



# Identification of novel splice site mutation IVS9 + 1(G>A) and novel complex allele G355R/R359X in Type 1 Gaucher patients heterozygous for mutation N370S☆



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## ABSTRACT

Gaucher disease is an autosomal recessive lysosomal storage disorder resulting from deficient glucocerebrosidase activity. More than 350 mutations that cause Gaucher disease have been described to date. Novel mutations can potentially provide insight into the glucocerebrosidase structure–function relationship and biochemical basis of the disease. Here, we report the identification of two novel mutations in two unrelated patients with type I (non-neuronopathic) Gaucher disease: (1) a splice site mutation IVS9 + 1G>A; and (2) a complex allele (*cis*) G355R/R359X. Both patients have a common N370S mutation in the other allele. The splice site mutation results from an intronic base substitution (G to A, c.1328 + 1, g.5005) at the donor splice site of exon and intron 9. The complex allele results from two point mutations in exon 8 of glucocerebrosidase (G to C at c.1180, g.4396, and T to C at c.1192, g.4408) substituting glycine by arginine (G355R) and arginine by a premature termination (R359X), respectively. In order to demonstrate that G355R/R359X are in *cis* arrangement, PCR-amplified glucocerebrosidase exon 8 genomic DNA from the patient was cloned into the vector pJET1.2 in *Escherichia coli* TOP10® strain. Out of the 15 clones that were sequence analyzed, 10 contained the normal allele sequence and 5 contained the complex allele G355R/R359X sequence showing both mutations in *cis* arrangement. Restriction fragment length polymorphism analysis using *Hph*I restriction endonuclease digest was established for the IVS9 + 1G>A mutation for confirmation and efficient identification of this mutation in future patients. Past literature suggests that mutations affecting splicing patterns of the glucocerebrosidase transcript as well as mutations in Gaucher complex alleles are detrimental to enzyme activity. However, compound heterozygosity with N370S, a mild mutation, will lead to a mild phenotype. The cases reported here support these past findings.

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## 1. Introduction

Gaucher disease (GD) is an autosomal recessive lysosomal storage disorder caused by a profound deficiency in the enzyme glucocerebrosidase [GBA (glucosylceramidase, acid beta-glucosidase) EC.3.2.1.45] (Brady et al., 1965). The deficiency in GBA results in the accumulation of its

lipid substrate glucocerebroside in reticuloendothelial cells with cardinal clinical findings of hepatosplenomegaly, pancytopenia from bone marrow infiltration and osteonecrosis. Three clinical forms or subtypes of GD have been reported: Type I (non-neuronopathic, OMIM 230800), Type II (acute neuronopathic, OMIM 230900), and Type III (sub-acute neuronopathic, OMIM 231000) (Beutler and Grabowski, 1995; Mikosch, 2011). Gaucher disease is a panethnic disease with an overall prevalence of 1/100,000, but higher prevalence in the Ashkenazi Jewish population of 1/855 with predominantly the N370S allele (Guggenbuhl et al., 2008).

Mutations in the *GBA* gene of patients with GD can result in deficiency or absence of GBA enzyme activity. Over 350 mutations causal to GD have been reported, including missense and nonsense mutations, insertions, deletions, complex recombinant alleles, and splice site mutations (Hruska et al., 2008). The four most common mutations found in the *GBA* gene are c.1226G>A (N370S), c.1448T>A (L444P), c.84dupG (84GG) and IVS2 + 1G>A (IVS2 + 1), and account for >90% of Ashkenazi

**Abbreviations:** bp, base pairs; EC, enzyme commission number; GD, Gaucher disease; GBA, glucocerebrosidase gene; GBA, glucocerebrosidase enzyme protein; Hb, hemoglobin; MRI, magnetic resonance imaging; OMIM, Online Mendelian Inheritance in Man entry number; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; RT-PCR, reverse transcription-PCR.

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Jewish population GD alleles, and 70% of general population GD alleles (Mao et al., 2001). Although studies of genotype–phenotype correlations have revealed significant heterogeneity, some consistent patterns have emerged for prognostic and therapeutic decisions. For example, a milder phenotype is associated with the N370S allele (Beutler and Gelbart, 1996). Alternatively, null (i.e., completely non-functional) alleles such as 84GG in combination with a severe mutation such as L444P in the second *GBA* allele, are commonly associated with severe clinical manifestations and symptoms of the central nervous system. To date, there has been no reported case of a Gaucher patient homozygous for two null alleles, e.g. 84GG/84GG, suggesting that this genotype is presumably lethal prenatally (Beutler and Grabowski, 1995).

This report describes the identification of a novel splice site mutation IVS9 + 1G>A, and a novel complex allele G355R/R359X, in two unrelated patients with Type I GD, who are both heterozygous for the common missense mutation, N370S. The entire *GBA* coding sequence of both patients was sequenced and the results were analyzed. We have also established a restriction fragment length polymorphism (RFLP) analysis using *Hph*I restriction endonuclease digest to confirm the presence of the IVS9 + 1 mutation and to screen for its presence in other patients. To demonstrate that mutations G355R and R359X are in *cis* arrangement, i.e. within the same allele on the same chromosome, *GBA* exon 8 in which the mutations are located was cloned and sequence analyzed.

## 2. Case reports

Patient 1 is of English ancestry and was diagnosed at 3 years of age with GD from a bone biopsy showing “Gaucher” cells but treatment was never sought. At 70 years of age, he pursued medical treatment of his GD. He had severe hepatosplenomegaly, thrombocytopenia (platelets  $15 \times 10^9/L$ ) with a history of easy bleeding and easy bruising from minor injuries, leukopenia (leukocytes  $2 \times 10^9/L$ ) and anemia [hemoglobin (Hb) 128 g/L], marrow replacement on spinal MRI (magnetic resonance imaging) and an elevated chitotriosidase [19,322 nmol/h/mL (normal 4–120, Integrated Genetics, LabCorp Specialty Testing Group, Santa Fe, NM, U.S.A.)]. His peripheral T-lymphocyte beta-glucosidase activity was 0.3 (reference 8.9–21.5 nmol/h/mg protein, Floyd Snyder, Alberta Children’s Hospital, Calgary, AB, Canada). He also had a monoclonal gammopathy of uncertain significance (MGUS) with only a mild elevation of immunoglobulin G kappa and free light chains.

Patient 2 was diagnosed in 1983 at age 20, when hepatosplenomegaly and mild thrombocytopenia led to a bone marrow biopsy that showed “Gaucher” cells. Acid beta-glucosidase level and mutation analysis were not done at that time. Over the next 30 years, her Hb and platelet count gradually decreased, the former from 125 g/L to 91 g/L by 2013, and the latter from  $84 \times 10^9/L$  to  $34 \times 10^9/L$ . She was then referred to our centre. Past history also included easy bruising, frequent nosebleeds, menorrhagia, and mild hypertension. Her spleen and liver were palpated 11 and 7 cm below the respective costal margins. Beta-glucosidase level was 1 nmol/mg protein (normal 8–16). Chitotriosidase level was 18,690 nmol/h/mL (normal 4–120). MRI of the abdomen showed liver and spleen volumes of 1802 and 1141 cm<sup>3</sup>, respectively, as well as the presence of cholelithiasis. MRI of the femurs showed the classic Erlenmeyer flask abnormality and evidence of marrow packing, but no necrosis or infarcts. The patient was begun on miglustat 30 mg/kg every 2 weeks; as of May 2015 her Hb was 129 g/L, platelets  $90 \times 10^9/L$ , and chitotriosidase 1125 nmol/h/mL.

## 3. Materials and methods

The methods for collecting dot blood samples on filter paper cards, (Devost and Choy, 2000) PCR amplification of *GBA* genomic DNA for sequence analysis using *GBA* specific primers, and RFLP analysis of *GBA* mutations are described in Supplementary information. In order to confirm that mutations G355R/R359X were in *cis* arrangement i.e. present

in the same allele, exon 8 was cloned into the sequencing vector pJET1.2. One Shot TOP10® chemically competent *E. coli* (Life Technologies, Carlsbad, CA) was transformed with pJET1.2/exon 8 and transformants were selected for on low sodium LB agar containing 100 µg/mL ampicillin. Colony PCR was performed on 24 clones, 15 of which contained the exon 8 insert and were sequenced analyzed by Europhins MWG Operon.

## 4. Results

Sequencing results were compared to functional *GBA* genomic sequence from GenBank (GI183011). The results revealed that in patient 1 at nucleotide position c.1226 (g.4824), there was a heterozygous A to G transition that resulted in asparagine codon (AAC) being substituted by that for serine (AGC) in *GBA* amino acid residue position 370 (N370S) (Fig. 1, left column and Fig. 2, panel B). At nucleotide position g.5005, c.1328 + 1, the sequence shows a heterozygous G to A transition (Fig. 1, right column and Fig. 2, panel A). Although this substitution is intronic, it is located at the intron–exon boundary and within the spliceosome recognition sequence (Mount, 1982), specifically the donor sequence. The chromatogram of both regions revealed a double peak depicting the normal and mutant sequence at the respective locations at c.1226 and c.1328 + 1, which is consistent with a heterozygous mutation (Fig. 2). These findings were confirmed by performing sequence analysis in both the forward and reverse directions (data not shown). Sequence analysis of the rest of the *GBA* coding region and intron–exon boundaries showed identity with the Genbank wild type sequence. RFLP analysis was employed to confirm each of the putative mutations. For N370S, mismatch PCR using primer set M370/PBII was performed, resulting in an amplified fragment of 105 bp. As first described by Beutler et al. (1990), the mutation N370S creates a cleavage site in this fragment and upon digestion with *Xho*I restriction endonuclease and electrophoresis in 6% polyacrylamide, DNA fragment lengths from patient 1 at 105, 86 and 19 bp were noted, while the normal control showed only a single band at 105 bp (data not shown). These results are consistent with the presence of heterozygous N370S mutation in the patient.

NEBCutter software (New England Biolabs) was used to determine various restriction sites in normal and IVS9 + 1G>A mutant alleles. The enzyme *Hph*I was determined to cleave in 2 positions on the normal allele, but 3 on the mutant allele as mutation IVS9 + 1G>A creates an additional *Hph*I cut sites. After digestion with *Hph*I, samples were run in an 8% acrylamide gel and visualized with ethidium bromide. The normal control has fragment lengths of only 145, 74 and 33 bp (Fig 3, lane 4). The fragment lengths for patient 1 were revealed to be 145, 107, 74, 38 and 33 bp, which is consistent with IVS9 + 1G>A in the heterozygous form (Fig 3, lane 3).

Sequence analysis of patient 2 showed that mutation N370S is present in the heterozygous form with two other heterozygous point mutations: G to C at c.1180, g.4396, and T to C at c. 1192, g.4408) substituting glycine by arginine (G355R) and arginine by a premature termination (R359X), respectively. To find out whether mutations R355R and R359X are present in *cis*- or *trans*-arrangement (i.e. within the same *GBA* allele or in different *GBA* alleles), PCR-amplified *GBA* exon 8 genomic DNA of patient 2 was cloned into the pJET1.2 vector in *E. coli* TOP10® strain and subjected to sequence analysis. Of the 15 clones sequenced, 5 contained the exon 8 mutations G355R and R359X showing both mutations in *cis* arrangement (i.e. on the same chromosome) as a G355R/R359X complex allele, while 10 contained the wild type exon 8 sequence indicating that the cloned fragments were from the other (exon 9 mutation N370S) Gaucher allele (Fig. 4). Genotyping of the Gaucher mutations N370S and G353R/R359X was also performed from dot blood genomic DNA of the asymptomatic mother and sister of patient 2. RFLP and DNA sequence analysis showed that the mother is heterozygous for mutation N370S and negative for G353R/R359X, while the sister was negative for both N370S and G353S/R359X, i.e.

	Normal allele sequence and amino acids	Mutant allele sequence and amino acids
Region containing N370S; g.5004-5018	5'— AAC CTC CTG TAC CAT—3' Asn Leu Leu Tyr His	5'— AGC CTC CTG TAC CAT—3' Ser Leu Leu Tyr His
Region containing IVS9+1 g>a; g.4813-4831	5'— GGC CAC TTC AG gtgagtg—3' Gly His Ala intronic	5'— GGC CAC TTC AG atgagtg—3' Gly His Ala intronic
Region containing G355R/R359X; g. 4396-4410	5'— GGC TCC TGG GAT CGA—3' Gly Ser Cys Asp Arg	5'— CGC TCC TGG GAT TGA—3' Arg Ser Cys Asp STOP

**Fig. 1.** Location of nucleotide and amino acid substitution in the *GBA* gene and polypeptide for patients 1 and 2. The mutation N370S in patients 1 and 2 is located at the beginning of exon 9. The IVS9 + 1g>a in patient 1 is located at the junction between exon 9 and intron 9. The complex allele G355R/R359X in patient 2 is located at the end of exon 8. Uppercase letters represent the coding region of *GBA*; lowercase letters represent the intronic region. Base and amino acid substitutions are shown in red.

normal homozygous. These findings indicated that the parents of patient 2 had transmitted mutation N370S and the complex G353S/R359X allele to her, and the normal *GBA* allele to her unaffected sister.

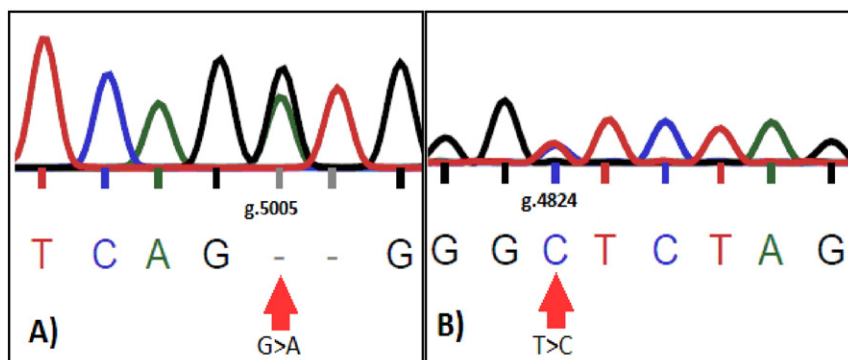
## 5. Discussion

There have been more than 350 mutations recorded to date, including 16 splice site mutations that cause Gaucher disease (<http://www.hgmd.org>). Many of these mutations, including IVS9 + 1(G > A) and G355R/R359X, are present in only one or a small number of individuals. A splice site mutation can have a range of consequences including exon skipping, activation of cryptic splice sites, creation of a pseudo-exon within a gene or intron retention (Nakai and Sakamoto, 1994). In the case of the highly prevalent splice site mutation IVS2 + 1 (G > A), exon 2 is entirely removed from the spliced mRNA (He and Grabowski, 1992). In order to assess the possible impact of the IVS9 + 1 (G > A) mutation found in patient 1 on *GBA* mRNA splicing, we used the RegRNA 2.0 program (<http://regrna2.mbc.nctu.edu.tw/>), the Neural Network program ([http://www.fruitfly.org/seq\\_tools/splice.html](http://www.fruitfly.org/seq_tools/splice.html)), and the Maximum Entropy program ([http://genes.mit.edu/burgelab/maxent/Xmaxentscan\\_scoreseq.html](http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html)) to predict how this mutation would affect RNA processing. All three programs indicate that the IVS9 + 1 mutated sequence would no longer be recognized as donor splice site (please refer to Supplementary Information for details of the *in silico* analyses). Additionally, the Neural Network and Maximum Entropy programs predicted that the mutation would result in a much lower splice site signal below recognition threshold. Thus, the IVS9 + 1 mutation will be very disruptive for *GBA* mRNA processing and enzyme function. An alternative possibility is the activation of cryptic donor splice sites. In a study describing another exon 9 splice site mutation (c.1389-1 G > A), the Neural Network and Maximum Entropy programs predicted the mutated sequence would no longer be recognized as an acceptor splice site. However, when RT-PCR analysis was performed on mRNAs from

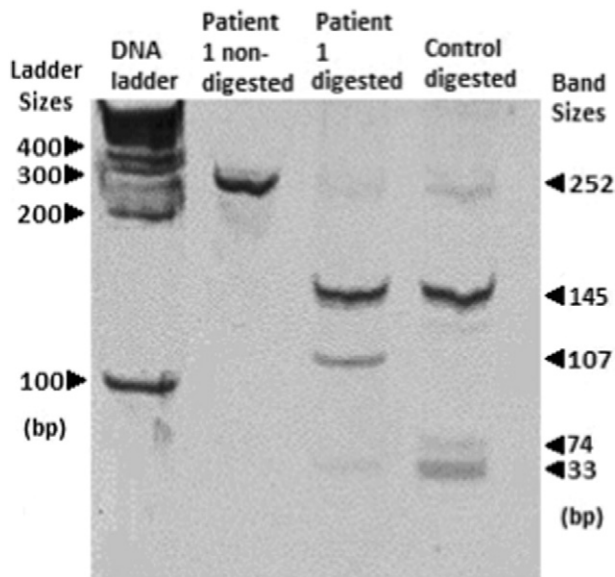
cultured fibroblasts, it was found that the mutation led to exon slippage of 4 nucleotides and activation of a cryptic acceptor splice site (Malini et al., 2014). We are unable to perform similar RT-PCR analysis of fibroblast *GBA* mRNAs from patient 1 because dried dot blood on filter paper cards is the only available cell source for our study.

The structure of *GBA* has been elucidated by x-ray crystallography and provides some insight to the role of the 497 residues making up the three domains of *GBA* polypeptide. Domain I (*GBA* amino acid residues 1–27 and 383–414) is a  $\beta$ -sheet containing two disulfide bridges and a glycosylation at N19, which is required for activity *in vivo*. Domain II (*GBA* amino acid residues 30–75 and 431–497) consists of two  $\beta$ -sheets resembling an immunoglobulin fold that may interact with saposin C, an essential activator of *GBA* (Aerts et al., 1990; Dvir et al., 2003; Tamargo et al., 2012). Domain III (*GBA* amino acid residues 76–381 and 416–430) consists of a ( $\beta/a$ )<sub>8</sub> TIM barrel containing the catalytic site: E340, the nucleophile, and E235, the acid/base catalyst (Dvir et al., 2003). Exon 9 codes for *GBA* amino acid residues 370–424, and therefore plays a major role in the formation of the both Domain I and the catalytic domain. An absence of exon 9 because of aberrant splicing would be detrimental to *GBA* function by abolishing its activity completely. In this case, however, the second Gaucher allele is the relatively mild mutation N370S (Beutler and Grabowski, 1995; Grace et al., 1990) which modulates the clinical expression to a mild to moderate Type 1 form. Other splicing mutations have been reported in compound heterozygosity with N370S in patients with Type 1 GD (Dominissini et al., 2006; Jack et al., 2014; Malini et al., 2014).

The novel complex allele G355R/R359X in patient 2 will result first in a substitution of glycine by arginine, followed by a protein truncation at *GBA* amino acid residue 359. Complex alleles are often the result of reciprocal or non-reciprocal recombination events with the pseudogene (Hruska et al., 2008). The human *GBA* pseudogene is 96% homologous to the *GBA* functional gene but contains a 55 bp deletion in exon 9 and a number of exonic point mutations as well as large deletions in several



**Fig. 2.** Sequence results for patient 1. Panel A: Forward trace chromatogram of the junction between exon and intron 9 indicated the G>A transition at nucleotide position g.5005 corresponding to IVS9 + 1G>A. Panel B: Reverse trace chromatogram of exon 9 indicating the T>C transition at nucleotide position g.4824 corresponding to N370S.



**Fig. 3.** RFLP analysis of patient 1 exon 9 using restriction enzyme *HphI*. From left: Lane 1, DNA ladder; lane 2, undigested DNA of patient 1 (252 bp fragment); lane 3, DNA of patient 1 digested with *HphI* (fragments of 145, 107, 74, 38 and 33 bp). Mutation IVS9 + 1G>A creates an additional *HphI* cut site, resulting in the cleavage of the 145 bp band to a 107 bp band and a 38 bp band; lane 4, Normal control digested with *HphI* (fragments of 145, 74 and 33 bp).

introns (Horowitz et al., 1989). In the case of G355R/R359X, however, neither mutation is pseudogene-derived, and therefore, there is no evidence to suggest this is the result of a recombination event. The missense mutation G355R has not previously been reported; however, two studies have characterized another mutation, G355D, in which glycine at GBA amino acid residue 355 is substituted by aspartic acid instead of arginine. In one study, G355D in combination with L444P resulted in the neuronopathic Type II GD (Tsai et al., 2001). In another study, homozygous G355D/G355D resulted in Type 1 GD (Ankleshwaria et al., 2014). These findings suggest that G355R may also have varying consequences, depending on the identity of the mutation in the second allele. *In silico* analysis of this mutation on the stability and function of GBA using the FoldX program (<http://foldxsuite.crg.eu>) and PolyPhen 2 program (<http://genetics.bwh.harvard.edu/pph2/>) predict that G355R would severely destabilize and damage the structure of GBA (please refer to Supplementary Information for details of the analysis). The nonsense mutation R359X has been described as a perinatal lethal mutation when in combination with other lethal mutations such as V398F (Stone et al., 1999), and as a Parkinsonism-associated mutation (Sunwoo et al., 2011). Because R359X results in premature termination of GBA translation, amino acid residues 359–497 that

constitute GBA Domain I (residue 383–414), Domain II (residue 431–497), and Domain III (residue 359–381) that constitute the catalytic and saposin activator binding domains (Dvir et al., 2003; Tamargo et al., 2012) will be missing in the truncated GBA polypeptide. In addition, the transcript resulting from nonsense mutation may also be prematurely degraded via nonsense-mediated mRNA decay, a post-transcriptional quality control process in eukaryotes that degrades transcripts carrying disease-causing premature termination codons (Turner and Choy, 2015; Popp and Maquat, 2013). These will be detrimental to GBA function and it is reasonable to postulate that the complex allele G355R/R359X might also be lethal when in combination with another severe or lethal Gaucher allele. However, the second Gaucher allele is the relatively mild mutation N370S which ameliorates the clinical severity and as such (as in the case of patient 1), patient 2 presents with Type 1 GD, which again demonstrates the rescuing properties of N370S as we previously reported (Jack et al., 2014). The nonsense mutation R359X has been reported in patients heterozygous for N370S, who also presented with Type 1 GD (Alfonso et al., 2007).

## 6. Conclusion

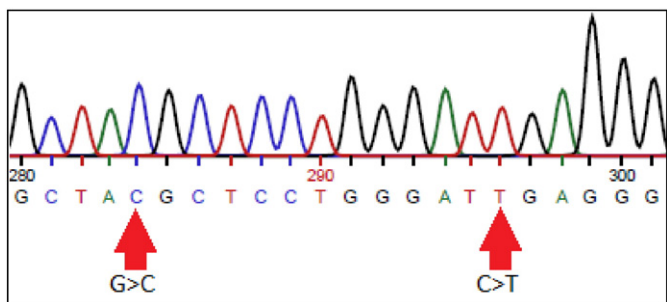
We have identified a novel splice site mutation (IVS9 + 1G>A) and a novel complex allele G355R/R359X in two unrelated patients with Type I GD, who are heterozygous for N370S. We have also developed a RFLP procedure that utilizes *HphI* restriction endonuclease digest for confirmation and efficient identification of the IVS9 + 1 mutation in future patients. *In silico* analysis of these novel Gaucher alleles showed that they are detrimental to either GBA splicing and/or structure and function. However, compound heterozygosity with mutation N370S in both patients has modulated the clinical expression to a mild to moderate Type 1 Gaucher phenotype.

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**Fig. 4.** Chromatogram of cloned sequence of GBA exon 8 from patient 2 showing the complex allele G355R/R359X in *cis* arrangement. The first arrow indicates the G>C transversion corresponding to G355R. The second arrow indicates the C>T transitions corresponding to G359X.

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