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Surfactant protein C mutations in sporadic forms of idiopathic interstitial pneumonias

Philipp Markart[1], Clemens Ruppert[1], Malgorzata Wygrecka[2], Reinhold Schmidt[1], Martina Korfei[1], Heinz Harbach[3], Ilka Theruvath[4], Ulrich Pison[4], Werner Seeger[1], Andreas Guenther[1§], and HeikoWitt[5§]

Departments of [1]Internal Medicine, [2]Biochemistry and [3]Anaesthesiology, Intensive Care Medicine and Pain Therapy, Faculty of Medicine, University of Giessen Lung Center, Departments of [4]Anaesthesiology and Intensive Care Medicine and [5]Gastroenterology, Charité, Campus Virchow-Klinikum, Universitatsmedizin Berlin, Germany.

[§] - both authors contributed equally to this manuscript

Corresponding author:

P. Markart, MD

Department of Internal Medicine, Faculty of Medicine, University of Giessen Lung Center

Klinikstr. 36, 35392 Giessen, Germany

Phone: +49-641-99-42502; Fax: +49-641-99-42509

e-mail: philipp.markart@innere.med.uni-giessen.de

Short title: SP-C mutations in sporadic IPF and NSIP

ABSTRACT

Interstitial pneumonias have been recently associated with mutations in the gene encoding surfactant protein C (*SFTPC*). In particular, *SFTPC* mutations have been reported in a number of familial forms of pulmonary fibrosis and in infants with interstitial lung diseases. In the present study, we searched for *SFTPC* mutations in adult patients with sporadic idiopathic interstitial pneumonia.

Thirty-five adult patients with sporadic idiopathic interstitial pneumonia and 50 healthy subjects were investigated for *SFTPC* mutations by direct DNA sequencing. Twenty-five patients suffered from idiopathic pulmonary fibrosis (IPF) and 10 patients from nonspecific interstitial pneumonia (NSIP).

We detected only two frequent non synonymous variants, T138N and S186N. Allele frequencies of both variations as well as of other identified non coding alterations did not differ significantly between the diverse patient groups and control subjects.

We conclude that *SFTPC* mutations are not common in sporadic cases of IPF and NSIP, suggesting that mutated *SFTPC* does not play an important role in the pathogenesis of these forms of idiopathic interstitial pneumonia.

Keywords: genetics, interstitial lung disease, interstitial pneumonia, pulmonary fibrosis, surfactant protein C

INTRODUCTION

Idiopathic interstitial pneumonias comprise seven different entities including idiopathic pulmonary fibrosis (IPF) and nonspecific interstitial pneumonia (NSIP) [1]. IPF is characterized by the histological appearance of usual interstitial pneumonia (UIP) and by a poor prognosis with a median survival of 2 to 3 years [2]. Of these, about 3 % are estimated to be familial [3].

Recent studies suggest that some cases of familial interstitial pneumonias are associated with mutations in the gene encoding surfactant protein C (*SFTPC*). Most of these mutations occur in the C-terminal BRICHOS domain of the proprotein [4]. This region is important for proper processing and folding of pro-SFTPC [5, 6, 7].

In 2001, Nogee *et al.* reported a *SFTPC* splice site mutation affecting the first base of intron 4 (c.460+1G>A) in a mother and her infant, which resulted in skipping of exon 4 and a truncated protein with a deletion of 37 amino acids of the carboxyterminal region [8]. The infant suffered from cellular NSIP, whereas the mother displayed desquamative interstitial pneumonitis (DIP). *In vitro* studies showed that the mutant causes apoptotic cell death by two mechanisms: on the one hand this mutant accumulated in the endoplasmic reticulum (ER) leading to the induction of ER stress characterized by an exaggerated unfolded protein response; on the other hand enhanced deposition of cellular aggregates of the mutated protein with an inhibition of proteasome activity indicating disruption of the ubiquitin/proteasome system, was demonstrated [4].

For the first time, a *SFTPC* mutation associated with UIP, the histological correlate of IPF, was demonstrated in 2002 [9]. In this study, Thomas *et al.* reported a heterozygous T to A transversion in exon 5 leading to the substitution of a highly conserved leucine residue by a glutamine residue at codon 188 (L188Q) in a large familial pulmonary fibrosis kindred. This kindred comprised 97 members. 11 members had pulmonary fibrosis. Six adults showed a

UIP pattern and three children a cellular NSIP pattern. An abnormal lamellar body formation and aberrant subcellular localization of pro-SFTPC was demonstrated in type II cells of affected family members. Furthermore, *in vitro* studies demonstrated exaggerated toxicity of the L188Q mutant [9].

In 2002, Nogee *et al.* analyzed *SFTPC* in infants with chronic lung diseases of unknown etiology and found heterozygous mutations in 11 out of 34 patients [10]. In another study investigating 34 sporadic or familial cases with unexplained respiratory distress, 2 heterozygous *SFTPC* mutations, I73T and R167Q, were identified and were found to be associated with pulmonary alveolar proteinosis with or without fibrosing lung disease [11].

In a recent study investigating *SFTPC* mutations in sporadic cases of adult IPF and NSIP, 13 out of 135 patients showed *SFTPC* variations that were not found in controls, but only one patient possessed an amino acid changing variant, I73T [12].

These data support the hypothesis that *SFTPC* mutations contribute to the pathophysiology of some types of interstitial lung diseases.

In the present study, we therefore investigated 35 adult patients with sporadic idiopathic interstitial pneumonia for *SFTPC* mutations.

METHODS

Study subjects

The study was approved by the local institutional review board and informed consent was obtained from all patients. We enrolled 25 German patients with IPF and 10 German patients with NSIP as defined by the American-European Consensus Criteria [1, 2]. In 15 patients, diagnosis was confirmed by open lung biopsy. In IPF, 9 out of 25 patients, in NSIP 6 out of 10 patients underwent open lung biopsy. Table 1 shows the demographic characteristics, 6 minute walk distance and results of Pulmonary Function Testing of the patient cohort. Fifty healthy German individuals without pulmonary diseases (medical staff and students) served as controls (23 female, 27 male; mean age 34.4 years, range 24-61 years).

DNA extraction and polymerase chain reaction (PCR)

Genomic DNA was extracted from blood leukocytes using spin columns (Qiagen). The *SFTPC* coding region of 35 patients and of 50 control subjects was analyzed by direct DNA sequencing. For sequencing, *SFTPC* was amplified by PCR in 3 fragments using the following oligonucleotides: 5'-GTTGGAACTGGTCCTTGCAGG-3' and 5'-TCCCCATA-CTCAGGCCTCTG-3' (promotor - intron 2), 5'-GCCTCATGACCTCATGCCTG-3' and 5'-AGCTTAGACGTAGGCACTGC-3' (intron 1 - exon 5), and 5'-GTCCCACAATAAGGGC-TGCAC-3' and 5'-CTGGGACAGAGGGCGAATGG-3' (intron 4 - 3'-UTR).

We performed PCR using 0.75 U AmpliTaq Gold polymerase (Applied Biosystems), 400 µM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, and 0.1 µM of each primer in a total volume of 25 µL. Cycle conditions were as follows: initial denaturation for 12 min at 95 °C; 48 cycles of 20 sec denaturation at 95 °C, 40 sec of annealing at 64 °C and 90 sec of primer extension at 72 °C; and a final extension for 2 min at 72 °C.

DNA sequencing

We digested PCR products with shrimp alkaline phosphatase (USB) and exonuclease I (USB) and performed cycle sequencing using BigDye terminator mix (Applied Biosystems). For sequencing, we used as oligonucleotides 5'-CCCAGGTTTGCTCTTGCTGG-3' and 5'-GAG-GAGGCAGGGCCCATCAC-3' (fragment 1); 5'-TCCAGCCCTAGGACGCCGTG-3', 5'-CTGTCTGGCATGTCCTGTGC-3', 5'-GATGGGTACCACTGGCTGAG-3', 5'-TGGGTC-AGGGAGAGAGCAGG-3', and 5'-CACTCCTCCCAGCAGCCCTG-3' (fragment 2); and 5'-GTCCCACAATAAGGGCTGCAC-3', 5'-GGGAGTGGGAAGTACCGGTC-3', and 5'-CTGGGACAGAGGGCGAATGG-3' (fragment 3). By doing so, the entire coding region including the 5'- and 3'-UTR and the promoter sequence until -400 were analyzed. Cycle conditions were as follows: initial denaturation for 3 min followed by 30 cycles of denaturation at 95°C for 20 sec, annealing at 60°C for 20 sec, and elongation at 60°C for 30 sec. The reaction products were purified with Sephadex G-50 (Amersham) and loaded onto an ABI 3100 fluorescence sequencer (Applied Biosystems).

Statistical analysis

Statistical analysis was carried out using Fisher's exact test. P-values less than 0.05 were considered to be statistically significant.

RESULTS

We identified two single nucleotide polymorphisms (SNPs) in the *SFTPC* coding region of the two patient groups and the control subjects: a c.413 C to A transversion in exon 4 leading to an exchange of threonine by asparagine at codon 138 (T138N) and a c.557 G to A transition in exon 5 resulting in an exchange of serine by asparagine at codon 186 (S186N). Allele and genotype frequencies of both variations did not differ significantly between the different patient groups and control subjects.

We found a strong linkage disequilibrium between the two coding SNPs, T138N and S186N, as previously described by a Finnish group: 138N was almost exclusively *in cis* with 186N [13]. As the same as for the allele frequencies of both SNPs, the frequencies of the estimated haplotypes did not differ significantly between the different patient groups and control subjects.

Additional variations located in the promotor, UTR or intronic regions were identified. Again, no significant differences in the allele frequencies between patients and controls were observed. Table 2 summarizes the allele frequencies of the detected variations in patients and control subjects.

DISCUSSION

In the present study, we investigated *SFTPC* mutations in 35 adult patients with sporadic forms of interstitial pneumonia. Twenty-five patients with IPF and 10 patients with NSIP, all of them sporadic and with first manifestation of the disease in adulthood, were investigated. Patients became symptomatic for their disease at an age of 60.8 ± 9.4 (IPF) and 49.9 ± 13.3 years (NSIP), respectively. Only two exonic SNPs that predict an amino acid change were detected, but no differences in the allele frequencies between controls and patients were observed. No *SFTPC* frame shift or splice site mutation was detected in any of the patients.

In general, data are rare regarding a possible genetic basis of sporadic forms of interstitial pneumonia. Polymorphisms in the genes coding for tumor necrosis factor α, interleukin-1 receptor antagonist, angiotensin-converting enzyme, and complement receptor 1 have been linked to sporadic cases of IPF [14, 15, 16]. Also transforming growth factor $ß_1$ polymorphisms have been associated with the progression of IPF [17]. So far, only one study investigated *SFTPC* mutations in adult patients with sporadic forms of IPF or NSIP. In this study, 89 patients with IPF and 46 with NSIP were analyzed, but only one IPF patient possessed a genetic sequence variation that predicted a change in the amino acid sequence (I73T) [12]. In a 13-month-old infant with severe respiratory insufficiency, the same mutation was associated with combined histological patterns of NSIP and pulmonary alveolar proteinosis. Functional analyses showed that expression of mutant proSP-C results in abnormal proprotein trafficking, leading to an accumulation of aberrantly processed proSP-C in the alveoli [18].

In contrast to sporadic forms of pulmonary fibrosis, *SFTPC* mutations might be more important in familial forms. Over 68 kindreds with familial idiopathic pulmonary fibrosis have been described so far [3, 19, 20]. These familial forms are most likely transmitted in an autosomal manner with reduced penetrance. Some cases of familial pulmonary fibrosis were

found to be associated with heterozygous *SFTPC* mutations [8, 9]. Interestingly, different histopathological types of pulmonary fibrosis were found in members of the same kindred who shared the identical *SFTPC* mutation. These different forms may represent pleiotropic manifestations of the same genetic defect [9]. Reduced penetrance is another feature of *SFTPC* associated familial forms of pulmonary fibrosis, suggesting that additional endogenous or exogenous factors contribute to the marked diversity of pulmonary fibrosis predisposed by *SFTPC* mutations [9].

There are several mechanisms how *SFTPC* mutations may contribute to the pathophysiology of pulmonary fibrosis. Recent *in vitro* studies indicate that an intracellular accumulation of incompletely processed and misfolded SFTPC may cause alveolar type II cell injury and apoptosis, thereby initiating fibrosis [4]. This is in line with the currently accepted hypothesis that chronic injury of the alveolar epithelium is the underlying pathogenic event for initiating the fibrotic response in IPF [21, 22]. While protein misfolding is one possible explanation for the development of fibrosis in patients with *SFTPC* mutations, it is also imaginable that fibrosis is caused by the lack of mature SFTPC: SFTPC deficient (*SFTPC* -/-) mice develop severe progressive pulmonary disorder displaying histological features consistent with interstitial pneumonitis [23]. Interestingly, mature SFTPC was absent in the lung tissue or bronchoalveolar lavage fluid of the infant with the recently described splice site mutation (c.460+1 G>A) [8]. Furthermore, SFTPC deficiency has been described in a kindred with interstitial pneumonitis without a detectable *SFTPC* mutation [24].

Next to mutations in *SFTPC*, mutations in another molecule associated with the pulmonary surfactant system were recently shown to occur in pediatric interstitial pneumonias: mutations in the ATP-binding cassette protein A 3 (*ABCA3*) have been associated with fatal respiratory failure in neonates without deficiency in the surfactant proteins B and C and with non-fatal

chronic interstitial pneumonitis in one older child [25]. Further studies are necessary to elucidate the role of *ABCA3* mutations in adult forms of interstitial pneumonias.

In summary, we did not identify pathogenic *SFTPC* mutations in 35 adult patients with sporadic IPF or NSIP, indicating that in contrast to familial pulmonary fibrosis mutated *SFTPC* represents a rare cause of sporadic IPF and NSIP.

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REFERENCES

1. American Thoracic Society, European Respiratory Society. American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias. *Am J Respir Crit Care Med* 2002: 165: 277-304.
2. American Thoracic Society. Idiopathic pulmonary fibrosis: diagnosis and treatment. International consensus statement. American Thoracic Society (ATS), and the European Respiratory Society (ERS). *Am J Respir Crit Care Med* 2000: 161: 646-664.
3. Hodgson U, Laitinen T, Tukiainen P. Nationwide prevalence of sporadic and familial idiopathic pulmonary fibrosis: evidence of founder effect among multiplex families in Finland. *Thorax* 2002: 57: 338-342.
4. Mulugeta S, Nguyen V, Russo SJ, Muniswamy M, Beers MF. Surfactant protein C precursor protein BRICHOS domain mutation causes endoplasmic reticulum stress, proteasome dysfunction, and caspase 3 activation. *Am J Respir Cell Mol Biol* 2005: 32: 521-530.
5. Beers MF, Lomax CA, Russo SJ. Synthetic processing of surfactant protein C by alveolar epithelial cells. *J Biol Chem* 1998: 273: 15287-15293.
6. Keller A, Steinhilber W, Schaefer K, Voss T. The C-terminal domain of the pulmonary surfactant protein C precursor contains signals for intracellular targeting. *Am J Respir Cell Mol Biol* 1992: 6: 601-608.
7. Kabore AF, Wang WJ, Russo SJ, Beers MF. Biosynthesis of surfactant protein C: characterization of aggresome formation by EGFP chimeras containing propeptide mutants lacking conserved cysteine residues. *J Cell Sci* 2000: 114: 293-302.
8. Nogee LM, Dunbar AE 3rd, Wert SE, Askin F, Hamvas A, Whitsett JA. A mutation in the surfactant protein C gene associated with familial interstitial lung disease. *N Engl J Med* 2001: 344: 573-579.

9. Thomas AQ, Lane K, Phillips J 3rd, Prince M, Markin C, Speer M, Schwartz DA, Gaddipati R, Marney A, Johnson J, Roberts R, Haines J, Stahlman M, Loyd JE. Heterozygosity for a surfactant protein C gene mutation associated with usual interstitial pneumonitis and cellular nonspecific interstitial pneumonitis in one kindred. *Am J Respir Crit Care Med* 2002: 165: 1322-1328.
10. Nogee LM, Dunbar AE 3rd, Wert SE, Askin F, Hamvas A, Whitsett JA. Mutations in the surfactant protein C gene associated with interstitial lung disease. *Chest* 2002: 121: 20S-21S.
11. Tredano M, Griese M, Brasch F, Schumacher S, de Blic J, Marque S, Houdayer C, Elion J, Couderc R, Bahuau M. Mutation of SFTPC in infantile pulmonary alveolar proteinosis with or without fibrosing lung disease. *Am J Med Genet A* 2004: 126: 18-26.
12. Lawson WE, Grant SW, Ambrosini V, Womble KE, Dawson EP, Lane KB, Markin C, Renzoni E, Lympany P, Thomas AQ, Roldan J, Scott TA, Blackwell TS, Phillips JA 3rd, Loyd JE, du Bois RM. Genetic mutations in surfactant protein C are a rare cause of sporadic cases of IPF. *Thorax* 2004: 59: 977-980.
13. Lahti M, Marttila R, Hallman M. Surfactant protein C gene variation in the Finnish population-association with perinatal respiratory disease. Eur J Hum Genet 2004: 12: 312-320.
14. Whyte M, Hubbard R, Meliconi R, Whidborne M, Eaton V, Bingle C, Timms J, Duff G, Facchini A, Pacilli A, Fabbri M, Hall I, Britton J, Johnston I, Di Giovine F. Increased risk of fibrosing alveolitis associated with interleukin-1 receptor antagonist and tumor necrosis factor-alpha gene polymorphisms. *Am J Respir Crit Care Med* 2000: 162: 755-758.

15. Morrison CD, Papp AC, Hejmanowski AQ, Addis VM, Prior TW. Increased D allele frequency of the angiotensin-converting enzyme in pulmonary fibrosis. *Hum Pathol* 2001: 32: 521-528.
16. Zorzetto M, Ferrarotti I, Trisolini R, Agli LL, Scabini R, Novo M, De Silvestri A, Patelli M, Martinetti M, Cuccia M, Poletti V, Pozzi E, Luisetti M. Complement receptor 1 gene polymorphisms are associated with idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2003: 168: 330-334.
17. Xaubet A, Marin-Arguedas A, Lario S, Ancochea J, Morell F, Ruiz-Manzano J, Rodriguez-Becerra E, Rodriguez-Arias JM, Inigo P, Sanz S, Campistol JM, Mullol J, Picado C. Transforming growth factor-beta 1 gene polymorphisms are associated with disease progression in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2003: 168: 431-435.
18. Brasch F, Griese M, Tredano M, Johnen G, Ochs M, Rieger C, Mulugeta S, Muller KM, Bahuau M, Beers MF. Interstitial lung diseases in a baby with a de novo mutation in the SFTPC gene. Eur Respir J 2004: 24: 30-39.
19. Marshall RP, McAnulty RJ, Laurent GJ. The pathogenesis of pulmonary fibrosis: is there a fibrotic gene? *Int J Biochem Cell Biol* 1997: 29: 107-120.
20. Bitterman PB, Rennard SI, Keogh BA, Wewers MD, Adelberg S, Crystal RG. Familial idiopathic pulmonary fibrosis: evidence of lung inflammation in unaffected family members. *N Engl J Med* 1986: 314: 1343-1347.
21. Pardo A, Selman M. Idiopathic pulmonary fibrosis: new insights in its pathogenesis. *Int J Biochem Cell Biol* 2002: 34: 1534-1538.
22. Selman M, King TE, Pardo A. Idiopathic pulmonary fibrosis: Prevaling and evolving hypotheses about its pathogenesis and implications for therapy. *Ann Int Med* 2001: 134: 136 -151.

23. Glasser SW, Detmer EA, Ikegami M, Na CL, Stahlman MT, Whitsett JA. Pneumonitis and emphysema in sp-C gene targeted mice. *J Biol Chem* 2003: 278: 14291-14298.

24. Amin RS, Wert SE, Baughman RP, Tomashefski JF Jr, Nogee LM, Brody AS, Hull WM, Whitsett JA. Surfactant protein deficiency in familial interstitial lung disease. *J Pediatr* 2001: 139: 85-92.

25. Shulenin S, Nogee LM, Annilo T, Wert SE, Whitsett JA, Dean M. ABCA3 gene mutations in newborns with fatal surfactant deficience. *N Engl J Med* 2004: 350: 1296-1303.

Table 1

Demographic characteristics, 6 minute walk distance and results of Pulmonary Function Testing of the patient cohort

	No. of patients	Lung biopsy open [%] TBB [%]	Sex [% female]	Mean age [years]	Smokers Current [% (py)] former [% (py)]	FVC [l (% predicted)]	D_LCO [mmol/minxkPa (% predicted)]	6 min walk distance [m]
IPF	25	36 96	32	61.9±13.3* 60.8 ± 9.4$^+$	4 (40) 28 (17)	2.3 ± 0.8 (61.2±20.4)	2.9 ± 1.1 (34.3±13.2)	347 ±150
NSIP	10	60 100	50	52.6±14.7* 49.9±13.3$^+$	0 (0) 40 (25)	2.2 ± 1.1 (55.3±27.1)	3.1 ± 0.8 (36.5 ±9.6)	408 ± 121

Data are given as mean ± SEM. FVC: = forced vital capacity; D_LCO = diffusion capacity of carbon monoxide; IPF = Idiopathic Pulmonary Fibrosis; NSIP = Nonspecific Interstitial Pneumonitis; py = packyears; TBB = transbronchial biopsy; * = Age at time of diagnosis; + = Age at which patients became symptomatic for their disease

Table 2

Allele frequencies of single nucleotide polymorphisms in patients and control subjects

Alteration	IPF (n=50)	NSIP (n=20)	All Patients (n=70)	Controls (n=100)
-271 G>A	0.02	0.10	0.04	0.04
-77 C>G	0.02	0.00	0.01	0.00
IVS1+35 G>A	0.02	0.10	0.04	0.04
IVS1-21 C>T	0.46	0.20	0.39	0.41
IVS2+14 G>A	0.04	0.00	0.03	0.02
c.413 C>A (**T138N**)	0.18	0.25	0.20	0.29
IVS4-8 C>G	0.18	0.20	0.19	0.19
c.557 G>A (**S186N**)	0.32	0.35	0.33	0.34
IVS5-39insA	0.32	0.35	0.33	0.35
c.717 G>A	0.32	0.35	0.33	0.35
c.767 C>T	0.22	0.30	0.24	0.14
c.768 G>A	0.30	0.35	0.31	0.33