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**Hypomorphic pathogenic variant in *SFTPB* leads to adult pulmonary fibrosis**

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Philippe Duquesnoy<sup>1</sup>, Mélanie Héry<sup>1</sup>, Aurélie Le Borgne<sup>2</sup>, Serge Amselem<sup>1,4</sup>, Marie Legendre<sup>1,4,\*</sup>,  
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### 33 **Running title**

34 Surfactant protein B pathogenic variant in adulthood

35

36 **Key words:** Surfactant protein B, familial lung fibrosis, interstitial lung disease, splice, mutation,  
37 pathogenic variant, hypomorphic variant

38

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42

### 43 **Conflicts of interest**

44 None to be declared

45

### 46 **Author contributions**

47 TD and NN performed the functional studies and wrote the manuscript that was reviewed by all the  
48 authors. ML contributed to the data analysis. PD provided his expertise in functional studies. GP and  
49 ALB were in charge of the patients. VN and FDL performed the molecular diagnosis under the  
50 supervision of ML and SA. AC performed the histological analyses.

51

## Abbreviations list

AEC2, alveolar epithelial cell (type 2); GGO, ground glass opacities; ILD, interstitial lung disease; PF, pulmonary fibrosis; SP-B, surfactant protein B; UIP, usual interstitial pneumonia; WT, wild-type.

## ABSTRACT (150/150 words)

Bi-allelic pathogenic variants in the surfactant protein (SP)-B gene (*SFTPB*) have been associated with fatal forms of interstitial lung diseases (ILD) in newborns and exceptional survival in young children. We herein report the cases of two related adults with pulmonary fibrosis due to a new homozygous *SFTPB* pathogenic variant, c.582G>A p.(Gln194=). *In vitro* transcript studies showed that this *SFTPB* synonymous pathogenic variant induces an aberrant splicing leading to three abnormal transcripts with the preservation of the expression of a small proportion of normal *SFTPB* transcripts. Immunostainings on lung biopsies of the proband showed an almost complete loss of SP-B expression. This hypomorphic splice variant has thus probably allowed the patients' survival to adulthood while inducing an epithelial cell dysfunction leading to ILD. Altogether, this report shows that *SFTPB* pathogenic variants have to be searched for in unexplained forms of ILD and pulmonary fibrosis in adults, especially in familial and/or early-onset forms of these diseases.

## INTRODUCTION

Surfactant protein (SP)-B deficiency has been associated with fatal forms of respiratory distress in newborns [1–3]. Patients usually present at term, after an unremarkable pregnancy, with immediate respiratory failure, diffuse alveolar or interstitial opacities and a fatal evolution towards refractory hypoxemia in a few days or weeks despite maximal levels of ventilation. In these newborns, most of the reported pathogenic variants are loss-of-function variations leading to premature stop codons, the most frequently reported one being the c.361delinsGAA, p.(Pro121Glnfs\*95) [4–7]. We herein report a homozygous *SFTPB* hypomorphic splice pathogenic variant in two related adults with pulmonary fibrosis (PF).

## CASES PRESENTATION

The proband, a non-smoker man born to a consanguineous union, presented with a fibrosing interstitial lung disease (ILD) with non-predominant features of alveolar hemorrhage evolving since the age of 34 years. He had no history of neonatal respiratory failure nor childhood respiratory symptoms. He first presented with dyspnea and cough, leading to a thoracic CT-scan showing mosaicism with ground glass opacities and emphysema (**Figure 1-A**). The lung biopsy (unavailable sample) reported lung fibrosis. His respiratory status deteriorated with, at the age of 42 years, a FEV1 of 2.21L (64%); FVC 3.13L (74%), TLCO 45% and an impaired 6-min walking test with a nadir of SaO<sub>2</sub> at 72% for 304m (50%). Despite treatments with oral corticosteroids, hydroxychloroquine, azathioprine (quickly stopped because of a lack of efficacy) and azithromycin, he required continuous oxygen therapy since the age of 44 years and benefited from a bi-pulmonary lung transplantation at the age of 51 years. The histopathology of the explanted lungs was consistent with a usual interstitial pneumonia (UIP) pattern (**Figure 1-B**). The genetic analysis by targeted-capture NGS sequencing of telomerase-related and surfactant-related genes identified no pathogenic variant in telomerase genes but a synonymous homozygous *SFTPB* variation: c.582G>A, p.(Gln194=). The patient had 7 children, also from a consanguineous union (**Figure 1A**, III.1, heterozygous for the reported variant), including 4 who died before one year of age from unknown cause and a boy who presented a neonatal respiratory distress at term with a pneumothorax. At 4 months, this boy was hospitalized for a *Respiratory Syncytial Virus* (RSV) bronchiolitis. At 10 months, alveolo-interstitial opacities have persisted and a working diagnosis of mild alveolar hemorrhage was first proposed based on a moderated anemia (Hb 11g/dl) and 30% of hemosiderin-laden macrophages at bronchoalveolar lavage cytology analysis. No CT-scan was available and he had no lung biopsy to confirm the diagnosis. After 2 years of corticosteroid treatment, he was stable. At 16 year-old, the CT-scan showed mild ground glass opacities, large areas of attenuations without emphysema or cysts. At last evaluation (aged 22), the patient presented a dyspnea on exertion with a distension (forced vital capacity 5.2L, 120% of expected values, FEV1 3.85L, 101%) and his CT-scan showed mild ILD with ground glass opacifications, mosaic attenuation with

area of decreased attenuation and subtle distortion (**Figure 1-A**). He was also found to be homozygous for the *SFTPB* c.582G>A variation.

## MATERIAL AND METHODS

### Transcript study

To assess the pathogenicity of the identified c.582G>A variation involving the last nucleotide of exon 5 of *SFTPB*, we generated *SFTPB* mini-gene constructs. As the studied variant is synonymous, a cDNA expression vector could not be used for protein functional studies. Moreover, the genomic DNA of *SFTPB* is long (NM\_000542.5, 13,436bp) and could hardly be used for the construction of a genomic expression vector. Thus, the genomic region spanning exons 4 to 6 of human *SFTPB* (NM\_000542.5) was cloned into the pcDNA3.1\_V5\_His\_TOPO vector resulting in plasmid pSFTPB\_WT. The *SFTPB* c.582G>A variation and the c.582+1G>T pathogenic variant (used as a positive control fully disrupting the donor splice site of intron 5, MaxEntScan score: 1.56) were introduced into pSFTPB\_WT by site-directed mutagenesis and were respectively designated pSFTPB\_mut and pSFTPB\_ctr.

After FuGene transfection of the plasmids in A549 cells, total RNA was isolated at day 2 (RNeasy mini kit, Qiagen). cDNAs were generated from mRNA using oligodT with the Transcriptor High Fidelity cDNA Synthesis Kit and PCR-amplified before agarose gel electrophoresis and Sanger sequencing.

### Immunohistochemistry assay

The lung biopsy from the proband was compared to three controls: a healthy child and adult as well as a SP-B-deficient neonate carrying a biallelic loss-of-function pathogenic variants in *SFTPB* (c.[75G>A];[361delinsGAA], p.[(Trp25\*)];[(Pro121Glufs\*95)]). Haematoxylin & eosin stainings were performed. Immunostaining assays were performed on a Leica Bond Platform using an anti-SP-B polyclonal antibody (HPA062148, Sigma, 1/20 targeting the 121-197 amino-acids) and an anti-SP-C



129 antibody (SC-13979, 1/100 targeting both SP-C and pro-SP-C). No frozen tissue was available to  
130 assess *ex vivo* RNA expression.

131 Submission of data to public databases

132 Variant and phenotypes of the proband and his son have been described in LOVD database under  
133 respective URL and <https://databases.lovd.nl/shared/individuals/00435123> and  
134 <https://databases.lovd.nl/shared/individuals/00435124>, and accession numbers 00435123 and  
135 00435124.

136 RESULTS

### 137 **Functional consequences of the *SFTPB* c.582G>A p.(Gln194=) variation**

138 The identified *SFTPB* c.582G>A variation has never been described in patients nor in the gnomAD  
139 variation database. *In silico* analysis predicts it to weaken the splice-donor site of intron 5  
140 (MaxEntScan score: 5.85 vs. 10.07).

141 Transcripts isolated from cells transfected with pSFTPB\_WT yielded a 363-bp amplicon  
142 corresponding to normal splicing and a 310-bp one resulting from the use of a cryptic splice-acceptor  
143 site in exon 6 (**Figure 2**). A similar assay performed with pSFTPB\_mut generated three molecular  
144 species in addition to the 363-bp expected *SFTPB* amplicon. The first two amplicons lead to premature  
145 Stop codons: a 464-bp one [p.(Asp195Valfs\*54)] resulting from the use of a cryptic splice-donor site  
146 located within intron 5 leading to the retention of the first 101-bp of this intron and a 121-bp one  
147 [p.(Asp132Glyfs\*39)] resulting from the use of the cryptic splice-acceptor site in exon 6. As *SFTPB*  
148 encompasses 11 exons, it is highly likely that, *in vivo*, these transcripts leading to premature Stop  
149 codons trigger the nonsense-mediated mRNA decay (NMD) pathway and lead to the absence of  
150 protein production. The third amplicon, a 174-bp one [p.(Asp132\_Gln194del)] lacking exon 5  
151 corresponds to an in-phase deletion located in the pro-protein (SP-B mature protein: amino acids 201-  
152 279). This deletion includes two major protein cleavage sites, certainly preventing the production of a  
153 mature SP-B protein (**Supplementary Figure 1**) [8, 9]. Whether or not this shorter peptide, if not

degraded, could trigger unfolded protein response and induce an epithelial cell dysfunction remains to be ascertained.

With a positive control of abnormal splicing – the c.582+1G>T variant predicted to fully disrupt the splice-donor site of intron 5 – we did not detect any normal transcript, but observed the same three aberrant transcripts.

Altogether, the c.582G>A pathogenic variant is therefore consistent with a drastic alteration of the expression of SP-B with, however, a residual expression of normal SP-B.

## **Lung tissue expression of SP-B and SP-C**

SP-B immunostaining revealed an almost complete loss of SP-B expression in the lung from the proband. A control experiment performed on a lung biopsy sample from a neonate with a SP-B deficiency due to a biallelic loss-of-function pathogenic variants in *SFTPB* showed a complete loss of SP-B expression (**Figure 1-B**). The *SFTPB* c.582G>A pathogenic variant also had an impact on SP-C metabolism, as observed by the higher expression of SP-C & pro-SP-C in both the proband and the SP-B deficient neonate compared to both controls.

## **DISCUSSION**

We herein report for the first time two adult patients from a single family with bi-allelic *SFTPB* pathogenic variants. Very rare cases of prolonged survivals have been described in SP-B deficiency after infant lung transplantation [10–12]. Only one case has been reported with a delayed presentation in a 4-month infant carrying the homozygous c.706C>T p.(Arg236Cys) missense pathogenic variant [13]. The patient was alive at the age of 8 years with oxygen supplementation. Three other patients with a prolonged survival also presented at birth with a severe respiratory distress. Two of them were homozygous for the c.465G>T p.(Gly155=) splice variant which creates a strong donor splice site within exon 5 (MaxEntScan score 9.80) and one of them was still alive at school age [14]. The third

one was homozygous for the missense c.770T>C, p.Leu257Pro pathogenic variant and died at 13 months from respiratory failure [15]. A mouse model of transgenic mice displaying a reduced expression of SP-B showed a different parenchymal aspect than in the described patient with an enlargement of the tissue airspaces at 6 weeks (corresponding to a young adult in human) [16]. Like in the family we are currently reporting, hypomorphic splice pathogenic variants may allow the persistence of a variable proportion of normal transcripts leading to a residual SP-B function which could explain the various age at onset (adult in the proband and neonate in his son) and the long-term survival of the patients. The possible epithelial cell dysfunction and the partial SP-B deficiency probably explain the fibrosing ILD in both patients [17]. Interestingly, ILD was initially diagnosed as mild alveolar hemorrhage in both patients. It is likely that the alveolar bleeding could be due either to chronic dysfunction of the alveolar epithelium eroding the alveolar capillaries or to pulmonary hypertension [18].

Genetic causes of familial ILD have been better understood over the past decade and are mostly related to telomerase-related genes and surfactant related genes pathogenic variants [19–21]. Pathogenic variants in surfactant genes that were hitherto associated with ILD in childhood are increasingly linked to young-adult onset ILD, and should be considered in atypical presentations of ILD particularly when a family history is identified [20, 21]. In the former patients, whatever the age of the patient is, a genetic analysis of the genes involved in surfactant metabolism could increase the number of described cases and the awareness on adult forms of surfactant genes pathogenic variants.

**Legal and ethical statement:** Written informed consents were obtained from the two patients. Ethical approval was obtained from the local authorities under the number 060916.

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206 The ILD cohort has been developed in collaboration with the Rare Disease Cohort (RaDiCo)-ILD  
207 project (ANR-10-COHO-0003), the Clinical research collaboration for chILD-EU and the COST  
208 Innovative Grant OpenILD CIG16125.

209

## 210 **Links:**

211 MaxEntScan [http://hollywood.mit.edu/burgelab/maxent/Xmaxentscan\\_scoreseq.html](http://hollywood.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html)

212 gnomAD <https://gnomad.broadinstitute.org/>

213 **Data Availability’ statement:** The datasets generated and/or analysed during the current study are  
214 available from the corresponding author on reasonable request.

215

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## Figure legends

### Figure 1. Family tree, CT scan and histological features

A. Genealogical tree of the described family. Consanguineous unions are indicated with double lines. Black symbols show subjects with pulmonary fibrosis. The age at the time of the study is mentioned within symbols. The arrow indicates the proband. The genotypes are provided with the reference nucleotide in green and the pathogenic variant in red.

The CT-scan of the proband (III.2) at 51 year-old (before lung transplantation) shows parenchymal distortion, ground glass opacities, centrilobular nodules and mosaic attenuation with area of decreased attenuation and pulmonary fibrosis. The CT-scan of his son (IV.7) at 16 year-old shows mild ground glass opacities, large areas of attenuation especially in the lingula and subtle distortion.

B. The native lung explant examination of the proband (III.2) using Haematoxylin and eosin (HE) staining shows fibroblastic foci alternating with less affected areas consistent with a usual interstitial pneumonia pattern. Pulmonary hemosiderosis was not observed nor pulmonary alveolar proteinosis. The SP-B-deficient neonate showed thickened septa with moderate pulmonary alveolar proteinosis and the adult and infant controls showed a normal lung parenchyma.

SP-B antibody targets pro-SP-B as it has been developed against amino acids 121 to 197. SP-B immunostaining (magnification x20 and x40) showed an almost complete loss of SP-B expression in the patient's type 2 alveolar epithelial cells (AEC)2, a complete loss of SP-B expression in SP-B-deficient neonate and a normal intracytoplasmic granular AEC2 SP-B expression in both adult and infant controls. Note that the anti-SP-B antibody does not target the peptide corresponding to the in-phase deleted transcript (p.(Asp132\_Gln194del)) which lacks most of the epitope (Figure 2).

SP-C antibody has been developed against full length SP-C (amino acids 1-197) and can thus detect both pro-SP-C and mature SP-C. SP-C immunostaining (magnification x20 and x40) showed a higher

degree of expression in the patient's and the SP-B-deficient neonate's type 2 alveolar epithelial cells than in adult and infant controls.

HE, haematoxylin and eosin; AEC alveolar epithelial cell

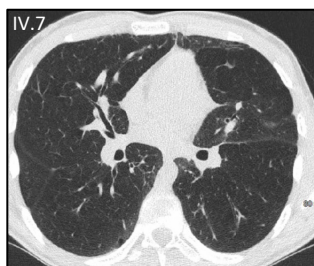
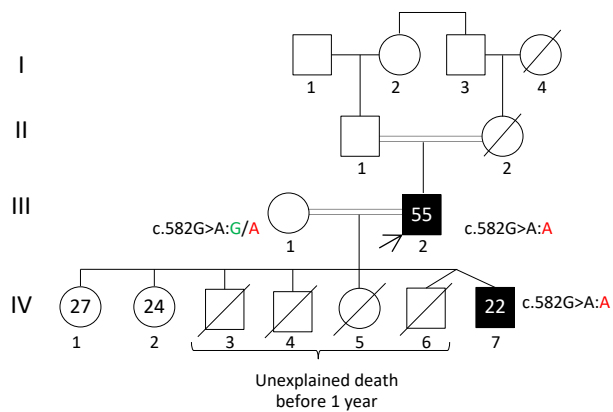
**Figure 2. In vitro assessment of *SFTPB* splicing defects associated with c.582G>A p.(Gln194=)**

*SFTPB* exons 4-6 pre-mRNA corresponding to plasmids pSFTPB-WT, pSFTPB\_mut and pSFTPB\_ctr are represented. The reference nucleotide is in green and the pathogenic variant is in red. The dotted line highlights a cryptic acceptor splice site in exon 6 (nucleotide 636).

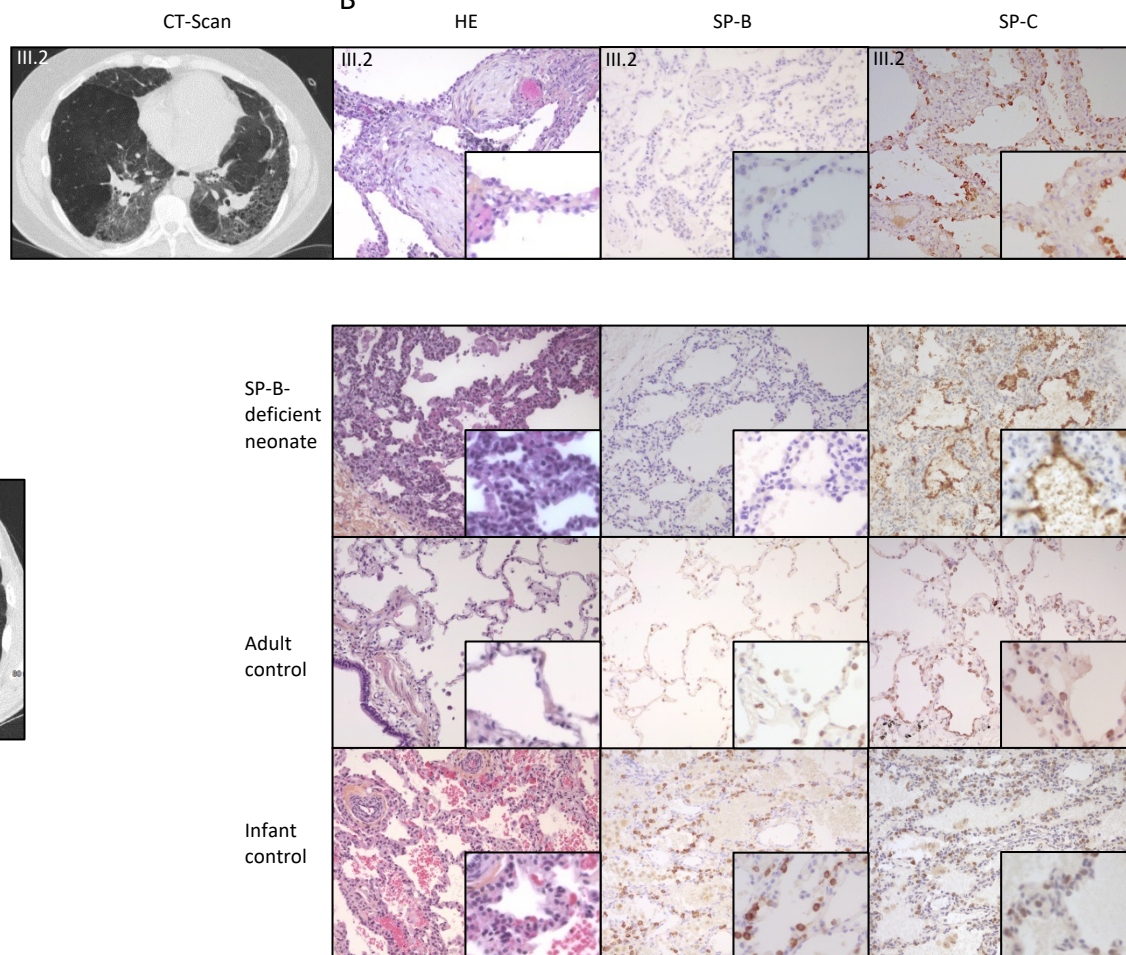
After transfection of A549 cells with the above-mentioned plasmids, the migration on a 1.5% agarose-BET gel of the RT-PCR products obtained from extracted RNAs is shown. The main amplicons characterized by Sanger sequencing and obtained from the normal and mutant constructs are represented on the right of the gel image. Two amplicons were observed for the pSFTPB\_WT: a 363bp (canonical splicing) and a 310bp (using exon 6 cryptic splice-acceptor site corresponding to putative p.(Asp195Glyfs\*39)). Both the c.582G>A (pSFTPB\_mut) and the c.582+1G>T (pSFTPB\_ctr) pathogenic variants resulted in three specific and aberrant amplicons, i.e. from top to bottom: one 464bp transcript related to the retention of the first 101bp of intron 5 and predicting a frameshift with a premature Stop codon in exon 6 (p.(Asp195Valfs\*54)), and two 174bp and 121bp amplicons lacking exon 5 and corresponding to the use of intron 5 canonical splice-acceptor site (p.(Asp132\_Gln194del) in-phase deletion), or exon 6 cryptic splice-acceptor site (p.(Asp132Glyfs\*39)) respectively. The data are representative from 3 independent experiments.

WT, wild type

**A**



**B**





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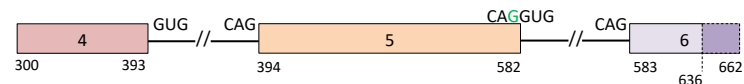
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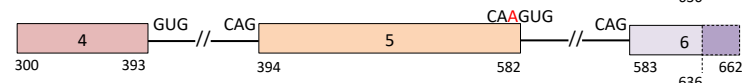
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HE, haematoxylin and eosin; AEC alveolar epithelial cell

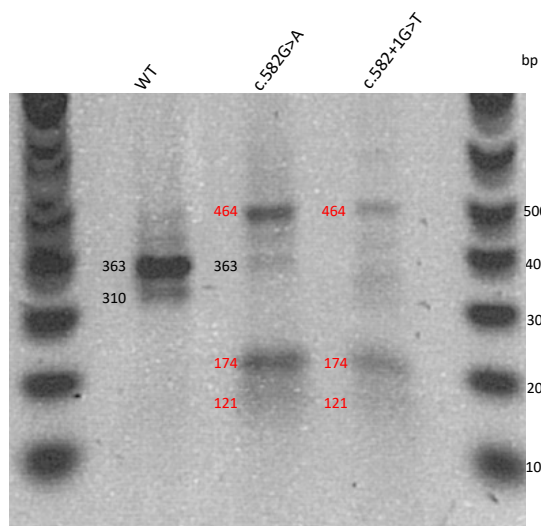
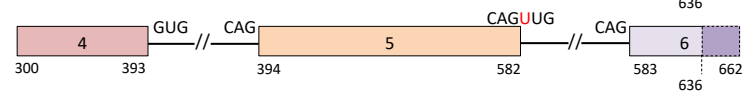
WT pre-mRNA



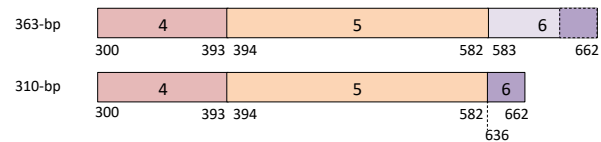
c.582G>A pre-mRNA



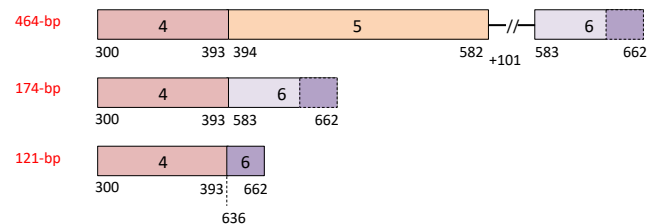
c.582+1G>T pre-mRNA



WT amplicons



Amplicons specific to the isoforms carrying the c.582G>A and c.582+1G>T pathogenic variants



**Figure 2. *In vitro* assessment of *SFTP*B splicing defects associated with c.582G>A p.(Gln194=)**

*SFTP*B exons 4-6 pre-mRNA corresponding to plasmids pSFTP<sub>B</sub>-WT, pSFTP<sub>B</sub>\_mut and pSFTP<sub>B</sub>\_ctr are represented. The reference nucleotide is in green and the pathogenic variant is in red. The dotted line highlights a cryptic acceptor splice site in exon 6 (nucleotide 636).

After transfection of A549 cells with the above-mentioned plasmids, the migration on a 1.5% agarose-BET gel of the RT-PCR products obtained from extracted RNAs is shown. The main amplicons characterized by Sanger sequencing and obtained from the normal and mutant constructs are represented on the right of the gel image. Two amplicons were observed for the pSFTP<sub>B</sub>\_WT: a 363bp (canonical splicing) and a 310bp (using exon 6 cryptic splice-acceptor site corresponding to putative p.(Asp195Glyfs\*39)). Both the c.582G>A (pSFTP<sub>B</sub>\_mut) and the c.582+1G>T (pSFTP<sub>B</sub>\_ctr) pathogenic variants resulted in three specific and aberrant amplicons, i.e. from top to bottom: one 464bp transcript related to the retention of the first 101bp of intron 5 and predicting a frameshift with a premature Stop codon in exon 6 (p.(Asp195Valfs\*54)), and two 174bp and 121bp amplicons lacking exon 5 and corresponding to the use of intron 5 canonical splice-acceptor site (p.(Asp132\_Gln194del) in-phase deletion), or exon 6 cryptic splice-acceptor site (p.(Asp132Glyfs\*39)) respectively. The data are representative from 3 independent experiments.

WT, wild type