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Association analysis of the surfactant protein-C gene to childhood asthma

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ABSTRACT

Objectives: This study aims to describe the molecular variability in the *SFTPC* gene in a childhood chronic respiratory disease, asthma, in the Tunisian population and to identify the implications based on a case-control study of p.Thr138Asn (T138N) and p.Ser186Asn (S186N) variants.

Methods: We used direct sequencing for the genotyping of the *SFTPC* gene within 101 asthmatic children. The study of T138N and S186N variants in 110 controls is conducted by the PCR-RFLP technique.

Results: The molecular study revealed 26 variants including 24 intronic variations and 2 exonic variations (T138N and S186N) with respective frequencies of 16.8% and 18.3%. We conducted a case-control study of the two identified exonic variations. A different genotypic and allelic distribution between the two groups was noted. Only the T138N polymorphism showed a significant association with asthma disease ($p < 10^{-3}$). Statistical analysis elaborated four haplotypes with the following frequencies in patients vs controls: 138Thr-186Ser (79.5% vs 57.6%), 138Thr-186Asn (3.7% vs 7.8%), 138Asn-186Thr (2.2% vs 20.2%) and 138Asn-186Asn (14.6% vs 14.4%). A significant difference ($p < 10^{-3}$) was highlighted in haplotype distribution. The 138Asn-186Ser (OR [95%CI] = 0.14[0.04-0.54], $p = 0.004$, $R^2 = 0.93$) and 138Thr-186Asn (OR [95%CI] = 0.35[0.12-0.54], $p = 0.047$, $R^2 = 0.88$) haplotypes showed a negative association with asthma which may constitute a protective factor against the disease.

Conclusion: In Tunisia, this work constitutes the first report interested in the *SFTPC* gene and highlights the genetic variability of the *SFTPC* gene in asthma. Therefore, the case-controls analysis may be useful in the study of surfactant proteins dysfunction in chronic respiratory disease at an early age.

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Introduction

Pulmonary surfactant is a complex lipoprotein mixture that coats alveolar surfaces and reduces surface tension at the air/liquid interface, enabling normal respiratory function. Mutations in the genes encoding the surfactant proteins (SP-A, SP-B, SP-C, and SP-D) have been associated with a wide spectrum of lung disease with considerable overlap in their clinical and histological presentation, primarily due to impaired surfactant function and gas exchange in the lung (1). Genetic analysis, family and clinical histories, expression of the surfactant proteins, and lamellar body ultrastructure may be helpful to understand these genetic disorders (2). Surfactant protein C (SP-C) is a hydrophobic integral membrane protein expressed exclusively by pulmonary type II epithelial cells. SP-C is synthesized as a large precursor proSP-C (197

amino acids, 21 kDa). ProSP-C is composed of four domains; a short N-terminal segment (residues 1-23), a transmembrane (TM) region constituting the main part of mature SP-C (residues 28-58), a linker region (residues 59-89) and a BRICHOS domain (residues 90-197). Post-translational processing of proSP-C results in mature SP-C of 35 amino acids (3.7 kDa) (3). Mature SP-C enhances, together with SP-B, the ability of surfactant phospholipids to decrease alveolar surface tension (4) and plays a role in the innate host defense of the lung (5). The deficiency of SP-C could also promote alveolar instability, associated with lung inflammation and injury (6,7). In vitro studies have highlighted the regulation of SP-C expression by pulmonary infection and inflammation suggesting immunological functions (7-9). The SP-C deficiency in patients and mouse models is associated with

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increased inflammation and delayed repair, but the mechanisms of SP-C regulated inflammation in response to injury are largely unknown (7).

The SP-C coding gene (*SFTPC*; OMIM number 178620) is located on chromosome 8p21, spanning 3.5 Kb. It is composed of 6 exons with only the first five coding for the propeptide (10). Since the first description of interstitial lung disease-causing mutation (11), more than sixty mutations have been identified as an autosomal dominant transmission with variable penetrance or sporadic trait caused by *de novo* mutation on one allele (12,13). A substitution of isoleucine for threonine in codon 73 (p.Ile73Thr) is the most common *SFTPC* mutation (14).

SFTPC mutations are associated with a wide array of lung diseases ranging from severe respiratory distress in infants to lethal and variable onset of respiratory symptoms (4,12). Two common non-synonymous variants p.Thr138Asn (T138N, rs4715) and p.Ser186Asn (S186N, rs1124) have been identified in respiratory disorders such as respiratory distress syndrome (RDS), infection with a syncytial respiratory virus (SRV), asthma, and chronic obstructive pulmonary disease (15–18).

Asthma is the most common chronic disease diagnosed among children worldwide according to the World Health Organization (WHO) (19). Asthma is a chronic inflammatory condition of the airways characterized by recurrent episodes of reversible airway obstruction and increased bronchial hyper-responsiveness (20). Asthma presentations combine the interactions of genetic predisposition, environmental factors, and epigenetic changes (21).

To further investigate the contribution of variants in the *SFTPC* gene to common respiratory chronic disease, for the first time in Tunisia, we used a complete sequencing of the *SFTPC* gene in 101 asthmatic children. The case-control study aims were to identify the association between the two exonic variants, p.Thr138Asn (T138N/exon 4) and p.Ser186Asn (S186N/exon 5), with asthma.

Subjects and methods

Subjects

A total of 101 unrelated consecutive Tunisian children with asthma were recruited from the Pediatric service at the Children Hospital “Bechir Hamza” of Tunis during 2014–2015. A questionnaire had been elaborated in the Biochemistry Laboratory to collect epidemiological (sex, age, geographic origin, consanguinity, and tobacco status) and clinical (clinical history, crisis frequency, hospitalization, atopic phenotypes, and last medications) data. The asthmatics mean age was 81.40 ± 48.53 months (ranging

from 4 months to 17 years old). Asthmatic children had been classified as follows; 78 subjects with mild persistent asthma, 19 subjects with moderate persistent asthma, and 4 subjects with severe persistent asthma. The atopic phenotype was defined by a positive skin test reaction characterized by a wheal of 3 mm in diameter, to a panel of common allergens in the presence of a positive histamine control and negative uncoated control. Bronchodilators, corticosteroids, and antihistamines were the treatments administrated to these patients to control asthma symptoms, and oxygen therapy aimed to attenuate the acute episodes of asthma. The smoking status, commonly associated with respiratory disease, was also noted in those patients. A control group of 110 Tunisian subjects (mean age 130.70 ± 124.70 months) with no antecedents of respiratory diseases or atopic complaints was included in this study. The controls visited the Children’s Hospital of Tunis services’ for other reasons than respiratory or atopic complaints (e.g. vaccination, fracture...). Patients and controls belonged to the same ethnic group: African white Caucasian.

Epidemiological and clinical data of asthmatic and control subjects are summarized in Table 1.

Inclusion and exclusion criteria

We included Tunisian asthmatic children (<18 years). The clinical profile was recorded including the severity of asthma, previous allergic rhinitis or conjunctivitis, atopic dermatitis, and any family history of respiratory diseases. The diagnosis of disease was conducted by a pediatric clinician who confirmed the positive asthma diagnosis using standard protocols i.e. the assessment of frequency and severity of symptoms (patient/parents reported) and the use of objective measurements such as spirometry, airway hyper-responsiveness and inflammatory markers according to the recommendations of GINA (Global Initiative for Asthma) and NAEPP (National Asthma Education and Prevention Program) guidelines (22,23). Children with non-respiratory disease (cardiovascular) comorbidity, malformation, or known genetic disorder were excluded from the study. The control group was selected carefully to elaborate on the case-control study. To qualify as a control subject, there should have been no history of respiratory disease or asthma and no first degree relative with a history of asthma.

Approval

This study was approved by the ethics committee of the Children’s Hospital of Tunis and the approval written from parents or legal guardians was obtained.

Table 1. Epidemiological and clinical data of our study groups.

Subjects		Controls	Asthmatics
Number of subjects		110	101
Age (months)	Mean $\pm$ SD	130.70 $\pm$ 124.70 months	81.40 $\pm$ 48.53 months
	Median	114 months	72 mois
	Min	1 months	4 moths
	Max	288 months (24 years)	204 months (17 years)
Sex n (%)	boy	70 (63.7%)	62 (61.4%)
	girl	40 (36.3%)	39 (38.6%)
	sex-ratio (male to femelle)	1.75	1.59
Consanguinity n (%)		0	36 (35.6%)
Family history n (%)		0	52 (51.5%)
Geographic origin n (%)	Northwest	54 (49.1%)	48 (47.5%)
	Northeast	24 (21.8%)	23 (23.8%)
	Central West	20 (18.2%)	17 (16.8%)
	Central East	9 (8.2%)	7 (6.9%)
	Southwest	2 (1.8%)	2 (2%)
	Southeast	1 (0.9%)	3 (3%)
Smoking status n (%)	passive smokers	0	63 (62.4%)
	non-smokers	110	37 (36.6%)
	smoker	0	1 (1%)
Atopic phenotypes n (%)	atopic allergy	0	37 (36.6%)
	rinitis		18 (17.8%)
	conjunctiivitis		5 (4.9%)
	dermatitis		4 (4.0%)
	rinitis-conjunctiivitis		7 (6.9%)
	rinitis - dermatitis		2 (2.0%)
	dermatitis-conjunctiivitis		1 (1.0%)
	no atopic allergy	110 (100%)	64 (63.4%)

n: effectif, %: percentage. Six areas defined the geographic origin of asthmatics. Northeast: Bizerte, Tunis, Ben Arous, Ariana, Manouba, Nabeul, and Zaghouan; Northwest: Beja, Jendouba, Le Kef, and Siliana; Central East: Sousse, Monastir, Mahdia, and Sfax; Central West: Kairouan, Sidi Bouzid, and Kasserine; Southeast: Gabes, Mednine, and Tataouine, Southwest: Gafsa, Tozeur, and Kebili.

Methods

DNA extraction

Genomic DNA was isolated from peripheral blood leucocytes collected on EDTA using a “Salting-Out” protocol (24). Each blood sample was collected in two EDTA tubes; the first one was conserved at -80°C and the second one was used for DNA extraction. The extracted DNA quality was controlled by the nanodrop technique and then stored in two copies at $+4^{\circ}\text{C}$ and -80°C . Before proceeding to PCR, the DNA sample was also controlled.

SFTPC gene amplification and sequencing

Primers described in Table 2 were elaborated by Primer 3 software. 300 ng of genomic DNA was combined with a PCR buffer (10X) containing 5 pmol each of forward and reverse primers, 0.15 mM MgCl_2 (50 mM), 0.0625 mM of each dNTP (2.5 mM) (Promega©, Madison, WI, USA), and 0.6 units of Taq DNA polymerase (5 U/ μl) (Thermofisher Invitrogen) in a total volume of 50 μl . The PCR reactions were cycled using the following protocol: 95°C for 5 min followed by 35 cycles consisting of 95°C for 30 s, 57°C for 40 s, 72°C for 1 min, with a final extension at 72°C for 7 min. Excess primers were removed using ExoSAP-IT (USB©, Cleveland, OH, USA)

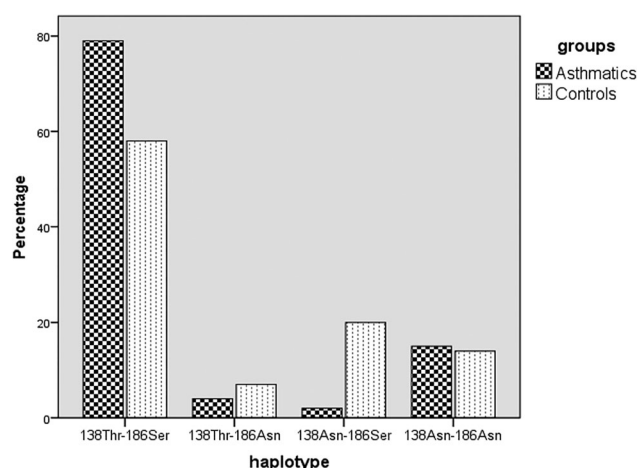
following the manufacturer’s protocol. Sequencing reactions were performed with Big Dye Terminator cycle sequencing kits from Applied Biosystems (Foster City, California, USA). Sequence reactions were performed using each PCR-Exosap product, 1 pmol of primer, 1.75 μl of Big Dye dilution buffer, and 0.5 μl of BigDye terminator. Reaction components were assembled in a 96-well multiplate and briefly pulsed in a centrifuge to mix. The cycling protocol consisted of 96°C for 10 min followed by 25 cycles consisting of 96°C for 1 min, 55°C for 5 s, and 60°C for 2 min. A second purification with BigDye XTerminator® Purification Kit was performed. Sequences were analyzed using SeqScape software and compared with the reference sequence NM_003018.3. Besides, each DNA sample was analyzed in both directions forward and reverse (Figure S1).

For genotyping of polymorphisms in exons 4 (T138N) and 5 (S186N) in controls, we used the PCR-RFLP technique (Table 2). Primer sequences were reported by Guillot et al. (25). Ten μl of PCR product was incubated with 20 U of Fsp BI (BfaI) and Sdu I (Bsp 1286I) restriction enzymes at 37°C for 4 h. The electrophoresis of products of PCR-RFLP was analyzed on 2.5% agarose gel in the presence of an undigested and heterozygous control (Figure S2). A meticulous adjustment of the protocol was developed to guarantee the results.

Table 2. Genotyping protocols of *SFTPC* gene. Sequences of primers used in amplification of *SFTPC* gene in asthmatics followed by PCR-RFLP technique of genotyping of T138N and S186N variants in controls.

Fragments	Forward primers	Reverse primers	Amplicons size
SFTPC_exon 1	ACCCAGGTTTGCTCTTGCTG	TGGATCTGGATAAGGAAACAGG	394bp
SFTPC_exon 2	TAGAATCCAGGCCACCTCCT	CGTGCCTCTTCTCTTAGC	475bp
SFTPC_exon 3	CTCTTGGGAAAGAGGGAAGC	GGGAGAGATGGATGTGGATG	327bp
SFTPC_exon 4	CAACCTAGAGGGAGGTGGCTA	GGAATAGGAGAGGGGCGAGTC	333bp
SFTPC_exon 5	GACTGCCCTCTCCTATTCC	GTACCGTCTGTGAGCTTCC	550bp
SFTPC_intron 5	CCGAGTGCCTACGTCTAAG	CCAAGCTCCCATTTCTCCAG	485bp
SFTPC_exon 6	GCTCAGACCGGTACTTCC	CAAACCCCACTTTGCTCTCC	375bp
SFTPC_6_insA	CCATCCTCAACATTCCTTGC	CAAACCCCACTTTGCTCTCC	334bp

Variant / Location	Forward and reverse primers	Amplicons size	Thermo Scientific™ Restriction enzyme	Restriction site	Fragments size after digestion
c.413 C > A (T138N) exon 4	5'..CTAGTATGACTCCCGTGCCC..3' 3'..TGAGGAACAGTGCTTTACAGG..5'	319bp	Fsp BI (Bfal)	5'..C▼TAG..3' 3'..GAT▲C..5'	ACT(Thr):1bp + 23bp + 78bp + 92bp + 125bp or AAT(Asn): 1bp + 23bp + 78bp + 217bp
c.557G > A (S186N) exon 5	5'..TCAGCTGAGTCCACTCACTACC..3' 3'...GTACCGTCTGTGAGCTTCC..5'	484bp	Sdul (Bsp 1286I)	5'..GDGCH▼C..3' 3'..C▲HCGDG..5'	AGC(Ser): 278bp + 69bp + 137bp or AAC(Asn): 347bp + 137bp

**Figure 1.** Histogram illustrating haplotypes distribution in patients and controls.

Thr: Threonine, Ser: Serine, Asn: Asparagine. Asthmatics vs Controls: 138Thr-186Ser 79.5% vs 57.6%; 138Thr-186Asn 3.7% vs 7.8%; 138Asn-186Ser 2.2% vs 20.2%; 138Asn-186Asn 14.6% vs 14.4%

Mutations nomenclature

Recommendations of the Human Genome Variation Society (<http://www.hgvs.org/mutnomen/>) were followed for mutation names, with +1 used as the A for the ATG translation initiation start site in the GenBank *SFTPC* reference sequence NM_003018.3. Exons are numbered as in NG_016968.1 (26).

The verification of sequence variation was carried out using the *Ensembl* database (https://www.ensembl.org/Homo_sapiens/Gene). The effect of substitution on the protein sequence was studied using the *ALAMUT* software (<http://www.interactive-biosoftware.com/doc/alamut-visual/2.7/SNPs>) and the Human Splicing Finder software available on (<http://www.umd.be/HSF/index.html>).

Data analysis

Hardy-Weinberg equilibrium (HWE) was tested in cases and controls, separately. The allele and genotype frequencies were calculated using *SPSS* software for windows version 20.0 (SPSS Inc., Chicago, IL, USA). Comparisons between qualitative variables were performed by the χ^2 test. The statement on power and sample size calculation was performed using *G*Power* software. Using two-side, 5% of significance level and an effect size of 30%, there is a 98% chance of correctly rejecting the null hypothesis of no difference between proportions with a total sample size of 211 participants. A minimum of 108 participants is required to detect an effect size of 30%, and a power of 80%. Genotype frequency comparisons under additive, dominant, and recessive models were performed using *Snptest* tool (<https://snptest.net/start.htm>). Odds Ratio (OR) and 95% Confidence Intervals (CI) were calculated whenever applicable. Linkage disequilibrium (D') and haplotype frequency based on T138N and S186N polymorphisms were determined using *THESIAS* (Testing Haplotype Effects In Association Studies) software to construct haplotypes from genotypes. A p values < 0.05 was considered statistically significant.

Results

SFTPC gene screening

The screening of the *SFTPC* gene in a group of 101 children with asthma identified twenty-six sequence variations, which are described and classified in the database (Table 3). Twenty-four of these variations

Table 3. Genetic variations identified in asthmatic patients.

SNP ID* Number	Variation	Nucleotide region	Allele frequency	MAF** (Ensembl database)	References
rs28438700	c.1-134 G > C	5' UTR	6,4 % (13)	0.07 (C)	(16)
rs79647630	c.1-92 C > G		1,5 % (3)	<0.01 (G)	
rs77253713	c.1-91 C > T		1,5 % (3)	<0.01 (T)	
rs377267823	c.1-67 C > T		0,5 % (1)	<0.01 (T)	
rs8192340	C.42 + 35 G > A	Intron 1	3,4 % (7)	0.03 (A)	(16)
rs8192326	c.43-87 C > T		1.0 % (2)	0.02 (T)	
rs13248346	c.43-21 T > C		45,0 % (91)	0.41 (T)	
rs79440568	c.43-7 G > A	splicing region	2,5 % (5)	0.01 (A)	(34,35)
rs8192327	c.201 + 14 G > A	Intron 2	4,4 % (9)	0.02 (A)	(16,38,39)
rs2070684	c.201 + 49 C > A		67,8 % (137)	0.32 (A)	
rs8192328	c.201 + 108 A > G		1.0 % (2)	0.01 (G)	
rs4715	c.413 C > A	Exon 4	16,8 % (34)	0.20 (A)	Missense variation p.Thr138Asn (T138N) (16,38–42)
rs79253830	c.435 + 39 C > T	Intron 4	1,5 % (3)	<0.01 (T)	(39,43)
rs8192330	c.435 + 71 G > A		1,5 % (3)	0.04 (A)	
rs181314084	c.435 + 95 T > C		2.0 % (4)	<0.01 (C)	
rs2070685	c.435 + 148 C > T		4,9 % (10)	0.15 (T)	
rs2070686	c.436-107C > T		32,7 % (66)	0.25 (T)	
rs2070687	c.436-8C > G	splicing region	31,2 % (63)	0.30 (G)	(16,38,39,44)
rs1124	c.557G > A	Exon 5	18,3 % (37)	0.24 (A)	Missense variation p.Ser186Asn (S186N) (16,38,39,42)
rs189992977	c.*10 + 63 G > A	Intron 5	0,5 % (1)	<0.01 (A)	
rs3216166	c.*19-39inA (-/A)		18,3 % (37)	0.33 (A)	
rs75518353	c.*36 A > G	Exon 6	0,5 % (1)	0.01 (G)	Silent variant p.Gln98=
rs7592	c.*123 G > A		27,2 % (55)	0.33 (A)	Silent variant p.Lys127=
rs1126931	c.*173 C > T		10,4 % (21)	0.10 (T)	Missense variation p.Ala144Val
rs1139547	c.*174 G > A		14,8 % (30)	0.24 (A)	Silent variant p.Ala144=
rs8192332	c.*286 C > T	3'UTR***	30,7 % (62)	0.31 (T)	

*SNP: Single Nucleotide Polymorphism, **MAF: Minor Allele Frequency, ***UTR: Untranslated Transcribed Region.

Polymorphisms are labeled either by their entry code in the public data base of SNPs (<http://www.ensembl.org>.) or by their positions based on the published SP-C genomic sequence.

are located in the non-coding regions of the *SFTPC* gene. We noted that twenty-five variations are nucleotide substitutions: 18 transitions (C/T or A/G) and seven transversions and one insertion of a nucleotide (A). Five of the variations are very rare and have a frequency lower than 1%.

Two variations are located in the vicinity of splicing regions of the *SFTPC* gene: c(0).43-7 G > A (rs79440568) upstream of exon 2 and c(0).436-8C > G (rs2070687) upstream of exon 5. The “Human Splicing Finder” analysis showed that only the c(0).436-8 C > G variation may probably affect the splicing. Indeed, the normal sequence carrying the wild-type allele “C” corresponding to a normal acceptor splice site starting at position c(0).436-12 (predicted score of 87.33) was affected by the mutated allele “G” which induces the creation of a new acceptor site that starts at nucleotide position c(0).436-19 (predicted score of 53.1 → 82.04). This change in the predicted score results in a 7 bp elongation inducing the frame-shift of the cDNA thus affecting the SP-C protein sequence. None of the other sequence variations identified in the non-coding regions should affect splicing, as assessed.

Only two variations have been identified in the coding region; c(0).413 C > A (rs4715) in exon 4 is a missense variation p.Thr138Asn (T138N) and

c(0).557 G > A (rs1124) in exon 5 corresponding also to a missense mutation p.Ser186Asn (S186N). Both variations are located in the BRICHOS domain of the pro-protein SP-C (pro-SP-C).

Case-control study

To explore the genetic contribution of these sequence variations, we conducted a case-control study (Table 4). The genotyping of c(0).413 C > A in patients and control groups has revealed a significant difference in allelic and genotypic distributions. It was noted that the mutated allele A is more frequent in controls than in asthmatics (34.5% vs 16.8%). The homozygous AA genotype was carried by only 3% of the patients vs 19.1% of the controls. Furthermore, a significant association was observed between the T138N polymorphism and asthma susceptibility for both, the smoking exposure, and the atopic phenotypes (Table 4).

The identification of c.557G > A polymorphism in our groups showed a similar distribution of alleles and genotype but we noted that the mutated genotype AA was more common in controls (10.9%) than in patients (5%).

The statistical analysis of segregation revealed a co-segregation between these two variations as well as an

Table 4. Case-control association for single nucleotide polymorphisms T138N and S186N.

SNPs*	Genotype and alleles	Individual numbers (frequency)		Chi ² test p-value	Unadjusted OR			Adjusted OR under additive model		
					Dominant model			by smoking status		
		Asthma	Controls		OR (95%CI)	p-value	OR (95%CI)	OR (95%CI)	p-value	p-value
c.413 C > A p.Thr138Asn T138N (rs4715)	CC	70 (69.3%)	55 (50.0%)	Chi ² = 15.525	1.00 ^{ref}	2 10 ⁻⁴	1.00 ^{ref}	1.00 ^{ref}	0.008	2 10 ⁻⁴
	CA	28 (27.7%)	34 (30.9%)	p < 10 ⁻³	0.65 (0.35-1.19)		0.44 (0.25-0.78)	0.28 (0.10-0.79)		0.55 (0.27-1.12)
	AA	3 (3.0%)	21 (19.1%)		0.11 (0.03-0.40)		0.13 (0.04-0.45)	0.27 (0.07-0.98)		0.06 (0.01-0.43)
	p (HWE)	0.921	0.0009	Chi ² = 17.146 p < 10 ⁻³						
c. 557 G > A p.Ser186Asn S186N (rs1124)	GG	69 (68.3%)	73 (66.4%)	Chi ² = 2.693	1.00 ^{ref}	0.25	1.00 ^{ref}	1.00 ^{ref}	0.34	0.15
	GA	27 (26.7%)	25 (22.7%)	p = 0.260	1.14 (0.61-2.16)		0.92 (0.51-1.63)	0.60 (0.22-1.62)		1.02 (0.49-2.10)
	AA	5 (5.0%)	12 (10.9%)		0.44 (0.15-1.32)		0.43 (0.14-1.25)	0.42 (0.09 - 1.99)		0.26 (0.06 - 1.24)
	p (HWE)	0.283	0.0003	Chi ² = 0.6 p = 0.493						

*SNP: Single Nucleotide Polymorphism, HWE p values > 0.05 considered significant for Hardy Weinberg Equilibrium (HWE), p < 0.05 is considered significant for association analysis; 1.00^{ref} refers to the reference genotype; OR odds ratio; CI confidence interval.

important linkage disequilibrium ($D' = 0.89$, $p < 10^{-3}$). Haplotypes were performed in cases and controls (Figure 1). Four haplotypes have been observed with the following frequencies in patients compared to controls: 138Thr-186Ser (79.5% vs 57.6%), 138Thr-186Asn (3.7% vs 7.8%), 138Asn-186Thr (2.2% vs 20.2%) and 138Asn-186Asn (14.6% vs 14.4%). It should be noted that the reference haplogroup is the most frequent haplotype, i.e. 138Thr-186Ser. The haplotype distribution was significantly different ($\chi^2 = 18.794$, $p < 10^{-3}$) between cases and controls. The haplotype analyses revealed a statistical association of haplotypes against asthma susceptibility. Indeed, the 138Thr-186Asn (OR [95%CI] = 0.35[0.12-0.54], $p = 0.047$, $R^2 = 0.88$) and 138Asn-186Ser (OR [95%CI] = 0.14[0.04-0.54], $p = 0.004$, $R^2 = 0.93$) haplotypes were more common in controls than in patients. Furthermore, a significant association was observed between the 138Asn-186Ser haplotype and asthma susceptibility for the two cofactors, the smoking exposure (OR [95%CI] = 0.14[0.02-0.78], $p = 0.027$), and the atopic phenotypes (OR [95%CI] = 0.15[0.04-0.53], $p = 0.0036$) (Table 4).

Discussion

Asthma is the most frequent chronic respiratory disease, affecting more than 300 million people worldwide. It's the most common chronic disease in children (27). Globally, asthma prevalence, severity, and mortality show a large geographical variation due to epidemiological (age, race/ethnicity, sex, and socio-demographic classes) and clinical (onset diagnosis, medical history, and health care) determinants. While asthma prevalence is higher in high-income countries, most asthma-related mortality occurs in low-middle income countries (27). In Tunisia, the general prevalence of asthma is estimated around at 3.6% with a higher rate in Tunis capital city (6.9%) (28).

Epidemiological and clinical data

A sex ratio (male to female) of 1.59 is defined for the asthmatic population; 61% of the studied patients were males, it is in accordance to the theory of the association of the male gender with early asthma, suggesting the potential role sex hormones in the disease's etiology (27). A high rate of consanguinity is estimated within patient groups (35.6%) referred to the general population in Tunisia (21% in 2008) (29). It is noteworthy that all the Tunisian regions are represented with a decreasing North-South gradient

probably due to the interaction of the environmental exposures (air pollution) and the geo-climatic conditions of our country (vegetation and humidity) to asthma presentation (27). Our findings show a high prevalence of asthmatic children who are exposed to parental smoking (62.4%). This subgroup includes a percentage of 57.1% of preschool-aged asthmatic patients (less than six years) with a ranging age of four months to about fifteen years. On the other hand, we found that asthmatics exposed to paternal smoking during the first two years of life were more likely to develop asthma disease. A positive association ($p = 0.024$) is noted between the age of patients and the smoking status, an important risk factor for the lung's development (30). The presentation of asthma in patients exposed to tobacco is earlier (76.63 ± 42.80 months) compared to the patients that were not-exposed (86.22 ± 53.91 months). Therefore, the risk of asthma was increased with exposure to tobacco smoke. Worse asthma symptoms and an increased rate of asthma exacerbations were reported regardless of the dose of exposure because even low levels of exposure can cause significant symptoms (31).

About half of our patients (51.5%) have a familial respiratory history, a significant predicting factor of asthma diagnosis. The medical history of asthmatic children includes classic asthma symptoms such as cough, wheezing, and breathlessness that are common to many respiratory conditions in childhood. Coexistence of atopic phenotypes is mentioned in 37 patients (36.6%): rhinitis (17.8% of patients), conjunctivitis (4.9%), dermatitis (4%), rhinitis-dermatitis (2%), rhinitis-conjunctivitis (6.9%) and dermatitis-conjunctivitis (1%).

The control group of 110 Tunisian subjects (mean age 130.7 ± 124.70 years) had a defined sex ratio of 1.75. The majority of Tunisian regions were represented with a larger percentage being from the North of the country.

SFTPC gene screening in asthma

In the current study, we investigated the implication of *SFTPC* variants in childhood asthma, a multifactorial disease with a challenging early diagnosis.

A total of twenty-six variations were reported within asthmatics, highlighting a greater variability for the non-coding regions (24 sequence variations) (Table 3). These results showed the genetic specificities of the asthmatic Tunisian population. Indeed, only 8 nucleotide substitutions were reported among 174 Danish asthmatic patients with 6 variations in common with our study (16). This finding may underline the genetic diversity due to the different ethnic

origin between the two populations. Tunisia is a North African country. The native background population is Berber. Nevertheless, Tunisian history has been marked by invasions and migratory waves of several populations and ethnic groups starting with the Phoenicians and followed by the Romans, the Vandals, the Arabs, the Ottomans, and the French (32).

Our results show that sequence variations in the coding region of *SFTPC* gene are not common and that it is a relatively conserved region. Furthermore, only two common non-synonymous variants T138N (rs4715) and S186N (rs1124) have been identified. These two variations are statistically associated with neonatal respiratory distress syndrome (17,18) and T138N-S186N haplotypes were associated with severe respiratory syncytial virus (RSV) infections (15). These variants are located in the C-Terminal region of the pro-SP-C which is crucial for the targeting and processing of pro-SP-C to mature protein (18). The two variations are located in the BRICHOS domain, a domain of approximately 100 amino acids, which as thus far been found as a constituent of 12 otherwise different protein families associated with degenerative and proliferative disease (25). Interestingly, other sequence variations identified in the BRICHOS domain reported previously as polymorphisms could contribute to abnormal function, alteration, or major deficiency of SP-C protein (3,25). On the other hand, the location of the p.Thr138Asn and p.Ser186Asn were adjacent to some mutations (p.Gln145His, p.Leu188Arg, and p.Leu188Gln) associated with severe respiratory phenotypes.

Recently, the c.435G > A, p.Gln145= mutation, located at the last base of exon 4, was considered as a variant of uncertain significance because it did not change the coding sequence of the pro-SP-C (33). At the same position, the missense mutation c.435G > C (p.Gln145His) has been reported to be deleterious not because of its amino acid substitution but because of its subsequent splicing defect and was associated with severe respiratory diseases (26).

We have also identified the c(0).43-7 G > A (rs79440568) variation in the skipping region of exon 2 that had been firstly described by Lawson et al. to be associated with idiopathic pulmonary fibrosis (34) and also by Tarocco et al. for its association with a neonatal respiratory distress case with an African ethnicity (35). A splicing defect has been suggested to be induced by the c(0).435-8 C > G variation that may contribute to a new acceptor site of the cDNA. Alternative splicing contributes significantly to the complexity of the human genome suggesting that 60% of genes have alternative splice forms (36). The

concept of splicing regulation by “enhancers” and “silencers”, stipulates that sequence elements within exons or introns can promote or inhibit splicing at nearby splice sites (26). Thus, an intronic or exonic mutation could be postulated to affect transcription and protein translation.

The role of SP-C in regulating inflammation is not fully understood. SP-C deficiency in patients and mouse models has been associated with increased inflammation and delayed lung repair (7,37). These findings report the functional and clinical significance of SP-C on surfactant metabolism, alveolar type II stability, and lung injury and may contribute to the understanding of the physiopathology of asthma.

Case-control study of T138N-S186N variants

To identify the implication of p.Thr138Asn (T138N) and p.Ser186Asn (S186N) in asthma disease, we conducted a case-control study (Table 4). HWE was tested in asthmatics and controls separately. Both polymorphisms were in HWE in the asthmatic group and showed deviation from HWE in controls. This could be caused by a selection bias resulting from the small sample size for the control group enlisted from the general population.

Formerly, only two studies have focused on the role of these variations in asthmatic patients (15,16). In the current study, the distribution analysis of both polymorphisms showed the advantage of wild type genotypes, as can be seen by the over-representation of the wide-type alleles in asthmatics versus controls groups with 83.2% vs 65.5% for the C allele of T138N and 81.7% vs 77.7% for the G allele of S186N, respectively. These results are in contrast with the studies of Puthothu et al. and Baekvad-Hansen et al. The German study reported that the heterozygous genotypes and the mutated alleles were more frequent in asthmatics than in controls (65% vs 68.8% for A allele of T138N and 62% vs 62.4% for A allele of S186N) (15). At the same time, Danish work reported, in asthmatics, the frequencies of 44.3% and 46.6% for T138N and S186N, respectively (16).

The genotypic and allelic distribution of T138N polymorphism has revealed a significant difference ($p < 0.05$) between our case-control. Noted that the mutated genotype of this variant is more represented in controls than in patients (19.1% vs 3%). The T138N polymorphism may constitute a protection against asthma's predisposition associated with the smoking exposure and atopic phenotypes. This association revealed in the present study is in contrast with

the results of Puthothu et al. and Baekvad-Hansen et al. (15,16). These divergences may be explained by the genetic specificity of each population and the difference between the number of participating patients, which could influence asthma susceptibility, control, and medical care.

No significant association for S186N polymorphism in genotypic and allelic distribution was noted in our work and the German study (15). The S186N allelic frequency is different from the result (46.6%) of Baekvad-Hansen et al. (16). The contribution of S186N polymorphism in respiratory distress syndrome according to gender has been reported (17). In our findings, the two polymorphisms show a statistical difference in genotype distribution ($p < 0.05$) between asthmatic males and asthmatic females with the advantage of the wild genotypes into both polymorphisms.

Association analysis has revealed four haplotypes in asthmatic and control groups with a different distribution ($p < 10^{-3}$). The 138Thr-186Ser haplotype is also over-represented.

The haplotypes 138Thr-186Asn and 138Asn-186Ser are markedly over-represented in controls than in patients and could constitute an independent protective factor against asthma. Therefore, these alleles' complex showed a negative association with the asthmatic patients and supply some protection for asthma. A similar observation was previously suggested for the 138Thr-186Asn haplotype in the German study (15). The same report noted that 138Thr-186Asn haplotype was associated with RSV infection (15). Furthermore, the 138Asn-186Ser haplotype was a protective factor against asthma predisposition related to smoking exposure and atopic allergy.

Unfortunately, our study is not without limitations and these should be mentioned: (1) This is a hospital case-control study, so the selection bias may not avoidable and the subjects may not be representative of the general population. (2) The sample size is limited compared to the prevalence of asthma. (3) The heterogeneity of asthma disease. Further prospective and multicentric studies, including a larger sample size, could contribute to confirm our findings with particular attention to genotype-phenotype correlation and environmental factors.

Conclusion

In the current study, the screening of the *SFTPC* gene identified twenty-six variations within Tunisian asthmatics children. The case-control study of T138N and S186N revealed an association of T138N

polymorphism to asthma susceptibility. Then, the analysis of these complex of alleles has showed that 138Thr-186Ser and 138Asn-186Ser haplotypes could confer a protective factor against asthma. Therefore, our results support the implication of *SFTPC* gene in this respiratory disease. Until now, the study of *SFTPC* haplotype is rare but it can be a useful tool in molecular genetics of complex traits of childhood asthma. Several hypotheses can be speculated in the role of SP-C protein in pulmonary disease pathogenesis, the integrity of epithelial cell II, the metabolism of surfactant, and SP-C interaction with other surfactant proteins (SP-A, SP-B, and SP-D) and its role in lung infection and inflammation.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Supplementary figures for

Association analysis of the surfactant protein-C gene to childhood asthma

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This supplement contains supplementary figures S1 and S2 for the Methods section.

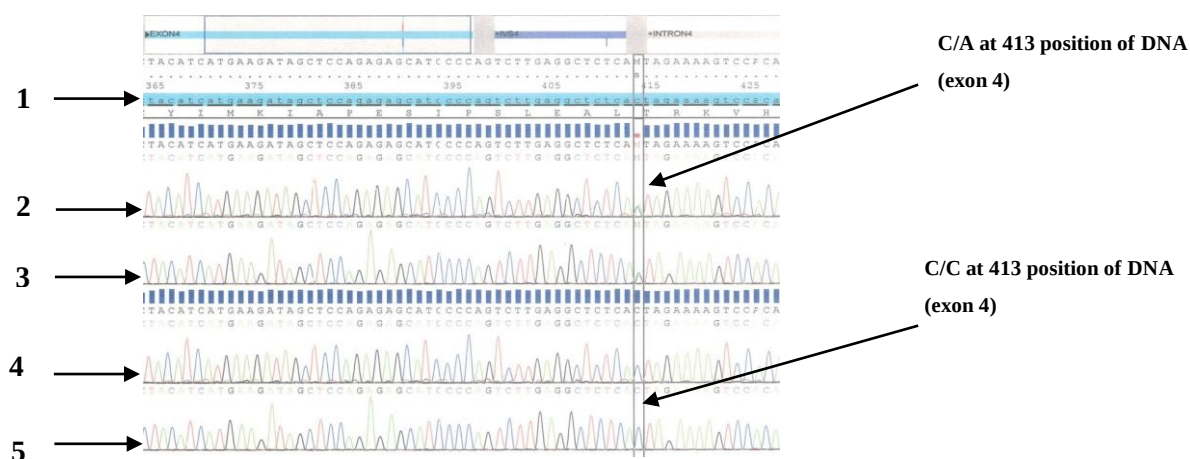


Figure S1: Sequencing profile of c.413C>A variant (T138N)

- 1 : reference sequence
- 2 : sequence of patient carrying the heterozygous at c.413C>A variant (reverse sens)
- 3 : sequence of patient carrying the heterozygous at c.413C>A variant (forward sens)
- 4 : sequence of patient carrying the homozygous wild type at c.413C>A variant (reverse sens)
- 5 : sequence of patient carrying the homozygous wild type at c.413C>A variant (forward sens)

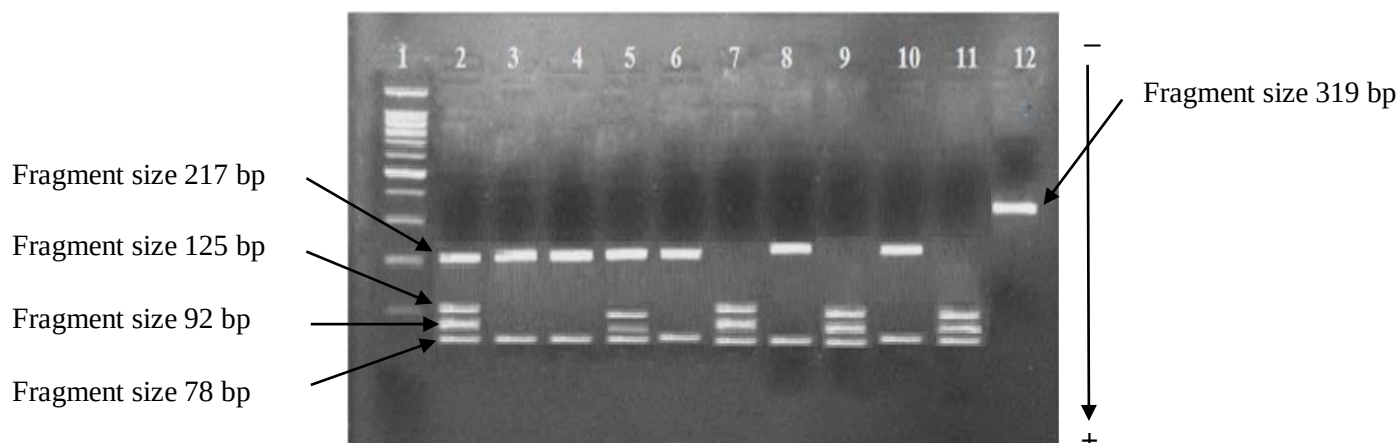


Figure S2: Electrophoretic profile of PCR-RFLP products of identification of c.413C>A variant (T138N)

- 1 – DNA ladder (100 bp DNA ladder Invitrogen)
- 2 – Heterozygous control
- 5 – Heterozygous profile C/A
- 3, 4, 6, 8, 10 – Mutated homouzygous profiles A/A
- 7, 9, 11 – Homouzygous wild-type profiles C/C
- 12– Undigested product