Highlights

- This is the first report showing that neonatal-onset pulmonary alveolar proteinosis is a phenotype correlated with poor outcomes in children with SP-C disorder.
- The pathogenic variations in the *SFTPC* gene did not accumulate in the BRICHOS domain in children, unlike in adults.
- *SFTPC* p.Leu45Arg might be associated with severe respiratory symptoms.
- Splicing disorder of *SFTPC* exon 4 might be the genotype predicting poor outcomes in patients with SP-C disorder.

Title: Neonatal-Onset Pulmonary Alveolar Proteinosis is a Phenotype Associated with Poor Outcomes in Surfactant Protein-C Disorder

Running title: Outcome of SP-C disorder

Key Words: *SFTPC*, surfactant protein-C, infant, outcome, pulmonary alveolar proteinosis, interstitial pneumonitis

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Disclosure

None of the authors have any conflicts of interest to declare.

Abbreviations

ABG; air bronchogram

BAL; bronchoalveolar lavage

CPP; crazy-paving pattern

CSD; consolidation

GGO; ground-glass opacification

HILD; hereditary interstitial lung disease

IP; interstitial pneumonitis

LT; lung transplant

MLPA; multiplex ligation dependent probe amplification

PAP; pulmonary alveolar proteinosis

SP-B; surfactant protein B

SP-C; surfactant protein C

Abstract

Background: Surfactant protein C (SP-C) disorder is a major component of hereditary interstitial lung disease (HILD) among Japanese. The correlation between clinical outcomes and the phenotype/genotype of SP-C disorder has not been evaluated comprehensively. The current study aimed to evaluate the phenotype/genotype correlated with poor outcomes in patients with SP-C disorder.

Methods: Sequencing analysis of *SFTPC* in 291 candidates with HILD was performed. The phenotype and genotype correlated with poor outcomes were examined. The log-rank test was used to compare the probability of good outcomes between two patient groups.

Results: Twenty patients were diagnosed with SP-C disorder. Of nine patients with neonatal-onset disease, four and five presented with pulmonary alveolar proteinosis (PAP) and interstitial pneumonitis (IP), respectively. The remaining 11 patients with late-onset disease had IP. In total, four and 16 patients had PAP and IP phenotypes, respectively. Four of nine patients with neonatal-onset disease died, and one survived after lung transplant. Further, 1 of 11 patients with late-onset disease died. Four patients with neonatal-onset PAP had a significantly lower probability of good outcomes than

the remaining patients. Two patients with neonatal-onset PAP had the p.Leu45Arg variant, one died and the another survived after lung transplant. Of eight patients with variants in the BRICHOS domain, one with frame shift variant located in exon 4, one with variant located at the splicing acceptor site of exon 4, and one with variant located at the splicing donor site of exon 4 died.

Conclusion: Neonatal-onset PAP was a phenotype predicting poor outcomes in patients with SP-C disorder. The p.Leu45Arg variant and splicing disorder of exon 4 might be genotypes predicting poor outcomes in patients with SP-C disorder.

Introduction

Hereditary interstitial lung disease (HILD) is a disease group characterized by respiratory insufficiency based on the responsible variants of genes that play a role in the lung surfactant system [1]. HILD includes pulmonary alveolar proteinosis (PAP), interstitial pneumonitis (IP), and abnormal lung development. If HILD is suspected, widespread ground-glass opacification (GGO) and individually irregular consolidation (CSD) on the dependent side or geographic opacification on computed tomography scan are useful [2]. In western countries, variants in *SFTPB* coding surfactant protein B (SP-B) and *ABCA3* coding ATP-binding cassette transporter A3 (ABCA3), both with autosomal recessive inheritance characteristics, are the major cause of HILD [3,4]. In Japanese, pathogenic variants in *SFTPB* and *ABCA*3 genes are rare. SP-C disorder caused by *SFTPC* variants was a relatively major condition [5]. *SFTPC* coding pro SP-C is located in chromosome 8, which is composed of 6 exons and is expressed specifically in type II alveolar epithelial cells [6]. Human SP-C is synthesized as a 197 amino acid integral membrane precursor protein consisting of a cytosolic N-terminal domain (residues 1-23) that directs intracellular trafficking of the proprotein, a hydrophobic membrane-spanning domain that comprises most of the biophysically active, secreted mature peptide (residues 24-58), and a luminal linker region (residues

59-89), followed by the C-terminal BRICHOS domain (residues 90-197) [7]. Normally processing of the 21-kD pro SP-C peptide (197 amino acids) to the smaller 4-kD mature SP-C（35 amino acids） occurs by successive proteolytic cleavage of the carboxyl terminus and then amino terminus. Maturation of SP-C from pro SP-C in type II alveolar epithelial cells is dependent on SP-B [8].

SP-C disorder was first reported as a familial case with IP in 2001 [9]. The phenotype of SP-C disorder significantly varies, with onset from the neonatal period to adulthood and with symptoms ranging from mild to severe. In adult patients with SP-C disorder, the p.Ile73Thr variant in the linker domain was the most common variant with favorable outcomes [10-14]. And some responsible variants in also BRICHOS domain were reported [15] [16]. Although IP is the main phenotype of SP-C disorder, some patients present with the PAP phenotype [17].

Based on the HILD cases in Japan since 2011, some patients with SP-C disorder have poor clinical outcomes. The current study aimed to evaluate the phenotype/genotype correlated with poor outcomes in patients with SP-C disorder.

Materials and Methods

From April 2011 to March 2022, candidates with HILD were identified via case consultation [5]. The Institutional Review Board of the Faculty of Medicine and Graduate School of Medicine, Hokkaido University, approved this study. Sequencing analyses of *SFTPC* were performed. Sanger sequencing was used until December 2017. Next-generation sequencing was utilized from January 2018 and a commercially available system since April 2020 [18]. Each *SFTPC* variant was considered pathogenic based on the American College of Medical Genetics and Genomics guideline [19]. We also collected data on clinical findings, sex, disease onset, phenotype (PAP or IP), diagnostic method, radiological finding, respiratory support and outcome (dead, survive with lung transplant, survive without lung transplant). The patients were divided into two groups. One group with poor outcomes included those who died and those who survived after lung transplant. The other group with good outcomes contained survivors without lung transplant. To compare the probability of good outcomes between the two groups, the Kaplan–Meier method and the log-rank test were used. The Statistical Package for the Social Sciences software version 26 (IBM Corp., Armonk, NY, the USA) was used, and a p value of <0.05 was considered significant.

Results

Sequencing analyses were performed on 291 candidates with HILD and 20 patients diagnosed with SP-C disorder. In total, four and 16 patients had the PAP and IP phenotypes, respectively (Figure 1). In the 271 patients without pathogenic *SFTPC* variant, we found associate variants in *FOXF1* (15 cases), *ABCA3* (nine cases), *NKX2-1* (six cases) and *OAS1* (three cases). Seven of the 15 patients with pathogenic *FOXF1* variants were diagnosed by copy number analysis using the multiplex ligation dependent probe amplification (MLPA) method. No associate variants were found in the remaining 238 patients. Of nine patients with neonatal onset, four and five presented with PAP and IP, respectively. The remaining 11 patients with late-onset disease had IP. Further, four of nine patients with neonatal onset died, and one survived after lung transplant. Further, one of 11 patients with late-onset disease died. Four patients with neonatal-onset PAP had a significantly lower probability for good outcomes than the remaining patients (Figure 2).

Clinical features of 20 Japanese patients with SP-C disorder were shown in Table 1. Radiological finding and respiratory support were at the timing of genetic analysis. Twelve and eight patients were male and female, respectively. Patients with PAP were diagnosed with bronchoalveolar lavage (BAL) or autopsy. Four of 16 patients with IP

received biopsy and the remaining 12 patients, including one with BAL, were diagnosed clinically. All patients were observed GGO in radiological finding. Nine patients needed mechanical ventilation at the timing of genetic analysis. Ten of 11 patients with late onset had missense variants. On the other hand, only five of nine patients with neonatal onset had missense variants. The origin of genetic variant was paternal, maternal, *de novo* and unknown in five, two, three and nine patients, respectively. The term of "*de novo*" mentioned potential *de novo*, neither of parents shared the pathogenic variant. One of two variants in a patient (Case 7) was maternal and the another was *de novo*. The locations of the variants were shown in Figure 3. Data of the Chinese children with SP-C disorder in a paper was superimposed on our data [10]. Square and circle means neonatal and late onset, respectively. Red and black frame line means Japanese children in this report and Chinese children in a previous report, respectively. Red and black letter means PAP and IP, respectively. Shadowed square and circle means patient with poor outcomes. Yellow and blue back ground means Japanese and Chinese children, respectively. Shadowed area shows BRICHOS domain. In our data, eight and 12 patients had variants in and out of BRICHOS domain, respectively. Five of twelve patients with variants out of BRICHOS domain had the p.Ile73Thr variant. In total, three of 11 patients with *SFTPC* variants out of BRICHOS domain had poor outcomes,

two died and one survived after lung transplant, one neonatal-onset PAP with p.Ser61Gly variant, and two neonatal-onset PAP with p.Leu45Arg. Three patients with variants in BRICHOS domain died, one neonatal-onset PAP with p.Gln145fs in exon 4, one late-onset IP with c.325-2A>T and one neonatal-onset IP with 435+2T>A.

Discussion

We analyzed 20 pediatric patients with SP-C disorder. Neonatal-onset PAP was a phenotype predicting poor outcomes in patients with SP-C disorder. Although main phenotype of SP-C disorder is IP, some patients show PAP phenotype [17]. In our series, all PAP patients had neonatal onset. SP-C disorder with PAP phenotype might show respiratory symptoms in early stage of life. Respiratory symptoms at birth of patients with neonatal-onset PAP might not due to PAP itself. Patients with SP-B or ABCA3 deficiency often showed respiratory failure at birth due to respiratory distress syndrome and then change into PAP. Liptzin et al [20] reported a series of three patients with pathogenic *SFTPC* variants, p.Gly182Arg, p.Leu188Gln and p.Cys189Trp. In the report, all three and two patients had neonatal onset and pathological findings compatible with PAP, respectively. All three patients had weaned from chronic

mechanical ventilation. Some patients with neonatal-onset PAP might be viable after appropriate therapies.

In our series, p.Ile73Thr was the most common causative variant, and this finding was similar to that of a previous reports [10-13]. Totally, 29 of 61 patients with pathogenic *SFTPC* variants had p.Ile73Thr (6/15 in ref. 10, 8/17 in ref. 11, 10/18 in ref. 12 and 5/11 in ref. 13). High incidence of p.Ile73Thr might be a consequence of favorable outcomes of the variant. In the Chinese child series two of six cases with p.Ile73Thr were dead. The discrepancy might depend on domestic clinical systems or that severe cases might have tendency to show symptoms in childhood. The p.Ile73Thr variant was sometimes reported as a reason of familial IP. In our series, the p.Ile73Thr variant was inherited from one of the parents in at least two of five patients.

Two patients with p.Leu45Arg had poor outcomes. The same variant was reported in a patient requiring lung transplant [21]. The p.Leu45Arg variant of *SFTPC* might be associated with severe respiratory symptoms. The variant is located in the region corresponding to mature SP-C, and introduces a positively charged amino acid in an extremely hydrophobic region. This variant is thus likely to both hinder pro SP-C folding and processing, and the ability of mature SP-C to interact with surfactant lipids.

In adult patient series, pathogenic *SFTPC* in BRICHOS domain were considered popular [15] [16]. In our data, twelve of 20 patients had variants out of BRICHOS domain. Variants in BRICHOS domain did not differ from those in a report on adult patients [22]. Of eight patients with variants in BRICHOS domain, one with frame shift variant located in exon 4, one with splicing disorder located at the splicing acceptor site of exon 4, and one with splicing disorder located at the splicing donor site of exon 4 died. Among cases of IP, only those with splicing abnormalities have a poor prognosis. A Chinese child patient with c.435+1G>A also was dead (Figure 3). Splicing of exon 4 might be a genotype predicting poor outcomes in patients with SP-C disorder. Splicing disorder of exon 4 or Δ4 might cause severe respiratory symptoms due to dominant negative effects [23].

We reported some correlations of genotype/phenotype with outcomes in patients with SP-C disorder. A previous study found no correlation between genotypes and phenotypes in children with SP-C disorder [11]. In their report, none of the patients died.

Combination therapy with methylprednisolone, hydroxychloroquine, and azithromycin is recommended for pediatric patients with IP [24]. There are no standard therapeutic strategies for neonatal-onset PAP. *SFTPC* is specifically expressed in type II epithelial

cells. Theoretically, lung transplant is effective in neonatal-onset PAP with SP-C disorder. However, this procedure is controversial among infants. In future, some experimental strategies, using induced pluripotent and embryonic stem cells or alveolar organoids might be in clinical use [25].

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Legends

Figure 1: Outcome of the patients enrolled in this study

We found pathogenic variants in 20 patients. Three of 4 patients with neonatal onset PAP died and the another had received lung transplant. Two patients with IP, one had neonatal onset and the another had late onset died.

LT; lung transplant

Figure 2: Probability of good outcomes

Patients with neonatal-onset PAP showed significant poor outcomes (p=0.044). Solid line shows the rate of good outcomes in patients with neonatal onset PAP. Dotted line shows that in the other patients.

Figure 3: Pathogenic variants found in *SFTPC*

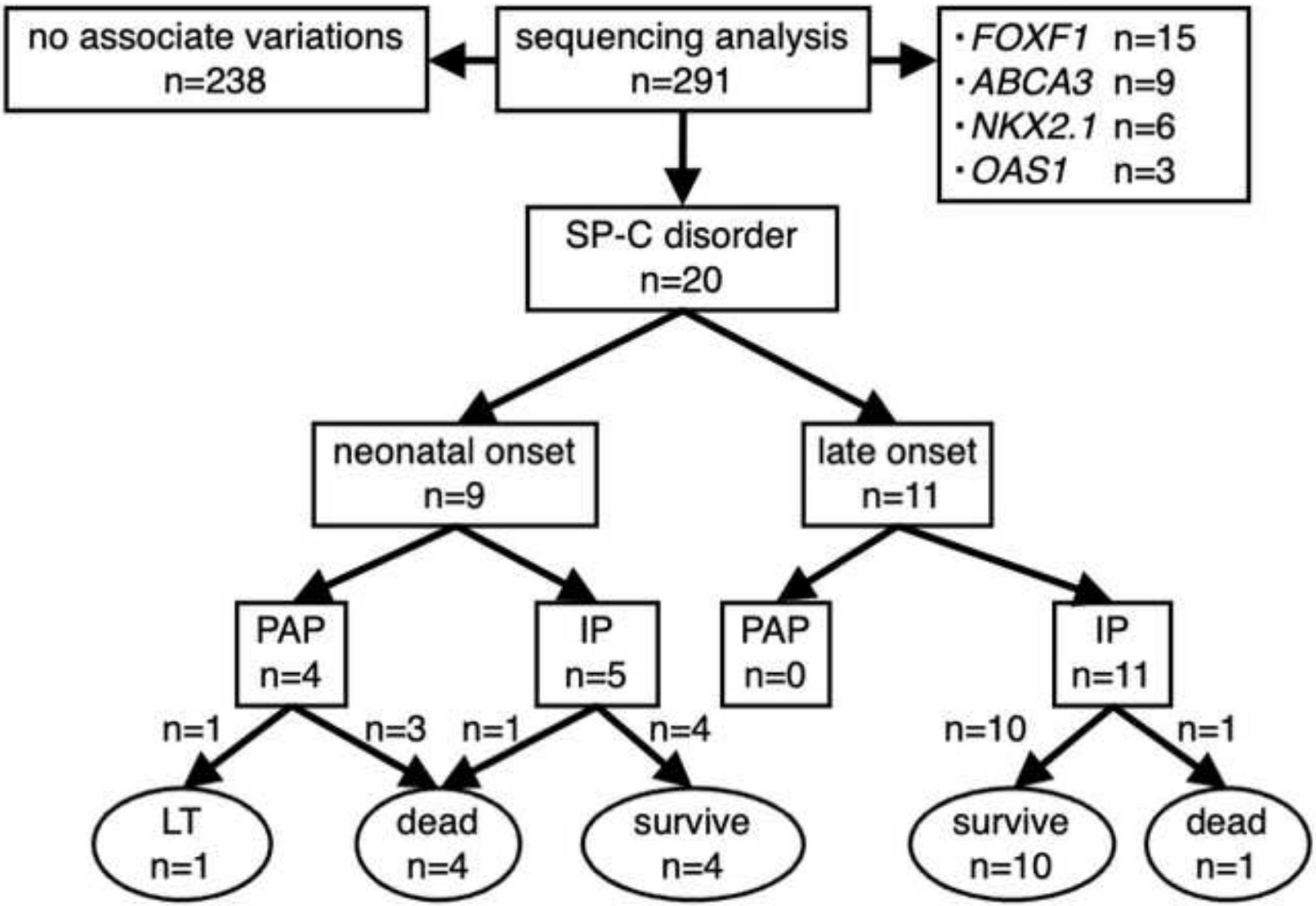
Square and circle means neonatal and late onset, respectively. Red and black frame line means Japanese children in this report and Chinese children in a previous report,

respectively. Red and black letter means PAP and IP, respectively. Shadowed square and circle means patient with poor outcomes. Yellow and blue back ground means Japanese and Chinese children, respectively. Shadowed area means BRICHOS domain.

Table 1 Clinical features of 20 Japanese patients with SP-C disorder

Radiological finding and respiratory support were at the timing of genetic analysis. The term of "*de novo"* mentioned potential *de novo*, neither of parents shared the pathogenic variant. PAP; pulmonary alveolar proteinosis, IP; interstitial pneumonitis, BAL; bronchoalveolar lavage, GGO; ground-glass opacification, CSD; consolidation, ABG; air bronchogram, CPP; crazy-paving pattern

Figure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sequencing analysis
n=291
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• ABCA3 n=9
• NKX2.1 n=6
• OAS1 n=3
SP-C disorder
n=20
neonatal onset
n=9
late onset
n=11
PAP
n=4
IP
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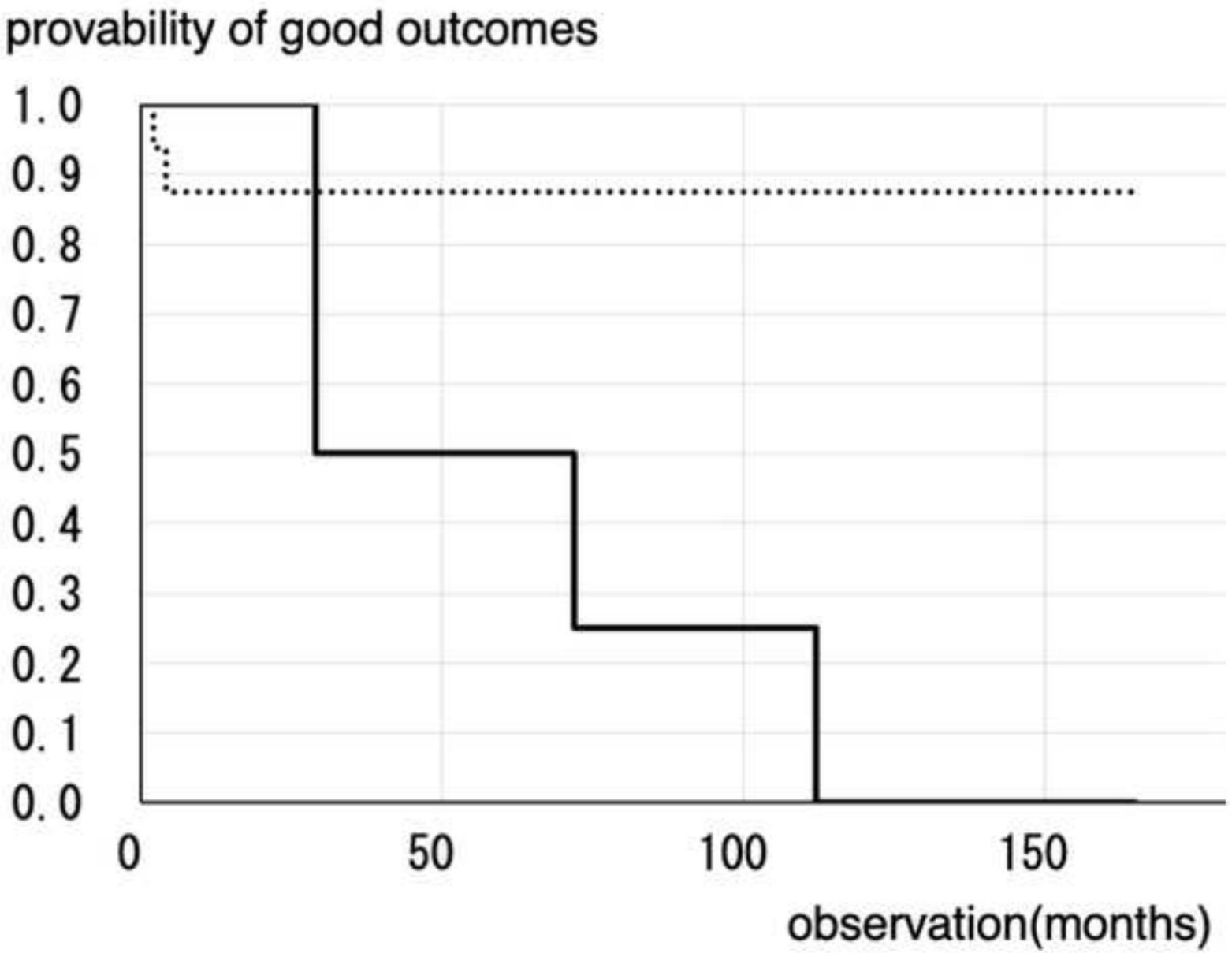
Figure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0.9
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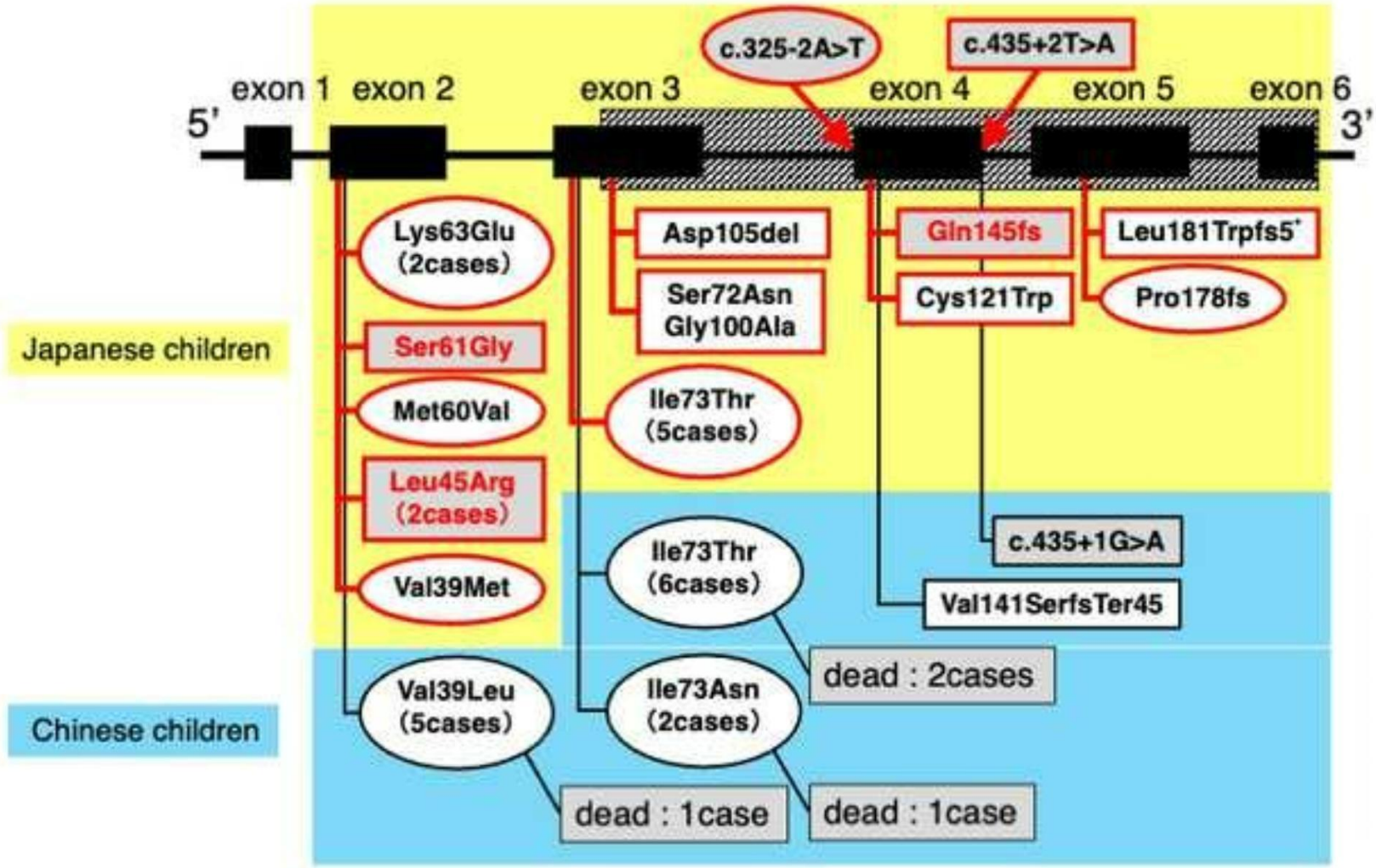
Figure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Leu181Trpfs5*
Ser72Asn
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Cys121Trp
Pro178fs
Japanese children
Ser61Gly
Met60Val
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(5cases)
Leu45Arg
(2cases)
c.435+1G>A
Ile73Thr
(6cases)
Val39Met
Val141SerfsTer45
dead : 2cases
Val39Leu
(5cases)
Ile73Asn
(2cases)
Chinese children
dead : 1case
dead : 1case

Table

Table 1 Clinical features of 20 Japanese patients with SP-C disorder

Patient	Sex	Age at onset	Onset period	Phenotype	Diagnostic method	Radiological finding	Respiratory support	Outcome	Genetic variant	Amino acid substitution	Origin
1	F	at birth	neonatal	PAP	BAL	GGO	mechanical ventilation	dead	c.134T>G	p.Leu45Arg	*de novo*
2	F	3.5hr	neonatal	PAP	BAL	GGO, CSD, ABG	mechanical ventilation	lung transplant	c.134T>G	p.Leu45Arg	*de novo*
3	M	at birth	neonatal	PAP	BAL	GGO	mechanical ventilation	dead	c.181A>G	p.Ser61Gly	*de novo*
4	F	6d	neonatal	PAP	autopsy	GGO, CSD, CPP	mechanical ventilation	dead	c.433delC	p.Gln145fs	unknown
5	F	12d	neonatal	IP	clinical course	GGO	supplemental oxygen	dead	c.435+2T>A	splicing disorder	unknown
6	F	2m	late	IP	biopsy	GGO	mechanical ventilation	dead	c.325-2A>T	splicing disorder	unknown
7	M	14d	neonatal	IP	biopsy	GGO	mechanical ventilation	survive	c.215G>A, c.299G>C	p.Ser72Asn, Gly100Ala	c.215G>A; paternal c.299G>C; *de novo*
8	M	at birth	neonatal	IP	clinical course	GGO, ABG	mechanical ventilation	survive	c.313_315del	p.Asp105del	unknown
9	F	10hr	neonatal	IP	biopsy	GGO	mechanical ventilation	survive	c.363C>G	p.Cys121Trp	maternal
10	M	1d	neonatal	IP	clinical course	GGO	mechanical ventilation	survive	c.541delC	p.Leu181Trpfs5*	maternal
11	M	1y9m	late	IP	clinical course	GGO	supplemental oxygen	survive	c.115G>A	p.Val39Met	unknown
12	F	3m	late	IP	clinical course	GGO	supplemental oxygen	survive	c.178A>G	p.Met60Val	unknown
13	M	5m	late	IP	clinical course	GGO	supplemental oxygen	survive	c.187A>G	p.Lys63Glu	maternal
14	M	5m	late	IP	clinical course	GGO	supplemental oxygen	survive	c.187A>G	p.Lys63Glu	maternal
15	M	1y5m	late	IP	clinical course	GGO	no therapy	survive	c.218T>C	p.Ile73Thr	unknown
16	M	1y7m	late	IP	BAL	GGO	supplemental oxygen	survive	c.218T>C	p.Ile73Thr	paternal
17	M	1y6m	late	IP	biopsy	GGO	supplemental oxygen	survive	c.218T>C	p.Ile73Thr	maternal
18	M	1y0m	late	IP	clinical course	GGO	supplemental oxygen	survive	c.218T>C	p.Ile73Thr	unknown
19	M	7m	late	IP	clinical course	GGO	supplemental oxygen	survive	c.218T>C	p.Ile73Thr	unknown
20	F	5y7m	late	IP	clinical course	GGO, cysts	no therapy	survive	c.533delC	p.Pro178fs	paternal

This manuscript has not been published elsewhere and is not under consideration by another journal. We have approved the manuscript and agree with submission to *Early Human Development*. There are no conflicts of interest to declare. The Institutional Review Board of the Faculty of Medicine and Graduate School of Medicine, Hokkaido University, approved this study.