PRENATAL DIAGNOSIS
Prenat. Diagn. **18:** 207–212 (1998)

RELIABLE CO-SEGREGATION ANALYSIS FOR PRENATAL DIAGNOSIS AND HETEROZYGOTE DETECTION IN GAUCHER DISEASE

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Received 5 March 1997
Revised 29 May 1997
Accepted 20 June 1997

SUMMARY

Mutations in the gene encoding *β*-glucocerebrosidase are the main cause of Gaucher disease. The identification of some of these mutations in prenatal tests is a good complement to enzymatic assay and allows diagnosis and, in some cases, prognosis of the disease to be made. DNA analysis is particularly useful for carrier detection since the results of biochemical analyses are often ambiguous. The main drawback of mutation analysis for prenatal diagnosis and carrier detection in Gaucher disease is that rare mutations account for more than 30 per cent of the mutant alleles in most populations. The individual detection of these mutations is too expensive and time-consuming for routine use. Here we present a diagnostic protocol based on co-segregation analysis, using highly polymorphic markers, to be applied when at least one disease allele does not correspond to the most common mutations. Because of the frequency of the N370S mutation and its relevance for prognosis, an improved PCR detection method is included.

KEY WORDS: Gaucher disease; co-segregation analysis; microsatellite prenatal diagnosis; mutation analysis; heterozygote detection

INTRODUCTION

Gaucher disease (GD) is the most prevalent form of sphingolipidosis and is caused mainly by mutations in the gene coding for *β*-glucocerebrosidase (GBA; EC 3.2.1.45). It is inherited in an autosomal recessive manner. Three clinical types have been distinguished on the basis of the absence (type 1) or presence and severity of neuronopathic manifestations (types 2 and 3) (Beutler and Grabowski, 1995).

Following the first prenatal enzymatic diagnosis accomplished by Schneider *et al.* (1972), analysis of *β*-glucocerebrosidase activity in cells derived from amniocentesis or chorionic villus biopsy is routinely performed for the identification of an affected fetus. More recently, mutation analysis of fetal DNA has provided new tools for the prenatal molecular diagnosis of GD (Dahl *et al.*, 1992; Zimran *et al.*, 1995).

GD is particularly prevalent among Ashkenazi Jews. In this population, a small number of mutations account for most of the mutant alleles. However, in other populations more than 30 per cent of the GD alleles correspond to a variety of rare mutations. Therefore, prenatal molecular diagnosis based on the identification of known mutations is not feasible in many cases.

Twelve internal polymorphisms have been described within the *GBA* gene (Beutler *et al.*, 1992). However, they are in strong linkage disequilibrium and only two major haplotypes,

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Contract grant sponsor: CICYT; Contract grant number: SAF 96-0329.

CCC 0197–3851/98/030207–06

named + and − have been found. Thus, these polymorphisms could be considered a single diallelic marker with only limited interest for co-segregation analysis.

We have recently localized the *GBA* gene in relation to a series of microsatellite markers (Cormand *et al.*, 1997). The fine genetic mapping of the *GBA* gene allows the use of highly informative markers for co-segregation analysis when the most common mutations have been ruled out, for one or both GD alleles, in a given family. Here we present two examples in which the patients bear uncommon mutations, to show the applicability of the method for prenatal diagnosis and detection of heterozygous carriers in families segregating GD. In addition, one of the families carrying the N370S (cDNA nt 1226 A→G) mutation was studied in order to demonstrate an improved polymerase chain reaction (PCR) method, which includes an internal control of digestion.

MATERIALS AND METHODS

Patients

Two Spanish GD families were examined to test the diagnostic method described in this paper. Both consisted of the parents, one affected child (proband), and a second child (or fetus) for whom prenatal or carrier diagnosis could have been performed as presented. The patient in family 1 (type 1) bore the common N370S mutation in one chromosome while the other allele corresponded to a rare GD mutation. The patient in family 2 presented with type 2 GD, and neither of the mutant alleles carried N370S or L444P (nt 1448 T→C), the two most frequent GD mutations in the Spanish population. Instead, one allele corresponded to the third most prevalent mutation: D409H (nt 1342 G→C), and the other to a rare mutation (Chabás *et al.*, 1996). In this case, prenatal enzymatic diagnosis showed that the fetus was affected, and the pregnancy was interrupted. The clinical aspects of these two patients were referred to as I.11 (family 1) and II.6 (family 2) in Cormand *et al.* (1995).

DNA isolation

Genomic DNA was prepared from peripheral blood leukocytes or a chorionic biopsy specimen (CVS) at gestational week 10 by a standard method (Miller *et al.*, 1988).

Analysis of mutation N370S

An improved PCR method to detect the N370S mutation, which included an internal control of digestion, was developed. The upstream primer contained a single mismatch (Beutler *et al.*, 1990) that creates an *Xho*I restriction site when the mutation is present. The downstream primer is as follows: 5′ GTAGATGCTAAGTC**CTCGAG**GATGGGACTGTCGACAAAGT 3′. The underlined sequence corresponds to nucleotides 5919–5938 of the gene, according to the sequence reported by Horowitz *et al.* (1989). Most of this 20 bp sequence is not present in the highly homologous pseudogene. The rest of the primer was added to incorporate an *Xho*I restriction site (bold). Fifty microlitres of PCR amplification mixture [containing 100 ng of genomic DNA, 0·2 mM dNTPs, 1·5 mM $MgCl_2$, 20 pmol of each primer, and 1 U of Dynazyme DNA polymerase (Finnzymes Oy) in the recommended buffer] was subjected to 35 cycles of 94°C for 40 s and 55°C for 30 s. A further 2 min extension at 72°C was added to the final cycle. Five microlitres of the PCR product was digested with 10 U of *Xho*I for 2 h at 37°C and subjected to electrophoresis on a 12 per cent acrylamide–bisacrylamide (19:1) gel (a similar result was obtained using agarose gels—NuSieve, 4 per cent). The size of the amplified fragment was 138 bp. When the N370S mutation is not present, the *Xho*I site in the downstream primer produces a fragment of 121 bp and a small fragment of 17 bp. If the N370S mutation is present, an additional *Xho*I site is created by the mismatched primer, giving one fragment of 104 bp and two of 17 bp.

Analysis of mutations L444P and D409H

Detection of L444P was carried out as described by Sidransky *et al.* (1992) and analysis of D409H is described elsewhere (Chabás *et al.*, 1995).

Microsatellite analysis

Microsatellite markers (Dib *et al.*, 1996) D1S305, D1S2777, D1S2721, D1S2140, D1S2624, D1S2715, D1S506, and D1S2635, located close to the *GBA* gene (Cormand *et al.*, 1997), were analysed. Detailed information on these markers is given in Table I. The recombination fraction and lod score values between the *GBA* gene and some of these markers are as follows: D1S305

Table I—Polymorphic markers useful for co-segregation analysis in Gaucher disease

Marker	Type	No. of alleles/ range in bp	Het*
D1S2715	(AC)*n*	9/150–168	0·85
D1S305	(AC)*n*	9/156–176	0·86
D1S2777	(AC)*n*	9/252–274	0·57
D1S2721	(AC)*n*	8/233–247	0·65
D1S2140	(AT)*n* . . . (TATC)*n*	7/232–260	0·71
D1S2624	(AC)*n*	5/203–211	0·71
D1S506	(AC)*n*	6/123–141	0·68
D1S2635	(AC)*n*	10/142–159	0·86
PKLR	(ATT)*n*	6/316–331	0·72
GBA	RFLP (*Hha*I)	2	0·36

*Heterozygosity was determined in most cases by observation of 28 unrelated individuals, according to CEPH–Généthon data.

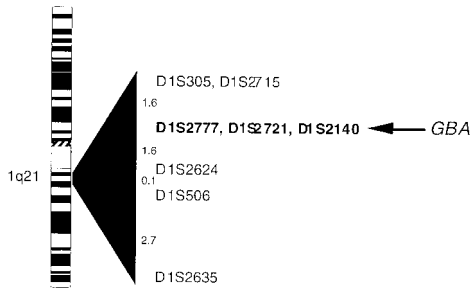

Fig. 1—Cytogenetic and genetic location of the *GBA* gene on chromosome 1. Markers useful for indirect diagnosis of Gaucher disease are shown, with those located at 0 cM of the *GBA* gene in bold (Cormand *et al.*, 1997). Distances between markers (in cM) are derived from the latest version of the Généthon map (Dib *et al.*, 1996)

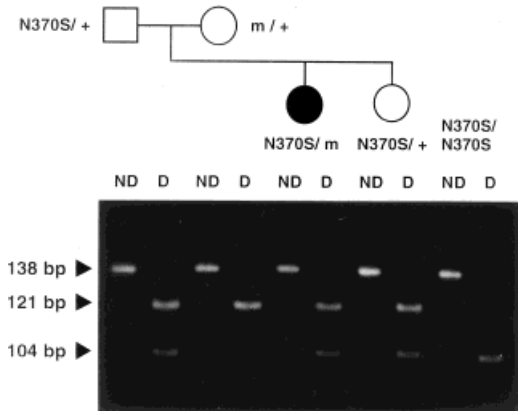

Fig. 2—Restriction analysis of amplified genomic DNA from members of family 1. A GD patient, homozygous for N370S, was included for comparison. Part of exon 9 of the *GBA* gene was amplified and incubated with *Xho*I. Digestion products were separated by electrophoresis in a 12 per cent polyacrylamide gel and visualized with ethidium bromide. + = wild-type allele; m = unknown mutated allele; ND = non-digested; D = digested

$\Theta_{max}=0{\cdot}023$ $Z_{max}=9{\cdot}08$; D1S2777 $\Theta_{max}=0{\cdot}00$ $Z_{max}=6{\cdot}32$; D1S2721 $\Theta_{max}=0{\cdot}00$ $Z_{max}=8{\cdot}43$; D1S2140 $\Theta_{max}=0{\cdot}00$ $Z_{max}=8{\cdot}73$; D1S2624 $\Theta_{max}=0{\cdot}25$ $Z_{max}=8{\cdot}49$. Genetic distances between the markers, shown in Fig. 1, are according to the Généthon map (Dib *et al.*, 1996) and were checked on its online version in May 1997. Marker D1S2140 was previously mapped at 0 cM from D1S2721 (Cormand *et al.*, 1997). All markers belong to the MapPairs set from Research Genetics and were analysed according to the manufacturer's recommendations.

RESULTS

General procedure

The molecular diagnostic protocol consisted in the assessment of mutations N370S, L444P, and D409H, followed by analysis of co-segregation between the disease and highly polymorphic markers.

Family 1

Analysis of the N370S mutation showed that the father, the affected individual, and her sister (for whom carrier detection or a hypothetical prenatal diagnosis could have been requested) were heterozygous for the mutation, as *Xho*I digestion of the PCR-amplified DNA produced two fragments of 121 and 104 bp, respectively (Fig. 2). Digestion of

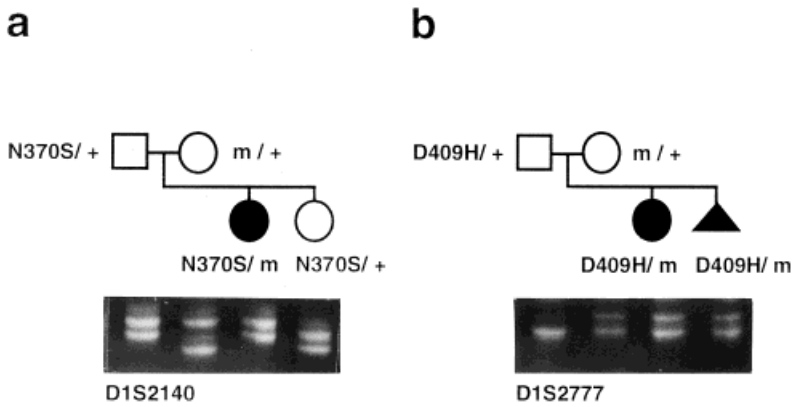

Fig. 3—Co-segregation analysis of Gaucher disease with microsatellite markers from chromosome 1q21 in families 1 (a) and 2 (b). The PCR products were resolved in a non-denaturing 6 per cent polyacrylamide gel and stained with ethidium bromide. + = wild-type allele; m = unknown mutated allele

DNA from the mother gave rise only to a 121-bp fragment, indicating that she was not a carrier of N370S. Undigested DNA appeared as an upper band (138 pb). The absence of this band in lanes corresponding to digested DNA shows that *Xho*I digestion had taken place on the analysed DNA. A sample from a GD patient homozygous for N370S was added for comparison and, as expected, a single band of 104 bp was observed.

Detection of the L444P and D409H mutations gave negative results in all the family members (data not shown).

Once the presence of N370S in the proband's sister had been established, it was necessary to determine whether she had inherited the same maternal GD allele as her affected sister. Eight polymorphic markers close to the *GBA* gene were tested, five of which (D1S2721, D1S305, D1S2635, D1S2140, and D1S506) were found to be informative. Figure 3a shows the results for the marker D1S2140. The father's genotype was 1-2, and the mother's 1-3. Both sisters had inherited the same allele (2) from the father, as expected (because they were both heterozygous for N370S) but received a different allele from the mother, which explains why the patient's sister was unaffected. The same information was obtained from other markers (data not shown).

Family 2

Mutation analyses for both N370S and L444P gave negative results in this family, while the affected individual, her father, and the fetus carried the D409H mutation in one allele (data not shown). Five out of the eight markers analysed (D1S2721, D1S2715, D1S2624, D1S2777, and D1S506) were informative for at least one of the parents. Figure 3b shows the results for the marker D1S2777, which was informative for the mother. The father was homozygous for allele 2, while the mother's genotype was 1-2. Both children had inherited the same maternal allele (1). Taken together, these data indicate that the fetus would have been affected.

DISCUSSION

Prenatal enzymatic diagnosis of Gaucher disease allows the identification of an affected fetus but it cannot differentiate between the various clinical types of the disease. Besides, carrier detection is not always reliable due to overlapping of the enzymatic activity between some heterozygotes and normal individuals (Beutler and Grabowski, 1995). DNA analysis partially overcomes these limitations: mutation detection, in some cases, could predict the severity of disease (for example, N370S undoubtedly indicates type I) and allows the unambiguous identification of a heterozygous carrier.

An important drawback in the use of mutation analysis for prenatal diagnosis and carrier detection in GD is that it exhibits a high degree of allelic heterogeneity. In addition to the most prevalent N370S and L444P (and D409H in the Spanish population), more than 60 mutations have

been reported as responsible for GD (Beutler and Gelbart, 1996). The identification of these uncommon mutations is too expensive and time-consuming for routine use.

The method presented here, based on co-segregation analysis using highly polymorphic markers, allows diagnosis without the need to identify the mutations. A recommended molecular diagnostic protocol would consist in testing the presence of the most frequent mutations, followed by co-segregation analysis when one or both alleles remain unidentified. The reliability of this method is based on the fine genetic localization of the gene (Cormand *et al.*, 1997) in relation to highly polymorphic markers (see Fig. 1). The use of markers known to be at a distance of 0 cM from the gene (i.e., for which no recombination with the *GBA* gene was found), together with close markers at each side of *GBA*, greatly reduces the risk of misdiagnosis due to recombination. The high number of markers mapped to this region ensures that at least some of them would be informative in a given family. Markers at increasing distances from the gene become less reliable because of the higher probability of recombination. In our examples, the marker D1S2635, 4·4 cM distal from *GBA*, recombined in one of the meioses analysed in family 1.

The *PKLR* gene is known to be close to the *GBA* gene and polymorphisms within *PKLR* could also be used for co-segregation analysis. Glenn *et al.* (1994) concluded, after an indirect approach, that the distance between these genes is probably less than 0·2 cM. Moreover, the 340 kb long YAC 887h8 from the CEPH library has been shown to bear both the *GBA* and the *PKLR* genes (Cormand *et al.*, 1997). Two polymorphisms have been described within the *PKLR* gene, a diallelic RFLP (Kanno *et al.*, 1992) and a trinucleotide repeat (Lenzner *et al.*, 1994). The latter, because of its informativeness, could be included as one of the markers for the indirect molecular diagnosis protocol described here (see Table I).

The detection of mutation N370S is important not only for the diagnosis, but also for the prognosis of the disease. The detection method, based on PCR amplification and *Xho*I digestion, was improved by adding an internal digestion control in one of the PCR primers. No fragment of 138 bp should remain after the digestion (see Fig. 2) if the enzyme worked properly. The presence of a 121-bp or a 104-bp band indicates the absence or the presence of N370S, respectively.

In the examples presented here, the second sister in family 1 was diagnosed as a carrier and the fetus in family 2 as affected. In fact, these two families had been previously subjected to extensive mutational analysis at the *GBA* gene. The genotype of the affected sib in family 1 was N370S/1098insA, while the unaffected sister was N370S/+ (Cormand *et al.*, 1996). The index case in family 2 was genotyped as D409H/R120W (nt 475 C→T) (Chabás *et al.*, 1996), as was the affected fetus. The use of other markers, for example D1S506 (data not shown), which was only informative for the father, would allow the indirect diagnosis in this family without performing D409H analyses. For the marker D1S506, the mother was homozygous for allele 1 and the father was heterozygous (1-2). Both the affected child and the fetus received the samel allele (2) from the father.

The use of DNA analysis for diagnostic purposes in a particular inherited disorder is only feasible when the disease-causing mutations can be easily determined or when intragenic or closely linked polymorphic markers are available. This approach is a good complement to classical prenatal enzymatic diagnosis and heterozygous detection. It is particularly useful in those cases where the enzymatic test gives ambiguous results, as in the detection of GD carriers, when only minimal amounts of sample are available, or in those laboratories where enzymatic analyses are not routinely performed. Moreover, it would be the most feasible prenatal diagnostic approach if the analysis of fetal cells in maternal blood (Cheung *et al.*, 1996) becomes widely used.

ACKNOWLEDGEMENTS

We thank Dr F. Mulas, Hospital Infantil La Fe, Valencia, for providing us with samples from family 2. We are also grateful to Robin Rycroft for revising the English. Bru Cormand is a recipient of a fellowship from CIRIT (Generalitat de Catalunya). This work was supported by CICYT (SAF 96-0329).

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