Identification of Recombinant Alleles Using Quantitative Real-Time PCR

Implications for Gaucher Disease

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Pseudogenes, resulting from duplications of functional genes, contribute to the functional complexity of their parental genes. The glucocerebrosidase gene (*GBA*), located in a gene-rich region on chromosome 1q 21, is mutated in Gaucher disease. The presence of contiguous, highly homologous pseudogenes for both *GBA* and *metaxin 1* at this locus increases the likelihood of DNA rearrangement. We describe a facile method to identify and analyze recombinant alleles in patients with Gaucher disease. Genomic DNA from 20 patients with recombinant *GBA* alleles and five controls was evaluated to identify DNA rearrangements or copy number variation using six probes specific for either the *GBA* gene or pseudogene. Quantitative real-time PCR was performed on genomic DNA, and Southern blot analyses using HincII together with sequencing confirmed the real-time results. Both *GBA* fusions and duplications could be detected. Different sites of crossover were identified, and alleles resulting from gene conversion could be distinguished from reciprocal recombinant alleles. Quantitative real-time PCR is a sensitive and rapid method to detect fusions and duplications in patients with recombinant *GBA* alleles. This technique is more sensitive, faster, and cheaper than Southern blot analysis, and can be used in diagnostic laboratories, and to detect other recombinant alleles within the genome. *(J Mol Diagn 2011, 13:401–405; DOI: 10.1016/j.jmoldx.2011.02.005)*

DNA rearrangement, occurring by chromosomal pairing, breakage, and crossover between two non-sister chromatids during meiosis, is a cellular process essential for genetic diversity. Recombination between homologous sequences may also introduce harmful mutations. DNA rearrangement is influenced by the degree of sequence homology between corresponding segments, and can be prompted by the presence of a pseudogene.

Gaucher disease (GD) (type 1: OMIM #230800, type 2: OMIM #230900, type 3: OMIM #2301000), the most common lipidosis and the most frequently inherited disorder among Ashkenazi Jewish individuals, presents with hepatosplenomegaly, anemia, thrombocytopenia, bone involvement, and neurological symptoms (type 2 and 3). This disorder is caused by the deficiency of the lysosomal enzyme glucocerebrosidase, which degrades glucocerebroside, a membrane glycolipid, to ceramide and glucose. The gene for glucocerebrosidase (*GBA*) (OMIM #606463) is located on 1q21, a gene-rich region encompassing seven genes and two pseudogenes within 85 kb.[1–3] *GBA*, spanning 7.6 kb, has 11 exons and 10 introns. A highly homologous 5.7-kb pseudogene (*GBA*P) is located 16 kb downstream, with the same organization of exons and introns.[3] Another gene, metaxin (*MTX*), encoding for a mitochondrial membrane protein, is convergently transcribed and located immediately downstream of *GBAP,* with a pseudogene (*pMTX*) located between *GBA* and *GBAP* (Figure 1A).[4,5]

GBA and *GBAP* share more than 96% exonic sequence homology, enhancing the likelihood of homologous recombination.[6] Such recombinations can result in deletions, duplications, inversions, and fusions leading to genetic disorders.[7] Recombination events between *GBAP* and *GBA* or *MTX* and *MTXP* are responsible for several different identified mutant alleles.[8,9] Crossover has been reported at sites ranging from intron 2 in *GBA* to *MTX* (Figure 1B), causing both reciprocal and nonreciprocal recombination.[8]
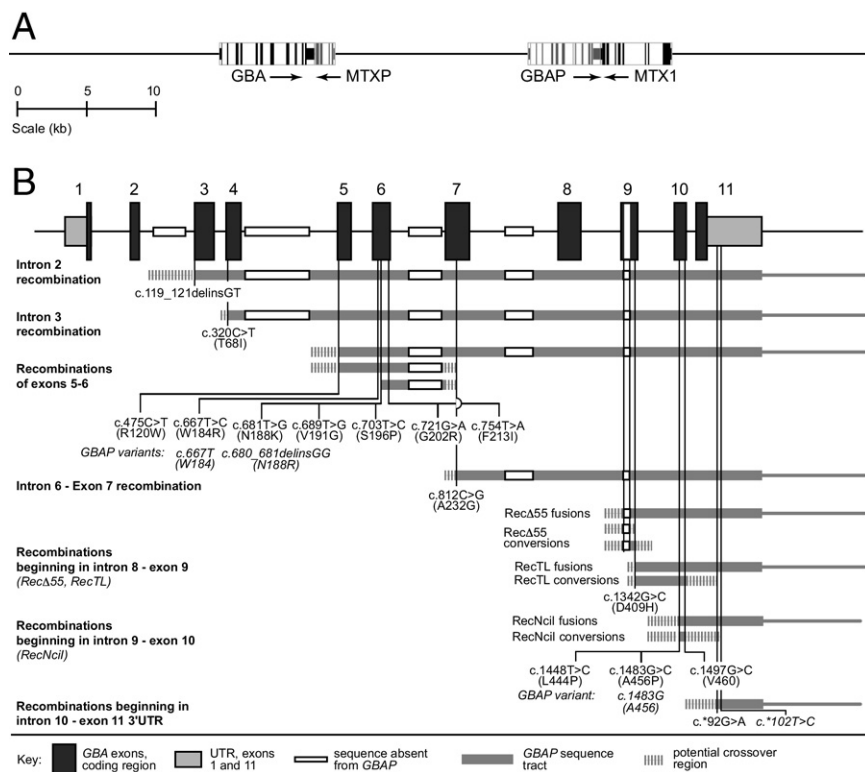
To accurately describe a recombinant allele, a combination of direct sequencing and Southern blot analyses, often performed using several restriction enzymes, has been required.[8] Since both of these

Figure 1. A: The location of glucocerebrosidase gene (*GBA*) and its pseudogene, *metaxin* and *metaxin* pseudogene on chromosome 1. **B:** Exonic and intronic structure of *GBA*, pseudogene-derived mismatches, and amino acid changes associated with the allele are indicated. Typical nonreciprocal (gene conversion) and reciprocal (gene fusion or duplication) recombinants are shown. The sequences absent in pseudogene are also shown. (Reproduced from Hruska et al, 2008.[9])

techniques are time consuming and expensive, they are not commonly used in diagnostic laboratories, and the detection of recombinant alleles in diagnostic settings has been challenging. We report the optimization of a facile method to identify and analyze recombinant alleles in patients with GD using quantitative real-time PCR (real-time qPCR).

Materials and Methods

Sample Preparation

DNA samples collected under National Institutes of Health institutional review board–approved protocols from 20 patients with recombinant *GBA* alleles and from five controls were extracted following standard protocols.

Real-Time Analysis

Six sets of primers and probes were designed targeting sequence present in the *GBA* gene, but absent from *GBAP*, synthesized by Applied Biosystems (Table 1) to detect any variation in the gene structure caused by a partial or complete deletion or duplication (Applied Biosystems, Foster City, CA). Six additional primer sets and probes were designed targeting *GBAP*. Each *GBA/GBAP* probe was labeled with FAM. A labeled β-globin probe was co-amplified as an internal control.

Quantitative real-time PCR reactions were optimized and performed using an Applied Biosystems StepOne-Plus real-time PCR System. Reactions in a 20-µL volume included 10 µL of TaqMan Universal PCR Master mix

(Applied Biosystems), 0.5 µL of each probe (10 µmol/L), 0.5 µL of β-globin probe (10 µmol/L), 1.8 µL of primer mix for each target (20 µmol/L), 1.8 µL of β-globin primer mix (20 µmol/L), and 10 ng of genomic DNA. Real-time PCR was performed with one cycle at 50°C for 2 minutes, one cycle at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Three replicates were used for each sample, and each reaction was performed twice.

The normal range for relative quantitation (RQ) was determined based on 2-ΔΔCt for each allele, using 10 samples with fusion alleles, and 10 samples with duplications compared to five normal controls. On the basis of these preliminary results, it was determined that the normal copy number ranged from 0.8 to 1.25 RQ, whereas fusion alleles were less than 0.5 RQ, and duplications were greater than 1.75 RQ (Figure 2A).

Southern Blot Analyses

HincII-digested genomic DNA (10 to 15 µg) was electrophoresed on a 0.6% I.D.NA agarose gel (FMC Bioproducts, Rockland, ME) and hybridized with ^{32}P-labeled *GBA* cDNA.[8]

Sequencing of GBA

Exons and flanking intronic regions of *GBA* were sequenced using a 3130 Genetic Analyzer (Applied Biosystems),[10] and data analyzed using Sequencing Analysis v.5.1.1 and SeqScape v.2.1.1 software programs (Applied Biosystems). Intronic regions of *GBA*

Table 1. *GBA* and *GBAP* primer and probe sequences

	GBA		*GBAP*
Intron 2		Intron 2	
Probe:	5′-CCCAGCTAATTTTTGTATTT-3′	Probe:	5′-CAGCTGTGTCCGTTCT-3′
Forward primer:	5′-ACAGGCGGCCACCACTAC-3′	Forward primer:	5′-TGAGGATTTTTGAAACCGTCTTC-3′
Reverse primer:	5′-CATGGTGAAACCCCGTCTCTA-3′	Reverse primer:	5′-GGTGAGGTCTGACAAGGATGTG-3′
Intron 4		Intron 4	
Probe:	5′-ATGATATACCTGCCTTGGC-3′	Probe:	5′-TCTTTGCCAATTCTTT-3′
Forward primer:	5′-CATGTTGACCAGGCTAGTCTTAAACTC-3′	Forward primer:	5′-GGATGACATAGAGCCACTGACACT-3′
Reverse primer:	5′-CGGTGGCTCACACTTGTAATTC-3′	Reverse primer:	5′-GGTGAGGTCTGACAAGGATGTG-3′
Intron 6		Intron 6	
Probe:	5′-CTGCCTCCTGAGTTCA-3′	Probe:	5′-TTCTCTTGCCAGGTTC-3′
Forward primer:	5′-CGCCATCTTCACTCACTGTAACC-3′	Forward primer:	5′-TCGGTCTGGCCCACTTTCT-3′
Reverse primer:	5′-AAGGCTGAGGCAGGAGAATTG-3′	Reverse primer:	5′-GTGCTCAGCATAGGCATCCA-3′
Intron 7		Intron 7	
Probe:	5′-CACTGCACTCCAGCC-3′	Probe:	5′-TTCTGGAGGCTAAGGC-3′
Forward primer:	5′-TTGCGGTGAGCCGAGATC-3′	Forward primer:	5′-GGCACGCCTATAGTCCCAAGT-3′
Reverse primer:	5′-CCCCCAGCCTAGATAGTTTTGTT-3′	Reverse primer:	5′-CCTGAAATTCTAGGCTCAACTAAACCT-3′
Exon 9		Exon 9	
Probe:	5′-ACAGTCCCATCATTG-3′	Probe:	5′-AGACATCACCAAGCAC-3′
Forward primer:	5′-CAATTGGGTGCGTAACTTTGTC-3′	Forward primer:	5′-CCGACTGGAACCCATCATTG-3′
Reverse primer:	5′-AAAACGTGTCCTTGGTGATGTCT-3′	Reverse primer:	5′-GAACATGGGCTGTTTGTAAAACG-3′
3′ UTR (*pMTX*)		3′ UTR (*MTX*)	
Probe:	5′-CCCATTGAGAAGAAGG-3′	Probe:	5′-AAGGAAGGCTCCGCC-3′
Forward primer:	5′-CCCGCTGGCCGTCAA-3′	Forward primer:	5′-GTCCAGCGCCCAATGC-3′
Reverse primer:	5′-TTGCGGCCCAACCTTTC-3′	Reverse primer:	5′-TTCTGGGAGCTGCTCCTAGTTT-3′
β-Globin			
Probe:	5′-CTCATGGCAAGAAAGTGCTCGGTGC-3′		
Forward primer:	5′-TGGGCAACCCTAAGGTGAAG-3′		
Reverse primer:	5′-GTGAGCCAGGCCATCACTAAA-3′		

with polymorphic sites were sequenced to confirm amplification of both alleles.

Results

Quantitative comparative (ΔΔCt) real-time qPCR identified both *GBA* fusion alleles, where the 5′ portion of the allele is derived from the gene and the 3′ from the pseudogene, and duplicated alleles, with an extra copy of the pseudogene using a normal sample as a calibrator. Moreover, unlike Southern blot analysis, this technique could discern mutant alleles resulting from gene conversion from those due to deletions, and detect the specific crossover site. All *GBA* fusions and duplications identified by Southern blot analysis were confirmed with real-time qPCR. However, real-time qPCR was more sensitive, enabling the identification and definition of several complex recombinant alleles as summarized in Table 2 and described below:

1) *Large deletions*: In Patient 5, the Southern blot analysis indicated a recombinant fusion allele (Figure
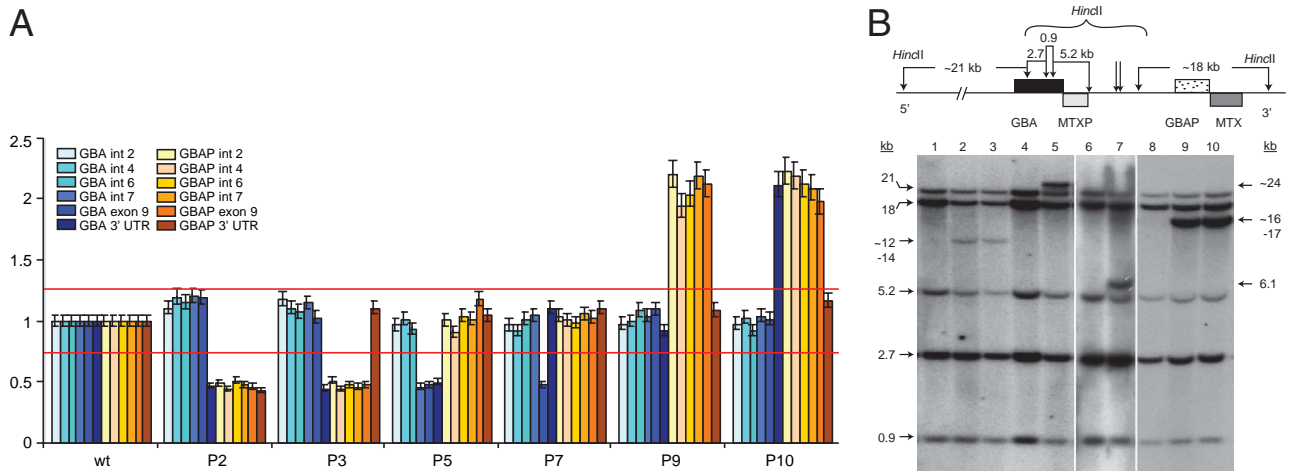
Figure 2. A: Results of quantitative real-time PCR of *GBA* and *GBAP* performed in samples from the patients listed in Table 2. The probes are in order, beginning from upstream of *GBA* to the 3′ end of *GBAP*. WT: Control sample with no known recombination, used to normalize the data; P2 and P3: patients with fusion alleles; P5: patient with a large deletion; P7: patient with recΔ55 [c.1263-1317del (55-bp deletion)]; P9 and P10: patients with *GBA* duplications. The *y* axis reflects relative quantitation. The **red lines** indicate the range in copy number found among controls. **B:** Southern blot using HincII-digested DNA samples, corresponding to the cases described in Table 2 (except P4, where insufficient DNA was available for real-time qPCR). Normal individuals have five bands as shown, whereas patients with recombinant alleles demonstrate extra bands.

Table 2.   The Genotypes of the Cases with Recombinations Studied

Patient	Phenotype	Genotype*	Site of cross over initiation	Southern blot	Real-time qPCR
1, 6, 8	Wild type	Normal controls (wt/wt)			
2	Type 2	p.Leu483Pro (L444P) + p.Glu365Lys (E326K)/ p.Leu483Pro (L444P)+ Rec F	*MTX*	Fusion in *MTX*	Fusion in *MTX*
3	Type 1	p.Arg502Cys (R463C)/Rec F	Intron 9	Fusion	Fusion of *GBA* and *GBAP* gene
4	Type 1	p.Asn409Ser (N370S)/Rec F	Exon 10	Normal	NA
5	Type 2	c.1505 + 2T>A (IVS10 + 2)/ Rec D	Exon 7	Fusion	Partial *GBA* deletion
7	Type 2	p.Arg296Gln (R257Q)/ RecΔ55 (c.1263-1317del)	Intron 8-exon 9	Missing exon 9 site on Southern blot	55-bp deletion (gene conversion)
9	Type 1	p. Arg209Cys (R170C)/ p.Leu483Pro (L444P)+ Rec G	3′ UTR (PMTX)	Duplication in pseudogene	Duplication in pseudogene
10	Type 1	c.222-224delTAC/Rec G	Intron 10-exon 11	Duplication in pseudogene	Duplication in pseudogene

GBA cDNA nucleotides (*c.*) are numbered designating the adenine of the first ATG translation initiation codon as the first nucleotide (GenBank reference sequence NM_000157). Amino acid designated with (*p.*) are based on the primary *GBA* translation product, including the 39-residue signal peptide. Recombinant alleles mentioned here are described in more detail in Tayebi et al and Hruska et al.[8,9]

   Rec D: recombination at the end of exon 7. *Rec F*: recombination at the end of intron 9 or beginning of exon 10, *Rec G*: recombination in intron10-exon 11 or the 3′ untranslated region.

   NA, not available; wt, wild type.

2, A and B), where the crossover site was approximated to be between the end of intron 6 and the beginning of exon 7. Real-time qPCR demonstrated that this patient actually had a partial deletion allele extending from intron 6 to the 3′ UTR (Figure 2A).

2) *Allelic conversion*: Real-time qPCR could detect non-reciprocal sequence exchanges, including gene conversions, which occur frequently with homologous sequences. Probes from different regions of the *GBA* locus were used to detect gene conversion events. For example, a common Gaucher mutation, rec Δ55 in exon 9, resulting from gene conversion, is easily identified by real-time qPCR, since the exon 9 probe detects only one copy (Figure 2, A and B, patient 7).

3) *Recombinations in the 3′ UTR region*: Because the number of DNA restriction sites is limited, different recombinant alleles with crossover sites that are close by look the same on Southern blot analysis. Figure 2B, lane 9, shows a duplication, with the site of initiation of the crossover in the 3′ UTR of *GBA*. A second patient (lane 10) appeared to have the same banding pattern. Using a probe in this region, real-time qPCR narrowed down the site of crossover initiation, demonstrating that these were two different mutant alleles. In lane 9, the 3′ UTR of *GBA* is normal, whereas in lane 10, the probe indicates that this region is duplicated (Figure 2A). Since an enhancer is found in the 3′ UTR, duplication of this region could impact the phenotype.[11]

Similarly, the cases in lanes 2 and 3 (Figure 2, A and B) both carry fusion alleles, resulting from recombination occurring in the 3′ UTR and intron 9. Although by Southern blot analysis both patients have the same bands, real-time qPCR confirms the presence of the *GBAP* 3′ UTR (MTX) probe in lane 3, although not in lane 2. The phenotypes of these two cases were extremely different, reflecting the different length of the deleted segment.

Discussion

We demonstrate that real-time qPCR is a more sensitive alternative method to detect recombinant alleles previously only identified with Southern blots and extensive sequencing. Reciprocal crossover between homologous regions can result in fusions with RQ values less than 0.5, and duplications with values of over 1.75. When different copy numbers are detected with two nearby probes, it can be concluded that the crossover lies in the region between the probes.

The presence of a highly homologous pseudogene sequence greatly complicates genotypic analysis in many disorders. In the case of Gaucher disease, the high degree of sequence homology between the exons of *GBA* and *GBAP* result in many of the mutant alleles encountered in patients, complicating the accurate identification and description of mutant *GBA* alleles. The development of real-time qPCR as a means to detect copy number variation provides a new tool for evaluating genomic duplications, deletions, and other rearrangements, facilitating the identification and definition of complex *GBA* alleles. Other advantages of this method over Southern blot analysis is that it requires far less DNA and can detect all known *GBA* recombinant alleles in a single reaction, whereas previously, two or more restriction enzyme digests were necessary.[8] Southern blot analyses are also limited by available restriction sites, leading to false-negative results, whereas with real-time qPCR, our ability to design more specific probes increases the sensitivity.

In Gaucher disease, the development of diagnostic kits has proven to be quite challenging due to the homologous nearby *GBA* pseudogene and the occurrence of recombinant alleles. The few kits commercially available screen for a limited number of mutations, and can generate false-positive and false-negative results, leading to diagnostic errors.[12,13]

Because of limitations in genotype–phenotype association in Gaucher disease, it has been postulated that other genes within the *GBA* locus, such as *MTX*, transcription factor binding sites, or enhancers may modify patient phenotype. Real-time qPCR may detect sequence rearrangements that impact gene function.

In the case of *GBA,* the design of primers and probes posed a major challenge, due to the high homology between *GBA* and *GBAP*. Multiple sets were optimized and tested, and the primers presented here were found to be the most useful for differentiating the various *GBA* recombinations. Our ability to compare the real-time results to traditional Southern blot analyses confirmed the value of this technique. However, as with Southern blot analysis, this approach may still miss some recombinations, especially in the upstream region of the gene, and may not discern whether recombinations result from double or reciprocal crossover.

We demonstrate that quantitative real-time PCR is a sensitive and rapid method to detect fusions and duplications in patients with recombinant *GBA* alleles. Since this technique is faster and less costly than Southern blot analysis, it may have wide applicability in diagnostic laboratories. Moreover, this technique may prove valuable in evaluating other genes in which mutations commonly result from recombination events.

Acknowledgments

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