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Common Mutations in the Surfactant Protein-C Gene in Iranian Patients with Diffuse Parenchymal Lung Disease

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Background: Recently, genetic mutations in surfactant protein C (SFTPC) have been linked to diffuse parenchymal lung diseases (DPLD). The present study investigated *SFTPC* mutations among Iranian patients with DPLD for the first time.

Materials and Methods: In this study, we examined 28 patients diagnosed with DPLD. Patients were divided into two groups: 23 cases (82.1%) had interstitial lung disease (ILD), 7 (30.4%) of which were categorized as familial ILD, and 5 cases (17.9%) had pulmonary alveolar proteinosis (PAP). Genetic variations in the *SFTPC* gene were detected by direct DNA sequencing.

Results: The mean (±SD) age of patients was 21.8 (± 17.1) years and 60.7% of the patients were male. Overall, 11 different mutations were detected in the *SFTPC* gene. Two novel mutations, c.202-43 G>A and c.416 G>C, were detected among patients. The c.201+49 C>T mutation showed a significant difference with the minor allele frequency (MAF) data. There was no significant difference between the most frequent mutations in Iranian patients and those of the general population in the world. The proximity analysis showed similarity between Iranian patients and patients of the African race. We did not find any correlation between *SFTPC* mutations and DPLD in the patients.

Conclusion: It seems that the rs2070684 (c.201+49 C>T) mutation could be used as a specific genetic marker for distinguishing the Iranian population from other human races in the world. There was a correlation between some intronic variations and the development of disease. A new missense mutation, c.416 G>C that encodes Arg139Thr, could probably damage the protein structure and/or function and cause the signs and symptoms of DPLD.

Keywords: Surfactant protein C; Interstitial lung disease; Pulmonary alveolar proteinosis; Gene mutation

INTRODUCTION

The surfactant protein C (*SFTPC*) gene encodes a 197-amino acid apoprotein that is secreted by type 2 alveolar epithelial cells. This protein plays a critical role in the appropriate function of alveoli by reducing surface tension and preventing alveolar collapse during exhalation (1). In preterm babies, the collapse of alveoli and respiratory distress syndrome can occur due to insufficient function of the surfactant protein, whereas term infants, older infants and adults suffering from respiratory distress syndrome and interstitial lung disease have an inherited surfactant deficiency (2).

SFTPC mutations can be either inherited as an autosomal dominant trait with variable penetrance and cause familial lung disease or as a *de novo* single mutation resulting in sporadic lung disease. The association of

SFTPC mutations with familial lung disease was first described in 2001 (3). Probably, *SFTPC* genetic mutations contribute to the pathogenesis of a broad spectrum of lung diseases with different clinical manifestations. Mutations in this gene can cause lethal neonatal respiratory distress syndrome or adult chronic interstitial lung disease (ILD) (4). Deficiency in surfactant production due to several genetic mutations has been demonstrated in patients with different interstitial lung diseases, such as usual interstitial pneumonia (UIP), desquamative interstitial pneumonia (DIP), nonspecific interstitial pneumonia (NSIP), lymphocytic interstitial pneumonia (LIP), and giant cell interstitial pneumonia (GIP) (5). On the other hand, abnormal accumulation of lipoprotein compounds within the pulmonary alveoli as a result of surfactant deficiency causes a rare lung disease named pulmonary alveolar proteinosis (PAP) (6). Although most cases of PAP have an autoimmune origin, the congenital form is associated with mutations in surfactant proteins (7). Therefore, the question arises whether surfactant protein deficiencies have a causative role in diffuse parenchymal lung disease (DPLD) pathogenesis. There is a hypothesis that pulmonary fibrosis occurs in genetically susceptible individuals who harbor specific mutations in some genes such as the *SFTPC* gene. To date, no study has evaluated the frequency of probable *SFTPC* mutations in Iranian DPLD patients. In the study, we investigated SFTPC genetic mutations in Iranian ILD and PAP patients for the first time. In addition, the frequency of *SFTPC* mutations in Iranian patients was compared with the reported global minor allele frequency (MAF) data.

MATERIALS AND METHODS

Patients

In this study, we included 28 patients with DPLD who were diagnosed by lung biopsy or based on the American Thoracic Society/European Respiratory Society 2011 criteria (8). Patients were divided into the following groups: 23 cases (82.1%) had ILD, and five cases (17.9%) had PAP. In the ILD group, 16 cases (69.6 %) had sporadic ILD, while seven cases (30.4%) had familial ILD. Demographic characteristics and clinical data, including sexual and family history, radiological reports, and lung biopsy results were obtained. All of the patients were evaluated for known and unknown mutations of the surfactant protein C gene by direct DNA sequencing.

DNA extraction

DNA was extracted from 2 ml of EDTA-treated whole blood using a silica-based extraction kit following the manufacturer's protocol (Yekta Tajhiz/IRAN). Briefly, 200 ul of whole blood was digested with proteinase K. After adding the binding solution and alcohol, the digested cells were transferred to silica-based columns and were washed twice with a washing buffer. DNA was then eluted by an elution buffer and stored at -20°C.

Polymerase chain reaction (PCR)

Five pairs of primers were designed to specifically amplify the five exons and related flanking intron of the *SFTPC* gene, using the sequence J03890 (NCBI nucleotide database) as a reference sequence based on recommendation of Hamvas and his colleagues (9) (Table 1).

Table 1. The sequence of primers used for amplification of five exons of the *SFTPC* gene

Primer name	Sequence of primers	Size of amplicon
SFTPC Exon 1 F	TTATCTGGGCTTCGGTTCTG	302 bp
SFTPC Exon 1 R	AAACAGGCCAGGGAGGATAG	
SFTPC Exon 2 F	CTGATCTCCTCAGCCCTTCC	398 bp
SFTPC Exon 2 R	CACTCCCCTTGGACAGTTTC	
SFTPC Exon 3 F	CACTCAGCCTCCCTGAACTC	346 bp
SFTPC Exon 3 R	GAGGGAGAGATGGATGTGGA	
SFTPC Exon 4 F	CCCTCTCCTCCAGACCTTTT	363 bp
SFTPC Exon 4 R	AGCCAATGAGGAACAGTGCT	
SFTPC Exon 5 F	GCACAGCCCCTCTTTACTGA	444 bp
SFTPC Exon 5 R	GTACCGGTCTGTGAGCTTCC	

For this purpose, 50 µg of extracted DNA was added to a 25 µL PCR master mix containing 10 mM Tris-HCl, 1.5 mM MgCl2, 0.2 mM of each dNTP, 20 pmol of specific primers for each exon and its related intron, 2.5% DMSO

plus 1 unit of Hot starTaq DNA polymerase (Pars gen, I.R.Iran). The thermal condition was provided by a thermocycler machine (Techne, England) as follows: initial denaturation at 95˚C for five minutes followed by 35 cycles composed of 95˚C and 60˚C each for 30 seconds, 72˚C for 60 seconds, and a final extension at 72˚C for 5 minutes.

Electrophoresis

To detect the desired bands, PCR products were run on 1.5% agarose gel containing Safe stain and were visualized under ultraviolet light (Figure 1). To prevent cross-contamination, all steps of the procedure were performed in separate fully equipped rooms with barrier tips. Distilled water was used instead of the target DNA as a negative control in each step of the procedure.

Figure 1. PCR products for exons 1 to 5 of the SFTPC gene

Sequencing

To detect any possible mutation in the exons and flanking intronic regions of the *SFTPC* gene, all of the products were sent to a private laboratory (Pouya Gostar Gene, I.R.Iran) for sequencing. The sequences were submitted to the National Center for Biotechnology Information (NCBI) and will release with the accession numbers MZ826289 to MZ826316. They were aligned with the BioEdit software (version 7.0.5) and compared with the reference sequences for Homo sapiens in GenBank.

Statistical analysis

The Chi-squared test, Pearson correlation coefficient, and linear regression test were used to investigate the association between *SFTPC* mutations and the two patient groups at a significance level of 0.05. Statistical analysis was performed using the SPSS software (version 22.0.1).

Ethical Approval

This work has been approved by the ethical committee of the National Research Institute of Tuberculosis and Lung Disease. Written informed consent was obtained from all participants prior to entering the study.

RESULTS

In this study, 60.7% of the patients were male and the mean (± SD) age of patients was 21.8 (± 17.1) years. Patients were classified into two main groups: 23 (82.1%) and 5 (17.9%) patients were diagnosed with ILD and PAP, respectively. In the PAP group, 40% were male and the mean (± SD) age was 5.8 (± 7.1) years. In the ILD group, the mean (± SD) age of patients was 28.3 (± 16.2) years and 66.7% were male. Among ILD patients, 7 (30.4 %) had a familial form of pulmonary fibrosis, while the remaining 16 patients (69.6%) had sporadic ILD. In brief, the relationships of familial ILD patients were as follows: three patients (father and his two sons), two patients (brother and sister), and two other unrelated patients with a family history of pulmonary fibrosis.

In general, 11 different mutations in five exons and flanking intronic regions of the *SFTPC* gene were detected in the patients, including two novel mutations c.202-43 G>A and c.416 G>C (Table 1). The latter mutation was identified as a missense mutation in exon 4 with 100% heterozygosity, and the former mutation was identified in intron 2 with 100% homozygosity.

Most of the detected mutations occurred in introns (72.7%). None, however, were reported as a pathogenic mutation in ClinVar. Three mutations occurred in the protein-coding region, leading to either amino acid substitution or truncation of the *SFTPC* transcript with potential functional relevance (c.413 C>A, c.416 G>C, c.557 G>A). Four commonly detected variants, c.201+14 G>A, c.413 C>A, c.436-8 C>G, and c.557 G>A, were reported as benign or benign/likely benign mutations in ClinVar.

The most common intronic mutation was c.201+49 C>A with a frequency of 92.9%, which showed homozygosity in 92.3% of the cases. The second most frequent intronic mutation was c.43-21 T>C with a heterozygosity rate of 56.6%. Both of these mutations have not been reported in ClinVar so far, and thus suggest no probable clinical relevance. The third most frequent mutation was c.557 G>A; a missense mutation in exon 5 that was reported as a benign mutation with a 63.2% rate of heterozygosity. The frequency of another mutation, c.413 C>A, was 39.3%. This mutation occurred in exon 4, and had a heterozygosity rate of 81.8%. Several other mutations including c.42+35 G>A, c.42+37 G>A, c.201+14 G>A, c.202-29 G>A, and c.436-8 C>G, which have been previously described as rare single nucleotide polymorphisms (SNPs) in the world, were also detected in this study. All of the detected mutations are summarized in table 2 along with their frequencies.

Another finding of this study was that the c.201+49 C>T mutation, with a transition of T to C (instead of A to C substitution), showed a significant difference with the MAF data.

Table 3 shows the frequency of allelic mutations in Iranian patients compared with the global MAF data.

Statistical analysis revealed that the frequency of rs8192340, rs78177348, rs13248346, rs4715, and rs1124 mutations in Iranian patients did not show a significant difference with the normal population of the world; however, a linear correlation was not observed between different populations (R^2=0.121, 0.087, 0.003, 0.112, and 0.181, respectively). Regarding the rs8192327 mutation, there was a strong linear correlation between Iranian patients and the normal population of the world (R^2=0.906). This polymorphic variation was more reliable for proximity analysis, revealing similarity between Iranian patients and the African race.

Table 2. The mutations detected in Iranian ILD and PAP patients and their frequencies

Mutation	Frequency n/N (%)	Mutation status in database
NM_003018.4: c.42+35 G>A	2/28 (7.2)	Not reported in ClinVar
(dbSNP:rs8192340)	Heterozygote: 100%	Intron 1 variant
NM_003018.4: c.42+37 G>A	1/28 (3.6)	Not reported in ClinVar
(dbSNP:rs78177348)	Heterozygote: 100%	Intron 1 variant
NM_003018.4: c.43-21 T>C	23/28 (81.5)	Not reported in ClinVar
(dbSNP: rs13248346)	Heterozygote: 13/23 (56.5)	Intron 1 variant
NM_003018.4(SFTPC): c.201+14 G>A)	1/28 (3.6)	Benign/Likely benign
(dbSNP: rs8192327)	Heterozygote: 100%	Intron 2 Variant
NM_003018.4: c.201+49 C>A or C>T	26/28 (92.9)	Not reported in ClinVar
(dbSNP: rs2070684)	Heterozygote: 2/26 (7.7)	Intron 2 variant
(NM_003018.4: c.202-43 G>A)	1/28 (3.6)	New variation
	Heterozygote: 0%	Intron 2 variant
NM_003018.4: c.202-29 G>A (dbSNP:rs1431474660)	1/28 (3.6)	Not reported in ClinVar
	Heterozygote: 100%	Intron 2 variant
NM_003018.4(SFTPC): c.413 C>A (p.Thr138Asn) (dbSNP: rs4715)	11/27 (39.3)	Benign/Likely benign
	Heterozygote 9/11 (81.8)	A missense mutation
NM_003018.4(SFTPC): c.416 G>C	1/28 (3.6)	New variation
(p.Thr139Arg)	Heterozygote: 100%	A missense mutation
NM_003018.4(SFTPC): c.436-8 C>G	2/28 (7.2)	Benign
(dbSNP: rs2070687)	Heterozygote: 1/2 (50)	436-8 C>G in intron 4 of SFTPC: This variant is not expected to have clinical significance
NM_003018.4(SFTPC): c.557 G>A (p.Ser186Asn)	19/28 (67.9)	Benign
(dbSNP: rs1124)	Heterozygote: 12/19 (63.2)	A missense mutation

Table 3. Frequency of allelic mutations in the *SFTPC* gene among Iranian patients compared with general population of the world

Mutation	Allelic frequency in Iranian patients	1000G MAF	ExAC MAF	Fisher test
NM_003018.4: c.42+35 G>A (dbSNP: rs8192340)	A= 0.03571428	A= 0.0347444	A= 0.0585685	0.295
NM_003018.4: c.42+37 G>A (dbSNP: rs78177348)	A= 0.01785714	A= 0.0107827	A= 0.0046196	0.328
NM_003018.4: c.43-21 T>C (dbSNP: rs13248346)	C= 0.5892857 T= 0.4107143	T= 0.405152	C= 0.493374	0.869
NM_003018.4 (SFTPC): c.201+14 G>A (dbSNP: rs8192327)	A= 0.01785714	A= 0.0239617	A= 0.0527658	0.378
NM_003018.4: c.201+49 C>A or C>T (dbSNP: rs2070684)	C= 0.1071429 A= 0.0000000 T= 0.8928571	C= 0.323682 A= 0.676318 T= 0.000000	C= 0.125800 A= 0.874200 T= 0.000000	0.287 0.000
NM_003018.4:c.202-43 G>A	A= 0.01785714	A new variation An intron 2 variant		0.000
NM_003018.4: c.202-29 G>A (dbSNP: rs1431474660)	A= 0.01785714	Not reported in ClinVar An intron 2 variant		0.098
NM_003018.4 (SFTPC): c.413 C>A (p.Thr138Asn) (dbSNP: rs4715)	C= 0.767857 A= 0.232143	A= 0.197484	A= 0.25228	0.264
NM_003018.4 (SFTPC): c.416 G>C (p.Thr139Arg)	C=0.01785714	New variation A missense mutation		0.000
NM_003018.4 (SFTPC):c.436-8 C>G (dbSNP: rs2070687)	C=0.92857	C= 0.74361	C=0.825843	0.078
NM_003018.4 (SFTPC):c.557 G>A (p.Ser186Asn) (dbSNP: rs1124)	G=0.535714 A=0.464286	A= 0.236022	A = 0.300094	0.148

On the other hand, there was similarity between Iranian patients and the Asian race considering the proximity analysis performed using the rs4715 and rs2070687 mutations. The frequency of two SNPs, rs1431474660 and rs2070687, showed borderline difference with the normal population of the world (p= 0.098 and 0.078, respectively).

Another intronic variation that was reported as a benign variation in ClinVar was c.436-8 C>G; this mutation had a frequency rate of 7.2% and a heterozygosity rate of 50%. The Fisher test showed a significant difference between the Iranian population and the normal population of the world (F=3.943, p=0.078; CI: 90%).

Notably, there was a significant difference between Iranian patients and the global variation in rs2070684. In the Iranian population, substitution of T to C was observed, whereas adenine to cytosine substitution is the dominant mutation in the world. The frequency of c.201+49 C>T variation was 0.8928571 in Iranian patients as opposed to a global frequency of 0.0000. There was no report of C>T transition in the world except for the Korean population (T=0.0003). Furthermore, the proximity analysis did not show any similarity between Iranian patients and the normal population of the world, even with the African and Asian races. Thus, it seems that this variation could be used as a specific genetic marker of the Iranian population.

There was a complete correlation between the rs8192327 and rs1431474660 mutations (r=1.000, p<0.001). In addition, rs8192327 had relative correlation with rs8192340 (r=0.694, p<0.001) and rs2070687 (r=0.420, p=0.026). On the other hand, there was a negative correlation between rs13248346 and the following rs2070687 (r=-0.450, p=0.016) and rs8192340 (r=-0.476, p=0.010). Also, there was a relative positive correlation between rs4715 and rs1124 (r=0.694, p<0.001). This study showed a strong positive correlation between rs8192340 and rs2070687 (r = 0.945, p < 0.001). Additionally, the study showed that there was a significant correlation between age and type of lung disease (PAP or ILD) (p=0.02).

DISCUSSION

In this study, we screened Iranian individuals with severe pulmonary disorders to identify genetic variations in the promoter and protein-coding regions of the *SFTPC* gene that have a possible effect on lung function. We identified two novel mutations; c.202-43 G>A, located in an intronic region, and c.416 G>C, which is located in a region encoding the mature protein. We also performed a large population-based cohort study to compare the frequency of the detected variations with the global MAF.

Previous studies have shown over 35 dominantly expressed genetic mutations in patients of all age groups, ranging from infancy to adulthood, suffering from acute and chronic lung diseases (3, 9-14). Our study included Iranian patients with a broad age range, with a newborn among the PAP patients and a 58-year-old patient in the ILD group. We observed a significant correlation between age and type of lung disease (PAP or ILD).

One study showed that about 55% of mutations in the *SFTPC* gene arise spontaneously and result in sporadic disease and the remainder is inherited (15). Nevertheless, we did not find any association between familial ILD and the detected mutations in our study.

Previous studies have reported that the most common mutation is the substitution of threonine for isoleucine in codon 73 (I73T); a non-BRICHOS mutation located in coding exon 3 of the *SFTPC* gene, which is associated with 25% of pulmonary diseases (1,9). Interestingly, this mutation was not observed in Iranian patients. Instead, the most frequent variation in our study was an intronic mutation, rs2070684 (c.201+49 C>T). In the global population, this mutation is reported as the transversion of C to A, but 92.3% of Iranian patients demonstrated a substitution of C to T. A similar variation was reported in Korean patients with a frequency of 0.0003, which was significantly different from our study. It seems that the rs2070684 SNP in the intronic region does not probably affect splicing because of its distance from the exon-intron junction. Thus, this mutation is unlikely to contribute to the pathogenesis of DPLD.

The results of the present study showed that there was no significant difference in the frequency of detected variations between Iranian patients and the normal population of the world. However, regression analysis did not show a linear correlation between Iranian patients and the normal population of the world. Detection of a common mutation could reflect either a shared ancestral origin or recurrent *de novo* events (16,17). A benign/likely benign variation in intron 2 of the *SFTPC* gene, rs8192327, was detected in our study as a heterozygote variation with a frequency of 3.6%. Except for rs8192327, none of the common variations showed a linear correlation with the MAF database. Regression analysis showed a linear correlation between rs8192327 variation in Iranian patients and the normal population of the world, making this variation a useful marker to explore the ancestry of the Iranian population. Proximity analysis showed that Iranian patients were similar to the African race, but distinct from European and Asian individuals. Therefore, the geographic distribution of the rs8192327 mutation suggested that this was an ancestral mutation that had spread throughout Africa and entered the Middle East as a result of migration. On the other hand, by using the rs2070684 (c.201+49 C>T) variation, the Iranian population could be clearly distinguished from the African race.

The second common variation with 81.5% frequency was a polymorphic variation, c.43-21 T>C (rs13248346), that had no significant difference with the normal population. Proximity analysis of this mutation showed similarity of the Iranian patients with the African population and their close ancestral origin.

The third frequent variant was a missense mutation (c.557 G>A, p.Ser186Asn) with a frequency of 67.9% and 63.2% heterozygosity, which was reported as a benign mutation in ClinVar. Statistical analysis showed no significant difference between Iranian patients and the normal population of the world regarding this variation, thereby excluding its significant effect on the pathogenesis of DPLD.

The fourth most commonly detected variation in Iranian patients was a benign/likely benign mutation (c.413 C>A) with 39.3% frequency and 81.8% heterozygosity. This mutation did not show a significant difference with the normal population of the world. In addition, there was no apparent correlation between this variation and developing DPLD, confirming its clinical insignificancy.

Another intronic variation that was reported as a benign variation in ClinVar was c.436-8 C>G; this mutation had a frequency rate of 7.2% and a heterozygosity rate of 50%. We suggest that this variation could have a possible role in the pathogenesis of DPLD, although the results of this study need to be confirmed by further investigations.

Another mutation detected in this study was c.202-29 G>A (dbSNP: rs1431474660) located in intron 2, which has not been reported in ClinVar to date. There was a borderline significant difference between Iranian patients and the normal population of the world. This mutation could have a causative role in DPLD by affecting splicing and subsequently, modifying the protein structure, due to its proximity to the exon. However, further molecular and cellular research is required to confirm this finding.

In one patient, a novel homozygote mutation in intron 2 of the *SFTPC* gene was detected; c.202-43 G>A. There is no data on the frequency of this mutation in the normal population, emphasizing further investigation to explain its significance in DPLD development.

Another novel mutation was a heterozygote missense mutation, c.416 G>C leading to Arg139Thr, detected in three of our patients with DPLD. This mutation was found in an adult man and two of his children who had also inherited this variant. To the best of our knowledge, the c.416 G>C (R139S) mutation and its resultant serine to arginine substitution has not been previously reported. The R139S variant is a semi-conservative amino acid substitution, which may affect the secondary protein structure since these residues differ in some properties (18). This substitution occurs at a position that is conserved across species; so, we believe this variant could probably damage the protein structure and/or function. Detection of this mutation in unrelated individuals with lung disease could reveal its role as a disease-causing mutation or a rare benign polymorphism.

The results of our study did not show any correlation between the detected mutations and DPLD, possibly because of a limited sample size. Therefore, we recommend a larger case-control, molecular and cellular research study to determine the possible association of the detected mutations with DPLD pathogenesis.

CONCLUSION

The results of our study suggest that the ancestral origin of the Iranian population and the African race might be the same. The rs2070684 (c.201+49 C>T) mutation is a specific genetic marker of Iranian patients and can be used to distinguish the Iranian population from other races in the world. We found that some intronic variations, c.202-43 G>A and c.202-29 G>A, might be correlated with the pathogenesis of DPLD. Also, we detected a novel missense mutation, c.416 G>C, that encodes Arg139Thr and could have probable harmful effects on the protein structure and/or function. We recommend larger molecular and cellular research studies to confirm our results.

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