Hereditary interstitial lung diseases manifesting in early childhood in Japan

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BACKGROUND: Genetic variations associated with interstitial lung diseases (ILD) have not been extensively studied in Japanese infants.

METHODS: Forty-three infants with unexplained lung dysfunction were studied. All 43, 22, and 17 infants underwent analyses of surfactant protein (SP)-C gene (SFTPC) and ATP-binding cassette A3 gene (ABCA3), SP-B gene (SFTPB), and SP-B western blotting, respectively. Two and four underwent assessment of granulocyte macrophage colony-stimulating factor–stimulating phosphorylation of signal transducer and activator of transcription-5 (pSTAT-5) and analyses of FOXF1 gene (FOXF1), respectively.

RESULTS: ILD were diagnosed clinically in nine infants: four, three, and two had interstitial pneumonitis, hereditary pulmonary alveolar proteinosis (hPAP), and alveolar capillary dysplasia with misalignment of pulmonary veins (ACD/MPV), respectively. Genetic variations considered responsible were detected in six (67%) of the nine infants with ILD: three with hPAP (*SFTPC* p.Leu45Arg and p.Gln145fs, and *ABCA3* p.Arg1583Trp/p.Val1495CysfsX21), two with interstitial pneumonitis (*SFTPC* p.Lys63Glu and p.Ser72Asn/p.Gly100Ala), and one with ACD/MPV (*FOXF1* p.Leu300ArgfsX79). None showed *SFTPB* mutations or defects in pSTAT-5. The 17 bronchoalveolar lavage or tracheal aspirates contained enough SP-B protein.

CONCLUSION: The SP-C abnormality was most prevalent, and SP-B deficiency was rare in Japanese infants with hereditary ILD.

Pulmonary diseases that require transient-assisted ventilation, such as respiratory distress syndrome due to lung immaturity, are often encountered by neonatologists working in neonatal intensive care units. However, although rare, there are genetic disorders of lung dysfunction manifesting in early childhood that cannot be explained by prematurity. These pulmonary diseases may be classified into two groups: disorder of alveolar type II epithelial cells (AEC2) and disorder of alveolar macrophages. Disorder of AEC2 includes surfactant protein (SP)-B deficiency (1), SP-C abnormality (2), and ATP-binding cassette A3 (ABCA3) deficiency (3). Disorder of alveolar macrophages includes abnormality in granulocyte macrophage colony–stimulating factor (GM-CSF) receptor (4,5) and dysfunction of macrophages associated with hypogammaglobulinemia (6). Although considerable overlapping exists, genetic disorders of SP-B and alveolar macrophages are likely to manifest hereditary pulmonary alveolar proteinosis (hPAP), while those of SP-C and ABCA3 are likely to manifest hPAP and/or interstitial pneumonitis (7). In addition, genetic disorders of thyroid transcription factor-1–associated thyroid dysfunction (8) and alveolar capillary dysplasia with misalignment of pulmonary veins (ACD/MPV) (9) often manifest interstitial lung disease (ILD) in early childhood. Thus, genetic abnormalities leading to hereditary ILD (HILD), including hPAP, interstitial pneumonitis, and ACD/MPV, vary.

As ILD is rare and as helpful measures such as high-resolution computed tomography, bronchoalveolar lavage (BAL), and/or lung biopsy are often difficult to perform in small infants, especially in neonates, it may often be difficult for neonatologists to determine the cause of these lung diseases. However, abnormalities in the SP-B gene (*SFTPB*), SP-C gene (*SFTPC*), ABCA3 gene (*ABCA3*), and FOXF1 gene (*FOXF1*) are known to be responsible for SP-B deficiency, SP-C abnormality, ABCA3 deficiency, and some of ACD/MPV (7,10), respectively. Therefore, analyses of these genes in addition to qualitative analyses of SP-B by western blotting in BAL or tracheal aspirates (11) are helpful to understand the associations between genetic abnormalities and disease phenotypes in these disorders. Assessment of GM-CSF–stimulating phosphorylation of signal transducer and activator of transcription-5 (pSTAT-5) also helps in the diagnosis of alveolar macrophage dysfunction (5).

Accordingly, we developed a new system to aid Japanese neonatologists working in the neonatal intensive care units to search for genetic causes of unexplained lung dysfunction in infants. Here, we report this system and preliminary results of an investigation on the causative genetic abnormalities involved in HILD.

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RESULTS

During the 2.5-year study period between February 2011 and July 2013, we had a total of 43 consultations from 34 institutions regarding the causes of lung dysfunction in 43 infants. Lung dysfunction manifested within 4 wk after birth in 34 infants (early onset) and 1 mo or later after birth in the remaining nine infants (**Table 1**). Clinically, nine patients were diagnosed as having ILD (**Table 2**): four with interstitial pneumonitis were based on findings in biopsied lung specimens except in one patient (case 4 in **Table 3**) in whom the diagnosis was based on a 2-y history of lung dysfunction, computed tomography, and serum KL-6 (12,13); three with hPAP were based on the results of microscopic BAL examination, and two with ACD/MPV were based on findings in biopsied or autopsied lung specimen. Others included four diagnoses responsible for or possibly associated with lung dysfunction for each one case: gastroesophageal reflux disease, severe combined immunodeficiency, Kabuki makeup syndrome, and congenital adrenal hypoplasia (14–16). One case with congenital adrenal hypoplasia showed delayed maturation of lung surfactant system due to deficiency of endogenous steroids.

The hPAP was suspected in five cases including three with early onset (cases 1, 2, and 3 in **Table 3**) and two with late onset (case 6 in **Table 3** and another case without proven genetic variations) based on computed tomography before the analyses shown in **Figure 1**. Genetic abnormalities likely to be responsible for lung dysfunction were detected in six of the nine patients with ILD (**Tables 2** and **3** and **Figure 1**). These abnormalities were found during the first-round examination for *SFTPC* and *ABCA3* in five patients (cases 1–5 in **Table 3**): *SFTPC* mutations (five different mutations) in four cases and compound heterozygous *ABCA3* mutations in one case (**Table 3**). Two other compound heterozygous *ABCA3*

mutations, i.e., *ABCA3* p.Pro73Leu/p.Gly1205Arg and *ABCA3* p.Thr761Met/p.Ala1362Val, were detected in two (cases 7 and 8 in **Table 3**) of the 34 infants without ILD: one full-term infant with p.Pro73Leu/p.Gly1205Arg manifested lung dysfunction immediately after birth, but recovered with transient assisted ventilation only, and another with p.Thr761Met/p.Ala1362Val born at gestational week 27 weighing 778 g manifested lung dysfunction at 8 mo after birth and is being treated currently with vasodilators and home oxygen.

Twenty-two patients underwent second-round examination (**Figure 1**). None of the 22 showed *SFTPB* mutations (**Figure 1**). The BAL or tracheal aspirates were available for western blotting analysis in 17 of the 22 infants and contained enough SP-B protein. Milky appearance of the BAL with eosinophilic materials on the microscopic examination supported the diagnosis of hPAP in three cases (cases 1, 2, and 3 in **Table 3**) but not in two late-onset cases with suspected hPAP (case 6 in **Table 3** and another). The pSTAT-5 was assessed in two infants: one with *ABCA3* mutations (case 3 in **Table 3**) and another with suspected hPAP by computed tomography, but not with BAL (case 6 in **Table 3**). Results indicated normally functioning macrophages in both cases.

Five infants had severe persistent pulmonary hypertension unresponsive to treatment. Four of them underwent analysis of *FOXF1*. Only case 6 with a diagnosis of ACD/MPV showed an abnormality in *FOXF1*, i.e., a novel frameshift mutation (**Table 3**). A female infant with ACD/MPV and birth weight of 3,024 g who died 4 days after birth from lung dysfunction due to pulmonary hypertension exhibited no abnormalities in *SFTPC*, *ABCA3*, and *SFTPB* but did not undergo examination for *FOXF1* because her family did not give informed consent to the analysis of *FOXF1*. This system was helpful to determine causative genetic variations in six (67%) of the nine patients with ILD and 14.0% of all 43 referrals.

Family members of five cases underwent genetic testing. Two *SFTPC* variations (cases 1 and 6) were derived from *de novo*, and one *SFTPC* variation (case 4) was inherited from

Table 1. Demographic characteristics of 43 patients

	Early onset	Late onset
Number of patients	34[a]	9[b]
Male sex	22 (65%)	5 (56%)
Onset of lung dysfunction	0 (0–14) d	6 (3–60) mo
Gestation length (weeks)	38 (28–41)	40 (27–41)
Apgar score < 7 (5 min)	6 (18%)	0 (0%)
Assisted ventilation	30 (88%)	5 (56%)
iNO	16 (47%)	2 (22%)
BAL/tracheal aspirates	15 (44%)	2 (22%)
CT/HRCT	22 (65%)	9 (100%)
Lung biopsy/autopsy	6 (18%)	3 (33%)
Poor outcome (death)	8 (24%)	2 (22%)

Early onset was defined as manifestation of lung dysfunction within 4 wk after birth. Median (range) or number (percentage) is indicated.
BAL, bronchoalveolar lavage; CT/HRCT, computed tomography or high-resolution computed tomography; iNO, inhaled nitric oxide.
[a]Clinically asphyxia and/or meconium aspiration syndrome were suspected in nine infants. [b]Presumptive clinical diagnosis was interstitial pneumonitis in four cases, respiratory failure associated with immune disorder in two cases, and pulmonary alveolar proteinosis in one case.

Table 2. Relationship between clinical diagnosis and genetic variations responsible for lung dysfunction

Clinical diagnosis		Number of infants with abnormality/ number of infants tested				
		SFTPC	*ABCA3*	*SFTPB*	pSTAT-5	*FOXF1*
Interstitial pneumonitis	(*n* = 4)	2/4	0/4	0/0	0/0	0/0
hPAP	(*n* = 3)	2/3	1/3	0/3	0/1	0/0
ACD/MPV	(*n* = 2)	0/2	0/2	0/2	0/1	1/2
Others[a]	(*n* = 34)	0/34	0/34	0/17	0/0	0/2
Overall	(*n* = 43)	4/43	1/43	0/22	0/2	1/4

ABCA3 variations seen in two cases (cases 7 and 8 in **Table 3**) were not included in this table.
ACD/MPV, alveolar capillary dysplasia with misalignment of pulmonary veins; hPAP, hereditary pulmonary alveolar proteinosis.
[a]Included four diagnoses responsible for or possibly associated with lung dysfunction for each one case: gastroesophageal reflux disease, severe combined immunodeficiency, Kabuki makeup syndrome, and congenital adrenal hypoplasia.

Table 3. Eight patients in whom genetic variations were found

Case	BW/GW	Onset	Diagnosis	AV	iNO	Genotype	SIFT/polyphen-2	Origin of variations	Treatment	Outcome
1	3,204/40	0 d	hPAP	Yes	No	*SFTPC* c.134T>C: p.Leu45Arg (het)	Damaging/damaging	*De novo*	Surfactant, steroids, and Hch	Undergoing treatment, 43 mo
2	2,600/40	6 d	hPAP	Yes	No	*SFTPC* c.433delC: p.Gln145fs (het)	Not done	Unknown[c]	Surfactant, steroids, and Hch	Died, 77 d
3	3,230/40	0 d	hPAP	Yes	Yes	*ABCA3* c.4747C>T: p.Arg1583Trp,	Damaging/damaging	Mother (asymptomatic)	Surfactant, steroids, and Hch	Died, 7 mo
						ABCA3 c.4483_4507del25: p.Val1495CysfsX21	Not done	Father (asymptomatic)		
4	3,060/41	5 mo	IP[a]	No	No	*SFTPC* c.187A>G: p.Lys63Glu (het)	Damaging/damaging	Mother (asymptomatic) and mother's father (pulmonary fibrosis)	Steroids and Hch	Undergoing treatment, 54 mo
5	3,520/38	2 wk	IP	Yes	No	*SFTPC* c.215G>A: p.Ser72Asn	Damaging/damaging	Unknown[c]	Steroids, CsA, CPM, and AZP	Undergoing treatment, 13 y
						SFTPC c.299G>C: p.Gly100Ala	Damaging/benign			
6	3,344/41	3 mo	ACD/MPV	Yes	Yes	*FOXF1* c.899delT: p.Leu300ArgfsX79 (het)	Not done	*De novo*	Steroids, vasodilator	Undergoing treatment, 24 mo
7	3,194/40	0 d	URD	Yes	No	*ABCA3* c.218C>T: p.Pro73Leu	Tolerated/benign[b]	Unknown[c]	None	Recovered
						ABCA3 c.3613G>A: p.Gly1205Arg	Tolerated/benign[b]			
8	778/27	8 mo	CLD, PH	Yes	No	*ABCA3* c.2282C>T: p.Thr761Met,	Damaging/damaging	Mother (asymptomatic)	Vasodilator	Undergoing treatment, 17 mo
						ABCA3 c.4085C>T: p.Ala1362Val	Tolerated/benign[b]	Father (asymptomatic)		

ACD/MPV, alveolar capillary dysplasia with misalignment of pulmonary veins; AV, assisted ventilation; AZP, azathioprine; BW/GW, birth weight/gestational week at delivery; CLD, chronic lung disease; CPM, cyclophosphamide; CsA, cyclosporine A; Hch, hydroxychloroquine; hPAP, hereditary pulmonary alveolar proteinosis; iNO, inhaled nitric oxide; IP, interstitial pneumonitis; PH, pumonary hypertension; URD, unexplained respiratory distress.
[a]Diagnosed based on medical history, computed tomography, and serum KL-6. [b]Three of the four variations in cases 7 and 8 were considered not to be causative for lung dysfunction. The variations at codon 73 and 1,205 in case 7 are not listed in dbSNP or the 1000 Genome Project database. The variation at codon 1,362 in case 8 was identified in more than 1.0% of Japanese according to the 1000 Genome Project. [c]Genetic testing of family members was not performed.

maternal family line (**Table 3**). Four *ABCA3* variations (cases 3 and 8) were inherited from both parents.

Clinical factors were compared between 6 and 37 infants with and without gene mutations, respectively (**Table 4**). Neither a low birth weight (<2,500 g) and Apgar score < 7 at 5 min, nor timing of onset of lung dysfunction differentiated these cases. However, the frequencies of infants with a positive BAL test result assisted ventilation > 30 days, and histologically proven ILD were significantly higher in the former six patients than that in the latter 37 patients.

DISCUSSION
The system presented here was useful for determination of genetic variations that were possibly causative for ILD in six (67%) of the nine patients and 14% of all 43 referrals with severe and unexplained lung dysfunction. These six HILD cases included four cases of SP-C abnormality, one case of ABCA3 deficiency, and one case of FOXF1 abnormality. None of these cases was affected by SP-B deficiency. As no infants had hypothyroidism, examination of thyroid transcription

factor-1 abnormality was not performed in this study. However, as infants with thyroid transcription factor-1 abnormality (*NKX2-1* mutation) do not necessarily have hypothyroidism (17), it is possible that some infants had thyroid transcription factor-1 abnormality in this study population.

Among the 12 genetic variations detected in the eight cases (cases 1–8 in **Table 3**), two in cases 1 and 6 were reported previously (18,19). To our knowledge, 10 other mutations in six cases (cases 2–5, 7, and 8) have not been reported to date, i.e., four *SFTPC* mutations of heterozygous p.Gln145fs, heterozygous p.Lys63Glu, p.Ser72Asn, and p.Gly100Ala, and six *ABCA3* mutations of p.Arg1583Trp, p.Val1495CysfsX21, p.Pro73Leu, p.Gly1205Arg, p.Thr761Met, and p.Ala1362Val. The pro-SP-C amino acids of codons 63, 72, and 100 are well preserved in many mammals and two *SFTPC* mutations, i.e., p.Lys63Glu (case 4) and p.Ser72Asn (case 5), were judged as "damaging" with both SIFT and polyphen-2. Although p.Gly100Ala was "damaging" with polyphen-2 only, but not with SIFT, mutations of p.Gly100Val and p.Gly100Ser are associated with lung dysfunction (20,21). Frameshift mutations were detected in case 2
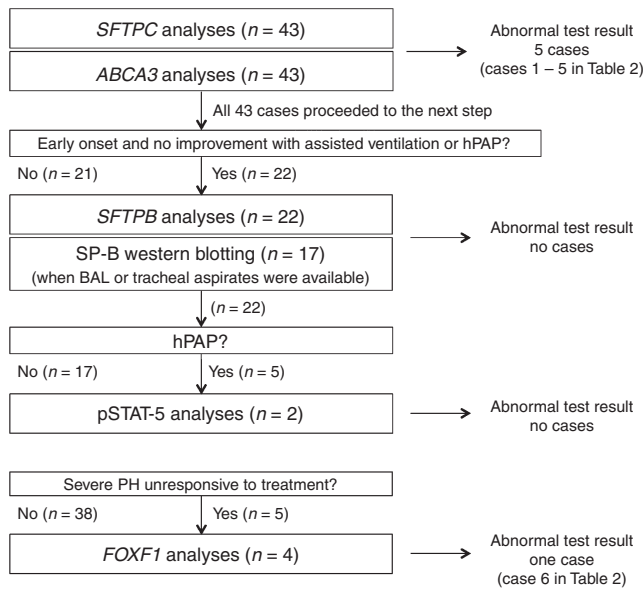
Figure 1. Flow diagram showing patient selection criteria for various examinations and test results. All 43 patients underwent analyses of *SFTPC* and *ABCA3* in the first step. Among 30 patients with early onset requiring assisted ventilation, 20 did not exhibit improvement in the respiratory status. These 20 and 2 with late onset and suspected hPAP (case 6 and another) underwent second-round examination for *SFTPB*. In 17 of the 22 patients, BAL or tracheal aspirates were available for SP-B western blotting analysis (**Table 1**). Milky appearance of the BAL with eosinophilic materials on the microscopic examination supported the diagnosis of hPAP in three cases (cases 1, 2, and 3), but not in two late-onset cases with suspected hPAP (case 6 and another). Two cases (cases 3 and 6) underwent assessment of pSTAT-5 because case 3 was not identified to have *ABCA3* mutation at that time and because case 6 was strongly suggested to be hPAP on computed tomography. Four of five patients with severe pulmonary hypertension unresponsive to treatment underwent analysis of *FOXF1*. One who died from pulmonary hypertension (PH) and ACD/MPV after 4 d of birth did not undergo analysis of *FOXF1*. ACD/MPV, alveolar capillary dysplasia with misalignment of pulmonary veins; BAL, bronchoalveolar lavage; hPAP, hereditary pulmonary alveolar proteinosis; SP-B, surfactant protein B.

(*SFTPC* p.Gln145fs) and case 3 (*ABCA3* p.Val1495CysfsX21), and such mutations can be associated with abnormal protein function. *SFTPC* p.His142fs was reported in a neonate with SP-C abnormality (22). The p.Arg1583Trp in case 3 was judged as "damaging" with both SIFT and polyphen-2. Therefore, we speculated that these six mutations were responsible for ILD in the four infants. Whether the three variations in cases 7 and 8 except *ABCA3* p.Thr761Met were causative for lung dysfunction is undetermined. The *ABCA3* p.Thr761Met in case 8 was judged as "damaging" with both SIFT and polyphen-2 occurring in one in 6,500 European and Americans according to the Exome Sequencing Project. Thus, the *ABCA3* p.Thr761Met may be responsible for lung dysfunction in case 8. More than 30 different mutations of *SFTPC* (2,7,18,20–27 and more than 70 different mutations of *ABCA3*) (3,7,21,28–30 have been reported in association with lung dysfunction. Thus, the first-round examination for *SFTPC* and *ABCA3* efficiently detected genetic abnormalities in five of the nine patients with ILD in this study.

The SP-B deficiency is one of the major causes of SP dysfunction disorders in Western countries: of 25 cases of hereditary SP dysfunction disorder in the UK, six (24%), seven

(28%), and 12 (48%) were SP-B deficiency, SP-C abnormality, and ABCA3 deficiency, respectively (21). In those with SP-B deficiency, more than 30 *SFTPB* mutations were reported) (1,3,7,11,21,31–33). However, none of the 22 cases tested had *SFTPB* mutations in this study (**Table 2** and **Figure 1**). There have been no reports of patients with diagnosis of SP-B deficiency in Japanese patients to date (34). The 121ins2 mutation responsible for SP-B deficiency, estimated to occur in ~1 in 1,000 Americans, is not found in Korean or South African populations (31). The ABCA3 deficiency accounts for 48% (12/25) of SP dysfunction disorders in the UK, while its deficiency was seen in only one of the six cases of HILD in this study. Thus, it was suggested that there are ethnic differences in the prevalence rates of SP-B deficiency and ABCA3 deficiency.

There were two patients with ACD/MPV in this study, and one of them had a *FOXF1* mutation (heterozygous p.Leu300ArgfsX79) which is listed in a recent report (19). Approximately 80% of ACD/MPV cases have anomalies of other organs, particularly of the cardiovascular, gastrointestinal, and genitourinary systems (35). The previous study (19) substantiates the suggestion that mutations in *FOXF1* lead to manifestation of ACD/MPV and that this transcription factor is involved in development of the pulmonary, cardiovascular, gastrointestinal, and genitourinary systems. Although rare, late presentation has been reported (36,37) with affected patients typically developing lung dysfunction and pulmonary hypertension a few hours after birth. Our case 6 with late onset at 3 mo after birth who has survived until age 2 y had no anomalies in other organs and is currently being treated with vasodilators and home oxygen. Another case with ACD/MPV in whom the analysis of *FOXF1* was not given exhibited a typical course and died 4 d after birth.

As our E-mail Network of Neonatologists covers almost all neonatologists who treat severe cases in neonatal intensive care

Table 4. Comparison of demographic characteristics between 6 and 37 patients

	Six patients[a]	Thirty-seven patients	*P* value
Preterm birth (<37 wk)	0 (0.0%)	6 (16%)	0.3813
Birth weight <2,500 g	0 (0.0%)	8 (22%)	0.2662
Male sex	3 (50%)	24 (65%)	0.3941
Apgar score <7 at 5 min	0 (0.0%)	7 (19%)	0.3195
Timing of onset			
Immediately after birth	2 (33%)	27 (73%)	0.0767
Within 4 wk after birth	4 (67%)	30 (81%)	0.3686
iNO	2 (33%)	16 (43%)	0.5034
Assisted ventilation	5 (83%)	30 (81%)	0.6922
More than 30 d	5 (83%)	10 (27%)	0.0146
Positive for PAP on BAL	3 (50%)	0 (0.0%)	0.0016
Histologically proven ILD	3 (50%)	3 (8.1%)	0.0272

BAL, bronchoalveolar lavage; ILD, interstitial lung disease; iNO, inhaled nitric oxide; PAP, pulmonary alveolar proteinosis.
[a]Six infants in whom causative genetic abnormalities were detected, not including two cases (cases 7 and 8 in **Table 3**) because it was uncertain whether detected variations were causative for their lung dysfunction.

units in Japan, most infants with severe and unexplained lung dysfunction may have been referred to us. However, it was possible that some neonates who died very early were not referred to us. Four of the nine infants with ILD including one with ACD/MPV but not with *FOXF1* analysis were born after the announcement of this system in February 2011, and three of the four were identified to have genetic abnormalities (cases 2, 3, and 6). As there were ~2,625,000 neonates during the 2.5-y study period in Japan, the prevalence of HILD was estimated to be at least one in 875,000 (3/2,625,000).

Neither clinical factors, such as a low Apgar score and timing of onset of lung dysfunction, nor use of inhaled nitric oxide differentiated HILD from patients with unproven gene mutations in this study. However, in patients in UK, respiratory distress at birth was the presenting symptom in all six infants with *SFTPB* mutations and in 10 of 12 infants with confirmed *ABCA3* mutations, while patients with *SFTPC* mutations were more likely to present with chronic cough, failure to thrive, or oxygen dependency during later stages (24). Indeed, three of the four patients with *SFTPC* mutations manifested lung dysfunction later, i.e., 6 d, 2 wk, and 5 mo after birth, while one infant with *ABCA3* mutations showed lung dysfunction immediately after birth in this study. In addition, gene mutations were efficiently detected in infants with assisted ventilation for more than 30 d, positive findings for pulmonary alveolar proteinosis in the BAL, and/or histologically confirmed ILD (**Table 4**). This information may be useful for clinicians in searching for specific mutations responsible for lung dysfunction.

In conclusion, 43 infants with severe and unexplained lung dysfunction were referred to us over a 2.5-y study period. Nine (21%) of these patients were diagnosed clinically as having ILD. Mutations of *SFTPC* in four cases, *ABCA3* in one case, and *FOXF1* in one case were considered to be responsible for ILD in six of the nine patients. None of 22 patients including 20 with early onset and no improvement of lung dysfunction and two with late onset and suspected hPAP had *SFTPB* mutations. These observations suggested that the prevalence of HILD is at least 1 per 875,000 Japanese infants, the SP-C abnormality is the most prevalent aberration, and SP-B deficiency is rare among Japanese infants with ILD.

METHODS

The present system was announced to Japanese neonatologists via an E-mail Network of Neonatologists in February 2011 after receiving approval from the Institutional Review Board of the Hokkaido University Graduate School of Medicine. Collaboration with the Japan Society for Premature and Newborn Medicine was begun in February 2012 to facilitate collection of cases with severe and unexplained lung dysfunction: the Japan Society for Premature and Newborn Medicine announced 3,300 neonatologists four times annually since February 2012 to register prospectively patients with unexplained sustained respiratory distress due to genetic disorder or unknown origin, and not patients with respiratory failure due to known reasons including infection, congenital heart disease, systemic bone disease, neuromuscular disease, malformations, pulmonary hypertension after birth asphyxia, and bronchopulmonary dysplasia caused by prematurity. Most Japanese neonatologists were members of the Japan Society for Premature and Newborn Medicine, and the members were working at ~90% of all facilities with neonatal intensive care units in Japan.

Patients

All of the 43 families provided detailed clinical information, and blood samples were obtained for genetic analysis with written informed consent. Our system offered a stepwise laboratory examination for patients with unexplained lung dysfunction (**Figure 1**). All 43 cases underwent first-round examination including analysis of *SFTPC* and *ABCA3*. Then, 22 cases including 20 with early onset requiring assisted ventilation and no improvement of respiratory status and two cases with late onset and suspected hPAP underwent second-round examination. Analysis of *SFTPB* was performed in these 22 cases. In 17 of them, the BAL or tracheal aspirates were available for SP-B western blotting analysis and microscopic examination. Among the 22 cases, two proceeded to third-round examination of pSTAT-5. Finally, four of five patients with severe persistent pulmonary hypertension unresponsive to treatment underwent analysis of *FOXF1*.

Analyses of the *SFTPC*, *ABCA3*, *SFTPB*, and *FOXF1* Genes

Genomic DNA was extracted from peripheral blood using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). PCR primers reported previously were used for *SFTPC* (23). PCR primers for *SFTPB*, *ABCA3*, and *FOXF1* were designed using Primer3 (38) to amplify all of the coding exons and their exon–intron boundaries. PCR was performed using a GeneAmp PCR System 2700 (Applied Biosystems, Foster, CA) with AmpliTaq Gold 360 PCR Master Mix (Life Technologies, Carlsbad, CA) in a 20-µl reaction mixture containing 40 ng of DNA template, and 0.5 µmol/l of each primer. PCR conditions were variable for each amplicon: incubation at 95 °C for 10 min followed by 40 cycles of three or two steps (depending on primer) of: (i) denaturation at 95 °C for 30 s, annealing at 58–60 °C for 30 s, and extension at 72 °C for 1 min or (ii) denaturation at 95 °C for 30 s and extension at 63 °C for 1 min with a final extension at 72 °C for 10 min. PCR products were analyzed by electrophoresis using 2.0% agarose gels and purified with a QIAquick Gel Extraction Kit (Qiagen). Purified products were subjected to nucleotide sequence analysis by a commercial sequencing service (FASMAC, Kanagawa, Japan). Nucleotide sequences were compared with the reported reference sequences: NM_003018.3 (*SFTPC*), NM_001089.2 (*ABCA3*), NM_198843.2 (*SFTPB*), or NM_001451.2 (*FOXF1*).

SP-B Western Blotting Analyses of BAL or Tracheal Aspirates

BAL and tracheal aspirates were used without centrifugation and lipid extraction. Samples corrected for protein concentration were suspended in Laemmli sample buffer. Tricine/sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed as reported previously (39), and aliquots of 20 µg of protein per lane were separated on 16% polyacrylamide gels. The proteins were transferred onto polyvinylidene fluoride membranes and examined by western blotting. Polyclonal antibody to human SP-B (Hycult, Uden, The Netherlands) was used as the primary antibody at a concentration of 1:50, and horseradish peroxidase–conjugated antirabbit immunoglobulin G (GE Healthcare, Little Chalfont, UK) was used as the secondary antibody at a concentration of 1:1,000. The blots were then visualized using Immobilon western chemiluminescent HRP substrate (Millipore, Billerica, MA).

Analyses of GM-CSF–Induced Phosphorylation of pSTAT-5

Peripheral blood mononuclear cells were suspended in Roswell Park Memorial Institute medium 10% fetal bovine serum at a concentration of 1×10^6 cells per ml and were incubated in the presence or absence of 20 ng/ml GM-CSF for 15 min. Whole cell lysates were prepared by homogenization in 1× sodium dodecyl sulfate sample buffer, separated by glycine/sodium dodecyl sulfate polyacrylamide gel electrophoresis according to the standard procedure, and transferred onto polyvinylidene fluoride membranes. Anti-STAT-5 (pY694) antibody (BD, San Diego, CA) was used at a final concentration of 0.5 µg/ml, and antiactin antibody (Sigma, St Louis, MO) was used at a final concentration of 1 µg/ml as a loading control.

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