Germline SFTPA1 mutation in familial idiopathic interstitial pneumonia and lung cancer

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Received: November 17, 2015. Revised and Accepted: January 14, 2016

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Abstract

Idiopathic interstitial pneumonias (IIPs) comprise a heterogeneous group of rare lung parenchyma disorders with high morbidity and mortality, which can occur at all ages. In adults, the most common form of IIPs, idiopathic pulmonary fibrosis (IPF), has been associated with an increased frequency of lung cancer. The molecular basis of IIPs remains unknown in most cases. This study investigates IIP pathophysiology in 12 families affected by IPF and lung cancer. We identified, in a multigenerational family, nine members carrying a heterozygous missense mutation with evidence of pathogenicity in SFTPA1 that encodes the surfactant protein (SP)-A1. The mutation (p.Trp211Arg), which segregates with a disease phenotype characterized by either isolated IIP/IFP, or IFP associated with lung adenocarcinoma, is located in the carbohydrate recognition domain (CRD) of SP-A1 and involves a residue invariant throughout evolution, not only in SP-A1, but also in its close paralog SP-A2 and other CRD-containing proteins. As shown through functional studies, the p.Trp211Arg mutation impairs SP-A1 secretion. Immunohistochemistry studies on patient alveolar epithelium showed an altered SP-A expression pattern. Overall, this first report of a germline molecular defect in SFTPA1 unveils the key role of SP-A1 in the occurrence of several chronic respiratory diseases, ranging from severe respiratory insufficiency occurring early in life to the association of lung fibrosis and cancer in adult patients. These data also clearly show that, in spite of their structural and functional similarities, SP-A1 and SP-A2 are not redundant.

Introduction

Idiopathic interstitial pneumonias (IIPs) (MIM263000) comprise a heterogeneous group of rare disorders that affect the distal part of the lung and are characterized by a progressive remodeling of the alveolar interstitium. IIPs are diseases that can be observed at all ages, although they are more frequently diagnosed in adults (1,2). The most common and severe form of IIPs is idiopathic pulmonary fibrosis (IPF) (MIM178500), a condition mainly observed in older adults with a median survival of 3–5 years after diagnosis (3–5). Noteworthy, compared with control populations, IPF has been shown to be associated with an increased risk of developing lung cancer (with reported prevalences ranging from 3 to 23%) (6).

The pathogenesis of IIP remains puzzling. The current understanding suggests a multiple hit model, with ongoing or repetitive insults of a vulnerable alveolar epithelium resulting in aberrant lung repair and progressive fibrosis (7–9). Observations of familial clustering of the disease have provided arguments for a genetic component to alveolar susceptibility and to the risk of IIP (10–13). To date, genetic factors involved in the development of IIP include genes encoding molecules of the telomerase complex (such as TERT, TERC, RTE1, DKC1, TINF2 and PARN) in adult IP (14–20), and genes encoding proteins that play a critical role in surfactant metabolism (SFTPB, SFTPC and ABCA3), mainly in pediatric cases (21,22). So far, little attention has been paid to the other surfactant proteins (SPs), despite their broad role in lung homeostasis. The most abundant SP is SP-A, which is structurally homologous to the collectins, a family of innate immune response components. It has indeed important roles, not only in surfactant production through its involvement in tubular myelin formation (23), but also in pulmonary host defense and in the repair processes of the lung parenchyma (24–27). In humans, SP-A is encoded by SFTPA1 (NM_005411) and SFTPA2 (NM_001098668). These two genes, located in chromosomal region 1q22 and composed of six exons (4 coding), are separated by a pseudogene (SFTPA3P). They encode two very similar 35–37 kDa proteins (SP-A1 and SP-A2) that share a highly conserved carbohydrate recognition domain (CRD). SP-A1 and SP-A2 are secreted in the alveolar space and assembled as a flower-like bouquet structure of six heterotrimers (28–30). In two families in which lung cancer and IPF were shown to co-segregate in an autosomal dominant manner, two heterozygous mutations have been identified in SFTPA2 (F198S and G231V) (31). More recently, four new SFTPA2 mutations have been reported in adult cases of IPF and lung cancer (32,33). The potential implication of SFTPA1 in IIP and lung cancer is, however, so far unknown. To further investigate the implication of SP-A in the pathogenesis of IIP, we sequenced SFTPA1 and SFTPA2 in a cohort of patients with familial forms of IIP and a personal and/or family history of lung cancer.

Results

Identification of a germline mutation in SFTPA1

Twelve patients met the patients’ selection criteria (Supplementary Material, Fig. S1). The study group of 12 patients belonged to 12 apparently unrelated families (Table 1, and Supplementary Material, Fig. S2). Patients were aged 30–78 years at the time of IIP diagnosis (median age: 57 years). The medical records documented the presence of a lung cancer in seven of them (four were smokers), and a first-degree family history of lung cancer in six patients. No SFTPA2 mutations were identified in all these patients. In two patients (Patients 1 and 4, Table 1), who presented with IPF and lung adenocarcinoma, the screening of SFTPA1 revealed two sequence variations in the heterozygous state. The first one is a C-to-T transition located within exon 6 (c.655C>T; rs4253527; NM_005411.4) and predicting an amino-acid substitution at codon 219 (p.Arg219Trp). This missense variation, which involves a residue that is not conserved throughout evolution and is predicted as a benign variation by the PolyPhen2 software, is reported in polymorphism databases with an allele frequency of 2–25% (Exome Variant Server, dbSNP, Ensembl, 1000 Genomes, ExAC), thereby arguing against its involvement in disease development. The other sequence variation is a T-to-C transition (c.631T>C; p.Trp211Arg; W211R) (Fig. 1A), also located in exon 6, which is predicted as probably damaging by PolyPhen-2. It has not been described in polymorphism databases (1000 Genomes, Ensembl, dbSNP, Exome Variant Server, ExAC). It predicts the replacement at codon 211 of an apolar residue (tryptophane) by a polar and charged amino acid (arginine). At the protein level (Fig. 1B), this nonsense variation (p.Trp211Arg; W211R) is located in the CRD of SP-A1 and involves a residue that is invariant throughout evolution not only in SP-A1 and SP-A2, but also in proteins with a SP-A-like or a lectin domain containing a CRD. Such a high degree of conservatism of tryptophan 211, therefore, strongly suggests that amino-acid substitutions at this position could be detrimental to protein function.
Intrafamilial segregation of the SP-A1 W211R variation

In order to study the intrafamilial segregation of the SFTPA1 W211R missense variation with the patients’ phenotype, the pedigrees of the two probands were then constructed (i.e. Patients 1 and 4 in Table 1, corresponding to individuals IV.3 and IV.16, respectively, in Fig. 2). Unexpectedly, additional information from the two family records and their history revealed similar geographic origins and professional orientations (mainly barge workers), and allowed us to assemble their members into a unique family (hereafter named family 1/4) with a common forefather (individual I.1). The genotyping of 13 intragenic non-coding single-nucleotide polymorphisms (SNPs) in the family showed that the individuals carrying the W211R variation share the same SFTPA1 haplotype (hereafter named H1), confirming the unicity of family 1/4 (Fig. 2 and Supplementary Material, Table S1). Phenotypic characteristics of the family 1/4 members that could be investigated are summarized in Table 2. In addition to Patients IV.3 and IV.16, eight individuals (II.4, III.1, III.2, III.8, IV.4, V.2, V.8 and V.11) had a diagnosis of IIP. These patients were aged 7 months to 69 years at onset of symptoms. Analysis of SFTPA1 and SFTPA2, that could be performed in four of them (IV.4, V.2, V.8 and V.11), showed the absence of SFTPA2 mutations, but the presence, in all of them, of the SFTPA1 W211R missense variation in the heterozygous state.

Table 1. Characteristics of the study group patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Family</th>
<th>Gender</th>
<th>Age at IIP diagnosis (years)</th>
<th>Personal history of lung cancer, no/yes (type of cancer)</th>
<th>First-degree family history of lung cancer, no/yes</th>
<th>Exposure, No/Yes (type)</th>
<th>SFTPA1 c.631T&gt;C mutation (W211R)</th>
<th>SFTPA1 c.655C&gt;T variant (R219W)</th>
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<td>1</td>
<td>1</td>
<td>M</td>
<td>45</td>
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<td>No</td>
<td>Yes (tobacco, barge worker)</td>
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<td>Yes</td>
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<tr>
<td>2</td>
<td>2</td>
<td>M</td>
<td>70</td>
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<td>No</td>
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<tr>
<td>3</td>
<td>3</td>
<td>M</td>
<td>40</td>
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<td>No</td>
<td>Yes (tobacco)</td>
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</tr>
<tr>
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<td>F</td>
<td>52</td>
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<td>No</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>5</td>
<td>5</td>
<td>F</td>
<td>43</td>
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<td>No</td>
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<tr>
<td>6</td>
<td>6</td>
<td>M</td>
<td>78</td>
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<tr>
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<td>M</td>
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<td>8</td>
<td>F</td>
<td>72</td>
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<td>No</td>
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<tr>
<td>9</td>
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<td>M</td>
<td>65</td>
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<tr>
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<tr>
<td>11</td>
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<td>M</td>
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<tr>
<td>12</td>
<td>12</td>
<td>F</td>
<td>62</td>
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<td>Yes</td>
<td>Yes (tobacco, firewood)</td>
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<td>No</td>
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</table>

IIP, idiopathic interstitial pneumonia; NA, not available.

Intrafamilial segregation of the SP-A1 W211R variation

In order to study the intrafamilial segregation of the SFTPA1 W211R missense variation with the patients’ phenotype, the pedigrees of the two probands were then constructed (i.e. Patients 1 and 4 in Table 1, corresponding to individuals IV.3 and IV.16, respectively, in Fig. 2). Unexpectedly, additional information from the two family records and their history revealed similar geographic origins and professional orientations (mainly barge workers), and allowed us to assemble their members into a unique family (hereafter named family 1/4) with a common forefather (individual I.1). The genotyping of 13 intragenic non-coding single-nucleotide polymorphisms (SNPs) in the family showed that the individuals carrying the W211R variation share the same SFTPA1 haplotype (hereafter named H1), confirming the unicity of family 1/4 (Fig. 2 and Supplementary Material, Table S1). Phenotypic characteristics of the family 1/4 members that could be investigated are summarized in Table 2. In addition to Patients IV.3 and IV.16, eight individuals (II.4, III.1, III.2, III.8, IV.4, V.2, V.8 and V.11) had a diagnosis of IIP. These patients were aged 7 months to 69 years at onset of symptoms. Analysis of SFTPA1 and SFTPA2, that could be performed in four of them (IV.4, V.2, V.8 and V.11), showed the absence of SFTPA2 mutations, but the presence, in all of them, of the SFTPA1 W211R missense variation in the heterozygous state.

The disease phenotypes of the six patients carrying the SP-A1 W211R variation are listed in Table 2. The W211R missense variation was first identified in a 53-year-old male patient (IV.3), who was a former smoker and worked as bargeman. He was
diagnosed at age 45 with IPF complicated at age 50 by lung adeno-
carcinoma (Fig. 3, IV.3 A and B). The second proband (IV.16) is a
58-year-old non-smoker woman, diagnosed with IPF at age 52
and with lung adenocarcinoma at age 54 (Fig. 3, IV.16 A and B). In-
dividual IV.4, who was a tobacco-smoker barge worker, was diag-
nosed at the age of 40 with IIP. She experienced worsening of her
IPF with no evidence of lung cancer during 18 years of follow-up
(Fig. 3, IV.4 A and B). For Patients V.2 and V.8, the diagnostic of IIP
was established when they were in their early thirties. The
youngest patient (V.11), who was the daughter of Patient IV.16,
presented at the age of 7 months with a severe respiratory insuf-
fi ciency. An infection was first suspected, but was not con-
fi rmed by microbiologic investigations. Her respiratory condition pro-
gressively deteriorated and she died 2 months later. A recent
pathological review of her lung biopsy performed when she
was 8 months was in favor of pre-existing fibrosing IIP (Fig. 4).
Genetic studies could then be undertaken from a stored frozen
white blood cell sample and revealed the presence of the SP-A1
W211R missense variation.

After informed consent and medical evaluation, genetic test-
ing could be performed in 12 other family members (Fig. 2). Ele-
ven of them were free of respiratory symptoms at the time of
the study and no abnormalities could be observed on their
chest high-resolution computed tomography (HRCT) scans
(III.4, IV.5, IV.7, IV.8, IV.11, IV.13, IV.17, V.1, V.3, V.9, V.10). One
member (IV.14), a 14-year-old girl, had a mental retardation and
frequent bronchitis. Review of her clinical and radiological record
was in favor of respiratory complications of her neurological con-
dition associated with aspiration and lung infection. The SP-A1
W211R missense variation was identified in three of them
(V.17, V.10 and V.14). The family study also revealed the pres-
ence, in the heterozygous state, of the SFTP A1 polymorphism
c.655C>T (R219W) in all the individuals carrying the SP-A1
W211R variation (Fig. 2). As shown by the haplotype analysis
(Fig. 2 and Supplementary Material, Table S1), the H1
haplotype (in red) corresponding to the allele that carries the W211R mutation. The asterisk (*) indicates that the haplotype analysis led to the identification of a H2
haplotype in the homozygous (or hemizygous) state.

![Pedigree of the family with the identified SFTP A1 mutation c.631T>C (W211R). The arrows indicate the two index cases (individuals 1 and 4, see Table 1). Individuals with IPF are indicated by a black symbol. Symbols that are half black and half checkerboard indicate individuals with IPF and lung cancer. A red outline indicates the presence of the SP-A1 W211R mutation in the heterozygous state, and a green outline indicates a wild-type sequence at codon 211. Each individual is identified by his generation (Roman numbers) and its number (Arabic number). SFTP A1 haplotypes are mentioned (see Supplementary Material, Table S1), the H1 haplotype (in red) corresponding to the allele that carries the W211R mutation. The asterisk (*) indicates that the haplotype analysis led to the identification of a H2 haplotype in the homozygous (or hemizygous) state.](1460.png)
fully penetrant, while excluding the implication of the R219W variation.

**Modeling of structural and electrostatic changes induced by the SP-A1 W211R mutation**

With the aim of assessing the effect of the W211R missense variation on SP-A1 conformation, we generated wild-type and mutated SP-A1 3D structures using the rat SP-A1 crystal structure (residues 201–248) as template. When compared with wild-type SP-A1 CRD domain, prediction of the 3D structure of the protein carrying the W211R missense variation disclosed no major conformational changes in the vicinity of the mutated residue, with moderate consequences on the overall domain structure (similar root mean square deviation (RMSD) values of wild-type (WT) and mutated SP-A1 domains: <0.8 using SuperPose Version 1.0) (Fig. 5A) (34).

However, electrostatic modeling of the protein domain surface showed a strong polarity change in the region surrounding residue 211, when compared with the corresponding wild-type SP-A1 domain (Fig. 5B), a result in keeping with the location of the lateral chain of residue 211 at the surface of this domain.

**Impact of the SP-A1 W211R mutation on protein production and secretion**

To further evaluate the functional consequences of the W211R substitution at the protein level, we compared the expression of wild-type SP-A1 (SP-A1_WT) and SP-A1 carrying the W211R variation (SP-A1_W211R) both in cellular lysates and in the medium of HEK293T cells transfected with the wild-type or mutated expression plasmid. Similar experiments were performed with SP-A1 proteins carrying the R219W variation (SP-A1_R219W) or the F198S mutation previously identified in SFTPA2.

As shown in Figure 6A, the SFTPA1 variants had no effects on protein expression. To determine whether the W211R missense variation identified in SP-A1 could impair protein secretion, similar experiments were performed on the medium of cells transfected with an expression plasmid encoding SFTPA1_W211R and the results were compared with those obtained on the medium of cells transfected with the other SFTPA1 expression plasmids. A single molecular species of expected molecular weight (35–37 kDa) was found in the secreted material from WT SP-A1-expressing cells. This molecular species was also present in the medium of cells expressing the R219W variant. In contrast, no SP-A1 protein could be detected in the medium of cells transfected with the SFTPA1 expression plasmids carrying the W211R or the F198S missense variation (Fig. 6C). Taken together, these data show that the W211R missense variation identified in family 1/4 is a deleterious mutation resulting in the absence of SP-A1 secretion. The key importance of residue 211 for a proper release of SP-A1 is also supported by the fact that similar data were obtained with a mutant form of SP-A2 carrying the W211R mutation (Fig. 6B and D).

**Surfactant protein A expression pattern in the lung tissue of a patient carrying the SP-A1 W211R mutation**

To provide further support to an altered parenchymal expression of SP-A1 in patients with the SP-A1 W211R mutation, tissue samples from the lung biopsy of Patient V.11 were studied by means of immunohistochemistry using an antibody directed against SP-A1.
of hematoxylin and eosin (HE) staining and immunohistochemistry analyses using a mouse anti-SP-A monoclonal antibody (Fig. 4, A and B). For comparison, similar studies were performed on lung samples from two age-matched controls, one without respiratory disease (Fig. 4, D and E) and one with a SFTPC mutation associated lung disease (Fig. 4, G and H). The lung parenchyma from Patient V.11 displayed a severe diffuse interstitial fibrosis with collapse of most alveolar spaces. This was associated with a discontinuous and mostly intra-cytoplasmic SP-A staining of the hyperplastic pneumocytes. This expression contrasted with the continuous linear layer of SP-A at the alveolar surface of the two control specimens, which has been previously reported as a labeling of the intra-alveolar surfactant material (35). To document whether the expression of other SP proteins could also be impaired, we performed immunohistochemistry analysis using an anti-SP-C antibody. The results showed that similar patterns of alveolar SP-C expression were found in the lung tissues from Patient V.11 and from the control individual without respiratory disease (Fig. 4, C and F). These findings contrasted with the altered pattern of SP-C expression in the tissue from the individual with a SP-C disorder (Fig. 4, I).

**Discussion**

In this study, we identified SFTPA1 as a novel gene involved in the development of IIP. Focusing on IIP patients with a personal and/or a family history of lung cancer, we identified a large family with several members heterozygous for the SFTPA1 c.631T>C (W211R) mutation.

A striking observation is the large variability in patient age at the time of diagnosis, ranging from infancy to elderly. This is well illustrated by the medical history of individual V.11 who developed early in life a severe respiratory disease with a dramatic and fatal outcome. In contrast, the diagnosis of IPF was established in her mother (IV.16) at the age of 52, thereby suggesting that altered SP-A1 expression could impact on lung parenchyma homeostasis from the early postnatal period to the elderly. We also found this mutation in asymptomatic family individuals at the time of the study, in keeping with an incomplete penetrance. Such phenotypic heterogeneity may be explained by the number of molecular processes involved in IIP pathophysiology. It is currently believed that IIP is the result of complex interactions between genetic and environmental factors, with exposures to
inhaled agents and virus being important risk factors (36,37). In familial IPF, cigarette smoking was reported as the strongest risk factor for disease development (10,38). In the present family, exposure to cigarette smoke was documented in several members. Also, some individuals shared similar professional situations as barge workers, suggesting an important dust exposure.

The genetic and functional data reported herein strongly support a pathogenic role of the W211R mutation identified in SFTPA1 and its involvement in the development of various forms of IIP. As for the R219W missense variation, it was found in all the individuals carrying the SFTPA1 c.631T>C (W211R) mutation, in close linkage disequilibrium with the disease-causing mutation. Although one cannot exclude the possibility that the R219W variation may act as a modifier factor in IIP, its high frequency in control populations and its presence in a healthy relative of the large family studied here (individual IV.8) argue against its involvement as a disease-causing mutation. In addition, functional assays performed in cells expressing SFTPA1_R219W or the SFTPA1 isoform carrying both the R219W and W211R variations, there was no detectable deleterious effect of the R219W variation expressed alone or in association with the W211R mutation (data not shown).

The present results indicate that the SP-A1 W211R mutation is associated with an impaired SP-A1 secretion and an abnormal expression pattern of SP-A in the lung parenchyma. SP-A1 contributes to the surfactant homeostasis, through its implication in the production and secretion of the phospholipid materials by the type 2 pneumocytes, as well as in the formation of the myelin structure at the air–liquid interface of the lung (39–41). It also participates, through several signaling pathways, in the regulation of the pulmonary immune response (24–27,42,43). In addition, it is implicated in the alveolar repair process including the fibroproliferative response (8,44). The altered secretion of SP-A1 in the alveolar space may, therefore, alter a number of lung alveolar functions, thereby explaining the spectrum of pathological consequences, ranging from a severe respiratory disease occurring early in life to the clinical association of lung fibrosis with lung cancer in adult patients. These data raise the question of the respective contribution of SP-A1 and SP-A2 in the various SP-A biological functions. SFTPA2, which encodes a protein that is 96% identical to SP-A1, was sequenced in all the study group patients, and no mutations were identified. When introduced into SP-A1, the F198S mutation, previously identified in SFTPA2 (27) and that was shown to interfere with SP-A2 secretion, had similar consequences on SP-A1 secretion. Reciprocally, when introduced into SP-A2, the W211R mutation also impaired SP-A2 secretion. Taken together, these data demonstrate that, in spite of these structural and functional similarities, SP-A1 and SP-A2 are not redundant. Accordingly, it has been speculated that the overall SP-A activity depends not only on the total amount of SP-A but also on the ratio of SP-A1 to SP-A2, with an inadequate ratio contributing to an altered parenchyma homeostasis (40).

![Figure 4](https://humanmoleculargenetics.oup.com/content/25/8/1457/F4)

**Figure 4.** SP-A expression on lung tissue from a patient carrying the SP-A1 W211R mutation. Histologic examination (×400) was performed on lung tissues from Patient V.11 with the W211R mutation (A–C), and from two age-matched individuals, one corresponding to a boy with normal lung (control individual) (D–F), and the other one to a female patient with a SFTPC mutation (G–I). HE staining was performed, and SP-A and SP-C expression were analyzed by immunohistochemistry. In Patient V.11, HE staining of the lung parenchyma (A) showed a severe diffuse interstitial fibrosis (*) with collapse of most alveolar spaces, and residual alveoli with type II pneumocytes hyperplasia, contrasting with the normal lung parenchyma structure of the control individual (D). SP-A staining of Patient V.11 tissue samples (B) documented a strong expression by hyperplastic pneumocytes of the collapsed alveoli (→), but this expression was heterogeneous and without the continuous secreted SP-A linear layer observed in the control individual (E). In Patient V.11, cytoplasmic SP-C expression by type II pneumocytes (→) was similar to that of the control individual (C and F). In the individual with a SFTPC mutation, HE staining (G) showed a typical IIP pattern characterized by diffuse septal thickening, type II pneumocyte hyperplasia and intra-alveolar macrophages. In this individual, SP-A analysis (H) revealed a pattern of expression located on the hyperplastic type II pneumocytes and associated with a thin continuous secreted SP-A linear layer on the alveolar border (→), similar to the expression observed in the control individual (E). As expected, in the tissue specimens from the individual with a SP-C disorder, SP-C expression (I) was dramatically decreased with a dot pattern (→), which contrasted with the homogeneous expression of SP-C on type II pneumocytes (→) observed in the materials from the control individual and Patient V.11 (F and C).
molecular investigations on SP-A1 and SP-A2 should help deciphering the influence of sequence variants found in each of these proteins in disease expression.

In summary, this study, which identifies SFTPA1 as a gene involved in IIP, shows that altered SP-A1 expression is associated with a large spectrum of phenotypic pulmonary manifestations.

Figure 5. Modeling of structural and electrostatic changes induced by the SP-A1 W211R mutation on the CRD. (A) 3D structures of wild-type and mutated SP-A1 proteins. The secondary structure is colorized according to the following code: beta-sheets in red, alpha helices in green and loops in yellow. Residue 211 is represented with sticks. Oxygen and nitrogen atoms are shown in red and blue, respectively. The arrow indicates Tryptophan 211 and Arginine 211 in the wild-type and mutant proteins, respectively. (B) Electrostatic properties of wild-type and mutated SP-A1 proteins. Electrostatic potentials are mapped on the surface of the SP-A1 3D structure. Blue color indicates regions of positive potential, whereas red depicts negative potential values. The position of residue 211 is indicated by a black arrow.

Figure 6. Effects of SFTPA1 and SFTPA2 variants on protein production and secretion. A 35–37-kDa molecular species consistent with the tagged SP-A1 or SP-A2 proteins is observed in lysates of cells expressing the wild-type or mutant SP-A isoforms (A and B). A similar 35–37-kDa molecular species is also observed in the medium of cells expressing the wild-type proteins, but not in the medium of the cells transfected with either the W211R or the F198S constructs in SP-A1 (C), or in SP-A2 (D) (representative of three independent experiments). NT, not transfected; WT, wild-type; EV, empty vector.
ranging from severe respiratory insufficiency occurring early in life to lung fibrosis and cancer in adult patients.

Materials and Methods

Patients’ selection

Patients with familial forms of IIP were retrieved from the database of the French national network of reference centers for rare lung diseases. A total of 220 patients (pediatric and adult) from 172 unrelated families were identified. Ascertainment of IIP diagnosis was performed by review of the medical records. The clinical features that include lung function, imaging and pathology were collected using a standardized form. From this IIP cohort, the criteria for patient enrollment into the SFTPA1-SFTPA2 study were as follows: a personal or a first-degree relative history of lung cancer; and the absence of identified mutations in SFTPB, SFTPC, ABCA3 and telomerase genes. The study was approved by the French ‘Comité de Protection des Personnes’, and each patient (or the parents for minor individuals) gave his/her written informed consent.

SFTPA1 and SFTPA2 sequencing

Patient DNA was isolated from blood using the Flexigene (Qiagen) method. The four coding exons of SFTPA1 and SFTPA2, and their flanking intronic junctions were sequenced. NM_005411.4 isoform was used for SFTPA1 analysis, and NM_001098668.2 isoform was used for SFTPA2 analysis. As SFTPA1 and SFTPA2 cDNA are 98% identical, PCR primers were designed in order to specifically amplify SFTPA1 and SFTPA2 (Supplementary Material, Table S2 and Supplementary Material, Fig. S3). PCR were performed with Master Mix GreenGoTaq (Promega) and sequenced with Big Dye Terminator v3.1 (Applied Biosystems) after an Exosap-IT purification step.

Modeling of structural and electrostatic changes induced by the SP-A1 W211R mutation on the CRD

The 3D structures of WT SP-A1 and SP-A1 carrying the W211R mutation were generated using the rat SP-A1 crystal structure (residues 201-248) from the Protein Data Bank (PDB ID: 3PAK) as template. Briefly, homology modeling was performed using Modeller software v.9.10. Accuracy of output structures from Modeller was further assessed using Procheck v.3.5.4 (45,46). Conformational changes were evaluated by a RMSD value comparison between the WT and the mutated SP-A1 domains using SuperPose Version 1.0 (34). Electrostatic modeling of the protein domain surface was generated with the use of the vacuum electrostatics PyMOL plug-in (v0.99).

Plasmid constructs

The WT SFTPA1 cDNA and the SFTPA2 cDNA, obtained from human universal RNA, were inserted into the pcDNA3 expression vector (Invitrogen, Carlsbad, CA) downstream of the CMV promoter. A 27 amino-acid HA tag was subsequently added to generate the plasmids pSFTPA1_WT. pSFTPA1_W211R was then obtained by site-direct mutagenesis. Following a similar procedure, additional SFTPA expression plasmids that were used as controls were generated: pSFTPA1_R219W, and pSFTPA1_F198S that encodes the mutation previously identified in the close paralog SFTPA2 (31); SP-A2-encoding plasmids carrying a 24 amino-acid FLAG tag and the corresponding same three sequence variations (i.e. pSFTPA2_W211R, pSFTPA2_219W and pSFTPA2_F198S) were also generated.

Transfections and western blotting

The above-mentioned plasmid constructs encoding various SP-A1 and SP-A2 isoforms were transfected into HEK293T human cells by the FuGENE method, and protein production was studied 48 h after transfection. Aliquots of 15 μg of proteins extracted from the cellular lysates and from the cell culture medium were analyzed by western blotting. To assess SP-A1 expression and secretion, the membranes were first incubated for 1 h, either with a monoclonal anti-HA high affinity antibody (Roche 3F10®), or a monoclonal anti-FLAG high affinity antibody (Sigma-Aldrich anti-FLAG M2® A8592), followed by a 1 h incubation with an anti-mouse peroxidase antibody (Southern Biotech 1070-05). For the data obtained on cell culture medium, as loading controls, the gels and the nitrocellulose membranes were stained, respectively, with Coomassie blue and Ponceau S solution (data not shown). Results are representative of three independent experiments.

Immunohistochemistry

Tissue samples from lung biopsies were analyzed by microscopic evaluation coupled with computerized image analysis of stained materials and studied by means of immunohistochemistry (35). A mouse SP-A monoclonal antibody (Abcam-32E12, 1/200) was used, according to manufacturer’s protocol. As a control, a rabbit polyclonal anti-SP-C antibody (Santa Cruz-13979, 1/100) was used, according to the manufacturer’s protocol.

Supplementary Material

Supplementary Material is available at HMG online.

Acknowledgements

We thank the patients and their families for their participation to the study; Jean-François Bernaudin for his valuable expertise in surfactant and lung physiology and Michel Peuchmaur and Capucine Picard for their help in collecting patient samples. We also thank Ecole Doctorale 394, Physiologie Physiopathologie et Thérapeutique, UPMC, Sorbonne Universités, Paris; and Assistance Publique Hôpitaux de Paris, Centre National de Référence des Maladies Respiratoires Rares RespiRare.

Conflict of Interest statement. None declared.

Authors’ contributions

N.N. designed the study, performed experiments, analyzed and interpreted the data, wrote and revised the manuscript; V.G., C.P. and H.N. were in charge of the involved patients, collected and analyzed the clinical data; L.Go., D.V., L.-J.C., T.C., R.B., B.C. and C.P. and H.N. were in charge of the involved patients, collected and interpreted the data, wrote and revised the manuscript; V.G., N.N. designed the study, performed experiments, analyzed and interpreted the data; S.A. and A.C. designed the study, analyzed and interpreted the data, wrote and revised the manuscript.
Funding

This work was supported by grants from the Institut National de la Santé et la Recherche Médicale (INSERM), the Leg’s Poix from the Chancellerie des Universités, Paris (grants 1305 and 1405), as well as fundings from the patient organisations Resipier c’est Grandir and Belleherbe Association.

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