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### **Insertion of a T Next to the Donor Splice Site of Intron 1 Causes Aberrantly Spliced mRNA in a Case of Infantile GM1-Gangliosidosis.**

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## RESEARCH ARTICLE

# Insertion of a T Next to the Donor Splice Site of Intron 1 Causes Aberrantly Spliced mRNA in a Case of Infantile G<sub>M1</sub>-Gangliosidosis

Amelia Morrone, Hans Morreau, Xiao Yan Zhou, Enrico Zammarchi, Wim J. Kleijer, Hans Galjaard, and Alessandra d'Azzo\*

MGC-Medical Genetics Center, Department of Cell Biology and Genetics (H.M., X.Y.Z., H.G., A.d'A.) and Department of Clinical Genetics (W.J.K.), Erasmus University, Rotterdam, The Netherlands, and Department of Pediatrics (A.M., E.Z.), University of Florence, Florence, Italy; Fax: 901-526-2907

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The lysosomal storage disorders G<sub>M1</sub>-gangliosidosis and Morquio B syndrome are caused by a complete or partial deficiency of acid  $\beta$ -galactosidase. Here, we have characterized the mutation segregating in a family with two siblings affected by the severe infantile form of G<sub>M1</sub>-gangliosidosis. In total mRNA preparations derived from the patients' fibroblasts at least two aberrantly spliced  $\beta$ -galactosidase transcripts (1 and 2) have been identified. Both transcripts contain a 20 nucleotide (nt) insertion derived from the 5' end of intron 1 of the  $\beta$ -galactosidase gene. Furthermore, in transcript 2 sequences encoded by exon II are deleted during the splicing process. Comparison of the 20-nt insertion with wild-type intronic sequences indicated that in the genomic DNA of the patients an extra T nucleotide is present immediately downstream of the conserved GT splice donor dinucleotide of intron 1. Both patients are homozygous for the T nucleotide insertion. We propose that this single base insertion is the mutation responsible for aberrant splicing of  $\beta$ -galactosidase pre-mRNA, giving rise to transcripts that cannot encode a normal protein. © 1994 Wiley-Liss, Inc.

KEY WORDS:  $\beta$ -Galactosidase, Pre-mRNA splicing, T nucleotide insertion, G<sub>M1</sub>-Gangliosidosis

## INTRODUCTION

The lysosomal enzyme  $\beta$ -D-galactosidase is required for removal of  $\beta$ -D-galactosyl residues from glycolipids, glycoproteins, glycosaminoglycans, and glycopeptides (Conzelmann and Sandhoff, 1987; O'Brien, 1989). Mutations in the human  $\beta$ -galactosidase locus on chromosome 3 (Shows et al., 1979) lead to absence or abrogation of the normal function of  $\beta$ -galactosidase, which results in lysosomal accumulation of undegraded substrates, particularly in the central nervous system (O'Brien, 1989; Suzuki, 1993). In tissues and cultured cells the majority of the active enzyme is found in complex with lysosomal neuraminidase (sialidase) and the protective protein/cathepsin A (Verheijen et al., 1982; 1985; Yamamoto et al., 1982; Yamamoto and Nishimura, 1987). It has been demonstrated that the latter is essential for intralysosomal activation and stabilization of the two glycosidases (d'Azzo et al., 1982; Hoogveen

et al., 1983; Verheijen et al., 1985; Van der Horst et al., 1989).

Absence or reduction of  $\beta$ -galactosidase activity results in two clinically and biochemically distinct metabolic storage disorders: G<sub>M1</sub>-gangliosidosis, a lipidosis, and Morquio-B syndrome, a mucopolysaccharidosis (Okada and O'Brien, 1968; O'Brien et al., 1976; Groebe et al., 1980; for reviews see O'Brien, 1989; Neufeld and Muenzer, 1989; Suzuki, 1993). G<sub>M1</sub>-Gangliosidosis patients suffer from a severe neurodegenerative disorder and, depending on the clinical symptoms, they are classified as having either the infantile or the mild

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\*To whom reprint requests/correspondence should be addressed: Department of Genetics, St. Jude Children's Research Hospital, P.O. Box 318, Memphis, TN 38101-0318.

Current address of H. Morreau: Department of Clinical Pathology, Erasmus University, Rotterdam, The Netherlands.

juvenile/adult type of the disease. Patients with the infantile form have extensive CNS involvement, dysmorphic features, and hepatosplenomegaly, leading to early death. The  $\beta$ -galactosidase activity in these cases is reduced to less than 1% of normal levels. Pathologic examination indicates excessive accumulation of G<sub>M1</sub>-ganglioside, particularly in neuronal tissues, as well as glycosaminoglycans and glycopeptides in visceral organs and other tissues (Suzuki, 1993 and references therein). The juvenile and adult variants of this disease have milder clinical manifestations, a prolonged life expectancy, and the residual  $\beta$ -galactosidase activity is 10–15% of normal levels (Suzuki et al., 1977; Wenger et al., 1980; Suzuki, 1993).

In human cultured fibroblasts the first immunoprecipitable form of  $\beta$ -galactosidase is a glycosylated precursor of 85 kDa that is processed, through a series of intermediates, into a 64-kDa mature enzyme (d'Azzo et al., 1982). The cDNAs and genes encoding human and mouse  $\beta$ -galactosidase have been isolated and characterized (Oshima et al., 1988; Morreau et al., 1989, 1991; Yamamoto et al., 1990; Nanba and Suzuki, 1990, 1991). It has been shown that the human gene can give rise to at least two alternatively spliced mRNAs: a major transcript of 2.5 kb encodes the classic, catalytically active  $\beta$ -galactosidase protein, and a minor species of about 2.0 kb gives rise to a nonlysosomal  $\beta$ -galactosidase-related protein which is inactive toward the artificial substrate used (Morreau et al., 1989; Yamamoto et al., 1990).

The characterization of the human  $\beta$ -galactosidase cDNA has enabled the identification of several mutations underlying distinct clinical forms of G<sub>M1</sub>-gangliosidosis and Morquio-B syndrome (Yoshida et al., 1991; Nishimoto et al., 1991; Oshima et al., 1991, 1992; Mosna et al., 1992). Most G<sub>M1</sub>-gangliosidosis patients, so far analyzed, are of Japanese origin. With the exception of two duplications found in either both or one of the alleles in two infantile Japanese patients, all other mutations are base substitutions, leading to single amino acid changes. They are different in clinically distinct patients, although in some cases only one allele has been identified. In this report we describe the genetic lesion present in two siblings affected with the severe infantile form of G<sub>M1</sub>-gangliosidosis. We have found that the insertion of an extra T nucleotide at the donor splice site of intron 1 leads to aberrant splicing of  $\beta$ -galactosidase pre-mRNA. Both patients are homozygous for the mutation, which leads to a complete lack of  $\beta$ -galactosidase protein.

## EXPERIMENTAL PROCEDURES

### Cell Culture

Human skin fibroblasts from the index patient, the fetus, and their parents were obtained from the European Cell Repository, Rotterdam (Dr. W.J. Kleijer). The index patient was originally diagnosed by Dr. Pirkko Ammälä (University Central Hospital, Helsinki, Finland). Cultured fibroblasts from the index patient and the affected fetus were provided by Prof. Pertti Aula (University Central Hospital, Turku, Finland). Fibroblasts were cultured in Dulbecco's modified Eagles–Hams F10 medium (1:1 vol/vol) with antibiotics and 10% fetal bovine serum.

### Oligonucleotides

For cDNA synthesis, PCR amplification, nucleotide sequencing, and hybridization studies several oligonucleotide primers were constructed on the basis of either human  $\beta$ -galactosidase cDNA (H $\beta$ Gal) or genomic sequences (Morreau et al., 1989; 1991). The oligonucleotide primers were synthesized on an Applied Biosystems 381A oligonucleotide synthesizer and purified as recommended by the manufacturer. The specific primers used in the experiments described in the text are the following:

- a. sense, 5' CGAATTCATGCCGGGGTTCC-TGGTTCGC 3' (nt 49–71, exon I)
- b. antisense, 5' CGAATTCCTCCCATTTC-CACTCTGCACAG 3' (nt 452–428, exon IV/exon III)
- c. sense, 5' GGCTTGCGCAATGCCACC 3' (nt 117–134, exon I/II)
- d. sense, 5' CTTGCGCgtaagtctgc 3' (nt 119–125, exon I/intron 1 + extra T)
- e. sense, 5' gggaccggGTATGTGCC 3' (intron 1/nt 256–304, exon III)
- f. antisense, 5' ggttccccgccagcctgt 3' (intron 1)
- g. sense, 5' CTTGCGCgtaagtctgc 3' (nt 119–125, exon I/intron 1)

### cDNA Synthesis

Total RNA was isolated from cultured fibroblasts as described by Auffray and Rougeon (1980). The entire coding sequence of the  $\beta$ -galactosidase mRNA was reverse transcribed into six overlapping cDNA fragments using specific antisense primers (Hermans et al. 1988). The primers were constructed on the basis of the sequence of the long  $\beta$ -galactosidase cDNA clone, H $\beta$ Gal (see above).

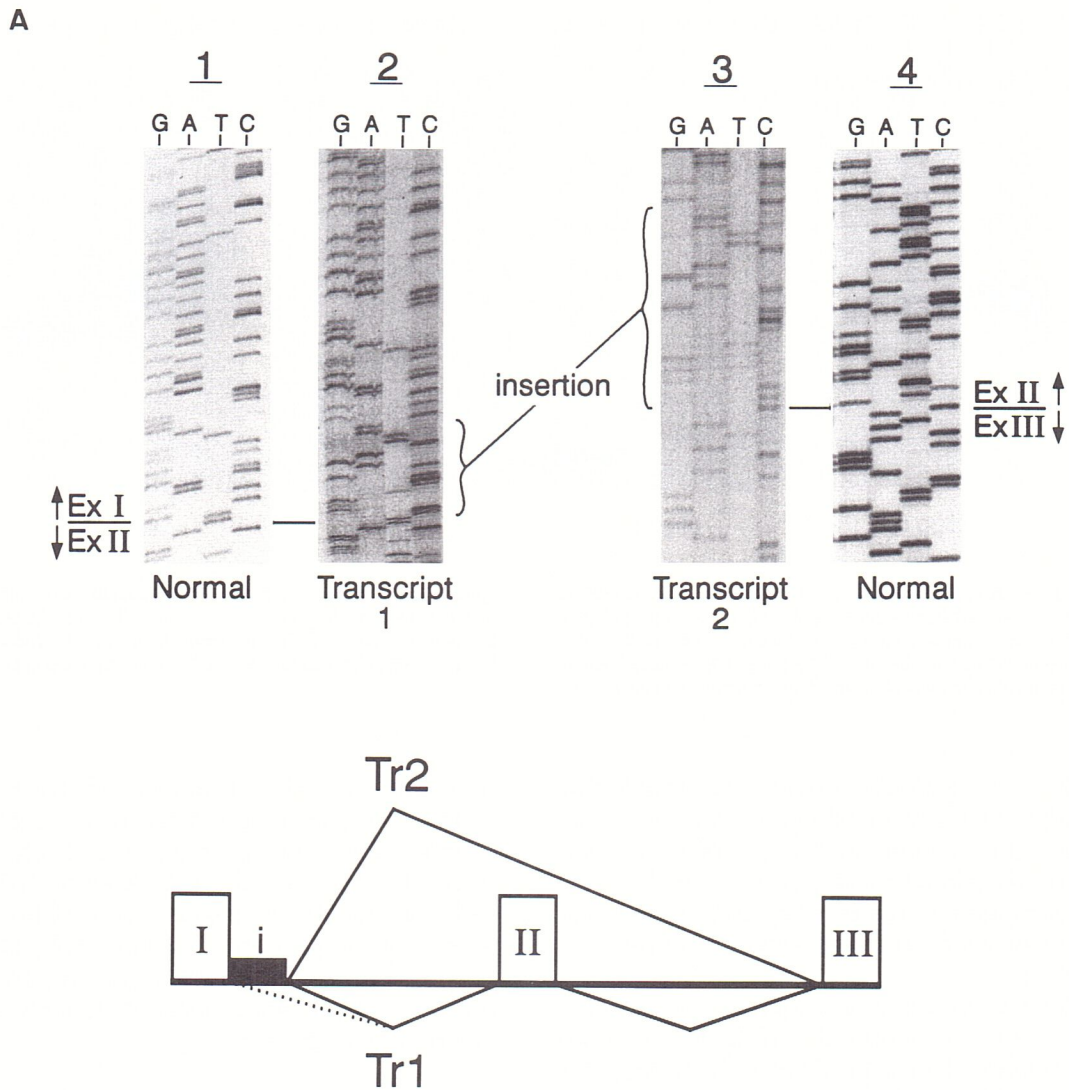


FIGURE 2. Partial nucleotide sequence of aberrant transcripts 1 and 2. (A) Total RNA was isolated from fibroblasts of the two patients, their parents, and an unaffected control, and reverse transcribed into cDNA with antisense primers located at different positions of  $\beta$ -galactosidase mRNA. These partial cDNA fragments were subsequently amplified, subcloned, and sequenced, or sequenced directly after an asymmetric polymerase chain reaction. A portion of the normal antisense sequence of  $\beta$ -galactosidase mRNA derived from ExII/ExI

and ExIII/ExII is shown. Antisense sequences of aberrant transcripts 1 and 2 are given, which include the 20-nt insertion, marked with a bracket. (B) Schematic representation of the splicing mechanism leading to aberrant transcripts 1 and 2. Exon sequences are indicated by roman numbers I/II and III. The black bar represents the 20-nt intronic sequence (i) present in transcript 1 and 2. Normal splicing is indicated by the dotted line.

site and 3' flanking sequences of intron 1 (Morreau et al., 1991). However, an additional T nucleotide is present in transcripts 1 and 2 of the patients, after the conventional GT dinucleotide of the splice donor site. The abnormal mRNAs must have arisen from aberrantly spliced pre-mRNA molecules, in a manner similar to that depicted schematically in Figure 2B. Apparently the normal splice donor site of intron 1 is no longer recognized, instead a cryptic site 21 nucleotides down-

stream is preferentially chosen. These transcripts were not amplified from mRNA preparations of both parents, indicating that they probably represent a minor pool. The 20-nt insertion causes a frameshift in the open reading frames (ORFs) of the aberrant mRNAs. The reading frame of transcript 1 terminates in an early stop codon. As a result, only a truncated peptide could be translated from transcript 1. In contrast, due to the deletion of exon II-encoded sequences in transcript 2, the

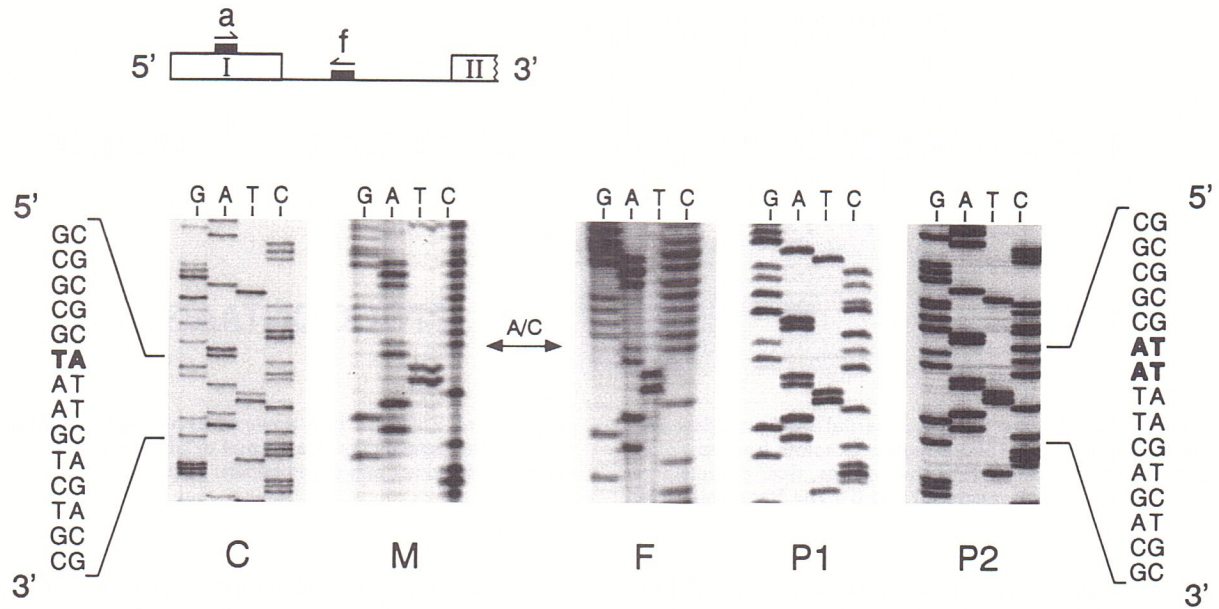


FIGURE 4. Partial nucleotide sequence of the  $\beta$ -galactosidase genes from two G<sub>M1</sub>-gangliosidosis patients (P1, P2), their parents (M, F), and a control (C). Genomic DNA was isolated and subjected to asymmetric PCR in the region containing

the mutation using the exonic primer (a) and the intronic primer (f). Portions of the antisense sequences of amplified fragments are shown.

are clearly homozygous for the T nucleotide insertion.

Finally, the 150-bp genomic fragment was again amplified, transferred onto nylon membranes, and hybridized with allelic-specific oligonucleotide probes either derived from the normal sequence (g) or carrying the T nucleotide insertion (d). As seen in Figure 5, this experiment convalidates that the two patients carry the same mutation in both alleles.

#### DISCUSSION

In this report we have described the genetic lesion present in both alleles of the  $\beta$ -galactosidase gene from two siblings affected with the severe infantile form of G<sub>M1</sub>-gangliosidosis. A single T nucleotide insertion, immediately after the conserved GT dinucleotide of the splice donor site of intron 1, is likely to interfere with the normal splicing process, leading to a preferential use of a more downstream cryptic splice site. In total RNA preparations from fibroblasts of both patients we have identified two aberrant transcripts, however, we cannot rule out the possibility that more alternatively spliced or erroneously processed mRNA forms may exist. The 20-nt insertion found in patients' mRNAs cannot account for the presence of a slightly bigger transcript detected in Northern blots. The origin of this species, which is not vis-

ible in neither of the parents' preparations, is at present unknown. Interestingly, the same mutation has recently been found in two compound heterozygous siblings, also of Scandinavian ancestry, having an adult form of G<sub>M1</sub>-gangliosidosis (S. Chakraborty and D.A. Wenger, personal communication). In this case only transcript 1 has been identified and represents a very small percentage of the total mRNA pool. The other allele carries a point mutation that is likely responsible for the mild clinical phenotype (Chakraborty et al., 1991).

The role of conserved structural elements in splicing of higher eukaryotic pre-mRNAs is well documented (for a review see Breathnach and Chambon, 1981). These elements consist of a 5' splice donor site, a 3' splice acceptor site, and a less conserved branch point sequence. Mutations at the GT/AG dinucleotides of 5' and 3' splice sites were shown to interfere with the normal splicing process both in site directed mutagenized genes and naturally occurring mutants (Padgett et al., 1986). It was empirically calculated that a T nucleotide in the third position of a normal splice donor site is found only in 5% of the cases, whereas an A nucleotide is most commonly present (70%; Padgett et al., 1986). Indeed an A is the base normally encountered in the third position of the

## REFERENCES

- Auffray C, Rougeon F (1980) Purification of mouse immunoglobulin heavy chain messenger RNAs from total myeloma tumor RNA. *Eur J Biochem* 107:303-314.
- Breathnach R, Chambon P (1981) Organization and expression of eucaryotic split genes coding for proteins. *Annu Rev Biochem* 50:349-383.
- Chakraborty S, Rafi MA, Wenger DA (1991) A point mutation in the acid  $\beta$ -galactosidase cDNA sequence of 2 adult patients with G<sub>M1</sub>-gangliosidosis. *Am J Hum Genet* (suppl) 49:451A.
- Conzelmann E, Sandhoff K (1987) Glycolipid and glycoprotein degradation. *Adv Enzymol Relat Areas Mol Biol* 60:89-216.
- d'Azzo A, Hoogveen A, Reuser AJ, Robinson D, Galjaard H (1982) Molecular defect in combined beta-galactosidase and neuraminidase deficiency in man. *Proc Natl Acad Sci USA* 79:4535-4539.
- Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 132:6-13.
- Groebe H, Krins M, Schmidberger H, von Figura K, Harzer H, Kresse E, Paschke E, Sewell A, Ullrich K (1980) Morquio syndrome (mucopolysaccharidosis IV-B) associated with beta-galactosidase deficiency. Report of two cases. *Am J Hum Genet* 32:258-272.
- Gyllensten UB, Erlich HA (1988) Generation of single-stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA-DQA locus. *Proc Natl Acad Sci USA* 85:7652-7656.
- Hermans A, Gow J, Sella L, von Lindern M, Hagemeijer, Wiedemann LM, Grosveld G (1988) Bcr-abl oncogene activation in Philadelphia chromosome-positive acute lymphoblastic leukemia. *Leukemia* 2:628-633.
- Hidaka Y, Pallela T, O'Toole TE, Tarlé SA, Kelley WN (1987) Human adenine phosphoribosyltransferase. Identification of allelic mutations at the nucleotide level as a cause of complete deficiency of the enzyme. *J Clin Invest* 80:1409-1415.
- Hoogveen AT, Verheijen FW, Galjaard H (1983) The relation between human lysosomal beta-galactosidase and its protective protein. *J Biol Chem* 258:12143-12146.
- Morreau H, Galjart NJ, Gillemans N, Willemsen R, van der Horst GTJ, d'Azzo A (1989) Alternative splicing of  $\beta$ -galactosidase messenger RNA generates the classic lysosomal enzyme and a  $\beta$ -galactosidase-related protein. *J Biol Chem* 264:20655-20663.
- Morreau H, Bonten E, Zhou XY, d'Azzo A (1991) Organization of the gene encoding human lysosomal  $\beta$ -galactosidase. *DNA Cell Biol* 10:495-504.
- Mosna G, Fattore S, Tubiello G, Brocca S, Trubia M, Gianazza E, Gatti R, Danesino C, Minelli A, Piantanida M (1992) A homozygous missense arginine to histidine substitution at position-482 of the beta-galactosidase in an Italian infantile GM1-gangliosidosis patient. *Hum Genet* 90(3):247-250.
- Murphy G, Kavanagh T (1988) Speeding-up the sequencing of double-stranded DNA. *Nucl Acids Res* 16:5198.
- Nanba E, Suzuki K (1990) Molecular cloning of mouse acid beta-galactosidase cDNA: Sequence, expression of catalytic activity and comparison with the human enzyme. *Biochem Biophys Res Commun* 173:141-148.
- Nanba E, Suzuki K (1991) Organization of the mouse acid beta-galactosidase gene. *Biochem Biophys Res Commun* 178:158-164.
- Neufeld EF (1991) Lysosomal storage diseases. *Annu Rev Biochem* 60:257-280.
- Neufeld EF, Muenzer J (1989) The mucopolysaccharidoses. In Scriver CR, Beaudet AL, Sly WS, Valle D (eds): *The Metabolic Basis of Inherited Disease*. New York: McGraw-Hill, pp 1565-1587.
- Nishimoto JE, Nanba E, Inui K, Okada S, Suzuki K (1991) G<sub>M1</sub>-gangliosidosis (genetic beta-galactosidase deficiency): Identification of four mutations in different clinical phenotypes among Japanese patients. *Am J Hum Genet* 49:566-574.
- Norden A, Tennant L, O'Brien JS (1974) Ganglioside G<sub>M1</sub> beta-galactosidase A: Purification and studies of the enzyme from human liver. *J Biol Chem* 249:7969-7976.
- O'Brien JS (1989) Beta-galactosidase deficiencies (G<sub>M1</sub>-gangliosidosis, galactosialidosis, and Morquio syndrome type B); ganglioside sialidase deficiency (mucopolidosis type IV). In Scriver CR, Beaudet AL, Sly WS, Valle D (eds): *The Metabolic Basis of Inherited Disease*. New York: McGraw-Hill, pp 1797-1806.
- O'Brien JS, Gugler E, Giedion A, Wiessmann U, Herschkowitz N, Meijer C, Leroy J (1976) Spondyloepiphyseal dysplasia, corneal clouding, normal intelligence and acid beta-galactosidase deficiency. *Clin Genet* 9:495-504.
- Okada S, O'Brien JS (1968) Generalized gangliosidosis: Beta-galactosidase deficiency. *Science* 160:1002-1004.
- Oshima A, Tsuji A, Nagao Y, Sakuraba H, Suzuki Y (1988) Cloning, sequencing, and expression of cDNA for human  $\beta$ -galactosidase. *Biochem Biophys Res Commun* 157:238-244.
- Oshima A, Yoshida K, Shimmoto M, Fukuhara Y, Sukuraba H, Suzuki Y (1991) Human  $\beta$ -galactosidase gene mutation in Morquio B disease. *Am J Hum Genet* 49:1091-1093.
- Oshima A, Yoshida K, Ishizaki A, Shimmoto M, Fukuhara Y, Sakuraba H, Suzuki Y (1992) G<sub>M1</sub>-gangliosidosis: tandem duplication within exon 3 of  $\beta$ -galactosidase gene in an infantile patient. *Clin Genet* 41:235-238.
- Padgett RA, Grabowski PJ, Konarska MM, Seiler S, Sharp PA (1986) Splicing of messenger RNA precursors. *Annu Rev Biochem* 55:1119-1150.
- Paschke E, Kresse H (1982) Morquio disease type B: Activation of GM1- $\beta$ -galactosidase by GM1-activator. *Biochem Biophys Res Commun* 198:568-578.
- Saiki RK et al. (1988) Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239:487-491.
- Sanger FG, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 74:5463-5467.
- Sankila EM, Tolranen R, van den Hurk JAJM, Cremers F, de la Chapelle A (1992) Aberrant splicing of the CHM gene is a significant cause of choroideremia. *Nature Genet* 1:109-113.
- Shows TB, Scrafford-Wolff LR, Brawn JA, Meisler M (1979) GM1-gangliosidosis: Chromosome 3 assignment of the beta-galactosidase A gene (beta-GAL A). *Somat Cell Genet* 5:47-58.
- Suzuki K (1993)  $\beta$ -galactosidase deficiency: GM1 gangliosidosis, Morquio B disease, and galactosialidosis. In Rosenberg R, Prusiner S, DiMauro S, Barchi R, Kunkel L (eds): *Molecular and Genetic Basis of Neurological Diseases*. Stoneham, MA: Butterworth-Einemann, pp 523-530.
- Suzuki Y, Nakamura N, Fukuoka K, Shimada Y, Uono M (1977)  $\beta$ -Galactosidase deficiency in juvenile and adult patients. Report of six Japanese cases and review of literature. *Hum Genet* 36:219-229.
- Van der Horst GTJ, Kleijer WJ, Hoogveen AT, Huijman JG, Blom W, van Diggelen OP (1983) Morquio B syndrome: a primary defect in beta-galactosidase. *Am J Med Genet* 16:261-275.
- Van der Horst GTJ, Galjart NJ, d'Azzo A, Galjaard H, Verheijen FW (1989) Identification and in vitro reconstitution of lysosomal neuraminidase from human placenta. *J Biol Chem* 264:1317-1322.