DE GRUYTER J Pediatr Endocrinol Metab 2016; 29(10): 1225–1228

Case Report

Maria Francisca Coutinho, Marisa Encarnação, Francisco Laranjeira, Lúcia Lacerda, Maria João Prata and Sandra Alves*

Solving a case of allelic dropout in the *GNPTAB* gene: implications in the molecular diagnosis of mucolipidosis type III alpha/beta

DOI 10.1515/jpem-2016-0173
Received May 5, 2016; accepted August 29, 2016

Abstract: While being well known that the diagnosis of many genetic disorders relies on a combination of clinical suspicion and confirmatory genetic testing, not rarely, however, genetic testing needs much perseverance and cunning strategies to identify the causative mutation(s). Here we present a case of a thorny molecular diagnosis of mucolipidosis type III alpha/beta, which is an autosomal recessive lysosomal storage disorder, caused by a defect in the *GNPTAB* gene that codes for the α/β-subunits of the GlcNAc-1-phosphotransferase. We used both cDNA and gDNA analyses to characterize a mucolipidosis type III alpha/beta patient whose clinical diagnosis was already confirmed biochemically. In a first stage only one causal mutation was identified in heterozygosity, the already described missense mutation c.1196C>T(p.S399F), both at cDNA and gDNA levels. Only after conducting inhibition of nonsense-mediated mRNA decay (NMD) assays and after the utilization of another pair of primers the second mutation, the c.3503_3504delTC deletion, was identified. Our findings illustrate that allelic dropout due to the presence of polymorphisms and/or of mutations that trigger the NMD pathway can cause difficulties in current molecular diagnosis tests.

Keywords: allelic dropout; genotype-phenotype correlation; GlcNAc-1-phosphotransferase; *GNPTAB*; ML III alpha/beta; molecular genetic testing; nonsense mediated mRNA decay; polymorphism.

Introduction

Mucolipidosis type III (ML III alpha/beta or ML III gamma, OMIM: 252600 and 252605, respectively) is a rare autosomal recessive disorder caused by deficiency of the (UDP)-*N*-acetylglucosamine: lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase (termed GlcNAc-1-phosphotransferase; EC 2.7.8.17). GlcNAc-1-phosphotransferase is a Golgi-resident 540-kDa hexameric transmembrane enzyme composed by three subunits, $\alpha_2\beta_2\gamma_2$, which catalyzes a crucial step in the formation of the mannose 6-phosphate (M6P) recognition marker that is responsible for the correct targeting of newly synthesized lysosomal enzymes from the Golgi to the lysosomal compartment [1]. The enzyme's polypeptide chains are coded by two genes: *GNPTAB* and *GNPTG*, which encode for the α/β-subunits and the γ-subunit, respectively. While mutations in both genes may cause ML III, alterations in the *GNPTAB* gene can also give rise to the more severe form ML II (ML II alpha/beta; OMIM: 252500) [2–4]. The *GNPTAB* gene, which contains 21 exons and spans 85 kb on chromosome 12q23.3, encodes an α/β-subunit precursor of 1256 amino acids with a predicted molecular mass of 144 kDa. Proteolytic processing of the α/β-precursor by the site-1 protease generates the α-and β- subunits, whose individualization is a prerequisite for the catalytic activity of the GlcNAc-1-phosphotransferase enzymatic complex [5]. Mutations that severely affect the α/β-subunit activity and/or the complex structure in such a way that its function is abolished result in the severe ML II alpha/beta

***Corresponding author: Sandra Alves,** Research and Development Unit, Department of Human Genetics, National Health Institute Dr. Ricardo Jorge, INSA, Rua Alexandre Herculano, 321 4000-055 Porto, Portugal, Phone: +351 223 401 100, Fax: +351223 401 109, E-mail: sandra.alves@insa.min-saude.pt; alvessandra@hotmail.com
Maria Francisca Coutinho and Marisa Encarnação: Research and Development Unit, Department of Human Genetics, National Health Institute Dr. Ricardo Jorge, Porto, Portugal
Francisco Laranjeira and Lúcia Lacerda: Unidade de Bioquímica Genética, Centro de Genética Médica Dr. Jacinto Magalhães, Centro Hospitalar do Porto, Porto, Portugal
Maria João Prata: i3S Instituto de Investigação em Saúde/IPATIMUP-Institute of Molecular Pathology and Immunology of the University of Porto, Porto, Portugal; and Department of Biology, Faculty of Sciences, University of Porto, Porto, Portugal

disease [6, 7], whereas mutations leading to the formation of a protein with residual phosphotransferase activity are associated to the milder form of the disorder. MLs are devastating diseases with an extremely high morbidity rate, a life expectancy that usually does not surpass the first decade of life for the severe ML II form and, unfortunately, with no therapy available at the moment. Actually, for families at risk (i.e. with confirmed genetic diagnosis) a meaningful help to cope with the disorder is to benefit from appropriate genetic counseling, promoting informed choices that are open by the possibility of screening fetuses whenever the causative mutations are known. Thus, the identification of genetic defects underlying ML III is a challenge with important medical implications.

Here we report the thorny molecular characterization of one ML III alpha/beta patient, who was a compound heterozygous for two distinct mutations in the *GNPTAB* gene.

Case presentation

The patient was diagnosed at 3 years of age. Biochemical studies confirmed an impairment of the GlcNAc-1-phosphotransferase through the demonstration that the activities of several lysosomal enzymes were increased in the serum and decreased in cultured fibroblasts. β-glucocerebrosidase activity was used as a reference since this enzyme is transported to the lysosomes in a M6P-independent fashion. Biological samples of the patient were obtained under parents' informed consent and all procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration.

Genomic and cDNA analysis

GNPTAB and *GNPTG* genomic and cDNA analyses were performed with specific primers according to previously reported conditions [8].

Cycloheximide treatment of control and patient fibroblasts

To perform nonsense-mediated mRNA decay (NMD) inhibition assays, patient fibroblast cell lines were cultured in the presence of two different concentrations of cycloheximide (1 and 2 mg/mL) for 3 h. Total RNA was isolated, cDNA synthesized and *GNPTAB* cDNA fragments amplified and sequenced.

Discussion

In order to search for the molecular defect(s) underlying the GlcNac-1-phosphotransferase impairment in the patient, cDNA analysis of the *GNPTAB* transcript was performed and the missense mutation c.1196C>T(p.S399F) [8] was identified in heterozygosity. No other alterations were detected through repeated amplification of the *GNPTAB* cDNA, even though both the patient clinical presentation and the biochemical results were incoherent with the expectations for a carrier individual. The discrepancy prompted further investigation with gDNA, carrying out amplification and sequencing of all 21 exons of *GNPTAB* and their intronic flanking regions as well of both the 5′ and 3′ untranslated regions of the gene. Once again, no other mutations were detected apart from the missense c.1196C>T(p.S399F) already identified in the cDNA.

Knowing that *GNPTAB* codes for only two (α- and β-) of the three subunits that form the active GlcNac-1-phosphotransferase, we questioned whether any mutation(s) in the *GNPTG* gene, which codes for the third subunit (γ-), could be contributing to the patient's phenotype, and so a gDNA analysis of all *GNPTG* exons and corresponding flanking regions was performed, failing also to reveal any pathogenic alteration.

Next, attention was again focused on the *GNPTAB* gene, exploring the presence of any mutation generating a mRNA transcript that could be a candidate for NMD. In order to test whether the NMD process was masking/degrading any mutant transcript, patient's fibroblasts were cultured with cicloheximide in a final concentration of 2 mg/mL for 3 h to inhibit the NMD process, rescuing eventual aberrant transcripts. After doing so, total RNA was isolated from the pelleted cells and the respective cDNA synthesized, providing an alternative approach to analyse the *GNPTAB* transcript. Examining the sequence of the six fragments, we successfully detected a second heterozygous mutation, c.3503_3504delTC, which was a relatively common deletion on exon 19 (Figure 1B and 1C). This was rather surprising, because the alteration escaped previous detection at the gDNA level (data not shown), despite the methodology to screen for it was very well-implemented in our laboratory, as demonstrated in previous works where we reported that the deletion was the most common mutation among Portuguese patients [8, 9]. One possible explanation to the failure to detect

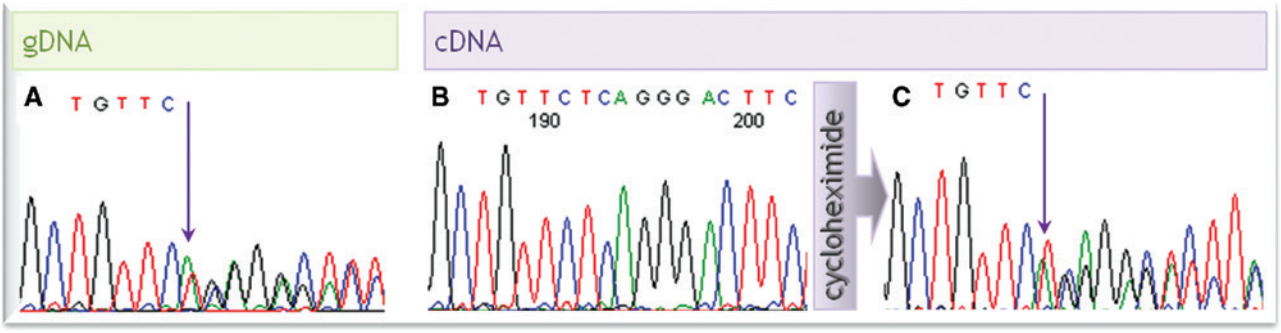

Figure 1: Electropherograms evidencing the detection of the mutation c.3503_3504delTC (p.L1168Qfs*5) mutation in heterozygosity in gDNA (A) and in cDNA obtained from patients fibroblasts untreated (B) and treated with 2 mg/mL of cycloheximide (C).

the mutation in this patient could rely in the *cis* presence in the allele carrying the deletion of an additional variation in any of the primer annealing regions, leading to the preferential amplification of the other allele when exon 19 and its boundaries were submitted to PCR. Accordingly, the next step was to design a second pair of primers surrounding the target exon but in an outer region relatively to the initially used primers' pair, perform PCR amplification in optimal conditions (primer sequences and PCR conditions available on request), and at last we were able to detect the deletion c.3503_3504delTC in heterozygosity (Figure 1A). Furthermore, one polymorphism was identified in the annealing region of the former reverse primer, confirming sequence variation as the cause of the observed allele-specific dropout. When the patient's parents were analyzed, we found out that the patient's mother was the carrier of the c.3503_3504delTC mutation, which in fact was detected with the two different pairs of primers, showing that the polymorphism was not inherited in *cis* with the mutant allele, but instead it was likely a *de novo* variant.

Having elucidated this particular case of allele-dropout, is worth mentioning that besides sequence variation, other factors do exist accounting for the amplification success, that might also cause differential amplification of two alleles. It has been demonstrated, for example, that differential DNA methylation can affect PCR, resulting in allele dropout due to preferential amplification of unmethylated alleles [10]. Secondary structures in the template DNA may also contribute to compromise the amplification efficiency [11].

Finally, and regarding cDNA characterization as a routine procedure in molecular diagnoses, as long as that persists much attention should be paid in the NMD process and its implications in the ability to pinpoint causative mutations. As NMD is a cellular quality control mechanism that recognizes abnormal mRNAs harboring premature termination codons (PTC) and degrades them, mutations that do trigger such mechanism may likely fail detection through cDNA analysis alone, thereby implying that gDNA screening should be always considered when cDNA provides negative or unsatisfactory results. Another case involving a ML III alpha/beta patient, who was studied in our lab can illustrate how NMD may lead to misdiagnosis. The patient was diagnosed at the age of 7 years presenting with polydistrophic dwarfism. Enzymatic assays revealed impaired activity of the GlcNAc-1-phosphotransferase. Like in the former patient, screening of *GNPTAB* in cDNA only enabled the identification of one missense mutation in heretozygozity, c.1208 (p.I403T) [9]. Yet, when screening the gDNA, a second pathogenic alteration was identified on exon 13: the nonsense mutation c.1999G>T(p.E667*) (data not shown). When the cultured fibroblasts from the patient were incubated with cicloheximide, in the novel cDNA sample synthesized using the extracted total RNA as template it was possible to detect the nonsense mutation (data not shown). Once again, NMD was masking the patient's genotype when inferred from cDNA without previous inhibition of NMD.

An important observation about the two NMD-triggering mutations here scrutinized, was their clear identification before in other patients in whom it was screened cDNAs obtained from non-cicloheximide-treated fibroblasts [9], which probably reflects the fact that NMD is not a mechanism 100% efficient. But yet in general, and as the two patients here studied demonstrate, NMD substantially accounts to degrade translationally abnormal mRNAs, implying therefore that the process should always be kept in mind when analysing cDNA sequences.

In conclusion, these two instances of tricky molecular diagnoses highlight the importance of relying in complementary genetic approaches to identify pathogenic mutations, in order to reduce the rate of genetically undiagnosed cases and consequent dramatic implications

especially for prenatal testing, whose value in the clinical setting goes hand in hand with quick and accurate results.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission. MFC participated in the conception and design of the study, acquisition of data, analysis and interpretation of data; article writing and in the approval of the final version of the manuscript to be published. ME participated in the acquisition of data, revision of the article for important intellectual content and in the approval of the final version of the manuscript to be published. FL participated in the acquisition of data, revision of the article critically for important intellectual content and in the approval of the final version of the manuscript to be published. LL participated in the acquisition of data, revision of the article critically for important intellectual content and in the approval of the final version of the manuscript to be published. MJP participated in the conception and design of the study; revision of the article for important intellectual content and in the approval of the final version of the manuscript to be published. SA participated in the conception and design of the study, analysis and interpretation of data, article writing and in the approval of the final version of the manuscript to be published.

Research funding: M.F. Coutinho is grantee from the FCT (SFRH/BPD/101965/2014).

Employment or leadership: None declared.

Honorarium: None declared.

Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

References

1. Braulke T, Bonifacino JS. Sorting of lysosomal proteins. Biochim Biophys Acta 2009;1793:605–14.
2. Raas-Rothschild A, Cormier-Daire V, Bao M, Genin E, Salomon R, et al. Molecular basis of variant pseudo-hurler polydystrophy (mucolipidosis IIIC). J Clin Invest 2000;105:673–81.
3. Tiede S, Storch S, Lübke T, Henrissat B, Bargal R, et al. Mucolipidosis II is caused by mutations in GNPTA encoding the alpha/beta GlcNAc-1-phosphotransferase. Nat Med 2005;11:1109–12.
4. Coutinho MF, Santos LD, Girisha KM, Satyamoorthy K, Lacerda L, et al. Mucolipidosis type II α/β with a homozygous missense mutation in the GNPTAB gene. Am J Med Genet A 2012;158A:1225–8.
5. Marschner K, Kollmann K, Schweizer M, Braulke T, Pohl S. A key enzyme in the biogenesis of lysosomes is a protease that regulates cholesterol metabolism. Science 2011;333:87–90.
6. Leroy JG, Ho MW, MacBrinn MC, Zielke K, Jacob J, et al. I-cell disease: biochemical studies. Pediatr Res 1972;6:752–7.
7. Kornfeld S, Sly WS. I-cell disease and pseudo-Hurler polydystrophy: disorders of lysosomal enzyme phosphorylation and localisation. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. Childs B, Kinzler KW, Vogelstein B, Assoc. Editors. The metabolic and molecular bases of inherited disease, 8th ed. New York: McGrawHill, 2001:3421–42.
8. Encarnação M, Lacerda L, Costa R, Prata MJ, Coutinho MF, et al. Molecular analysis of the GNPTAB and GNPTG genes in 13 patients with mucolipidosis type II or type III – identification of eight novel mutations. Clin Genet 2009;76:76–84.
9. Coutinho MF, Encarnação M, Gomes R, da Silva Santos L, Martins S, et al. Origin and spread of a common deletion causing mucolipidosis type II: insights from patterns of haplotypic diversity. Clin Genet 2010; 80:273–280.
10. Tomaz RA, Cavaco BM, Leite V. Differential methylation as a cause of allele dropout at the imprinted GNAS locus. Genet Test Mol Biomarkers 2010;14:455–60.
11. Buchan JC, Archie EA, Van Horn RC, Moss CJ, Albert SC. Locus effects and sources of error in noninvasive genotyping. Mol Ecol Notes 2005;5:680–3.