Electronic letter

A simple non-isotopic method to show pitfalls during mutation analysis of the glucocerebrosidase gene

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EDITOR—Partial or total glucocerebrosidase (GBA) gene deletions and genetic rearrangements between the functional *GBA* gene and its highly homologous pseudogene (ψ*GBA*) have been reported as pitfalls in diagnostic mutation analysis leading to incorrect genotyping in Gaucher disease (GD, MIM 230800, 230900, 231000). In particular, homozygosity for 1226G/1226G has been misdiagnosed in the presence of both deletions and complex alleles.[1–6] Because of the necessity selectively to amplify functional *GBA* sequences from unprocessed pseudogene (ψ*GBA*) ones, currently available PCR based techniques make use of primers either in or around the 55 bp pseudogene gap.[7–12] Therefore, if a deletion or a recombination event is present in sites where one of the primers has to bind, the use of these oligonucleotides that hybridise only with the other allele may produce an apparently homozygous genotype, which can lead to incorrect genotyping and unreliable genetic counselling for patients and their families. Thus, as soon as the rapid screening techniques proved occasionally to be misleading in diagnostic mutation analysis, the laboratories that make use of PCR based methods were warned to pay particular attention to this fact.

To ascertain accidental misgenotyping in a panel of GD patients, molecularly characterised by conventional techniques,[13] we have adapted a simple non-isotopic PCR based method from the isotopic one described by Beutler and Gelbart.[1] The procedure is based on simultaneous *GBA* and ψ*GBA* amplification and subsequent comparison of light intensity between the PCR fragments of the gene and the internal control pseudogene. The approach has been used to re-examine 34 patients who were previously genotyped as homozygotes (15 for 1448C (L444P), 14 for 1226G (N370S), one for 1342C (D409H), and four for unique mutations) as well as all patients who had one or both alleles still unidentified, 42 and three respectively. This latter group was reanalysed to try to discover whether deletions or rearrangements had been the cause of the failed identification.

Of a total of 79 GD patients, the PCR based approach allowed us to select six samples (fig 1A). In the six PCR products, visualised by ethidium bromide staining, the *GBA* band was markedly less intense than that of the ψ*GBA*, whereas in normal conditions the *GBA* and ψ*GBA* products gave two bands of approximately the same intensity. To address the hypothesis that one allele could be partially deleted in the six samples, a long template PCR was used to differentiate gene from pseudogene, as already described by Tayebi *et al*.[2] Intriguingly, in three of the six selected patients
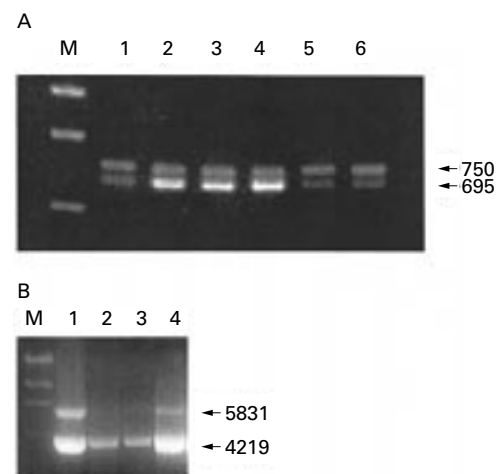

Figure 1 Simultaneous gene-pseudogene PCR amplification. (A) GBA-ψGBA exon 9; the 5' ACCTACACTCTCTGGGGACC 3' (forward) and 5' TTCAGCCCACTTCCCAGACC 3' (reverse) primers are designed on sequences that are homologous to both the gene and the pseudogene, flanking the 55 bp pseudogene gap. By using ethidium bromide staining to visualise the two PCR products (750 bp of the gene and 695 bp of the internal control pseudogene), it is possible to observe in the control approximately the same intensity of gene and pseudogene bands (lanes 1, 5, 6), whereas, conversely, in the case of allele deletion, the band derived from the gene is less intense than the band from the pseudogene (lanes 2, 3, 4). M: marker, φX 174 DNA HaeIII digested. (B) Similar results were obtained by Long Template PCR amplifying both gene and pseudogene from intron 1 using forward 5' AGGAGAGTAGTTGAGGGGTG 3' primer to exon 11 by reverse 5' CTTTAATGCCCAGGCTGAGC 3' primer. PCR reactions were prepared according to the Expand Long PCR system protocol (Boehringer Mannheim). The PCR products containing GBA (5831 bp) and ψGBA (4219 bp) were loaded on a 0.8% agarose gel and visualised using ethidium bromide staining. Lane M: λ DNA size standard. Lanes 1 and 4: PCR products amplified from control DNA. Lanes 2 and 3: PCR products amplified from patients with allele deletion.

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the analysis confirmed a consistent genomic material deficiency of the whole gene in comparison with the pseudogene (fig 1B), but simultaneously showed, in the other three patients, an extra shorter fragment besides the expected *GBA* and *ψGBA* products.

To characterise the nature of this latter alteration, the shorter fragment was cloned and sequenced. A new recombinant allele was identified in two patients who had been diagnosed as having the 1226G/1226G genotype and in one with both alleles still unidentified.[5] On the other hand, in the other three patients, to characterise the deletion encompassing the amplified sequences (intron 1-exon 11), a quantitative fluorescent PCR based technique was adapted from the method described by Woodward *et al*.[14] Two *GBA*:ψ*GBA* ratio values were examined for each sample in exons 2-3 (results not shown) and exon 9 (fig 2). Two pairs of 20 bp primers (one labelled with the fluorescent phosphoramidite (FAM) at the 5' end) were designed on gene/pseudogene homologous sequences flanking pseudogene gaps making it possible to generate two distinct products. The primers encompassing the deleted 55 bp portion at exon 9 were 5' FAM-ACCTACACTCTCT GGGGACC 3' (forward) and 5' TTCAGCCC ACTTCCCAGACC 3' (reverse). The primers around the pseudogene gap in intron 2 were 5' FAM-TGAGTGACTGAGACCAACTT (forward) and 5' GAAGCTTTTAGGGATGCA GG 3' (reverse). An automated DNA sequencer was used to quantify the fluorescently labelled PCR products. Electropherograms

showed two peaks representing gene and pseudogene fragments; dosage estimates were obtained by comparison of the area under the peak correlating to the quantity of fluorescent signal incorporated by the labelled primer. As expected, the *GBA*:ψ*GBA* ratio was 1:1 in the control, whereas in the three suspected patients it was 0.5:1, corresponding to one and two copies of the gene and pseudogene, respectively. Using such a technique, the complete gene deletion was confirmed in three patients previously genotyped as 1226G/1226G, and in five unaffected relatives who were classified as non-carriers by conventional screening methods. Nevertheless, it must be admitted that quantitative assay can be affected by several factors, such as non-uniform amplification of the *GBA* and *ψGBA* fragments as well as the quality of genomic DNA.

In conclusion, the simple non-isotopic PCR based method, used by us to ascertain accidental misgenotyping, enabled us to select six (five apparently homozygous for 1226G/ 1226G and one with both alleles still undetected) of the 79 previously genotyped patients, so that they could be correctly genotyped at a later time. It must, however, be admitted that the approach is not diagnostically useful because of inherent limitations in its use. As a matter of fact, the method based on simultaneous *GBA* and *ψGBA* amplification and comparison of light intensity between the PCR fragments allows only an approximate valuation of the amount of template requiring further specific investigation. Nevertheless, it proved to be suitable for pointing out pitfalls, especially when it is not possible to quantify the number of *GBA* gene copies, and the parents are not available for carrier analysis. Recently, the method prompted us to further investigations in another patient with apparent homozygosity for 1226G, leading to the characterisation of an allele with the partial deletion Δ55. Furthermore, the set up of the quantitative fluorescent PCR, confirming the complete gene deletion in three samples, was useful for assigning the correct genotype to the patients and to discriminate between carriers and non-carriers as well.

Therefore, we emphasise the need for additional techniques in order to refine genotyping for reliable genetic counselling for the patients and their families.

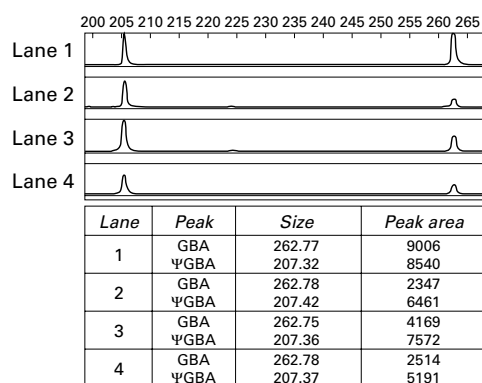| Lane	Peak	Size	Peak area
1	GBA	262.77	9006
	ΨGBA	207.32	8540
2	GBA	262.78	2347
	ΨGBA	207.42	6461
3	GBA	262.75	4169
	ΨGBA	207.36	7572
4	GBA	262.78	2514
	ΨGBA	207.37	5191

Figure 2   Quantitative fluorescent PCR results in exon 9. The amplification was carried out using two pairs of 20 bp primers designed on gene/pseudogene homologous sequences flanking pseudogene gaps. PCR reactions were carried out in a volume of 10 μl containing 100 ng of DNA, 1 × AmpliTaq Gold PCR buffer (Perkin-Elmer), 200 μmol/l of each dNTP, 1 μmol/l of each primer, 1.5 mmol/l MgCl₂, and 0.75 U of AmpliTaq Gold enzyme. Amplification conditions were 94°C for 10 minutes, 18 cycles of one minute at 94°C, one minute at 54°C, and one minute at 72°C, with a final extension of 10 minutes at 72°C. Products were analysed on an ABI 373A DNA sequencer using GENESCAN software. Electropherograms showed two peaks representing the two PCR products, GBA (262 bp) and ψGBA (207 bp). Dosage estimates are obtained by comparison of the area under the peak correlating to the quantity of fluorescent signal incorporated by the labelled primer. Lane 1: PCR products from DNA control; the GBA:ψGBA ratio was about 1:1 (9006:8540). Lanes 2, 3, 4: PCR products from DNA patients with GBA reduced copy number; the GBA:ψGBA ratio was about 0.5:1, in particular, 2347:6461 (lane 2), 4169:7572 (lane 3), 2514:5191 (lane 4).

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