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Kathrin Heidinger · Inke R. König · Anette Bohnert · Anja Kleinsteiber · Anne Hilgendorff · Ludwig Gortner · Andreas Ziegler · Trinad Chakraborty · Gregor Bein

Polymorphisms in the human surfactant protein-D (*SFTPD*) gene: strong evidence that serum levels of surfactant protein-D (SP-D) are genetically influenced

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Abstract The collectin surfactant protein-D (SP-D) plays a significant role in innate immunity. Epidemiological studies described associations between single nucleotide polymorphisms (SNPs) of the human gene coding surfactant protein-D (*SFTPD*) and infectious pulmonary diseases. Studies on twins indicated very strong genetic dependence for serum levels of SP-D. The aim of this study was to determine the genetic influence of sequence variations within the *SFTPD* gene on the constitutional serum SP-D levels. We sequenced the 5′ untranslated region (5′UTR), the coding region and the 3′ region of the *SFTPD* gene of 32 randomly selected blood donors. Six validated SNPs were genotyped with sequence-specific probes (TaqMan 7000) in 290 German blood donors. Serum SP-D levels were analysed by ELISA, and the association of *SFTPD* haplotype estimates with the quantitative phenotype serum SP-D level was determined. One single *SFTPD* haplotype (allele frequency 13.53%) revealed a negative association with serum SP-D levels ($P<0.0001$). This was confirmed in a second prospectively collected group of blood donors ($n=160$, $P=0.0034$). The discovery of a frequent negative variant of the *SFTPD* gene provides a basis for genetic analysis of the function of SP-D in the resistance against pulmonary infections and inflammatory disorders in humans.

Keywords Surfactant protein-D · Single nucleotide polymorphism · Serum concentrations · Haplotypes

K. Heidinger (✉) · A. Bohnert · A. Kleinsteiber · G. Bein
Institute of Clinical Immunology and Transfusion Medicine,
University of Giessen,
Langhansstrasse 7,
35392 Giessen, Germany
e-mail: Kathrin.S.Heidinger@immunologie.med.uni-giessen.de
Tel.: +49-641-9941523
Fax: +49-641-9941509

I. R. König · A. Ziegler
Institute of Medical Biometry and Statistics,
University at Lübeck,
23538 Lübeck, Germany

A. Hilgendorff
Department of Paediatrics, University of Giessen,
35392 Giessen, Germany

L. Gortner
Department of Paediatrics, University of Saarland,
66421 Homburg/Saar, Germany

T. Chakraborty
Institute for Medical Microbiology, University of Giessen,
35394 Giessen, Germany

Introduction

Surfactant protein-D (SP-D) is a member of the collagenous subfamily of calcium-dependent lectins (collectins) that includes pulmonary surfactant protein-A (SP-A) and the serum protein mannose-binding lectin (MBL) (Crouch 1998; Wright 1997; Eggleton and Reid 1999). Strong indications prove that the collectins play a significant role in innate immunity.

SP-D is involved in the innate response to inhaled microorganisms and contributes to immune and inflammatory regulation within the lung. SP-D binds to microbial surfaces and induces effector mechanisms such as aggregation and virus neutralisation, attraction and activation of phagocytes and promotion of phagocytosis (Crouch 1999).

SP-D is an M_r 43,000 glycoprotein (Persson et al. 1989) synthesized and secreted by pulmonary alveolar type II cells and nonciliated airway cells (Wong et al. 1996). However, recent results indicate that SP-D in humans is widely distributed on mucosal surfaces (Madsen et al. 2000).

The gene for S-PD, *SFTPD*, is located on the long arm of Chromosome 10q22.2–23.1, as are the genes for SP-A and MBL. Four coding SNPs of *SFTPD* have been described previously (Crouch et al. 1993). One of the *SFTPD* polymorphism resides in the N-terminal region at position 11, where a methionine is exchanged for a threonine (position 31 from transcription start site). The three other coding variants are located in the collagen-encoding region.

Up to this present study, allelic variations have not been associated with the levels of SP-D in bronchoalveolar lavage fluid or in serum. However, data of a recently published study on twins indicate strong genetic dependence of serum SP-D levels. Biometric model fitting estimated the heritability of the serum SP-D level to 0.91 (Husby et al. 2002).

Polymorphic marker loci for *SFTPD* also have been characterized (DiAngelo et al. 1999), and a recent study on patients with tuberculosis indicates that the *SFTPD* allele threonine 11 (position 31 from transcription start site) is associated with the susceptibility to this disease (Floros et al. 2000). Interestingly, the opposite *SFTPD* allele coding for methionine 11 was associated with severe respiratory syncytial virus bronchiolitis in infants (Lahti et al. 2002).

Discovering that *SFTPD* (−/−) mice spontaneously develop emphysema with increased metalloproteinase activity, as well as increased levels of oxidants in the absence of infections (Wert et al. 2000), suggests that changes in the level of lung collectins may account for the pathogenesis of various diseases. It is likely that the decreased levels of SP-D, which have been observed in different clinical situations, increase the risk of infections (Holmskov et al. 2003). Clinically, serum SP-D levels have been used as biomarkers for lower respiratory tract infections, acute lung injury and type II cell hyperplasia as seen in pulmonary fibrosis and other forms of interstitial lung diseases (Takahashi et al. 2000; Asano et al. 2001; Ohnishi et al. 2002).

In this study, we aimed at examining the association between *SFTPD* SNPs and estimated haplotypes found within a general Caucasoid population and serum levels of the SP-D protein. Here we report the discovery of one single *SFTPD* haplotype with a highly significant negative association with serum SP-D levels.

Materials and methods

Subjects

A total of 32 healthy voluntary blood donors were randomly selected for resequencing of the *SFTPD* gene, including the 5′ and 3′ flanking regions. Six validated SNPs were genotyped with sequence-specific probes (TaqMan 7000) in 290 German healthy blood donors without any signs of pulmonary dysfunctions (age 19–68 years, mean 34 years, 207 male, 83 female) after they gave their informed consent. To confirm associations, we analysed a second prospectively collected group of 160 blood donors (age 18–64 years, mean 34 years, 88 male, 72 female). The study protocol was approved by the Medical Ethics Committee of the University of Giessen.

Blood samples

Blood samples were drawn in K_2-ethylenediaminetetraacetic acid tubes (Sarstedt, Nümbrecht, Germany). The samples were centrifugated at 800 *g* for 10 min. Plasma and buffy coat were separated immediately. Plasma was stored in aliquots at −70°C and was defrosted in a 37°C water bath before use.

DNA was extracted from peripheral blood leukocytes, using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, Minn., USA) according to the manufacturer's instructions. DNA was dissolved in DNA hydration solution and stored at −70°C until use.

DNA sequence analysis

DNA sequencing of 800 bp upstream of the ATG-translation initiation codon, the complete coding region, and the 3′ region of the *SFTPD* gene was performed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, Calif., USA), using an ABI Prism Big Dye Terminator DNA sequencing kit according to the manufacturer's instructions. Sequence alignment and SNP validation were analysed using SeqScape software (ABI).

Genotyping of *SFTPD* sequence variants

We selected six SNPs with an allele frequency exceeding 10% in the German population. The validated SNPs were genotyped with sequence-specific probes (ABI PRISM 7000 Sequence Detection System, TaqMan). Genotyping primers and probes are listed in Tables 1 and 2.

PCR was carried out in a volume of 25 µl containing 20 ng genomic DNA, forward and reverse primers in final concentrations of 900 nM, fluorescence-labelled, sequence-specific minor groove binders probes in final concentrations of 200 nM, and MasterMix Buffer (containing polymerase, $MgCl_2$, dNTPs including dUTP and

Table 1 Primer sequences for the genotyping of polymorphisms of the surfactant protein-D (*SFTPD*) gene

Primer description, forward (F) and reverse (R)	Primer sequences for PCR
rs1923537 F	CCT AGT CCT TTT TAA GCC CAT TTG
rs1923537 R	GGA TGT AGC ATA AAC CAA GAG AAG TG
rs1923538 F	TCC CAT TGG AGA GTT TCT GAT TTC
rs1923538 R	ACT ACC CAA GCA TTA AGA ATC ATG TG
rs1923539 F	CCC ACT CCC CAA GTT TAG TTG AT
rs1923539 R	TTG GAA ACT CCT GTG GAA ATG AG
rs2243639 F	ATT GAC AGC TCC AAG CAG GC
rs2243639 R	AGG TGT CCC TGG TGA GCG T
rs721917 F	CCT GGA AGC AGA AAT GAA GAC CTA
rs721917 R	CCA CTC TCC ACT GAG CAC ATG
rs2255326 F	TCG AAC TCC TGA CTT CAA GTG ATC
rs2255326 R	GAG AGG GTT TGG AAA AGA AGT ATG TG

Table 2 Probe sequences for detecting the *SFTPD* single nucleotide polymorphisms (SNPs)

Probe description	Probe sequences for TaqMan analysis
rs2255326 Vic	TGA GCG ACC GTG CC
rs2255326 Fam	AGC GAC TGT GCC C
rs721917 Vic	CAG AAC AAC GCC CAG TG
rs721917 Fam	CAC AGA ACA ATG CCC A
rs1923537 Fam	CTC TGA ACA AAG GGT T
rs1923537 Vic	CTC TGA ATA AAG GGT TC
rs1923538 Fam	CAG CCG TCT TTA GGA
rs1923538 Vic	TGT GCA GCC ATC TT
rs1923539 Fam	CCA GAG GTG GGC AT
rs1923539 Vic	ACC AGA GAT GGG C
rs2243639 Vic	TGC CCC TGT GTT TC
rs2243639 Fam	CCC TGC GTT TCC A

UNG). PCR was performed in the following steps according to the universal protocol supplied by ABI:

1. Contamination control 50°C, 2 min.
2. Heat activation 96°C, 10 min.
3. Annealing/extension 40 cycles of 95°C for 15 s and 60°C for 1 min.

Fluorescence emission was measured on an ABI PRISM 7000 Sequence Detection System at 60°C. Known DNA samples of each genotype and water samples were used as positive and negative controls for each run.

Measurement of serum SP-D concentrations by enzyme immunoassay

The concentrations of SP-D in sera were measured by a specific ELISA kit, using mAbs against SP-D 7C6 and horseradish peroxidase (HRP)-conjugated mAb 6B2 and recombinant SP-D as a standard. The commercially available test kit was used for the measurement of SP-D according to the manufacturer's instructions (Yamasa, Tokyo, Japan). The 96-microwell plate coated with SP-D Abs was incubated with 100 µl of 11-fold diluted plasma at 4°C for 24 h. The wells were then washed and incubated at 25°C for 2 h, with 100 µl 111-fold diluted HRP-conjugated anti-SP-D Ab. The wells were washed again, and 100 µl substrate mixture (11 ml tetramethylbenzidine solution + 50 µl 1% H_2O_2) was added. Incubation was then performed at 25°C for 15 min. Finally, 100 µl 1 N sulphuric acid was added to terminate the peroxidase reaction, and the absorbance at 450 nm was measured.

The quantitative serum SP-D levels were calculated from a standard curve, employing serial dilutions of recombinant SP-D and an analytical sensitivity of 1.56 ng/ml.

Statistical analysis

For description, median SP-D serum levels were compared between men and women, using the two-sided exact Mann–Whitney *U*-test. To evaluate deviation from the Hardy–Weinberg equilibrium, observed and expected genotype frequencies were compared by a Monte Carlo simulation-based goodness-of-fit test.

To investigate the relation between the quantitative serum SP-D level and different genotypes, median, mean SP-D level and standard deviation (SD) are reported. Descriptive exact *P*-values from a Kruskal–Wallis test are also reported.

Linkage disequilibrium between pairs of polymorphic positions was estimated adopting a Malecot model as implemented in LDMAP (Maniatis et al. 2002).

Frequencies of haplotypes were estimated with the expectation–maximization algorithm (Excoffier and Slatkin 1995). Median values of SP-D were compared across the estimated haplotype frequencies, using the score test with simulated *P*-values from 10^6 replications (Schaid et al. 2002).

Results

Identification of *SFTPD* gene variants

We screened the 5′UTR, the coding region and the 3′ region of the *SFTPD* gene for possible sequence variations in 32 healthy volunteers and validated six known SNPs. One nucleotide substitution was characterized in the 5′UTR (−759A>G), another substitution was found in the exon 2 (92A>G), which leads to an amino acid exchange from methionine to threonine at position 31. Furthermore, a nucleotide substitution was characterized in exon 5 (4694C>T), leading to an amino acid exchange from threonine to alanine at position 180. The others are located in the 3′UTR (11208A>G; 11384A>G; 11466G>A). Additional SNPs were not identified. These six SNPs were genotyped with sequence-specific probes (TaqMan 7000) in at least 248 German blood donors and, four of the six SNPs in a second independent group of at least 158 German blood donors. We determined the allele frequencies of these polymorphisms as shown in Table 3. The rate of homozygosity and heterozygosity for all polymorphism showed no deviation from the Hardy–Weinberg equilibrium, indicating the state of random assortment.

Table 3 Allele frequencies of *SFTPD* polymorphisms

Sequence variations (position, substitution)	Typed donors (*n*)	Rare allele frequency (%)
−759A>G	448	27.57
92A>G	446	42.42
4694C>T	407	39.73
11208A>G	447	38.74
11384A>G	289	39.44
11466G>A	285	24.92

Quantitative serum SP-D levels

The mean serum SP-D level for the first group was 84.86 ng/ml (SD, 44.94 ng/ml; minimum, 17.0 ng/ml; maximum, 302.11 ng/ml; median, 76.21 ng/ml; *n*=290) as measured by ELISA. Comparable results were obtained in the second group. The mean serum SP-D level for the second group was 73.79 ng/ml (SD, 38.73 ng/ml; minimum, 17.60 ng/ml; maximum, 288.65 ng/ml; median, 64.61 ng/ml; *n*=160).

Separate analysis for each gender indicated that male subjects showed higher serum SP-D levels than females (descriptive, *P*=0.024). The mean serum SP-D level for male subjects was 89.56 ng/ml (SD, 48.18 ng/ml; median, 79.17 ng/ml; *n*=207), the mean serum SP-D level for females was 73.15 ng/ml (SD, 33.06 ng/ml; median, 66.99 ng/ml; *n*=83). The second group again revealed a trend towards higher serum SP-D levels in males (mean serum SP-D levels, 78.36 ng/ml; SD, 41.92 ng/ml; median, 70.93 ng/ml; *n*=88) than in females (mean serum SP-D level, 68.20 ng/ml; SD, 33.88 ng/ml; median, 60.93 ng/ml; *n*=72).

Association of *SFTPD* polymorphism with serum SP-D levels

Differences in median serum levels of SP-D for carriers of *SFTPD* polymorphisms are shown in Table 4. There was an association between two of the six examined SNPs (92, A→G and 11208, A→G) and serum levels of SP-D in the first cohort showing that, under a model of dominant inheritance, carriers of the *G* allele at position 92 had lower levels of serum SP-D compared to those who were homozygous for the *A* allele (Mann–Whitney *U*-test, *P*=0.001). Prospective analysis of the second independent group of blood donors confirmed the initial observation (Mann–Whitney *U*-test, *P*=0.031). In addition, carriers of the *G* allele at position 11208 showed higher median values of SP-D in serum (Mann–Whitney *U*-test, *P*=0.0039).

A comparable association between this polymorphism and the serum SP-D levels was also observed in the second group (Mann–Whitney *U*-test, *P*=0.027).

Linkage disequilibrium and definition of putative human *SFTPD* haplotypes

Analysis of the linkage disequilibrium units among the six *SFTPD* SNPs revealed that the genetic region consists of a single haplotype block, with high levels of LD towards the end (Table 5).

Nine putative human *SFTPD* haplotypes with a frequency of at least 1% were estimated, of which the four most common possessed a cumulative frequency of 74.47% (Table 6).

Table 4 Differences in quantitative serum SP-D levels and different genotypes, mean SP-D levels, standard deviation and median are given. Association of SNPs with levels of the serum SP-D (descriptive exact *P*-values from Kruskal–Wallis tests) are shown as described

SNP (position, substitution)	Genotype	Number	Mean value (ng/ml)	Standard deviation (ng/ml)	Median (ng/ml)	*P*-value
−759A>G		289				0.1882
	GG	146	82.51	47.50	73.70	
	AG	118	86.04	42.58	77.64	
	AA	25	94.41	40.81	85.82	
92A>G		288				0.0040
	AA	95	97.36	50.81	88.87	
	AG	133	79.12	41.44	72.03	
	GG	60	76.76	37.15	70.93	
4694C>T		248				0.3638
	TT	41	93.61	46.37	86.45	
	CT	115	86.51	47.46	75.27	
	CC	92	84.77	45.09	79.73	
11208A>G		288				0.0039
	AA	109	75.59	40.98	69.55	
	AG	134	89.21	48.89	81.13	
	GG	45	95.83	38.45	84.45	
11384A>G		289				0.3275
	GG	45	91.57	46.49	81.45	
	AG	142	84.94	45.66	75.38	
	AA	102	82.03	43.53	75.66	
11466G>A		285				0.0552
	GG	165	80.27	44.01	72.50	
	AG	100	91.39	48.91	79.73	
	AA	20	91.42	30.50	95.43	

Association of *SFTPD* haplotypes with serum SP-D levels

The association of putative *SFTPD* haplotype estimates with the quantitative phenotype serum SP-D level was calculated using a score test assuming ordinally scaled values. Overall, SP-D serum levels differed across the estimated haplotypes (Monte Carlo, *P*<0.0001). One single *SFTPD* haplotype, *SFTPD*03* (allele frequency, 13.53%), revealed a negative association with serum SP-D levels (Monte Carlo, *P*<0.0001) as shown in Table 6. We attempted to

Table 5 Linkage disequilibrium units map (*LDU map*)

SNP (position, substitution)	Absolute position (kb)	kb map	LDU map
−759A>G	−759	0	0
92A>G	92	0.851	0.400775
4694C>T	4694	5.452	0.888735
11208A>G	11208	11.967	0.952663
11384A>G	11384	12.143	0.952663
11466G>A	11466	12.225	0.952663

Table 6 Putative human *SFTPD* haplotypes (group 1, *n*=290) with frequencies ≥1% and the association of haplotype estimates with quantitative phenotype SP-D serum levels

Haplotype	Hap. freq (%)[a]	−759A>G	92A>G	4694C>T	11208A>G	11384A>G	11466G>A	Hap. score	Sim. *P*-value[b]
*SFTPD *01*	35.93	1[c]	1	1	1	1	1	0.5739	0.5686
*SFTPD *02*	18.09	2[d]	2	2	2	2	2	1.9542	0.0530
*SFTPD *03*	13.53	1	2	2	1	2	1	−7.1568	<0.0001
*SFTPD *04*	6.94	1	1	2	2	2	1	2.3560	0.0213
*SFTPD *05*	6.86	1	1	2	1	2	1	−1.4096	0.1630
*SFTPD *06*	4.78	2	2	2	2	2	1	−1.5547	0.1254
*SFTPD *07*	4.71	1	2	2	2	2	2	1.4250	0.1639
*SFTPD *08*	2.51	2	1	1	1	1	1	0.3022	0.7651
*SFTPD *09*	1.50	1	2	2	2	2	1	−0.0033	0.9974

[a]Frequencies of haplotypes were estimated with the expectation–maximization algorithm
[b]Serum SP-D levels were compared across the estimated haplotype frequencies, using a score test assuming ordinally scaled values with simulated *P*-values from 10^6 replications
[c]Frequent allele
[d]Rare allele

Table 7 Putative human *SFTPD* haplotypes (group 2, *n*=160) with frequencies ≥1% and the association of haplotype estimates with quantitative phenotype SP-D serum levels

Haplotype	Hap. freq. (%)[a]	−759A>G	92A>G	4694C>T	11208A>G	Hap. score	Sim. *P*-value[b]
SFTPD*01	30.67	1[c]	1	1	1	−0.5807	0.5658
SFTPD*02	18.28	2[d]	2	2	2	2.4278	0.0166
SFTPD*03	8.75	1	2	2	1	−3.0304	0.0034
SFTPD*04	11.47	1	1	2	2	1.3393	0.1878
SFTPD*05	17.95	1	1	2	1	0.4470	0.6584

[a]Frequencies of haplotypes were estimated with the expectation–maximization algorithm
[b]Serum SP-D levels were compared across the estimated haplotype frequencies, using a score test assuming ordinally scaled values with simulated *P*-values from 10^6 replications
[c]Frequent allele
[d]Rare allele

confirm this observation by analysing a second independent group of blood donors (*n*=160). Analysis of this second cohort confirmed the initial observation of the negative association of the *SFTPD* haplotype *SFTPD*03* with the serum SP-D levels (Monte Carlo, *P*=0.0034) (Table 7).

Discussion

Currently, there is a growing interest on the role of SP-D in host defense against infections. This present study has been the first to elucidate the genetic basis for the variation in the SP-D serum protein level.

Associations between polymorphism of the *SFTPD* gene and serum SP-D levels were identified as well as nine putative frequent human *SFTPD* haplotypes, including one single *SFTPD* haplotype, *SFTPD*03* (allele frequency 13.53%), which revealed a negative association with serum SP-D levels (*P*<0.0001).

Mean serum levels of SP-D in this study were in accordance with serum SP-D levels of healthy subjects from Caucasian populations in previously published studies using the same commercially available SP-D ELISA (Greene et al. 2002; Janssen et al. 2003). In the Japanese population, lower mean serum levels of SP-D were reported (Nagae et al. 1997; Takahashi et al. 2000; Ihn et al. 2002; Greene et al. 2002). A direct comparison of the serum SP-D levels among ethnic groups could be a matter of particular interest.

However, higher mean serum SP-D levels were reported in healthy subjects of Caucasian origin, employing another not-commercially available ELISA (Husby et al. 2002; Leth-Larsen et. al. 2003).

Polymorphisms of the *SFTPD* gene were associated with the risk of severe respiratory syncytial virus (RSV) bronchiolitis in susceptible infants (Lahti et al. 2002). In this study from an ethnic homogenous Finnish population, the *SFTPD* allele coding for methionine 11 (position 31 from transcription start site) was associated with severe RSV bronchiolitis in infants (*P*=0.033). Floros et al. (2000) reported association of the opposite *SFTPD* allele coding for threonine 11 (position 31 from transcription start site) with tuberculosis within the Mexican population (*P*=0.002). Different alleles appear to be protective in the two studies. One possible reason for these discrepant results is that false-positive results have occurred by chance (type I error) (Colhoun et al. 2003). The *P*-value from the study of Floros et al. (2000) would have been near or above 0.05 if it had been corrected for multiple testing and there were no replication in either study. Thus, the *P*-values reported in both studies are far greater than the *P*-values needed to

assure a low posterior error rate (Colhoun et al. 2003; Freimer and Sabatti 2004; Thomas and Clayton 2004; Wacholder et al. 2004).

To our knowledge, we demonstrated and validated for the first time that a specific haplotype is significantly associated with decreased serum SP-D levels.

Serum concentrations of SP-D are largely determined genetically as shown by a recently published study on twins: model fitting showed that the estimated heritability was 0.91 (95% CI 0.83–0.95) for SP-D with additive genetic and non-shared environmental factors (Husby et al. 2002). Based on the genotypic data, we used an expectation–maximization algorithm to estimate the frequencies of putative *SFTPD* haplotypes (frequency >1%) and have suggested a nomenclature according to haplotype frequency. Four common putative haplotypes with a cumulative frequency of >74% were identified.

We were able to define one single *SFTPD* haplotype, *SFTPD*03* (allele frequency 13.53%), which revealed a negative association with serum SP-D levels. We conclude that this is not due to a possible effect of the missense variation at position 92, which may affect binding of the mAbs that were used for ELISA, based quantification of serum SP-D levels, since the G at position 92 is also included in several haplotypes without association to the serum SP-D level. Moreover, we were able to confirm the association of the haplotype *SFTPD*03* with low SP-D serum levels in a second independent cohort of blood donors.

Serum SP-D has been shown in clinical investigations as a promising biomarker for acute lung injury and type II cell hyperplasia as seen in pulmonary fibrosis and other forms of interstitial lung disease (Takahashi et al. 2000; Asano et al. 2001; Ohnishi et al. 2002; Greene et al. 2002). The elevation of SP-D is related to the extent of the parenchymal lung disease and predicts survival in idiopathic pulmonary fibrosis (Greene et al. 2002). Serum SP-D is also increased in patients with acute ARDS (Greene et al. 1990) and patients with clinical pneumonia (Leth-Larsen et al. 2003). In these conditions, elevated SP-D serum levels were partly accounted to leakage from the alveolar space, since alveolar type II cells are regarded as the main cellular source of SP-D. Although the lung is the major site of SP-D expression, it is likely that the protein has more generalized roles in host defence and the acute response to infection and tissue injury (Crouch 2000).

In patients with acute pneumonia, the SP-D serum concentration may increase up to 22-fold within a few days (Leth-Larsen et al. 2003). It is likely that decreased serum levels of SP-D which have been observed in different clinical situations are associated with the risk of infections (Holmskov et al. 2003): temporal changes in serum SP-D concentrations occur during infections, and infections superimposed on other pulmonary disease states may radically interfere with the outcome of cross-sectional measurements of serum SP-D concentrations.

In conclusion, here we describe a frequent *SFTPD* haplotype that is associated with low serum SP-D levels. Since the investigated SNPs belong to a single haplotype block, we did not identify the 'functional' SNP that may affect the serum protein level by allele specific regulation of transcription or other mechanisms (Knight 2003).

However, the genetic discovery of a frequent negative variant of the *SFTPD* gene provides an excellent basis for analysis of the function of SP-D in the resistance against pulmonary infections and inflammatory disorders in humans.

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