Human SP-A: genotype and single-strand conformation polymorphism analysis

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Krizkova, Livia, Ramasamy Sakthivel, Samuel A. Olowe, Peter K. Rogan, and Joanna Floros. Human SP-A: genotype and single-strand conformation polymorphism analysis. *Am. J. Physiol.* 266 (*Lung Cell. Mol. Physiol.* 10): L519–L527, 1994.—We have previously characterized two surfactant protein A (SP-A) cDNAs termed 1A and 6A, as well as a 6A allelic variant termed 6A^1. These sequences are quite heterogeneous at the 3′ untranslated region (3′UT). Differences between 6A and 6A^1 alleles include an 11-bp insertion/deletion 407 bases downstream from the start of the translation termination codon and a base pair polymorphism (C or G) in exon 1 (position 1,193; White, Damm, Miller, Spratt, Schilling, Hawgood, Benson, and Cordell. *Nature Lond.* 317: 361–363, 1985). The 11-bp (GCCCACTGCCT) segment is present in 6A^1 and absent in 6A. The 6A/6A genotype, in a small number of specimens, showed a trend toward a higher frequency in the black Nigerian population compared with Caucasians. In this report, we examine the frequency of the 6A genotype in a larger number of samples from Caucasians and black Nigerians as well as the meiotic stability of the 3′UT heterogeneity. Slot-blot analysis and allele-specific oligonucleotide probes have confirmed that the 6A/6A genotype is more frequent in the Nigerian population. Single-strand conformation polymorphisms in the 3′UT appear to be stably inherited by members of a three-generation family, suggesting that these nucleotide variants represent natural polymorphisms in the population.

surfactant protein A; respiratory distress syndrome; race; 3′ untranslated region; allelic variation

PULMONARY SURFACTANT, a lipoprotein complex, is essential for normal lung function. It consists primarily of lipids (~90%) and of proteins (5–10%) including surfactant proteins (SP)-A, -B, -C, and -D. The human SP-A locus has been localized on chromosome 10 (3) as assessed by chromosomal hybridizations of SP-A cDNAs corresponding to SP-A gene I and II. Two SP-A genomic sequences as well as their corresponding cDNAs have been cloned and characterized (7, 12, 28). The two cDNAs termed 1A and 6A (7) are homologous to the genomic sequences SP-A gene II and I reported by Katyal et al. (12) and White et al. (28), respectively. A human SP-A pseudogene sequence and a 6A allelic variant sequence, termed 6A^1, have been reported by Korfhagen et al. (13) and Rishi et al. (24), respectively. Two characteristic changes between 6A^1 and 6A alleles have been previously identified. 6A^1 contains a GTC instead of CTC (position 1,193; Ref. 28) and an 11-bp insertion, 407 bases downstream from the start of the translation termination codon (position 3,738, Ref. 28) in the 3′ untranslated (3′UT) region (24).

Deficiency of surfactant production in prematurely born infants can result in respiratory distress syndrome (RDS) (2) which can lead to death in ~10% of the cases. A number of epidemiological (5, 10, 22), biochemical (18, 26, 27), and genetic studies (16) suggest that developmental as well as genetic factors contribute to the pathogenesis of RDS. Gender (5, 23) and race (5, 8, 18) have also been implicated as confounding factors in RDS. For example, in Nigeria the rate of RDS is very low compared with whites or other African populations (17). In a preliminary report, we have suggested that the SP-A 6A/6A genotype appears with higher frequency in the black Nigerian population (24).

Sequences in the 3′UT region of SP-A are polymorphic among individuals (24). The nature of this heterogeneity and possible mechanisms involved in its generation have been examined elsewhere (Floros, Rishi, Veletza, and Rogan, unpublished observations). In this report, we suggest that polymorphisms in this 3′UT region are meiotically stable and confirm the elevated frequency of the 6A genotypes in a larger number of genomic DNAs from black Nigerians.

MATERIALS AND METHODS

Genomic DNA was isolated from whole blood specimens using an adaptation of a previously described method (15). Reagents for the polymerase chain reaction (PCR) were purchased from Perkin-Elmer Cetus. Oligonucleotides used as primers for the PCR and the sequencing reactions were commercially synthesized by Research Genetics, Huntsville, AL and the Macromolecular Core at the Pennsylvania State University College of Medicine. Restriction enzymes and other enzymes were purchased from either Bethesda Research Laboratories or from New England Biolabs, and reagents for dideoxy sequencing from United States Biochemicals. The Pfu DNA polymerase used for PCR-single-strand conformation polymorphism (SSCP) analysis was purchased from Stratagene and mutation detection gel (MDE) gel from Hydrolink.

Slot-Blot Analysis

PCR and slot blots were performed as previously described (24) using gene and 6A allele specific oligonucleotides. Oligonucleotides 97 and 98 are gene specific. Oligonucleotide 97 hybridizes to all known 6A sequences and not to 1A sequences and it serves therefore as positive control (Fig. 6). Oligonucleotide 98 hybridizes to 1A sequences and not to 6A and it has served as negative control (24). Oligonucleotide 96 is allele specific of the 6A variants and hybridizes to 6A^1 but not to 6A. Oligonucleotide 96 hybridizes as expected to 1A sequences as well. Cloned 1A and 6A cDNAs were used as positive and negative controls with the above three (96, 97, 98) oligonucleotides. A 1.7-kb DNA fragment, which contains 6A genomic sequences was amplified using oligonucleotides 7 and 19. The

Table 1. *Frequency of 6A genotypes in Nigerian, Caucasian, and American black groups*

	6A/6A	$6A^1/6A$	$6A^1/6A^1$
Nigerian	70 (44)	21 (13)	8 (5)
Caucasian	45* (37)	44* (36)	11 (9)
Amer. black	31* (12)	36 (14)	33† (13)

Values are given in percent; N = 62 Nigerians, 82 Caucasians, and 39 American blacks. Nos. in parentheses indicate no. of specimens identified with the particular genotype. χ^2 P = 0.001; *Z test $P \leq$ 0.007 when compared with corresponding Nigerian group; †Z test P = 0.007 when compared with corresponding Caucasian or Nigerian group.

7/19 PCR amplified product was purified by a 1.5% low-melt agarose in TAE buffer [0.04 M tris(hydroxymethyl)aminomethane(Tris)-acetate, 0.001 M, EDTA)]. After visualization of DNA under low ultraviolet light, gel slices containing the 1.7-kb fragment were excised, melted at 65–70°C for 30–40 min, and the agarose was removed with two to three phenol extractions followed by two to three ether extractions. The gel-purified DNAs were ethanol precipitated and resuspended in 10 mM Tris·Cl (pH 7.6)-1 mM EDTA (pH 8.0). An aliquot of the gel-purified PCR product was subjected to slot-blot analysis and hybridized with a α-^{32}P-labeled hSP-A cDNA probe. The intensity of each slot band was quantitated by densitometry. Based on the densitometric readings, equal amounts of each 7/19 gel-purified product were immobilized onto duplicate nitrocellulose filter along with the appropriate 1A and 6A cDNA controls (24), prehybridized at 65°C for 1 h in 5× Denhardt's and 100 μg/ml salmon sperm DNA, and then hybridized with ^{32}P-end-labeled allele and gene-specific oligonucleotides [sp act 4–6 × 10^8 counts per minute (cpm)/μg] in 0.3 M NaCl, 0.06 M Tris pH 8.0, 2 mM EDTA, and 1% sodium dodecyl sulfate (SDS) for 2 h at 47°C. Washes were carried out at room temperature for 5 min (twice) in 0.3 M NaCl, 0.06 M Tris (pH 8.0), 2 mM EDTA followed by a 1-h wash at 45°C in the same solution containing 0.1% SDS. The filters were then exposed at −70°C to X-AR film for 6–24 h. Densitometric analysis was carried out as described (24). Briefly, the densitometric readings for the gene-specific oligonucleotides 97 and 98 served as positive and negative controls, respectively, for the 6A genotypes. Values a and b were calculated as follows using densitometric readings of the band intensities in the autoradiograms where oligonucleotides 96 and 97 were used as probes, respectively: a = [test sample − background (6A cDNA)]/[positive control (1A cDNA) − background (6A cDNA)], b = [test sample − background (1A cDNA)]/[positive control (6A cDNA) − background (1A cDNA)]. Then the ratio (a/b) was obtained for each sample. Because oligonucleotide 97 hybridizes to both 6A alleles, value b was used to normalize the a value and obtain the genotype of each specimen. Samples with a/b value ranging from 0.00 to 0.25 were denoted to represent 6A/6A genotype, from 0.251 to 0.75 to represent $6A^1/6A$, and those from 0.75 to 1 or >1 to represent $6A^1/6A^1$. χ^2 Analysis was used to determine the significance of the observations in Table 1. The Z test comparison of proportions (9) was also used to determine whether the proportions of any two groups within a category are significantly different.

PCR-SSCP Analysis

Genomic DNAs from a three-generation family were amplified according to Fig. 2 as discussed in RESULTS AND DISCUSSION and then analyzed by SSCP analysis. In addition to family history, paternity was verified by Southern analysis with the variable number of tandem repeat probe, D15S86 (1). This probe can detect a high degree of stable polymorphisms (14).

PCRs were performed with primers listed in Table 2. Each reaction contained 400 ng of genomic DNA, 400 ng of each

Table 2. *List of the specifications of the oligonucleotides used in present study*

Oligo No.	Oligonucleotide Sequence 5′ → 3′	Strand	Position (From TGA)
7*	TCCAGCCTGAGTGCTCTTGG	Sense	7
10	GAGGCATTTAGGCCATGGGACAGGG	Sense	1
11	CAAAGACTCAAAGGAGTGAATCGTACA	Antisense	641
12	TGTACGATTCACTCCTTTGAGTCTTTG	Sense	615
13	CAGGTCCAGGAAGATGGGTTTGGATCC	Antisense	1,251
19*	GGGGAATTCAGGGTCGCTGTGCCCATGTT	Antisense	1,681
46	ACTCCCCTTGCAAACTCTCC	Sense	198
47	CACGTGCCAGGTCTTAGGAT	Sense	853
57	CACAGTGGAGATTCTCTGGC	Sense	492
68	CAGGTCGACTGCCACAGAGACCTCAGAGT	Antisense	531
96*	TCCTTTGACACCATCTC	Antisense	1,181
97*	GACATGGCATTTCTCCA	Antisense	1,547
98*	GACATGGTGTTTCTCCA	Antisense	1,547
104	CAGGTCGACATCTTCAGGCAAGTGGGGCT	Antisense	1,422
105	CCCGGGTACCGGACAGGGAGGACGCTCT	Sense	18
117	CACAATGTCACGCGGACCAGT	Sense	1,118
173	CACTGCACCCCAGGCAGCCA	Sense	221
174	TGGCTGCCTGGGGTGCAGTG	Antisense	240
175	GTTCCTTCACTTACAGATGG	Sense	298
176	GTGGGCATTCACATCACCCC	Sense	769
177	GGGGTGATGTGAATGCCCAC	Antisense	788
178	ACAGAGGCTATTGACTGAGC	Sense	1,030
179	GCTCAGTCAATAGCCTCTGT	Antisense	1,049
208	TCTGCAGTGGGGGGCTCTTC	Antisense	712
238	CTACCTGGCCTTCTAACCT	Antisense	571

*These oligonucleotide sequences are located at the 5′ flanking sequences (no. 7) and within the intervening sequence (no. 19) following coding exon 2. Oligonucleotides 96, 97, and 98 were characterized by Rishi et al. (24) and are specific for haplotypes $6A^1$ (no. 96) and 6A (no. 97) and gene II (no. 98). The location of all the oligonucleotides noted with an asterisk is noted after the numbering system of White et al. (28). The location of the remaining oligonucleotides is identified from the start of the translation termination codon.

primer, 100 μM each of the four deoxynucleotides, 1 × *reaction buffer 1* recommended for Pfu DNA polymerase [1 × buffer: 20 mM Tris·Cl pH 8.2, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.1% Triton X-100, 10 ng/μl nuclease-free bovine serum albumin (BSA)], 2.5 U Pfu DNA polymerase (Stratagene), and 1 μl of [α-^{32}P]dCTP (3,000 Ci/mmol) in a volume of 100 μl. The reaction was allowed to proceed in a thermocycler (Perkin-Elmer Cetus) for one cycle at 95°C for 5 min, 54°C for 5 min, and 72°C for 2 min and then for 30 cycles at 95°C for 1 min, 54°C for 2 min, and 72°C for 2 min. Oligonucleotides 104 and 105 were used to amplify specific SP-A templates. Initially oligonucleotide 105 was shown to be specific for gene I or 6A cDNA (7, 24, 28), however, recently it was shown to also recognize certain sequence variants of the gene II. The 104/105 PCR product was then gel purified and an aliquot (5 ng) was used as a template to reamplify smaller fragments that were more suitable for SSCP analysis (150–400 bp). Routinely an aliquot of the PCR product was evaluated for purity by gel electrophoresis and a small aliquot of the PCR reaction was used for SSCP gel analysis as follows: 2 μl out of a 100-μl reaction was mixed with 8 μl sequencing stop solution (95% deionized formamide, 20 mM EDTA, pH 8.0, 0.05% bromophenol blue, 0.05% Xylene cyanol), heated at 94°C for 2 min then placed into an ice bath for 2 min and loaded onto an MDE gel. The MDE gel is a Tris borate-EDTA, pH 8.0, sequencing gel that contains 25% MDE gel mix. Electrophoresis was performed at room temperature for 14 h at either 6 or 8 W for segments of < 200 bases and 200–400 bases long, respectively.

After electrophoresis, the gel was transferred to Whatman 3MM paper and dried on a vacuum slab dryer. Autoradiography with Kodak X-Omat AR film at −70°C with an intensifying screen for 12–72 h was usually sufficient to allow detection of the PCR product (4). PCR-SSCP procedure was repeated three to five times for all members of the three-generation family.

RESULTS AND DISCUSSION

Differences in the frequency of 6A genotypes between black Nigerians and Caucasians were suggested in a preliminary study by Rishi and colleagues (24). Here, we further investigate the differences in the frequency of the 6A genotypes and suggest that the 3′UT heterogeneity in these genotypes is inherited in a Mendelian fashion.

Frequency of SP-A Gene I (6A) Genotypes Among Various Groups

To study the frequency of 6A genotypes we took advantage of the one base polymorphism (C or G position 1, 193, Ref. 28) in exon 1. The C and G variants identify haplotypes 6A and 6A^1, respectively. Toward this goal, a 1.7-kb segment of the human SP-A gene I (6A) was amplified from black Nigerian, Caucasian, and American black genomic DNAs with oligonucleotides 7 and 19 (see Table 2). The Nigerian samples were composed primarily of aboriginal groups of the tribes Yoruba and Ibo, although a small number of specimens from other tribes were also included. Equal amounts of the 7/19 PCR products were analyzed by slot-blot analysis using allele and gene-specific oligonucleotide probes as previously described (24). The oligonucleotide specificity has been previously examined (24) and it is also shown in Fig. 6. The results are shown in Table 1. The frequency of the 6A/6A genotype is higher in the Nigerian population (70 vs. 45% in Caucasians). The difference in the proportions of this genotype is significant assuming that the gene pools of these populations can be treated as random variables (Z test, $P = 0.003$). Furthermore, assuming that the 6A and 6A^1 alleles are in equilibrium, the distribution of these alleles (based on 125 individuals; present study and data reported in Ref. 24) in the Caucasian population, appears to be codominant and unbiased according to Hardy-Weinberg equation ($p^2+q^2+2pq = 1$). In the Caucasian population the predicted frequency of heterozygotes is identical to the observed frequency (0.4). In the Nigerian population, however, the frequency of heterozygotes is significantly underrepresented. If, for example, for the sake of argument, we assume that the 6A, 6A^1 alleles in the Nigerian

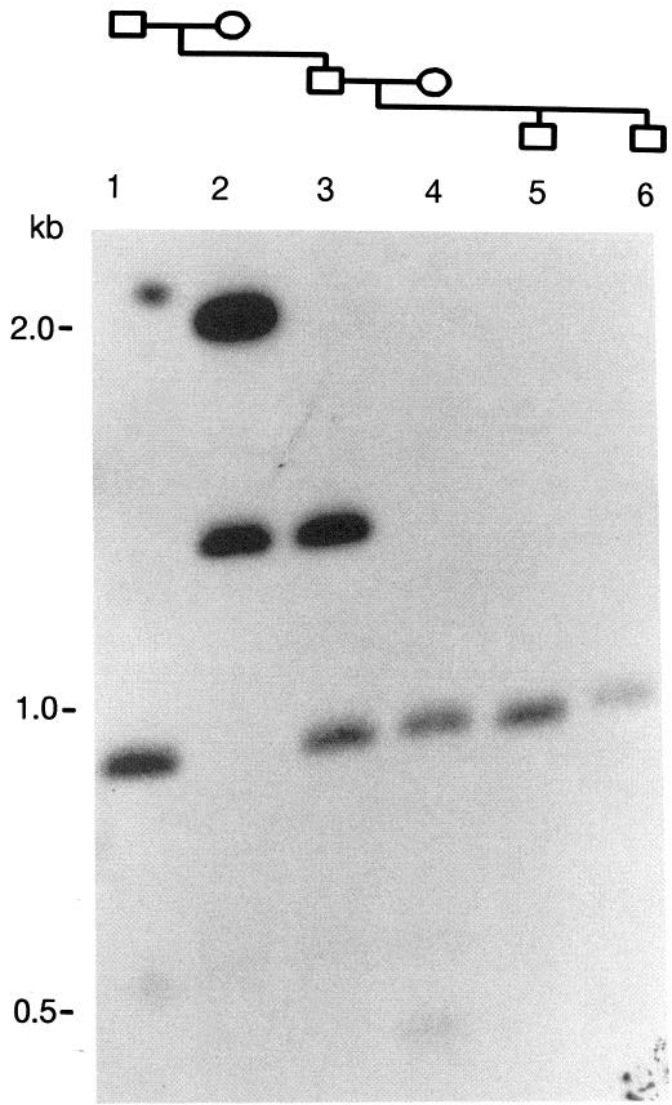

Fig. 1. Southern blot analysis. Ten micrograms of genomic DNA from each family member were digested with *Alu* I and hybridized with a ^{32}P-labeled D15S86 VNTR probe. *Lanes 1-6* contain *Alu* I digested genomic DNA of each family member as indicated. Squares, males; circles, females. *Lanes 1* and *2* contain DNAs from 1st generation, father and mother, respectively. *Lanes 3* and *4* contain DNAs from 2nd generation, son and wife, respectively. *Lanes 5* and *6* contain DNAs from 3rd, sons of *individuals 3* and *4*.

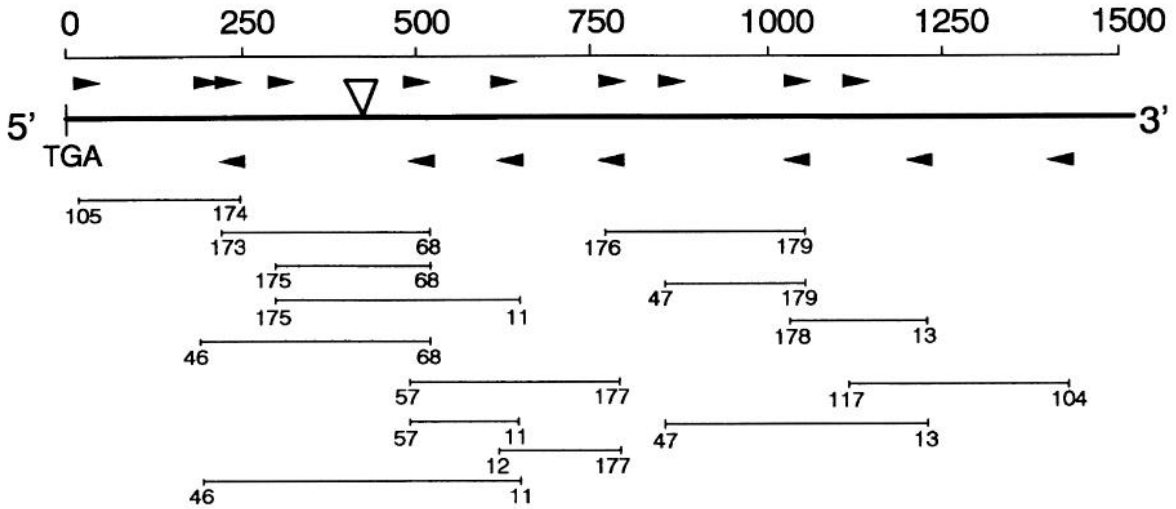

Fig. 2. 3′ Untranslated region of surfactant protein A (SP-A). Arrows, position of oligonucleotides used in study and TGA position is noted. Polymerase chain reaction products used in single-strand conformation polymorphism (SSCP) analysis along with corresponding oligonucleotides that generated them are shown below arrows. Position of 11-bp insertion/deletion is noted by a triangle. Top bar, a scale in bases.

population are in Hardy-Weinberg equilibrium, the expected frequency of heterozygotes is 63%, or 52 of a total of 82 individuals. If we assume 6A and $6A^1$ to be binomially distributed, the SD of ± 5 individuals is calculated for a population of this size. The 95% confidence interval of the number of estimated heterozygotes covers a range of 36–67 individuals. Since only 17 heterozygotes are actually observed (a number outside of the range predicted by Hardy-Weinberg statistics), the observed data suggest population bias.

We reasoned that these findings either represent a genetically isolated population or are one of the factors that contribute to the lower probability of RDS observed in the prematurely born black infants. To distinguish between these two possibilities we examined genomic DNAs derived from Caucasian and American black populations. These results, shown in Table 1, indicate similar frequencies of the 6A/6A and $6A^1/6A$ genotypes in American blacks and Caucasians. The difference in the frequency of these genotypes between the Nigerian population and the other groups may either indicate a founder effect or that the American blacks examined here did not derive a significant amount of their genetic pool from these tribes. Alternatively, it could reflect a selective advantage with regard to RDS. In this circumstance, it is possible that this variant may be associated with factors that predispose this Nigerian group toward earlier developmental lung maturity as previously suggested by Olowe and Akinkugbe (18). Another perhaps less likely explanation is that this genotype came to fixation in the Nigerian population after the conclusion of the transatlantic trade in enslaved peoples. The elevated frequency of the $6A^1/6A^1$ genotype in American blacks may reflect regional enrichment of this allele (possibly a founder effect) in their ancestors.

SSCP Analysis

Meiotic stability. To determine whether the 3′UT variability is meiotically stable or is a consequence of a

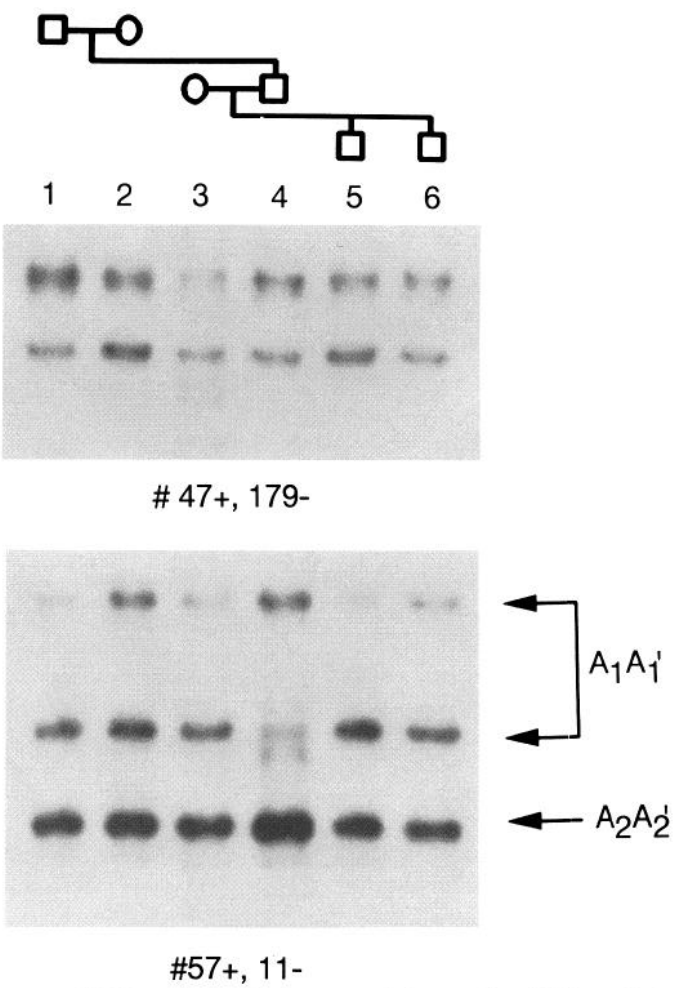

Fig. 3. SSCP analysis of polymerase chain reaction (PCR) products from each family member as indicated by pedigree (see Fig. 1). *Top*, 47+/179− PCR products (196 bp); *bottom*, 57+/11− products (149 bp) following electrophoresis on a mutation detection enhancement nondenaturing sequencing gel. Bands in each *lane* depict number of conformants detected for given DNA region.

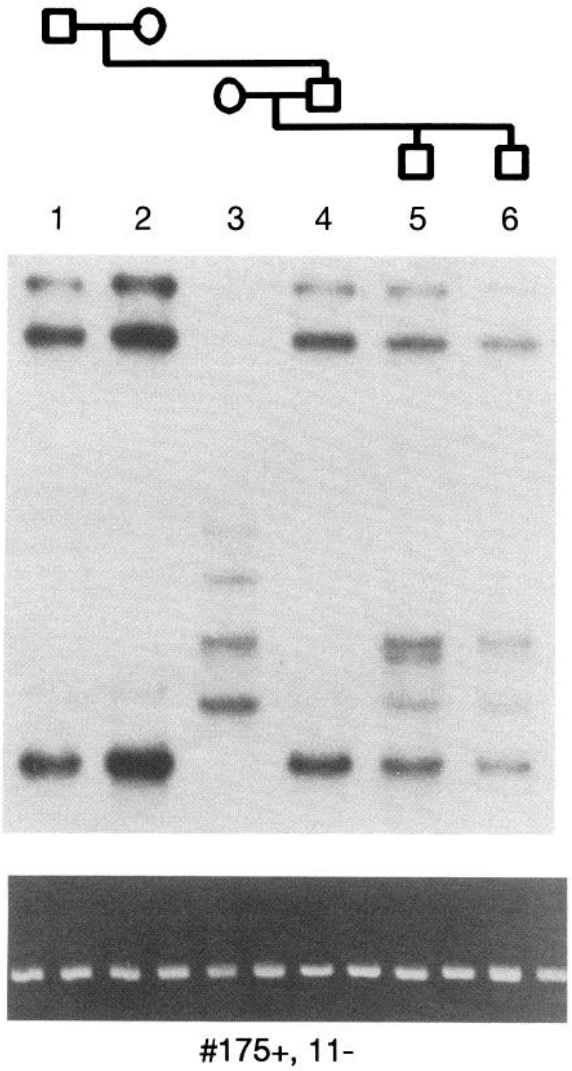

Fig. 4. SSCP analysis of 175+/11− PCR products (343 bp). *Top*: PCR product from each family member (Fig. 1) following electrophoresis on an mutation detection enhancement (MDE) gel as described in Fig. 3. *Bottom*: 175+/11− PCR products on a 1% agarose gel before electrophoresis on an MDE gel.

high frequency of mutation, we analyzed a three-generation family by SSCP (19, 21). Paternity in this family was confirmed by hybridizing *Alu* I genomic digests with the variable number of tandem repeat probe, D15S86 (1, 14). The results are shown in Fig. 1. As expected in true paternity, the inheritance of the various bands follows a Mendelian pattern. For example, *individual 3* (*lane 3*) inherited the 0.9-kb band from his father (*lane 1*) and the 1.5-kb band from his mother (*lane 2*). The offspring (*lanes 5* and *6*) of *individual 3* both inherited the 0.9-kb fragment shared by both parents.

SSCP has been shown to be a sensitive indicator of sequence and length variation, when DNA segments of ~100–400 bp in length are amplified by PCR, heat denatured, and run on a high-resolution nondenaturing acrylamide gel (20). Under these conditions each single-stranded DNA fragment assumes a unique secondary folded structure determined in part by its nucleotide sequence. The nucleotide changes are detected as a different mobility band shift for each allele. These bands reflect differences in the folded conformations of each DNA strand. Since in these experiments both DNA strands are ^{32}P-labeled, for each single copy locus per haploid genome one would expect to see a maximum of two conformants (two bands, one for each strand) and for a single copy locus per diploid one would expect to see a maximum of four conformants (4 bands). However, since not all conformants are individually resolved and the possiblity of some heteroduplex formation cannot be totally excluded, odd numbers of bands are also visible in this type of analysis.

Genomic 6A (SP-A gene I) sequences of each family member were amplified with oligonucleotides 104 and 105. Subsequently each 104/105 PCR product (1,405 bp) was used as template to amplify smaller segments (150–400 bp) as shown in Fig. 2. Fourteen pairs of oligonucleotide primers were used to amplify specific DNA segments in order to screen the 3′UT region of 6A (Table 2).

The double-stranded PCR products were initially examined by nondenaturing agarose gel electrophoresis and ethidium bromide staining. In all samples, a single band of the appropriate size was seen and no mobility differences were detected among the individual samples (Fig. 4, *bottom*). On SSCP analysis of the PCR products, different single-strand conformation polymorphic patterns were detected (*top*). Regardless of the number of the conformants and/or polymorphic patterns, in all

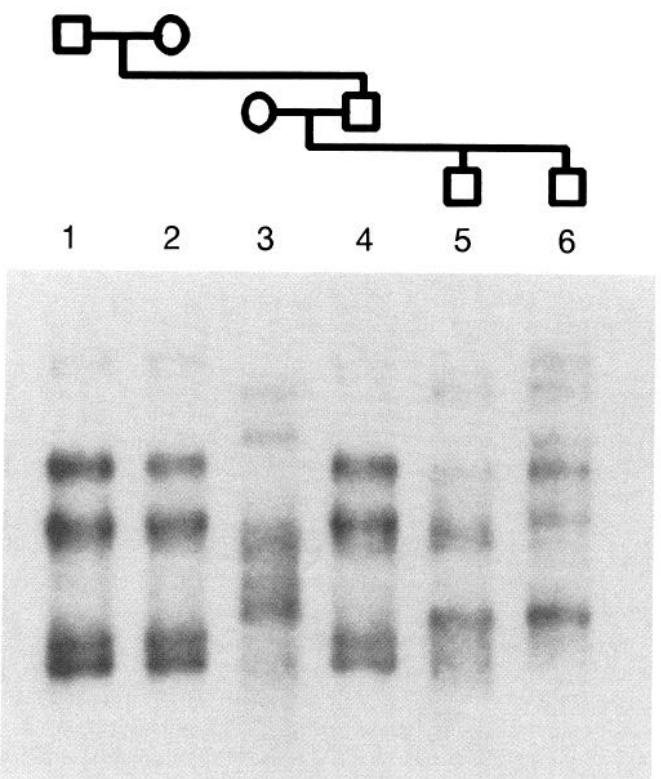

Fig. 5. SSCP analysis of 173+/68− PCR products (340 bp). *Lanes*, number of conformants detected for 173+/68− DNA region for each family member as designated by pedigree, after electrophoresis on a MDE nondenaturing sequencing gel.

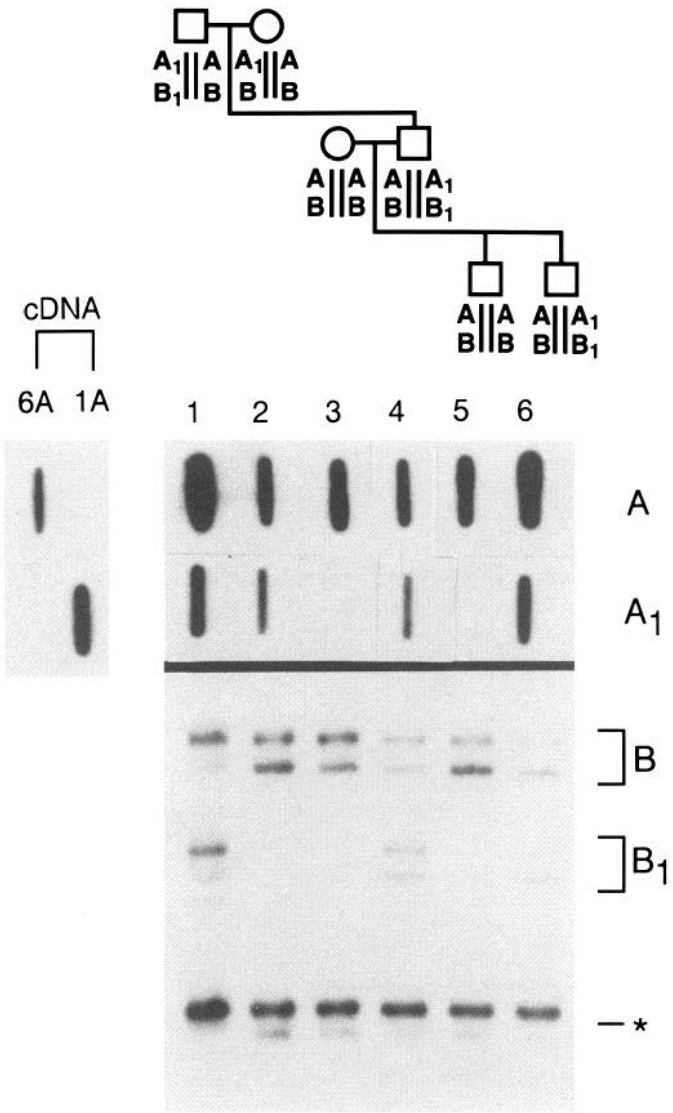

Fig. 6. Segregation of linked polymorphisms in 6A gene in 3-generation family. Coupled polymorphisms for 2 loci are depicted *below* each individual. Locus A refers to a point mutation in exon 1 detected by oligonucleotides 96 and 97. Oligonucleotide 96 is specific for 1A and haplotype $6A^1$ (allele A_1). Oligonucleotide 97 hybridizes with all known 6A variants (alleles A_1 and A). Specificity of oligonucleotides 96 (*bottom*) and 97 (*top*) is shown using control cDNA templates containing sequences of alleles A_1 (1A cDNA) and of A (6A cDNA). Segment defined by oligonucleotides 57+/177− contains a SSCP defined as locus B. Since alleles in this locus defined as B and B1 are transmitted through 2 generations in this family, this locus was chosen to be compared with and assess linkage with genotype analysis of exon 1. Star, an invariant band.

cases these conformants showed Mendelian inheritance (Figs. 3–6).

Figure 3 (*top*) depicts two conformants for each family member. Each band represents each strand conformant of homozygous 47+/179− segment. A similar pattern was observed in all family members for oligonucleotides 105+/174−, 176+/179−, 47+/13−, and 178+/13−, suggesting conservation of these segments. These segments include SP-A nucleotide sequences (from TGA) 18 to 240, and 769 to 1,251. Direct sequence analysis of several genomic clones (not including the family members examined here, our unpublished observations) showed that the DNA sequence from 18 to 221 is less variable and therefore consistent with the SSCP data, but the region from 769 to 1,251 is considerably more variable. The region from 769 to 1,251 may not differ in this family either at all or in ways that cannot be detected by SSCP. It is possible that when multiple changes within a given region exist, SSCP analysis is not sensitive enough to identify them. For example, mutations lying in loop formations or at the 5′ or 3′ end of the fragment studied are less likely to be detected (25). A possible explanation is that multiple nucleotide changes affect single-strand folding in ways that may lead to minor differences in the overall migration of the conformants.

Figure 3 (*bottom*) shows three conformants of the 57+/11− PCR product from each family member. The presence of the third band suggests that either the copy of the 57+/11− PCR product differs in each allele or suggests the presence of an additional 6A gene. One

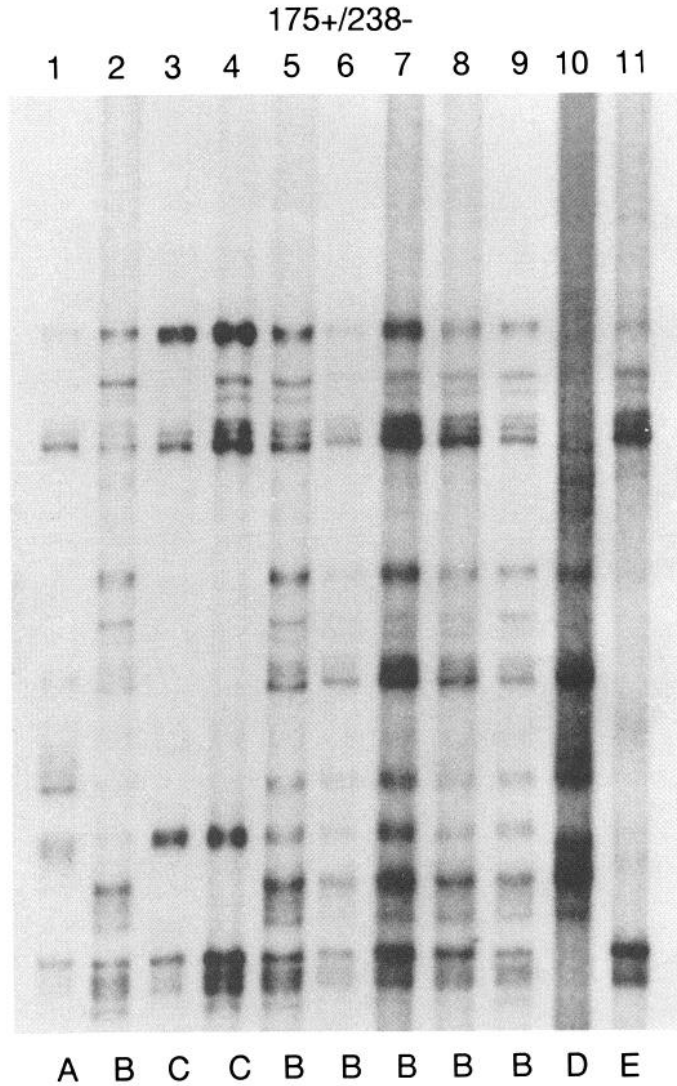

Fig. 7. SSCP analysis of 175+/238− PCR products (273 bp) of genomic DNAs from 13 unrelated individuals according to Fig. 3. Oligonucleotide 238 is specific for 6A (SP-A gene I).

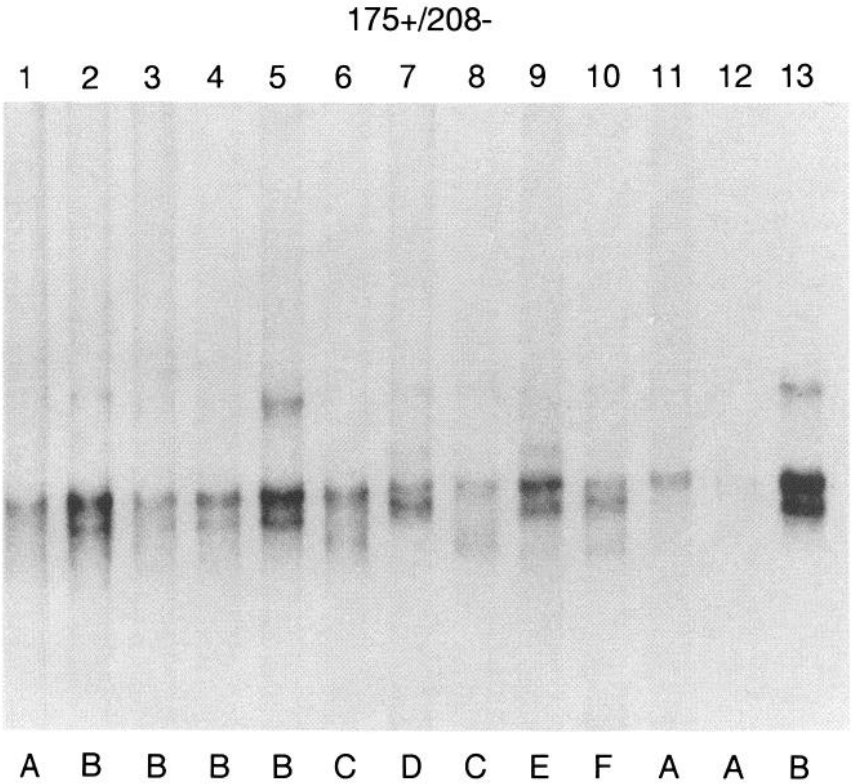

Fig. 8. SSCP analysis of 175+/208− PCR products (414 bp) of genomic DNAs from 15 unrelated individuals according to Fig. 3. Oligonucleotide 208 is specific for 1A (SP-A gene II).

interpretation of Fig. 3, *bottom*, could be as follows. Each one of the top two bands may represent one allelic copy of this segment (A_1A_1') and the bottom band may represent an additional copy of the $57^+/11^-$ segment, which is identical on both alleles (A_2A_2'), resulting in only one visible conformant as it is represented by a more intense band. Three conformants were also observed for segments generated with oligonucleotides 12+/177− and 57+/177− that span nucleotides 492–788 and with oligonucleotides 117+/104− spanning nucleotides 1,118–1,422.

The more complex patterns identified by SSCP were those of the segments that included the 11-bp insertion/deletion. The segments that span the region from nucleotide 221 to 641 were generated with oligonucleotides 175+/11−, 173+/68−, 46+/68−, 175+/68−, and 46+/11. Conformants of the 175+/11 and of the 173+/68− PCR products are shown in Figs. 4 and 5, respectively. In Fig. 4, *individuals 1, 2,* and *4* have three visible conformants and are similar to that of Fig. 3*B*. *Individual 3* has four bands corresponding to distinctly different sequences for each strand. This is the maximum number of possible single-strand conformants (one band/strand in a diploid genome). *Individuals 5* and *6*, however, have five bands suggesting the presence of a second 6A sequence. This sequence may represent a yet undetected pseudogene sequence homologous to 6A (gene I) or it may represent sequences homologous to variant 1A (gene II) sequences that may share homology with oligonucleotide 105 as discussed earlier. A third possibility is that some of the conformants may represent truncated PCR product due to replicative slippage of the polymerase, which can occur in regions where nucleotide repeats exist (25). Figure 5 shows the most complex pattern and it also suggests the presence of additional copies as more than four conformants are detected (see, for example, *lane 6*).

To determine whether there is a link between the information obtained by SSCP analysis and that of the SP-A genotypes, we assessed the SP-A genotype for each family member and compared them with a 3′UT segment (57+/177−, Fig. 2) analyzed by SSCP. This segment (37+/177−) was chosen because the alleles in this locus defined as BB_1 are transmitted through two generations in this family, making it possible to follow these particular haplotypes. The results are shown in Fig. 6. Family *members 3* and *5* have two AB alleles resulting in 6A/6A genotype and B-type conformants. *Members 1, 4,* and *6* have one AB and one A_1B_1 allele, resulting in a $6A/6A^1$ genotype and B/B_1 conformants. In this family the results of SSCP analysis are directly correlated with the oligonucleotide based genotypes. Together these joint changes suggest the existence of haplotypes encompassing the majority of the coding region.

The information obtained from the SSCP analysis suggests that the 3′UT heterogeneity is meiotically stable. This finding suggests that the 3′UT polymorphisms are not the result of spontaneous mutations generated in one generation, but are likely to occur and/or accumulate over a longer period of time. Since mutations that do not change the conformation of single strands, such as those lying within loop or long stable stem formations, or at the 5′ or at the 3′ end of the particular fragment, may not be readily detected by SSCP analysis, the possibility remains that certain

mutations could be spontaneous and therefore accumulate over a period of one generation.

Detection of heterogeneity. We also used SSCP analysis to detect genetic heterogeneity among individuals in each SP-A gene. Oligonucleotide 238 specific for the SP-A 6A gene (28) and oligonucleotide 208 specific for the SP-A 1A gene (12) were designed and each was used in combination with an oligonucleotide corresponding to a sequence common to both genes (oligonucleotide 175) in a PCR-SSCP experiment. The results for both genes are depicted in Figs. 7 and 8 and suggest that heterogeneity exists in both SP-A genes. Five of the possible haplotypes (Fig. 7, *A-E*) are identified for 6A (gene I) in this group of samples. For example, haplotype B appears to be a composite of haplotypes A and C, since most of the bands found in B can be traced to either A or C. Haplotype D appears to be related to B, and E is similar to A. Similarly, for gene 1A (gene II) in a group of 13 samples, we identified six possible haplotypes (Fig. 8, *A-F*). Haplotypes B-F, although they are distinct conformants, are related through A, since A appears in all haplotypes. It is likely that among the individuals shown in Figs. 7 and 8 not all possible polymorphisms are detected. SSCP is a more rapid means than sequencing of assessing genetic variability for the SP-A 3′UT region, although it may be less comprehensive in some instances.

In summary, we have shown that differences in the SP-A 6A (gene I) genotypes exist between black Nigerians and Caucasians. The PCR-SSCP analysis indicates that the pattern of inheritance of the 3′UT heterogeneity follows Mendelian inheritance, suggesting that most if not all of the nucleotide changes are meiotically stable and not artifacts of the amplification or cloning procedures. Since genotypic differences were not observed between American blacks and Caucasians, it is likely that the higher frequency of the homozygous 6A/6A genotype in the Nigerian population is consistent with genetic isolation or a founder effect in this group. Interestingly, among humans the mRNA level of another surfactant protein, SP-C, is considerably less variable (6) compared with SP-A, and the SP-C genomic sequences show little polymorphism (11). Whether the prevelance of this allelic variant in the Nigerian population has a selective advantage for pulmonary disease or yet for unknown abnormalities remains to be determined.

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REFERENCES

1. **Armour, J. A., S. Povey, S. Jeremiah, and A. J. Jeffreys.** Systematic cloning of human minisatellites from ordered array charomid libraries. *Genomics* 8: 501–512, 1990.
2. **Avery, M. E., and J. Mead.** Surface properties in relation to atelectasis and hyaline membrane disease. *Am. J. Dis. Child.* 97: 517–523, 1959.
3. **Bruns, G., H. Stroh, G. M. Veldman, S. A. Latt, and J. Floros.** The 35-kDa pulmonary surfactant-associated protein is encoded on chromosome 10. *Hum. Genet.* 76: 58–62, 1987.
4. **Dean, M., and B. Gerrard.** Helpful hints for the detection of single-stranded conformation polymorphisms. *Biotechniques* 10: 332–333, 1991.
5. **Farrell, P. N., and R. E. Wood.** Epidemiology of hyaline membrane disease in the United States: analysis of national mortality statistics. *Pediatrics* 58:167–176, 1976.
6. **Floros, J., D. S. Phelps, D. E. deMello, J. Longmate, H. Harding, B. Benson, and T. White.** The utility of postmortem lung for RNA studies; variability and correlation of the expression of surfactant proteins in human lung. *Exp. Lung Res.* 17: 91–104, 1991.
7. **Floros, J., R. Steinbrink, K. Jacobs, D. Phelps, R. Kriz, M. Reeny, L. Sultzman, S. Jones, H. W. Taeusch, H. A. Frank, and E. F. Fritsch.** Isolation and characterization of cDNA clones for the 35-kDa pulmonary surfactant-associated protein. *J. Biol. Chem.* 261: 9029–9033, 1986.
8. **Fujikura, T., and L. A. Froehlich.** The influence of race and other actors on pulmonary hyaline membranes. A report from the collaborative study of cerebral palsy. *Am. J. Obstet. Gynecol.* 95: 572–578, 1968.
9. **Glantz, S. A..** *Primer of Biostatistics* (3rd ed.). New York: McGraw-Hill, 1992.
10. **Graven, S. N., and H. R. Misenheimer.** Respiratory distress syndrome and the high risk mother. *Am. J. Dis. Child.* 109: 489–493, 1965.
11. **Hatzis, D., G. Deiter, D. E. deMello, and J. Floros.** Human surfactant protein C: Genetic homogeneity and expression in RDS; comparison with other species. *Exp. Lung Res.* 20: 57–72, 1994.
12. **Katyal, S. L., G. Singh, and J. Locker.** Characterization of a second human pulmonary surfactant-associated protein SP-A gene. *Am. J. Respir. Cell Mol. Biol.* 6: 446–452, 1992.
13. **Korfhagen, T. R., S. J. Glasser, M. D. Bruno, M. J. McMahan, and J. A. Whitsett.** A portion of the human surfactnat protein A (SP-A) gene locus consists of a pseudogene. *Am. J. Respir. Cell Mol. Biol.* 4: 463–469, 1991.
14. **Mascari, M. J., W. Gottlieb, P. K. Rogan, M. G. Butler, D. A. Waller, J. A. L. Armour, A. J. Jeffreys, R. L. Ladda, and R. D. Nicholls.** The frequency of uniparental disomy in Prader-Willli syndrome. Implications for molecular diagnosis. *N. Engl. J. Med.* 326: 1599–1607, 1992.
15. **McCabe, E. R., B. S. Huang, W. K. Seltzer, and M. L. Law.** DNA microextraction from dried blood spots on filter paper blotters: potential applications to newborn screening. *Hum. Genet.* 75: 213–216, 1987.
16. **Myrianthopoulos, N. C., J. A. Churchill, and A. J. Baszynski.** Respiratory distress syndrome in twins. *Acta Genet. Med. Gemellol.* 20: 199–204, 1971.
17. **Nelson, G. H., and J. C. McPherson, Jr.** Respiratory distress syndrome in various cultures and a possible role of diet. In: *Pulmonary Development.* New York: Dekker, 1985, vol. 27, p. 159–178. (Lung Biol. Health Dis. Ser.)
18. **Olowe, S. A., and A. Akinkugbe.** Amniotic fluid lecithin/sphingomyelin ratio: comparison between an African and a North American community. *Pediatrics* 62:38–41, 1978.
19. **Orita, M., H. Iwahana, H. Kanazawa, K. Hayashi, and T. Sekiya.** Detection of polymorphisms of human DNA gel electrophoresis as single-strand conformation polymorphisms. *Proc. Natl. Acad. Sci. USA* 86: 2766–2770, 1989.
20. **Orita, M., T. Sekiya, and K. Hayashi.** DNA polymorphism in Alu repeats. *Genomics* 8: 271–278, 1990.
21. **Orita, M., Y. Suzuki, T. Sekiya, and K. Hayashi.** Rapid and sensitive detection of point mutation and DNA polymorphisms using the polymerase chain reaction. *Genomics* 5: 874–879, 1989.
22. **Perelman, R. H., and P. M. Farrell.** Analysis of causes of neonatal death in the United States with specific emphasis on fatal hyaline membrane disease. *Pediatrics* 70: 570–575, 1982.
23. **Perelman, R. H., M. Palta, R. Kirby, and P. M. Farrell.** Discordance between male and female deaths due to the respiratory distress syndrome. *Pediatrics* 78: 238–244, 1986.
24. **Rishi, A., D. Hatzis, K. McAlmon, and J. Floros.** An allelic variant of the 6A gene for human surfactant protein A. *Am. J. Physiol.* 262 (*Lung Cell. Mol. Physiol.* 6): L566–L573, 1992.

25. **Shriver, M. D., G. Siest, and E. Boerwinkle.** Length and sequence variation in the apolipoprotein B intron 20 Alu repeat. *Genomics* 14: 449–454, 1992.
26. **Snyder, J. M., J. E. Kwuan. J. A. O'Brien, C. R. Rosenfeld, and M. J. Odom.** The concentration of the 35 kDa surfactant apoprotein in amniotic fluid from normal and diabetic pregnancies. *Pediatr. Res.* 24: 728–734, 1988.
27. **Torday, J., L. Carson, and E. E. Lawson.** Saturated phosphatidylcholine in amniotic fluid and prediction of the respiratory distress syndrome. *N. Engl. J. Med.* 301: 1013–1018, 1979
28. **White, R. T., D. Damm, J. Miller, K. Spratt, J. Schilling, S. Hawgood, B. Benson, and B. Cordell.** Isolation and characterization of the human surfactant apoprotein gene. *Nature Lond.* 317: 361–363, 1985.