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MLPA-based approach for initial and simultaneous detection of *GBA* deletions and recombinant alleles in patients affected by Gaucher Disease

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ABSTRACT

The chromosomal region, in which the *GBA* gene is located, is structurally subject to misalignments, reciprocal and nonreciprocal homologous recombination events, leading to structural defects such as deletions, duplications and gene-pseudogene complex rearrangements causing Gaucher Disease (GD).
Interestingly deletions and duplications, belonging to the heterogeneous group of structural defects collectively termed Copy Number Variations (CNVs), together with gene-pseudogene complex rearrangements represent the main cause of pitfalls in GD mutational analysis. In the present study, we set up and validate a Multiplex Ligation-dependent Probe Amplification (MLPA)-based approach to simultaneously investigate the potential occurrence of CNVs and complex rearrangements in 8 unrelated GD patients who had still not-well-characterized or uncharacterized alleles. The findings allowed us to complete the mutational analysis in 4 patients, identifying a rare deletion (g.-3100_+834del3934) and 2 novel recombinant alleles (g.4356_7031conJ03060.1:g.2544_4568; g.1942_7319conJ03060.1:g.1092_4856).
These results demonstrate the diagnostic usefulness of MLPA in the detection of *GBA* deletions and recombinations. In addition, MLPA findings have also served as a basis for developing molecular approaches to precisely pinpoint the breakpoints and characterize the underlying mechanism of copy number variations.

1. Introduction

Gaucher Disease (GD), the autosomal recessive inherited deficiency of the lysosomal enzyme acid β-glucosidase (EC:3.2.1.45), encoded by the glucocerebrosidase gene (*GBA*; MIM #606463; GenBank accession #J03059.1), leads to the progressive storage of undigested glucosylceramide and its lyso-derivative glucosylsphingosine. Although GD encompasses a continuum of clinical traits from perinatal lethality to asymptomatic conditions, it has been traditionally classified into three main clinical forms on the basis of presence and rate of progressive neurological symptoms: type 1 GD (MIM #230800), the most common phenotype, is classically considered the non-neuropathic variant; type 2 GD (MIM #230900) is associated with early death due to a severe neurodegenerative impairment; type 3 GD (MIM #231000), already known as the chronic neuropathic form, comprises a wide heterogeneous group of clinical manifestations including different stages of onset (from childhood to early adulthood) and diverse combinations of mild/severe systemic disease with variable neurological involvement [1,2].

The *GBA* gene comprises 11 exons and 10 introns spanning 7.6-kb of sequence. It is located on chromosome 1q21, a gene-rich region encompassing seven genes and two pseudogenes within an 85-kb genomic portion [3,4]. A highly homologous 5.5 kb pseudogene (*GBAP1*) is located 16 kb downstream with a similar organization in exons and introns as the functional gene [3]. Metaxin gene (*MTX1*), encoding for an outer mitochondrial membrane protein involved in the preprotein import complex, and its pseudogene (*MTX1P1*) are contiguous to the *GBAP1* and *GBA* genes, respectively, but in the opposite direction [5]. *GBA* and *MTX1* genes share 96% and 98.9% of sequence homology with their respective pseudogenes, likely originating from a duplication event of this chromosomal region [4,5].

To date, >430 variants in the *GBA* gene have been reported as causing of the disease (see the Human Gene Mutation Database, www.hgmd.org) [6]. These include all kind of point mutations (missense/

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nonsense, splicing and regulatory), small deletions/insertions, indels and structural variants such as large insertions, gross deletions/duplications, collectively termed Copy Number Variations (CNVs), and gene-pseudogene complex rearrangements.

The above structural lesions can be promoted by high sequence homology of repeated DNA fragments, such as *Alu* motifs, and duplicated portions, such as low-copy repeats elements, gene clusters and pseudogenes, both representing local genomic instability hotspots. Potential outcomes comprise misalignments, reciprocal/nonreciprocal homologous recombination events and DNA rearrangements (deletions, duplications, inversions and fusions), that are more likely to occur when repeated/duplicated regions are not adjacent but in close proximity on the same chromosome [7].

Deletions and duplications belonging to the heterogeneous group of large structural defects can result in novel breakpoint junctional events that, in a diploid genome, lead to copy number variants (CNVs) at a given locus. The CNV size ranges from a hundred to millions of base pairs of DNA [8].

Gene-pseudogene complex rearrangements, yielding recombinant alleles, arise from either nonreciprocal (gene conversion) or reciprocal homologous recombination [7]. Gene conversion, representing the main form of such phenomenon, mediates the transfer of genetic information from an intact homologous sequence ("donor") that is copied into an analogous region containing double-strand breaks ("acceptor") created by a topoisomerase-like enzyme during meiosis. In GD, such finely regulated pathway leads to the whole or partial replacement of *GBA* sequence ("acceptor") with *GBAP1* sequence ("donor") [7,9]. The reciprocal homologous recombination entails the occurrence of a single crossover event resulting in the formation of fusion alleles, deletions or duplications between *GBA* and *GBAP1* and, although rarely, between *GBA*/*GBAP1* and *MTX1*/*MTX1P1* [10].

Additionally, *Alu*-*Alu*-mediated DNA rearrangements, known to be causative of human genetic diseases [11], have been described even in GD as underlying mechanism of gene-pseudogene recombinations [12,13] and deletion [14].

Deletions and duplications, together with *GBA*-*GBAP1* complex rearrangements, represent the main cause of pitfalls in GD mutational analysis as the conventional polymerase chain reaction (PCR)-based molecular testing makes use of primers designed in pseudogene gaps to discriminate the functional *GBA* gene from the unprocessed *GBAP1* pseudogene. Therefore, if a structural defect affects a primer binding sequence, the use of these oligonucleotides, preventing the annealing (drop-out allele), hybridise only with the other allele giving the appearance of homozygosity. Hence, the detection of CNVs and *GBA*-*GBAP1* rearrangements, requiring several complementary approaches to the traditional ones, turns out to be complicated and time-consuming in GD diagnostic routine.

Some years ago, we started to make an inventory of the *GBA* sequence variations, including various structural defects, in large series of Italian GD patients by using conventional and supplemental methods [15–18]. In the present study, we set up and validate a Multiplex Ligation-dependent Probe Amplification (MLPA)-based approach (MRC-Holland, Amsterdam, Netherlands) to simultaneously investigate the potential occurrence of CNVs and complex rearrangements in DNAs extracted from 8 unrelated GD patients from our series who had still not-well-characterized or uncharacterized alleles. The results allowed us to totally (4 patients) or partially (2 patients) complete the mutational analysis. This study, integrated with the previous published ones, provides a more complete picture of the molecular basis of GD in Italy.

2. Material and methods

2.1. Cohort of the study

Thirty-eight individuals were analysed of whom 19 were patients (pts) affected by GD and the remaining 19 were unrelated healthy controls (negative references). Among the GD series, 11 pts (positive references) carried already known genomic variants in compound heterozygous or in homozygous state. The previously analysed genotypes included genomic lesions (i) affecting some MLPA probe signals (MLPA detectable variants), such as complex recombinant alleles g.7319_7368conJ03060.1:g.4856_4905 (RecNciI), g.6764_6820con J03060.1:g.4356_4357 (RecΔ55), g.4641_J03060.1:g.2828 (Complex I-Rec 7), c.1448T>C;c.1497G>C (RecFs) and 2 missense variants c.1448T>G (L444P), c.754T>A (F213I); or (ii) involving nucleotides beyond those regions (MLPA undetectable variants) consisting of missense/nonsense variants, such as the common c.1226A>G (N370S), c.681T>G (N188K), the rare c.599T>A (I161N) and the novel c.516C>A (Y133*) (Table 1). The remaining 8 GD pts, belonging to 7 families, were included in the MLPA analysis as they harboured still not-well-characterized (6 pts) or unknown (2 pts) alleles. Clinically, seven of them were classified as type 1 and one as type 3. They were selected on the basis of previous results obtained from different conventional molecular tests, based on a preliminary PCR-amplification with the sets of primers "A" and "B" designed to discriminate between *GBA* and *GBAP1* sequences (Table 2). Whenever possible, the molecular testing was refined by transcript sequencing of PCR-product amplified with the set of primers "D" (Table 2) and/or performed with alternative techniques for pointing out possible structural defects. The additional detection methods were mostly based on the simultaneous amplification of *GBA* and *GBAP1* using the set of primers "C" (Table 2) potentially revealing abnormally sized PCR-product(s) (in addition to the expected *GBA* and *GBAP1* ones), resulting from complex recombinant alleles, which could be thus cloned and sequenced.

The initial results of the mutation analyses carried on the 8 patients are summarized as follows:

Pt #1. *GBA* genomic sequencing revealed the common c.1226A>G (N370S) missense variant in heterozygous state but cDNA analysis displayed an apparent homozygosis. This inconsistency suggested a possible drop-out allele mechanism preventing the expression of such allele.

Pts #2, #3, #4 and #5–6 sibs. Although the genomic molecular analysis showed the c.1226A>G (N370S) in apparent state of homozygosis, the same analysis extended to the respective parents did not confirm the variant in paternal (pt #2) and maternal (pt #3, pt #4 and pts #5–6) samples. The preliminary DNA profiling performed by analysis of variable number of tandem repeats (VNTR) loci confirmed the equal contribution of both parents in the four families. All these findings, thus, suggested the occurrence of deletions and/or complex rearrangements in these patients.

Pts #7 and #8. Conventional molecular testing identified the heterozygous missense variants c.1226A>G (N370S) in pt #7 and c.809C>G (T231R) in pt #8, whereas their respective second allele remained still unknown. As RNA samples were not available, MLPA analysis was planned to reveal potential occurrence of a drop-out allele mechanism or a partial deletion or a complex rearrangement affecting genomic regions far from the missense variants.

2.2. Ethical aspects

The patients' samples were obtained from the "Cell Line and DNA Biobank from Patients affected by Genetic Diseases" [19]. Following ethical guidelines, the samples were collected for analysis and storage in the Biobank with the patients' (and/or a family member's) written informed consent, using a form approved by the Regional Ethics Committee.

2.3. Molecular analysis

Genomic DNA was extracted from whole peripheral blood using Nucleon BACC3 Kit (Amersham Biosciences, UK), QIAamp DNA blood Mini Kit (Qiagen, Germany) and REALPURE Spin Kit (REAL life-science solutions, Spain).

Total RNA was extracted from the lymphoblast cell line of pt #1 using Rneasy Mini kit (Qiagen, Germany) and reverse transcribed by

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