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Genetic Screening for the Next Decade: Application of Present and New Technologies

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Molecular genetic technology is diffusing from the research laboratory to the clinical laboratory, where it has already begun to influence prenatal diagnosis and counseling. In the very near future, this technology will be applied more generally, using population-based screening strategies. Pilot programs are beginning to evaluate the technical feasibility and efficacy of recombinant DNA techniques for newborn screening follow-up. DNA-based population screening is being considered for heterozygous carriers of an autosomal recessive disorder such as cystic fibrosis in order to identify carrier couples at risk of having an affected child. We will review the current DNA methodologies in the context of three genetic disorders: sickle-cell disease, Duchenne muscular dystrophy, and cystic fibrosis. We will then consider the requirements for implementation of these new technologies. We will conclude that implementation will require two key factors: machines and people. Machines are required to automate molecular genetic procedures, which are currently personnel-intensive, so that the expense can be reduced and the procedures made more cost-effective. The people who are required are health professionals knowledgeable in the clinical aspects of the target disorders, as well as in the DNA laboratory testing. These professionals will be able to facilitate sample acquisition and information exchange among the laboratory, the primary health care provider, and the families requesting consultation.

One of the primary goals of basic research in human genetics is to improve our understanding of the underlying molecular mechanisms responsible for human disease. The recent explosion of information in this area has provided medical genetics with the opportunity to achieve improved diagnosis of patients affected with genetic diseases and improved ascertainment of individuals heterozygous for autosomal recessive disorders.

We will review three genetic disorders, sickle-cell disease (SCD), Duchenne muscular dystrophy (DMD), and cystic fibrosis (CF), and discuss the recent advances in molecular genetic technology as they relate to screening and diagnosis for these diseases. The application of the techniques of molecular biology to neonatal screening relies on our ability to manipulate DNA from dried blood specimens on filter paper blotters. These blotters, frequently referred to as "Guthrie cards" after their originator, Dr. Robert Guthrie, have proven their value in newborn screening due to ease of sample collection and transport and to stability of a variety of analytes [1–3]. Examples of analytes routinely analyzed from Guthrie cards include phenylalanine for detection of phenylketonuria (PKU), thyroxine (T_4) and thyroid stimulating

Abbreviations: ASO: allele-specific oligonucleotide CF: cystic fibrosis DMD: Duchenne muscular dystrophy HRP: horseradish peroxidase PCR: polymerase chain reaction PKU: phenylketonuria RFLP: restriction fragment length polymorphism SCD: sickle-cell disease T_4: thyroxine TSH: thyroid stimulating hormone

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hormone (TSH) for congenital hypothyroidism, and hemoglobin for sickle-cell disease and other hemoglobinopathies. The illustrations which we will provide will demonstrate that DNA is another analyte which can be analyzed, using these dried blood specimens. In addition, we will consider the requirements for implementation of this molecular genetic technology so that it may have an even broader effect in clinical medicine.

SICKLE-CELL DISEASE

Sickle-cell disease is a common genetic disorder occurring with an incidence of 1 in 400 births among individuals in the U.S. of African-American origin [3]. Universal newborn screening for sickle-cell disease was recommended by an NIH Consensus Development Conference [4], because of the evidence that prophylactic treatment with penicillin is effective in preventing the infant mortality associated with this disorder [5].

Knowledge of sickle-cell disease has consistently been at the forefront in our understanding of the molecular pathogenesis of human disease, and this phenomenon has been true also in the area of molecular genetic diagnosis. In 1978, Kan and Dozy demonstrated the use of a restriction fragment length polymorphism (RFLP) for the prenatal diagnosis of sickle-cell disease [6,7]. They showed that there was linkage disequilibrium between the sickle-cell mutation and a 13 kb *HpaI* restriction fragment: 87 percent of individuals with the hemoglobin S allele had this restriction fragment. This fragment was, however, only a linked marker for the sickle-cell mutation; it required knowledge of the RFLP pattern of the proband and was not informative in all families. In 1982, it was shown that the sickle-cell mutation resulted in an alteration in the restriction recognition sequence for *MstII* [8,9]. Detection of the presence or absence of this restriction site by *MstII* digestion provided a sensitive and specific DNA test which did not require analysis of other family members and would identify all individuals with the β^S allele. In 1983, Conner et al. utilized allele-specific oligonucleotide (ASO) probes for detection of the β^S allele by differential hybridization [10]. By controlling the conditions of hybridization and washing, the presence of the β^A and/or B^S alleles could be determined with the appropriate labeled ASOs hybridized to Southern blots [11]. Electrophoresis of the human DNA was required to separate the specific diagnostic band from other cross-hybridizing sequences [10].

In 1985, Saiki et al. described the polymerase chain reaction (PCR) and demonstrated the application of this new enzymatic amplification technique for the diagnosis of sickle-cell disease [12]. This methodology permitted amplification of a specific DNA region of interest, such as that spanning the sickle-cell mutation in the β-globin gene. There would be approximately 220,000-fold enrichment in the region of interest at the end of 20 PCR cycles with 85 percent efficiency of amplification per cycle. This process resulted in marked diminution of the cross-hybridization that had previously been observed using ASOs, since now the amplified region of interest represented the majority of the DNA present. This process permitted one to hybridize the ASOs directly to dot blots of the PCR reaction products or, alternatively, to separate the PCR reaction products on a gel and stain directly with ethidium bromide. The diagnostic application of PCR was facilitated by the introduction of a thermostable (*Taq*) DNA polymerase which permitted automation of this process [13].

Our group had been interested in the analysis of DNA on the dried blood specimens used for newborn screening [14]. PCR amplification of microextracted DNA from the specimens facilitated genotypic analysis of SCD [15,16]. One could successfully distinguish AA homozygotes, SA heterozygotes, and SS homozygotes using ASO probes labeled with ^{32}P [15] or horseradish peroxidase (HRP) [17], as well as by direct ethidium bromide staining of gels after digestion with restriction enzyme [16,18]. Chehab and Kan recently presented an elegant fluorescence assay for detection of the sickle-cell mutation which readily lends itself to automation [19].

We have proposed that DNA follow-up to newborn screening would permit rapid genotypic confirmation of positive newborn screening specimens and clarification of those which were questionable after the initial protein-based evaluation [15]. We are currently involved in a project, in collaboration with the newborn screening laboratory in the Texas Department of Health, to evaluate this approach. Preliminary results indicate that PCR-based strategies can be effective in providing genotypic confirmation and clarification with a very low recall rate for second specimens [18]. Such an approach would reduce one of the major expenditures for newborn screening programs, the personnel time required to contact the families to obtain the confirmatory liquid blood specimens [15]. In addition, we feel that contacting the family in a more timely fashion with the knowledge of the genotype would convey to them the appropriate sense of urgency and immediacy regarding the diagnosis of SCD and might reduce the frequency of noncompliance with antibiotic prophylaxis [15].

DUCHENNE MUSCULAR DYSTROPHY

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder with incidence estimates of 1:3,000 to 1:5,000 male live births or 1:11,500 overall births [3]. This disorder is characterized by progressive deterioration of the muscles, resulting in death in the second or third decade. Recent advances in the cloning and characterization of mutations in the DMD locus at Xp21 have improved remarkably the diagnostic capability for this disorder [20,21]. Utilizing the DMD cDNA probes, approximately 56 percent of patients reveal detectable deletions [21]. Because of the frequency of deletions, this disorder is particularly amenable to rapid diagnosis by PCR, since deletions which remove one or both of the priming sites would prevent amplification. Multiplex PCR permits the amplification of many sequences simultaneously and results in detection of 80 percent to 90 percent of all DMD gene deletions [22,23]. Thus, utilizing nine sets of PCR primers, the vast majority of DMD deletions can be detected, and nearly 50 percent of all DMD patients will have a recognizable deletion. This procedure greatly facilitates the diagnosis of these patients and in many cases can replace biopsy confirmation of the DMD diagnosis. In the DNA laboratory, multiplex PCR replaces the personnel-intensive efforts required to perform Southern blotting with multiple probes. Multiplex PCR should accelerate implementation of DNA technology for diagnosis of DMD in the general medical community and would facilitate rapid genotypic confirmation in at least 50 percent of patients ascertained by infant screening programs for DMD.

CYSTIC FIBROSIS

Cystic fibrosis is an autosomal recessive disorder with an average incidence among Caucasians of 1:2,000 births and a heterozygote frequency of approximately 1:22

individuals [3]. Death usually results in the second to fourth decades from obstructive pulmonary disease and infection.

In September of 1989, we received the exciting news that the gene for cystic fibrosis (CF) had been cloned and the mutation determined [24–26]. The common Caucasian CF mutation is referred to as ΔF508, because it results in the loss of three nucleotides in frame and, consequently, the deletion of a phenylalanine residue, amino acid 508, in the CF protein. Knowledge of this mutation permitted PCR amplification and hybridization with ASOs for analysis of the presence or absence of the ΔF508 mutation among homozygotes and heterozygotes for this disorder [27]. The ΔF508 mutation was present on 75.8 percent of the CF chromosomes. The results of this analysis indicated that population-based screening would identify approximately 57 percent of the non-Ashkenazic white couples at risk for CF [27]. A recent NIH-sponsored workshop recommended that, at this time, population-based carrier screening should not be undertaken and that mutation analysis should be used in families only where there was an affected individual with this disorder [28].

Pilot studies on the efficacy of newborn screening for cystic fibrosis are in progress [3]. Although reports of PCR amplification for detection of the ΔF508 mutation in dried blood specimens are not yet available, such samples have been used for haplotype analysis in this disorder. In one remarkable example from the United Kingdom, a specimen that had been stored for 17 years was obtained for amplification and restriction enzyme digestion [29]; the specimen, obtained in 1971 and analyzed in 1988, permitted informed genetic counseling for the sibling of that deceased patient with CF. This experience showed that such newborn screening specimens can be quite valuable as a molecular genetic resource. It also showed that, in addition to providing a simple form of sample acquisition and transport, dried blood specimens on filter paper blotters also provide a convenient medium for storage of DNA in a stable form.

IMPLEMENTATION OF MOLECULAR GENETIC TECHNOLOGY FOR THE FUTURE

The key to implementation and broad-ranging diffusion of this technology will be twofold: machines and people. Automated equipment is essential in order to reduce the current labor-intensive molecular genetic techniques. In our current trials to interface recombinant DNA methodologies with newborn screening, the major costs are the salaries of the technical personnel who perform the DNA microextractions and analyses. The ability to automate this technology would make it available at an acceptable cost. In addition, equipment automation will reduce personnel involvement in the repetitive manipulations associated with these procedures, in turn decreasing the opportunity for human error [30]. Just as there is the need for quality assurance and quality control in other aspects of neonatal screening [31,32], there also will be the need for analogous standards to be adopted for the molecular genetic components of such programs.

But, having said that we should automate in order to reduce personnel costs and human error, why have I said that the second important factor in implementation of this technology is people? The people who are needed are individuals knowledgeable in both the clinical sector and the DNA laboratory, who are able to operate at the interface of these two arenas. These health professionals will facilitate the movement

of samples to the DNA laboratory and will ensure that the information from the laboratory is transferred to the physician and the patient in a clear, effective manner.

In summary, the successful implementation of this technology will require research and innovation. Research will be required in order to provide automated and reliable methods. Innovation will be required in order to introduce this novel technology effectively and to train the health professionals who will facilitate implementation of molecular genetic technologies for improved patient care.

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