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Genetic basis of respiratory distress syndrome

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1. ABSTRACT

Respiratory distress syndrome (RDS) is a multifactorial developmental disease caused by lung immaturity and presenting as high-permeability lung edema ("hyaline membrane disease"). It is characterized by a transient deficiency of alveolar surfactant during the first week of life. During the first few days of life, the alveolar surfactant pool size increases up to that in the controls. The allelic variants of the genes encoding the surfactant proteins (SP) *SP-A1*, *SP-A2*, *SP-B,* and *SP-C* have been associated with RDS. The main *SP-A* haplotype, interactively with the *SP-B* Ile131Thr polymorphism and with constitutional and environmental factors, influence the risk. Case reports on mutations with partially functional SP-B have been published. The genetic susceptibility factors depend on the degree of prematurity at birth, consistent with sequential differentiation of the lung and gestation-dependent differences in clinical presentation. The preferentially type 2 cell expressed genes involved in critical functions (such as ATP-binding cassette transporter, *ABCA3*), those involved in susceptibility to acute lung damage, and those with known susceptibility to other severe lung diseases (such as G protein-coupled receptor for asthma susceptibility, *GPR154 alias GPRA*) will possibly serve as candidate genes in future studies. RDS associated with near-term and term births may have a different genetic predisposition and pathogenesis compared to RDS after very preterm birth. As we learn more about the molecular consequences of allelic variation, new therapies based on a new generation of surfactant diagnostics and individualized therapies may follow.

2. INTRODUCTION

Despite the fact that perinatal respiratory adaptation has, through numerous generations, evolved into an effective process of adaptive sequences, respiratory failure has been the most common cause of death in early infancy. As a result of the development of antenatal and neonatal special care, including antenatal glucocorticoid therapy, exogenous surfactant therapy, and advanced ventilation management using continuous distending pressures, the prognosis has improved dramatically. However, RDS continues to be a serious, sometimes fatal disease that also is a major risk factor of bronchopulmonary dysplasia (BPD) and intraventricular hemorrhage. The current estimate of the global death rate ranges between 0.1 and 0.3 million annually (1).

Early seminal studies suggested that the deficiency in extracellular surfactant is the major pathogenetic factor precipitating RDS (2) that is associated with the degree of prematurity. However, some infants born at term develop RDS, while others born extremely premature have no RDS. The risk of RDS of an unborn fetus can be predicted by measuring the surfactant that has been secreted from the fetal lung into the amniotic fluid (3). There is a very large individual variation in the timing of surfactant maturation associated with constitutional, environmental, and/or genetic factors (4, 5). In RDS, the small alveolar surfactant pool in the alveolar lining begins to increase shortly after birth, and within a few days after the birth, the amount of this extracellular surfactant pool becomes similar to that present in age-matched controls (6).

Table 1. Adjusted risk factors for RDS and factors protecting from RDS in preterm infants

Risk	Protection
Degree of prematurity	Antenatal glucocorticoid one day to one week before birth at <34 weeks
Cesarean birth with labor vs. vaginal birth	Presenting multiple vs. non-presenting multiple
Cesarean birth without labor vs. Cesarean birth with labor	Smaller twin pair
Very preterm twin vs. very preterm singleton (<30 weeks)	Preterm twin vs. preterm singleton (>32 weeks)
Acute birth asphyxia	Chorioamnionitis resulting in extremely preterm birth
Infant of diabetic mother	Female gender
Hydrops fetalis	Chronic retroplacental bleeding
	Hyperthyroidism

Apart from surfactant deficiency, a number of interactive constitutional, environmental, and/or genetic factors disturb neonatal respiratory adaptation or delay the infant's recovery from RDS. Severe lung diseases, most notably acute lung injury (ALI), also called acute RDS (ARDS), and BPD associate with surfactant defect (7, 8) or surfactant dysfunction (9) that appears to be more persistent than in RDS.

Very premature infant (<32 weeks of gestation) used to die, regardless of the RDS, and susceptibility to RDS in very preterm infants has hence not been under evolutional selection pressure. In the present brief review, we discuss the pathogenesis and the known genetic susceptibility factors. According to the current view, RDS manifesting after preterm to even term births, are somewhat separate disease entities with different risk profile and clinical features than RDS among infants born very premature.

3. EPIDEMIOLOGY AND DIAGNOSTICS

The genetic susceptibility to RDS is likely to be influenced by the development of prevention and treatment practices. The reported incidence of RDS is 0.5 to 1.5%, the major determinant being the prematurity rate. The risk factors are listed in Table 1. The incidence of RDS among extremely immature infants has increased parallel to early survival, whereas the incidence among less preterm infants has decreased as a result of the general coverage of the high-risk population with antenatal glucocorticoid treatment and evaluation of fetal lung maturity before elective near-term deliveries (10-12).

Chest X-rays reveal diffuse reticulogranularity, whiteout, or ground-glass appearance of lung fields. Poor lung compliance, small residual lung volume, and surfactant deficiency are characteristic findings shortly after birth. The natural course reveals progressive respiratory failure with tachypnea, retractions, cyanosis, and apnea during the first day (progression), a more stable respiratory failure during the following 1-3 days (plateau), and gradual recovery with at least 50% mortality (10). As a result of advanced management (surfactant therapy, techniques based on continuous distending airway pressure) the treatment failures are rare and symptoms are mostly brief in duration (13, 14). The clinical course and treatment requirements are generally different for the groups of infants of extremely low gestational age (ELGA, <28 weeks of pregnancy), those born very preterm (28-31 weeks), preterm (32-34 weeks), near-term (35-36 weeks) or term (≥37 weeks).

A non-exhaustive list of differential diagnoses includes meconium aspiration, microbial pneumonias, ALI, BPD, lung hypoplasia, alveolar-capillary dysplasia, congenital pulmonary alveolar proteinosis, persistence of pulmonary hypertension (PPHN), and patent ductus arteriosus (PDA) with congestive cardiac failure. Wet lung syndrome (transient tachypnea), is a sequence of relatively mild respiratory distress, spontaneously resolving within two days. It mimics those cases of surfactant deficiency that are treated with exogenous surfactant shortly after the birth and did not develop RDS.

4. PATHOGENETIC FACTORS IN RDS

RDS is a multifactorial syndrome. On the basis of current knowledge, it is unlikely that any overriding genetic susceptibility factors will be found. The degree of prematurity strikingly influences the risk, undermining other risk factors. Genetic susceptibility factors appear different in extremely preterm and near-term infants. Genetic factors are diluted by a number of stress factors related to high-risk births.

4.1. Structural immaturity of the lung

During the 7th week of gestation, the embryonic lung bud lined by endoderm appears as a diverticulum of the foregut, dividing into branches in the surrounding mesodermal tissue. During the pseudoglandular stage (8-17 weeks), the centrifugal division of airway buds proceeds variably 16-22 times, and various airway cells, including Clara cells with 10 kDa CC10 protein, begin to differentiate.

The canalicular stage (16-25 weeks) is characterized by the formation of 2-3 generations of respiratory bronchioli and the alveolar duct. By 20-22 weeks, elongated type 1 alveolar epithelial cells and cuboidal type 2 alveolar epithelial cells with lamellar bodies are increasingly seen. The thinning of the interstitium between the epithelium and the increasingly robust underlying capillaries, lead to the formation of a future blood-gas barrier sufficient to potentially sustain gas exchange from 20-22 weeks on. During the saccular stage from 24 weeks towards the term, the terminal future airspaces enlarge, while they are delineated with rather thick septal walls and a double capillary network.

At the beginning of the alveolar stage from 30-36 fetal weeks onward, the terminal saccules of the alveolar ducts form small crests subdividing the walls. These secondary septa elongate producing primitive alveoli, which further develop into cup-shaped alveoli with single

capillary layers and double capillary supply. The formation of new alveoli continues during two years after birth (15).

4.2. Abnormal pulmonary adaptation due to immaturity

In normal respiratory adaptation after birth, the fetal lung fluid is replaced with a similar volume of air, pulmonary circulation increases 6- to 10-fold within a few minutes, and the interstitial fluid decreases by >70% within a few hours. One to a few forceful expirations with cry and forceful inspirations with high negative transpulmonary pressures are required. Gestation-related structural immaturity includes a compliant chest cage with inadequate capacity to generate the requisite high negative transpulmonary pressures, a tendency to apnea, and poor connective tissue support of airspaces surrounded by liquid-rich interstitial spaces.

The deficiency of active ion transport for the clearance of lung liquid due to the immaturity of ion transport channels, which is normally activated during labor, delays the clearance of lung fluid (16, 17). Immature lung epithelium actively secretes chloride *via* Cl^- cannels with low reabsorption of Na^+ channels. The transitional stage involves transition in the direction of secretion of water towards liquid reabsorption. The net increase in Na^+ movement from lumen to alveolar epithelium prevents secretion of Cl^- through Cl^- channels. Finally, the adult stage of predominant Na^+ reabsorption involves fully active amiloride-sensitive epithelial Na^+ channels containing alpha, beta and gamma subunits (18).

Depending on the degree of prematurity, copious fluid and macromolecule leakage is induced across the alveolar epithelium shortly after birth. A few hours to several days after birth, a large left-to-right shunt through the PDA increases the risk of cardiac failure and severe lung edema. Inappropriate innate response, defects in the antioxidant system, and other abnormalities in the host defense are likely to perturb the respiratory function by a number of mechanisms, setting the stage to the development of BPD (19).

The failure to decrease the interfacial surface tension at the air-liquid interface of the small airways and airspaces is the major immediate cause of generalized atelectasis, increased work of breathing, and inadequate gas exchange in RDS. High minimum surface tension during inspiration (low stability of the surface film) and increased surface tension during expiration (slow rate of surface adsorption, high equilibrium surface tension) also contribute toward lung edema and obstruction of the small airways. Besides surfactant deficiency, the protein-rich, high-permeability edema fluid inhibits, inactivates and dilutes the surfactant complex. The primary surfactant deficiency and the lung damage additively increase the generalized atelectasis, edema and the formation of fibrin-rich deposits on epithelial surface.

4.3. Regulation of the maturity of fetal lung

A number of hormones and cytokines influence the maturity of fetal lung and the differentiation of the surfactant system. The reader is referred to a recent review (20). Most significantly, antenatal glucocorticoid treatment in imminent preterm birth increases the expression of the surfactant proteins and enzymes required for surfactant phospholipids. In addition, glucocorticoid induces clearance of fetal lung liquid, increases the size of peripheral airways, and decreases the high-permeability lung edema induced shortly after very preterm birth (21). These adaptive events may be influenced by genetic factors. Therefore events, such as antenatal acceleration of the fetal lung development need to be considered as potential modifiers that influence the genetic susceptibility to RDS.

5. SURFACTANT COMPONENTS IN RDS

Alveolar surfactant is synthetized and processed for secretion in type 2 alveolar cells. The surfactant system is highly conserved, and analogous components are found in bird lung and fish swim bladder. The major component is phosphatidylcholine (PC), which contains an exceptionally high percentage of fully saturated dipalmitoyl species, unsaturated PC, and acidic phospholipids. Of the latter, phosphatidylinositol (PI) is present in RDS and in surfactant from extremely preterm lung, whereas phosphatidylglycerol (PG) increases at the expense of PI during advancing gestation. Some cholesterol and other minor components are present. The surfactant proteins (SP) SP-A, SP-B, and SP-C are bound to the surfactant complex, whereas SP-D is mostly not bound to the complex. Apart from the surface tension-lowering functions of SP-B, SP-C, and SP-A (22, 23), all SP are involved in other host defense functions (24).

Surfactant components measured in the amniotic fluid predict the risk of RDS when elective preterm or near-term births are planned. The available methods are based on the concentration of surfactant phospholipids, on the basis of the sedimentable surfactant aggregate, or on the basis of internal standard (saturated PC/sphingomyelin ratio) (4, 25). The concentrations of SP-A and SP-B increase in amniotic fluid as a function of gestation. The SP-A (35 kDa protein) concentration accurately predicts the risk of RDS (26), and it appears in amniotic fluid somewhat later than SP-B (27). The gestational trend for SP-D is less distinct than that for SP-A (28).

Within a few hours after birth, the surfactant pool sizes (6, 29) and the concentrations of surfactant in epithelial lining fluid (25) are strikingly lower in RDS infants than in controls. The surfactant pool size has been measured after the administration of a stable isotope-labeled phospholipid or non-endogenous surfactant phospholipid tracer.

The fractional synthesis rate is slower and the apparent half-life longer than after term birth or after the first postnatal week. Exogenous surfactant tended to increase the secretion times (30), whereas antenatal steroid tended to increase the fractional synthesis rate of PC (31). Unlike the SP-B and SP-A mRNA or the proteins, which appear in this order in canalicular human lung, SP-C

mRNA and proprotein are detectable before differentiation of type 2 alveolar cells (32). The lipid-soluble surfactant components (phospholipids, SP-B and SP-C) are processed from ER *via* Golgi complex and multivesicular bodies to lamellar bodies that secrete its contents into the alveolar lining in a process stimulated by ventilation (33). SP-A that appears to be processed by the constitutive pathway by-passing the lamellar bodies, associates with the surfactant aggregates in the alveolar lining (22). A predisposition to a RDS-like syndrome and disproportionately low SP-C content has been observed in Belgian white and blue calves susceptible to RDS (34). In preterm infants, the alveolar lining contains deficient quantities of several surfactant components (25, 9, 35).

SP-B or stretches of amino acids that mimic SP-B, together with surfactant lipids (apparently an analog of SP-B), remarkably improve the surface properties of phospholipids (36). Synthetic surfactant containing the KL4 peptide and surfactant-associated lipids appears to perform similarly to animal-derived surfactants in the treatment of RDS (37). Being an extremely hydrophobic protein, SP-C disrupts the phospholipid aggregates, increasing the spreading of lipids and recruiting the lipids into the surface films (22). SP-C or its analogs, combined with surfactant lipids, appears to be effective in the treatment of respiratory failure.

6. LOSS OF FUNCTION MUTATIONS INVOLVING SURFACTANT PROTEINS

PC containing fully saturated species and the hydrophobic proteins (SP-B or SP-C) are essential components for appropriate surface tension lowering function. PI or PG remarkably improves the surface activity (22). Since phospholipids are essential membrane components, loss of function mutations involving them are non-viable.

Genetic deletion of SP-A does not result in respiratory failure in term-born mice (38). SP-A improves the surface activity of the lipid extract of surfactant, particularly at low surfactant concentrations (23, 39). SP-A forms tubular myelin aggregates together with SP-B, phospholipids, and Ca++. SP-B and SP-A aggregates show extremely rapid surface adsorption, and the films thus formed can be compressed repeatedly to near-zero surface tension, retaining their surface activity despite inhibitors or oxidants. SP-A and SP-D influence the secretion and the alveolar pool size of the surfactant complex.

SP-A, a member of the collagen-containing lectin (collectin) subgroup of mammalian C-type lectins, including SP-D, mannose-binding lectin (MBL), and conglutinin, are multifunctional proteins involved in host defense (24). Mice with inactivated SP-A gene by gene targeting are susceptible to group B *Streptococci*, respiratory syncytial virus (RSV), and *Pseudomonas*. SP-A and SP-D bind a number of viruses and enhance the binding of bacteria to alveolar macrophages.

Lack of SP-B expression causes respiratory death in mice at birth (40). Similarly, mutations causing a lack of SP-B result in fatal respiratory failure (estimated incidence 1 to 1.5 x 10^6 births). Initially, the disease resembles RDS or occasionally a milder form of respiratory disease similar to wet lung disease. Eventually, the disease progresses to therapy-resistant respiratory failure, unless lung transplantation is performed. The pathologic diagnosis of the present form of interstitial lung disease is mostly congenital pulmonary alveolar proteinosis. Analysis of the material present in alveolar lavage reveals excess of SP-A, SP-D, and phospholipids, but deficient PG and no SP-C. However, large quantities of incompletely processed proSP-C protein (between 6 and 12 kDa) are found in lung tissue and in airway specimens. Electron microscopy reveals abnormal type 2 cell morphology with few abnormal lamellar bodies. In adult mice, inflammatory lung disease generally develops when the tetracycline responsible promoter is silenced to allow 25% of normal SP-B secretion. Mice with heterozygous deletion have 50% expression levels and are sensitive to oxygen toxicity, whereas human carriers of the mutant were reportedly healthy. Milder forms of disease allowing some SP-B production (5 to 10% of the controls) and prolonged survival have been described. Genetically decreased levels of SP-B combined with superimposed O_2-induced injury reveals the distinct contribution of SP-C to pulmonary function in SP-B deficiency (41).

Genetically modified NIH Swiss black mice lacking SP-C expression do not develop neonatal lung disease resembling RDS. However, surfactant from these animals has a high surface tension upon compression, producing unstable films at low lung volume. However, SP-C-deficient (SP-C -/-) 129/Sw mice by the age of 3-6 months developed a severe pulmonary disorder consistent with interstitial pneumonitis. There was progressive emphysema, monocytic infiltrates, epithelial cell dysplasia with hyperplasia of type 2 cells, and increased production of matrix metalloproteinases (MMP-2 and MMP-9) in alveolar macrophages *in vitro* (42, 43).

Dominant mutations of SP-C with an altered single amino acid have been shown to cause severe disease with variable penetrance, ranging from non-specific interstitial pneumonitis, desquamative interstitial pneumonitis, or pulmonary alveolar proteinosis to no clinical disease or late manifestation of the disease. SP-C proprotein containing 191-197 amino acids, is processed through a regulated secretory pathway to an extremely hydrophobic 35 amino acid-containing proteolipid. Most of the mutations described locate on the carboxyterminal proprotein, with one mutation described within the mature peptide sequence (44). The COOH domain (Phe94-Ile197) of pro-protein presents as the BRICHOS domain, present in several proteins, all associated with various degenerative or proliferative diseases, such as British Danish Dementia or chondrosarcoma. It has been proposed that the dominant mutation in SP-C represents a dominant-negative effect or a toxic gain function. It may be related to incomplete processing and to a response and ER stress in an attempt to

destroy the protein that cannot be transmitted to the Golgi complex. This results in inflammatory activation and death of type 2 alveolar cells (44).

7. EVIDENCE OF GENETIC PREDISPOSITION TO RDS

Genetic susceptibility to RDS could be due to rare mutations or due to much more common polymorphisms, although the latter are likely to be predominant. According to the current hypothesis, constitutional, environmental, and genetic factors influencing the risk are interactive, the polymorphism being intertwined with constitutional or environmental risk factors. When large population samples are studied, accurate definitions of the disease and treatment practices are mandatory, since highly efficient and targeted management practices, such as antenatal glucocorticoid therapy (12) or surfactant supplementation, may influence the risk and its genetic component.

The population-based genetic differences manifest as differences in haplotype maps or differences in the polymorphisms of individual genes. Isolated populations originating from a small founder population as recently as several hundred to thousand years ago are genetically more homogenous and may show limited variation of the causative genes compared to old major populations. Selection pressures in early infancy played a significant role since 10 to 50% of all infants used to die during the first year of life, which is also currently the case among underprivileged populations. Hereditary protection against a common severe respiratory disease during early life was a highly significant selection advantage. However, since the mortality of very preterm infants approached 100%, the genetic factors associating with term or near-term birth without the risk of RDS were likely to be selected for.

In genetic research on diseases associated with prematurity, twin studies appear useful, because nearly 50% of twins are born preterm, and the degree of prematurity as a major confounder is equal. Monozygotic (MZ) twins have identical genomes, whereas dizygotic (DZ) twins share 50% of their genes. Thus, the difference in concordance for a disease between MZ and DZ pairs gives an estimate of the genetic contribution to the risk: Hereditability = (concordance rate in MZ pairs - concordance rate in DZ pairs) x 2.

There are several caveats in twin studies. The lower mean gestation at birth (*i.e.* the higher risk and the higher concordance) of MZ compared to DZ twins artificially increases the concordance difference. The gender discordance decreases concordance, since male gender is a constitutional risk factor. Weight discordance also influences the risk of RDS, the heavier twin being more predisposed to RDS (45). These problems can be overcome with proper patient selection. However, the RDS risk is lower for the presenting infant compared to the non-presenting infant (46), and in fact, twins have a different risk profile of RDS compared to singletons (45). These epidemiologic findings tend to undermine the genetic component of RDS.

The genetic factors are so intertwined with the environment that it is difficult to differentiate between environmental and genetic factors. A striking example is the disproportionately high concordance for childhood leukemia in MZ compared to DZ twins. This appears to be due to the spreading of the clonal progeny from one to the other co-twin *via* the vascular anastomoses within the monochorionic placenta (47). Another example is the dilution of concordance by fetal confinement to the cervix, which mostly takes place in the early fetal period. It was shown that the tendency of the presenting fetus to have a lower risk of RDS than the co-twin was dependent on a specific *SP-B* genotype of the presenting twin. However, the *SP-B* Ile131Thr genotype of the non-presenting twin had no detectable influence on the risk (48).

Candidate gene analysis is the principal method of testing the risk related to individual gene variants. Using large pedigrees, whole-genome linkage analysis has identified susceptibility genes predisposing to multifactorial disease (49). Triads involving the sick child and the parent(s) with informative alleles have been used for the evaluation of the transmission of the disease gene. A similar method can be used to study the transmission of an allele that protects from the disease (50). The transmission disequilibrium test (TDT) evaluates the transmission rates of different alleles. A significant TDT result – *i.e.* a significantly higher transmission rate of the putative disease allele compared to the other - confirms the heredity of the trait, decreasing the problems of unrecognized confounding factors in the population (51).

8. DEFINITION AND IDENTIFICATION OF SPECIFIC GENES

In the case of multifactorial diseases like RDS, the impact of a single gene is often small and difficult to extract. The potential phenotypic effects of common polymorphisms arise interactively with environmental and constitutional factors and are expected to cause only subtle variations at the molecular level, such as reduced expression or altered ligand binding. Most of the genetic sequence variants that contribute to variability in complex human traits will have small effects that are not readily detectable with population samples typically used in genetic association studies. Furthermore, due to diversity, correct definition of the phenotype among disease subtypes and severities is essential and far from simple.

If multiple generations with affected subjects were available for studies, linkage analysis could be useful in mapping the chromosomal locations of the causative or disease-associating genes. It should be noted that even a very significant positive finding in linkage analysis or a candidate gene study is always merely an indication of an association, not necessarily a causal relationship, between a genetic marker and a disease. Causality cannot be proven conclusively without experimental evidence. So far, candidate gene analysis has been the sole approach in attempts to study the genetic determinants of RDS. Using large pedigrees,

Table 2. The surfactant gene polymorphisms that have been most widely used for RDS candidate gene association studies

Gene (official symbol)	dbSNP[1]	Major/minor allele[2]	Amino acid(s)	Location in the gene	Location in the protein
SP-A1 (SFTPA1)	rs1059047	T/C	19 Val/Ala	Exon 1	N-terminal domain
	rs1136450	G/C	50 Val/Leu	Exon 1	Collagenous domain
	rs1136451	A/G	62 Pro	Exon 2	Collagenous domain
	rs1059057	A/G	133 Thr	Exon 4	CRD[3]
	rs425357	C/T	219 Arg/Trp	Exon 4	CRD
SP-A2(SFTPA2)	rs17880809	A/C	9 Asn/Thr	Exon 1	N-terminal domain
	rs17886395	G/C	91 Ala/Pro	Exon 2	Collagenous domain
	rs17884713	C/T	140 Ser	Exon 4	CRD
	rs1965708	C/A	223 Gln/Lys	Exon 4	CRD
SP-B (SFTPB)	rs1130866	T/C	131 Ile/Thr	Exon 4	N-terminal propeptide
	- (Δi4, length variation)	invariant/deletion(s)/insertion(s)	-	Intron 4	-
SP-C (SFTPC)	rs8192341	C/A	138 Thr/Asn	Exon 4	N-terminal propeptide
	rs1124	G/A	186 Ser/Asn	Exon 5	C-terminal propeptide
SP-D (SFTPD)	rs721917	T/C	30 Met/Thr[4]	Exon 1	N-terminal
	rs17885900	G/A	180 Ala/Thr[5]	Exon 4	Collagenous

[1] Most SNPs have more than one rs entry, [2] Ethnic differences in allele frequencies, [3] CRD, Carbohydrate recognition domain, [4] Amino acid 11 of the mature protein, [5] Amino acid 160 of the mature protein

Table 3. The most frequent *SP-A1* and *SP-A2* haplotypes or "alleles" $6A^n$ and $1A^n$ as defined by the tagging SNPs, their frequencies, and the typical *SP-A1-SP-A2* haplotypes as a result of strong linkage disequilibrium between the two adjacent genes

Gene	Haplotype name	Haplotype	Frequency[1]	Major SP-A haplotypes
SP-A1	6A	CCGGC[2]	0.04	6A-1A, 6A-$1A^5$
	$6A^2$	TGAAC	0.60	$6A^2$-$1A^0$
	$6A^3$	TCAAC	0.28	$6A^3$-$1A^1$, $6A^3$-$1A^2$
	$6A^4$	TCGAT	0.09	$6A^4$-$1A^5$
SP-A2	1A	CCCC[3]	0.04	
	$1A^0$	AGCC	0.57	
	$1A^1$	CGTA	0.16	
	$1A^2$	CGCC	0.13	
	$1A^3$	AGTA	0.03	
	$1A^5$	CCTC	0.01	

[1] In the Finnish population, from (109), [2] SP-A1 haplotype definitions based on alleles at amino acids 19, 50, 62, 133, and 219, [3] SP-A2 haplotype definitions based on alleles at amino acids 9, 91,140, and 223

whole-genome linkage analysis has been done successfully for several multifactorial diseases, followed by studies of positional candidate genes in loci of interest. In neonatal respiratory diseases, however, dominant impact of the major environmental risk factor (preterm birth) and the lack of families with affected probands in multiple generations have prevented the use of linkage analysis (52).

Another approach for identifying potential candidate genes involves genome wide association studies, using new techniques, including yeast-two hybrid screening, microarray gene expression or proteomic profiling. However, very large patient populations are required for adequate power. The flood of data and analytical methods, however, raise many new challenges (53). Thus far this approach has not been used in studies of neonatal respiratory disease.

The identification of candidate genes associating with RDS as a multifactorial disease is a simple task compared to establishing a causal association of a specific genotype with the disease. *In vitro* or *in vivo* evidence of the functional characteristics of the gene, whether obtained in animal studies or in human disease, are particularly relevant. Haplotype analysis and proteomics will help to illustrate the relevant approach. These and other aspects of the choice of the candidate gene are discussed elsewhere (54, 55).

9. GENES ENCODING SURFACTANT PROTEINS AND THE SUSCEPTIBILITY TO RDS

9.1. Human SP genes and their polymorphisms

The genes encoding SP-A, -B, -C, and -D have been regarded as the prime candidates for susceptibility to RDS, and the studies have so far nearly exclusively focused on them. All of them contain a number of polymorphic sites, as found in e.g. Entrez dbSNP (http://www.ncbi.nlm.nih.gov/SNP/index.html), but the association studies have mostly been performed using a few single nucleotide polymorphisms (SNPs) and a length variation summarized in Table 2.

The human SP-A protein is composed of trimeric subunits formed by two similar but distinct polypeptide chains, SP-A1 and SP-A2, which are encoded by two closely linked, highly homologous genes, *SFTPA1* and *SFTPA2*, each spanning a 5 kb region on chromosome 10q22.2-q23.1 (56). A few tagging exonic SNPs have been used to define the intragenic *SP-A1* vs. *SP-A2* haplotypes as "alleles" $6A^n$ vs. $1A^n$, as illustrated in Table 3**.** As a result of the strong linkage disequilibrium between the two adjacent genes, the most common *SP-A1-SP-A2* haplotypes based on this nomenclature are as follows: $6A^2$-$1A^0$, $6A^3$-$1A^1$, $6A^3$-$1A^2$, 6A-1A, $6A^4$-$1A^5$, and $6A^4$-$1A^2$ (57).

The other member of the collectin family SP-D is encoded by a single 11 kb gene *SFTPD* on chromosome

Table 4. A condensed summary of the most significant associations between the *SP* genes and RDS in the Finnish population, illustrating the importance of the major confounding factors in determining the genetic susceptibility of a complex phenotype[1]

Confounding factor	Subgroup	SP-A1-SP-A2	SP-B	SP-A & SP-B
Degree of prematurity	very preterm singletons (GA<32 weeks)	$6A^2$-$1A^0$: + $6A^3$-$1A^1$: -	131 Ile/Thr: 0 Δi4: 0	$6A^2$-$1A^0$: +; restriction of the SP-A association to SP-B Thr/Thr homozygotes
	near term singletons (GA>32 weeks)	$6A^2$-$1A^0$: 0 $6A^3$-$1A^1$: 0	131 Ile/Thr: 0 Δi4: 0	no interactive association
Degree of prematurity in multiple gestation	very preterm multiples	$6A^2$-$1A^0$: 0	n.a.	no interactive association
	near-term multiples	$6A^2$-$1A^0$: -	n.a.	SP-A1 $6A^2$: -, restriction of the SP-A1 association to SP-B Thr allele carriers
Birth order in multiple gestation	presenting (first-born) twins	n.a.	131 Thr: +	n.a.
	non-presenting twins	n.a.	131 Thr: 0	n.a.

[1] +: increased risk; -: decreased risk; 0: no association; n.a., not analyzed

10q23.3 in the collectin locus approximately 100 kb centromeric from *SFTPA2* (58). Two nonsynonymous SNPs in the exons 1 and 4 have been studied for disease associations.

The 9.5 kb gene encoding SP-B, *SFTPB*, is located on the chromosome 2p12-p11.2 (59, 60). Apart from a nonsynonymous coding SNP in exon 4, noncoding polymorphisms have been included in genetic studies.

The short SP-C gene (*SFTPC*) of only 3.5 kb is located on chromosome 8p21 (61). For some time, it was regarded as non-variable, but it is now known to be as polymorphic as any other gene.

9.2. Association between SP gene polymorphisms and RDS

The allelic variation of the SP genes as potential risk factors in the etiology of RDS has been investigated in several case-control studies (12, 62-68), family-based association studies (50, 64), and twin studies (48, 69, 70). Most of the published studies have been performed in the Finnish population with no racial admixture. Both simplified and multi-parameter analytical approaches in different settings have been used in attempts to identify alleles or allele combinations that, intertwined with a large number of non-genetic constitutional or environmental elements, would play a role in the development of the disease. There are remarkable ethnic-racial differences in the epidemiology of RDS and allele frequencies. Therefore, ethnically homogenous populations are more favourable for studies of complex traits and easier to control for stratification by non-genetic factors than those with variegated genetic background. Indeed, multi-parameter data mining analyses using different statistical approaches and study settings have revealed the extensive complexity of disease-contributing genetic factors.

A condensed overview of the associations evident between the *SP* genes and RDS is summarized in Table 4. The *SP-A1, SP-A2,* and *SP-B* genes have been investigated most extensively and found to associate with RDS. The *SP-C*, but not the *SP-D* gene, has also shown an association, but one that has not yet been replicated in other studies.

The major haplotypes of *SP-A1* and *SP-A2* or the combined $6A^2$-$1A^0$ haplotype have been shown to be associated with an increased risk of RDS in independent populations with a tendency to an additive impact of the homozygous haplotype (12, 66). The risk was confined to infants born very preterm. A Finnish study of near-term twin infants revealed, surprisingly, that the major haplotype was underrepresented in RDS (69). As some 50% of twins are born preterm, the $6A^2/1A^0$ haplotype can be regarded as a neonatal survival factor for twins.

Extremely preterm infants typically develop RDS. The *SP-A1/SP-A2* haplotype $6A^2$-$1A^0$ revealed excess transmission to affected very preterm infants, while $6A^2$ decreased the transmission to "hypernormals" (no RDS despite very preterm birth) (50). On the other hand, the *SP-A1* $6A^3$ haplotype tended to be transmitted to the "hypernormal" phenotype in singletons.

The *SP-B* variations do not seem to be directly associated with RDS in singletons (12, 66). However, a remarkable gene-gene interaction involving the *SP-B* Thr131 and *SP-A* haplotypes has repeatedly been evident (66, 70). In this interaction, the *SP-B*131Thr allele defines the population influenced by the *SP-A* haplotypes. The *SP-A1* $6A^2$ haplotype was associated with an increased risk of RDS in very preterm infants. The Thr131 allele of *SP-B* was associated with RDS in a population of twin infants presenting to the cervix prior to birth but not in the non-presenting twins (48). This gene-environment interaction hence decreased the concordance even in MZ twins. According to an epidemiologic evaluation, the genetic risk *via* SP-B – SP-A interaction was associated with the intrauterine mass rather than with the length of pregnancy at birth.

The specific association of *SP-B* i4 variants with RDS remains open. Studies on the US population suggest an association with RDS (62, 65), whereas neither the *SP-B* i4 deletion nor the insertion variants associated with RDS in the Finnish population (12, 66). However, the deletion variant alleles have repeatedly been associated with BPD in the Finnish (67) and German (71) Caucasian populations.

Among very preterm infants, the *SP-C* Asn138 allele and the 186Asn allele associated with RDS (68). Similar to the mutations of *SP-C*, which increased the risk of interstitial lung disease, gender was also a confounder.

The *SP-C* Asn138 and 186Asn alleles also associated with the risk of spontaneous extremely preterm birth of female fetuses. These intriguing observations need to be confirmed and tested in other populations.

9.3. Studies on mechanisms underlying the association between SP polymorphisms and RDS

Polymorphisms of the *SP-A1, SP-A2*, *SP-B*, and *SP-C* genes associate with RDS. However, the molecular mechanisms underlying these associations remain unknown. Specific alleles affect the different risk categories, as defined by the degree of prematurity or uterine size, multiple pregnancy and gender. In near-term singleton infants, the alleles studied did not influence the risk, a finding that may be due to a lack of power of the available studies or to genetic susceptibility factors other than SP polymorphism.

In RDS, surfactant deficiency lasts for 1 to 5 days, and this gap is currently bridged by the high dose of exogenous surfactant (6, 25). According to preliminary information, the secretion of SP-B tended to be slower in very preterm infants (mean age 11 days) than in more mature individuals (72). In immature fetal lung, the intracellular processing of surfactant phospholipids before secretion proceeds slowly within days, instead of within hours. Therefore the intracellular transport may be rate limiting in immature lung. Gene variants encoding the hydrophobic proteins may influence intracellular processing and trafficking, as the alleles influence the pro-proteins rather than the conserved mature SP-B and SP-C in the alveolar lining.

The *SP-B* Thr131 allele associates with an increased risk of RDS in the presenting twin infant. These infants are exposed to an inflammatory stress by cervical endotoxins and cytokines. After the neonatal period, the carriers of 131Thr are reportedly susceptible to acute RDS due to pneumonia (73). SP-B is a member of the saposin family of proteins, which are involved in membrane perturbation, lipid binding or transport, and immunity (74). The Thr131 variant allows the amino terminus of pro-SP-B to become glycosylated, which may influence the regulated intracellular functions *via* chaperone-modulated pro-protein folding and processing (potentially SP-A and its putative receptor, calreticulin) (52, 66, 70).

The 3'UT gene variants of *SP-A* may influence transcription, splicing, and mRNA stability and the glucocorticoid and the cytokine responses *in vitro* (75, 76). Preterm infants with RDS who responded poorly to antenatal glucocorticoid tended to have the *SP-A1* $6A^2$ haplotype (untranslated exon 4 in $6A^2$ different from the other haplotypes) (12). Glucocorticoid and cytokines regulate interactively both the expression of surfactant proteins (77) and the responses of the fetal monocyte-macrophage lineage (78). The fetal inflammatory response is acutely suppressed and later activated, depending on the length of time after antenatal glucocorticoid.

The major *SP-A* haplotype serves as a hereditary risk factor for RDS among very preterm infants, whereas the same haplotype protects against RDS among near-term twin infants. This reversal of the genetic effect remains unexplained. During the antenatal period, the surfactant components appear successively, and the expression of SP are influenced by proinflammatory cytokines and corticosteroid. In very immature animal lung, the proinflammatory cytokine and glucocorticoid induces the expression of SP-A and SP-B, whereas toward term, cytokines decrease the expression of surfactant proteins (79). As there are *SP-A* haplotype-specific differences in the cytokine and glucocorticoid responses (75), these differences could translate into differences in the risk of RDS.

10. OTHER CANDIDATE GENES

10.1. Introduction

It is to be expected that variations in tens or even hundreds of genes other than SP genes are more or less subtle determinants of individual susceptibility to RDS, but are yet to be identified. Genes encoding the other structural components or involved in the processing of pulmonary surfactant as well as those encoding the proteins specifically involved in the synthesis or intracellular transport of phospholipids could play a role. These include the lung-specific SP-processing proteases pepsinogen C, cathepsin H, and napsin A (80-84). In addition, transcription factors involved in the regulation of embryogenesis and functional differentiation of the distal lung may be considered as candidate genes. The transcription factors known to be important in the cell- or tissue-specific expression of SPs include HNF-3 (FOXA), TTF-1 (Nkx2.1), C/EBPalpha and likely others (85-89). The knowledge of the rate-limiting factors of ontogeny would be helpful in defining the most likely candidate genes. In case the phenotype resulting from the knock-out of a single gene mimics RDS without other serious pathologies, the gene concerned is a prime candidate gene influencing the susceptibility to RDS.

Besides factors influencing the synthesis, transport, secretion, recycling and metabolism of the surfactant complex, activities involved in epithelial permeability, lung water clearance and the caliber of the air spaces may profoundly influence the respiratory adaptation and the risk of RDS. However, the studies investigating the genetic susceptibility remain a future challenge. For instance the potential role of genes encoding epithelial Na^+ channel as a candidate gene remains unknown (90).

10.2. G protein-coupled receptor for asthma susceptibility (GPR154, GPRA)

A novel asthma susceptibility gene (*GPR154 alias GPRA*, for G Protein-coupled Receptor for Asthma susceptibility, also known as NPSR or neuropeptide S receptor) was identified on chromosome 7p14-15 by means of positional cloning (91). Three evolutionary, closely related *GPR154* haplotypes have been shown to associate with an increased risk of asthma-related traits among Finnish, Canadian, and German patients (91-93). GPR154 agonist, neuropeptide S (NPS), co-localizes with GPR154 in the bronchial epithelium (94). The function of GPR154 in the airways is still largely unknown, but the expression

patterns of GPR154 and NPS in terminally differentiated epithelia may relate to a role in innate immunity. GPR154 and its ligand NPS are likely to have diverse biological functions (95, 96).

The results of a very recent case-control pilot study on preterm infants suggest that there is an association between *GPR154* and neonatal RDS (97). Immunohistochemistry showed a similar staining pattern of the GPR154-A and –B isoforms in lung tissue as has been observed in asthma. Interestingly, and contrary to the associations between *SP* gene polymorphisms and RDS in very preterm infants, the association between RDS and *GPR154* haplotypes was observed only in preterm infants born after 32 weeks of gestation. These preliminary findings showed that near-term RDS and asthma populations may share the same susceptibility and protective *GPR154* haplotypes. This preliminary observation among Finnish infants offers a novel alternative for a genetic RDS susceptibility factor other than an *SP* polymorphism, but requires further examination.

10.3. ATP-binding cassette transporter A3 (ABCA3)

The *ABCA3* gene encodes a 1704 amino acid protein, which belongs to the superfamily of ATP-binding cassette (ABC) transporters. They are conserved transmembrane proteins which use the hydrolysis of ATP to energize diverse biological systems and to transport various substrates across cellular membranes. Several members of the ABC superfamily are involved in the transport of phospholipids and sterols (98). Three ABC transporters - ABCA1, ABCA3, and cystic fibrosis transmembrane conductance regulator (CFTR) - are known to be expressed in the lung and to play an important role in lung function or in pulmonary lipid metabolism (99). Absent ABCA1 in -/- mice results in respiratory distress, alveolar proteinosis and alterations in surfactant composition (100). ABCA1 controls the rate-limiting step in serum high-density lipoprotein (HDL) particle assembly, and mutation in *ABCA1* causes Tangier disease. However, pulmonary disease is not commonly associated with Tangier disease.

In the lung, ABCA3 is exclusively expressed in lung alveolar type II cells, where it is robustly localized at the limiting membrane of the lamellar bodies (101, 102). Evidence is beginning to accumulate to support an essential role for ABCA3 in lipid transport and lamellar body biogenesis (103, 104). Loss-of-function mutations in the *ABCA3* gene have been identified as a cause of fatal surfactant deficiency in newborns (105). Ultrastructural examination of the lung tissue of these infants demonstrates the presence of abnormal lamellar bodies. *ABCA3* missense or splice site mutations are not always associated with fatal neonatal disease, but have also been shown to be the cause of less severe respiratory distress and pediatric interstitial lung disease in some infants (106). Furthermore, immunohistochemical staining has revealed markedly reduced staining for mature SP-B while proSB-B staining appeared normal, indicating a potential impairment of proSP-B processing in ABCA3 deficiency (106).

Based on several *in vivo* and *in vitro* observations, ABCA3 seems to be essential for normal surfactant homeostasis. Therefore, common *ABCA3* polymorphisms, which could have a minor effect on protein expression or function, can be regarded as one of the most fascinating novel candidate risk factors for the development of RDS (107). dbSNP lists 175 polymorphisms within the gene, and it remains to be studied whether any of them form disease-associating haplotypes.

11. ConclusionS and PerspectiveS

Candidate gene studies have revealed several genetic determinants of RDS, which are dependent on confounding factors - degree of prematurity, multiple birth and order of birth - reflecting the heterogeneity in the rate limiting factors of surfactant synthesis. In very preterm infants, alveolar surfactant deficiency due to lack of differentiation of the surfactant system is a central factor in pathogenesis and variants of genes encoding surfactant proteins are significant determinants of susceptibility. However, extremely immature fetuses are very likely to develop RDS, regardless of the prominence of protective genotypes, diluting or diminishing the genetic impact. With advancing gestation, the phenotype of RDS and the profile of genetic polymorphism influencing the risk become altered. The mechanisms of these paradoxical findings remain unknown. There are gestation-dependent differences in hormone- and cytokine- induced influences on the expression and mRNA stability of SPs (20,108). *SP* genotype-specific differences in the cytokine or hormone responses may translate into differences in the risk of RDS.

Many challenges remain for the future. The antenatal development and growth of the lung reveals functional differentiation of specific cells and profound remodeling of the extracellular matrix. The recent evidence on genetic susceptibility supports the view that the phenotypes of RDS in very premature *vs*. near-term infants may represent even partially separate disease entities. The new concept that integrates genetic, environmental and constitutional factors complicates the genetic analysis, requiring large, well defined population samples. Other multifactorial lung diseases may additionally share some genetic susceptibility factors with RDS (97) and as yet unknown genetic factors, exerting a global influence on the risk of RDS cannot be ruled out, either. Introduction of genetic diagnostics for neonatal lung disease would require either a tight association with the susceptibility or the availability of focused therapies, capable of neutralizing the disease-promoting genotype.

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