Population-Based Screening for Rare Mutations: High-Throughput DNA Extraction and Molecular Amplification from Guthrie Cards

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ABSTRACT

To determine the population-based frequency of a rare mutation (the 121ins2 mutation in the surfactant protein B gene), we developed high-throughput techniques to extract reliably and rapidly amplifiable DNA from Guthrie cards. Using a 3-mm punch from each of 10,044 Guthrie cards obtained from the Missouri Department of Health, we extracted DNA with deionized water by heating in the presence of 2% Chelex in a 96-well format. Average yield of DNA from each punch was $52.6 \pm 21 \mu\text{g}$. Using 36mer primers and a 10- μL reaction volume, we amplified a 354-bp fragment of the surfactant protein B gene that contained the mutation and identified the mutation by its susceptibility to restriction enzyme digestion with *Sfi*I. The procedure required 5 h per 96 samples but only 2 h of technician time. The

amplification rate on the first attempt was 99.2%. Based on detection of eight individuals heterozygous for the mutation (confirmed by direct sequencing), we estimate the allele frequency to be 0.8/1000 individuals, an estimate not significantly different from previous estimates based on independent methods. High-throughput DNA extraction and amplification will permit establishment of DNA banks as well as efficient estimation of population-based genotype frequency for both rare and common genetic disorders. (*Pediatr Res* 50: 666–668, 2001)

Abbreviation

SP-B, surfactant protein B

The availability of information about genetic variation from the Human Genome Project permits associating specific genetic variants or markers with disease phenotypes from analysis of affected individuals and families. To determine the influence of genotype on disease expression, population-based estimates of both the frequency of specific genetic variants and the disease phenotype are required. For studies of rare genetic variants, high-throughput, efficient, low-cost methods for DNA extraction and amplification are necessary to screen the number of samples necessary to achieve statistically valid estimates of allele frequency.

Inherited deficiency of SP-B is one such rare genetic cause of lethal respiratory distress syndrome in full-term newborn infants (1, 2). It is most commonly caused by a homozygous, frameshift, loss of function mutation in codon

121 of the surfactant protein B gene (121ins2) (3, 4). The mutation introduces an *Sfi*I restriction enzyme site that permits detection of the mutation with restriction enzyme digestion. Using clinical ascertainment and molecular analysis of DNA extracted from cord blood, we previously estimated the frequency of the 121ins2 allele to be 1/1000 individuals (95% confidence interval, 0.03–5.6/1000) and 0.15/1000 individuals (95% confidence interval, 0.08–0.25/1000), clinical and molecular estimates, respectively (5). These estimates were limited by the accuracy of birth certificate data and by the risk of amplification of maternal DNA from cord blood samples.

Guthrie cards obtained through universal screening programs for newborn metabolic disease are an attractive source of DNA for large-scale, population-based estimates of genetic disorders (6–12). However, techniques reported to date have been labor and resource intensive and are not adapted for high throughput. We therefore developed high-throughput methods for molecular ascertainment of the frequency of the 121ins2 mutation using DNA extracted from Guthrie cards.

Received February 16, 2001; accepted June 5, 2001.

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Supported in part by a grant from the National Institutes of Health (HL/HD 54187; F.S.C.) and the Eudowood Foundation (L.M.N.).

METHODS

We used random, anonymous, archived Guthrie cards obtained between 1993 and 2000 by the Missouri Department of Health as part of its screening program for metabolic diseases. The unique identification number on each card permitted determination of birth certificate information for identified heterozygotes. We adapted previously described techniques for DNA extraction to enable high-throughput analysis (6–8, 13–15). Specifically, we placed a 3-mm-diameter punch from each blood spot into individual wells of a 96-well polypropylene microplate (Robbins Scientific Corp., Sunnyvale, CA, U.S.A.). We flamed the paper punch between samples to avoid DNA cross-contamination (16). Each 96-well plate also contained DNA from individuals known to lack the 121ins2 mutation or to be homozygous or heterozygous. Because Hb is an inhibitor of molecular amplification, we removed Hb by sequential incubations with 200 μ L of distilled water at room temperature for 45 min and 30 min on an orbital shaker at 150 rpm (17, 18). We subsequently rinsed each well with 200 μ L of distilled water. Hb removal was more complete as judged by amplification and by color of the blood spot if this process was performed within 2 wk of punching the Guthrie card. After sealing each 96-well plate with aluminum foil and vortex mixing for 10 s, we extracted DNA from each punch in 200 μ L of a solution of 2% (wt/vol) Chelex 100 chelating resin (Bio-Rad, Richmond, CA, U.S.A.) and 1 mM EDTA, pH 8.0. We centrifuged each plate at $500 \times g$ for 3 min, heated each plate in a microtiter hot block to 65°C for 30 min, vortex mixed, and heated to 100°C for 25 min (15, 19, 20). We spectrophotometrically measured the DNA concentration in selected wells. The plates were then stored at –20°C until amplification.

We performed PCR amplification in 10- μ L reaction volumes in each well of a 96-well plate as previously described: 5 μ L of supernatant containing DNA template; 1.6 μ L deoxynucleotides (final concentration 200 μ M); 0.5 μ L of 5% DMSO; 0.75 U (0.1 μ L) *Taq* polymerase (Sigma Chemical Co., St. Louis, MO, U.S.A.), and 2 μ L of Buffer J [300 mM Tris HCl, 75 mM (NH₄)₂SO₄, and 10 mM MgCl₂, Invitrogen, Carlsbad, CA, U.S.A.] (5). To increase the specificity of amplification of the target sequence, we used 36-monomer primers (sense: 5' TAA CTC CTT GGC ACT CGT GAA CTC CAG CAC CCT G 3'; antisense: 5' GCT GGC TGG GGT GCT GTG TGT GTG GCT CCC CCA TG 3') to amplify a 354-bp fragment of the SP-B gene (nucleotides 1320–1674) that contains the 121ins2 mutation. We amplified extracted template in a thermocycler (MJ Research, Watertown, MA, U.S.A.) as follows: 3.5 min at 95°C for initial denaturation, 30 cycles denaturation at 95°C for 30 s each, annealing at 68°C for 45 s, elongation at 72°C for 80 s, and a final elongation at 72°C for 3 min.

We performed restriction enzyme digestion with *Sfi*I as previously described (3, 5). Briefly, we digested amplicons in each well with 2.5 U (5 μ L) *Sfi*I in H buffer (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.) by incubating at 37°C for 90 min. We added 5 μ L of loading buffer and subjected each digest to metaphor/agarose-ethidium bromide gel electrophoresis. Each gel was photographed under UV light and

scanned into a database. Heterozygotes were identified by inspection of the gel. The identification number on the Guthrie card was forwarded to the Missouri State Department of Health, where one of us (J.B.) extracted the demographic data from the birth certificate database. No one investigator had access to all the information.

The Washington University School of Medicine Human Subjects Committee approved this study.

RESULTS

We found that increasing the time of the second incubation during DNA extraction increased the DNA yield from each blood spot significantly: in 24 samples, the DNA yield after 10 min of incubation at 100°C was 30.8 ± 10 μ g, and after 25 min, 52.6 ± 21 μ g ($p < 0.001$ by paired *t* test). The DNA was reliably amplifiable: only 174 of 10,044 (1.7%) failed to amplify on the first attempt. After a second attempt, 172 of the 174 samples successfully amplified, for an overall amplification success rate >99.9%. The oldest Guthrie card from which DNA was successfully amplified was 6 y old. Extraction from and amplification of each 96-well plate required 5 h, but only 2 h of technician time. We estimate the cost of the reagents for these analyses to be approximately 35 cents per sample.

We identified eight samples heterozygous for the 121ins2 mutation (Fig. 1), which were confirmed by direct sequencing. We therefore estimated a gene frequency in this population of 0.8/1000 individuals (95% confidence interval, 0.2/1000–1.4/1000), an estimate not significantly different from the previous molecular estimate from New York City and clinical estimate from Missouri (0.3/1000 and 1/1000, respectively, $p = 0.3$ by Fisher's exact test) (5). There were six male and two female infants; seven were white and one was black; all had normal birth weights. Respiratory distress syndrome was not noted in the birth certificate information of these eight infants.

Figure 1. Representative *Sfi*I digest for the 121ins2 mutation in DNA amplified from Guthrie cards. Pictured is a gel with 48 of the 96 wells from one microplate. Each lane contains amplified DNA from one individual. Lanes with normal, heterozygous, and homozygous controls are labeled. One subject heterozygous for the 121ins2 mutation is identified on this plate (arrow).

DISCUSSION

Statistically valid estimates of the frequency of rare alleles require screening of large populations. Availability of previous estimates of the frequency of the most common mutation that causes SP-B deficiency (121ins2) provided the opportunity to test the validity and throughput characteristics of methods for DNA extraction and amplification from Guthrie cards (5). Potential amplification of maternal DNA in cord blood samples and under-ascertainment of affected infants from birth certificate data limited the usefulness of methods used for previous estimates. Guthrie cards obtained from state-based neonatal screening programs provide a potentially useful source for population-based screening: demographics of the population can be obtained from birth certificates (with appropriate protections for confidentiality), the sample is not biased by clinical or institutional variables, and the likelihood of maternal DNA contamination in samples obtained at 2–3 d of life is low. Previous methods for DNA extraction and amplification from Guthrie cards have been limited by lack of high-throughput capability (6–15). In this analysis of the largest sample population to date, we report that adequate amounts of DNA for multiple determinations can be extracted from a single 3-mm punch and that inhibitors of molecular amplification, including Hb, albumin, and heavy metals, can be adsorbed with 2% Chelex. The time required for these methods (approximately 5 h per 96 samples) permits a single technician with access to two thermocyclers to process up to 1000 samples per week.

The lack of statistical difference between the estimate of the 121ins2 gene frequency in this report and our previous report may be related to the limited statistical power of our study, but also suggests that this high-throughput method provided a reasonable estimate of this rare allele. Additional studies evaluating the 25,000 samples necessary to detect confidently differences in this and other rare alleles between populations are underway. Because the 121ins2 mutation accounts for approximately two thirds of the mutant alleles responsible for SP-B deficiency, we can extrapolate our estimate to suggest that SP-B deficiency occurs with a frequency of approximately 1 in 2 million births in the United States (4). Regional differences in ethnic distribution may contribute to differences in apparent disease frequency. From the birth certificate diagnoses, which historically have been limited in the ability to detect clinical conditions of the newborn, the infants in this cohort heterozygous for 121ins2 did not exhibit respiratory symptoms at birth, which is consistent with previous observations (2, 3, 21, 22).

The small reaction volume for molecular amplification (10 μ L), the simplicity of reagents for DNA extraction, the low average technician time (3 min per sample), and the quality of the extracted DNA make these methods attractive for the development of DNA banks for use in determination of the population-based frequency of genetic variants (23). Detection

systems that use DNA chip-array technology may be more cost-effective and efficient than restriction enzyme digestion for identification of new or known mutations.

Acknowledgments. The authors thank Corrine Hamvas, Jennifer Heeley, Robert Jones, Qua Mei Lei, Robin Peterson, George Roffman, Thomas Sandbothe, and Jasmine Wong for technical assistance.

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