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

2

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16

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33 **Running title**

34 Surfactant protein B pathogenic variant in adulthood

35

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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

52 **Abbreviations list**

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

55

56 **ABSTRACT (150/150 words)**

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

68

69 **INTRODUCTION**

70 Surfactant protein (SP)-B deficiency has been associated with fatal forms of respiratory distress in
71 newborns [1–3]. Patients usually present at term, after an unremarkable pregnancy, with immediate
72 respiratory failure, diffuse alveolar or interstitial opacities and a fatal evolution towards refractory
73 hypoxemia in a few days or weeks despite maximal levels of ventilation. In these newborns, most of
74 the reported pathogenic variants are loss-of-function variations leading to premature stop codons, the
75 most frequently reported one being the c.361delinsGAA, p.(Pro121Glufs*95) [4–7]. We herein report
76 a homozygous *SFTPB* hypomorphic splice pathogenic variant in two related adults with pulmonary
77 fibrosis (PF).

78 CASES PRESENTATION

79 The proband, a non-smoker man born to a consanguineous union, presented with a fibrosing interstitial
80 lung disease (ILD) with non-predominant features of alveolar hemorrhage evolving since the age of 34
81 years. He had no history of neonatal respiratory failure nor childhood respiratory symptoms. He first
82 presented with dyspnea and cough, leading to a thoracic CT-scan showing mosaicism with ground
83 glass opacities and emphysema (**Figure 1-A**). The lung biopsy (unavailable sample) reported lung
84 fibrosis. His respiratory status deteriorated with, at the age of 42 years, a FEV1 of 2.21L (64%); FVC
85 3.13L (74%), TLCO 45% and an impaired 6-min walking test with a nadir of SaO₂ at 72% for 304m
86 (50%). Despite treatments with oral corticosteroids, hydroxychloroquine, azathioprine (quickly
87 stopped because of a lack of efficacy) and azithromycin, he required continuous oxygen therapy since
88 the age of 44 years and benefited from a bi-pulmonary lung transplantation at the age of 51 years. The
89 histopathology of the explanted lungs was consistent with a usual interstitial pneumonia (UIP) pattern
90 (**Figure 1-B**). The genetic analysis by targeted-capture NGS sequencing of telomerase-related and
91 surfactant-related genes identified no pathogenic variant in telomerase genes but a synonymous
92 homozygous *SFTPB* variation: c.582G>A, p.(Gln194=). The patient had 7 children, also from a
93 consanguineous union (**Figure 1A**, III.1, heterozygous for the reported variant), including 4 who died
94 before one year of age from unknown cause and a boy who presented a neonatal respiratory distress at
95 term with a pneumothorax. At 4 months, this boy was hospitalized for a *Respiratory Syncytial Virus*
96 (*RSV*) bronchiolitis. At 10 months, alveolo-interstitial opacities have persisted and a working diagnosis
97 of mild alveolar hemorrhage was first proposed based on a moderated anemia (Hb 11g/dl) and 30% of
98 hemosiderin-laden macrophages at bronchoalveolar lavage cytology analysis. No CT-scan was
99 available and he had no lung biopsy to confirm the diagnosis. After 2 years of corticosteroid treatment,
100 he was stable. At 16 year-old, the CT-scan showed mild ground glass opacities, large areas of
101 attenuations without emphysema or cysts. At last evaluation (aged 22), the patient presented a dyspnea
102 on exertion with a distension (forced vital capacity 5.2L, 120% of expected values, FEV1 3.85L,
103 101%) and his CT-scan showed mild ILD with ground glass opacifications, mosaic attenuation with

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

106

107 MATERIAL AND METHODS

108 Transcript study

109 To assess the pathogenicity of the identified c.582G>A variation involving the last nucleotide of exon
110 5 of *SFTPB*, we generated *SFTPB* mini-gene constructs. As the studied variant is synonymous, a
111 cDNA expression vector could not be used for protein functional studies. Moreover, the genomic
112 DNA of *SFTPB* is long (NM_000542.5, 13,436bp) and could hardly be used for the construction of a
113 genomic expression vector. Thus, the genomic region spanning exons 4 to 6 of human *SFTPB*
114 (NM_000542.5) was cloned into the pcDNA3.1_V5_His_TOPO vector resulting in plasmid
115 pSFTPB_WT. The *SFTPB* c.582G>A variation and the c.582+1G>T pathogenic variant (used as a
116 positive control fully disrupting the donor splice site of intron 5, MaxEntScan score: 1.56) were
117 introduced into pSFTPB_WT by site-directed mutagenesis and were respectively designated
118 pSFTPB_mut and pSFTPB_ctr.

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

123 Immunohistochemistry assay

124 The lung biopsy from the proband was compared to three controls: a healthy child and adult as well as
125 a SP-B-deficient neonate carrying a biallelic loss-of-function pathogenic variants in *SFTPB*
126 (c.[75G>A];[361delinsGAA], p.[(Trp25*);[(Pro121Glufs*95)]). Haematoxylin & eosin stainings
127 were performed. Immunostaining assays were performed on a Leica Bond Platform using an anti-SP-B
128 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 *SFTP*B c.582G>A p.(Gln194=) variation**

138 The identified *SFTP*B 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 pSFTP_B_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 pSFTP_B_mut generated three molecular
144 species in addition to the 363-bp expected *SFTP*B 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 *SFTP*B
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

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

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

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

161

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

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

169

170 DISCUSSION

171 We herein report for the first time two adult patients from a single family with bi-allelic *SFTPB*
172 pathogenic variants. Very rare cases of prolonged survivals have been described in SP-B deficiency
173 after infant lung transplantation [10–12]. Only one case has been reported with a delayed presentation
174 in a 4-month infant carrying the homozygous c.706C>T p.(Arg236Cys) missense pathogenic variant
175 [13]. The patient was alive at the age of 8 years with oxygen supplementation. Three other patients
176 with a prolonged survival also presented at birth with a severe respiratory distress. Two of them were
177 homozygous for the c.465G>T p.(Gly155=) splice variant which creates a strong donor splice site
178 within exon 5 (MaxEntScan score 9.80) and one of them was still alive at school age [14]. The third

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

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

198

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

201

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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

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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269 101

270

271

272 **Figure legends**

273

274 **Figure 1. Family tree, CT scan and histological features**

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

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

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

288 SP-B antibody targets pro-SP-B as it has been developed against amino acids 121 to 197. SP-B
289 immunostaining (magnification x20 and x40) showed an almost complete loss of SP-B expression in
290 the patient's type 2 alveolar epithelial cells (AEC)2, a complete loss of SP-B expression in SP-B-
291 deficient neonate and a normal intracytoplasmic granular AEC2 SP-B expression in both adult and
292 infant controls. Note that the anti-SP-B antibody does not target the peptide corresponding to the in-
293 phase deleted transcript (p.(Asp132_Gln194del)) which lacks most of the epitope (Figure 2).

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

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

298 HE, haematoxylin and eosin; AEC alveolar epithelial cell

299

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

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

304 After transfection of A549 cells with the above-mentioned plasmids, the migration on a 1.5% agarose-
305 BET gel of the RT-PCR products obtained from extracted RNAs is shown. The main amplicons
306 characterized by Sanger sequencing and obtained from the normal and mutant constructs are
307 represented on the right of the gel image. Two amplicons were observed for the pSFTPB_WT: a
308 363bp (canonical splicing) and a 310bp (using exon 6 cryptic splice-acceptor site corresponding to
309 putative p.(Asp195Glyfs*39)). Both the c.582G>A (pSFTPB_mut) and the c.582+1G>T
310 (pSFTPB_ctr) pathogenic variants resulted in three specific and aberrant amplicons, i.e. from top to
311 bottom: one 464bp transcript related to the retention of the first 101bp of intron 5 and predicting a
312 frameshift with a premature Stop codon in exon 6 (p.(Asp195Valfs*54)), and two 174bp and 121bp
313 amplicons lacking exon 5 and corresponding to the use of intron 5 canonical splice-acceptor site
314 (p.(Asp132_Gln194del) in-phase deletion), or exon 6 cryptic splice-acceptor site
315 (p.(Asp132Glyfs*39)) respectively. The data are representative from 3 independent experiments.

316 WT, wild type

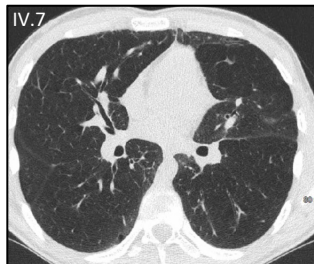
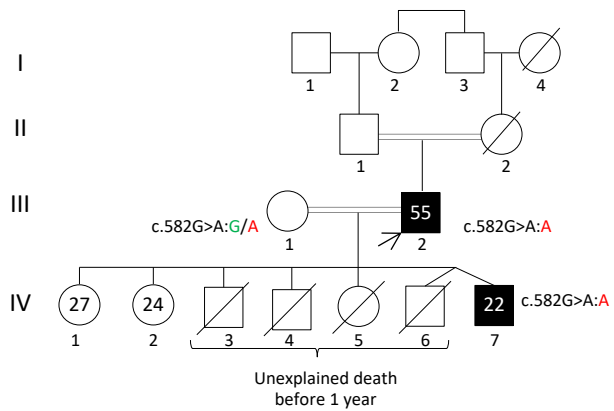
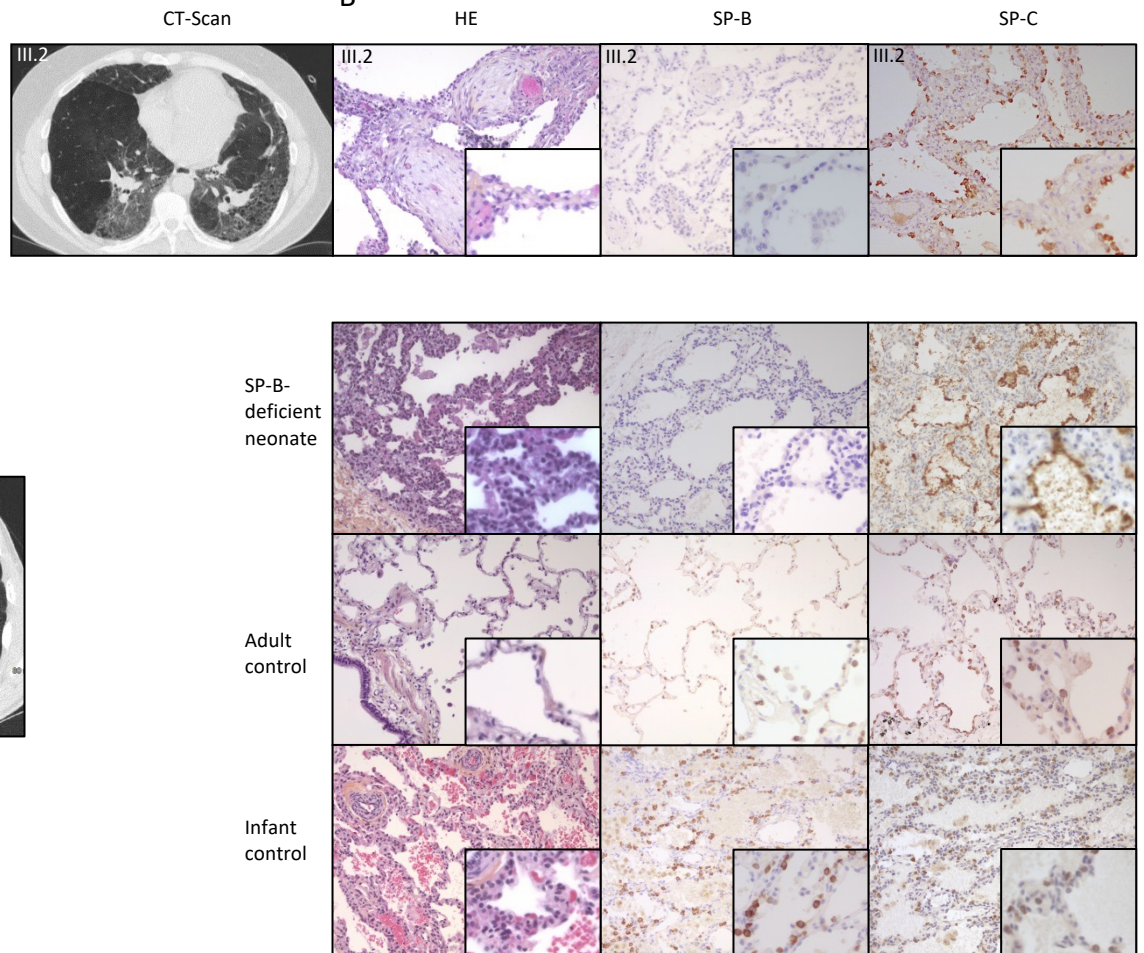
A**B**

Figure 1. Family tree, CT scan and histological features

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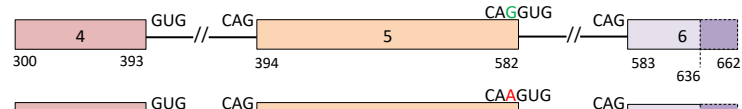
B. The native lung explant examination of the proband (III.2) using Haematoxylin and eosin (HE) staining shows fibroblastic foci alternating with less affected areas consistent with a usual interstitial pneumonia pattern. Pulmonary hemosiderosis was not observed nor pulmonary alveolar proteinosis. The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

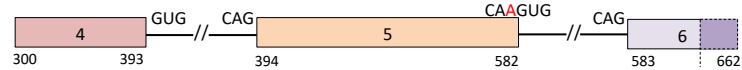
SP-C antibody has been developed against full length SP-C (amino acids 1-197) and can thus detect both pro-SP-C and mature SP-C. SP-C immunostaining (magnification x20 and x40) showed a higher degree of expression in the patient's and the SP-B-deficient neonate's type 2 alveolar epithelial cells than in adult and infant controls.

HE, haematoxylin and eosin; AEC alveolar epithelial cell

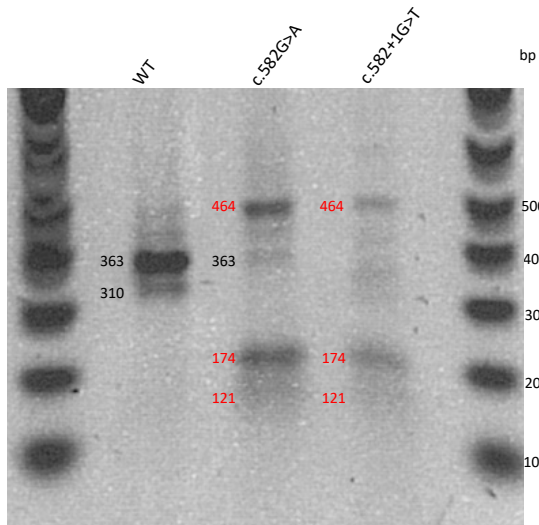
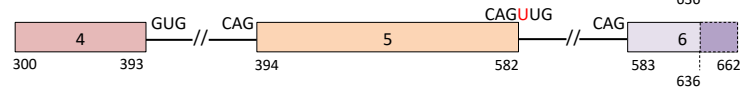
WT pre-mRNA



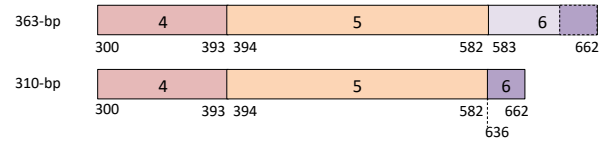
c.582G>A pre-mRNA



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

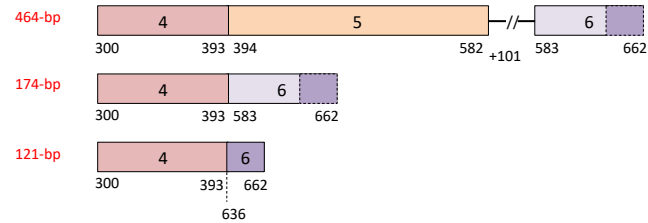


Figure 2. *In vitro* assessment of *SFTP*B splicing defects associated with c.582G>A p.(Gln194=)

*SFTP*B exons 4-6 pre-mRNA corresponding to plasmids p*SFTP*B-WT, p*SFTP*B_mut and p*SFTP*B_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 p*SFTP*B_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 (p*SFTP*B_mut) and the c.582+1G>T (p*SFTP*B_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