur in genome instability syndromes and has been described in patients with dyskeratosis congenita with *TR* mutations.²² In these cases, homologous recombination mediates reversion to the wild-type allele in the blood.²² In our study, however, we found evidence suggesting an acquired clonal event in the blood. The mechanisms by which this occurred are unclear, but because the acquired deletion abolished the expression of the missense mutation, it may have given a growth advantage to the hematopoietic clone where it arose (Fig 3D). The selective growth advantage may have arisen because *TINF2* heterozygous null mutations are better tolerated than exon 6 point mutations in vivo. Functional correction has been documented previously in Fanconi anemia where acquired insertions and deletions in hematopoietic clones restore mutant protein function.²³ Our report indicates that this form of mosaicism may also occur in telomere disorders, and this possibility should be considered in the genetic evaluation and counseling of individuals at risk.

Although the mechanisms by which *TINF2* mutations cause telomere shortening remain to be fully elucidated, several pieces of evidence point to a dominant negative genetic model. First, the human mutations cluster in a discrete hotspot. Moreover, *TINF2* mutations have been suggested to interfere with telomere elongation by telomerase in cell culture systems.²⁴ The example we report here of an acquired deletion in the blood-abolishing expression of the mutant *TINF2* further supports this evidence. Congruent with this model, *TINF2* heterozygous mice that carry an analogous *TINF2* hotspot mutation acquire telomere shortening when bred successively for multiple generations.²⁵ Altogether, these observations suggest that *TINF2* exon 6 missense mutations likely interfere with normal telomere function and that their effect on telomere length is likely more deleterious than that of heterozygous null alleles. Additional human cell line experiments are needed to fully test this model. Although the reversion may have protected against the bone marrow failure phenotype in the proband, to date, reversion has not been detected before the age of 40 years.²² It is, therefore, possible that the missense mutation in this family represents a hypomorphic allele.

The short telomere length in the proband is consistent with the possibility that the reversion may have occurred later in life, although telomere elongation may take up to two generations to be restored in wild-type offspring of telomerase mutation carriers.^{26,27}

The presentation in this family highlights an association between short telomeres and decreased fertility in humans. Telomere shortening causes infertility and germ cell apoptosis in male mice^{28,29} and has been linked to decreased fertility in female mice.³⁰ Our retrospective observations in telomerase mutation carriers with pulmonary fibrosis suggest that infertility, both male and female, may be a first presentation of telomere syndromes in some cases. As in the index family here, the infertility phenotype may be more pronounced in later generations because of genetic anticipation, the pattern of earlier, more severe disease seen in autosomal dominant families with telomere syndromes.^{31,32} This association between telomere short-

ening and infertility has implications for the care of patients with telomere syndrome because affected patients may need earlier referral for reproductive assistance. A family history of infertility in association with pulmonary fibrosis may also raise suspicion for an inherited telomere syndrome. A telomere syndrome diagnosis should, in turn, be considered in the workup of unexplained infertility, especially in the context of other telomere syndrome features.

Conclusions

The genetic diagnosis of telomere-mediated pulmonary fibrosis has important implications for treatment. In the lung transplant setting, patients with pulmonary fibrosis are prone to otherwise rare complications caused by immunosuppressive medications that include excessive transfusion support for cytopenias, renal failure, and GI bleeding.³³ The link between *TINF2* and FPE, therefore, has implications for genetic evaluation as well as for patient care.

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Author contributions: M. A. is guarantor of the manuscript and takes responsibility for the integrity of the data and the accuracy of the data analysis. J. K. A., S. E. S., and M. A. designed the experiments and interpreted the data; J. K. A., S. E. S., C. L. W., M. H., V. S. H., and M. A. performed experiments; and M. A. wrote the manuscript.

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