Original Article

Prediction Markers for Respiratory Distress Syndrome: Evaluation of the Stable Microbubble Test, Surfactant Protein-A and Hepatocyte Growth Factor Levels in Amniotic Fluid

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Surfactant treatment in infants with respiratory distress syndrome (RDS) has decreased neonatal mortality. With the advent of this therapy, it has become important to predict accurately the fetal lung maturity of a fetus before delivery. We evaluated the stable microbubble test (SMT), surfactant protein-A (SP-A) and hepatocyte growth factor (HGF) in amniotic fluid as predicting markers for RDS. Of 55 amniotic fluid samples obtained by amniocentesis from women less than 37 weeks pregnant, the SMT values were as follows: sensitivity 76.5%, specificity 84.2%, positive predictive value 68.4%, negative predictive value 88.9% and overall accuracy 81.8%. For SP-A, the values were 88.2%, 65.8%, 53.6%, 92.6% and 72.7%, respectively. If we used both SMT and SP-A, we could diagnose with 100% accuracy that a case with measurements of SMT ≥ 2 and SP-A ≥ 420 ng/ml would not complicate with RDS (24/24). However, the RDS diagnostic accuracy of HGF does not equal to those of SMT and SP-A levels. We concluded that the rapidity, simplicity and reliability of SMT was very useful during 24-36 weeks of gestation as a bedside procedure to predict fetuses likely to develop RDS. We also noted the additive effect of SP-A in improving the accuracy of lung maturity diagnosis.

Key words: respiratory distress syndrome, stable microbubble test, surfactant protein-A, hepatocyte growth factor

R espiratory distress syndrome (RDS) is one of the most life-threatening conditions for neonates. Positive clinical responses in infants with RDS to a bovine lung source surfactant were first reported in 1980 [1]: the surfactant treatment improved oxygenation in all infants with severe RDS. Since then, the use of surfactants has become an accepted standard for treatment of RDS in neonates requiring mechanical ventilation. At least 36 randomized, placebo-controlled trials, involving approximately 7000 neonates, have shown that exogenous surfactant is effective in the treatment of pediatric RDS [2]. Surfactant supplementation decreased neonatal mortality by about 33% and increased the chance of survival without chronic lung disease by about 17% [2]. Routine surfactant treatment at birth in all neonates of less than 30 weeks' gestation decreases RDS, but it also results in unnecessary treatment in 37 to 54% of these cases [3-5]. When fetal lung maturity was diagnosed prior to treatment, only 18% of patients were treated [6].

If no evaluation of lung maturity is performed, intensive therapy may be needed to improve immature lung

function, thereby increasing the lungs' susceptibility to injury by a host of factors, including immaturity of the host defense system, infections, barotrauma, brain damage and hyperoxia. Thus, it is very important to accurately predict fetal lung maturity and to promote maturation of fetal lung function before birth. RDS continues to be one of the major causes of neonatal mortality and morbidity. It has been reported that peri-natal mortality caused by dyspnea in Japan in 1999 was 7.16% in infants with birth weights over 500 g and 4.99% in those over 1000 g [7]; in 1995 the figure for the latter group was 5.2% in the United States [8]. These results indicate that predicting the possibility of RDS before birth is still a vital concern. Many tests for evaluation of lung maturity have been reported, including measurement of lecitin/sphingomyelin (L/S) ratio, phos-phatidylglycerol, form stability test, stable microbubble test (SMT), fluorescent polarization, amniotic fluid absor-bancy, surfactant-albumin ratio and surfactant-associated proteins A, B and C (SP-A, -B, -C) [9]. Efficacies have been reported in each test, but the drawbacks of some include complicated procedures and delayed results, making them unacceptable for emergency or daily bedside testing. With the advent of surfactant replacement ther-apy, there is an increasing need for a rapid test to predict development of RDS.

SMT on amniotic fluid, first reported by Pattle et al. [10], is recognized as a rapid, simple and reliable procedure that can identify infants who are likely to develop RDS. SP-A, the major surfactant protein (about 5% of surfactant), is a water-soluble glycoprotein with immunomodulatory and surface activity-promoting prop-erties [11]. SP-A possesses specific receptors on the surface of type II cells [12] and is secreted by type II alveolar cells. Its concentration in amniotic fluid increases during the third trimester of pregnancy, paralleling the increase in surfactant phospholipids during that period [13]. Therefore, measurement of SP-A concentration in amniotic fluid has been used to assess lung maturation [14]. HGF was first reported in the serum of partially hepatectomized rats [15], and was identified as hepatocyte-specific. Now it is considered multifunctional with mitogenic, motogenic, and morphologic influences on a variety of epithelial cells. Recent studies suggest that HGF has strong mitogenic and motogenic effects on pulmonary epithelium [16, 17].

The incidence of babies born before 28 weeks of gestation and thereby likely to need treatment has been increasing; rapid and accurate evaluation of fetal lung maturity has been required to improve their prognosis. In the present study, we aimed to re-evaluate the efficacy of SMT and assess whether other chemical indicators such as SP-A and HGF could improve the RDS prediction rate.

Material and Methods

Fifty-five samples of amniotic fluid were obtained from cases of less than 37 weeks (24–36 weeks) pregnancy, who were undergoing labor and admitted to the Depart-ment of Obstetrics in the Okayama University Medical School Hospital. All amniotic fluid samples were obtained by amniocentesis less than 24 h before delivery.

Immediately after amniocentesis, SMT was perform-ed on uncentrifuged specimens according to the procedure of Pattle et al. [10]. Bubbles formed by agitation with a Pasteur pipette were examined in hanging drops under a 10 X-power microscope. After either a count of the bubbles or a general survey of the hanging drops, the fluid was given a stable microbubble rating. Cases with fewer than 2 microbubbles/mm² were defined as immature because the incidence of RDS is known to increase in cases with microbubbles $< 2/\text{mm}^2$ [10]

The residual amniotic fluid was centrifuged and the supernatant stored at $-20\,^\circ\text{C}$ until use in assays for SP-A and HGF. SP-A was measured by enzyme-linked immunoassay (ELISA) using an SP-A kit from Teijin Chemicals, Ltd. (Tokyo, Japan); HGF levels were measured by ELISA using a human HGF Kit from Otsuka Pharmaceutical Co. (Tokushima, Japan). Sam-ples were assayed in duplicate and the coefficient varia-tions of inter- and intra-assay of both kits were under 5 percent. The predefined cut-off value of SP-A for the kit was 420 ng/ml. There were no reports concerning am-niotic HGF level as a parameter for fetal lung maturation. HGF levels decreased as pregnancy progressed but showed no large variations after 32 weeks of gestation. So we evaluated whether HGF level predicted RDS after 32 weeks of gestation. We used 2.0 ng/ml as a cut-off value of amniotic fluid HGF.

Clinical diagnoses of RDS were made according to the following clinical features and chest X-ray findings. Clinical features: 1) Gradual increase in respiratory rate (over 60 respirations/min), 2) Intercostal retraction, 3) Expiratory moan or grunt, sternal and costal retraction, flaring of nares, and 4) Cyanosis when infant is in room

air. Chest X-ray examinations: 1) A reticulogranular pattern of the lung can be expected, or opacification of both lung fields in severe cases, and 2) The radiolucent bronchial airway extends beyond the heart border because of an air-filled bronchial tree outlined by opacified perihilar areas.

Statistical analysis. Statistical analyses were performed using Student's unpaired test. Analyses were performed using a Statview statistical package for Macintosh (HULINKS, Cary, NC, USA). $P < 0.05$ was defined as statistically significant.

Results

The distribution of RDS and non-RDS cases is shown in Fig. 1. Cases of RDS were distributed between 25 to 35 weeks of gestation. On the other hand, cases of non-RDS were mainly distributed after 32 weeks of gestation. Background and data of SMT, SP-A and HGF of RDS and non-RDS cases are shown in Table 1. There were significant differences in gestational age, birth weight, SMT, SP-A and HGF between the 2 groups.

Distribution of the SMTs shows that the numbers of microbubbles increase with the period of the pregnancy (Fig. 2). Four cases with over 2 bubbles/mm² showed RDS; on the other hand 6 cases with fewer than 2 bubbles/mm² did not complicate with RDS. Distribution of SP-A levels in amniotic fluid is shown in Fig. 3. The level of SP-A also increased with the progress of the

pregnancy. Thirteen of 28 cases with SP-A levels under 420 ng/ml did not complicate with RDS; on the other hand 2 cases with SP-A levels over 420 ng/ml displayed RDS. In summary, SMTs performed with a sensitivity of 76.5% (13/17), specificity of 84.2% (32/38), positive predictive value of 68.4% (13/19), negative predictive value of 88.9% (32/36) and overall accuracy of 81.8% (45/55). SP-A levels showed a sensitivity of 88.2% (15/17), specificity of 65.8% (25/38), positive predictive value of 53.6% (15/28), negative predictive value of 92.6% (25/27) and overall accuracy of 72.7% (40/55). These data suggest that the RDS diagnostic accuracy of SMT is better than that of SP-A.

The details of RDS cases showing false negative SMTs or SP-A levels are shown in Table 2. Four cases

Table 1 Backgrounds of patients

	RDS (n = 17)	non-RDS (n = 38)
Age	28.9 ± 5.2	30.1 ± 4.1
Gestational week	29.8 ± 3.1	33.2 ± 2.7**
Birth weight (g)	1335 ± 577	1913 ± 488**
SMT (No)	3.6 ± 6.9	18.1 ± 19.4**
SP-A (ng/ml)	214.2 ± 190.9	490.7 ± 250.8**
HGF (ng/ml)	9.20 ± 6.15	4.33 ± 5.27*

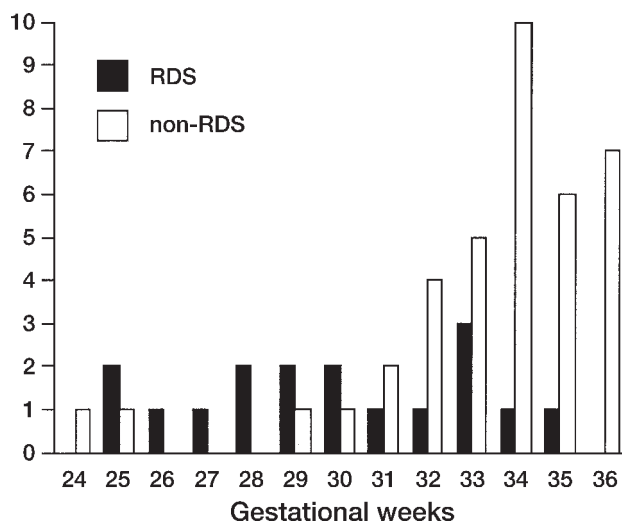Values are mean ± S.D., *$P < 0.01$, **$P < 0.001$ *vs.* RDS.

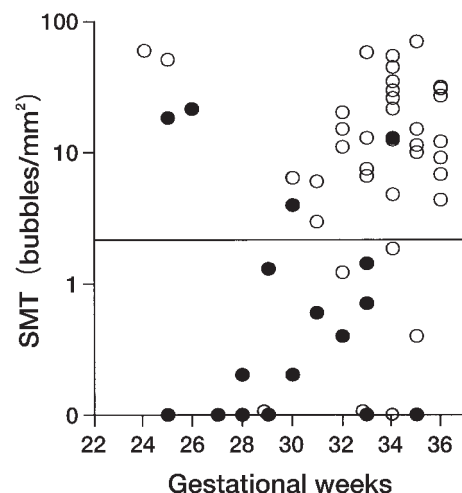**Fig. 1** Incidence of RDS and non-RDS cases by gestational weeks.

Fig. 2 Relationship between SMT and RDS. Cases of RDS (●) and non-RDS (○) are indicated. Number of microbubbles increases as pregnancy progresses. Line indicates the cut-off value. There are 4 false negative cases. There is no significant correlation between SMT value and gestational week (r = 0.0014, $P = 0.97$).
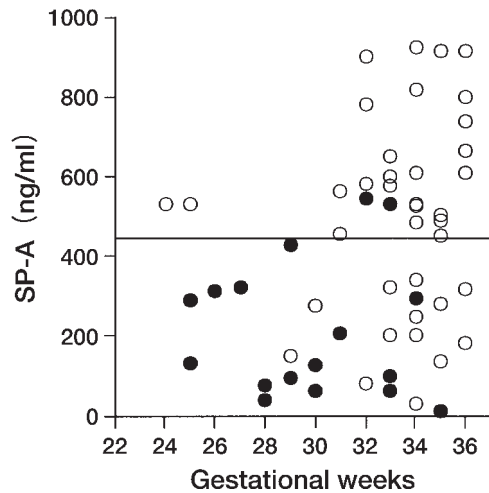
Fig. 3   Relationship between SP-A level in amniotic fluid and RDS. Cases of RDS (●) and non-RDS (○) are indicated. SP-A level increases as pregnancy progresses. Line indicates the cut-off value (420 ng/ml). There are 2 false negative cases. There is significant positive correlation between SP-A level and gestational week (r = 0.94, $P < 0.05$).
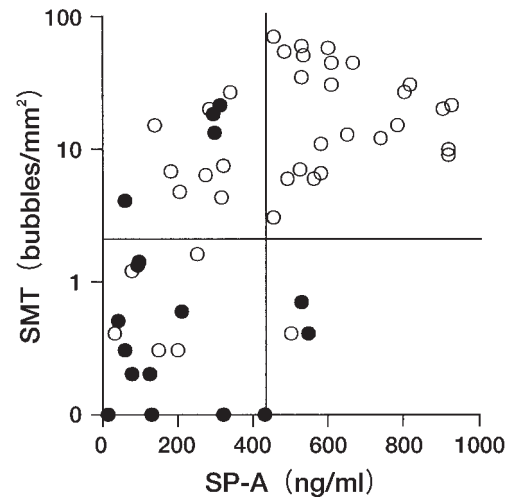

Fig. 4   Relationships between SMT, SP-A and RDS. Cases of RDS (●) and non-RDS (○) are indicated. The diagnostic accuracy of RDS with SMT < 2 and SP-A < 420 ng/ml is 11/16 (68.8%) and that of non-RDS with SMT ≧ 2 and SP-A ≧ 420 ng/ml is 24/24 (100%).

Table 2   Background of false negative cases

A. Cases of RDS with false negative SMT

No	Week	Complications	Birth weight (g)	Apgar score (1 min/5 min)	SMT (bubbles/mm²)	SP-A (ng/ml)	HGF (ng/ml)
1	25W6	PROM	950	5/7	18.4	292.1	13.69
2	26W0	PROM	645	4/6	21.4	312.5	15.24
3	30W5	preeclampsia, DM, oligohydroamnios	795	5/7	4.0	61.6	9.60
4	34W1	preeclampsia	1632	4/6	13.0	295.8	0.65

B. Cases of RDS with false negative SP-A

No	Week	Complications	Birth weight (g)	Apgar score (1 min/5 min)	SMT (bubbles/mm²)	SP-A (ng/ml)	HGF (ng/ml)
1	32W1	brain tumor, oligohydroamnion	1946	5/8	0.4	546.7	1.79
2	33W5	malignant lymphoma	2166	8/9	0.7	529.5	1.88

PROM, premature rupture of membrane; DM, diabetes mellitus.

of RDS with a false negative SMT (Table 2-A) were complicated with premature rupture of membranes, preeclampsia and diabetes. These 4 cases could be predicted RDS before birth combined with measurement of SP-A and/or HGF. Two cases of RDS with false negative SP-A levels were terminated because of maternal complications of brain tumor or malignant lymphoma (Table

2-B). These 2 cases could be predicted as RDS before birth combined with measurement of SMT and/or HGF.

The relationship between SMT, SP-A and RDS is shown in Fig. 4. If we use both SMT and SP-A, 68.8% (11/16) of cases with SMT < 2 and SP-A < 420 ng/ml had RDS, on the other hand 100% (24/24) of cases with SMT ≧ 2 and SP-A ≧ 420 ng/ml were non-RDS cases.

Distribution of HGF levels in cases of with and without RDS is shown in Fig. 5. HGF levels significantly decreased as pregnancy progressed and showed significant negative correlation with gestational week (r = 0.740, $P < 0.001$). In addition, HGF levels in non-RDS were significantly lower than those in RDS (Table 1). The changes were dramatic before 32 weeks but showed no large deviation after 32 weeks of gestation. Hence, we evaluated the efficacy of HGF as a predicting marker of RDS in cases after 32 weeks of gestation (n = 38) using cut off values 3.0, 2.5 and 2.0 ng/ml and found that 2.0 ng/ml was most reliable (Table 3). HGF levels showed a sensitivity of 66.7% (4/6), specificity of 53.1% (17/32), positive predictive value of 21.1% (4/19), negative predictive value of 89.5% (17/19) and overall accuracy of 55.3% (21/38). This data suggests that the RDS diagnostic accuracy of HGF does not equal those of SMT and SP-A levels, even in cases after 32 weeks of gestation.
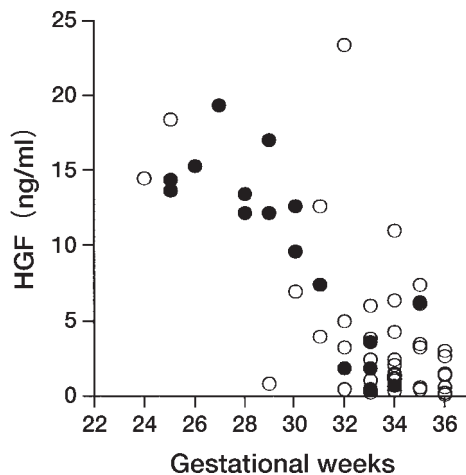

Fig. 5   Relationship between HGF level in amniotic fluid and RDS. Cases of RDS (●) and non-RDS (○) are indicated. HGF levels in amniotic fluid do not show remarkable changes with pregnancy. There is significant negative correlation between HGF level and gestational week (r = 0.74, $P < 0.001$).

Table 3   Disribution of HGF after 32 weeks of gestaion

	HGF (ng/ml)		Total
	< 2.0	≧ 2.0	
RDS	4	2	6
non-RDS	15	17	32
Total	19	19	38

Discussion

Surfactant deficiency at birth is the main precipitating factor for RDS. Pulmonary surfactant is synthesized and stored in type II alveolar epithelial cells as lamellar bodies, which are released by exocytosis into the alveolar space, where they are transformed into tubular myelin, a lattice-like structure that gives rise to the surfactant monolayer that lines the alveolus [18]. Pulmonary surfactant is composed of approximately 80% glycerophospholipid, 10% cholesterol and 10% protein by weight. Dipalmitoylphosphatidylcholine, the most abundant surfactant glycerophospholipid, is the primary component responsible for the surface tension-lowering properties of the surfactant [18].

The lecithin/sphingomyelin (L/S) ratio is a well known and widely used measure for assessing fetal lung maturity and the risk of RDS when early delivery is under consideration [19]. This indicator depends on the flow of fetal lung fluid into the amniotic fluid changing the amniotic phospholipid composition in concert with changes in fetal lung maturation. The L/S ratio for a normal pregnancy is less than 0.5 at 20 weeks' gestational age. A value of 2.0 is achieved by 35 weeks' gestational age, and empirically RDS is unlikely if the L/S is more than 2.0 [19]. The sensitivity and specificity of the L/S ratio increases when phosphatidylglycerol is also determined [20]. But an L/S ratio measurement is not simple; it is a difficult test to perform and interpret, and care at each step of sample handling is critical for consistent results [21].

SMT on amniotic fluid is recognized as a rapid, simple and reliable procedure that can identify infants who are likely to develop RDS. The test takes 5 to 10 min to perform, is cheap and easy, and is not affected by blood, but may be by meconium. In this test, a 'strong' rating (microbubble ≧ 20/mm²) indicates that idiopathic RDS will not occur after delivery, and that the L/S ratio will indicate maturity. Complete absence of stable microbubbles suggests a high risk of respiratory trouble for the newborn infant, as does a 'weak' (microbubble 2–10/mm²) or 'very weak' (microbubble < 2/mm²) rating in the 30 to 37 week gestational age group. The incidences of RDS in 'weak', 'very weak' or 'zero' were 17%, 80%, 50% [10] or 0%, 14.5%, 80% [22], respectively for 2 different studies. These results had good compatibility with our data. Our SMT data using amniotic fluid showed a positive predictive value of 68.4%, and a negative

predictive value of 88.9%, indicating that it was necessary to combine the tests to increase the positive predictive value.

After birth, SMT is performed with gastric aspirate and is also useful to predict RDS [23, 24]. Chida et al. [23] reported that an SMT with a predefined cut-off value of less than 5 bubbles/mm^2 for amniotic fluid and less than 10 bubbles/mm^2 for gastric aspirate signified the risk of RDS with the positive predictive value of 100% and 96% and with the negative predictive value of 91% and 84%, respectively.

Chida and Fujiwara evaluated the clinical usefulness of SMT in predicting the development of RDS by comparison with other tests in amniotic fluid samples obtained under 12 h before delivery from 40 pregnancies of between 23–35 weeks gestation. These tests included the L/S ratio, disaturated phosphatidylcholine/sphingomyelin (DSPC/S) ratio, concentrations of lecithin, DSPC, and SP-A, -B and -C [25]. The overall diagnostic accuracy of SMT was similar to that of other tests. However, both SMT and SP-B,C concentrations had positive predictive values of 100% [25]. SP-A is the most abundant of the specific surfactant proteins and is synthesized primarily in Type II pneumocytes [26]. SP-A positive cells appear at around gestational week 21 in large bronchi, around week 25 in medium bronchi and around week 26 in the alveoli, and after then increase rapidly in number [27]. Tracheal fluid SP-A levels in neonates with RDS soon after birth was 1/8 to 1/9 lower compared with neonates without RDS, and initial SP-A concentrations correlated inversely with severity of RDS [28].

In normal pregnancies, the concentration of SP-A in the amniotic fluid at less than 30 weeks gestation is very low. It then increases approximately 6.5-fold from 34 to 36 weeks of gestation and approximately 15.5-fold at 37 weeks of gestation [20]. Thus, it accurately predicts lung maturity. Hallman et al. reported that immature levels (less than 0.6 μg/ml) predicted 59% of all cases of RDS with an accuracy of 91%, and mature levels (greater than 3.0 μg/ml) predicted 68% of all infants who would not have RDS with an accuracy of 100% [20]. Satoh et al. reported that the sensitivity, specificity and accuracy of SP-A for prediction of RDS were 100%, 83% and 88%, respectively. These results were compatible with those for L/S ratio, disaturated phosphatidylcholine determination and the SMT [29]. Our SP-A data using amniotic fluid before birth showed a sensitivity

of 88.2%, specificity of 92.6% and overall accuracy of 72.4%, respectively. A little difference between Satoh's data and ours may depend on the difference of cut off value and distribution of cases.

After birth, SP-A measurement in tracheal aspirates is also useful for diagnosis of RDS, assessment of the eventual outcome of the pulmonary disease, and evaluation of prognosis after exogenous surfactant therapy [28]. Moya et al. [28] reported that SP-A and SP-C were lower in neonates with RDS than in control infants. Initial SP-A concentrations correlated inversely with severity of RDS. Boo et al. reported that SMT of tracheal aspirates had a higher overall accuracy for the diagnosis of RDS than did measurement of SP-A levels (94.6% vs. 82.4%) [24]. Our results also showed SMT of amniotic fluid had a higher overall accuracy for the diagnosis of RDS than did measurement of SP-A levels (81.8% vs. 72.7%). However, if we used both tests, we could diagnose cases with SMT $\geq$ 2 and SP-A $\geq$ 420 ng/ml as non-RDS (24/24, 100%). It is known that maternal hypertension, preeclampsia, maternal cardiovascular disease, placental infarction, intrauterine growth restriction, prolonged rupture of membrane, Rh isoimmunization and smoking affect fetal lung maturation [9, 30, 31]. Indeed, 5 of 6 RDS cases that showed mature levels of SMT or SP-A involved such complications (Table 2), suggesting that further precise evaluations of fetal lung maturity using multiple markers were required in such cases.

It has been reported that some hormones and growth factors including dexamethasone, retinoids, epidermal growth factor (EGF), fibroblast growth factor (FGF) and HGF promote fetal lung growth and differentiation [16, 17, 32–35]. HGF and its receptor, the product of c-MET proto-oncogene, are highly expressed in both fetal and adult lung [34]. HGF has strong mitogenic and motogenic effects on pulmonary epithelial cells [16, 17] and stimulates [^{3}H] thymidine incorporation into type II cells in primary cultures [17, 34]. HGF mRNA is detectable from day 14 in fetal rat lung by RT-PCR [34]. The mean level of HGF in amniotic fluid was 12.4 $\pm$ 4.5 ng/ml (second trimester) and 10.5 $\pm$ 6.6 ng/ml (third trimester) [36]. This indicates that HGF exerts its effects on type II cells as a potent mitogen and plays a crucial role in the morphogenesis of both alveolar and bronchial epithelia in rat fetal lung.

We measured HGF in amniotic fluid and investigated its possibility as a predicting marker of RDS. However,

HGF levels in the RDS group were significantly higher than those of the non-RDS group and were not so effective in predicting RDS. This may be because HGF shows significant negative correlation with gestational week and there is no difference in amniotic HGF levels between the second and the third trimesters [36] or that HGF impacts the lungs mainly in the postnatal period. Recently, Kagoshima *et al*. [35] reported that HGF mRNA in lung was either undetectable or very low during late gestation and the neonatal period, and increased markedly to reach a maximum at 3–4 weeks postnatally. HGF mRNA and the HGF receptor increased within a few weeks of birth, suggesting that it may play roles in organ growth, organ maturation and the maintenance of tissue homeostasis during the postnatal period, presumably through its potential to act as mitogen, motogen and morphogen [35].

We conclude that the rapidity, simplicity and reliability of SMT using amniotic fluid during 24–36 weeks of gestation is very useful as a bedside test to identify fetuses who are likely to develop RDS after birth. We also concluded that the SP-A level has an additive effect in improving the accuracy of the diagnosis of lung maturity.

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