A New Diagnostic Test for Gaucher Disease Suitable for Population Screening

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A new test for the diagnosis of Gaucher disease is described. The test is designed to screen large numbers of clinical specimens from high-risk populations. It consists of duplex PCR amplification of genomic DNA followed by hybridization to alkaline phosphatase-conjugated allele-specific oligonucleotide probes (ASOs). High melting temperature PCR primers were used to increase specificity and eliminate the need for a separate annealing step. All hybridization and washing steps were performed at one temperature. Chemiluminescent detection of signals is fast, and results are easily interpreted directly from x-ray films. Currently, the test is being used in our laboratories to screen Ashkenazi Jewish populations in whom Gaucher disease is common.

Gaucher disease (GD) is a lysosomal storage disease caused by mutations in the gene coding glucocerebrosidase.⁽¹⁻³⁾ The most common variant of the disease is type 1, which has an incidence rate of 1 in 40,000 and a carrier frequency of 1 in 100. In the Ashkenazi Jewish population the carrier rate is 1 in 10, and ~1 in 450 persons is affected.⁽⁴⁾ The disease is suspected in patients with painless splenomegaly, and diagnosis is confirmed by measurement of glucocerebrosidase activity in leukocytes, fibroblasts, amniocytes, or chorionic villi. Most carriers of GD (heterozygotes) have enzyme levels of 50% of normal values. However, in 20% of obligate heterozygotes, carrier detection by enzyme assay has proved unreliable.⁽¹⁾

Several PCR-based methods for the detection of GD mutations have been described in recent years. These generally fall into two categories: PCR-mediated mutation detection⁽⁵⁾ or hybridization of allele-specific oligonucleotide probes (ASOs) to PCR-amplified genomic regions that contain loci for these mutations.⁽⁶⁾ PCR-mediated mutation detection methods are lengthy and expensive and are not suitable for large-scale population screening. The ASO approach, however, is simpler, faster, and relatively inexpensive.

In this report we describe a new test for detection of the N370S,⁽⁷⁾ 84gg,⁽⁸⁾ L444P,⁽⁹⁾ and IVS2+1^(10,11) mutations. The test involves a two-temperature duplex PCR reaction followed by isothermal dot blot hybridization using alkaline phosphatase-conjugated ASOs. It is specifically designed for population screening and will identify >95% of heterozygote carriers as well as compound

heterozygotes. Starting with DNA extracted from whole blood, the test takes 1 day to finish and the results are easily interpretable.

MATERIALS AND METHODS

DNA Extraction from Whole Blood

Whole blood samples (200 μ l) were diluted with 1 ml of distilled water and mixed by gentle inversion for 45 min at room temperature. The tubes were centrifuged at low speed (4500 rpm) in a microcentrifuge for 3 min at 4°C. Cell pellets were washed once with 1 ml of distilled water followed by one to two times in 1 ml of Dulbecco's PBS. Pellets were resuspended and mixed with 250 μ l of 5% Chelex 100 suspension (Bio-Rad, Hercules, CA), incubated at 56°C for 1 hr, vortexed, and boiled for 10 min. The Chelex resin was removed by centrifugation at high speed (14,000 rpm) in a microcentrifuge for 3 min, and DNA preparations (~5 μ g) were stored at 4°C. Of these preparations, 20–25 μ l (~500 ng DNA) was used in 50- μ l PCR reactions.

Oligonucleotide Synthesis and Purification

PCR primers were synthesized on a Cruachem P250 DNA synthesizer (Cruachem, Sterling, VA), and purified on Sep-Pak columns (Millipore, Milford, MA) using the manufacturer's instructions, and their sizes were verified by electrophoresis of ³²P-end-labeled oligonucleotides on 20% denaturing polyacrylamide gels. Alkaline phosphatase-conjugated hybridization probes (Table 1) were custom synthesized by Synthetic Genetics (San Diego, CA).

TABLE 1 Hybridization Probes

Hybridization probes	Sequence
370 normal	CCTAGAACCTCCTGT
370 mutant	CCTAGAGCCTCCTG
444 normal	AACGACCTGGACGC
444 mutant	ACGACCCGGACGC
84gg normal	CAGGATTGCTTCTAC
84gg mutant	AGGATTGGCTTCTAC
IVS normal	GCATCAGGTGAGTG
IVS mutant	GCATCAGATGAGTGA

Duplex PCR Reaction

Reactions contained PCR buffer [33 mM Tris-HCl (pH 8.3), 16.7 mM (NH₄)₂SO₄], 3 mM MgCl₂, 200 μM each of the four deoxynucleotide triphosphates, 20–25 μl DNA, 50 pmoles each of GD1F and GD1R, 10 pmoles each of GD2F and GD2R, and 1.25 units of AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CT) in a final volume of 50 μl. Temperature cycling was carried out in a Perkin-Elmer Thermal Cycler Series 9600. After 2 min at 94°C, 30 cycles of amplification were carried out, each consisting of a 15-sec denaturation at 94°C and a 50-sec annealing/extension at 72°C. A final 5-min extension at 72°C was performed.

Acrylamide Gel Electrophoresis

PCR reactions (7.5 μl) were analyzed on 5% acrylamide gels (Bio-Rad Laboratories). Reaction products were UV-visualized by ethidium bromide staining.

Hybridization With Labeled ASOs

PCR reactions were diluted with 50 μl of TE buffer [10 mM Tris-HCl (pH 8), 1 mM EDTA] containing bromophenol blue at 5 μg/ml. This allowed easy visualization of the DNA dots on the hybridization membrane. Of this mixture, 7.5 μl was spotted on nylon membranes (Applied-Bio, Pleasanton, CA). Membranes were UV cross-linked (254 nm, 120 mJ/cm²) twice for 10 sec each in a Spectrolinker XL1000 (EM Corp., Chestnut Hill, MA) and dried at 80°C for 30 min. DNA was denatured by soaking the membranes for 20 min in 0.2 N NaOH, 0.1% SDS, neutralized in 2× SSC [1× SSC is 0.15 M NaCl, 15 mM sodium citrate (pH 7)], and prehybridized in a hybridization oven (Bellco, Vineland, NJ) at 40°C for 1 hr in 5× SSC, 0.2% SDS, 2 mg/ml of Geneprint

(Promega, Madison, WI), and 1× Denhardt's solution [0.02% (wt/vol) each of Ficoll 400, polyvinylpyrrolidone, and fraction V of bovine serum albumin]. The prehybridization solution was decanted and replaced with the hybridization buffer (5× SSC, 0.2% SDS, 1× Denhardt's solution, 0.1–2 pmoles/ml labeled probe), and incubation continued for 1 hr at 40°C. Membranes were washed for 10 min in 0.8× SSC and 0.1% SDS twice at room temperature, and once at 40°C. A final wash was performed for 10 min in 2× SSC at room temperature.

Detection of Hybridization Signals

Hybridization membranes were washed twice, for 15 min each, in 100 mM Tris-HCl (pH 7.5) and 150 mM NaCl, followed by two 5-min washes in 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 50 mM MgCl₂. Finally, blots were dipped briefly in LumiPhos 530 solution (Lumigen, South Port, MI), wrapped in plastic wrap, and incubated at 37°C for 15 min. Blots were then exposed to x-ray film (Kodak XOMAT-AR) at room temperature for 10–60 min.

RESULTS

The PCR reaction was designed to coamplify two genomic DNA fragments containing the four most common mutations found in the Ashkenazi Jewish population (Fig. 1A). A PCR primer pair (GD1F and GD1R) amplifies a 1024-bp DNA fragment containing the loci for N370S and L444P mutations. This fragment also contains loci for four other less common missense mutations: V394L, D409H, D409V, and P415R.^(12,13) In addition, the site for a rare 55-bp deletion at nucleotides 5879–5933^(14,15) is amplified in this fragment. The second primer pair (GD2F and GD2R) amplifies a 701-bp fragment containing the loci for 84gg and IVS2 + 1 mutations. To prevent amplification of the highly homologous pseudogene sequences,⁽¹⁶⁾ the 3' ends of the PCR primers were chosen in regions where nucleotide mismatches between the functional and pseudogenes exist. (Fig. 1B)⁽¹⁵⁾ Moreover, all primers have a theoretical *T_m* of 78–80°C, thus eliminating the need for a separate annealing step, which reduces the time required to complete the PCR cycles. Figure 2 shows the amplification

products of the duplex PCR reaction using clinical specimens. The two fragments amplified efficiently and reproducibly and contained sequences derived from the functional gene only (see below).

The objective of this test was to simplify the logistics of screening for multiple mutations for a large number of clinical specimens. To achieve this goal, we designed our ASO probes (Table 1) with a *T_m* of 44–46°C. Hence, regardless of the ASO being used, the hybridization reactions were carried out at the same temperature (40°C). Also, all washings were performed at 40°C. The hybridization and washing procedures required only one hybridization oven set at this temperature. Figure 3 shows the detection of carriers for all four mutations discussed here. Signals are very specific and no cross-hybridization can be detected among normal and mutant ASOs. The inability of the PCR primers to amplify pseudogene sequences homologous to the two amplified fragments of the functional gene is also demonstrated in Figure 3. The L444P (T → C mutation at nucleotide 6433) and the IVS2 + 1 mutations (G → A mutation at nucleotide 1067) are both present naturally in the glucocerebrosidase pseudogene.⁽¹⁴⁾ Yet, mutant ASOs for these two alleles failed to hybridize to amplified DNA from normal subjects, indicating the absence of pseudogene-derived products.

DISCUSSION

Although screening for genetic disorders such as GD is still controversial, it is very useful for high-risk groups, people with a family history of the disease, and symptomatic patients.^(8,16–18) There are a number of published reports on the detection of GD mutations by PCR and ASO hybridization.⁽⁶⁾ These generally involve individual, three-temperature PCR reactions for each mutation tested, followed by ASO hybridization at various temperatures depending on the probe used. Another test was described recently in which a duplex PCR reaction similar to the one described here is followed by hybridization to individual ASOs at different temperatures.⁽¹⁹⁾ ASO hybridization at different temperatures obviously is cumbersome and time-consuming, especially when several mutations are being analyzed. Thus, none of these tests is

FIGURE 1 Duplex PCR amplification strategy. (A) The relative locations of the PCR primers is shown. Thin lines represent sequences that are deleted in the pseudogene. (B) Nucleotide sequence alignment of the PCR primers with the functional and pseudogenes. Note that the 3'-terminal nucleotide of all four primers does not match with the pseudogene at that position, thus, preventing pseudogene sequences from being amplified. (*) Nucleotide mismatches.

well-suited for screening large numbers of clinical specimens.

In this report we describe a new PCR/ASO-based test for detection of GD mutations, which is designed specifically for mass screening of specimens. DNA is

first extracted from a small volume of whole blood, and segments of the DNA where mutation sites reside are amplified in a single tube by a two-temperature duplex PCR. By eliminating the need for annealing at lower temperature

we reduced the time required for the reaction and, more importantly, increased the specificity of the PCR primers. Specificity is increased further by PCR primers that do not amplify pseudogene sequences. As mentioned earlier, the IVS2+1, L444P, and deletion mutations exist naturally in the pseudogene.^(14,15) Coamplification of any pseudogene sequences therefore will complicate interpretation of results. This problem was avoided by having the PCR primers end with a nucleotide mismatch between the functional gene and pseudogene, thus, preventing primer extension of the pseudogene sequence by *Taq* DNA polymerase.

An important feature of this test is that detection of normal and mutant alleles is carried out by hybridization to ASOs at a single temperature (40°C). Also, all subsequent washings are performed at 40°C. Moreover, testing for cystic fibrosis (CF) mutations that are common in Jewish populations is carried

FIGURE 2 Acrylamide gel electrophoresis of the PCR amplification products. Samples of PCR reactions from several clinical specimens (lanes 1–8) were analyzed on 5% nondenaturing acrylamide gel as described in Materials and Methods. The two amplified fragments are indicated by arrows. (Lane 9) *Hae*III restriction digest of phage ϕ X174 DNA.

FIGURE 3 ASO hybridization analysis of GD mutations. Products of the duplex PCR reactions were spotted on nylon membranes, fixed by UV cross-linking, dried, and hybridized to alkaline phosphatase-conjugated ASOs (see Materials and Methods). Duplicate membranes were spotted for each mutations analyzed. One membrane in each set was hybridized to wild-type ASO (*top*), the other membrane was hybridized to mutant ASO (*bottom*). (A) Detection of heterozygous carriers. Patients A1, B4, and C3 are carriers for the N370S mutation. Patients D1, H1, and L1, on the other hand, are carriers for the 84gg, IVS2 + 1, and L444P mutations, respectively. The rest of the positions are normal subjects with the exception of position J1, which is a negative control (no DNA). (B) Detection of homozygous (N2) and compound heterozygous (Q2) affected patients. Patients M2, M4, N1, O3, and P2 are carriers for N370S. Patient P3 is a carrier for L444P.

out in our laboratories by ASO hybridization at 40°C. Because GD is often requested along with CF tests, our test has significantly simplified the logistics of

routine screening of hundreds of clinical specimens for both genetic disorders.

The use of alkaline phosphatase-conjugated probes in this test eliminates the

need for a second blocking step and additional incubation with antibody. Total analysis time (post-PCR) for all four mutations is ~3 hr. Finally, results obtained by this test are easy to interpret. As the data presented here show, normal subjects and heterozygous carriers can be identified easily. Homozygous patients (where only the normal ASO would hybridize), as well as compound heterozygous patients (where both normal and mutant ASOs would hybridize), also can be identified on x-ray films (Fig. 3). Identification of carriers is particularly useful in providing genetic counseling for high-risk individuals.

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