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BRIEF REVIEW

Prenatal Diagnosis of Hemoglobin Disorders by DNA Analysis

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PRENATAL DIAGNOSIS of hemoglobinopathies has been largely performed by globin chain analysis of fetal blood samples.[1] Detection of these disorders through study of fetal DNA has distinct advantages over this now traditional approach. For one, fetal DNA samples may be routinely obtained with greater safety from amniotic fluid cells acquired by simple amniocentesis in midtrimester. Second, whenever possible, direct identification of the primary mutation in the DNA, rather than a phenotypic consequence referable to it, is desirable. Finally, early diagnosis during pregnancy will be increasingly possible with current and developing technology. In this review, I update the current status of prenatal detection of hemoglobin disorders by DNA analysis.

DNA ANALYSIS

Options for Diagnosis

A number of approaches are available by which DNA may be employed for genetic diagnosis. In the simplest situation, the primary defect in DNA leading to a disorder may be identified by direct assay.[2] This should be a goal of all current and future research in this area. This strategy rests on an understanding of the molecular basis of a given disorder. Second, neutral DNA variations (polymorphisms) in the vicinity of the gene of interest may be identified within a family and used to trace mutant genes by linkage analysis.[3] This approach obviates the necessity of knowing the particular mutation involved, but is limited in practice by the availability of useful polymorphisms. Whatever method actually chosen, the detection of mutations directly or by linkage is founded on restriction mapping (or gene mapping). In this general procedure, restriction enzymes are used to fragment DNA reproducibly. The resulting fragments are electrophoresed in agarose, transferred to filter sheets, and hybridized with radioactive cloned DNA segments of interest.[4] DNA fragments harboring specific sequences are then recognized as autoradiographic bands on x-ray film.

Direct DNA Analysis of Defects

It is useful to consider several classes of genetic lesions and their potential for direct detection. In those conditions where extensive genetic material is deleted from the genome, diagnosis is rather simple. For example, the absence of alpha globin genes in the DNA of fetuses with homozygous alpha thalassemia (hydrops fetalis) is readily observable by gene mapping.[2] Only rather uncommon forms of β-thalassemia are caused by gene deletions.[5,6] A notable exception is one variety of β^0-thalassemia which is seen among Asian Indians, in which 600 base pairs (bp) of the 3′ end of the β-gene is deleted.[5,7]

A more common situation is exemplified by sickle cell anemia and β-thalassemia, where the genetic defects are more subtle. Specific assays must then be designed.

Sickle cell anemia provides a particularly illustrative example. The glutamic acid to valine substitution in the sixth codon of the β^S chain results from an A–T nucleotide change in the DNA. The sickle mutation alters the sequence of a region normally recognized and cleaved by three different restriction enzymes,

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Mnl I, Dde I, and Mst II. For purely technical reasons, the enzyme Mst II is the most useful,[8-10] yielding DNA fragments from normal and sickle cell DNAs that are most easily detected in standard gene mapping experiments. With this enzyme and an appropriate DNA probe prepared from the 5′ portion of the β-globin gene, the genotype of individuals with respect to this particular mutation may be assessed directly. The approach is sufficiently sensitive for use with the small amount of amniotic cell DNA isolated directly from cells aspirated from amniotic fluid during the second trimester.[9,10] This test is now in rather wide use, with excellent results.

β-Thalassemia presents a rather different situation. Unlike sickle cell anemia, which is a single disorder caused by a single DNA alteration, recent work has shown that β-thalassemia is a collection of different mutations with similar phenotypic consequences.[11,12] We cannot hope to find one or even a few restriction enzymes that would fortuitously recognize all the mutations. Of the more than two dozen point mutations leading to β-thalassemia that have been defined, only a handful reside at recognition sites for restriction enzymes. To make matters even more complex, the most common point mutations in Mediterraneans (those at intervening sequence-1 [IVS–1], position 110, and in codon 39) are not detectable in this manner. Nevertheless, some rather uncommon β-thalassemia mutations are detectable with an appropriate restriction enzyme[11,12] and could be identified in fetal DNA specimens in a manner analogous to that now used for sickle cell anemia.

Recent molecular biologic techniques provide an alternative and powerful approach to the detection of these common forms of β-thalassemia, as well as virtually any other point mutation in the DNA. Under appropriate experimental conditions, short synthetic DNA fragments (oligonucleotides or oligomers) can hybridize to their homologous sequences but not to heterologous sequences, i.e., those with any degree of mismatch. For example, Wallace and coworkers[13,14] have shown that a 19-nucleotide long probe (19-mers) directed to the normal β-globin sequence in the region of the β^S mutation can hybridize efficiently with the normal gene but not with DNA containing the sickle mutation. With high specific activity labeling of the synthetic probe, normal and sickle sequences can be distinguished upon hybridization of restriction-enzyme-digested total human DNA.[14] The method is sufficiently sensitive to be used with 15 μg or less of DNA per gel lane. Upon assay with both the mutant and corresponding mutant synthetic probes, the genotype of an individual with respect to the mutation can be established without reference to additional data.

In a similar manner, we have used oligonucleotides to devise an assay for the direct detection of the most common variety of β^+-thalassemia among Mediterraneans.[15] Similarly, Pirastu et al.[16] have done the same for another common mutation, that in codon 39, which represents the predominant defect in Sardinia.[17] Three additional β-thalassemia mutations have been detected with other oligonucleotide probes (unpublished studies). Pirastu et al.[16] have also used their oligonucleotide assay successfully in several pregnancies at risk for β-thalassemia. It is apparent that virtually any thalassemia mutation could be diagnosed directly in fetal DNA by an appropriate oligonucleotide assay.

The actual use of oligonucleotide probes for direct mutant gene detection is depicted in Fig. 1 for illustrative purposes. In part A, the hybridization specificity of synthetic probes is shown. Under appropriate conditions, these probes react strongly only with homologous (i.e., perfectly matched) sequences. Therefore, the normal β-gene sequence can be distinguished from each of three β-thalassemia mutants situated within a span of only six nucleotides. In part B, analysis of genomic DNAs demonstrates how pairs of probes can be employed to establish the genotype with respect to a particular mutation. By hybridization of the same DNAs separately (or sequentially) to a mutant probe and a corresponding normal probe, the genotype can be directly determined.

As with all newly developed technology, practical considerations and potential difficulties of the oligonucleotide approach must be addressed. First, in order to detect globin DNA fragments or other single-copy sequences in blot hybridization of total DNA, the synthetic probe must be rendered highly radioactive. Our own experience indicates that this is the most troublesome part of the methodology and tends to depend, at least in part, on the quality of the synthesized DNA probe. The higher the specific activity of the probe, the greater the sensitivity and the less fetal DNA that is required for analysis. This is especially important if one wishes to perform diagnoses with the amount of DNA reliably obtained from simple aspiration of amniotic fluid cells. Second, the discrimination between normal and mutant sequences depends on careful control of the hybridization and washing conditions. Fortunately, the suitable conditions can be established initially with cloned mutant and normal genes and then extended to total genomic digests. This has not been a problem in general. Cloned mutant genes should be freely available to laboratories wishing to establish specific assays in their own locales. Third, in order to utilize this approach effectively, one must know something regarding the mutations for which the prospective family at risk for β-thalassemia may carry.

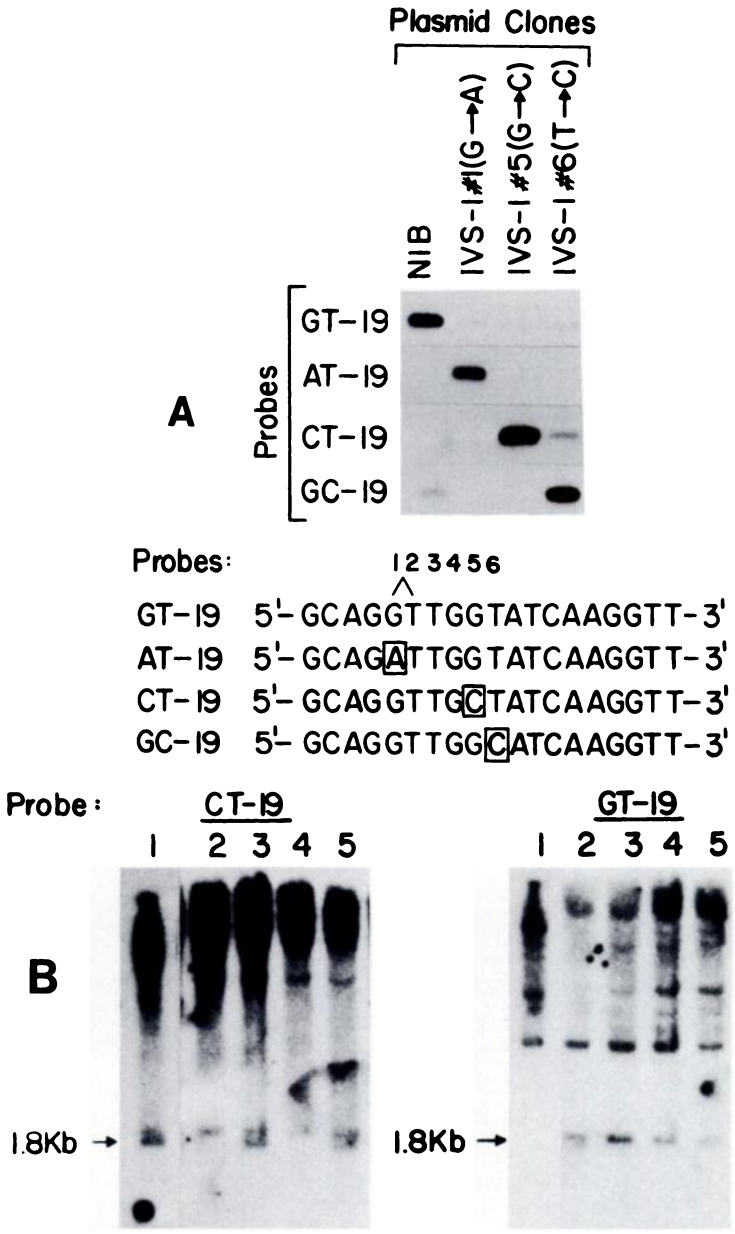

Fig. 1. Genotyping DNA samples by hybridization with oligonucleotides. (A) Four different 19-nucleotide-long synthetic DNAs (whose sequences are indicated below the autoradiogram) were used as hybridization probes to four different cloned β-genes. The probe GT-19 represents the normal β-gene sequence at the end of the first coding block and the beginning of IVS-1. AT-19, CT-19, and GC-19 correspond to β-thalassemia mutants with the nucleotide substitutions shown in boxes. These mutations are, respectively, at IVS-1 positions 1, 5, and 6. Plasmid subclones of the normal β gene and these mutants were digested with the restriction enzyme Bam HI, electrophoresed in agarose, blotted, and then hybridized under controlled conditions with the synthetic probes. Hybridization of the appropriate restriction fragment (1.8 kb in length) is presented for each DNA against each probe. The homologous sequences, that is, normal β gene with GT-19, and IVS-1 #1 with AT-19, etc., hybridize strongly, whereas mismatched sequences do not. (B) CT-19 and GT-19 probes were hybridized to Bam HI digests of patient genomic DNA samples (numbered 1–5). The critical region at 1.8 kb is indicated by the arrow. The CT-19 probe, which detects the IVS-1 position 5 mutation, hybridizes to DNAs 1, 3, and 5. The hybridization seen in all DNAs in the left panel, just above 1.8 kb, is a nonglobin band present in all samples. The normal probe (GT-19) yields a positive signal in DNAs 2, 3, 4, and 5 (right panel). DNA 1 is homozygous for the IVS-1 #5 β-thalassemia mutation. DNAs 3 and 5 are heterozygous for it (as hybridization with the normal probe is seen). DNAs 2 and 4 have at least one β-gene with a normal sequence in the region scored by the oligonucleotide GT-19 and do not have the IVS-1 position 5 defect.

For example, use of the oligonucleotides for the common Mediterranean mutations in analyses of Asian samples would be largely futile, as specific mutations are heavily concentrated in particular ethnic groups.[11,12] Systematic cloning of DNA samples of various ethnic groups, coupled with judicious use of oligonucleotides to survey patient panels, will soon provide rather accurate estimates of mutant gene frequencies worldwide.

Indirect DNA Assays—Linked Polymorphisms

An alternate method for the detection of mutant genes is based on the use of DNA polymorphisms to trace chromosomes or alleles within a family.[3] DNA polymorphisms have been identified in nearly all large segments of the human genome examined carefully to date. At present, more than a dozen restriction enzyme sites have been located in the β-globin gene cluster that are polymorphic.[12,18] Some of these sites are highly polymorphic, i.e., there is a reasonable likelihood that different alleles will be identified within a family. Kan and Dozy[3] first reported the association of a mutant gene, that for β^S-globin, with a restriction enzyme polymorphism. The strong association of a particular gene with a polymorphism is an example of what is termed "linkage disequilibrium." The apparent multicentric origin of sickle mutations, perhaps combined with recombination of DNA within the β-gene cluster

over time, obviates the widespread use of this approach without careful family studies. Although specific β-thalassemia genes are in linkage disequilibrium with various restriction sites,[11,12,19] there is no single DNA polymorphism that is generally useful for analysis of families at risk. Instead, the most frequent polymorphic sites can be assessed in various family members and their linkage phase with respect to mutant alleles determined prior to study of fetal DNA. The search for appropriate polymorphisms within a family and their linkage with the normal and mutant genes in that family is a labor-intensive process. This aspect tends to limit the widespread use of the approach for prenatal diagnosis. Nevertheless, as exemplified by the nearly 100 pregnancies monitored by this strategy by the group at Johns Hopkins,[20] it is feasible on a referral basis.

Earlier Acquisition of Fetal DNA Samples for Diagnosis

In part, the development of improved methods for prenatal diagnosis of hemoglobin disorders has relied on advances in sample acquisition, as well as in cell and molecular techniques. The acquisition of sufficient fetal cells earlier in pregnancy would facilitate prenatal diagnosis by DNA analysis and also improve the acceptance of the approach in general. Diagnosis dependent on amniotic fluid cells can be performed only during midtrimester (14–20 wk). Recently, it has been reported that chorionic villus biopsy specimens can provide adequate tissue samples within the first trimester (8–12 wk) of pregnancy.[21-23] These samples are generally free of maternal contamination. Considerable amounts of fetal DNA (often as great as 50 μg) may be obtained in this manner. Such samples have already been used for prenatal diagnosis of both sickle cell anemia and thalassemia.[22] Although small series have not described any greatly increased risk of this procedure in relation to routine amniocentesis, adequate trials have yet to be performed. These are in progress, and it should not be long before we have some notion of the potential risks to this approach. It appears likely already that the use of early fetal samples will increase in the near future.

In addition to the major advantage of earlier diagnosis, such chorionic biopsy specimens will provide greater impetus for the development of specific assays for all common varieties of β-thalassemia, as well as other single-gene disorders. The acquisition of greater than 20 μg of DNA in this manner will substantially facilitate detection of mutant genes with synthetic oligonucleotide probes.

CONCLUDING REMARKS

The prenatal diagnosis of hemoglobin disorders by DNA analysis has developed rapidly over the past 5 yr. Progress in molecular biologic techniques and our understanding of the genetic basis of thalassemias permits design of specific direct detection assays for virtually any mutant allele. These assays may most effectively be targeted for specific geographic regions or ethnic groups, based on the frequencies of particular mutations in each population. If coupled with earlier diagnosis, we can expect considerably wider use of these diagnostic tests. The hemoglobin disorders constitute an important model for the transfer of new technologies to genetic practice. We can be assured that similar approaches to prenatal genetic diagnosis of other conditions will follow.

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