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Surfactant protein C G100S mutation causes familial pulmonary fibrosis in Japanese kindred

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Abstract

Several mutations in surfactant protein C (SP-C) gene (*SFTPC*) have been reported as causing familial pulmonary fibrosis (FPF). However, the genetic background and clinical features of FPF are still not fully understood.

We identified one Japanese kindred, in which at least six individuals over three generations were diagnosed with pulmonary fibrosis. We examined the patients radiologically and histopathologically and sequenced their *SFTPC* and *ABCA3* genes. We also established a cell line stably expressing the mutant gene.

All the patients had similar radiological and histopathological characteristics. Their histopathological feature was that of the usual interstitial pneumonia (UIP) pattern, showing numerous fibroblastic foci even in areas without abnormal radiological findings on chest HRCT. No child had respiratory symptoms in the kindred. Sequencing of *SFTPC* showed a novel heterozygous mutation, c.298G>A (G100S), in the BRICHOS domain of proSP-C, which co-segregated with the disease. Meanwhile in *ABCA3* gene, no mutation was found. *In vitro* expression of the mutant gene revealed that several endoplasmic reticulum stress-related proteins were strongly expressed.

Thus, the mutation increases endoplasmic reticulum stress and induces apoptotic cell death compared with wild-type SP-C in alveolar type II cells, supporting the significance of this mutation in the pathogenesis of pulmonary fibrosis.

Keywords

Endoplasmic reticulum stress, Familial pulmonary fibrosis, Mutation,

Surfactant protein C

Introduction

Familial pulmonary fibrosis (FPF) is characterised by cases of idiopathic interstitial pneumonia in two or more first-degree relatives [1]. Marshall *et al*. estimated that familial cases account for 0.5–2.2% of all individuals with IPF [1]. Several kindreds with FPF have been reported, and the familial form is likely to be transmitted in an autosomal dominant inheritance mode [1-3]. Recent studies have revealed that several cases of FPF are associated with mutations in *SFTPC* [4, 5]. *SFTPC*, located at 8p21.3, has six exons and encodes a hydrophobic peptide called surfactant protein C (SP-C). The first reported *SFTPC* mutation, IVS4+1G>A, located at the first base of intron 4, disrupted the donor splice site and resulted in the skipping of exon 4 and the deletion of 37 amino acids from the C-terminal region of the proprotein of SP-C (proSP-C) [6]. Twenty-six *SFTPC* mutations have since been identified [6-19] (Supplementary Table 1), and all are heterozygous mutations in affected individuals. However, only a few reports described familial cases including several affected individuals [6-11] (TABLE 1).

SP-C is synthesised as a 197 amino acid proSP-C that undergoes multiple processing steps to form mature SP-C. It is finally released into the alveoli in association with other surfactant proteins and phospholipids [4, 5, 20]. Mature SP-C, consisting of 35 amino acids corresponding to Phe24-Leu58 of proSP-C, is encoded within exon 2 of *SFTPC* and

is stored in the lamellar body, from where it is secreted into the alveolar space. In the lung, proSP-C is expressed only in alveolar type II epithelial cells and the N-terminus of proSP-C is in the cytosol with the mature SP-C domain anchoring it in the membrane [4, 5, 20]. Furthermore, proSP-C contains a domain known as BRICHOS, which is thought to be involved in proteolytic processing and protecting the peptide from aggregation [21], corresponding to residues Phe94-Ile197 in the C-terminal domain of proSP-C. About three-quarters of all mutations that have been reported in *SFPTC* from interstitial lung diseases are detected in the BRICHOS domain. It has been reported that a BRICHOS mutant protein increased the amount of insoluble aggregates and resulted in apoptosis following an ER stress response [22].

Here, we investigated the clinical features of one Japanese FPF kindred with a heterozygous mutation, G100S, in the BRICHOS domain of proSP-C (SP-C^{G100S}). Furthermore, we also verified that the mutant protein SP-C^{G100S} elicited the activation of caspase 3 following the induction of ER stress.

Materials and Methods

Subjects

Pedigree and DNA samples

The family we encountered is shown in FIGURE 1a. Patient IV-1 was the proband, a Japanese girl who was referred to our hospital at the age of 18 for further assessment of an abnormal shadow in the lung field that was noticed at a school medical health check. Patient IV-2 is a younger brother of the proband (FIGURE 1a). In a routine preoperative chest radiological examination (an orthopaedic surgery for congenital dysplasia of the femur), abnormal chest shadows were noticed, and further analysis was performed in our department after the operation at the age of 16. Patient IV-3 is a younger sister of the proband. After the family history of the proband was taken, we assessed her chest by radiographic examination at the age of 14, in accordance with her and her father's requests. The three individuals in generation IV were delivered without any problem and showed normal development. None of them have histories of coughing, shortness of breath, or environmental exposures, and all were free from other respiratory symptoms. Patient III-3 was the proband's mother. She had been diagnosed with idiopathic pulmonary fibrosis (IPF) at the age of 34; she died at age 41 from lung fibrosis. Patient II-4 is a grandmother of the proband who developed a cough at the age of 63 and was

diagnosed with interstitial pneumonia. Patient III-1 is an aunt of the proband. She had no respiratory symptoms. After the family history was taken, we performed a chest radiological examination on patient III-1, at her request. It was supposed that three more family members, I-1, II-1 and II-2, died from lung disease between 35 to 45 years old following a few years of illness.

Written informed consent was obtained from the patients and their family members before participating in this study. Genetic counselling was performed for patients before and after genetic analyses. Genomic DNAs were extracted from individuals' peripheral blood (II-3, II-4, III-1, III-2, IV-1, IV-2 and IV-3) or formalin-fixed paraffin-embedded lung tissue (III-3) using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). This study was approved by the institutional review boards of Nagasaki University.

Lung biopsy and lung histopathology

For histopathological diagnosis, a lung biopsy was performed by a video-associated thoracoscopic surgery (VATS) technique under general anaesthesia. Tissue sections were prepared from formalin-fixed paraffin-embedded samples. Haematoxylin and eosin-stained sections were prepared following conventional procedures. Pathology slides were observed by two trained pulmonary pathologists.

Mutation analysis

We performed PCR-based mutation analysis of *SFTPC* (NM_003018.3) from eight specimens, composed of six affected individuals (II-4, III-1, III-3, IV-1, IV-2 and IV-3) and two unaffected individuals (II-3 and III-2). Subsequently, we also sequenced ATP-binding cassette, sub-family A member 3 (*ABCA3*; NM_001089), which was postulated to be a gene that modifies disease severity of FPF caused by *SFTPC* mutations [23]. All exons and intron-exon boundaries of the two genes were sequenced on a 3130xl automated sequencer (Applied Biosystems, Foster City, CA, USA) using BigDye Terminator version 3.1 (Applied Biosystems). DNA sequences were analysed using Variant Reporter and Sequencing Analysis (Applied Biosystems). Genomic sequences were obtained from the University of California, Santa Cruz (UCSC) genome browser (assembly: March 2006; NCBI36/hg18). PCR primers were designed with the assistance of Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi). Primer sequences are available on request.

***In silico* analysis**

The determination of whether an amino acid substitution is a recognised polymorphism was carried out using the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/). Predicted protein functions caused by an amino acid substitution were examined using PolyPhen (http://genetics.bwh.harvard.edu/pph/) and SIFT (http://sift.jcvi.org/). Comparison of genomic alignments of human and other species were accessed using online software, the ECR browser (http://ecrbrowser.dcode.org/) and the UCSC genome browser (http://genome.ucsc.edu/).

Functional analysis of mutant protein

SP-C cDNA constructs

A cDNA encoding the full-length human SP-C ($SP\text{-}C^{1\text{-}197}$) was cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) to generate $SP\text{-}C^{1\text{-}197}$/pcDNA3.1. A QuikChange® II Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA, USA) was used to generate mutant $SP\text{-}C^{G100S}$ in a single polymerase chain reaction with two primers: 5'-ATCGGCTCCACTAGCCTCGTGGTGT-3' (forward) and 5'-ACACCACGAGGCTAGTGGAGCCGAT-3' (reverse). The mutation site is underlined.

Cell culture and transfection

Human embryonal kidney (HEK) 293T cell line was obtained from Amrican Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (Gibco) at 37 °C in 5% CO_2. The culture media were supplemented with 10% foetal bovine serum (Biofluids). A549 cells (ATCC) over-expressing wild type or mutant proSP-C was constructed as follows. HEK293T cells were transiently transfected with murine leukemia virus gag–pol (2 μg) (TaKaRa Bio, Shiga, Japan), proSP-C-encoding retroviral vector (2 μg), and VSV-G expression plasmids (2 μg), which were obtained from Dr. L. Chang through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAIS, NIH, USA [24] by the FuGene HD reagent (30 μl) (Roche Applied Science, Mannheim, Germany). The cells were washed 24 h after transfection and cultured for 24 h in fresh medium. Culture supernatant of the transfected cells was inoculated into A549 cells. The inoculated cells were selected by puromycin (2.5 μg/ml). The puromycin-resistant cell pool was utilized in this study.

RNA isolation and real-time RT-PCR

Total RNA from the stably transfected A549 cells were isolated using FastPure RNA Kit (TaKaRa Bio, Shiga, Japan) and reverse transcribed into cDNA using PrimeScript RT

reagent Kit with gDNA Eraser (TaKaRa Bio). We performed real-time quantitative RT-PCR using Thunderbird SYBR qPCR Mix reagent (Toyobo, Osaka, Japan). PCR amplification was run on a LightCycler 480 Real-Time PCR system (Roche Diagnostics, Mannheim, Germany). All samples were measured in triplicates.

Western blot analysis

Cells were solubilised in RIPA buffer with PhosSTOP Phosphatase inhibitor cocktail (Roche Applied Science). Furthermore, cells treated with proteasome inhibitor MG-132 (Merck Ltd, Lutterworth, UK) for 16 h were also solubilised in the same manner. Total protein extracts were separated by 5–15% Tris-HCl gels (BioRad laboratories, Richmond, CA) electrophoresis and transferred to PVDF membranes. The membranes were blocked in blocking buffer (1× PBS, 0.1% Tween-20 with 5% w/v non-fat dry milk) for 1 h at room temperature and incubated with primary antibodies at 4°C overnight. After washing in 1× PBS with 0.1% w/v Tween-20, membranes were incubated with horseradish peroxidase-linked secondary antibodies for 1 h at room temperature. Detection was performed by enhanced chemiluminescence with ECL-Plus (GE Health Care, Buckinghamshire, England). Primary antibodies to BiP, IRE1α and Cleaved caspase-3 were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti

phosphorylated PERK (phospho-PERK) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). β-actin was measured as a loading control for each sample using anti-β-actin antibodies (Santa Cruz Biotechnology).

Statistics

Data are presented as means ± SE. The *t*-statistic was used to determine significant differences between two groups. One-way analysis of variance was used to determine significant differences among groups.

Results

Clinical presentation of patients

High-resolution CT (HRCT) findings of the proband, patient IV-1, revealed a reticulonodular shadow and intralobular fine linear opacity predominantly in both upper lung fields. Centrilobular micronodule lesions were observed mainly in subpleural lesions (FIGURE 1b). The HRCT findings of patients II-4, III-1, III-3, IV-2 and IV-3 are presented in Supplementary Figure 1a and are similar to those found in the proband. All affected individuals showed similar radiological findings, i.e. upper lung field dominant

shadow. Additionally, IV-1, IV-2, and IV-3 showed moderate cystic changes, mainly in the upper lobes, as shown in a previous report of adult FPF [11].

VATS lung biopsy was performed for diagnosis and pathological assessment. Haematoxylin and eosin-stained samples from the proband showed features of the usual interstitial pneumonia (UIP) pattern with marked fibroblastic foci and mild infiltration of lymphoid cells (FIGURE 1c). In addition, mild to moderate airway-centred fibrosis/inflammation, along with peribronchiolar metaplasia, were observed. No granulomas were seen. Interestingly, all histological samples of the patients (III-3, IV-2 and IV-3) showed a similar UIP pattern (Supplementary Figure 2).

The clinical findings and information are summarised in TABLE 2. Briefly, for the proband and her siblings, serum biomarkers, pulmonary function, and respiratory condition were almost normal, and no airway inflammation was observed in their broncho-alveolar lavage fluid (BALF). Because they had kept pet birds in their home, we measured serum antibodies to the avian antigen, but those were negative for these siblings. Thus, chronic hypersensitivity pneumonitis was clinically ruled out. Based on radio-pathological findings and family history, familial interstitial pneumonia was diagnosed.

Mutation analysis and in silico analysis

Two genes, *SFTPC* and *ABCA3*, were analysed. We detected a base alteration, c.298G>A, in exon 3 of *SFTPC* causing a GGC to AGC change that results in a glycine to serine change at codon 100 (FIGURE 2a). This variant segregated with the disease in this family (FIGURE 2a) and was not present among 576 ethnically-matched control alleles. The ECR browser and the UCSC genome browser indicated that codon 100 of *SFTPC* is conserved among mammals (FIGURE 2b). Furthermore, *in silico* analysis using SIFT and Polyphen predicted a damaging effect on the protein by this one amino acid change (position-specific independent counts score, 1.722; SIFT score, 0.03).

Expression of proSP-C in A549 cells

To prove comparable expression of proSP-C, we performed western blotting of cell lysates of SP-C^{WT} and SP-C^{G100S} stably expressed A549 cells. The amount of proSP-C was increased in SP-C^{G100S} stably expressed A549 cells compared with SP-C^{WT} stably expressed cells (FIGURE 3a). However, the expression levels of SP-C mRNA from these two cell pools assessed by real-time quantitative RT-PCR were equivalent (FIGURE 3b).

SP-C^{G100S} causes ER stress, resulting in apoptosis

We performed western blotting analysis to detect the expression of proSP-C, BiP, phospho-PERK, IRE1α and cleaved caspase-3 to determine whether the expression of the G100S SP-C mutant induces ER stress in epithelial cells compared with wild type. The activations of BiP, IRE1α and cleaved caspase-3 were increased in mutant cells compared with wild-type cells (FIGURE 3c, 3d). After MG-132 treatment, SP-C^{G100S} stably expressed A549 cells showed increases in BiP, phospho-PERK, IRE1 and cleaved caspase-3 that significantly exceeded the increases seen in SP-C^{WT} stably expressed A549 cells (FIGURE 3c, 3e).

Discussion

In the present study, we described a novel pathogenic *SFTPC* variant, which is associated with familial pulmonary fibrosis in a Japanese kindred who had abnormal HRCT findings when aged from their mid-teens to forties. This pedigree included six individuals with similar radiological findings and histopathological characteristics of the UIP pattern. Notably, all the patients were asymptomatic until they were at least 15 years old, and there was no child with respiratory symptoms. Furthermore, we also verified that expression of the mutant protein, SP-C^{G100S}, resulted in caspase 3 activation following the induction of ER stress.

Glycine at codon 100 of *SFTPC*, which was mutated to serine in this kindred, is in the BRICHOS domain of proSP-C. This mutation is novel and is the first reported pathogenic mutation of *SFTPC* in an Asian kindred, proving that pulmonary fibrosis caused by *SFTPC* mutations is a worldwide phenomenon. Recent reports showed that the BRICHOS domain of proSP-C had chaperone-like properties that prevent the transmembrane region of proSP-C from aggregating. Mutation of this region in proSP-C triggered induction of intracellular aggregate formation, ER stress, and accumulation in endosomal-lysosomal compartments [22, 25, 26]. To further characterise the mutant protein SP-C^{G100S}, we showed that unfolded protein response (UPR) proteins, including BiP (chaperone proteins), phospho-PERK and IRE1α (proximal sensor for UPR), were upregulated in A549 cells stably transformed with SP-C^{G100S}, eventually resulting in apoptotic cell death. These results are consistent with previous observations in several studies of other mutations in the BRICHOS domain, including SP-$C^{\Delta exon4}$ and SP-C^{L188Q} [22, 26, 27, 28]. Recently, Sisson *et al*. reported that targeted injury of type II alveolar epithelial cells induced pulmonary fibrosis in mice [29]. Collectively, these observations lead us to conclude that SP-C^{G100S} is a pathogenic mutation leading to cell death, which leads to pulmonary fibrosis. Categorising *SFTPC* mutations inducing lung fibrosis by functional analysis of the mutant protein might help in tailoring treatment for IPF

patients. Rosen and Waltz have reported that hydroxychloroquine was useful in treating a case of interstitial lung disease in a child with an *SFTPC* mutation in the BRICHOS domain [16]. They predicted that hydroxychloroquine caused inhibition of the intracellular processing of proSP-C, thereby reducing the dominant negative effect elicited by mutant proSP-C. It is possible that the suitability of a treatment for interstitial lung diseases with *SFTPC* mutations depends upon the location of the mutation. Hydroxychloroquine might be a suitable treatment for our cases with SP-C^{G100S}.

Intriguingly, A549 cells transfected with SP-C^{G100S} contained more proSP-C protein than SP-C^{WT}, despite the SP-C mRNA levels being equivalent. This result was inconsistent with the previous report by Bridges et al., showing that the mutant protein of SP-$C^{\Delta exon4}$ was barely detectable in contrast to the wild-type protein in the stably expressed HEK293 cell lines [27]. We also confirmed the minimal accumulation of proSP-C^{G100S} when HEK293 cells were transfected with SP-C^{G100S} (data not shown). Therefore, the observed difference is likely to be due to the difference in cell origins, not the features of mutations. Our experiments also showed that the expression of the 26-kDa isoform of the mutant SP-C^{G100S} was weaker than that of wild type in A549 cells. Formation of the 26-kDa isoform requires palmitoylation of proSP-C [30] and a 21-kDa isoform is considered to be the proprotein of pre-proteolytic processing [5, 25]. Taken

together, we speculate that the palmitoylation process in the mutant proSP-C^{G100S} was impaired and unpalmitoylated proprotein accumulated in human alveolar epithelial cells (A549). We believe that the slow degradation of unpalmitoylated proprotein in A549 cells is a better reflection of the process actually taking place in the patients presented in this report.

To date, more than 20 mutations have been described in *SFTPC*. Although studies of *SFTPC* mutations have focused on cases of children with interstitial lung diseases, there have been a few studies focusing on pedigrees with adult FPF [11, 31]. They found five kindred with *SFTPC* mutations, including two new mutations, M71V and IVS4+2T>C, in adult FPF patients. They showed histopathological patterns of UIP and non-classifiable HRCT patterns with nodular septa thickening and multiple lung cysts in combination with ground glass or diffuse lung involvement on chest HRCT. The present study, similarly focusing on a pedigree with adult FPF, highlighted some outstanding characteristics of this kindred with SP-C^{G100S}. Our patients presented with a histopathological pattern of UIP and the HRCT findings had features of nodular septa thickening and multiple lung cysts. These findings, however, were seen predominantly in the upper lobes. In particular a small number of lung cysts were present only at the apex, a feature that was inconsistent with the above report.

Interestingly, the age of phenotypic appearance (by "phenotypic appearance" we mean that radiological and histopathological findings are positive, even if presymptomatic) of all six patients was school-age or older—not at the neonatal or infancy stage, as is commonly reported for other *SFTPC* mutations. These observations caused us to make two speculations. Firstly, SP-C^{G100S} is directly involved in the severity of the disease; the late onset and slow progress of respiratory symptoms might be unique to this mutation. However, *SFTPC* mutation can have pleiotropic effects across different families, so other families with SP-C^{G100S} mutation need to be investigated carefully to confirm more characteristics of this mutation. IL-8 production in IPF patients is increased [32], but BALF findings of the proband and her siblings showed no inflammatory cells response (TABLE 2) and no IL-8 response in BALF (data not shown). This could be related to the relatively modest radiological change and late onset. SP-C^{G100S} might lead to chronic cell death, but does not induce acute inflammation, eventually resulting in respiratory symptoms and progression to lung fibrosis. Second, any genetic modifier shared with their patients might suppress the progression of the disease caused by SP-C^{G100S}, indicating an indirect involvement of SP-C^{G100S} in the severity of the disease. Bullard *et al*. implied that *ABCA3* mutations modified the severity of lung disease associated with *SFTPC* mutations [23]. In the present study, we detected no mutations in

ABCA3. However, considering the late onset of our patients through three generations, it is likely that inherited genetic and epigenetic factors might have homogeneously and moderately suppressed the cytotoxicity induced by SP-C^{G100S}.

Despite the fact that intralobular reticular opacities were barely observed in the lower lobe on chest CT, histopathological findings of fibrotic changes were found, similar to the findings of the upper lobe, where both radiological and histopathological abnormalities were seen. Recent studies suggested that fibrotic changes might be present in family members with *SFTPC* mutations who have little evidence of disease [31, 33]. In the present study, histopathological examination revealed a UIP pattern in the lower lobe in which no radiological finding was observed. Several reports showed that individuals carrying other *SFTPC* mutations including I73T have not developed the symptoms even in adulthood [8, 34]. These observations suggest that individuals with no clinical symptoms, no radiological findings and no phenotypic appearance, but who carry *SFTPC* mutations, might have pathologically recognisable fibrosis, and their lesions might be slowly progressing.

In conclusion, we detected a new pathogenic mutation in *SFTPC*. The functional analyses in this study suggest that this mutant protein, SP-C^{G100S}, elicits ER stress leading to apoptotic cell death. Our study indicates that this mutation is pathogenic and caused

slow progression of pulmonary fibrosis in this kindred. We could not confirm the reason for this slow progression; it could be a characteristic of SP-C^{G100S} or could be due to the influence of other genes or epigenetic modifications. Functional understanding of the misfolded SP-C protein is important to determine treatment approaches for FPF, which might help in tailor-made treatment based on genotype.

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Figure legends

FIGURE 1

Pedigree of a family with familial pulmonary fibrosis (FPF), and radiological and histopathological findings of the proband.

a) Pedigree of the family with FPF. Black squares and circles indicate individuals diagnosed with pulmonary fibrosis. Gray squares indicate individuals who had died due to respiratory failure; however, detailed information was not available. Asterisks indicate individuals whose DNA was available and used in direct sequencing. An arrow indicates the proband.

b) High-resolution CT (HRCT) image of the proband. Reticulonodular opacity, predominantly in both upper lung fields, and intralobar opacity in the subpleural area were observed in the HRCT image from the proband. No honeycombing lesions could be seen. A magnified CT image is shown in the right column.

c) Haematoxylin and eosin-stained tissue samples from the proband (right lung S8). Haematoxylin and eosin-stained samples revealed a usual interstitial pneumonia (UIP) pattern, including patchy peripheral accentuated fibrosis, marked fibroblastic foci (arrow), smooth muscle hyperplasia (arrow head) and abrupt changes to adjacent normal

lung areas. Bar: 1mm (i), 200μm (ii). Biopsies were performed from right lung S2 and S8. Pathological findings were similar in S2 and S8.

FIGURE 1

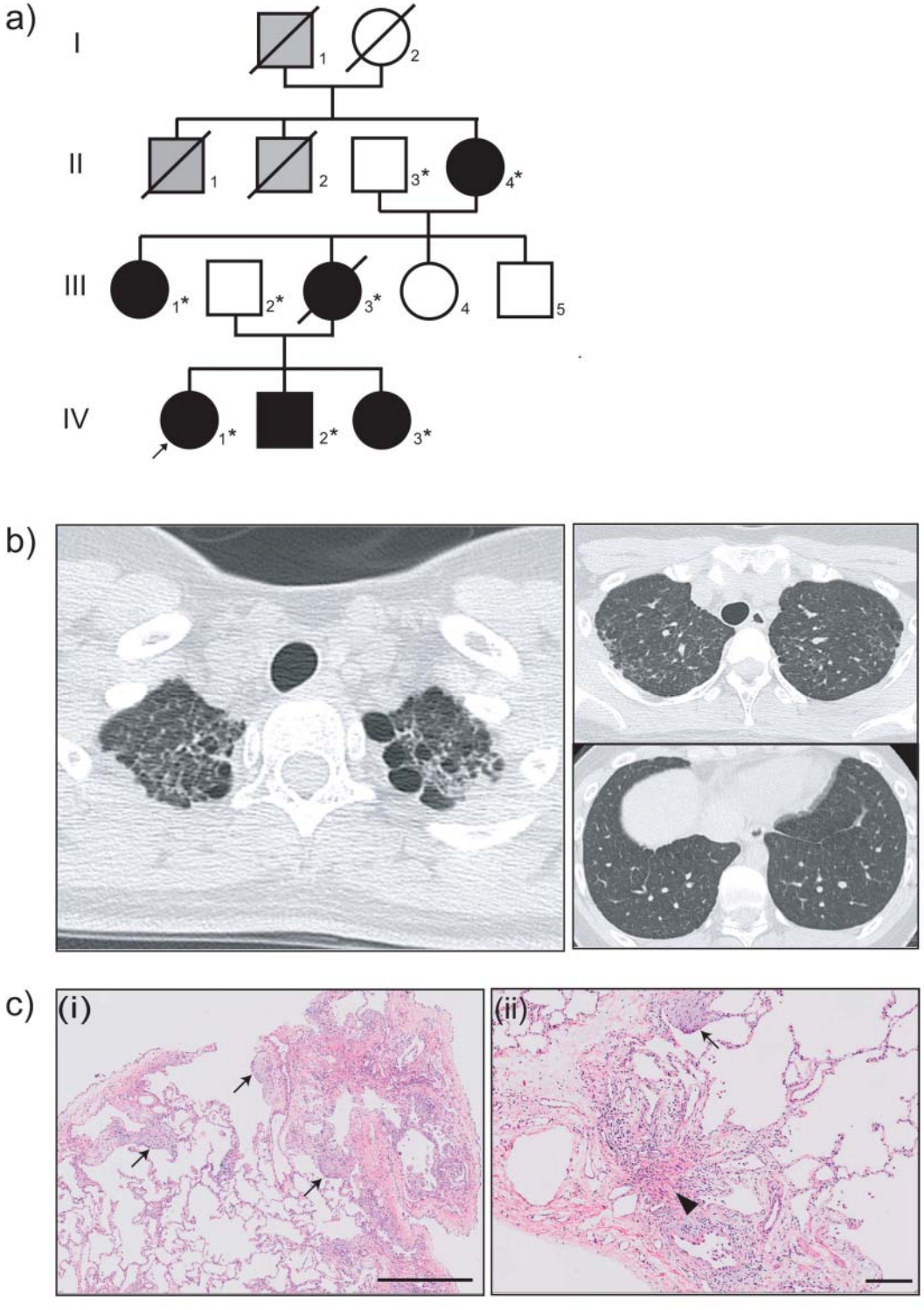
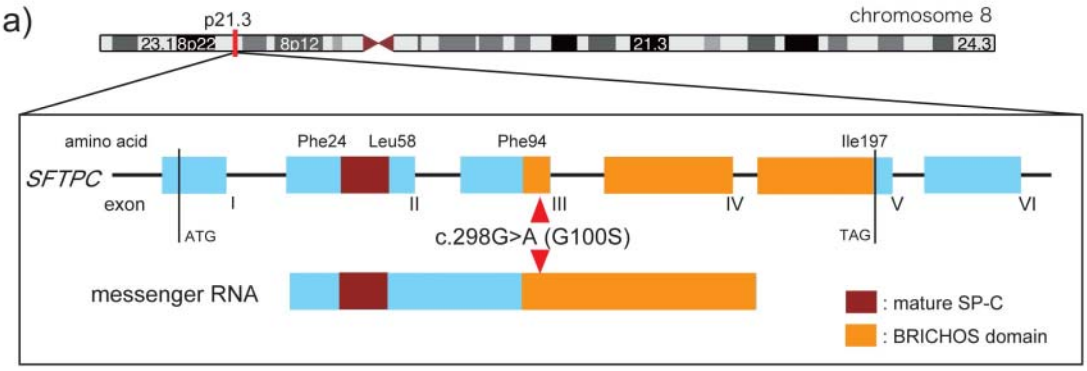


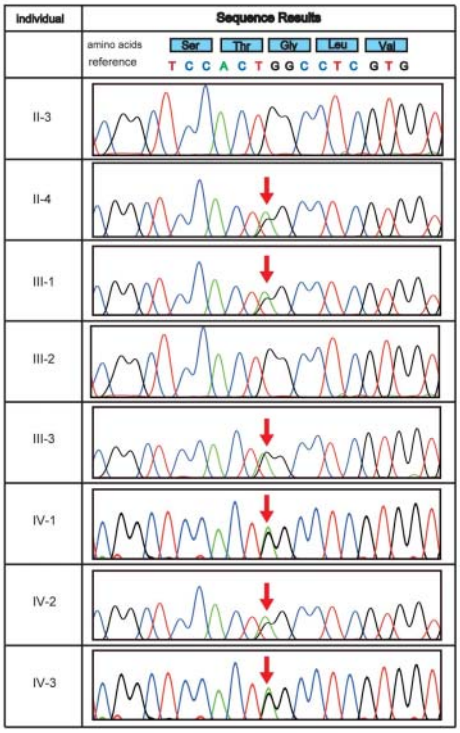
FIGURE 2

Amino acid substitution identified in surfactant protein C (SP-C) in individuals with familial pulmonary fibrosis (FPF). a) Location of *SFTPC*. Red triangle indicates the location of c.298G>A (G100S) of *SFTPC*, which is in the BRICHOS domain (upper and middle column). Results of direct DNA sequencing in eight individuals. Red arrow indicates the location of the nonsynonymous substitution (c.298 G>A) (lower column). **b)** The highly conserved orthologous protein sequences of SP-C across eight species in mammals are illustrated. The area surrounded by the red line indicates the location of codon 100 of *SFTPC*.

FIGURE 2

a)





b)

DNA sequence (Human)	A T C	G G C	T C C	A C T	G G C	C T C	G T G	G T G	T A T
Human	I	G	S	T	G	L	V	V	Y
Chimpanzee	I	G	S	T	G	L	V	V	Y
Gorilla	I	G	S	T	G	L	V	V	Y
Mouse	I	G	S	T	G	I	V	V	Y
Rat	I	G	S	T	G	I	V	L	Y
Cow	I	G	S	T	G	T	V	V	Y
Dog	I	G	S	T	G	I	V	V	Y
Opossum	I	G	S	S	G	T	V	V	Y

Species

FIGURE 3

Surfactant protein C G100S mutation (SP-C^{G100S}) elicits the induction of endoplasmic reticulum (ER) stress that leads to apoptotic cell death.

a) Western blotting for proSP-C in whole cell lysate of A549 cells stably expressing wild-type SP-C (SP-C^{WT}) or SP-C^{G100S}. b) Expression of SP-C mRNA in the two cell pools. Normalized expression levels are shown relative to beta 2 microglobulin as an internal control gene. c) Immunoblot analyses using antibody against BiP, IRE1α, phospho-PERK and cleaved caspase-3 in whole cell lysate of A549 cells stably expressing SP-C^{WT} and SP-C^{G100S} and empty vector. Cell lysate treated with Tunicamycin (Tm) or TNFα was used as a positive control. Data were obtained from cell pools from at least three separate experiments. Error bars represent SE. The band intensity values were normalized with β-actin and empty vector band intensities. *$p < 0.05$, **$p < 0.01$. Tm = tunicamycin.

FIGURE 3

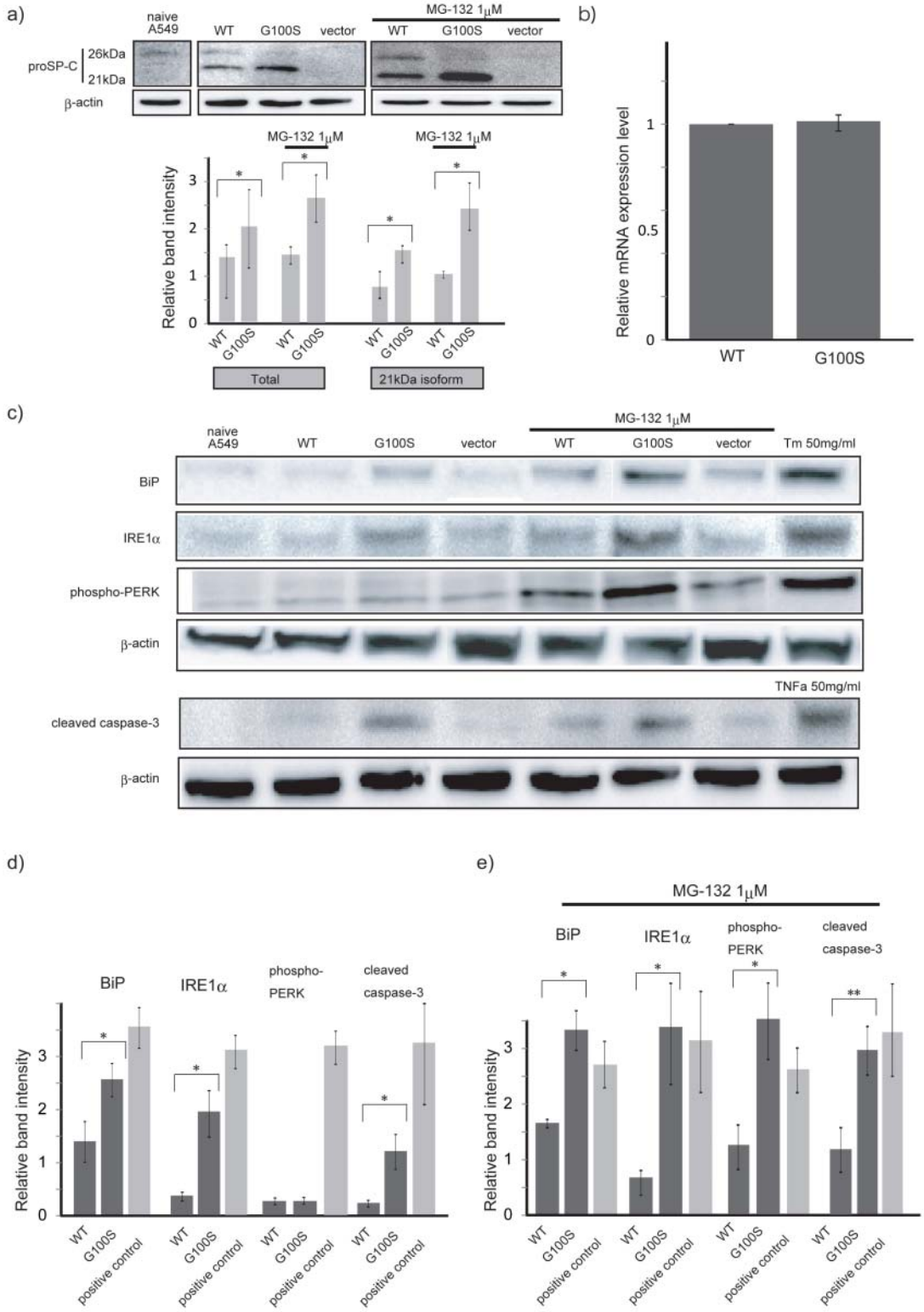

a)
naive A549
WT
G100S
vector
MG-132 1μM
WT
G100S
vector
proSP-C
26kDa
21kDa
β-actin
MG-132 1μM
MG-132 1μM
Relative band intensity
WT
G100S
Total
21kDa isoform
b)
Relative mRNA expression level
WT
G100S
c)
naive A549
WT
G100S
vector
MG-132 1μM
WT
G100S
vector
Tm 50mg/ml
BiP
IRE1α
phospho-PERK
β-actin
TNFa 50mg/ml
cleaved caspase-3
β-actin
d)
BiP
IRE1α
phospho-PERK
cleaved caspase-3
Relative band intensity
WT
G100S
positive control
e)
MG-132 1μM
BiP
IRE1α
phospho-PERK
cleaved caspase-3
Relative band intensity
WT
G100S
positive control

TABLE 1.

Published SP-C mutations found in large families.

Mutation	References	Families with ILD	Pathology
MET71VAL	Van Moorsel et al. 2010 [11]	1 family	UIP/DIP_adults
ILE73THR	Cameron et al. 2004 [8]	3 families	NSIP
	Abou Taam et al. 2009 [10]	1 family	unspecified
	Van Moorsel et al. 2010 [11]	3 families	UIP_adults NSIP/DIP_child
IVS4+1,G>A,	Nogee et al. 2001 [6]	1 family	NSIP_child DIP/UIP_adults
IVS4+2,T>C	Van Moorsel et al. 2010 [11]	1 family	NSIP/DIP_children UIP_adults
LEU188GLN	Thomas et al. 2002 [7]	1 family	NSIP_children DIP/UIP_adults
CYS189TYR	Guillot et al. 2009 [9]	1 family	NSIP
LEU194PRO	Guillot et al. 2009 [9]	1 family	NSIP

ILD: interstitial lung disease

NSIP: non-specific interstitial pneumonia

DIP: desquamative interstitial pneumonia

UIP: usual interstitial pneumonia

TABLE 2. Patient profile and laboratory data

	patient					
Data obtained	II-4	III-1	III-3**	IV-1	IV-2	IV-3
sex	female	female	female	female	male	female
Age at present (yr)	68	46	41(died)	18	16	14
Age of diagnosis	66	46	34	18	16	14
Age of first evidence	57	44	34	18	16	14
Age of first symptoms	63	None	34	None	None	None
Serum biomarker						
KL-6 (U/ml) (<500)	1560	386	N/A	245	309	332
LDH (IU/l) (119-229)	303	147	166	161	141	144
PFT						
%VC (%) (>80)	42.5	101.9	65.3	72.2	85	96.6
$FEV_{1.0}$% (%) (>70)	92.9	92.8	83.3	84.1	90.3	85
%DLCO (%) (>80)	38.5	72.2	N/A	69.3	N/A	65.2
BGA (Room air)						
PaO_2 (Torr) (75-100)	68.3*	83.7	90.6	113	109	111
A-aDO_2 (Torr) (<10)	24.2*	16.2	6.1	-8.5	-14	-10.8
BAL						
Cell count (x10^5/ml)	1.21	N/A	3.85	2.4	2	1.4
AM (%)	54.2	N/A	80	90	86	91
Lym (%)	10.1	N/A	17.3	7.5	12	5.8
Neut (%)	34.5	N/A	1.1	2.5	1	2.4
Eo (%)	1.2	N/A	1.6	0	1	0.8
CD4/CD8 ratio	0.25	N/A	0.6	1.7	1.6	1.5
Histological pattern	UIP	N/A	UIP	UIP	UIP	UIP

Abbreviations: A-aDO2 = alveolar-arterial oxygen tension difference, AM = alveolar macrophage, BAL = Bronchoalveolar lavage, BGA = Blood gas analysis, Eo = eosinophil, $FEV_{1.0}$% = forced expiratory volume percent, Lym = lymphocyte, Neut =

neutrophil, N/A = not available, PFT = Pulmonary function test, PaO_2 = arterial oxygen partial pressure, %VC =vital capacity percent, UIP = usual interstitial pneumonia. *These data are from the first diagnosis, **Data from patient III-3 are based on those from the first diagnosis