UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL – UFRGS
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOLOGIA MOLECULAR

ESTUDO ABRANGENTE SOBRE A DEFICIÊNCIA DA GlcNAc-1-FOSFOTRANSFERASE (MUCOLIPIDOSES II E III): DO DIAGNÓSTICO MOLECULAR A PROSPOSTAS DE TRATAMENTO

Renata Voltolini Velho

Orientadora: Profa. Dra. Ida Vanessa D. Schwartz
Co-orientadora: Profa. Dra. Ursula Matte

Porto Alegre, maio de 2015.

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Renata Voltolini Velho

Tese de doutorado submetida ao Programa de Pós-Graduação em Genética e Biologia Molecular da Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção do grau de Doutor em Ciências (Genética e Biologia Molecular).

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LISTA DE ABREVIATURAS

Asp - aminoácido asparato

CFTR - Regulador de condutância transmembranar da fibrose cística

CPT1A - Carnitina palmitoil 1 A

DMAP1 -correpressor transcricional DNA metiltransferase 1

DMD - Distrofia muscular de Duchenne

DNA – Ácido desoxirribonucleico

DP6 - Distância percorridaem 6 minutos

DUF 3184 - Proteína de função desconhecida denominada DUF 3184

EIM - Erros Inatos do Metabolismo

ERGIC-53 -proteína de membrana do compartimento intermediário Golgi-retículo endoplasmático

FC -Fibrose cística

FEV1 -volume expiratório forçado em 1 segundo

GlcNAc -N-acetilglucosamina

GlcNAc-1-fosfato -N-acetilglucosamina-1-fosfato

GlcNAc-1-fosfotransferase - N-acetilglucosamina-1-fosfotransferase

***GNPTAB* –** gene que codifica a proteína precursora das subunidades alfa e beta da enzima N-acetilglucosamina-1-fosfotransferase

***GNPTG* -** gene que codifica a proteína da subunidade gama da enzima N-acetilglucosamina-1-fosfotransferase

Golgi – Complexo de Golgi

HEK – Célula embrionária de rim humano

HeLa – Célula de tumor ovariano humano

kDa – kilodaltons

LEIM-HCPA – Laboratório de Erros Inatos do Metabolismo do Hospital de Clínicas de Porto Alegre

Lys – Aminoácido lisina

m –Metro

M6P –Manose-6-fosfato

mg/ kg –Miligramas/ kilogramas

ML – Mucolipidose (s)

ML II – Mucolipidose (s) tipo II

ML III – Mucolipidose tipo III

MPS I – Mucopolissacaridose tipo I ou Síndrome de Hurler

mRNA – Ácido ribonucleico mensageiro

NAGPA -GlcNAc-1-fosfodiéster N-acetilglucosaminidase

NMD -*Nonsense mediated RNA decay*

PTC – Códon (s) de parada prematuro (s)

RE – Retículo endoplasmático

RNA –Ácido ribonucleico

s – Segundo

S1P – Proteína*site-1-protease*

SGM-HCPA -Serviço de Genética Médica do Hospital de Clínicas de Porto Alegre

TCH - Transplante de células-tronco hematopoiéticas

TMO - Transplante de medula óssea

TRE - Terapia de reposição enzimática

UDP-GlcNAc – uridina difosfato-N-acetilglucosamina

UFRGS -Universidade Federal do Rio Grande do Sul

UTR -Região não traduzida

α –subunidade alfa da enzima N-acetilglucosamina-1-fosfotransferase

α-MM - α-metilmanosídeo

β – subunidade beta da enzima N-acetilglucosamina-1-fosfotransferase

γ – subunidade gama da enzima N-acetilglucosamina-1-fosfotransferase

RESUMO

Introdução: Os lisossomos são organelas ácidas em que muitos tipos de macromoléculas, incluindo proteínas, carboidratos, ácidos nucléicos e lipídios são entregues para a degradação. A biogênese dos lisossomos requer a contínua substituição das enzimas lisossomais solúveis e das proteínas de membrana lisossomal. O direcionamento das hidrolases ácidas depende dos resíduos de manose-6-fosfato (M6P) que são reconhecidos por receptores específicos que medeiam o seu transporte para os lisossomos. O papel chave na formação destes resíduos é desempenhado no Complexo de Golgi pela enzima GlcNAc-1-fosfotransferase que contêm seis subunidades, $\alpha_2\beta_2\gamma_2$. Este complexo enzimático é codificado por dois genes, *GNPTAB* e *GNPTG*. Mutações nestes genes resultam em duas doenças lisossômicas, as mucolipidoses (ML) tipo II e III, caracterizadas bioquimicamente pela perda de múltiplas enzimas lisossomais, devido à formação deficiente dos resíduos de M6P.

Objetivos: 1) Verificar a patogenicidade, por meio de ensaios *in vitro*, das seguintes mutações em *GNPTAB* previamente identificadas em pacientes brasileiros com ML II e III alfa/beta: c.1208T>C, c.1514G>A, c.1723G>A, c.1759C>T, c.1931_1932CA>TG, c.2269_2273delGAAAC, c.2808A>G e c.3668_3670delCTA; 2) Caracterizar o perfil de mutações de *GNPTG* presente em uma amostra de pacientes com ML III gama; 3) Analisar o efeito de gentamicina e cloranfenicol sobre a atividade das enzimas α-manosidase, β-glicuronidase e β-galactosidase em fibroblastos de pacientes com ML III gama heterozigotos ou homozigotos para mutações sem sentido em *GNPTG*.

Metodologia: Estudo transversal, amostragem por conveniência, incluindo pacientes com diagnóstico clínico e bioquímico de ML II/III. Mutações em *GNPTAB* previamente identificadas em pacientes brasileiros com ML II/III alfa/beta foram estudas em relação a expressão de mRNA, proteína, atividade enzimática e localização intracelular da GlcNAc-1-fosfotransferase. Pacientes também foram avaliados quanto a variações no gene *GNPTG* e seus possíveis impactos a nível de mRNA. O grupo de pacientes com ML III gama foram avaliados para medir sua independência funcional “Functional Independence Measure” (FIM). Estudos de expressão de *GNPTAB* e *GNPTG* também foram desenvolvidos para maior compreensão da relação destes gen[illegible]

desenvolvimento de um possível tratamento realizado nos fibroblastos de pacientes com ML III gama também foram objeto de estudo.

Resultados: Mutações sem sentido e que alteram a fase de leitura da enzima GlcNAc-1-fosfotransferase não são corretamente transportados do retículo endoplasmático (RE) ao aparelho de Golgi, devido a interrupção, a falta dos sinais de exportação localizados nas porções N- e C- terminais da proteína. O não transporte ao Golgi e consequentemente, a ausência da clivagem proteolítica da proteína precursora das subunidades α/β, são responsáveis pela falta de atividade enzimática. Além dessas, mutações com sentido trocado na porção luminal da enzima também podem ter o transporte ao Golgi prejudicado, o que sugere a presença de um local de contato para uma proteína necessária para a exportação eficiente do RE. O estabelecimento de um ensaio radioativo para medir a atividade enzimática da GlcNAc-1-fosfotransferase confirmou que o transporte para o Golgi e a clivagem proteolítica nas subunidades α- e β- maduras é um pré-requisito para a atividade enzimática. As mutações em *GNPTAB* tiveram sua patogenicidade confirmada. Em relação a análise de *GNPTG*, as técnicas moleculares empregadas permitiram a identificação de três novas mutações patogênicas (c.244_247dupGAGT, c.328G>T e c.233+5G>C) e de uma outra variação (c.-112C>G). Após incessante investigação do trio cuja mutação causal c.244_247dupGAGT foi encontrada, a mesma foi atribuída como um evento *de novo* que ocorreu em um único óvulo ou de um mosaicismo germinativo no óvulo materno. Ao investigar o potencial efeito das mutações sem sentido através de mRNA, não foi possível identificar a mutação c.328G>T (p.E110X), no lugar desta, todos os pacientes apresentaram a sequência selvagem de *GNPTG*, evento denominado edição de RNA (c.328G@T). Porém, níveis de mRNA muito próximos a zero foram obtidos o que confirma a patogenicidade das mutações em *GNPTG* bem como o diagnóstico dos pacientes. Em pacientes ML II e III alfa/beta e em ML III gama, a redução dos níveis de mRNA de *GNPTAB* e *GNPTG*, respectivamente, pode ser facilmente explicada pela natureza das mutações identificadas nos pacientes. O que intriga, porém, é a expressão do gene não mutado. A divergência entre os resultados encontrados pode estar relacionada ao tipo de amostra, sabendo-se que as ML II e ML III são tecido-específicas. Fibroblastos de três pacientes com ML III gama portadores de mutações sem sentido foram tratados com geneticina e

cloranfenicol. Este estudo de conceitos não demonstrou a possibilidade ou eficácia de um tratamento ser desenvolvido baseado na utilização de compostos não antibióticos atuarem sobre a tradução alternativa ou *read through* em pacientes com ML II e III.

Conclusão: Estudos de caracterização genética, clínica e populacional sempre contribuirão para a elucidação dos mecanismos da doença e concomitantemente, melhor atendimento ao paciente. Com isto, é de grande importância que esforços e recursos sejam destinados a pesquisas nesta área para o completo entendimento das ML II e III e dos processos biológicos envolvidos. Este é o primeiro estudo brasileira a realizar o diagnóstico molecular de ML III gama; o primeiro a relatar uma mutação de novo e também, a ocorrência de edição de mRNA em pacientes com ML III gama.

ABSTRACT

Introduction: Lysosomes are acidic organelles into which many types of macromolecules including proteins, carbohydrates, nucleic acids and lipids are delivered for degradation. The biogenesis of lysosomes requires a continuous substitution of soluble lysosomal enzymes and lysosomal membrane proteins. The targeting of lysosomal enzymes depends on mannose 6-phosphate residues (M6P) that are recognized by M6P-specific receptors mediating their transport to lysosomes. The key role in the formation of M6P residues plays the Golgi-resident GlcNAc-1-phosphotransferase complex consisting of six subunits, $\alpha_2\beta_2\gamma_2$. This enzyme complex is encoded by two genes, *GNPTAB* and *GNPTG*. Mutations in these genes result in two lysosomal storage diseases, mucolipidosis (ML) type II and III, biochemically characterized by the missorting of multiple lysosomal enzymes due to impaired formation of M6P residues, and general lysosomal dysfunction.

Objectives: 1)To analyze the pathogenicity, through in vitro tests, of *GNPTAB* mutations previously identified in Brazilian patients with ML II and III alpha/beta: c.1208T>C, c.1514G>A, c.1723G>A, c.1759C>T, c.1931_1932CA>TG, c.2269_2273delGAAAC, c.2808A>G and c.3668_3670delCTA; 2) Characterize *GNPTG* mutational profile in a ML III gamma patients group; 3) Analyze the effect of gentamicin and chloramphenicol on the activity of α-mannosidase, β-glucuronidase and β-galactosidasein fibroblasts of ML III gamma patients with *GNPTG* nonsense mutations.

Methods: Cross-sectional study, with convenience sample, including patients with a clinical and biochemical diagnosis of ML II/III. *GNPTAB* mutations previously identified in ML II/III alpha/beta Brazilian patients were studied in relation to mRNA and protein expression, enzyme activity and intracellular localization of GlcNAc-1-phosphotransferase. Patients were also evaluated to *GNPTG* variations and possible mRNA impacts. The ML III gamma patients group was also evaluated with the Functional Independence Measure (FIM). *GNPTAB* and *GNPTG* expression studies were developed to better understand the relationship between them. Geneticin and chloramphenicol were used to treat ML III gamma fibroblasts.

Results: Nonsense and frameshift mutations of GlcNAc-1-phosphotransferase failed to reach the Golgi apparatus due to the interrupted cooperative ER export signals localized both in the N- and C-terminal cytosolic tails and lacked proteolytic activation of the α/β-subunit precursor. In addition, luminal missense mutations of the GlcNAc-1-phosphotransferase can also impair the transport to the Golgi apparatus, suggesting the presence of a protein contact site required for efficient ER export. The establishment of a radioactive assay to measure the activity of the GlcNAc-1-phosphotransferase confirmed that the transport to the Golgi and proteolytic cleavage into mature α- and β-subunits is prerequisite for enzymatic activity. Regarding *GNPTG* analysis, molecular techniques employed allowed the identification of three new pathogenic mutations (p.F83X, p.E110X, c.233+5G>C) and another variation (c.-112C>G).

After research, p.F83X was attributable to a *de novo* event which occurred in only one ovum, or to germline mosaicism in the mothers' ova. The potential effect of p.E110X mutation in mRNA was investigated. However it was not possible to identify transcripts carrying this mutation since all patients appear to present only the wild type sequence, an event called mRNA editing (c.328G@T). Nevertheless, low mRNA levels confirm the *GNPTG* mutations pathogenicity and the patients' diagnosis. In ML II/III alpha/beta and ML III gamma patients, low *GNPTAB* and *GNPTG* mRNA expression levels, respectively, can easily be explained by the nature of the mutations. Interestingly, it is the non-mutated gene. Divergence between results may be related to the type of sample, given that ML II and III are tissue-specific diseases. Fibroblasts from three ML III gamma patients were treated with geneticin and chloramphenicol with no effect being observed. This pilot study does not support the feasibility or effectiveness for the development of a treatment based on the use of non-antibiotic compounds acting on the read through of either ML II or III patients.

Conclusions: Genetic, clinical and population characterization studies always contribute to the elucidation of disease mechanisms and concomitantly, lead to better patient care. It is very important that efforts and resources to be focused in this area for the complete understanding of ML II and III as well as the biological processes involved. This is the first study to perform molecular diagnosis in ML III gamma Brazilian patients. Also it is the first to report a *de novo* mutation and the occurrence of mRNA editing in ML III gamma patients.

1 INTRODUÇÃO

Os lisossomos, primeiramente descritos por De Duve et al. em 1955, são organelas ácidas em que muitos tipos de macromoléculas, incluindo proteínas, carboidratos, ácidos nucléicos e lipídios são entregues para a degradação. A biogênese dos lisossomos requer a contínua substituição das enzimas lisossomais solúveis e das proteínas de membrana lisossomal (BRAULKE; RAAS-ROTHSCHILD; KORNFELD, 2013). O direcionamento das hidrolases ácidas depende dos resíduos de manose-6-fosfato (M6P) que são reconhecidos por receptores específicos que medeiam o seu transporte para os lisossomos (BRAULKE; BONIFACINO, 2009). O papel chave na formação destes resíduos é desempenhado no Complexo de Golgi pela enzima N-acetilglucosamina-1-fosfotransferase (GlcNAc-1-fosfotransferase) que contêm seis subunidades, $\alpha_2\beta_2\gamma_2$. Este complexo enzimático é codificado por dois genes, *GNPTAB* e *GNPTG* (RAAS-ROTHSCHILD et al., 2000; TIEDE et al., 2005b). Mutações nestes genes resultam em duas doenças lisossômicas, as mucolipidoses tipo II (ML II) e III (ML III), caracterizadas bioquimicamente pela perda de múltiplas enzimas lisossomais, devido à formação deficiente dos resíduos de M6P (BRAULKE; RAAS-ROTHSCHILD; KORNFELD, 2013).

1.1 GlcNAc-1-fosfotransferase

1.1.1 Estrutura molecular da enzima GlcNAc-1-fosfotransferase

Em 1996, Bao et al. demonstraram que a GlcNAc-1-fosfotransferase é uma enzima hexamérica residente no Complexo de Golgi (Golgi) de 540 kDa ($\alpha_2\beta_2\gamma_2$). A proteína precursora das subunidades α/β é sintetizada como uma proteína de membrana tipo III, *N*-glicosilada de 190 kDa e 1256 aminoácidos (TIEDE et al., 2005b). Para o transporte vesicular do retículo endoplasmático (RE) para o aparelho de Golgi, a proteína precursora das subunidades α/β requer um motivo combinatório nos domínios N- e C-terminais citosólicos (FRANKE; BRAULKE; STORCH, 2013). Através de uma clivagem proteolítica realizada pela enzima *site-1-protease* (S1P) localizada na porção *cis* do Golgi, entre os resíduos Lys-928-Asp-929, há a ativação catalítica das subunidades α e β geradas com tamanho de 928 e 328 aminoácidos e massas de 145 e 45 kDa,

respectivamente (KOLLMANN et al., 2010; KUDO et al., 2005; MARSCHNER et al., 2011).

A subunidade α é uma proteína de membrana tipo II com uma porção N-terminal, um domínio transmembrana e um luminal contendo 19, 22 e 886 aminoácidos, respectivamente. O domínio luminal compreende 17 potenciais sítios de *N*-glicosilação e exibe uma complexa e conservada estrutura com diferentes domínios de função desconhecida (Figura 1- TIEDE et al., 2005). Utilizando a base de dados do *NCBI,* foi possível identificar uma região de homologia com uma proteína de função desconhecida (DUF 3184, aminoácidos 318-429), e com o domínio *Notch* (aminoácidos 433-469 e 500-535). A proteína *Notch* é um receptor transmembrana de sinalização intercelular. Processado no Golgi e estabilizado pelo cálcio, atua como um fator transcricional após clivagem proteolítica na membrana plasmática (GURUHARSHA; KANKEL; ARTAVANIS-TSAKONAS, 2012). Os domínios *Notch* da subunidade α contêm resíduos de ligação para o cálcio e exibem um elevado teor de cisteína, 12 do total de 19 presentes na proteína precursora das subunidades α/β, sugerindo uma estrutura secundária complexa.

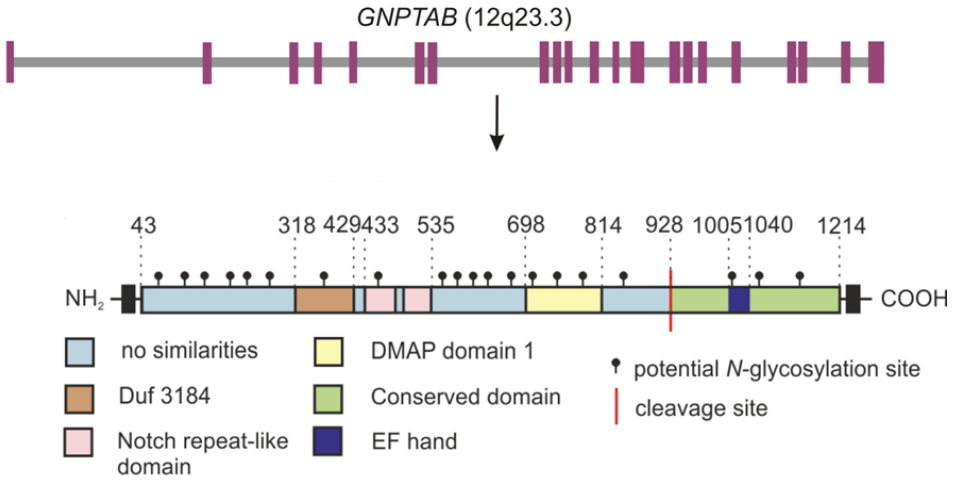

Figura 1. Representação esquemática da estrutura molecular da proteína precursora das subunidades α/β da GlcNAc-1-fosfotransferase humana. A posição dos éxons no gene *GNPTAB* humano estão representados em barras roxas e os íntrons, pela linha cinza. Os diferentes domínios homólogos são descritos de acordo com o alinhamento realizado usando o banco de dados *NCBI* (NM_024312.4). Potenciais sítios de *N*-glicosilação (•) são indicados. Domínios transmembrana estão representados pelos retângulos pretos. A numeração inicia pela primeira metionina da sequência codificante. O sítio de clivagem (aa 928/929) está marcado pela linha vermelha.

Outro domínio (aminoácidos 699-814) apresenta similaridades com o correpressor transcricional DNA metiltransferase 1 (DMAP1). O domínio DMAP1 foi recentemente descrito como região de ligação para catepsina D e α-L-iduronidase (QIAN et al., 2013) sugerindo que esta região represente o local de ligação de enzimas lisossomais para a subsequente marcação com os resíduos de M6P.

A subunidade β madura é uma proteína de membrana tipo I com 3 possíveis sítios de *N*-glicosilação, um domínio citoplasmático com 21 aminoácidos, um domínio transmembrana contendo 23 resíduos e, um luminal com 284 aminoácidos. Embora a sequência que compreende a região luminal da subunidade β seja altamente conservada, não foram encontradas homologias com outras proteínas. Na porção que compreende os aminoácidos 1005-1040 há uma estrutura *hand EF* (Figura 1) que é um domínio estrutural hélice-laço-hélice encontrado numa grande família de proteínas de ligação ao cálcio.

A reação enzimática catalisada pela GlcNAc-1-fosfotransferase é cálcio dependente, no entanto, não se sabe se o Ca^{2+} liga-se aos resíduos *hand EF* da subunidade β. O papel dos domínios para a atividade catalítica da GlcNAc-1-fosfotransferase, oligomerização das subunidades ou interações com outras proteínas não é clara.

A subunidade γ da GlcNAc-1-fosfotransferase humana (Figura 2) representa uma proteína *N*-glicosilada solúvel de 36 kDa composta por 305 aminoácidos (RAAS-ROTHSCHILD et al., 2000). Alguns pesquisadores acreditam que a subunidade γ pode estar envolvida na estabilização da conformação das subunidades α e β e também pode ser capaz de ligar-se mais eficientemente às enzimas lisossomais (LEE et al., 2007; QIAN et al., 2010). Além disso, a expressão de γ parece regular a expressão da proteína precursora das subunidades α/β de uma forma compensatória (POHL et al., 2009).

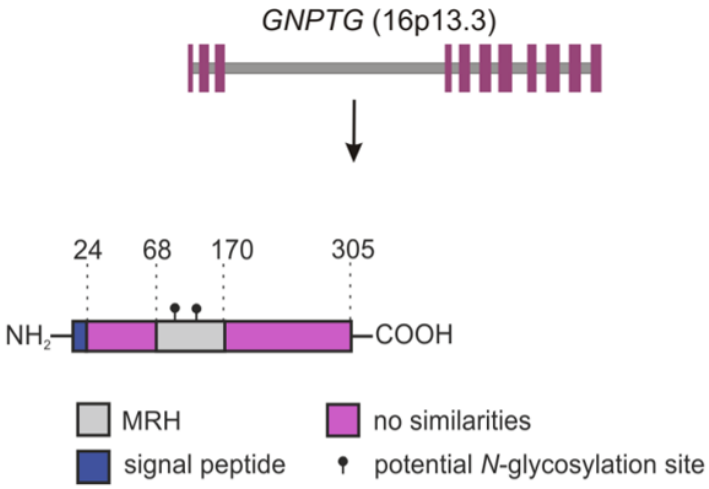

Figura 2. Representação esquemática da estrutura molecular da proteína precursora da subunidade γ da GlcNAc-1-fosfotransferase humana. A posição dos éxons no gene *GNPTG* humano estão representados em barras roxas e os íntrons, pela linha cinza. Os diferentes domínios homólogos são descritos de acordo com o alinhamento realizado usando o banco de dados NCBI (NM_032520.3). Potenciais sítios de N-glicosilação (•) são indicados.

1.1.2 Atividade enzimática da GlcNac-1-fosfotransferase

A GlcNAc-1-fosfotransferase catalisa a transferência do GlcNAc-1-fosfato a partir de UDP-GlcNAc para grupamentos hidroxil na posição C6 de resíduos terminais de manose das cadeias de oligossacarídeos das enzimas lisossomais, gerando um fosfodiéster intermediário. Em seguida, o resíduo GlcNAc é hidrolisado pela enzima GlcNAc-1-fosfodiéster N-acetilglicosaminidase (NAGPA) e os resíduos de manose-6-fosfato (M6P) são expostos (Figura 3 A). As subunidades α e β são responsáveis pela atividade catalítica, possuem os sítios de ligação para o substrato UDP-GlcNAc e são responsáveis por reconhecer uma porção comum das enzimas lisossomais (BRAULKE; RAAS-ROTHSCHILD; KORNFELD, 2013). A precisa localização do centro catalítico desta enzima ainda é desconhecida.

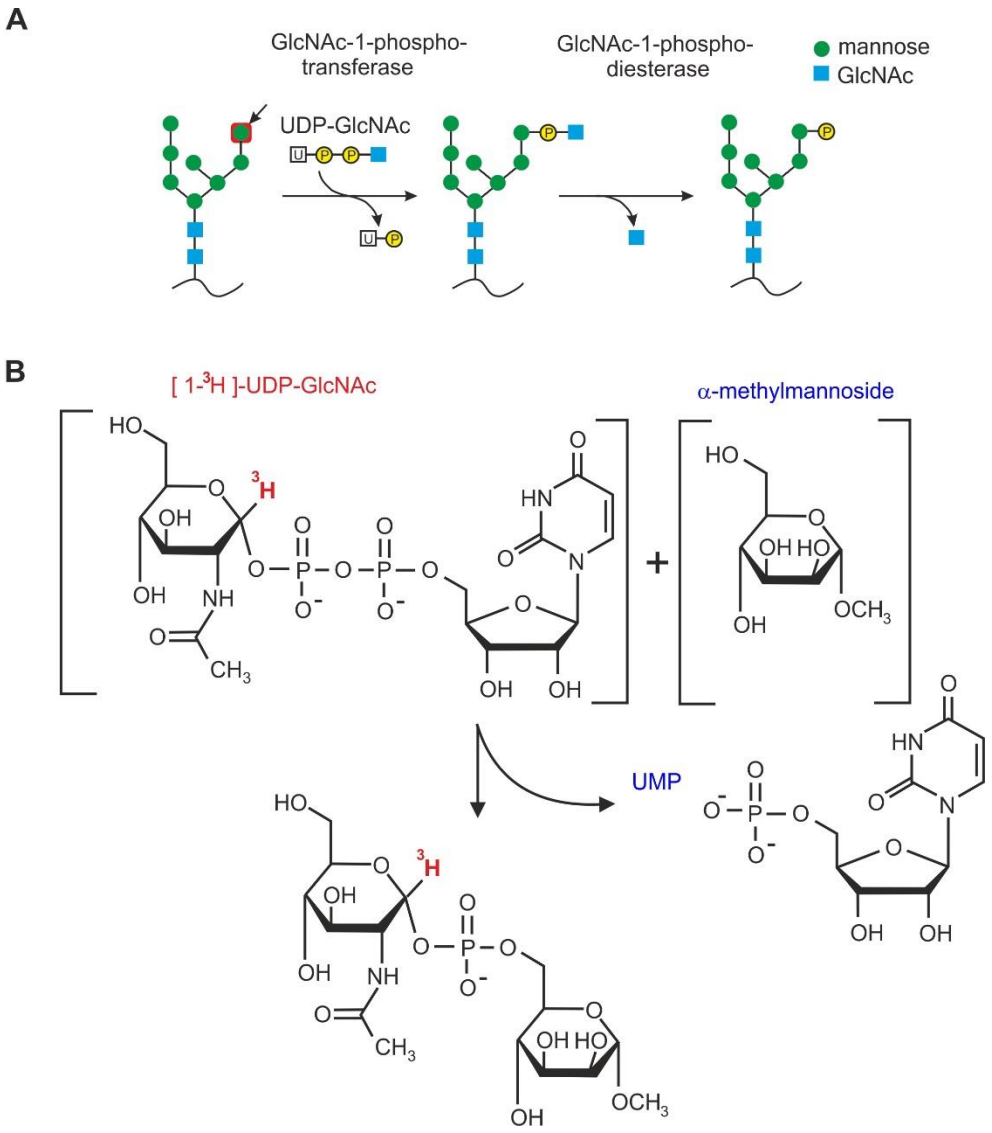

Figura 3. Reação enzimática da GlcNAc-1-fosfotransferase. A. Representação esquemática da reação enzimática catalizada pela GlcNAc-1-fosfotransferase e GlcNAc-1-fosfodiesterase. GlcNAc-1-fosfotransferase transfere GlcNAc-1-fosfato do grupamento UDP-GlcNAc para grupamentos hidroxil na posição C6 de resíduos terminais de manose das cadeias de oligossacarídeos das enzimas lisossomais, gerando um fosfodiéster intermediário. Em seguida, o resíduo GlcNAc é hidrolisado pela GlcNAc-1-phosphodiesterase e os resíduos de M6P são expostos. **B.** Para o ensaio in vitro da GlcNAc-1-fosfotransferase, [^{3}H]UDP-GlcNAc e α-metilmanosídeo são usados como doador e aceptor de fosfato, respectivamente. Durante a reação, UMP e [^{3}H]GlcNAc-fosfodiester são produzidos.

A atividade endógena da GlcNAc-1-fosfotransferase pode ser diretamente determinada *in vitro* usando extrato celular e os substratos [^{32}P]UDP-GlcNAc ou [^{3}H]UDP-GlcNAc como doadores de fosfato e α-metilmanosídeo (ou uma enzima lisossomal recombinante não fosforilada se disponível) como aceptor do

grupamento fosfato (Figura 3 B; BEN-YOSEPH; BAYLERIAN; NADLER, 1984; REITMAN; LANG; KORNFELD, 1981). O ensaio *in vitro* pode também ser realizado em extratos celulares superexpressando a enzima selvagem ou constructos mutantes de GlcNAc-1-fosfotransferase (QIAN et al., 2013).

1.1.3 Relação dos genes *GNPTG* e *GNPTAB*

Muitas divergências surgem quando a relação entre os genes *GNPTAB* e *GNPTG* é estudada. Em recente pesquisa desenvolvida por Encarnação *et al.* (2009), os resultados da quantificação relativa dos níveis de mRNAs de *GNPTAB* e *GNPTG* indicam que as mutações presentes em *GNPTAB* estão associadas com uma diminuição nos níveis de mRNA do mesmo e, em alguns casos, de *GNPTG*, quando comparados com os valores médios controles. Em pacientes com alterações na sequência de *GNPTG,* ambos os níveis de mRNA, *GNPTG* e *GNPTAB,* foram significativamente menores também. Os autores explicam que a baixa expressão dos genes *GNPTAB* ou *GNPTG* quando a mutação encontra-se no gene codificador da outra subunidade, pode estar relacionada com a existência de um mecanismo de *feedback* entre os genes que codificam as subunidades α/β e γ da enzima GlcNAc-1-fosfotransferase, o que vai de encontro aos dados publicados por Ho *et al.* (2007).

Ao investigar a interação entre as subunidades α/β/γ em linhagens de células humanas com ML, o grupo de pesquisa chinês liderado por Ho et al. (2007) mostrou que quando a expressão das subunidades α/β está diminuída nestas linhagens, há uma indução na expressão de γ. Em fibroblasto de camundongos normais, ao suprimir as subunidades α/β também foi verificada uma indução de γ. Por outro lado, a supressão de γ resultou em uma resposta diferenciada. Primeiramente, as subunidades α/β foram reprimidas, e logo após, foram superexpressas. Esses dados sugerem que um fino balanço entre as expressões das subunidades α/β/γ possa ser um importante fator na patogênese das MLs.

Outro grupo indica uma função regulatória de *GNPTG* sob *GNPTAB* no processo de formação do marcador M6P. Em três linhagens celulares de pacientes com ML III gama onde os níveis de mRNA foram verificados, houve uma redução de *GNPTG* (25 e 60%) e uma indução das subunidades α/β

(*GNPTAB*). Similarmente, a elevada expressão de *GNPTG* pareceu induzir a baixa regulação de *GNPTAB.* Estes dados sugerem um mecanismo compensatório entre *GNPTG/GNPTAB* (POHL et al., 2009).

1.2 GlcNAc-1-fosfotransferase- doenças relacionadas

Mucolipidoses tipo II (ML II) e III (ML III) são doenças autossômicas recessivas causadas por um defeito genético na enzima GlcNAc-1-fosfotransferase. Mutações no gene que codifica as subunidades α e β (*GNPTAB*) causam ML II ou, com condição clínica mais branda, ML III alfa/beta. ML III gama resulta de mutações no gene *GNPTG* que codifica a subunidade γ da GlcNAc-1-fosfotransferase (CATHEY et al., 2008; RAAS-ROTHSCHILD; POHL; BRAULKE, 2013).

1.2.1 Características clínicas

O termo ML refere-se a uma combinação de sintomas de mucopolissacaridores e esfingolipidoses observados clinicamente. Os pacientes com ML II apresentam face grosseira, hipertrofia gengival, macroglossia, hérnias inguinais, infiltrações cutâneas, limitação articular, surdez, atraso psicomotor e baixa estatura. A ML II pode estar associada à ocorrência de fraturas intra-útero e a achados radiológicos e bioquímicos compatíveis com raquitismo ou hiperparatireoidismo neonatal. É fatal durante a infância, em geral na primeira década, devido à ocorrência de complicações cardiopulmonares (CATHEY et al., 2010; KORNFELD; SLY, 2001; RAAS-ROTHSCHILD; POHL; BRAULKE, 2013).

ML III é uma forma atenuada da doença, com posterior aparecimento dos sintomas clínicos e progressão mais lenta o que permite a sobrevivência até a idade adulta (BRAULKE; RAAS-ROTHSCHILD; KORNFELD, 2013). Geralmente, o primeiro sinal de pacientes com ML III é rigidez articular que leva ao diagnóstico durante a infância. As dismorfias faciais são menos graves e os indivíduos afetados atingem estatura superior a 1,50 m. A inteligência pode ser normal, mas a maioria das crianças tem dificuldade de aprendizado, o que pode estar relacionado com a diminuição da acuidade auditiva (CATHEY et al., 2010; RAAS-ROTHSCHILD et al., 2004; ROBINSON et al., 2002).A variabilidade dos

sintomas clínicos das ML correspondem aos diferentes níveis de atividade residual de GlcNAc-1-fosfotransferase, o que resulta em um amplo espectro de gravidade (DIERKS et al., 2009; TIEDE et al., 2005b).

1.2.2 Perfil mutacional das Mucolipidoses II e III

Mais de 140 mutações já foram descritas em pacientes com ML II e III alfa/beta além de outras 29 descritas em pacientes com ML III gama (HGMD, 2015), totalizando aproximadamente 170 mutações. A grande maioria são de ocorrência única, porém a mutação presente no éxon 19 do gene *GNPTAB,* c.3503_3504delTC, é a mutação mais comum ocorrendo em 37,5% dos alelos de 21 pacientes brasileiros (CURY et al., 2013). É também a mutação patogênica mundialmente mais frequente associada a ML II e III alfa/beta e descrita como fundadora na população franco-canadense. *GNPTG* não apresenta uma mutação mais frequente.

Dentre os tipos de mutação descritas nas ML II e III, as sem sentido são muito frequentes em *GNPTAB* correspondendo a 15,49%. Já em *GNPTG,* essas mutações correspondem a 10,34%.

A pesquisa de mutações possibilita estabelecer correlações genótipo-fenótipo indicando um prognóstico mais seguro da doença e, através de estudos de expressão, permite compreender a patogenicidade das mutações além de propiciar descobertas em relação à função das subunidades da enzima GlcNAc-1-fosfotransferase. Além disso, entender o perfil mutacional de uma doença, permite o desenvolvimento de propostas de tratamento mutação-específica, uma das novas possibilidades dentro da inovação de fármacos, de tratamentos.

Tabela 1. Perfil mutacional das Mucolipidoses II e III

Tipo de mutação	Número de mutações nos genes

	GNPTAB	***GNPTG***
Sentido trocado	38	5
Sem sentido	22 (15,49%)	3 (10,34%)
Splicing	15	6
Pequenas deleções	35	7
Pequenas inserções	27	6
Grandes deleções	1	2
Grandes inserções	2	-
Pequenas *indels*	2	-
Total	**142**	**29**

Fonte: HGMD, 2015

1.2.3 Epidemiologia

A incidência das mucolipidoses II e III varia de acordo com a população estudada. Considerando os recém- nascidos vivos, estima-se 1 caso para 120.000 em Portugal, 1 para 152.000 no Japão, 422.000 na Austrália e 642.000 na Holanda (RAAS-ROTHSCHILD; POHL; BRAULKE, 2013). Acredita-se que em algumas populações esta incidência seja maior, como na região de Quebec, no Canadá, onde estima- se que a incidência de pacientes com ML II seja de 1/6.184 recém- nascidos vivos (PLANTE et al., 2008). Não foram encontrados estudos na população geral e na população brasileira.

1.2.4 Diagnóstico

O primeiro passo para estabelecimento diagnóstico é a suspeita clínica. Para a conclusão definitiva da condição é necessária a demonstração da atividade intracelular deficiente da GlcNAc-1-fosfotransferase, ou a identificação de mutações patogênicas em *GNPTAB* ou *GNPTG* (BRAULKE; RAAS-ROTHSCHILD; KORNFELD, 2013). Porém estes são testes complexos e disponíveis apenas em seletos centros de pesquisa. Assim, a atividade da GlcNAc-1-fosfotransferase é geralmente avaliada indiretamente através da medição da atividade das hidrolases lisossomais no plasma e em fibroblastos, uma vez que pacientes com ML II e III apresentam níveis elevados de hidrolases

no plasma e outros fluídos corporais e redução destas enzimas no compartimento intrecelular (particularmente em fibroblastos). É possível a análise das atividades das hidrolases em papel-filtro mas este, permanece sendo um método de triagem. A excreção urinária de glicosaminoglicanos geralmente está normal ou levemente aumentada (RAAS-ROTHSCHILD; POHL; BRAULKE, 2013).

O diagnóstico pré-natal pode ser realizado através da identificação de uma atividade aumentada das enzimas lisossomais em líquido amniótico e sua diminuição em cultivo de amniócitos, ou ainda, por meio da atividade da GlcNAc-1-fosfotransferase em células fetais cultivadas e a pesquisa de mutações específicas na amostra de DNA fetal (ENCARNAÇÃO et al., 2009; POHL et al., 2010).

1.2.5 Tratamento

O único tratamento específico disponível para os pacientes com esta doença é o transplante de células-tronco hematopoiéticas (TCTH). Tais procedimentos foram realizados em um número relativamente pequeno de pacientes com ML II e sua eficácia não foi ainda comprovada. O maior estudo incluiu 22 pacientes (LUND et al., 2014), com taxa de sobrevida de 27% no último *follow-up*; os sobreviventes apresentavam atraso global de desenvolvimento e, na maioria das vezes, necessitavam gastrostomia e traqueostomia com ventilação mecânica. Mesmo assim, o TCTH permanece como uma alternativa para os pacientes com ML II diagnosticados precozmente. Não existem relatos de TCTH para ML III.

Um estudo *in vitro* com genisteína (inibidor da síntese de glicosaminoglicanos) demonstrou a redução do acúmulo de heparan sulfato em fibroblastos de pacientes com ML II (OTOMO et al., 2012), mas ensaios clínicos não foram realizados até o momento.

O êxito da terapia de reposição enzimática (TRE) em outras doenças lisossômicas como a doença de Gaucher, Mucopolissacaridoses I, II e VI bem como para a doença de Fabry sinalizam um caminho para o tratamento das ML II e III (BEUTLER; GRABOWSKI, 2001; CONNOCK et al., 2006; HARMATZ et al., 2006; MUENZER et al., 2006). Todavia, tendo em vista que a enzima

deficiente nas ML II e III é uma proteína hexamérica e insolúvel, a TRE como hoje se conhece, parece ser uma possibilidade bastante remota. O uso concomitante das enzimas recombinantes atualmente existentes (alfa-L-iduronidase, iduronato-sulfatase, arilsulfatase B e alfa-galactosidase A) ou de filtrados de células normais poderia ser um caminho para o tratamento, como já demonstraram Otomo e colaboradores (2011). Entretanto, deve-se levar em consideração o alto custo do tratamento, o possível desenvolvimento de anticorpos (BIGGER; SAIF; LINTHORST, 2015; KIM; MESSINGER; BURTON, 2015; LANGEREIS et al., 2015; SUN et al., 2014) e o fato destas enzimas não serem capazes de passar a barreira hemato-encefálica quando administradas em doses terapêuticas (BALDO; GIUGLIANI; MATTE, 2014; OTOMO et al., 2011; OU et al., 2014; VOGLER et al., 2005).

Assim, até o momento, a intervenção adotada é essencialmente sintomática e paliativa, baseada no atendimento global ao paciente e no aconselhamento genético (BRAULKE; RAAS-ROTHSCHILD; KORNFELD, 2013; VELLODI, 2005).

1.3 Proposta de tecnologias para tratamento

1.3.1 Ação de aminoglicosídeos e outras drogas sobre a tradução ribossômica

Mutações que introduzem códons de parada prematuros (PTCs) apresentam implicações em numerosas doenças hereditárias e vários tipos de cânceres (MORT et al., 2008; OMIM, 2014). Estima-se que mutações sem sentido representam 5 a 70% dos casos individuais de doenças genéticas e são responsáveis por ~11% de todas as mutações que causam doenças hereditárias conhecidas e presentes no banco de dados *Human Gene Mutation* (HGMD-HGMD, 2015; RYAN, 2014).

PTCs originam-se de mutações sem sentido, mutações que alteram a fase de leitura ou de processamento errôneo o qual gera isoformas de mRNA que podem vir a produzir uma proteína não funcional, truncada ou deletéria (KANDASAMY et al., 2011; LINDE; KEREM, 2008). Normalmente, estas mutações resultam na ativação de um processo de controle celular denominado *nonsense mediated RNA decay* (NMD) onde mRNAs que contêm PTC são

rapidamente degradados. Este mecanismo bloqueia a produção de proteínas truncadas que podem trazer efeitos negativos ao funcionamento celular (ALMEIDA et al., 2012; SILVA; ROMÃO, 2009). Estratégias que induzam a leitura através do PTC, também conhecido como *read through*, onde um aminoácido aleatório é incorporado no local, oferecem a possibilidade de reduzir a patologia decorrente deste tipo de mutação. Neste sentido, há pelo menos, dois cenários em que terapias que focam em PTCs podem ser efetivas: (i) doenças em que um nível muito inferior ao normal de atividade da proteína em questão pode ser terapêutico (ex. hemofilia e fibrose cística); e (ii) doenças em que a proteína terapêutica possua uma meia vida longa e então, acumule-se em níveis significativos via supressão das PTCs (ex. distrofia muscular de Duchene) (HAINRICHSON; NUDELMAN; BAASOV, 2008; KANDASAMY et al., 2011).

Aminoglicosídeos como gentamicina, amicacina e tobramicina têm importantes aplicações clínicas no tratamento de infecções causadas por bactérias Gram-negativas e, recentemente descoberto, no tratamento experimental de doenças recessivas com mutações sem sentido (HAINRICHSON; NUDELMAN; BAASOV, 2008; JANA; DEB, 2006; PELTZ et al., 2013; VECSLER et al., 2011). Essas moléculas ligam-se à porção 16S ou 18S do RNA ribossômico em procariotas e eucariotas, respectivamente, induzindo uma alteração conformacional (HALVEY; LIEBLER; SLEBOS, 2012; HU; GATTI, 2008). Em procariotos, a ligação de aminoglicosídeo é altamente específica e mediada pela adenina 1408 da porção 16S, inibindo a síntese proteica. No entanto, em eucariotos, a sequência de nucleotídeos correspondente é uma guanina e a ligação, menos eficiente, o que resulta em um *readthrough* traducional pela inserção de um aminoácido em lugar do PTC (KEELING; BEDWELL, 2002a; SÁNCHEZ-ALCUDIA et al., 2012).

Antibióticos aminoglicosídeos foram as primeiras pequenas moléculas que apresentaram promissores resultados *in vitro* e *in vivo* em doenças como fibrose cística (FC), distrofia muscular de Duchenne (DMD), síndrome de Hurler (MPS I), diabetes, entre outros (DIOP; CHAUVIN; JEAN-JEAN, 2007a; GOLDMANN et al., 2010a; NUDELMAN et al., 2009). Alguns ensaios com relevância clínica direta têm sido realizados em humanos com mutações PTC. Os resultados mais promissores vieram do uso tópico nasal de gentamicina em pacientes com FC. Após o tratamento, os indivíduos com mutações PTC

apresentaram melhora considerável em alguns parâmetros, incluindo a resposta ao isoproterenol, um medicamento simpaticomimético que atua ao nível dos receptores beta adrenérgicos melhorando o fluxo de ar. Além disso, a proteína CFTR (proteína deficiente em FC) de comprimento normal foi detectada nas células epiteliais nasais de dois indivíduos tratados (CLANCY et al., 2006; SERMET-GAUDELUS et al., 2007; WILSCHANSKI et al., 2003). Em pacientes com DMD causada por mutações sem sentido, evidências demonstram que a administração intravenosa de gentamicina suprimiu as mutações sem sentido, conforme determinado pela análise da distrofina de comprimento normal em biópsias musculares (POLITANO et al., 2003). Estes estudos demonstram a capacidade de pequenas moléculas como os aminoglicosídeos induzirem *read through* (Figura 4) restaurando parcialmente proteínas de tamanho normal (BURKE; MOGG, 1985; KANDASAMY et al., 2011; SÁNCHEZ-ALCUDIA et al., 2012).

O cloranfenicol foi o primeiro antibiótico de amplo espectro a ser utilizado clinicamente e por seus graves efeitos secundários (relacionados à toxicidade sobre a síntese proteica mitocondrial no baço e medula óssea) teve seu uso drasticamente limitado. Trabalhos clássicos sobre o modo de ação de cloranfenicol demonstraram inequivocamente que este inibe a formação de ligações peptídicas (CUNDLIFFE, 1981). Estudos posteriores sobre o alongamento peptídico permitiram concluir que a ação do cloranfenicol não ocorria através do impedimento da ligação dos aminoacil-RNAt, mas sim, impedindo o reconhecimento por parte da peptidil-transferase sobre o seu substrato aceptor, concluindo-se que a droga se liga diretamente ao centro catalítico da peptidil-transferase (o que posteriormente foi visto por cristalização de proteínas), inibindo a formação peptídica. No entanto, a possibilidade do cloranfenicol ter outros efeitos sobre a síntese proteica foi muito pouco estudada, havendo uma descrição publicada demonstrando a sua capacidade de produzir repressão fenotípica na mesma medida que estreptomicina em modelos bacterianos. Essa observação foi estendida e confirmada por Thompson e colaboradores que demonstraram que esses antibióticos quando interagem com a peptidil-transferase da região da subunidade 50S do ribossomo podem afetar os mecanismos de *frameshifting* e *readthrough* dos códons de parada na subunidade 30S (THOMPSON et al., 2002).

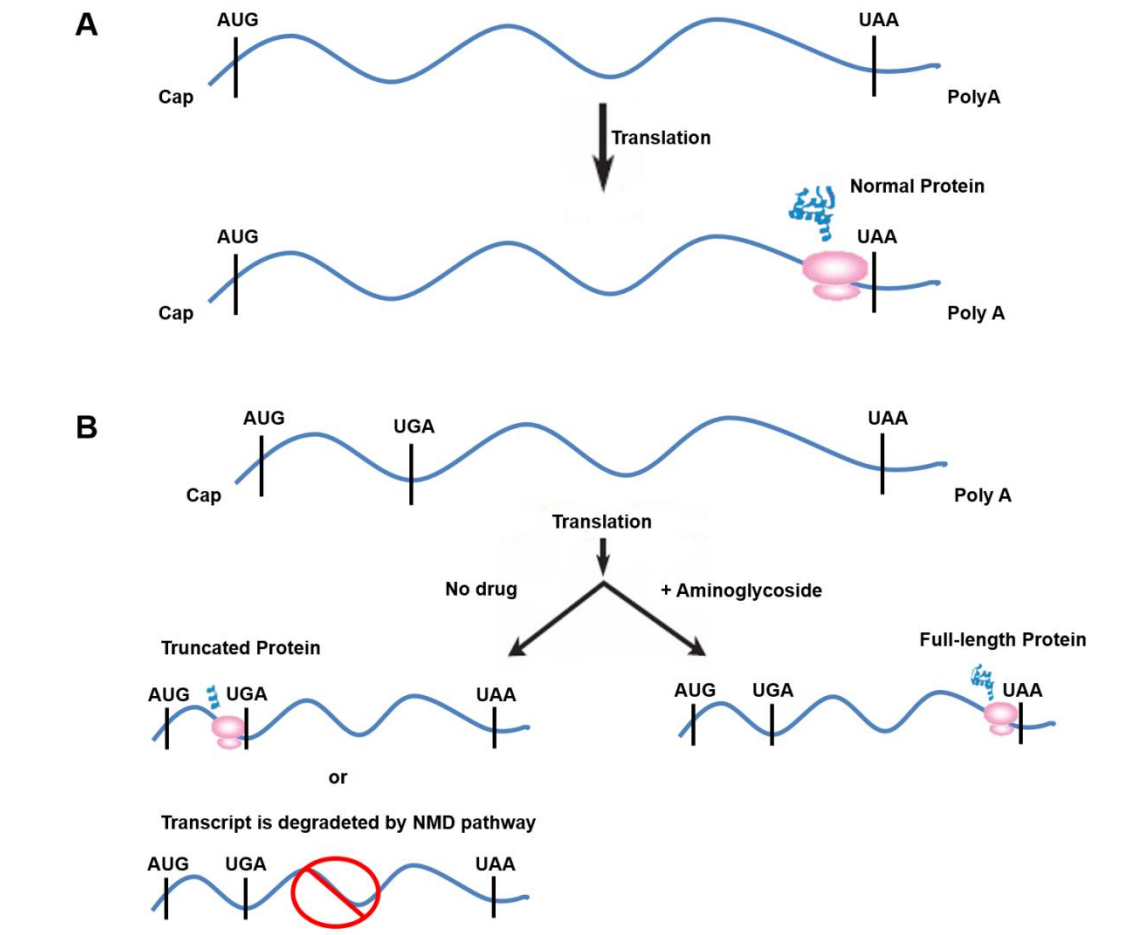

Figura 4. Representação esquemática da estratégia de regulação gênica. As posições relativas do CAP, sequência de poliadenilação, dos códons de iniciação e terminação nos mRNAs são mostrados. (A) Em células selvagens, mRNAs normais codificam proteínas funcionais, de tamanho normal. (B) Em células mutantes, os mRNAs que contêm PTC, são traduzidos na ausência de aminoglicosídeos gerando proteínas truncadas ou o transcrito é degradado via NMD. A presença do aminoglicosídeo permite a incorporação de um aminoácido aleatória em lugar do PTC (*read through*) do mRNA mutante o que resulta em uma proteína de comprimento normal.

A substância recentemente identificada, PTC124 ou ataluren, é um não aminoglicosídeo composto que induz *read through*, preservando o códon de terminação natural e oferecendo vantagens de não ter efeitos tóxicos evidentes além de ser disponível oralmente (PELTZ et al., 2013; RYAN, 2014; WELCH et al., 2007). Esta droga mostrou resultados promissores para a distrofia muscular de Duchenne, distrofinopatia, fibrose cística, síndrome de Hurler, deficiência de carnitina palmitoil 1A (CPT1A), com o potencial de tratar uma gama mais ampla

de doenças genéticas (FINKEL, 2010; PELTZ et al., 2013; ROWE et al., 2012; RYAN, 2014; SERMET-GAUDELUS et al., 2010).

Um conjunto de estudos clínicos foi realizado com ataluren em pacientes com FC e DMD. O estudo internacional fase IIb, randomizado, duplo-cego, controlado por placebo avaliou os efeitos da terapia com ataluren sobre a capacidade ambulatorial em 174 pacientes (≥5 anos de idade) com DMD, durante 48 semanas (FINKEL, 2010; FINKEL et al., 2013a; MCDONALD et al., 2013a, 2013b). Os pacientes foram estratificados com base em sua idade (<9 e ≥9 anos), uso de corticosteróides (sim ou não), distância percorrida em 6 minutos (DP6) (<350 ou ≥350 m) e randomizados 1: 1: 1 entre os grupos placebo, baixa ou elevada dose de ataluren sendo todos os tratamentos, administrados três vezes ao dia. Este estudo mostrou que ataluren nas doses de 10, 10, 20 mg/ kg foi mais eficaz do que o placebo aumentando significativamente o DP6 e apresentando tendências positivas para os testes de função muscular temporizada (caminhada/ corrida de 10 m, subir e descer escadas). Ataluren mostrou atividade e segurança neste estudo de curto prazo, apoiando sua posterior avaliação (10, 10, 20 mg/ kg e 20, 20, 40 mg/ kg) em estudos futuros semelhantes (FINKEL et al., 2013a; MCDONALD et al., 2013a, 2013b).

Em pacientes com FC ≥ 6 anos de idade, um estudo duplo-cego de fase III foi utilizado para avaliar a segurança e eficácia de ataluren em 48 semanas. Os pacientes foram estratificados por idade, uso crônico de antibióticos inalatórios e volume expiratório forçado em 1 s (FEV1). Na semana 48, a diferença média entre ataluren e placebo no parâmetro FEV1 foi de 3,0% e a taxa de exacerbação pulmonar foi 23% menor para ataluren (KEREM et al., 2014a; PELTZ et al., 2013). Estes resultados foram mais evidentes em pacientes que não utilizaram cronicamente antibióticos inalatórios tais como colistina, tobramicina e aztreonam. No entanto, analisando os efeitos desses antibióticos sobre FEV1 e as taxas de exacerbação pulmonar, verifica-se um efeito antagonista de tobramicina sobre ataluren, o que foi confirmado por ensaios *in vitro*. Os perfis de segurança foram semelhantes para ataluren e placebo (KEREM et al., 2014a; PELTZ et al., 2013; ROWE et al., 2012).

PTC124/ ataluren (Translarna™ - PTC Therapeutics, Inc.) é uma molécula pequena, de administração oral, não-tóxica que tem como alvo mutações sem sentido (FINKEL et al., 2013a; RYAN, 2014). Este composto é a primeira droga

desenvolvida especificamente para o tratamento de doenças causadas por mutações sem sentido. Além disso, oferece a possibilidade de sua validação como supressor de mutações sem sentido em um grande número de doenças genéticas. Translarna^{TM} obteve sua primeira aprovação condicional para uso em DMD no ano de 2014 (RYAN, 2014).

1.4 Processos biológicos

1.4.1 Nonsense-mediated mRNA Decay

Nonsense-mediated mRNA decay (NMD) ou decaimento de mRNA mediado por mutação sem sentido é um mecanismo responsável pelo reconhecimento e degradação de RNA mensageiro quando este carrega um códon de terminação prematuro (MÜHLEMANN et al., 2008). O principal objetivo do NMD é bloquear a produção de proteínas truncadas, o que acarreta efeitos negativos sobre a função celular (SILVA; ROMÃO, 2009). Evidências deste mecanismo encontram-se no fato de que em diversos organismos com RNA mensageiro contendo uma mutação sem sentido, a quantidade deste RNA é mais baixa do que o normal (ZHANG; MAQUAT, 1997).

O mecanismo desta rota está envolvido com a deposição de um complexo proteico a 20-24 nucleotídeos 5' antes de uma junção éxon-éxon, que ocorre durante o *splicing*. Durante a primeira tradução, o ribossomo exclui estes complexos, possibilitando a leitura de todo o RNA mensageiro. Quando há uma mutação de terminação prematura a 50-54 nucleotídeos 5' de uma destas junções, o ribossomo não exclui o complexo, o que ativa a rota NMD (INÁCIO et al., 2004). Além desta "regra" da proximidade com junções éxon-éxon, há trabalhos mostrando que quanto mais próximo o códon de terminação está do códon de iniciação AUG, há maior degradação de RNAs mensageiros que carregam a mutação (INÁCIO et al., 2004). Sendo assim, estes mecanismos mostram que mutações sem sentido em diferentes locais do gene podem gerar mRNAs com graus variados de estabilidade.

1.4.2 Edição de RNA

O termo edição de RNA descreve um fenômeno biológico pelo qual a sequência de uma molécula de RNA é alterada em relação à sequência de DNA correspondente. Existem vários mecanismos moleculares mecanicamente e evolutivamente relacionados e responsáveis pela edição de RNA. Pós- ou co-transcricionalmente, a diversidade destes mecanismos podem aumentar a diversidade proteômica incluindo inserções, deleções ou modificações de um ou mais nucleotídeos mas não processamentos como *splicing*, poliadenilação ou degradação de moléculas de RNA (BRENNICKE; MARCHFELDER; BINDER, 1999; FARAJOLLAHI; MAAS, 2010; RAMASWAMI et al., 2013a).

A edição de RNA pode desenvolver um importante papel na expressão. Ao alterar um nucleotídeo dentro da sequência codificadora (CDS), o mecanismo pode alterar um aminoácido ou até introduzir um códon de parada; em íntrons, pode afetar o *splicing* e gerar produtos alternativos; na edição de regiões não traduzidas (UTRs), pode levar a retenção deste RNA no núcleo e, consequentemente, sua baixa expressão; e, em microRNAs, a edição de RNA pode redirecionar a atuação desta molécula (FARAJOLLAHI; MAAS, 2010; MAAS; GOMMANS, 2009; WEDEKIND et al., 2003).

Desde a descoberta original em tripanossomas há quase 30 anos (BENNE et al., 1986), o processo de edição de RNA foi encontrado em todos os reinos, incluindo plantas, animais, fungos, protistas, bactérias e até vírus. Entretanto, as evidências da existência e funcionalidade deste processo em seres humanos é muito recente (FARAJOLLAHI; MAAS, 2010; XU; ZHANG, 2014). Um grande número das edições de RNA foram recentemente descobertas por meio da análise *in silico* de discrepâncias entre sequências genômicas e seus RNAs correspondentes. E, os dados até agora encontrados, estão espalhados por uma série de trabalhos de pesquisa complementares, com diferentes abordagens e objetivos. Com o objetivo de compilar dados cada vez mais numerosos, alguns grupos criaram bancos de dados de livre acesso, tais como: REDIdb – onde não há dados em relação à edição de RNA humano (PICARDI et al., 2007); DbRES – contém informações sobre apenas 34 casos de edição na espécie *Homo sapiens* (HE; DU; LI, 2007); RADAR – apresenta edições de RNA de adenina para iosina identificados em humanos, camundongos e moscas (RAMASWAMI; LI, 2014), e; DARNED - banco de dados

totalmente livre que reúne aproximadamente 42 mil edições de RNA de todos os tipos descritas somente em humanos (KIRAN; BARANOV, 2010). Ao analisar estes bancos de dados, encontramos 113 alterações em *GNPTAB* e 102 em *GNPTG,* mas nenhuma relacionada às ML II e III alfa/beta ou ML III gama (KIRAN; BARANOV, 2010).

2 JUSTIFICATIVA

Embora tenha evoluído significativamente nos últimos anos, o conhecimento acerca da estrutura e funções da enzima GlcNAc-1-fosfotransferase bem como das ML II e III, doenças causadas pela deficiência desta, ainda é muito restrito na literatura internacional. Poucos grupos pesquisam essa doença uma vez que ela é extremamente rara e, neste contexto, o grupo de pesquisa coordenado pela Dra. Ida Schwartz realiza pesquisas desde 2009.

Ao realizar a genotipagem do gene *GNPTAB* em pacientes brasileiros diagnosticados bioquimicamente com ML II e III (CURY et al., 2013), algumas questões surgiram. Dentre os pacientes com diagnóstico bioquímico porém sem variações em *GNPTAB*, questiona-se se estes pacientes teriam mutações em *GNPTG.* Em relação aos pacientes com mesmo genótipo, mas diferente fenótipo, seria possível que *GNPTG* agisse como um gene modificador? Qual a relação de expressão entre os genes? A terapia de *read through*, já testada para várias doenças monogênicas, mas jamais para uma onde dois genes, *GNPTAB* e *GNPTG,* que codificam uma mesma enzima- poderia funcionar? Tendo em vista que mutações frequentes e algumas novas foram identificadas em *GNPTAB* nos pacientes brasileiros*,* qual o efeito de cada uma destas sobre a GlcNAc-1-fosfotransferase? Estas análises poderão fornecer novas perspectivas sobre a fisiopatologia das ML II e III bem como em relação à compreensão da doença e da variabilidade nos fenótipos individuais.

As pesquisas realizadas pelo grupo da Dra. Ida Schwartz conta com o importante apoio dos laboratórios e serviços que compõem o HCPA. O laboratório do SGM-HCPA (LEIM-HCPA) é um serviço de referência internacional para o diagnóstico dos Erros Inatos do Metabolismo (EIM), além disso, está integrado com o Serviço de Genética Médica do Hospital de Clínicas de Porto Alegre (SGM-HCPA) e com o Departamento de Genética da Universidade Federal do Rio Grande do Sul (UFRGS), propiciando aos pacientes atendimento multidisciplinar por uma equipe experiente em EIM. O LEIM-HCPA, com auxílio da Rede MPS Brasil, concentra uma casuística significativa, se não a maior do país, em pacientes com diagnóstico de ML.

Embora raras e pouco conhecidas, as ML II e III impõem singular morbidade e mortalidade aos acometidos, geralmente crianças. Impõem

também, às suas famílias, regime de sofrimento permanente e, ao Estado, regime de gastos elevados com suporte paliativo. Neste sentido, a partir dos dados obtidos, espera-se também ser possível o delineamento de estratégias mais efetivas para o diagnóstico e o tratamento dos pacientes com ML II e III.

3 OBJETIVOS

Objetivos Gerais

I. Verificar a patogenicidade, por meio de ensaios *in vitro*, das seguintes mutações em *GNPTAB* previamente identificadas em pacientes brasileiros com ML II e III alfa/beta: c.1208T>C, c.1514G>A, c.1723G>A, c.1759C>T, c.1931_1932CA>TG, c.2269_2273delGAAAC, c.2808A>G e c.3668_3670delCTA;

II. Caracterizar o perfil de mutações de *GNPTG* presente em uma amostra de pacientes com ML III gama;

III. Analisar o efeito de gentamicina e cloranfenicol sobre a atividade das enzimas α-manosidase, β-glicuronidase e β-galactosidase em fibroblastos de pacientes com ML III gama heterozigotos ou homozigotos para mutações sem sentido em *GNPTG.*

Objetivos específicos:

i. Inserir as mutações c.1208T>C, c.1514G>A, c.1723G>A, c.1759C>T, c.1931_1932CA>TG, c.2269_2273delGAAAC, c.2808A>G e c.3668_3670delCTA em sequência selvagem do vetor de expressão GNPTAB-pcDNA6/V5-His através da técnica de mutagênese sítio dirigida;

ii. Expressar as sequências mutantes de *GNPTAB* obtidas em linhagem de células HEK e HeLa;

iii. Identificar os efeitos das mutações em nível de mRNA e proteína por expressão e análises de localização;

iv. Avaliar o impacto de cada mutação sobre a atividade da GlcNAc-1-fosfotransferase;

v. Avaliar a relação entre os genes *GNPTG* e *GNPTAB* em pacientes com ML II e III alfa/beta e gama;

vi. Identificar possíveis modulares do fenótipo clínico de pacientes com ML II e III.

vii. Quantificar a atividade de hidrolases lisossomais em fibroblastos de pacientes antes e após o tratamento com os antibióticos;

viii. Analisar os níveis de mRNA de *GNPTG* em fibroblastos de p
antes e após o tratamento com gentamicina e cloranfenicol.

4 RESULTADOS

O trabalho desenvolvido nesta tese resultou em sete artigos científicos, sendo: um de revisão já aceito para publicação (apresentado parcialmente na introdução, e em sua íntegra no item Resultados), dois já publicados, um submetido e, três em fase final de preparação e/ou revisão para ser submetido à publicação.

4.1 Artigo 1

Analyses of disease-related *GNPTAB* mutations define a novel GlcNAc-1-phosphotransferase interaction domain and an alternative site-1 protease cleavage site

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Original Article

ORIGINAL ARTICLE

Analyses of disease-related *GNPTAB* mutations define a novel GlcNAc-1-phosphotransferase interaction domain and an alternative site-1 protease cleavage site

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Abstract

Mucolipidosis II (MLII) and III alpha/beta are autosomal-recessive diseases of childhood caused by mutations in *GNPTAB* encoding the α/β-subunit precursor protein of the GlcNAc-1-phosphotransferase complex. This enzyme modifies lysosomal hydrolases with mannose 6-phosphate targeting signals. Upon arrival in the Golgi apparatus, the newly synthesized α/β-subunit precursor is catalytically activated by site-1 protease (S1P). Here we performed comprehensive expression studies of *GNPTAB* mutations, including two novel mutations T644M and T1223del, identified in Brazilian MLII/MLIII alpha/beta patients. We show that the frameshift E757K*fs*X1 and the non-sense R587X mutations result in the retention of enzymatically inactive truncated precursor proteins in the endoplasmic reticulum (ER) due to loss of cytosolic ER exit motifs consistent with a severe clinical phenotype in homozygosity. The luminal missense mutations, C505Y, G575R and T644M, partially impaired ER exit and proteolytic activation in accordance with less severe MLIII alpha/beta disease symptoms. Analogous to the previously characterized S399F mutant, we found that the missense mutation I403T led to retention in the ER and loss of catalytic activity. Substitution of further conserved residues in stealth domain 2 (I346 and W357) revealed similar biochemical properties and allowed us to define a putative binding site for accessory proteins required for ER exit of α/β-subunit precursors. Interestingly, the analysis of the Y937_M972del mutant revealed partial Golgi localization and formation of abnormal inactive β-subunits generated by S1P which correlate with a clinical MLII phenotype. Expression analyses of mutations identified in patients underline genotype–phenotype correlations in MLII/MLIII alpha/beta and provide novel insights into structural requirements of proper GlcNAc-1-phosphotransferase activity.

[†]The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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Introduction

Lysosomal enzymes catalyze the degradation of a variety of endogenous and exogenous macromolecules. They require mannose 6-phosphate (M6P) residues on their oligosaccharides for efficient targeting of lysosomes. The formation of M6P is initiated in the lumen of the *cis*-Golgi apparatus by the transfer of an *N*-acetylglucosamine (GlcNAc)-1-phosphate from UDP-GlcNAc to distinct mannose residues generating GlcNAc-1-phosphodiester (1–3). This reaction is catalyzed by a hexameric GlcNAc-1-phosphotransferase complex consisting of two α-, β- and γ-subunits (4). In a second step, the masking GlcNAc residues are removed in the *trans*-Golgi network by the uncovering enzyme that exposes terminal M6P modifications (5). The M6P residues allow the binding of newly synthesized lysosomal enzymes to M6P-specific receptors which mediate their segregation from the secretory pathway and delivery to the endosomal/lysosomal compartment (6).

The α- and β-subunits of GlcNAc-1-phosphotransferase complex are synthesized as a common α/β-subunit precursor protein encoded by the *GNPTAB* gene that is located on chromosome 12q23.3 (7,8). The *GNPTG* gene, located on chromosome 16p13.3, encodes the γ-subunit (9). Non-sense, frameshift and splice site mutations in *GNPTAB* cause the severe lysosomal storage disorder mucolipidosis type II (MLII; MIM #252500; also called I-cell disease) which is associated with a complete loss of GlcNAc-1-phosphotransferase activity (10). In contrast, missense mutations in *GNPTAB* showing residual GlcNAc-1-phosphotransferase activity result in a milder course of the disease (MLIII alpha/beta; MIM #252600) (10,11). The MLIII gamma form (MIM #252605) is caused by defects in the *GNPTG* gene (11). To date, more than 140 different *GNPTAB* mutations have been described.

Biochemically, the complete or partial loss of the GlcNAc-1-phosphotransferase activity leads to missorting and hypersecretion of multiple lysosomal enzymes. The subsequent intracellular deficiency of lysosomal enzymes results in the accumulation of non-degraded storage material in lysosomes (12). MLII patients suffer from severe psychomotor retardation and show coarse facial features, gingival hypertrophy, shortened neck, joint contractures, osteopenia, *dysostosis multiplex* and very short stature. Death occurs due to cardiopulmonary complications within the first decade of life (11,13). MLIII alpha/beta is presented by progressive joint stiffness, claw hands, carpal and tarsal tunnel syndrome, scoliosis and decreased mobility of knees and hip joints (11).

The α/β-subunit precursor is a type III membrane protein of 1256 amino acids and two transmembrane domains. Its exit from the endoplasmic reticulum (ER) requires a combinatorial sorting motif located in the N- and C-terminal cytoplasmic tails (14). Upon arrival in the *cis*-Golgi apparatus, the α/β-subunit precursor is proteolytically cleaved between amino acids K928 and D929 into the mature α- and β-subunits by the site-1 protease (S1P), which also plays an essential role in cholesterol homeostasis (15,16). The proteolytic cleavage of α/β-subunit precursor protein is a prerequisite for the catalytic activity of the GlcNAc-1-phosphotransferase and therefore plays an important role in the biogenesis of lysosomes.

The luminal α-subunit exhibits a conserved modular structure, and recently the DNA methyltransferase-associated protein (DMAP) domain has been described as the binding site for lysosomal enzymes (17). The α- and β-subunits mediate the catalytic function of GlcNAc-1-phosphotransferase (16), which appears to be modulated by the γ-subunits (18).

Here, we used the subcellular localization determined by double immunofluorescence microscopy, and the capability to be enzymatically activated by the Golgi-localized S1P to analyze the effect of eight selected disease-causing *GNPTAB* mutations found in Brazilian MLII and MLIII alpha/beta patients. We have presented evidence that in addition to the loss of combinatorial cytosolic targeting motifs (14), luminal missense mutations located in the 'stealth' region 2 of the α-subunit impair the transport of the α/β-subunit precursor to the Golgi apparatus. This region most likely represents a contact site for a yet unknown accessory transport protein. Finally, a stretch of amino acids in the N-terminus of the β-subunit is essential for precise S1P-mediated cleavage and activity of the GlcNAc-1-phosphotransferase.

Results

We examined the biological significance of eight selected disease-causing *GNPTAB* mutations found in MLII and MLIII alpha/beta patients in Brazil. Two of the mutations, T644M and T1223del, were novel and found in heterozygosity in the patient described in Materials and Methods (Supplementary Material, Table S1, patient #6). In addition, three missense mutations (I403T, C505Y and G575R), an in-frame deletion mutation of 36 amino acids (Y937_M972del), a non-sense mutation (R587X) and a frameshift mutation (E757K*fs*X1), which were identified previously (19,20) have been included in this study (Fig. 1). These mutations were introduced into the wild-type (WT) cDNA of human *GNPTAB*-myc constructs and transiently expressed in HEK-293, HeLa or S1P-deficient SRD-12B cells. The cells were analyzed for expression, subcellular localization of the GlcNAc-1-phosphotransferase and correlated to the activity.

Expression of mutant α/β-subunits of the GlcNAc-1-phosphotransferase

First of all, cell extracts were analyzed by western blot using a monoclonal antibody raised against the α-subunit (21) or

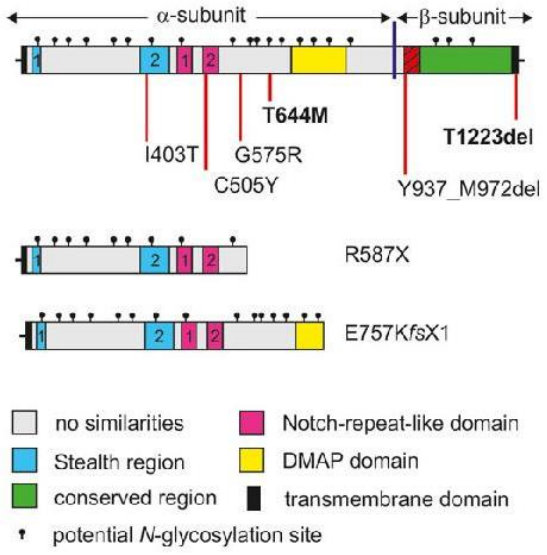

Figure 1. Schematic presentation of the domain organization of the full-length WT and mutant α/β-subunit precursor protein of the GlcNAc-1-phosphotransferase. The positions of the four missense and two deletion mutations are indicated in the upper scheme. The novel mutations identified in the MLIII alpha/beta patient are marked in bold. The C-terminally truncated α/β-subunit precursor mutants R587X and E757K*fs*X1 are shown below. The positions of potential N-glycosylation sites (filled circle) and the S1P cleavage site between amino acid K928 and D929 (in blue) are indicated.

myc-tag to detect the α- and β-subunits, respectively, as well as the α/β-subunit precursor proteins of human GlcNAc-1-phosphotransferase. In cells expressing the WT protein, a 190 kDa α/β-subunit precursor and 45 kDa β-subunits were detectable (Fig. 2A, lane 2, B, lane 1) which were not present in extracts of non-transfected control cells (Fig. 2A, lane 1). The reactivity of the monoclonal antibody with the mature α-subunit of a calculated molecular mass of 145 kDa was weak, most likely due to the high N-glycosylation, which could be improved by deglycosylation of the samples. After treatment of cell extracts with peptide-N-glycosidase F (PNGase F), hydrolyzing all N-linked oligosaccharides, the molecular masses of the α/β-subunit precursor and α- and β-subunits shifted to ∼170, 125 and 38 kDa (Fig. 2A, lane 3, B, lane 2), respectively. In extracts of cells overexpressing the missense mutant I403T, the α/β-subunit precursor protein but no cleaved α- and β-subunits were observed (Fig. 2A, lanes 4 and 5). The mutants C505Y, G575R (Fig. 2A, lanes 6–9) and T644M (Fig. 2B, lanes 3 and 4) were cleaved into mature α- and β-subunits. Densitometric analysis revealed, however, that only 20, 10 and 30% of the C505Y, G575R and T644M mutants were proteolytically processed in comparison to 80% of the WT α/β-subunit precursor. The non-sense R587X mutant led to the expression of a truncated 70 kDa glycosylated and 60 kDa non-glycosylated polypeptide which could be detected with the anti α-subunit antibody but not with the anti-myc antibody (Fig. 2A, lanes 10 and 11). The frameshift mutant E757K*fs*X1 was expressed as 120 kDa glycosylated and 80 kDa non-glycosylated polypeptide (Fig. 2B, lanes 5 and 6). In extracts from HEK-293 cells overexpressing the Y937_M972del mutant, the loss of 36 amino acid residues was hardly detectable by changes in the electrophoretic mobility of the α/β-subunit precursor (Fig. 2B, lanes 7 and 8). However, we unexpectedly observed no β-subunits with increased electrophoretic mobility due to the deletion but with approximately the same mass as the WT β-subunit (Fig. 2B, lanes 7 and 8). In contrast, we observed in other experiments even a lower migrating β-subunit (discussed below). The mutant T1223del α/β-subunit precursor was cleaved into mature α- and β-subunits (Fig. 2B, lanes 9 and 10).

Densitometric analysis of western blots from five independent experiments revealed that the expression levels of the deletion mutants were comparable to the WT polypeptide, whereas the amounts of all other mutants were reduced by 50–75% (Fig. 3A). To determine whether variations of mutant α/β-subunit precursor protein result from differences in transcript levels, the mRNA levels of overexpressed WT and mutant *GNPTAB* constructs were analyzed by real-time polymerase chain reaction (PCR). The transcript levels of the four missense and two deletion mutants were comparable to WT (Fig. 3B), whereas the amounts of R587X and E757K*fs*X1 mRNA were decreased by 40%, suggesting that these mutant mRNAs are unstable (Fig. 3B).

Subcellular localization of mutant GlcNAc-1-phosphotransferase

Proteolytic S1P-mediated activation of the α/β-subunit precursor of GlcNAc-1-phosphotransferase into mature subunits occurs in the *cis*-Golgi apparatus (8,21). To examine the subcellular localization of mutant GlcNAc-1-phosphotransferase subunits, HeLa cells overexpressing WT or mutant α/β-subunit precursor polypeptides were analyzed by immunofluorescence microscopy using anti α-subunit antibodies. Cells were co-stained for the ER marker protein disulfide isomerase (PDI) or the *cis*-Golgi marker protein GM130. WT α/β-subunits were found to co-localize with GM130 but not with PDI (Fig. 4). In accordance with the lack of processing of the mutant α/β-subunit precursor I403T (Fig. 2A), it completely co-localized with PDI (Fig. 4). The majority of the

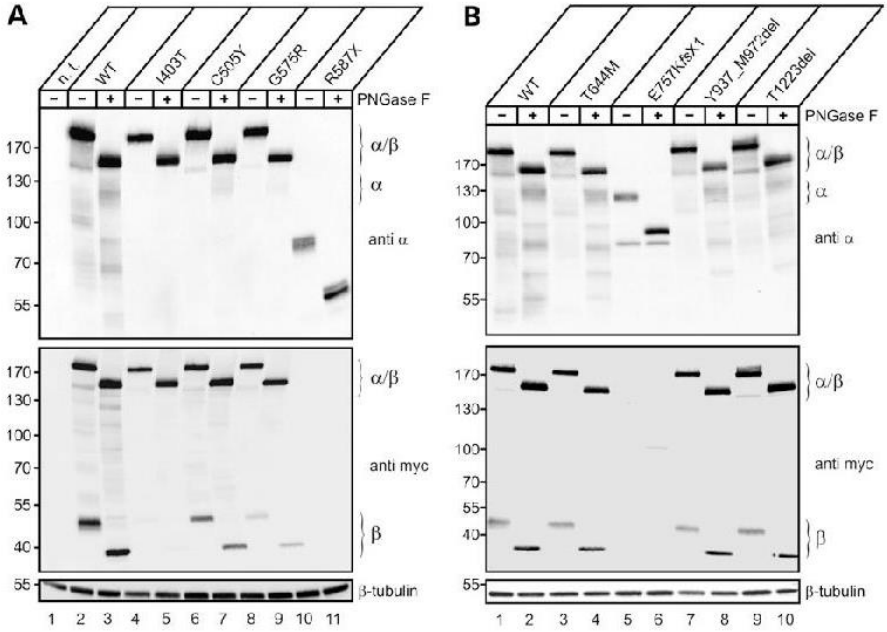

Figure 2. Expression and proteolytic cleavage of WT and mutant α/β-subunit precursor constructs of GlcNAc-1-phosphotransferase. (**A**, **B**) HEK-293 cells were transfected with cDNA encoding WT or the indicated mutant α/β-subunit precursor constructs. Twenty-four hours after transfection, cell extracts were incubated for 1 h in the presence (+) or absence (–) of PNGase F followed by SDS–PAGE (10% acrylamide) under reducing conditions and western blot analysis detecting α/β-subunit precursor and α-subunits (anti α) or α/β-subunit precursors and β-subunits (anti-myc). β-Tubulin and extracts of non-transfected cells were used as loading and negative control, respectively. The positions of molecular mass marker proteins (in kDa), α/β-subunit precursor, α- and β-subunits are indicated.

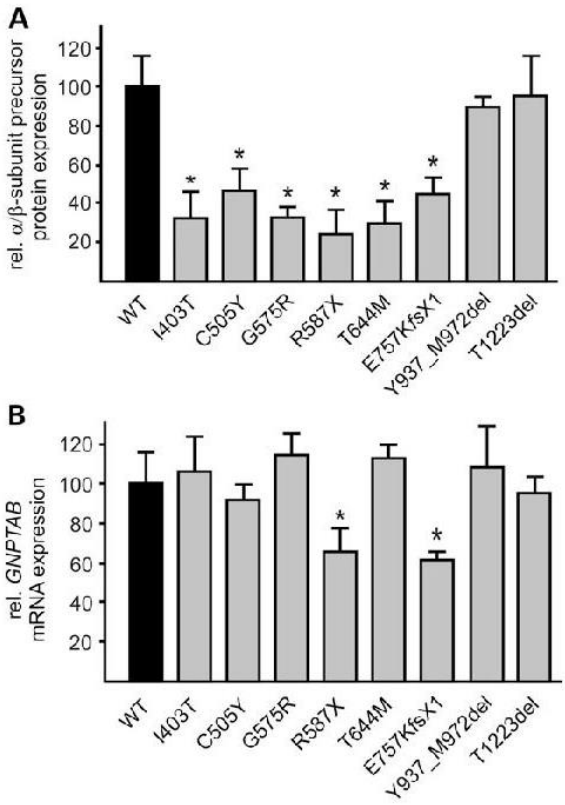

Figure 3. Relative expression of mutant α/β-subunit precursor GlcNAc-1-phosphotransferase. (**A**) Densitometric evaluation of western blots analyzing the mutated α/β-subunit precursor protein expression from five independent transfections were performed and normalized to β-tubulin level. The expression of the WT α/β-subunit precursor was set as 100%. (**B**) The relative mRNA levels of overexpressed WT and mutant *GNPTAB* were determined by real-time PCR and normalized to *ACTB* mRNA expression (mean ± SEM, $n = 5$, $^*P < 0.05$). The mRNA expression of WT α/β-subunit precursor corrected by the endogenous *GNPTAB* in HEK-293 cells was assigned as 100%.

C505Y, G575R and R587X mutants co-localize with PDI and only a small amount of the G575R mutant reached GM130-positive Golgi structures (Fig. 4). As expected by the presence of cleaved α- and β-subunits (Fig. 2B), the mutant T644M was correctly targeted to the Golgi apparatus and showed co-localization with GM130 but not with PDI (Fig. 4). The frameshift mutant E757KfsX1 and the deletion mutant Y937_M972del completely co-localized with PDI. Of note, when the mutant Y937_M972del has been expressed in HEK-293 cells, partial co-staining with the Golgi marker protein GM130 was observed (Supplementary Material, Fig. S2). The deletion mutant T1223del was correctly transported to the *cis*-Golgi apparatus and showed co-localization with GM130 (Fig. 4).

Enzymatic activity of mutant GlcNAc-1-phosphotransferase

The type of mutation in the *GNPTAB* gene and the position of the mutation in the α/β-subunit precursor protein affect the GlcNAc-1-phosphotransferase activity and determine the clinical phenotype of the patients (11,22). To examine genotype–phenotype correlations in this cohort of Brazilian MLII and MLIII alpha/beta patients, the GlcNAc-1-phosphotransferase activity was measured using α-methylmannoside (α-MM) as a phosphate acceptor. The expression of the WT α/β-subunits precursor of GlcNAc-1-phosphotransferase has led to a 13-fold increase in GlcNAc-1-phosphotransferase activity compared with non-transfected cells. Less than 2% of WT GlcNAc-1-phosphotransferase activity was measured in cells expressing R587X and E757K*f*sX1 mutants (Fig. 5) which are retained in the ER (Fig. 4). The activities of mutant GlcNAc-1-phosphotransferase I403 T, C505Y and G575R were also significantly reduced to 4–6% of WT. The deletion of 36 amino acids in the β-subunit (Y937_M972del) led to a complete ER localization in HeLa cells (Fig. 4), and a partial localization in the Golgi apparatus in HEK-293 cells (Supplementary Material, Fig. S2) associated with an irregularly cleaved α/β-subunit precursor protein showed total loss of GlcNAc-1-phosphotransferase activity (Fig. 5). The mutants T644M and T1223del that were correctly transported and proteolytically cleaved into mature α- and β-subunits exhibited 50 and 85% of GlcNAc-1-phosphotransferase activity, respectively, of the WT enzyme. When the GlcNAc-1-phosphotransferase activities of mutated forms were related to the amounts of β-subunits determined by densitometric evaluation of western blots (Supplementary Material, Fig. S3), a similar activity pattern was observed, indicating that C505Y, G575R, T644M and T1223del show residual activity of 7, 3, 50 and 85%, respectively, whereas Y937_M972del (as well as all mutants retained in the ER) led to the total loss of GlcNAc-1-phosphotransferase activity. These data are consistent with the milder and less progressive MLIII alpha/beta than MLII phenotype of the patients carrying the T644M and T1223del mutations.

Defining a novel luminal ER export domain in the α-subunit

In addition to mutant I403T, another close-by mutation, S399F, has been described that is also located in the highly conserved stealth domain 2 of the α-subunit (Supplementary Material, Fig. S4) and prevent the exit from the ER and the subsequent S1P-mediated cleavage in the Golgi apparatus (21). To examine whether other residues in this domain play a similar role for ER exit, six conserved amino acid residues located proximal or distal to S399/I403 were selected randomly (I346, W357, L380, E389, D408, and Y421) and substituted by alanine (Supplementary Material, Fig. S4). The expression analysis revealed that all mutants are stable (Fig. 6). However, the mutant α/β-subunit precursor proteins W357A and I346A were not or only partially cleaved, respectively, by S1P (Fig. 6, lanes 3 and 4). All other tested mutants were cleaved by S1P and could exit the ER. The data suggest that at least amino acid residues at positions I346, W357, S399 and I403 in the stealth domain 2 play an important role for the export of the α/β-subunit precursor from the ER. Most likely, these residues form an interaction site for protein(s) promoting the α/β-subunit precursor trafficking to the Golgi apparatus.

S1P-mediated abnormal cleavage of the Y937_M972del mutant GlcNAc-1-phosphotransferase

To analyze whether the irregular cleavage of the Y937_M972del mutant α/β-subunit precursor was catalyzed by S1P, the mutant construct was expressed in S1P-deficient SRD-12B CHO cells. The loss of S1P resulted in a reduction of the endogenous GlcNAc-1-phosphotransferase activity by 75% of the parental control CHO cells (Fig. 7A). Residual activity of GlcNAc-1-phosphotransferase is caused by the non-homogeneous composition of the SRD-12B cells due to incomplete amphotericin B selection (23). Re-expression of S1P in SRD-12B cells rescued the cleavage of

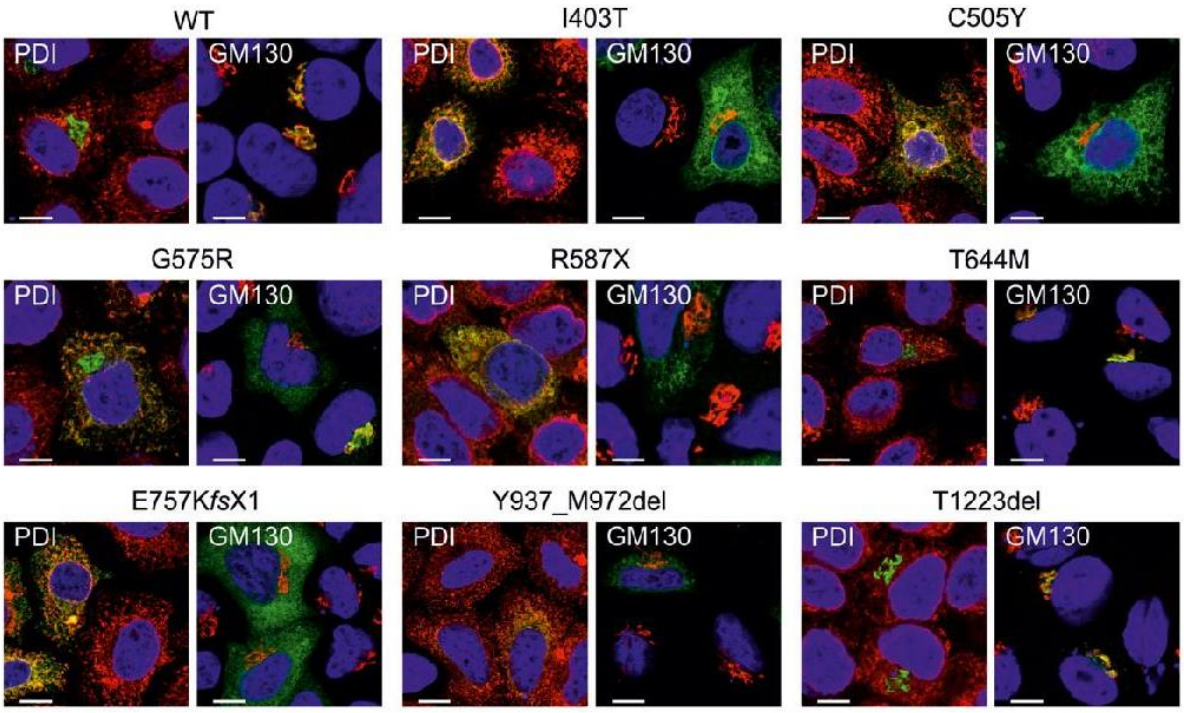

Figure 4. Intracellular localization of WT and mutant α/β-subunit precursor constructs of GlcNAc-1-phosphotransferase. HeLa cells were transfected with cDNA encoding WT or mutant α/β-subunit precursor constructs. Prior fixation, cells were treated with 100 μg/ml cycloheximide for 40 min to block further protein translation and allow protein export from the ER, followed by staining with monoclonal antibodies against the α-subunit (green), the *cis*-Golgi marker protein GM130 (red) or the ER marker protein PDI (red). Nuclei were visualized by DAPI staining (blue). Magnified views of merged images (indicated by rectangles in Supplementary Material; Fig. S1) are shown. Yellow indicates colocalization. Scale bar: 5 μm.

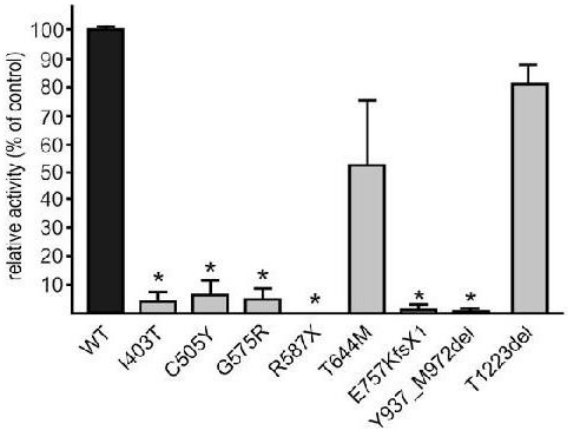

Figure 5. GlcNAc-1-phosphotransferase activity of WT and mutant α/β-subunit precursor constructs. The activity towards α-MM corresponding to 100 μg protein extracts of HEK-293 cells overexpressing full-length WT or mutant α/β-subunit constructs was measured for 60 min. The activity of overexpressed WT (9034 ± 1566 pmol/mg/h corrected by the endogenous GlcNAc-1-phosphotransferase activity) was set to 100%. Data are average values of three independent experiments, and error bars represent SEM. *$P < 0.005$.

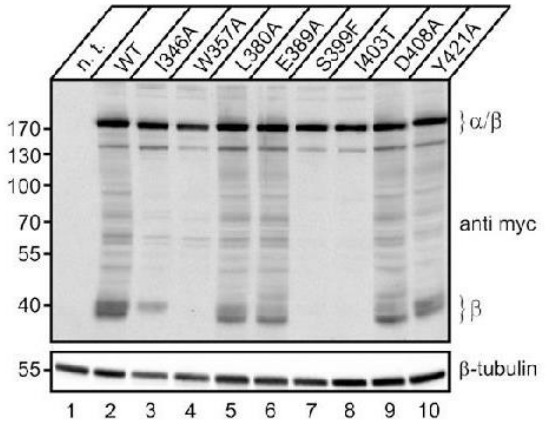

Figure 6. Proteolytic cleavage of luminal α/β-subunit precursor mutants of GlcNAc-1-phosphotransferase. HEK-293 cells were transfected with cDNA encoding WT or the indicated mutant α/β-subunit precursor constructs. Twenty-four hours after transfection, cell extracts were analyzed by SDS–PAGE (10% acrylamide) under reducing conditions and western blot detecting α/β-subunit precursor and β-subunit (anti-myc). β-Tubulin and extracts of non-transfected cells were used as loading and negative control, respectively. The positions of molecular mass marker proteins (in kDa), α/β-subunit precursor and β-subunits are indicated.

the α/β-subunit precursor (15) and the activity of endogenous GlcNAc-1-phosphotransferase activity to 75% of control CHO cells (Fig. 7A and B). Western blot analysis revealed that the non-glycosylated Y937_M972del mutant α/β-subunit precursor exhibits a slightly faster mobility than the WT protein in extracts of both SRD-12B cells and HEK-293 cells used as positive control (Fig. 8). Surprisingly, a reduction rather than an increased

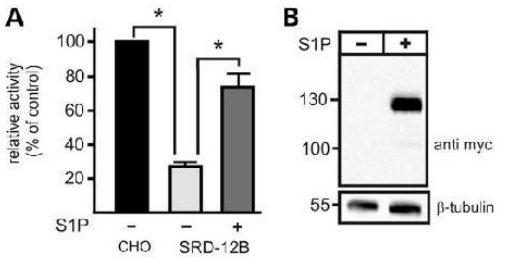

Figure 7. Endogenous GlcNAc-1-phosphotransferase activity in S1P-deficient cells. (**A**) The activity towards α-MM corresponding to 100 µg protein extracts of SRD-12B and CHO cells was measured for 60 min. The activity in CHO cells was set to 100%. Overexpression of myc-tagged human S1P rescued GlcNAc-1-phosphotransferase activity. Data are average values of three independent experiments, and error bars represent SEM. *$P < 0.05$. (**B**) SRD-12B cells were transfected with cDNA encoding human myc-tagged S1P. Twenty-four hours after transfection, cell extracts were analyzed by SDS–PAGE (10% acrylamide) under reducing conditions and western blot against the myc-tag of S1P. β-Tubulin western blot was used as a loading control. Extracts of non-transfected SRD-12B cells were used as negative control. The positions of molecular mass marker proteins (in kDa) are indicated.

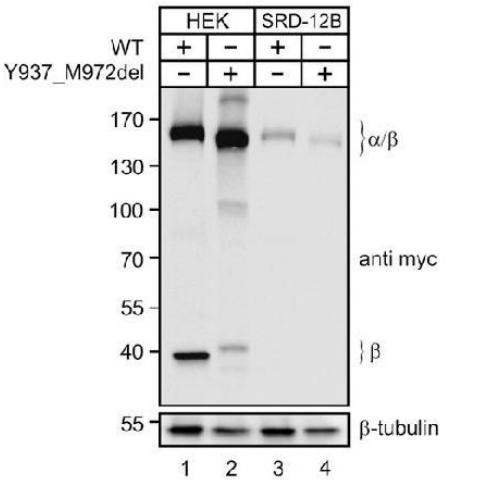

Figure 8. Proteolytic cleavage of Y937_M972del mutant α/β-subunit precursor. HEK-293 and SRD-12B cells were transfected with cDNA encoding WT or mutant α/β-subunit precursor constructs. Twenty-four hours after transfection, cell extracts were prepared and incubated for 1 h in the presence of PNGase F, followed by SDS–PAGE (8–16% acrylamide gradient) under reducing conditions and western blot analysis of the β-subunit (anti-myc). β-Tubulin western blot was used as a loading control. The positions of molecular mass marker proteins (in kDa) are indicated.

electrophoretic mobility of the β-subunits was observed, suggesting the generation of a new cleavage site located in the α-subunit. In contrast to HEK-293 cells (Fig. 8, lanes 1 and 2), no cleavage of the Y937_M972del mutant could be observed in SRD-12B cells (Fig. 8, lanes 3 and 4). The data demonstrate that the abnormal cleavage of the Y937_M972del mutant α/β-subunit precursor is mediated by S1P, but fails to activate the GlcNAc-1-phosphotransferase.

Discussion

The comprehensive expression analyses of mutations identified in the *GNPTAB* gene of Brazilian patients allow predictions on the severity and clinical course of the diseases, MLII and MLIII alpha/beta, and provide novel insights into the complex domain structure of α/β-subunits of GlcNAc-1-phosphotransferase and their role in substrate binding and catalytic activity. Several molecular defects result in the total loss of GlcNAc-1-phosphotransferase activity and severe MLII, such as mutations affecting (i) the catalytic center, (ii) UDP-GlcNAc or lysosomal enzyme binding sites, (iii) the transport of the α/β-subunit precursors from the ER to the Golgi apparatus and (iv) the capability of the α/β-subunit precursors to be proteolytically cleaved by S1P in the *cis*-Golgi cisternae. At present, 33 of the 144 known *GNPTAB* mutations have been studied by expression analysis in HeLa or HEK-293 cells (14,17,21,24,25).

Here we show that the mutations leading to a frameshift and the synthesis of C-terminally truncated α/β-subunit precursor forms, R587X, E757*Kfs*X1 and L1168*Qfs*X5, cause the complete loss of GlcNAc-1-phosphotransferase activity and a severe MLII phenotype in homozygosity (patient #1, Supplementary Material, Table S1) or in compound heterozygosity with another deleterious mutation (patient #2). The loss of ER exit signals in the cytoplasmic domain of the β-subunit (14) in these truncated α/β-subunit precursors is responsible for the retention in the ER and the subsequent inability for S1P-mediated proteolytic activation. In contrast, both mutations T644M and T1223del identified in the patient #6 altered the amino acid sequence in the region between the Notch-repeat-like domain 2 and DMAP domain and the second transmembrane domain (Fig. 1), respectively, partially impaired Golgi localization, proteolytic activation and GlcNAc-1-phosphotransferase activity. These data are consistent with the milder course of the disease and justify the classification as MLIII alpha/beta (Supplementary Material, Table S1). Of note, the substitution of T644 by methionine changes a potential N-glycosylation in the α-subunit. At present, it is unclear whether N642 is glycosylated *in vivo* and the loss of this N-glycosylation site affects the function of the GlcNAc-1-phosphotransferase complex.

The 31-year-old MLIII alpha/beta patient #5 was found to be compound heterozygous for the non-sense and missense mutations R587X and C505Y, respectively. The premature termination after R586 in the α-subunit explains both the ER localization and the absence of GlcNAc-1-phosphotransferase activity. The substitution, however, of cysteine 505 by tyrosine in the Notch-repeat-like domain 2 (Fig. 1) impaired the ER-Golgi transport and proteolytic activation which is consistent with the residual GlcNAc-1-phosphotransferase activity (Supplementary Material, Table S1). During the preparation of the manuscript, Kornfeld and co-workers (25) reported the analysis of two missense mutations affecting cysteine residues in the Notch-repeat-like domain 2 of the α-subunit. Our data are in agreement with their finding on the C505Y mutation. Although comparative activity measurements in cysteine mutants of the Notch-repeat-like domain 1 (C442Y, C461G and C468S) suggested that this domain is involved in the recognition of lysosomal enzyme binding (25), it is unclear whether the Notch-repeat-like domain 2 harboring C505 residue also has a role in lysosomal enzyme binding.

We previously reported that the mutation S399F located in the stealth domain 2 (Fig. 1) is retained in the ER and not cleaved to mature α- and β-subunits (21). Interestingly, the Brazilian patient #3 carries the heterozygous mutation I403T that behaves in an analogous manner as S399F (21) (Figs 2 and 4) lacking catalytic activity as shown by Kornfeld and co-workers (25). When we examined the substitutions of other randomly selected residues by alanine, I346A and W357A were found to be not or partially cleaved by S1P (Fig. 6). Two other mutations in the stealth domain

2 have been reported, R334Q and R334L (10,26), which are localized in the ER and therefore lack cleavage and catalytic activity (25). Thus, it is likely that at least the amino acid residues R334, I346, W357, S399 and I403 in stealth domain 2 form a putative surface structure capable of interacting with a yet unknown protein in the lumen of the ER required for proper sorting to the ER exit sites, cargo incorporation into the nascent vesicles and/or transport to the Golgi apparatus. Several proteins functioning as subunits or luminal chaperones in the trafficking of transporter proteins or transcription factors from the ER to other organelles have been described (27–30). The identification of potential accessory proteins which bind to signal structures of the stealth domain 2 in the α-subunit is currently under investigation.

Of particular interest was the Y937_M972del mutant which was partially transported to the Golgi apparatus and abnormally cleaved (Fig. 8). The regular cleavage site of the α/β-subunit precursor between K928 and D929 is located in the consensus recognition motif of S1P (R/K)X(hydrophobic)Z↓, where X represents any amino acid and Z preferentially L or T. For cleavage, at least 20 amino acid residues proximal to the cleavage site are required to allow the interaction of S1P with the α/β-subunit precursor protein (15). Obviously, an intact amino acid sequence covering more than nine residues distal to the cleavage site is also necessary for effective S1P-mediated cleavage. Although no further S1P consensus sequences in the α-subunit are known, the experiments in S1P-deficient SRD-12B cells have demonstrated that S1P is the responsible protease for the generation of abnormal cleavage of the α-subunit preventing the proper activation step. The identification of the abnormal cleavage site remains to be investigated.

The data presented here demonstrate the importance of comprehensive expression analyses of missense and deletion mutations in the *GNPTAB* gene in the context with a thorough clinical evaluation and help to define genotype–phenotype correlations in order to improve the predictions of the clinical course of MLII and III alpha/beta. In addition, the expression analysis represents an important approach to gain new insights into the composition and complexity of the modular domain structure of the α- and β-subunits, as well as in the function of the GlcNAc-1-phosphotransferase and the homeostasis of lysosomes.

Materials and Methods

Reagents

Penicillin/streptomycin (P/S), α-methylmannoside (α-MM), adenosine 5′-triphosphate (ATP) disodium salt, uridine 5′-diphosphate *N*-acetylglucosamine (UDP-GlcNAc) sodium salt, bovine serum albumin (BSA), 4′,6′-diamidino-2-phenylindol (DAPI), protease inhibitor cocktail, cholesterol, sodium mevalonate, sodium oleate, amphotericin B and other common laboratory reagents were obtained from Sigma. UDP-[^{3}H]GlcNAc was purchased from American Radiolabeled Chemicals. QAE™ Sephadex A-25 was from GE Healthcare. Fetal bovine serum (FBS) was from PAA Laboratories. Easy-DNA gDNA Purification Kit, Dulbecco's modified Eagle's medium (DMEM), F12 Ham and GlutaMAX™ were from Life Technologies. Transfection reagent JetPEI® was purchased from Peqlab. Phusion® polymerase and GeneJET Plasmid Miniprep Kit, GeneJET PCR Purification Kit, dNTPs and prestained protein ladder Page-ruler™ were from Thermo Scientific. Oligonucleotides used for sequencing and mutagenesis were synthesized by MWG Biotech. PNGase F was from Roche Applied Sciences. Mowiol® and Roti®quant Protein Assay were from Roth. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gradient gels (8–16%) from NuSep were used.

Antibodies

The monoclonal rat antibody against the human α-subunit of the GlcNAc-1-phosphotransferase was described recently (21). Monoclonal antibodies against myc-tag, PDI and GM130 were purchased from Cell Signalling, Biomol and BD Biosciences, respectively. The monoclonal antibody against β-tubulin was obtained from the Developmental Studies Hybridoma Bank. Secondary antibodies conjugated to HRP, anti-mouse Alexa Fluor® 546 and anti-rat Alexa Fluor® 488 were purchased from Dianova and Life Technologies, respectively.

Clinical manifestation, biochemical and genetic diagnosis of an MLIII alpha/beta patient

The male patient (#6, Supplementary Material, Table S1) was the only son of non-consanguineous parents of Brazilian origin. The full-term pregnancy was carried with no problems occurring. Birth weight (3610 g), length (50 cm) and head circumference were normal. He first presented at 1 year and 6 months of age with stiffness of small hand and finger joints. At the age of 2 years, he had a generalized bone dysplasia with thoracic deformities, coarse facies, depressed nasal bridge, scoliosis, *pectus carinatum* and claw hands, as well as gingival hypertrophy and language delay. There is no history of recurrent infections. Neither myopenia nor hearing problems were found. The activities of α-L-iduronidase, iduronate 2-sulfatase and β-glucoronidase in leukocytes were normal excluding MPSI, II and VII. Strongly elevated activities in plasma and reduced activities of several lysosomal enzymes in cultured fibroblasts of the patient led to the diagnosis of MLII or MLIII. Genomic sequencing of *GNPTAB* and *GNPTG* revealed no alterations in *GNPTG*, whereas the patient was found to be heterozygous for the novel mutations c.1931_1932CA>TG (T644M) and c.3668_3670delCTA (T1223del) and therefore diagnosed as MLIII alpha/beta. The patient was re-evaluated at 9 years. At that time, he attended regular school and had no cognitive impairment. At physical examination, he presented with 97 cm of height (<3 percentile) and 17 kg of weight (<3 percentile), thickening of metopic suture, claw hands joint contractures of shoulders, elbows, hands hip and knees, and radiological evidence of *dysostosis multiplex*. There were no corneal clouding or organomegaly, but the boy had diathesis of the *rectus abdominis*, leading to abdominal protuberance. Ophthalmological examination was normal. There was mitral insufficiency on echocardiogram.

GNPTAB/GNPTG mutational analysis

Genomic DNA from the patient (#6, Supplementary Material, Table S1) and his parents were extracted from peripheral blood leukocytes with the Easy-DNA gDNA Purification Kit, and the 21 or 11 exons that comprise the *GNPTAB* or *GNPTG* gene, respectively, were amplified as described previously (19,31). Sample sequencing was performed using the automatic ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems). The sequences of the *GNPTG* and *GNPTAB* genes used as reference was GenBank accession numbers NG_016985.1 and NM_024312.4, respectively.

Generation of mutant *GNPTAB* cDNA constructs

The pathogenic *GNPTAB* mutations c.1208T>C (I403T), c.1514G>A (C505Y), c.1723G>A (G575R), c.1759C>T (R587X), c.1931_1932CA>TG (T644M), c.2269_2273delGAAAC (E757KfsX1), c.3668_3670delCTA (T1223del) (Fig. 1) as well as structural *GNPTAB* mutations (I346A, W357A, L380A, E389A, D408A and Y421A) were inserted into the

C-terminally myc-tagged full-length WT *GNPTAB* construct (14) by site-directed mutagenesis using mutagenic primers and Phusion® polymerase. Mutagenic primers were designed using the web-based program PrimerX (www.bioinformatics.org/primerx). The mutation c.2808A>G was not associated with an amino acid change but effect the splicing. This mutation creates a similar sequence to the canonical donor splice site, and consequently cDNA analysis of the patient RNA revealed the presence of an abnormal transcript lacking the last 108 bp of exon 14 resulting in an in-frame deletion of 36 amino acids of the β-subunit (Y937_M972del) (19). To introduce this mutation into the *GNPTAB* construct, 48 bp primers were used. The generation of mutant *GNPTAB* construct S399F was described recently (21). All nucleotide primers used for cloning and site-directed mutagenesis are listed in Supplementary Material, Table S2. The DNA plasmids were commercially sequenced to confirm proper introduction of the mutations (SeqLab). Mutation nomenclature follows the HSVG rules and is based on GenBank accession number NM_024312.4, with nucleotides numbered using 1 as the A of the ATG starting codon.

Cell culture and transfections

HEK-293 and HeLa cells were maintained in DMEM supplemented with 10% FBS, GlutaMAX™ and P/S at 37°C and 5% CO_2. SRD-12B cells and their parental CHO control cells were cultured in a 1:1 mixture of DMEM and nutrient mixture F12 Ham with 5% FCS and P/S. SRD-12B cells were supplemented with 5 μg/ml cholesterol, 50 μM sodium mevalonate and 20 μM sodium oleate. Weekly selection with amphotericin B of SRD-12B cells was performed as described elsewhere (23).

Cells grown on 6-cm plates or on glass cover slips were transiently transfected with cDNAs coding for human WT or mutant α/β-subunit precursor-myc fusion proteins using JetPEI® reagent according to the manufacturer's instructions. All constructs were transfected in parallel under identical conditions, and cell extracts were analyzed by western blot, immunofluorescence microscopy, mRNA expression and GlcNAc-1-phosphotransferase activity 24 h after transfection.

GlcNAc-1-phosphotransferase activity assays

HEK-293 cells as well as SRD-12B and their parental CHO control cells were lysed in PBS containing 1% Triton X-100 and protease inhibitor cocktail for 15 min on ice. After centrifugation at 10 000*g*, supernatants were used for measurement of the protein content by the Roti®quant Protein Assay. Aliquots of cell extracts (100 μg protein) were adjusted to 50 mM Tris–HCl (pH 7.4) containing 10 mM $MgCl_2$, 10 mM $MnCl_2$, 2 mg/ml BSA, 2 mM ATP, 75 μM UDP-GlcNAc and incubated with 1 μCi UDP-[^{3}H]GlcNAc and 100 mM α-MM at 37°C for 1 h (24). After incubation, 1 ml of 2 mM Tris–HCl (pH 8.0) was added to the samples and applied to QAE sephadex A-25 column (1 ml of resin equilibrated with 2 mM Tris–HCl, pH 8.0) and the columns were washed twice with 2 ml and once with 1 ml of 2 mM Tris–HCl (pH 8.0). The bound [^{3}H] GlcNAc-P reaction product was eluted twice with 2 ml and once with 1 ml of 30 mM NaCl in 2 mM Tris–HCl (pH 8.0). Each elution was collected and measured in 5 vol. of scintillation liquid. The values obtained from non-transfected cell lysates were subtracted from the values obtained from cell lysates transfected with WT or the mutants to correct for endogenous enzyme activity and non-specific background counts. Depending on the mutation, the GlcNAc-1-phosphotransferase activity was related to the steady-state expression of the β-subunit determined by densitometric analysis of western blots using Imager ChemiDoc XRS (Biorad) and Image Lab software.

Other methods

Isolation of total RNA, cDNA synthesis, and real-time PCR was performed as described previously (32). Confocal microscopy of transfected cells, preparation of cell extracts, enzymatic deglycosylation of proteins, and SDS–PAGE followed by western blot analysis were performed as described recently (21).

Statistical analysis

Results are expressed as mean ± SEM. Differences between mean values are determined using Student's *t*-test. *P*-values <0.05 were considered significant.

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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4.2 Artigo 2

A *de novo* or germline mutation in a family with Mucolipidosis III gamma : Implications for molecular diagnosis and genetic counseling

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Short Communication

A *de novo* or germline mutation in a family with Mucolipidosis III gamma: Implications for molecular diagnosis and genetic counseling

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ABSTRACT

Mucolipidosis III (ML III) gamma is a very rare autosomal-recessive disorder characterized by the abnormal trafficking and subcellular localization of lysosomal enzymes due to mutations in the *GNPTG* gene. The present study consists of a report of a Brazilian compound heterozygote patient with ML III gamma resulting from one mutant paternal allele and one allele that had most likely undergone a *de novo* or maternal germline mutation. This is the first report of a *de novo* mutation in ML III gamma. This finding has significant implications for genetic counseling.

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1. Introduction

Mucolipidosis II (ML II disease, inclusion cell disease or I-cell disease) and III (ML III, pseudo-Hurler polydystrophy) are autosomal recessive disorders caused by defects in the GlcNAc-1-phosphotransferase (EC 2.7.8.17) complex, which is composed by three subunits: α, β, and γ. Mutations in the gene encoding the α- and β-subunits (*GNPTAB*) lead to ML II alpha/beta (OMIM #252500), or to the less clinically severe

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condition, ML III alpha/beta (OMIM #252600). ML III gamma (OMIM #252605) is caused by mutations in the gene encoding the γ-subunit of GlcNAc-1-phosphotransferase [1,2], and is thought to be the mildest form of the disease. Very few cases of ML III gamma are reported in the literature, maybe because the disease is underdiagnosed due to its relatively mild and unspecific clinical findings, which is suggested by a recent report of ML III gamma patients diagnosed through next generation sequencing [3]. To date, approximately 28 mutations have been reported in the *GNPTG* gene. A large number of these mutations are unique or rare [4].

De novo mutations are not rare events and the perception they are potentially important in genetic diseases, even in autosomal recessive conditions, have major implications for genetic counseling [4,5]. The present study consists of a report of a Brazilian compound heterozygote patient with ML III gamma resulting from the inheritance of one mutant paternal allele and one maternal allele that had most likely undergone a *de novo* or germline mutation.

2. Materials and methods

2.1. Case report

The proband, a female born at term to young (maternal and paternal age at conception was 27 years old) and non-consanguineous parents, has been described previously [6]. She was referred for clinical genetic evaluation due to large joint contractures. On physical examination, the patient presented contractures and restrictions of movement, especially in the hands, feet and shoulders, and heart systolic murmur, audible mainly at the left sternal border. The two-dimensional color Doppler echocardiography revealed the presence of mild thickening of aortic valve leaflets with mild regurgitation. All other clinical parameters were within normal limits for the patient's age. The exam was performed through the subcostal window, as thoracic deformity prevented the use of standard echocardiographic measures of pulmonary artery systolic pressure. Electroneuromyography of the upper limb was normal, and showed no electrophysiological evidence of peripheral neuropathy. The somatosensory evoked potential of the upper and lower limbs was also normal. The patient was diagnosed as having ML III when she was 8 years old (Table 1), and is currently stable. At the time of the study, she was 16 years old and attended regular school. Previous *GNPTAB* sequencing showed no alterations.

2.2. GNPTG analysis and maternity testing

Genomic DNA was extracted from leukocytes (patient and both parents), buccal cells (patient and mother) and fibroblasts (patient) after informed consent was given.

GNPTG was amplified in five fragments containing exons 1 to 2, 3, 4 to 7, 8 to 9 and 10 to 11 as described by Persichetti et al. [7] with modifications. The fragment which comprises exons 4 to 7, where the mutation c.244_247dupGAGT is located, was also amplified using a second pair of primers [8]. Samples were submitted to DNA sequencing, performed on an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA). All samples were amplified and sequenced twice. The sequence of the *GNPTG* gene used as a template was GenBank accession no. NG_016985.1.

Table 1
Biochemical characterization of a patient with Mucolipidosis III gamma.

Enzymes	Sample	Patient	References values
Arylsulfatase A (EC 3.1.6.8)	Plasma	+	Negative
α-L-Iduronidase (EC 3.2.1.76)	Plasma	176	32–52 nmol/h/ml
β-Glucuronidase (EC 3.2.1.31)	Plasma	475	30–300 nmol/h/ml
α-Mannosidase (EC 3.2.1.24)	Plasma	1,548	17–56 nmol/h/ml
Iduronate-sulfatase (EC 3.1.6.12)	Plasma	1894	122–463 nmol/h/ml
Total β-hexosaminidases (EC 3.2.1.52)	Plasma	12,675	1000–2857 nmol/h/ml
α-Mannosidase (EC 3.2.1.24)	Fibroblasts	16	60–400 nmol/h/mg
β-Galactosidase (EC 3.2.1.23)	Fibroblasts	132	394–1440 nmol/h/mg
β-Glucuronidase (EC 3.2.1.31)	Fibroblasts	8.6	62–361 nmol/h/mg
α-Fucosidase (EC 3.2.1.51)	Fibroblasts	2.1	46–221 nmol/h/mg

Total RNA extraction was performed on a whole blood sample of the patient and her relatives, as well as three controls using the Paxgene blood RNA Kit (Qiagen, Germany), and conversion to cDNA was made using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instructions. The *GNPTG* mRNA levels were determined by qRT-PCR using 2× SYBR Green PCR Master Mix (Applied Biosystem) with the Mx3000P (Stratagene, Amsterdam, NL). *GAPDH* was chosen as housekeeping gene. Primers and conditions were performed as described by Ho et al. [9] with modifications. The relative quantification of the RNA was normalized to the level of *GAPDH* mRNA in the same cDNA using the comparative CT method ($2^{-\Delta\Delta CT}$).

For maternity testing, DNA from each sample was analyzed by PCR multiplex reaction using the AmpFlSTR® Identifiler® PCR Amplification kit (Applied Biosystems®), which enables the analysis of the 15 STR markers (STRs) using fluorescent primers, according to the manufacturer's instructions. PCR products were then resolved by capillary electrophoresis in an ABI3130xl genetic analyzer (Applied Biosystems®) using GeneScan™ 500 LIZ® as an internal marker and alleles were identified through GeneMapper® Software® v1.2 (Applied Biosystems®).

2.3. Statistical analysis

Values obtained for the relative quantification of *GNPTG* mRNA in patient, parents and control samples were compared using Student's test (IBM SPSS Statistics version 20). P values lower than 0.05 were considered statistically significant.

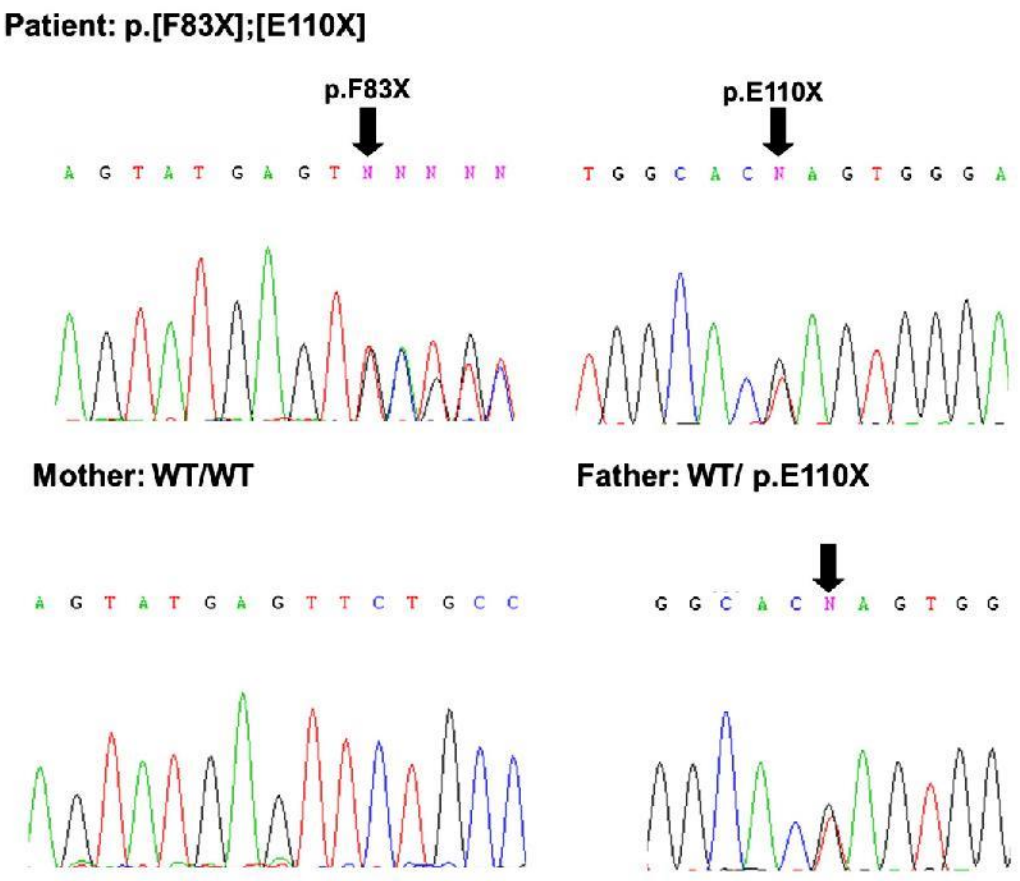

Fig. 1. *GNPTG* gene sequencing of the patient, her mother and father (leukocytes). WT = wild type.

3. Results

The genotype of the proband was c.[244_247dupGAGT];[−112C > G; 328G > T] (or p.[F83X];[E110X]), as determined by leukocyte DNA sequencing. To confirm this result, parents were also analyzed. Paternal allele sequencing identified a heterozygous p.E110X mutation, which had been previously found by our group in two other unrelated Brazilian ML III patients always *in cis* with the −112C > G mutation; sequencing of 100 control alleles did not show the presence of both mutations (data not shown). Surprisingly, no mutation was detected in maternal leukocytes (Fig. 1), even using different pairs of primers. Based on these findings, buccal cells were collected from both individuals, and fibroblasts were obtained from the patient in order to investigate the possibility of mosaicism for the c.244_247dupGAGT mutation in the patient and her mother.

Quantitative RT-PCR revealed that the amount of *GNPTG* mRNA found in blood samples of the patient, her father and her mother was approximately 1.6%, 54% and 88.5% of the levels found in controls (Fig. 2).

The probability of maternity was determined as 99.9999% by assessment of 15 different DNA markers in the patient and her parents.

4. Discussion

On the basis of the present results, we propose that the new mutation c.244_247dupGAGT is attributable to a *de novo* event which occurred in only one ovum, or to germline mosaicism in the mothers' ova. Although there is the possibility of mosaicism in the patient, this is an unlikely alternative, since more than one tissue was analyzed in the present study. Unfortunately, the possibility of germline mutation or germline mosaicism could only be confirmed in this case through ovarian biopsy.

We also consider very unlikely the possibility of preferential amplification of the normal allele (e.g., allele dropout of the mutant allele) in the mother due to the presence, *in cis*, of any genetic variation in the annealing region of one of the primers used to amplify exons 4 to 7 of *GNPTAB*, since we use two different pairs of primers to amplify this fragment, both showing the same results. Besides that, RNA studies showed a decreased *GNPTG* mRNA in patient and her father, but not in her mother, supporting the hypothesis of a *de novo* or germline mutation.

On average, 74 *de novo* single-nucleotide variants (SNVs) and three novel indels are believed to occur in an individual's genome per generation. The rates at which these phenomena occur are strongly influenced by factors such as parental sex and age, and DNA sequences located next to the mutation [5,9,10]. In the present case, maternal and paternal age at conception was not advanced, but just upstream to c.244_247dupGAGT mutation, an indel (TMP_ESP_16_1411876 DELETION) has already been described [11], a finding which suggests this region of the *GNPTG* is prone to mutations (e.g., a hot spot site).

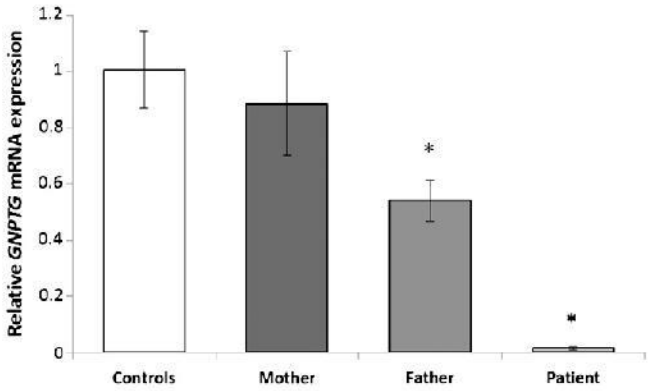

Fig. 2. Quantitative mRNA studies. The relative mRNA level of *GNPTG* was determined in a blood sample of the patient and her relatives, as well three controls, by real-time PCR and normalized to *GAPDH* mRNA expression. The final values are the mean values of three real-time PCRs made from two RNA preparations for each individual, and expressed as the fold change ± SD. The values fond in father and patient were found to differ from those found in controls. *$p < 0.001$ Student's test.

As expected, *de novo* germline mutations have been described more frequently in dominant disorders such as achondroplasia, Apert syndrome and multiple endocrine neoplasia [12] than in recessive disorders. Studies of autosomal recessive disorders have also detected *de novo* germline mutations in one patient with Ataxia-telangiectasia and in two patients with Gaucher disease [13]. From a genetic counseling perspective, the recurrence risk for an autosomal recessive disorder changes from 25% – if both parents are carriers of a pathogenic mutation – to a negligible value if only one parent is a carrier and there is no germline mosaicism in the other parent. There are few studies estimating the rates of germline mosaicism in recessive disorders, but for X-linked recessive disorders such as Duchenne muscular dystrophy and hemophilia A, maternal germline mosaicism is believed to occur in up to 5% [14] and 11% of mothers [15], respectively.

This is the first report of a *de novo* mutation in ML III gamma, and suggests that this kind of event probably occurs more often than currently recognized in recessive disorders. The present findings have major implications for genetic counseling, and strongly recommend that the carrier status of non-consanguineous parents of a child with-recessive disorders should always be confirmed through DNA analysis.

Conflict of interest

The authors declare no conflict of interest.

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4.3 Artigo 3

Enigmatic *in vivo* GlcNAc-1-phosphotransferase (*GNPTG*) transcript correction to wild-type in two Mucolipidosis III gamma siblings homozygous for nonsense mutations

Artigo a ser submetido ao periódico *Journal of Inherited Metabolic Diseases.*

Enigmatic *in vivo* GlcNAc-1-phosphotransferase (*GNPTG*) transcript correction to wild-type in two Mucolipidosis III gamma siblings homozygous for nonsense mutations

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Abstract

Mucolipidosis (ML) III gamma is a rare autosomal-recessive disorder caused by pathogenic mutations in the *GNPTG* gene. *GNPTG* encodes the γ-subunit of GlcNAc-1-phosphotransferase, which catalyzes mannose 6-phosphate targeting signal synthesis on soluble lysosomal enzymes. Biochemically MLIII gamma patients are characterized by missorting of lysosomal enzymes. In this report, we describe the occurrence of mRNA editing in two MLIII gamma patients. *Case Reports:* Patients A and B (siblings) presented at the adult age with a typical clinical picture of ML III gamma, mainly compromising bone and joints, high levels of lysosomal enzymes in plasma and lower levels in fibroblasts. Both were found to be homozygous for c.-112C>G and c.328G>T mutations in gDNA analysis of *GNPTG*. Analysis of cDNA, however, showed normal genotypes for both patients. Low *GNPTG* mRNA expression was observed in both patients. *Conclusions*: mRNA editing can explain the differences found in patients A and B regarding gDNA and cDNA analysis, and the mild clinical phenotype associated with homogozity for a nonsense mutation. Our results suggest that mRNA editing can be more frequent than expected in monogenic disorders and that *GNPTG* analysis should be performed on gDNA.

Keywords: *GNPTG*; lysosomal storage disorder; GlcNAc-1-phosphotransferase; Mucolipidosis II and III; mRNA editing

Introduction

Lysosomes, first described by De Duve and collaborators in 1955, are acidic organelles into which many types of macromolecules, including proteins, carbohydrates, nucleic acids and lipids are delivered for degradation. The targeting of most lysosomal enzymes depends on mannose 6-phosphate (M6P) residues that are recognized by M6P-specific receptors mediating their transport to lysosomes (Braulke et al 2013; Braulke and Bonifacino 2009). The key role in the formation of these residues is played by the Golgi-resident GlcNAc-1-phosphotransferase (EC 2.7.8.17) complex, which is composed of three different subunits encoded by *GNPTAB* and *GNPTG* (Braulke et al 2013). *GNPTAB* (MIM# 607840) located on chromosome 12q23.3, encodes the α- and β-subunits, while the γ-subunit is encoded by the gene *GNPTG* (MIM# 607838), located on chromosome 16p13.3 (Raas-Rothschild, 2000; Tiede et al 2005). *GNPTG* has 11 exons and spans 11.13 kb.

Pathogenic mutations in *GNPTG* are associated with Mucolipidosis (ML) type III gamma formerly known as pseudo-Hurler polydystrophy (MIM# 252600), a very rare autosomal-recessive disorder characterized by the abnormal trafficking and subcellular localization of lysosomal enzymes. There is not a frequent type of mutation in *GNPTG* gene, and missense/nonsense/splice site mutations have already been described (HGMD 2015 - Figure 1). There are few reports about this disease, and all of the studies include a small number of patients (Raas-Rothschild et al 2000; Raas-Rothschild et al 2004; Tiede et al 2004; Tiede et al 2005; Encarnação et al 2009; Persichetti et al 2009; Pohl et al 2009; Zarghooni and Dittakavi 2009; Gao et al 2011; Liu et al 2014; Velho et al 2014). The clinical manifestations include claw hand deformity, joint contractures, short stature, and

scoliosis. Mild coarsening of the face, astigmatism, mild retinopathy, and cardiac valve involvement have also been reported. Moderate to severe dysostosis multiplex and Legg-Calvé-Perthes disease maybe evident upon radiological examination (Raas-Rothschild et al 2004; Persichetti et al 2009).

The need for prognostic information and genetic counseling has fuelled the drive to establish genotype-phenotype associations in ML III gamma. However, close associations between mutant genotypes and clinical phenotypes have invariably been elusive, suggesting that other factors may be responsible for the clinical heterogeneity found in genetic diseases (Lualdi et al 2010).

Pre-mRNA processing and post-transcriptional modification can, together with mRNA decay, exert a profound influence on both the efficiency and fidelity of human gene expression (Buratowski 2008; Preker et al 2008; Peters and Steward 2003; Trapnell et al 2013). In addition, it has become increasingly clear that mRNA editing is a frequent phenomenon and may make a significant contribution to human transcriptome diversity (Li et al 2009; Trapnell et al 2013). Since the original discovery of mRNA editing in trypanosomes nearly 30 years ago (Benne et al 1986), mRNA editing has been found across all kingdoms of life, including plants, animals, fungi, protists, bacteria and viruses (Farajollahi and Maas 2010). The aim of this study is to report the occurrence of mRNA editing in ML III gamma disease.

Material and Methods

This study was approved by the institutional review board of Hospital de Clínicas de Porto Alegre (HCPA), Brazil. Two adult patients, who were siblings and presented with clinical and biochemical diagnoses of ML III, were enrolled.

Clinical evaluation

Clinical data were retrieved from medical files. Functional capacity was prospectively evaluated through the Functional Independence Measure (FIM) tool, always by the same examiner. The FIM is validated for the Brazilian population (Mancini and Mello 2007; Riberto et al 2004) and evaluates functional capacity for self-care, sphincter control, mobility, communication, and social integration. It consists of two sub-scores (mobility ranging from 13 to 91 points, and cognition ranging from 5 to 35 points). The total score ranges from 18 (worse) to 126 (better) points. Patient functionality is classified as follows: a) complete dependence (total assistance) = 18 points; b) modified dependence (assistance in up to 50% of tasks) = 19-60 points; c) modified dependence (assistance in up to 25% of tasks) = 61-103 points; d) complete or modified independence = 104-126 points.

Goniometry was used to evaluate the following joints for all possible passive movements: shoulder, elbow, wrist, hip, knee, and ankle (Gajdosik & Bohannon 1987), always by the same examiner.

GNPTG and GNPTAB analysis

Genomic DNA was extracted using Easy-DNA gDNA Purification kit (Life Technologies, USA) from peripheral blood leukocytes of affected individuals and their parents. *GNPTG* and *GNPTAB* genes amplification was performed as

described by Persichetti et al (2009) and Cury et al (2013), respectively. Total RNA was extracted from patient's cultured fibroblasts and blood samples using an RNeasy Mini Kit (Qiagen, Germany). RT-PCR amplification was performed using specific primers to amplify the c.328G>T allele (forward 5'-AGTATGAGTTCTGCCCGTTCC and reverse 5'-CAGGGTTGGGTACACTAGCAA). Samples were submitted to automated DNA sequencing performed on an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, USA). The sequences of the *GNPTG* and *GNPTAB* genes used as references were, respectively, GenBank accession no. NG_016985.1 and NM_024312.3.

The presence of each mutation was always confirmed in two independent experimental assays and also in the patients' relatives. In addition, the c.-112C>G mutation was investigated in 200 controls alleles from random samples of blood bank donors by DNA sequencing.

Testing the authenticity of the GNPTG alleles and transcripts

To exclude the possibility of artifactual nucleotide misincorporation during reverse transcription, all experiments were repeated twice using two different reverse transcriptases, a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) and SuperScript III (Life Technologies, USA). To minimize the risk of eventual artifactual nucleotide misincorporation by DNA polymerase *in vitro* during PCR amplification, PCR was performed with two different polymerases, DreamTaq DNA Polymerase (Thermo Scientific, USA) and Phusion High-Fidelity DNA Polymerase (Thermo Scientific, USA).

Quantitative real time PCR

The *GNPTAB* and *GNPTG* mRNA levels were determined by quantitative real time PCR using 2x SYBR Green PCR Master Mix (Applied Biosystem, USA) with Mx3000P (Stratagene, NL). *GAPDH* was chosen as a housekeeping gene. Primers and reaction conditions were determined based on Ho et al (2007). The relative quantification of RNA was normalized to the level of *GAPDH* mRNA in the same cDNA using the comparative CT method ($2^{-\Delta\Delta CT}$).

Statistical analysis

Values obtained for the relative quantification of *GNPTAB* and *GNPTG* mRNA in patients and controls samples were compared using Student's test (IBM SPSS Statistics version 20). P values lower than 0.05 were considered statistically significant.

Results

Clinical and biochemical evaluation

A summary of the clinical, functional and mobility characteristics of the patients are presented in Table 1.

Patient A, male, is the third sibling from non-consanguineous parents. First symptoms started at age of 10 years (claw hands). He developed disabling hip pain due to Legg-Calvé-Perthes disease, and prostheses were inserted bilaterally at 34 and 36 years of age. An umbilical hernia was repaired at the age of 37, and severe carpal tunnel syndrome was corrected by wrist surgery at the age of 40. At the last visit, he was 42 years old and currently working on the family farm, having no complaints or difficulties in his daily tasks. He did not finish high school

but does not appear to have cognitive impairment. A physical exam showed mild coarse facial features, systolic murmur, no organomegaly, claw hands and severe joint contractures of the shoulders, elbows and knees. In addition to those symptoms, he had some difficulty in moving the right leg due to an external rotation of the knee. On echocardiography he had supravalvular aortic stenosis, minimal physiological mitral regurgitation, mild tricuspid regurgitation, systolic pulmonary artery pressure at 29 mmHg, without other alterations. The neurological exam was normal. A polysomnography was performed and showed mild apnea hypopnea index (11.5).

Patient B, male, second sibling from non-consanguineous parents, was diagnosed at the age of 38 years, after the ML III diagnosis of his brother (Patient A). Claw hands and shoulder contractures were noted since adolescence. He developed disabling hip pain due to Legg-Calvé-Perthes disease, and prostheses were inserted bilaterally at 38 and 40 years of age, respectively. At his last visit, he was 44 years old and currently working on the family farm, without complaints or difficulties in his tasks. Similar to his brother, he did not finish high school but does not appear to have cognitive impairment. A physical exam showed coarse facial features (even milder than Patient A) and, mild joint restrictions in the hands, elbow, shoulder and knees. The neurological exam was normal. The echocardiogram and polysomnography were normal.

The biochemical diagnosis of patient A and B were performed at the age of 34 and 40 years, respectively, by measuring the activities of lysosomal enzymes in plasma and in cultured fibroblasts. Both patients showed a marked increase of plasma lysosomal enzyme activities. In cultured fibroblasts of patient A and B the

lysosomal enzyme activities were decreased in comparison to control fibroblasts (Table 2).

GNPTG and GNPTAB mutations analysis

Because the clinical and biochemical data suggested that the patients are affected by MLIII, genomic sequencing of *GNPTG* and *GNPTAB* were carried out. The *GNPTG* mutational profile was characterized by two mutations in homozygosis: c.328G>T (p.Glu110X) and c.-112C>G (Table 3). Both mutations have already been reported by our group in another ML III gamma patient, not related to patients A and B (Velho et al. 2014). No pathogenic mutations were detected on the *GNPTAB* gene.

The nonsense mutation c.328G>T occurs in exon 6. The corresponding translation product contains a premature stop codon (p.Glu110X) resulting in a predicted truncated protein of 109 amino acids. The mutation c.-112C>G, located at the 5'UTR, was also not found in 200 controls allele's samples.

To assess the potential effects of the *GNPTG* gene mutations at the mRNA level, RT-PCR and cDNA sequence analyses were performed. However, in both patients the normal wild type *GNPTG* cDNA sequence was evident with regard to position c.328G>T. Possible artifactual sample contamination was excluded because (i) the samples were analyzed separately, (ii) the primers were designed to specifically amplify the c.328G>T allele, (iii) experimental reproducibility following re-sampling and re-analysis of the patients, and, (iv) no evidence of contamination was ever detected in negative controls.

To exclude the issue of potential artifactual misincorporation, several further independent experiments were performed employing various different RT-PCR

amplification systems to prove the authenticity of the *GNPTG* alleles and transcripts. Control experiments revealed no evidence of artifactual nucleotide misincorporation *in vitro* during either PCR or reverse transcription. Finally, BLAST analysis revealed no sequence with homology to the *GNPTG* gene fragment containing exons 4 to 7 in the human genome.

GNPTG and GNPTAB expression in ML III gamma patients

The expression of *GNPTG* mRNA in the fibroblasts of the patient A and B were reduced by 99 % in comparison with the mRNA levels in fibroblasts of a healthy individuals (Figure 2) suggesting instability of the mutant *GNPTG* mRNA due to nonsense-mediated mRNA decay. In both patients an increase of 3 and 15-fold (patient A and B, respectively) in *GNPTAB* levels compared with the median control values were observed.

Discussion

In this study, we analyzed the entire coding regions of *GNPTG* and *GNPTAB* genes in two ML III gamma patients. Both patients exhibited a mild clinical phenotype and a normal neurological evaluation, which is supported by the findings of FIM. Patients A and B presented FIM total scores compatible with complete or modified independence. When we analyzed cognitive and mobility FIM sub-scores, the patients achieved the maximum score in the FIM cognitive and close to the maximum scores in the FIM mobility. All of these data support previous observations that mutations in the *GNPTG* gene are predictive of a milder ML III phenotype and a better prognosis than *GNPTAB* gene mutations (Braulke et al 2013), probably due to the function of each GlcNAc-1-

phosphotransferase subunit. The α- and β-subunits harbor the binding sites for the substrate UDP-GlcNAc, recognize a common protein determinant of lysosomal enzymes and contain the catalytic activity (Braulke et al 2008; Qian et al 2010). The function of the γ-subunit remains to be elucidated, but it has been speculated that it might be involved in the stabilization the α- and β-subunits conformation that is able to bind lysosomal enzymes efficiently (Lee et al 2007; Pohl et al 2009). However, due to the nonsense-mediated mRNA decay (NMD), we did not expect milder phenotypes in patients who are homozygous for nonsense mutations at genes involved in autosomal recessive diseases

The difference between the results of gDNA (identification of c.328G>T) and cDNA sequencing (no identification of c.328G>T) can be explained by mRNA editing (c.328G@T). This editing could also potentially contribute for the milder phenotype presented by both patients. Recently, a large number of mRNA editing instances in humans have been identified using bioinformatic screens and high-throughput experimental investigations utilizing next-generation sequencing technologies (Brennicke et al 1999; Farajollahi and Maas 2010; Knoop 2011; Peng et al 2012; Ramaswami and Li 2014; Ramaswami et al 2013). U-to-G mRNA editing, which would be required to account for the appearance of wild-type *GNPTG* mRNA in our patients has previously been reported to occur in *Acanthamoeba castellanii* (Brennicke et al 1999), human glutamate receptor (GluR7 - Novo et al 1995), PR (PRDI-BF1-RIZ) domain zinc finger protein 1 (*PRDM1* - Tam et al. 2006), and in Mucopolysaccharidosis type II (Hunter Syndrome - Lualdi et al 2010). In the DARNED, a database of mRNA editing in humans, more than one hundred instances of mRNA editing have been reported in *GNPTG*, but none are related to ML III gamma (Kiran and Baranov 2010).

The nonsense mutation generates premature termination codon; therefore, the observed reduction in *GNPTG* mRNA levels might be explained by the involvement of the transcripts in the NMD pathway. NMD usually reduces the level of premature termination codon (PTC)-bearing transcripts but does not eliminate them completely (Linde and Kerem 2008). NMD has been observed in Mucopolisaccharidosis type I (Almeida et al. 2012), Niemann-Pick type C (Macías-Vidal et al 2009) and *GNPTG* (Persichetti et al 2009).

Herein, the 5'UTR mutation (c.-112C>G) found in *cis* with the nonsense mutation can also have some effect on the level of transcription of *GNPTG* (Albers et al 2012; Almeida et al 2012; Bhuvanagiri et al 2010; Rebbapragada and Lykke-Andersen 2009). Mutations located in the 5′UTR may have effects on mRNA expression and, therefore, can sometimes be pathogenic. For instance, the presence of 5'UTR mutations have been reported as causing TAR (thrombocytopenia-absence of radius) syndrome, an autosomal recessive disease; in patients harboring a previously associated microdeletion in 1q21.1, researchers identified two different low-frequency variants in the regulatory region of *RBM8A*. The combination of either variant with the original microdeletion is sufficient to cause this disorder (Albers et al. 2012). Interestingly, the promoter region of *GNPTG* is overlapped to by *TSR3* - 20S rRNA homolog (*Saccharomyces cerevisiae*) gene, which is transcribed in the opposite direction as the *GNPTG*; so, c.-112C>G can affect the processing of 20S rRNAs (Li et al 2009).

In patients A and B, the relative mRNA expression of the gene encoding α/β-subunits precursor of GlcNAc-1-phosphotransferase (*GNPTAB*) was found to be up-regulated. This up-regulated expression suggest that a compensatory

mechanism is preventing the complete missorting of newly synthesized lysosomal enzymes (Pohl et al 2009). Patient B has higher levels of *GNPTAB* than patient A and this may be associated to the less severe clinical phenotype presented by patient B.

In conclusion, we suggest that *GNPTG* analysis must be performed on gDNA due to the instability of mRNA containing premature stop codons and the occurrence of mRNA editing. mRNA editing could play an important role in modulating the association between mutant genotype and clinical phenotype.

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TABLE 1. Summary of clinical, functional and mobility characteristics of patients with Mucolipidosis III gamma.

	Patient A	Patient B	Reference Values
Gender	M	M	
Parental consanguinity	No	No	
Age at diagnosis (years)	34.7	40.0	
Age at inclusion (years)	42.0	44.0	
Height (cm)	148.0	152.0	
Height Z score*	-3.77	-3.00	
Education/ Occupation	Elementary school/ Farmer	Elementary school/ Farmer	
FIM total	120	122	18 to 126**
FIM Mobility	85	87	13 to 91**
FIM Cognitive	35	35	5 to 35**
Shoulder Abduction	101	106	180°
Shoulder Flexion	126	137	180°
Shoulder Internal Rotation	32	35	90°
Shoulder External Rotation	40	53	90°
Shoulder Extension	68	67	80°
Elbow Extension	49	39	0°
Elbow Flexion	121	126	160°
Wrist Extension	54	20	70°
Wrist Flexion	32	64	80°
Hip Extension	16	16	45°
Hip Flexion	99	70	125°
Hip Abduction	23	18	45°
Hip Adduction	24	22	30°
Hip Internal Rotation	39	23	60°
Hip External Rotation	21	15	70°
Knee Flexion	126	130	130°
Knee Extension	10	11	0°

*According to the World Health Organization (http://www.who.int/growthref/who2007_height_for_age/en/index.html).

**As higher the punctuation, as better.

TABLE 2. Mucolipidosis III gamma: Biochemical characterization of patients included in this study.

Biochemical Investigation	**Sample**	**Patient A**	**Patient B**	**Reference Values**
Arylsulfatase A	Plasma	+	+	-
β-Glucuronidase	Plasma	1037	1899	30-300 nmol/h/mL
Total β– Hexosaminidases	Plasma	36533	17489	1000-2857 nmol/h/mL
Iduronate-sulfatase	Plasma	1707	1644	122-463 nmol/4h/mL
α–Mannosidase	Fibroblasts	13	NA	60-400 nmol/h/mg
β–Galactosidase	Fibroblasts	178	130	394-1440 nmol/h/mg
β–Glucuronidase	Fibroblasts	4.5	15	62-361 nmol/h/mg
α–Iduronidase	Fibroblasts	90	NA	74-148 nmol/h/mg
Iduronate-sulfatase	Fibroblasts	12	4.8	35-80 nmol/4h/mg

NA: not analyzed; +: present; -: absent;

TABLE 3. Mutations identified in Brazilian patients with Mucolipidosis III gamma.

Patient	Pathogenic mutations identified in *GNPTG*		Non-pathogenic mutations identified in *GNPTG*
	cDNA	protein	
A	c.[328G>T];[328G>T]	p.[Glu110X];[Glu110X]	c.[-112C>G];[-112C>G]
Mother	c.[328G>T];[N]	p.[Glu110X];[N]	c.[-112C>G];[N]
Father	c.[328G>T];[N]	p.[Glu110X];[N]	c.[-112C>G];[N]
B	c.[328G>T];[328G>T]	p.[Glu110X];[Glu110X]	c.[-112C>G];[-112C>G]
Mother	c.[328G>T];[N]	p.[Glu110X];[N]	c.[-112C>G];[N]
Father	c.[328G>T];[N]	p.[Glu110X];[N]	c.[-112C>G];[N]

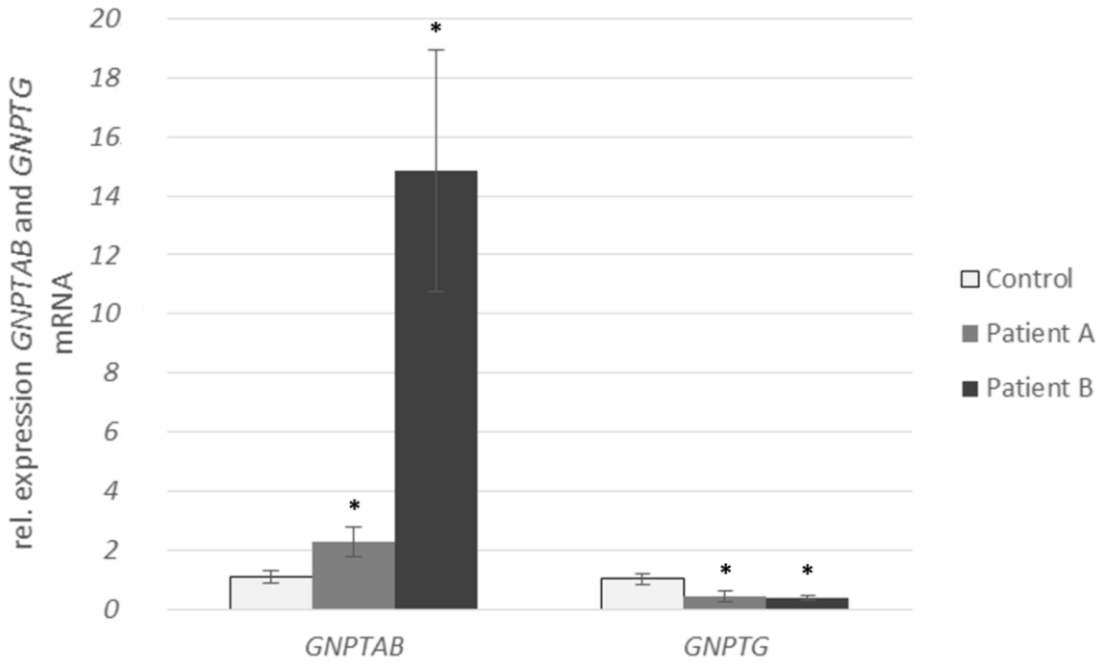

Figure 1. *GNPTAB* and *GNPTG* expression in Mucolipidosis III gamma patients. The relative levels of *GNPTG* and *GNPTAB* mRNA expression were determined in fibroblasts of ML III gamma patients by real-time PCR normalized to *GAPDH* expression. Analysis of control and ML III patients are the mean of triplicate PCRs obtained from three independent RNA preparations. Data are expressed as the mean fold change in respective gene expression ± SD (*$p < 0.001$).

4.4 Artigo 4

First case of Mucolipidosis III gamma diagnosed after screening in dried blood spots

Artigo submetido ao periódico Journal of Inherited Metabolic Diseases Reports.

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FIRST CASE OF MUCOLIPIDOSIS III GAMMA

DIAGNOSED AFTER SCREENING IN DRIED BLOOD SPOTS

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To the Editor,

In a recently published paper in *JIMD Reports*, Cobos et al (2015) confirmed that enzymatic analysis performed on dried blood spot samples (DBS) is a reliable tool for investigating patients at risk of mucopolysaccharidosis (MPS) I, II and VI or mucolipidosis (ML) II/III alpha/beta. They reported activities of α-L-iduronidase, iduronate-2-sulphatase and arylsulphatase B found in DBS collected from 200 patients with MPS-like phenotype. Of these samples, thirty-seven showed low enzymatic activity: 17 for MPS I, 11 for MPS II and 9 for MPS VI. Interestingly, high activity of at least two enzymes was found in 8 samples, suggesting MLII/III diagnosis. *GNPTAB* gene analysis was carried out using the same DBS in these cases, and MLII/III alpha/beta diagnosis was confirmed in 5/8 patients; no mutations were found in the other three patients. *GNPTG,* the other causative gene for MLIII, was not analyzed.

MLII/III is caused by the deficient activity of GlcNAc-1-phosphotransferase, which is encoded by the *GNPTAB* (α- and β-subunits) and *GNPTG* (γ-subunit) genes. Pathogenic mutations in *GNPTAB* lead to MLII, or to the milder condition, MLIII alpha/beta. MLIII gamma is the rarest form, and arises from mutations in *GNPTG* gene. It is believed that MLIII alpha/beta is associated with a more severe clinical picture than MLIII gamma (Raas-Rothschild et al. 2013). We herein report the first diagnosis of MLIII gamma performed using DBS samples.

A Chilean patient, who was born to non-consanguineous parents with no family history of genetic disease, was evaluated by a medical genetic service from Santiago de Chile. The patient presented with bone and joint disease at the age of seven; however no corneal clouding or cognitive impairment was noted, and

levels of glycosaminoglycans in urine were normal. Therefore, a DBS sample was sent to South Brazil mainly to screen for oligosaccharidoses. The activities of α-manosidase, α-L-iduronidase and total β-hexosaminidase were above the upper reference ranges (Table 1), raising the suspicion of ML II/III. The genomic DNA from patient and mother was extracted and *GNPTAB* and *GNPTG* genes were sequenced to further investigate the biochemical suspicion. No pathogenic mutations were found in *GNPTAB,* and a novel probable disease-causing mutation (c.233+5G>C or IVS4+5G>C) was identified in homozygosis in *GNPTG.* The mother was found to be a carrier. This mutation was not found on reference databases such as the 1000 Genomes Project, HGMD or dbSNP (1000 Genomes 2015; HGMD 2015; dbSNP 2015). According to the GENSCAN Web Service results, it would strongly interfere with *GNPTG* mRNA splicing (Figure 1).

This report confirms the importance of DBS for biochemical screening and for subsequent DNA-based diagnosis of MLIII gamma, even across countries. The use of DBS for biochemical screening of MLII/III has already been reported (Chamoles et al 2001; Reuser et al 2011; Cobos et al 2015; Uribe and Giugliani 2013; Cury et al 2013). However, only Cury et al (2013) and Cobos et al (2015) confirmed the positive biochemical screening by DNA tests (e.g., by *GNPTAB* sequencing). Our findings emphasize the need to analyze *GNPTG* in every DBS found both to be positive for MLII/III by enzyme assays and negative for *GNPTAB* mutations.

Acknowledgments:

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Table 1. Biochemical investigation of the patient included in this study.

Biochemical characterization	Enzyme activity	Reference range
β-Galactosidase* (EC 3.2.1.23)	35	35 – 126 nmol/h/mL
α-Mannosidase (EC 3.2.1.24)	1153	39 – 102 nmol/h/mL
α-L-Iduronidase (EC 3.2.1.76)	35	1.1 – 9.5 nmol/h/mL
Chitotriosidase (EC 3.2.1.14)	17	0 – 44 nmol/h/mL
Total β-Hexosaminidase (EC 3.2.1.52)	273	29 – 133 nmol/h/mL

* The measurement of β-Galactosidase activity was performed to assess the integrity of the patient's sample.

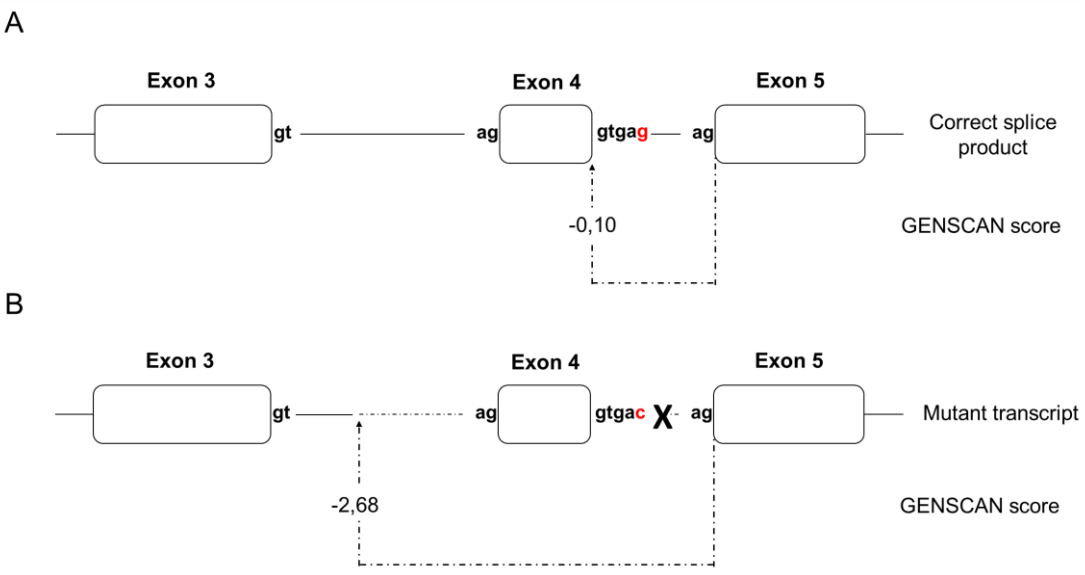

Figure 1. Schematic representation of exons and introns 3, 4 and 5 of the *GNPTG* gene and the scores obtained with the GENSCAN Web Server - normal sequence (A) and the mutated (B) forms obtained in the presence of c.233+5G>C, located at intron 4. Red letter: mutation position; Dotted line: new splicing. X: splicing replaced.

4.5 Artigo 5

GNPTAB and *GNPTG* genes expression in Mucolipidosis II/III patients

Artigo em fase de submissão ao periódico *Molecular Genetics and Metabolism*.

GNPTAB and *GNPTG* genes expression in Mucolipidosis II/III patients.

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Abstract

GlcNAc-1-phosphotransferase is the enzyme deficient in mucolipidosis (ML) II/III. *GNPTAB* gene encodes α/β-subunits and *GNPTG* encodes the γ-subunit of GlcNAc-1-phosphotransferase. Contradictory results in relation to *GNPTAB* and *GNPTG* mRNA expression in fibroblasts came from different studies. Aims: To analyze *GNPTAB* and *GNPTG* genes expression in fibroblasts (MLII= 2; MLIII gamma= 3) and blood (MLII= 6; MLIII alpha/beta= 1; MLIII gamma= 3) samples of ML II/III patients and controls (fibroblast=1; blood samples=3). Methodology: Total RNA was extracted from cultured fibroblasts and blood samples. *GNPTAB* and *GNPTG* mRNA levels were determined by qRT-PCR. Results: ML II/III alpha/beta and ML III gamma patients showed low *GNPTAB* and *GNPTG* mRNA levels, respectively. In ML III gamma patients the levels of mRNA *GNPTAB* were significantly increased. In ML II/III alpha/beta fibroblasts, one patient present a significant increase in *GNPTG* mRNA level. However, in blood samples, ML II and III alpha/beta patients can be distinguished based in *GNPTG* expression. MLII patients present low *GNPTG* mRNA level and MLIII alpha/beta, a high *GNPTG* mRNA expression. Discussion/Conclusions: This is the first study using blood samples to analyze both *GNPTAB* and *GNPTG* expression. The reduction of the *GNPTAB* and *GNPTG* mRNA levels in ML II/III alpha/beta and ML III gamma patients, respectively, can be easily explained by the nature of the mutations. The divergence between the results in blood and fibroblasts might be related to the sample's type and size. In blood is possible to distinguish the phenotype between MLII and MLIII alpha/beta and in ML alpha/beta or gamma fibroblasts, the expression of the non-affected subunit of GlcNAc-1-phosphotransferase appears to be altered in a compensatory mechanism.

Keywords: *GNPTAB*; *GNPTG*; GlcNAc-1-phosphotransferase; mRNA expression; blood; fibroblasts;

Introduction

Newly synthesized lysosomal hydrolases are specifically modified in the Golgi apparatus with mannose 6-phosphate (M6P) residues. GlcNAc-1-phosphotransferase (EC 2.7.8.15) is the enzyme that catalyzes the first step in the synthesis of the M6P. This enzyme transfers GlcNAc-1-phosphate to C6-position of mannose residues on high-mannose type oligosaccharides using UDP-GlcNAc as substrate [1, 2]. After, the GlcNAc-1-phosphodiester N-Acetylglucosaminidase enzyme (NAGPA, EC3.1.4.45) remove the terminal GlcNAc to expose the M6P residues. These residues function as recognition marker required for the M6P receptor mediated transport to lysosomes [2, 3].

The GlcNAc-1-phosphotransferase forms a complex composed of three subunits: $\alpha_2\beta_2\gamma_2$. *GNPTAB* gene (MIM#607840) encodes the α/β-subunit precursor who is proteolytically processed by the site-1-protease (S1P, EC 3.4.21.112) into the individual subunits which seems a prerequisite for the catalytic activity of the enzyme [4, 5]. The *GNPTG* gene (MIM#607838) encodes the γ-subunit [6]. While it is thought that the α- and β-subunits comprise the binding sites of the UDP-GlcNAc substrate, and the catalytic activity [2, 7], little is known about the role of the γ-subunits for the GlcNAc-1-phosphotransferase complex.

Mutations in *GNPTAB* gene result in two autosomal recessive lysosomal diseases, mucolipidosis type II (MLII, MIM#252500) and III alpha/beta (MLIII,

MIM#252600), while mutations in *GNPTG* gene result in ML III gamma (MIM#252605). MLII and MLIII alpha/beta and gamma are biochemically characterized by the missorting of multiple lysosomal enzymes due to impaired formation of M6P residues, and general lysosomal dysfunction [2, 8, 9]. MLII alpha/beta is a fatal disease with death occurring in the first decade of life. Patients have severe psychomotor retardation, short stature, coarse facial features, gingival hypertrophy, macroglossia, inguinal hernias, cardiopulmonary complications, and hearing loss. MLIII alpha/beta or gamma are attenuated form of the disease, with later onset, slower progression and less severe clinical symptoms, which enables survival into adulthood [2, 10].

Contradictory results in relation to *GNPTAB* and *GNPTG* mRNA expression in patients with ML II and III came from different studies. In 2007, Ho et al. [11] found low levels of *GNPTAB* expression and up-regulation of *GNPTG* in fibroblasts of 6 MLII and III alpha/beta patients; in one MLIII gamma fibroblasts, expression of alpha/beta and gamma subunits was all at low levels. A different pattern of expression was found by Encarnação et al. [12]: out of 8 MLII patients, none presented increased *GNPTG* mRNA levels; in 4 ML III alpha/beta patients, low levels of *GNPTAB* and *GNPTG* expression are shown; an in nine MLIII alpha/beta patients, no significant alteration of the mRNA *GNPTG* levels was observed. A discordant result was found in MLIII gamma patients by Pohl et al. [13]: in these patients, the relative expression of *GNPTG* was decreased and *GNPTAB* was increased in comparison with the expression of control cells. Therefore, as further studies are needed in order to obtain a better understanding betweenα/β- and γ-subunits expression, we analyzed *GNPTAB* and *GNPTG* genes expression and its relation in fibroblasts and blood samples of ML II/III patients and controls.

Materials and methods

Patients' samples

Fibroblasts and blood samples of controls and ML II and III patients (Table 1) were obtained under informed consent and the project was approved by the institutional review board of Hospital de Clínicas de Porto Alegre, Brazil. All patients have been previously sequenced to *GNPTAB* and *GNPTG* genes in gDNA. Fibroblasts were maintained in culture for 24 h with Dulbecco's Modified Eagle's Medium (DMEM- Gibco, USA) supplemented with 10% fetal bovine serum (FBS- Gibco, USA), 1% penicillin/streptomycin (P/S- Gibco, USA) and 2 mM L-glutamine (Gibco, USA).

RT-qPCR

Total RNA was extracted from cultured fibroblasts and blood sample using RNeasy Mini Kit (Qiagen, DE) and conversion to cDNA was made using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). The *GNPTAB* and *GNPTG* mRNA levels were determined by qRT-PCR using 2x SYBR Green PCR Master Mix (Applied Biosystem, USA) with the Mx3000P (Stratagene, NL). *GAPDH* was chosen as housekeeping gene. Primers and conditions were performed as described by Ho et al. [11] with modifications. The relative quantification of the RNA was normalized to the level of *GAPDH* mRNA in the same cDNA using the comparative CT method ($2^{-\Delta\Delta CT}$).

Statistical analysis

Statistical analysis was performed by the IBM SPSS Statistics version 22 using Student's test. Statistical differences among groups were considered when $p < 0.05$.

Results

Results on mRNA relative quantification are show in Fig. 1. In all patients with mutations in *GNPTAB* gene, a significant decrease in *GNPTAB* mRNA levels were observed in blood and fibroblasts samples (Fig. 1A and B). However, the data obtained for the *GNPTG* mRNA levels are contradictory. In fibroblasts (Fig. 1B), the patient A present a significant decrease in mRNA *GNPTG* levels and patient B present *GNPTG* in normal level. Nevertheless, in blood samples (Fig. 1A), the patients B, C and D, both homozygotes to p.L1168QfsX5, show a decrease in *GNPTG* expression. In the same way, in the patient E (p.L1168QfsX5;S385L) and F (p.I403T;Q278X) both *GNPTAB* and *GNPTG* mRNA levels were significantly decreased. In the patient G, heterozygote to the mutations p.L1168QfsX5 and p.S399F, *GNPTG* mRNA levels was increased compared with controls.

The values obtained in blood and fibroblasts samples indicate that the identified mutations in *GNPTG* are associated with a significant decrease in mRNA *GNPTG* levels compared with the mean controls values (Fig. 1C and D). On the other hand, in these same patients the *GNPTAB* mRNA levels were significantly increased. If we analyze only the *GNPTAB* mRNA levels in blood or fibroblasts samples, we can see a significant difference between the types of sample. The patients H (p.F83Ter;E110Ter) and I (p.E110Ter;E110Ter) presents an increase of *GNPTAB* expression of the 2.16 and 1.82 in blood. In fibroblasts,

patients H, J and I present an increase to 14.31, 6.44 and 13.23 in *GNPTAB* mRNA expression, respectively (Fig. 1C and D).

Discussion

In our study, the reduction of the *GNPTG* mRNA levels found in MLIII gamma patients can be easily explained by the nature of the mutations, as both p.F83Ter and p.E110Ter generate premature stop codons and are predictably target of the mRNA nonsense-mediated decay (NMD). In another study [14], our group showed that the alleles p.E110Ter (patients I and J) pass for a process call mRNA editing. In this case, the mRNA is normal, but still are decrease compared with mean values controls. On the other hand, the significantly increase *GNPTAB* mRNA levels observed might be related to a compensatory mechanism between the α/β- and γ-subunits where the *GNPTG* down-regulation induces *GNPTAB* up-regulation [13].

In MLII and III alpha/beta patients, the reduction of the *GNPTAB* mRNA levels are in agreement with the *GNPTG* findings. The type of the mutations, a deletion in homozygosis and heterozygosis and a nonsense mutation in heterozygosis, explain the *GNPTAB* expression. However, the levels of *GNPTG* expression observed in blood might be related to the existence of feedback mechanism between *GNPTAB* and *GNPTG* genes already described [11, 12] and to the phenotype. Analyzing gene expression, we can easily see the difference between *GNPTG* expression in blood of MLII and MLIII alpha/beta patients. MLII patients (patients B, C, D, E and F) presenting with a decrease in *GNPTG* mRNA levels; however, MLIII alpha/beta patient (patient G) showed an increase of *GNPTG* expression. In fibroblasts, the genotype p.L1168QfsX5;L1168QfsX5

showed decreased *GNPTG* mRNA (patient A) and no variation (patient B) compared with mean values controls.

This is the first study using blood samples to analyze *GNPTAB* and *GNPTG* expression. The divergence between the results in blood and fibroblasts might be related to the size of and type of the sample. In blood, we have neutrophils, eosinophils, basophils, lymphocytes, monocytes and macrophages, different types of leukocytes compared with fibroblasts where only one cell type is present. Besides that, the plasma reflect a pool of cells and which are secreting substances to plasma. Moreover, fibroblasts contribute little to the pathogenesis of ML II and III. Different cellular types can express differently *GNPTAB* and *GNPTG* since MLII and MLIII are tissue-specific [15]. Expression analysis in blood samples can give more significant information of patients.

Another interesting point raised by this work is the possibility to improve a ML alpha/beta phenotype increasing the *GNPTG* expression. Increasing *GNPTG* expression could be a treatment to ML alpha/beta patients.

Together, the data present here suggest a different *GNPTAB* and *GNPTG* expression levels in blood and fibroblasts samples in ML patients. In blood it is possible to distinguish the phenotype between MLII and MLIII alpha/beta and in fibroblasts of patients with ML alpha/beta or gamma, the expression of the non-affected subunit of GlcNAc-1-phosphotransferase appears to be altered in a compensatory manner mechanism.

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Table 1. Brazilian patients with ML II and III included in this study.

Patient	Sex	Phenotype	Genotype		Sample	
			Protein	cDNA	Blood	Fibroblast
A	M	ML II alpha/beta	p.L1168QfsX5;L1168QfsX5	c.3503_3504delTC;3503_3504delTC	No	Yes
B	M	ML II alpha/beta	p.L1168QfsX5;L1168QfsX5	c.3503_3504delTC;3503_3504delTC	Yes	Yes
C	M	ML II alpha/beta	p.L1168QfsX5;L1168QfsX5	c.3503_3504delTC;3503_3504delTC	Yes	No
D	M	ML II alpha/beta	p.L1168QfsX5;L1168QfsX5	c.3503_3504delTC;3503_3504delTC	Yes	No
E	F	ML II alpha/beta	p. L1168QfsX5;S385L	c.3503_3504delTC;1154C>T	Yes	No
F	F	ML II alpha/beta	p.I403T;Q278Ter	c.1208T>C;832C>T	Yes	No
G	M	ML III alpha/beta	p. L1168QfsX5;S399F	c.3503_3504delTC;1196C>T	Yes	No
H	F	ML III gamma	p.F83Ter;E110Ter	c.244_247dupGAGT;328G>T	Yes	Yes
I	M	ML III gamma	p.E110Ter;E110Ter	c.328G>T;328G>T	Yes	Yes
J	M	ML III gamma	p.E110Ter;E110Ter	c.328G>T;328G>T	Yes	Yes

M – Male; F- Female;

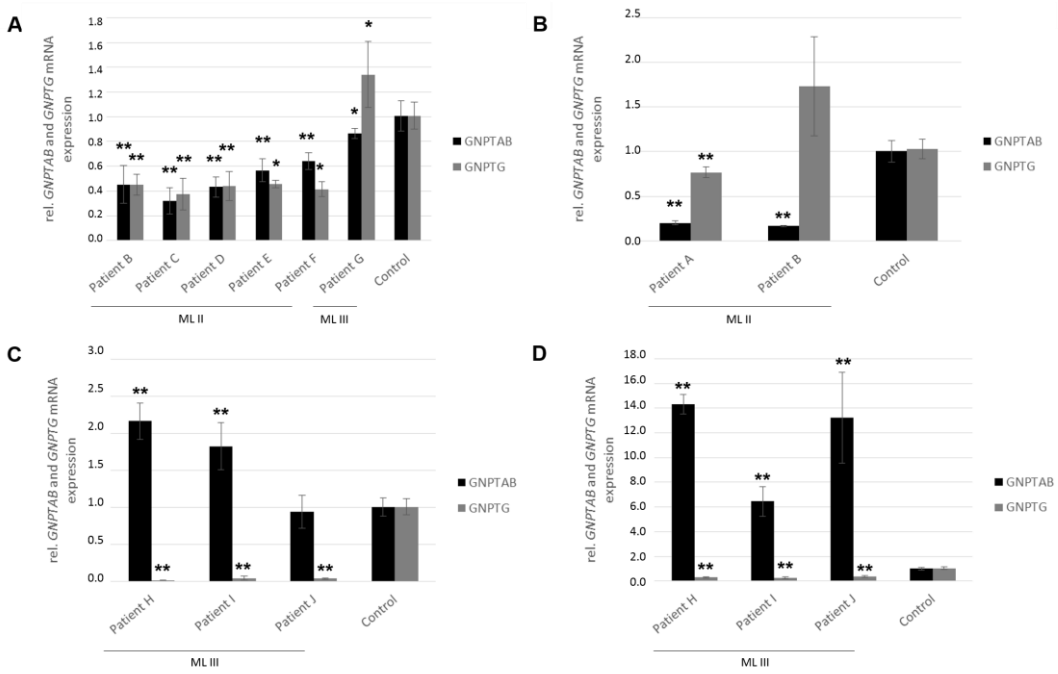

Figure 1. *GNPTAB* and *GNPTG* genes expression in MLII and III patients. Relative mRNA expression in blood (A) and in fibroblasts (B) samples of ML II and III alpha/beta patients. Relative mRNA expression in blood (C) and in fibroblasts (D) samples of ML III gamma patients. The relative levels of *GNPTG* and *GNPTAB* mRNA expression were determined in blood and in fibroblasts samples of ML II and III patients by real-time PCR normalized to *GAPDH* expression. Analysis of controls, and ML II/ III patients are the mean of triplicate PCRs obtained from three independent RNA preparations. Data are expressed as the mean fold change in respective gene expression ± SD (*$p < 0.05$ and **$p < 0.001$).

4.6 Artigo 6

New approaches to the treatment of orphan genetic disorders: Mitigating molecular pathologies using chemicals

Artigo aceito para publicação no periódico Anais da Academia Brasileira de Ciências, *Special Issue on Hot Topics in Biomedical Sciences*em 2015.

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Para: re.voltolini@hotmail.com

> 08-Mar-2015

Dear Dr. Voltolini Velho:

It is a pleasure to accept your manuscript entitled "New approaches to the treatment of orphan genetic disorders: Mitigating molecular pathologies using chemicals" in its current form for publication in the Anais da Academia Brasileira de Ciências. The comments of the reviewer(s) who reviewed your manuscript are included at the foot of this letter.

Thank you for your fine contribution. On behalf of the Editors of the Anais da Academia Brasileira de Ciências, we look forward to your continued contributions to the Journal.

Sincerely,
Dr. Alexander Kellner
Editor-in-Chief, Anais da Academia Brasileira de Ciências
editor-in-chief@abc.org.br

Associate Editor
Comments to the Author:
(There are no comments.)

Review for the special issue Hot Topics in Biomedical Sciences

New approaches to the treatment of orphan genetic disorders: Mitigating molecular pathologies using chemicals

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Mitigating molecular pathology using chemicals

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Abstract

With the advance and popularization of molecular techniques, the identification of genetic mutations that cause diseases has increased dramatically. Thus, the number of laboratories available to investigate a given disorder and the number of subsequent diagnostics have increased over time. Although it is necessary to identify mutations and provide diagnosis, it is also critical to develop specific therapeutic approaches based on this information. This review aims to highlight recent advances in mutation-targeted therapies with chemicals that mitigate mutational pathology at the molecular level, for disorders that, for the most part, have no effective treatment. Currently, there are several strategies being used to correct different types of mutations, including the following: the identification and characterization of translational readthrough compounds, such as PTC124; antisense oligonucleotide-mediated splicing redirection; mismatch repair; and exon skipping. These therapies and other approaches are considered in this study.

Keywords: antisense oligonucleotide-mediated splicing redirection mutations; exon skipping; mismatch repair; mutation-targeted therapies; translational readthrough

Resumo

Com o avanço e a popularização de técnicas moleculares, a identificação de mutações genéticas que causam doenças aumentou drasticamente. Deste modo, o número de laboratórios disponíveis para investigar uma determinada doença e, consequentemente, o número de diagnósticos, tende a aumentar ao longo do tempo. No entanto, tanto quanto identificar mutações é necessário o desenvolvimento de específicas abordagens terapêuticas. Com base nesses avanços, esta revisão tem como objetivo destacar as terapias químicas que tem como alvo os diferentes tipos de mutação e que podem diminuir a patologia ao nível molecular de doenças para as quais, na maioria das vezes, não existe um tratamento eficaz. Atualmente existem diversas estratégias em uso para correção de diferentes tipos de mutações. Dentre eles podemos citar: identificação e caracterização de compostos de readthrough traducional como o PTC 124, redirecionamento antisense de splicing mediado por oligonucleotídeos, reparação por imparidade e “salto de éxon”. Estas e outras abordagens são consideradas neste estudo.

Palavras-chave: readthrough traducional; redirecionamento antisense desplicing mediada por oligonucleotídeo; reparação por imparidade; “salto de éxon”; terapias mutação-alvo;

Introduction

Many human diseases have an identified genetic basis. Whereas some of these diseases may be attributed to mutations at multiple loci, a significant number of diseases are believed to be caused by mutations in single genes – monogenic diseases. These monogenic disorders can be subdivided into autosomal dominant, autosomal recessive and X-linked diseases. Although each of these conditions is relatively rare, the many different types of monogenic diseases together affect a substantial population. The World Health Organization (WHO, 2014) estimated the global prevalence of all single gene diseases to be approximately 10 in every 1000 births. Some of these diseases can be very severe and even fatal (WONG; CHIU, 2010).

Genetic mutations can be classified into four major groups: missense, nonsense, splicing and frameshift. Missense mutations are typically single nucleotide changes that either alter the amino acid in translated proteins (nonsynonymous) or do not alter the amino acid (synonymous or ‘silent’). Nonsense mutations are point mutations in a sequence that create a UAA, UAG, or UGA codon in the coding region of the mRNA, resulting in a premature translation termination and, usually, a nonfunctional or rapidly degraded protein. Splicing mutations can originate in multiple ways that result in the disruption of a critical sequence of nucleotides by the mutation and the creation of aberrant or cryptic splice sites that translate to aberrant proteins. Frameshift mutations most commonly result from the deletion or insertion of a number of nucleotides that alters the reading frame for any subsequent downstream codons (HU; GATTI, 2008; MITUI et al., 2009; ORRO et al., 2008).

The existence of common groups of mutations prompted the hypothesis of common group-specific molecular pathogenesis. From this hypothesis, it follows that therapeutic strategies developed against a common mutation group will be effective against similar mutations, regardless of the gene (HU; GATTI, 2008). In this review, we highlight mutation-targeted therapies with chemicals that mitigate mutational pathology at the molecular level, mainly for recessive disorders for which there is no effective treatment (Table 1).

Aminoglycosides and others drugs that promote stop codon readthrough

Mutations that introduce premature stop codons (PTCs) into the coding sequences of genes have been implicated in numerous inherited diseases and several cancers, and at least 2,400 different genetic disorders have at least one causative nonsense allele (MORT et al., 2008; OMIM, 2014). In general, it has been estimated that nonsense mutations account for 5 to 70% of the individual cases of genetic disorders and are responsible for ~11% of all known Human Gene Mutation Database (HGMD) lesions that cause inherited disorders and as many as 20% of the protein-coding region located, single base-pair mutations that cause these diseases (HGMD 2014; Ryan 2014). PTCs can originate from nonsense mutations, frameshift mutations, or from aberrant splicing that generates mRNA isoforms that lead to the production of nonfunctional, truncated or deleterious proteins (KANDASAMY et al., 2011; LINDE; KEREM, 2008). Typically, these mutations result in the activation of nonsense mediated RNA decay (NMD), whereby PTC-containing mRNAs are targeted for rapid degradation. This mechanism blocks the production of truncated proteins, which leads to negative effects on cell function (ALMEIDA et al., 2012; SILVA; ROMÃO,

2009). Strategies for inducing stop codon readthrough offer the possibility of reducing the pathology caused by this type of mutation. There are at least two different scenarios in which PTC therapies may be maximally effective: (i) diseases in which a much lower than normal level of the key protein's activity may be therapeutic (e.g., hemophilia and cystic fibrosis); and (ii) diseases in which the therapeutic protein has a very long half-life and thus can accumulate to significant levels via PTC suppression (e.g., Duchene muscular dystrophy) (HAINRICHSON; NUDELMAN; BAASOV, 2008; KANDASAMY et al., 2011).

Aminoglycosides such as gentamicin, amikacin and tobramycin have important clinical applications in the treatment of serious Gram-negative bacterial infections and, recently discovered, in the experimental treatment of recessive diseases with nonsense mutations (HAINRICHSON; NUDELMAN; BAASOV, 2008; JANA; DEB, 2006; PELTZ et al., 2013; VECSLER et al., 2011). These molecules bind to the decoding site of the 16S or 18S ribosomal RNA in prokaryotes and eukaryotes, respectively, inducing a local conformational change (HALVEY; LIEBLER; SLEBOS, 2012; HU; GATTI, 2008). In prokaryotes, the binding of aminoglycoside is highly specific and is mediated through adenine 1408 in the 16S ribosome, thus leading to the inhibition of protein synthesis. However, in eukaryotes, the corresponding nucleotide is a guanine and the binding is less efficient, resulting in translational readthrough by the insertion of an amino acid at the stop codon (KEELING; BEDWELL, 2002a; SÁNCHEZ-ALCUDIA et al., 2012).

Aminoglycoside antibiotics were the first small-molecule drugs that gave promising results, and this approach has been validated by numerous in vitro and in vivo experiments in different disorders, including cystic fibrosis (CF), Duchene

muscular dystrophy (DMD), Hurler's syndrome (MPS I), and diabetes, among others (DIOP; CHAUVIN; JEAN-JEAN, 2007a; GOLDMANN et al., 2010b; NUDELMAN et al., 2009). With direct clinical relevance, some trials of aminoglycoside therapy have been carried out in humans with PTC mutations. The most promising results came from topically administered gentamicin in CF patients. After treatment, nasal potential difference measurements, including the response to isoproterenol, improved considerably in individuals with PTC mutations. Furthermore, full-length CFTR protein was detected in the nasal epithelial cells of two treated individuals (CLANCY et al., 2006; SERMET-GAUDELUS et al., 2007; WILSCHANSKI et al., 2003). In patients with nonsense mutation-mediated DMD, there was evidence that intravenous gentamicin administration suppressed nonsense mutations, as determined by the analysis of full-length dystrophin in muscle biopsies (POLITANO et al., 2003). These experiments demonstrated the ability of selected aminoglycoside structures to induce mammalian ribosomes to read through disease-causing PTCs (Figure 1) and partially restore full-size functional proteins (BURKE; MOGG, 1985; KANDASAMY et al., 2011; SÁNCHEZ-ALCUDIA et al., 2012).

However, readthrough efficiency depends on the PTC tested (UGA > UAG > UAA); on the nucleotide context of the mutation, particularly the fourth nucleotide immediately after the stop codon (C > U > A ≥ G); and on other factors involved in readthrough regulation (BIDOU et al., 2004; DIOP; CHAUVIN; JEAN-JEAN, 2007a; KIMURA et al., 2005). In addition, the efficiency of aminoglycoside therapy depends on the molecular mechanism by which the nonsense mutation cause the defects and on the level of active protein required to reduce clinical symptoms. Moreover, results are impaired by the need for regular intravenous

administration and the toxic side effects (nephrotoxicity and ototoxicity) of long-term treatment (FINKEL, 2010). With the aim of identifying alternative compounds that do not elicit toxic side effects, several studies have been successfully carried out using high throughput screens or designed aminoglycosides (DU et al., 2009; HAINRICHSON; NUDELMAN; BAASOV, 2008; KANDASAMY et al., 2011; KARIJOLICH; YU, 2014; KEELING et al., 2014; PELTZ et al., 2013; VECSLER et al., 2011).

The recently identified compound PTC124, or ataluren, is a non-aminoglycoside, readthrough-inducing compound that shows potent PTC suppression while preserving the natural termination codon and offers the advantages of having no obvious toxic effects and being orally bioavailable (Welch et al. 2007; Peltz et al. 2013; Ryan 2014). This drug has shown promising results for Duchenne muscular dystrophy, dystrophinopathy, cystic fibrosis, Hurler syndrome, and Carnitine Palmitoyltransferase 1A Deficiency (CPT1A), with the potential of treating a wider range of genetic disorders (Finkel 2010; Sermet-Gaudelus et al. 2010; Rowe et al. 2012; Peltz et al. 2013; Ryan 2014).

A set of clinical studies have been conducted with ataluren in CF and DMD. A phase IIb international, randomized, double blind, placebo-controlled study evaluated the effects of ataluren therapy on ambulatory ability in 174 patients (≥5 years of age) with DMD for 48 weeks (FINKEL et al., 2010, 2013a; MCDONALD et al., 2013c, 2013d). Patients were stratified based on their age (<9 versus ≥9 years), use of corticosteroids (yes versus no), and baseline 6-min walk distance (6MWD) (<350 or ≥350 m) and were randomized 1:1:1 to placebo, ataluren low dose, or ataluren high dose with all regimens given three times per day. This study showed that ataluren 10, 10, 20 mg/kg was more effective than placebo,

increasing the mean 6MWD by 31.3 m over the 48 week period and representing a 48% reduction in the risk of the 6MWD worsening by 10%. Timed function tests of muscle function (walking/ running 10 m, up and down stairs) also revealed positive trends for ataluren 10, 10, 20 mg/ kg, as evidenced by less decline over the 48 weeks. Ataluren showed activity and safety in this short-term study, supporting the further evaluation of ataluren 10, 10, 20 mg/kg and 20, 20, 40 mg/kg in similar future studies (FINKEL et al., 2013b; MCDONALD et al., 2013a, 2013b).

In CF patients ≥ 6 years of age, a phase III 48-week, double-blind study was utilized to assess the safety and efficacy of ataluren (CLANCY et al., 2006; KEREM et al., 2014a; ROWE et al., 2012). Patients were stratified by age, chronic inhaled antibiotic use and percent-predicted forced expiratory volume in 1 s (FEV1). At Week 48, the difference in the mean relative change from baseline in percent-predicted FEV1 between ataluren and placebo was 3.0%, and the mean pulmonary exacerbation rate was 23% lower for ataluren (KEREM et al., 2014b; PELTZ et al., 2013). These results were more evident in patients who did not chronically take inhaled antibiotics. In this study, several inhaled antibiotics were used chronically by patients, including colistin, aztreonam and tobramycin. However, analyzing the effects of these different inhaled antibiotics on percent-predicted FEV1 and pulmonary exacerbation rates indicated that tobramycin antagonized the effect of ataluren, which was confirmed by in vitro assays. The safety profiles were similar for ataluren and placebo (KEREM et al., 2014b; PELTZ et al., 2013; ROWE et al., 2012).

PTC124/ataluren (Translarna – PTC Therapeutics, Inc.) is a small molecule, orally available, non-toxic compound that targets nonsense mutations (Finkel et

al. 2013b; Ryan 2014). Ataluren allows cellular machinery to read through premature stop codons, enabling the translation process to produce full-length proteins. This compound is the first drug developed specifically to treat diseases caused by nonsense mutations. Additionally, it offers the prospect of validating nonsense suppression as a potential therapeutic approach in a large number of genetic disorders. Notably, a conditional first approval for the use of this drug was achieved for DMD in 2014 (Ryan 2014).

Use of therapeutic oligonucleotides in splicing mutations

RNA mis-splicing diseases account for up to 15% of all inherited diseases, ranging from neurological to myogenic to metabolic disorders. With the great increase in performing genomic sequencing for individual patients, the number of known mutations that affect splicing has risen to 50–60% of all disease-causing mutations (BARALLE; LUCASSEN; BURATTI, 2009; HAMMOND; WOOD, 2011; WANG; COOPER, 2007). Thus, correction or redirection of pre-mRNA splicing in a mutation-specific context represents a potential gene therapy modality with applicability to many inherited disorders.

Splicing mutations can be grouped into a minimum of five types. Type I is the classical splicing mutation that results in the deletion of an entire exon. Types II, III, and IV are nonclassical splicing mutations that result in pseudoexon inclusion or partial exon deletion. Type V involves the branch point; although such mutations exist, they have not been targeted for therapy (HU; GATTI, 2008). Exon inclusion, exon exclusion and exon skipping to restore the open reading frame and cryptic splicing are techniques used to correct frameshift mutations (SPITALI; AARTSMA-RUS, 2012).

Antisense oligonucleotide (AON) therapies have been used to correct pre-mRNA splicing in many disease models. Modified oligonucleotides can alter the pathogenic splice signals that are activated by mutations, resulting in close to normal levels of mRNAs that encode functional protein; however, the therapeutic principles for each AON varies considerably (Figure 2) (WILTON; FLETCHER, 2011).

The chemicals that are used to work in animal models include peptide nucleic acids (PNAs), alternating locked nucleic acids (LNAs) and deoxynucleotide oligonucleotides, fully modified (non-gapmer) 2′-substituted oligonucleotides and phosphorodiamidate morpholino (PMO)-based oligomers (KOLE; KRAINER; ALTMAN, 2012). By sterically blocking mutations and regulatory sequences within the pre-mRNA transcript, AONs have been used to induce the skipping or inclusion of exons, to block pseudoexons from recognition and to influence the alternative splicing of pathogenic isoforms (HAMMOND; WOOD, 2011).

Antisense oligonucleotides have been used to successfully modulate RNA splicing in CF (CFTR gene) (FRIEDMAN et al., 1999), β-thalassemia (β-globin gene) (SUWANMANEE et al., 2002), Hutchinson–Gilford progeria syndrome (LMNA gene) (SCAFFIDI; MISTELI, 2005) and DMD (Dystrophin gene) (CIRAK et al., 2011; KINALI et al., 2009; VAN DEUTEKOM et al., 2007). These compounds have also been used to modify alternative splicing in the SMN2 (spinal muscular atrophy) (HUA et al., 2010; NLEND NLEND; MEYER; SCHUMPERLI, 2010), Bcl-x (TAYLOR et al., 1999), and C-myc genes (Giles et al. 1999), suggesting that such antisense oligonucleotides are capable of crossing the blood–brain barrier in amounts sufficient to restore neural repair.

The data from the first clinical trial in DMD patients were highly encouraging, and technical advances in the development of methods for both directed exon exclusion and directed exon inclusion suggest that such antisense oligonucleotide splice-correction therapies may have broad application (ANTHONY et al., 2014; DICK et al., 2013; WOOD; GAIT; YIN, 2010).

Missense mutations

Targeted gene alteration is a promising method for correcting single-base mutations. In this technique, endogenous DNA repair pathways of the cell are activated, leading to specific genetic correction of single-base mutations in the genome. This strategy can be implemented using single-stranded oligonucleotides (ssODNs), chimeric RNA/DNA oligonucleotides (RDOs), small DNA fragments (SDFs), triplex-forming oligonucleotides (TFOs), adeno-associated virus vectors (AAVs) and zinc-finger nucleases (ZFNs) (JENSEN et al., 2011).

Gene editing using ssODNs can alter single nucleotides and induce stable alterations at the genomic level. Several studies have tested technique optimizations. ssODNs are oligonucleotides that are <200 bp in length and are designed to anneal to the lagging strand to generate a 100-fold greater ‘editing’ efficiency than those that anneal to the leading strand. The majority of editing events (~70%) occur by the incorporation of the ssODN within the lagging strand during replication (FALGOWSKI et al., 2011). Bertoni et al. (2009) showed that ssODNs that contain a methyl-CpG modification and are capable of binding to the methyl-CpG binding domain protein 4 (MBD4) are able to induce >10-fold

higher levels of gene correction than ssODNs lacking these specific modifications.

Chimeric RNA/DNA oligonucleotides are a class of oligonucleotides used for gene targeting with a correction efficiency of approximately 50%; however, the reproducibility of these studies has been limited in mammalian systems (SARGENT; KIM; GRUENERT, 2011). TFOs are ssODNs that are typically 10–40 nt in length and bind to specific regions in duplex DNA as a third strand to form a triple helix. TFOs act in polypurine or polypyrimidine regions of DNA and bind DNA via Hoogsteen hydrogen bonds (PAUWELS et al., 2014; SARGENT; KIM; GRUENERT, 2011).

Gene therapy remains a great promise for the treatment of genetic diseases. Recombinant adeno-associated virus vectors (AAV) have been successfully used to transduce a variety of genes in different cellular types in vitro and have been validated in small and large animal models (in vivo). There are three critical elements in gene transfer: the gene, the target tissue and the vector. The gene is the active therapeutic agent, but the virus-derived vector is the determinant of the therapeutic success and of the toxicity profile (POTTER et al., 2014). AAV vectors are currently being used in Phase I/II clinical trials for many diseases, such as CF, Pompe disease, α-1 antitrypsin deficiency, muscular dystrophy, Batten's disease, Parkinson's disease, Leber's congenital amaurosis, hemophilia, and choroideremia (MACLAREN et al., 2014; MENDELL et al., 2014; MITTERMEYER et al., 2012; SMITH et al., 2013; ZHONG et al., 2012). Furthermore, this treatment is already licensed in Europe for lipoprotein lipase deficiency (MINGOZZI; HIGH, 2011).

More recently, the use of zinc-finger nucleases has become a powerful strategy. These nucleases are engineered to introduce site-specific double-stranded DNA breaks in the genome. Site-specific alterations of the genome are then accompanied by homology-directed repair of the double-stranded DNA breaks using a linear double-stranded donor DNA fragment carrying the desired alteration. This strategy has been shown to be highly efficient at disrupting genes in several cell types and model organisms, making it a good choice for novel therapeutic applications (GRANJA et al., 2014). Although this approach can be remarkably effective, one obstacle of this approach is that zinc-finger nucleases need to be designed and constructed for every specific location to be modified. Thus, zinc-finger nucleases are most useful for repairing frequently occurring mutations (AARTS; TE RIELE, 2011).

A new approach that combines zinc-finger nucleases with adeno-associated viral vectors has been studied. Based on the enzyme's ability to create a site-specific DNA double-strand break, Händel et al. (2012) demonstrated that ZFN-encoding AAV expression vectors can be employed to induce large chromosomal deletions or to disrupt genes.

Current strategies combine different techniques for sequence-selective double-strand DNA targeting, including triplex forming oligonucleotides, synthetic hairpin polyamides, engineered zinc finger proteins and peptide nucleic acids. TFOs bind as a third strand in the major groove of dsDNA. In the classical ‘pyrimidine motif’, thymine/cytosine containing TFOs hybridize to the complementary adenine/guanine bases of the target in a parallel orientation via Hoogsteen base pairing (HANSEN; BENTIN; NIELSEN, 2009).

Many of these strategies reviewed here are commonly used for missense, nonsense and frameshift mutations.

Frameshift mutations

Frameshift mutations change the reading frame, thus inducing a completely different translation from the original one. These mutations often inactivate genes by producing truncated, nonfunctional proteins, resulting in the onset of diseases (MORITA et al., 2011).

The use of oligonucleotides in targeted sequence conversion has been developed for introducing sequence alterations, including deletions, insertions, and base-substitutions, into genomic DNA (DE SEMIR; ARAN, 2006; PAREKH-OLMEDO; KMIEC, 2007). Studies have demonstrated that both single-stranded DNA (ssDNA) fragments containing the sense sequence and the tailed duplex (TD) DNA fragments prepared by annealing an oligonucleotide to the ssDNA fragment have the ability to correct single-base substitution mutations. However, the ssDNA fragments correct mutations with low efficiency. Because the correction efficiencies of single-base substitution mutations by the TD fragments are higher, it is important to consider and examine the ability of the TD fragments to correct frameshift mutations (MORITA et al., 2011).

Another approach for frameshift mutations therapy is the use of engineered zinc-finger proteins to recognize a unique chromosomal site, which can be fused to a nuclease domain. Moreover, double-strand breaks induced by the resulting zinc-finger nuclease can create specific sequence alterations by stimulating homologous recombination between the chromosome and an extrachromosomal DNA donor (RAHMAN et al., 2011; URNOV et al., 2005).

Urnov et al. (2005) showed that zinc-finger nucleases designed against an X-linked severe combined immune deficiency (SCID) mutation in the IL2Rγ gene yielded higher than 18% of the gene-modified human cells, even without selection.

The targeted gene correction technique employs a site-specific DNA lesion to promote a homologous recombination that eliminates the mutation in a gene of interest. However, double-strand breaks that are typically used to initiate corrections can also result in genomic instability if deleterious repair occurs rather than gene correction, possibly compromising the safety of targeted gene correction (DAVIS; MAIZELS, 2011).

Cell therapy combined with gene therapy is a broadly expanding field in the correction of frameshift mutations and other mutations. Using a transgenic/knockout sickle cell anemia mouse model containing the human β^S-globin gene, Chang et al. (2006) prepared embryonic stem (ES) cells from blastocysts that had the sickle cell anemia genotype and carried out homologous recombination with DNA constructs containing the β^A-globin gene. Hematopoietic cells differentiated from these ES cells produced both hemoglobin A and hemoglobin S, having the potential to correct the sickle and β-thalassemia mutations.

The development of induced pluripotent stem (iPS) cells allowed stem cell therapies to advance to new frontiers. Using a humanized sickle cell anemia mouse model, Hanna et al. (2007) showed that mice can be rescued after transplantation with hematopoietic progenitors obtained in vitro from autologous iPS cells. This was achieved after correcting the human sickle hemoglobin allele with gene-specific targeting. Ye et al. (2009) treated fibroblasts from patients with

β-thalassemia caused by frameshift mutations. They showed that iPS cells could be produced from the somatic cells of these patients and that the mutations could be corrected with gene targeting. Cells differentiated into hematopoietic cells can be returned to the patient. The innovation of reprogramming somatic cells into induced pluripotent stem cells provides many possible new approaches for treating β-thalassemia and other genetic diseases.

Nature provides an interesting model of mutation reversion that should be thoroughly explored. Revertant mosaicism is a naturally occurring phenomenon that involves the spontaneous correction of a pathogenic mutation in a somatic cell. Recent studies suggest that this is not a rare event and that reversion could be clinically relevant to phenotypic expression and patient treatment. Indeed, revertant cell therapy represents a potential ‘natural gene therapy’ because in vivo reversion obviates the need for further genetic correction. Revertant mosaicism has been observed in several inherited conditions, including epidermolysis bullosa, a heterogeneous group of blistering skin disorders (LAI-CHEONG; MCGRATH; UITTO, 2011).

Several structural modifications and techniques are being developed to optimize the therapeutic responses to these new treatments. However, it is also apparent that much remains to be learned regarding the organization of these modifications. Eventually, principles with predictive value should emerge to guide the use of these new approaches.

Conclusions

Translational research for rare diseases is clearly a resource-intensive undertaking in terms of cost and time. Therefore, the central aspects of any broadly successful approach for this class of diseases will be creating and employing generalizable methodologies whenever possible. The identification of novel, non-toxic and efficient therapeutic agents and advances in the stability and delivery of antisense oligonucleotide splice-correction therapies are necessary. Although the approaches proposed in this paper are credible and feasible, the prospect of a rapid configuration of numerous effective disease therapies should be viewed in perspective. Among the many genetic disorders that have been or will shortly be molecularly characterized, only a small number will be tractable with the approaches reviewed here; however, the generation of information will at least help to move a larger number of disorders closer to the day of effective therapy.

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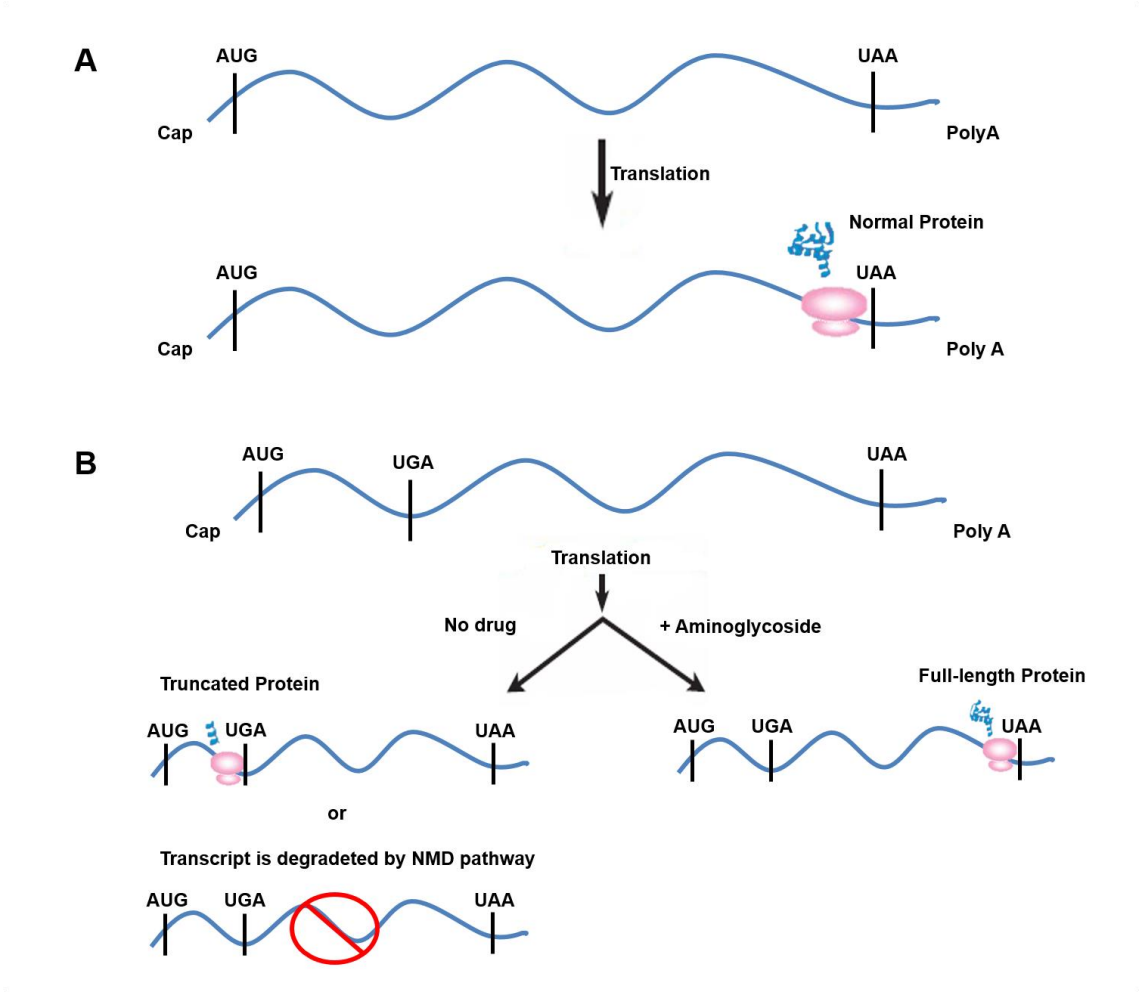

Figure 1. Schematic diagram of gene regulation strategy. The relative positions of caps, polyadenylation sequences, and initiation and termination codons within mRNAs are shown. (A) In wild type cells, normal mRNAs encode complete proteins. (B) In mutant cells, the mRNAs contain a premature stop codon, and truncated proteins are translated in the absence of aminoglycoside or the transcript is degraded by the NMD pathway. The presence of aminoglycoside allows the incorporation of a random amino acid at the terminal stop codon of the mutant mRNA. Full-length proteins result from the aminoglycoside-induced readthrough.

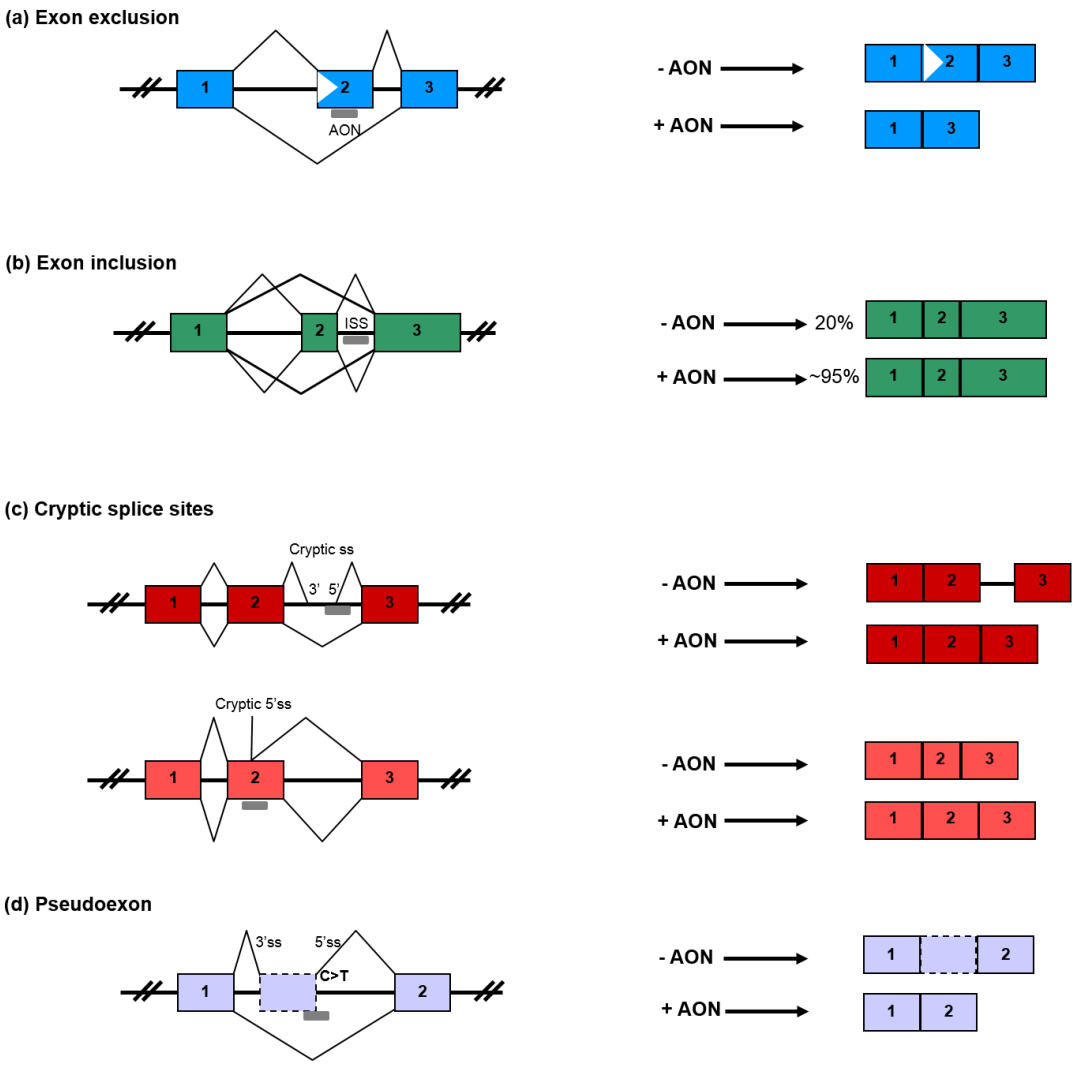

Figure 2. Antisense oligonucleotides (AONs) therapies are used to correct pre-mRNA splicing. (A) Exon exclusion: Large-scale deletions can result in out-of-frame mRNA transcripts. AONs directed toward an exon splice enhancer element within the mutated exon result in exclusion of the exon and, thus, an alternative in-frame transcript. (B) Exon inclusion: A mRNA transcript can lose one exon owing to negative splicing regulatory elements within and surrounding this exon and produce a less functional protein. Treatment with AON directed toward an intron splice silencer (ISS) can increase the inclusion of the exon from 20% to approximately 95%, as seen in the SMN2 gene (HUA et al., 2010). (C) Cryptic

splice sites: This can result in inclusion of introns or partial deletions of exons from mRNA. The hypothetical intronic mutation illustrated creates an intronic 5' splice site (ss) and activates a cryptic 3' ss within intron 2. AONs targeting the mutated intron 5' ss blocks recognition by the splicing machinery and generates transcripts without the aberrant intron element. Similarly, a mutation within exon 2 creates a cryptic 5' ss preferentially utilized over the natural splice site. AONs directed to the mutant cryptic site cause steric blockage of the splicing machinery and redirect splicing to the natural 5' ss. (D) Pseudoexon: A C–A mutation within intron 1 strengthened the 50ss of a pseudoexon (dashed box), which maintains a strong 3' ss. AONs directed to the pseudoexon 5' ss and inclusion of C–A mutation restored the natural transcript (Adapted from Hammond and Wood 2011).

4.7 Artigo 7

Geneticin and Chloranfenicol: a promising treatment for Mucolipidosis III gamma? A proof of concept.

Artigo em preparação.

Geneticin and Chloramphenicol: a promising treatment for Mucolipidosis III gamma? A proof of concept.

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Abstract

Mucolipidosis (ML) III gamma is an autosomal-recessive disorder caused by mutations in *GNPTG* gene, which encodes GlcNAc-1-phosphotransferase. This enzyme catalyzes the formation of mannose 6-phosphate targeting signals on soluble lysosomal enzymes. MLIII gammapatients are biochemically characterized by high levels of lysosomal enzymes in plasma, and low levels in fibroblasts. ML III gamma could be a potential therapy for this disorder since it can associated with nonsense mutations and these drugs can cause stop codon read through (SCRT). Aims: To evaluate the effect of geneticin and chloramphenicol treatment upon *GNPTG* gene expression and lysosomal enzyme activities in fibroblasts from MLIII gamma patients bearing nonsense mutations. Methodology: Fibroblasts from three MLIII gamma patients (two homozygous for p.E110Ter and one p.F83Ter;E110Ter) and one normal control were treated with geneticin or chloramphenicol for 24 h, or keeped without treatment. *GNPTG* mRNA quantification and lysosomal enzyme assays were performed and compared. Results: *GNPTG* mRNA quantification in non-treated fibroblasts samples indicate nonsense and frameshift mutations are associated with a significant decrease in mRNA *GNPTG* levels compared with the median controls values. Geneticin treatment led to a statistical increase in the levels of mRNA *GNPTG* of the patients A and C. Chloramphenicol had a positive effect upon mRNA of *GNPTG* level of patients B and C. Geneticin and chloramphenicol presented effect upon intracellular alpha-mannosidase activity in patient A. Both treatments do not have positive effect upon beta-glucuronidase and beta-galactosidase activities. Conclusions: Geneticin and chloranfenicol may increase the mRNA expression of *GNPTG* and improve some secondary enzyme abnormalities that occur in MLIII. The mechanisms by which occur is not completely known.

Keywords: Mucolipidosis III gamma; *GNPTG;* GlcNAc-1-phosphotransferase; treatment; enzyme activity;

Introduction

The autosomal recessive lysosomal disorders mucolipidosis type II (MLII, inclusion cell or I-cell disease) and type III (MLIII, pseudo-Hurler polydystrophy disease) are caused by defects in the GlcNAc-1-phosphotransferase complex(EC 2.7.8.17)which is composed by three subunits: α, β, and γ. Mutations in the gene encoding the α- and β-subunits (*GNPTAB*) lead to MLII, or to the clinically milder condition, MLIII alpha/beta. MLIII gamma arises from mutations in the *GNPTG* gene encoding the γ-subunit[1, 2]. MLII patients have severe psychomotor retardation, short stature, coarse facial features, gingival hypertrophy, macroglossia, inguinal hernias, cardiopulmonary complications, and hearing loss. It is a fatal disease with death occurring in the first decade of life. MLIII is an attenuated form of the disease, with later onset, slower progression and less severe clinical symptoms, which enables survival into adulthood[3].

The only specific treatment available is the transplant of hematopoietic stem cells (HSCT). This procedure was performed in a relatively small number of ML II patients and their effectiveness is not proven yet. The largest study included 22 patients [4], with 27% survival rate at the last follow-up; survivors had overall delay of development, and often requires complex medical support, such as gastrotomy tubes for nutrition and tracheostomy with mechanical ventilation. Meanwhile, HSCT remains an alternative for patients diagnosed early with ML II. There are no reports of HSCT to ML III.*In vitro* study with genistein (glycosaminoglycan synthesis inhibitor) demonstrated a reduction in the accumulation of heparan sulfate in fibroblasts of patients with ML II [5] but clinical trials have not been performed yet. These hurdles have led researchers to develop new treatment approaches for ML II and III.

One potential treatment has arisen from studies that described the mechanism of stop codon read through (SCRT) since MLIII gamma can be associated with nonsense mutations. This therapeutic strategy pharmacologically suppresses translation termination at premature termination codons (PTC) in order to restore expression of functional protein [6–9]. Aminoglycoside antibiotics such as gentamicin and geneticin were the first small-molecule drugs with promising results, and this approach has been validated by numerous *in vitro* and *in vivo* experiments in different disorders, including cystic fibrosis (CF), Duchene muscular dystrophy (DMD), Hurler's syndrome (MPS I), and diabetes, among others [10–13]. Aside from aminoglycosides, chloramphenicol is also efficient for SCRT and maybe also acts as pharmacological chaperone[14, 15].

The aim of this study was to evaluate the effect of treatment with geneticin and chloramphenicol upon enzyme activity and gene expression of *GNPTG* in fibroblasts from patients with ML III gamma carrying nonsense mutations.

Materials and methods

Fibroblasts from threeadults ML III gamma patients (Patients A, B, C; Table 1) described by our group elsewhere[16, 17]and one normal control were included in the study. All patients did not show any pathogenic mutation in *GNPTAB* and had been previously investigated regarding their cDNA sequence of *GNPTG* which surprisingly did not show any alteration (Table 1) [17].

The cells were obtained under informed consent and the project was approved by the institutional review board of Hospital de Clínicas de Porto Alegre, Brazil.

Experimental design and cell culture

Fibroblasts were maintained in culture with Dulbecco's Modified Eagle's Medium (DMEM - Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 1% penicillin/streptomycin (P/S) (Gibco, USA) and 2 mM L-glutamine (Gibco, USA). Cells (2x 10^5)were plated in a 6-well-plate and were kept in culture for 24 h with medium supplemented with 200 µg/mL of geneticin (Sigma-Aldrich, DE), 200 µg/mL of chloramphenicol (Acros Organics, USA) or without treatment (n=6 wells for enzyme activity and n=3 wells for qRT-PCR analysis).

mRNA analysis

RNA extraction was performed with RNeasy mini kit (Qiagen, DE) and the conversion to cDNA was made using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). *GNPTG* mRNA levels were determined by qRT-PCR using 2x SYBR Green PCR Master Mix (Applied Biosystem, USA) with the Mx3000P (Stratagene, NL). *GAPDH* was chosen as housekeeping gene. Primers and conditions were performed as described by Ho et al. [18]with modifications. The relative quantification of the RNA was normalized to the level of *GAPDH* mRNA in the same cDNA using the comparative CT method ($2^{-\Delta\Delta CT}$).

Enzyme assays

GlcNAc-1-phosphotransferase activity was measured indirectly through the measurement of 3 lysosomal enzymes (alpha-mannosidase, beta-galactosidase, beta-glucuronidase) in the supernatant and in the intracellular medium either without or after 24 h of treatment with geneticin or chloramphenicol. Alpha-

mannosidase activity was measured using fluorimetric substrate 4-Methylumbellyferyl-alpha-D-mannopyrano (Sigma-Aldrich, DE) [19]; beta-galactosidase was measured using 4-Methylumbellyferyl-beta-D-galactoside (Sigma-Aldrich, DE) [20] and; beta-glucuronidase using 4-Methylumbellyferyl-beta-D-glucoronide (Sigma-Aldrich, DE) [21]. Results were calculated as nmol/h/mg protein or nmol/h/mL and compared to non-treated normal cells.

Statistical analysis

Statistical analysis was performed by the IBM SPSS Statistics version 22 using parametric and non-parametric tests. The comparisons between control, geneticin and chloramphenicol groups were made for all the patients. Statistical differences among groups were considered when $p<0.05$.

Results

mRNA quantification without treatment (Fig. 1)

The values obtained in the non-treated fibroblasts samplesindicate nonsense and frameshift mutations are associated with a significant decrease in mRNA *GNPTG* levels compared with the median controls values.

mRNA quantification and enzymatic assays pos-treatment (Table 2 and Fig 1)

Geneticin treatment led to a statistical increase in the levels of mRNA *GNPTG* of the patients A and C (Figure 1A and B). Chloramphenicol had a positive effect upon mRNA of *GNPTG* level of patientsB and C. These two treatments had not effect in the normal fibroblast's mRNA expression.

Geneticin and chloramphenicol presented effect (Table 2) upon intracellular alpha-mannosidase activity in the patient A. Both treatments do not have positive effect upon beta-glucuronidase and beta-galactosidase activities in patients A, B and C. On the same way, chloramphenicol enhance the intracellular alpha-mannosidase, beta-glucuronidase and beta-galactosidase activities in the normal fibroblast. Geneticin increased the intracellular beta-glucuronidase and beta-galactosidase activities in these cells.

Discussion

Several therapeutic approaches have been investigated for patients with lysosomal diseases (LD), but most of these diseases remain without an effective treatment option, especially for neurological manifestations. In this scenario, MLII and III are include. Experimental therapies are being developed and tested, such as reduction of substrate synthesis, the use of chaperones, agents for induction of gene therapy and the alternate translation.

This is the first study showing the effect of treatment with geneticin and chloramphenicol on MLIII gamma patients' cells. Many works shown good results in monogenic diseases, but never in one, where two genes encode the deficient protein. In our study, a statistically significant increase in mRNA levels in two patients was observed after geneticin treatment. However, the mechanism of increased is unknown.

It is known that read through efficiency is inversely correlated with translation-termination efficiency. The highest read through efficiency is for the UGA stop codon, exactly the created by c.244_247dupGAGT (p.F83Ter), followed by UAG[10, 20], the stop codon created by c.328G>T (p.E110Ter). The translation-

termination efficiency can explain a better result in mRNA and enzyme activity level to the patient A, a heterozygote for the p.F83Ter and p.E110Ter in compared with patient C, a homozygote for the mutation p.E110Ter.

In cases of efficient nonsense-mediated mRNA decay (NMD),a mechanism where mRNAs containing PTC are degraded, the level of nonsense transcripts is markedly reduced, being insufficient to generate enough functional proteins even when gentamicin is provided. It is worth noticing that the position of the premature termination codon is only one among the factors that determine NMD efficiency, as patients with the same genotypes and different NMD levels have been described [23]. This can explain our discrepant results in patients with the same genotype, B and C.

Chloramphenicol also increase mRNA levels and show effect upon enzyme activity. This is not surprise, since Thompson et al. [14] have shown that it could be used to induce SCRT and Mayer et al. [15]that it would be acting as a pharmacological chaperone. Several chemical compounds have been used as pharmacological chaperones being able to restore the biological activity of misfolded proteins in many human diseases[24].

Another point to be analyzed is mRNA editing. Already report for our group [17], mRNA editing occurs in patients B and C what is confirmed by the pre-treatment mRNA sequencing. In all patients the normal wild type *GNPTG* cDNA sequence was evident with regard to position c.328G>T (p.E110Ter). In this case, we do not expect an increase in enzymatic assays since geneticin and chloramphenicol are SCRT or chaperons drugs and, patients not present mRNA with stop codon mutations. The patients present a normal mRNA, but in really low levels. The

results lead to believe that aminoglycosides are acting in another way, but no SCRT.

There was no effect of geneticin or chloramphenicol on mRNA levels in but on enzyme activities of normal cells. This result is different of those described by Keeling and Bedwell [25] and similar to that found by Valenzano et al. [26]who showed a decrease on enzyme activity in normal controls after treatment.

Therapies that enhance residual enzyme activity could be beneficial for certain LDs, as it is known that even a small increase (15 to 20%) in enzyme activity can be sufficient to induce clinical improvement [27]. Considering this and the large allelic heterogeneity observed in ML II and III patients, we consider that this approach is not a potential therapy that can be used. This preliminary and concept study must be repeated with other drugs aiming better results and clinical application. NMD inhibitors should be used too with the aim to prove the NMD occurrence and aiming better results.

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Table 1. Brazilian patients with ML III gamma included in this study.

Patient	Phenotype	Genotype (*GNPTG* gene)		cDNA*
		Protein	cDNA	
A	ML III	p.F83Ter;E110Ter	c.244_247dupGAGT;328G>T	N/N
B	ML III	p.E110Ter;E110Ter	c.328G>T;328G>T	N/N
C	ML III	p.E110Ter;E110Ter	c.328G>T;328G>T	N/N

N= wild sequence; *cDNA sequenced without treatment;

Table 2. Enzymatic activities after geneticin or chloramphenicol treatment in Mucolipidosis III gamma and control fibroblasts.

Treatment	Sample	Enzymes	Patients			Control
			A	B	C	
Chloramphenicol	Intracellular	β-glucuronidase	-	-	-	↑
		β-galactosidase	-	-	-	↑
		α-mannosidase	↑	-	-	↑
	Supernatant	β-glucuronidase	-	-	-	-
		β-galactosidase	-	-	-	↓
		α-mannosidase	-	-	-	-
Geneticin	Intracellular	β-glucuronidase	-	-	-	↑
		β-galactosidase	-	-	-	↑
		α-mannosidase	↓	-	-	-
	Supernatant	β-glucuronidase	-	-	-	-
		β-galactosidase	-	-	-	-
		α-mannosidase	-	-	-	-

(↑) Statistical significance increase; (↓) Statistical significance decrease; (-) statistical non-significance;

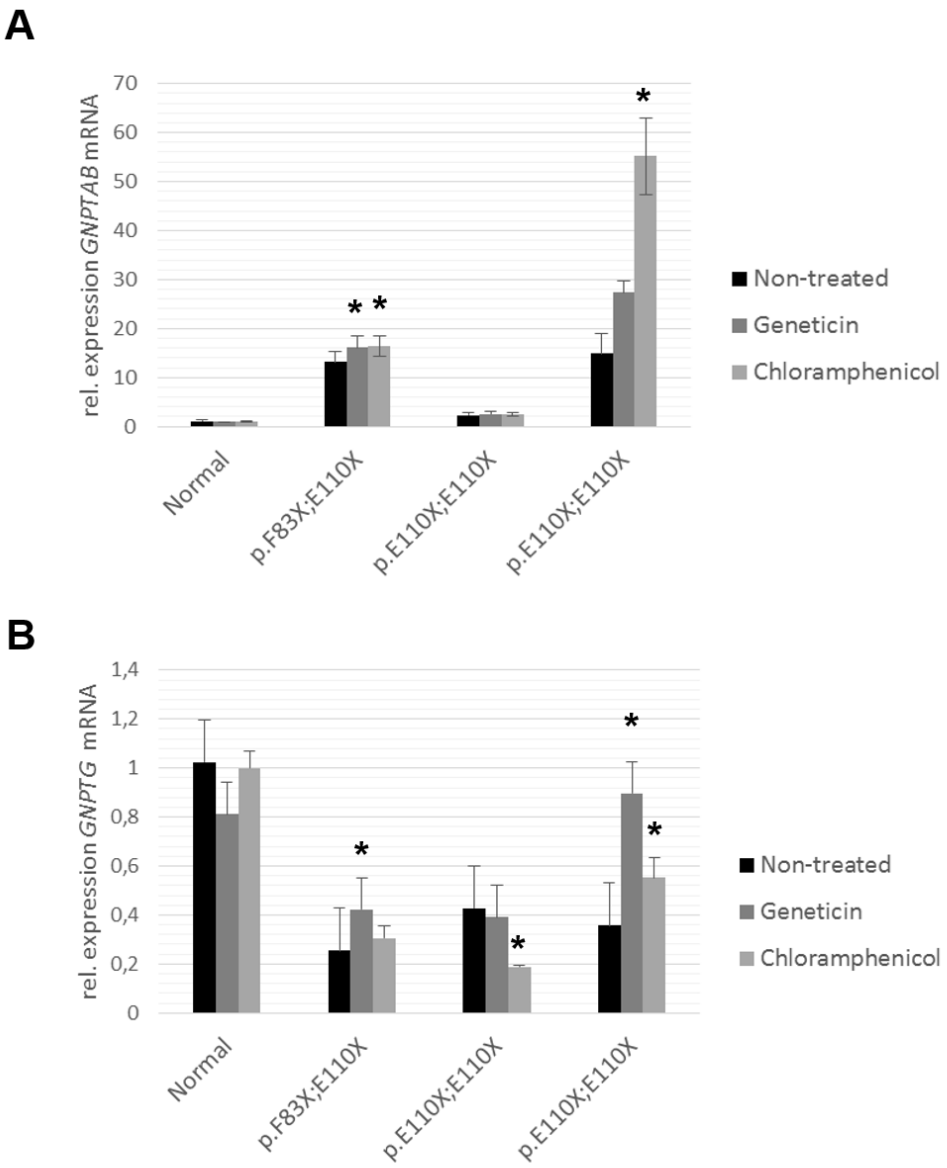

Figure 1. *GNPTG* mRNA expression in Mucolipidosis III gamma fibroblasts with or without geneticin or chloramphenicol treatment. The relative levels of *GNPTG* mRNA expression were determined in fibroblasts of ML III gamma patients by real-time PCR normalized to *GAPDH* expression. Data are expressed as the mean fold change in respective gene expression ± SD (*$p < 0.05$).

5 DISCUSSÃO

As Mucolipidoses II e III são doenças raras, multissistêmicas, herdadas de forma autossômica recessiva e causadas pela deficiência da enzima GlcNAc-1-fosfotransferase. Codificada pelos genes *GNPTAB* e *GNPTG*, a GlcNAc-1-fosfotransferase é uma enzima hexamérica ($\alpha_2\beta_2\gamma_2$) chave para a formação do marcador de reconhecimento M6P em enzimas lisossômicas recém sintetizadas, marcação essa, necessária para a internalização destas hidrolases nos lisossomas (BRAULKE; RAAS-ROTHSCHILD; KORNFELD, 2013). Mais de 140 mutações já foram descritas em pacientes com ML II e III alfa/beta além de outras 30 descritas em pacientes com ML III gama (HGMD, 2015). A pesquisa de mutações possibilita estabelecer correlações genótipo-fenótipo indicando um prognóstico mais seguro da doença e, através de estudos de expressão, permite compreender a patogenicidade das mutações além de propiciar descobertas em relação à função das subunidades da enzima GlcNAc-1-fosfotransferase.

Neste âmbito, esta tese apresenta os efeitos de 8 mutações em *GNPTAB* identificadas em pacientes brasileiros em relação à estabilidade, localização subcelular, clivagem proteolítica e atividade enzimática da GlcNAc-1-fosfotransferase.

Em um paciente do sexo mascuino com MLIII alfa/beta, as mutações novas p.T1223del (c.3668_3670delCTA) e p.T644M (c.1931_1932CA>TG) foram encontrados em heterozigose em *GNPTAB*. Localizado na porção luminal da subunidade α, a mutação de sentido trocado p.T644M altera o potencial motivo de N-glicosilação ^{642}NST^{644}o que pode contribuir para a interação dependente de glicosilação entre as subunidades α e β. Já a deleção p.T1223del leva à remoção de um aminoácido no domínio transmembrana da subunidade β. Ambas as mutações resultam em níveis de mRNA e de expressão proteíca comparáveis aos níveis do controle, são corretamente transportados do RE ao Golgi e proteoliticamente clivados. No entanto, a atividade enzimática dos mutantes da enzima GlcNAc-1-fosfotransferase, p.T1223del e p.T644M, foi de 80% e 40%, respectivamente, em relação a atividade enzimática do selvagem. Uma vez que o ensaio enzimático utiliza α-metilmanosídeo (α-MM) como aceptor de fosfato, em vez de enzimas lisossomais, mutações no sítio de ligação para enzimas lisossomais não pode ser detectada enzimaticamente. Neste âmbito,

novas investigações são necessárias para compreender de que forma o mutante p.T644M afeta a enzima GlcNAc-1-fosfotransferase. Em contraste, a mutação p.T1223del afeta o comprimento do segundo domínio transmembrana da proteína precursora das subunidades α/β. Estudos demonstram que o comprimento e os resíduos polares dos domínios transmembranares influenciam a oligomerização e exportação do RE da proteína de membrana ERGIC-53 (NUFER et al., 2003). Tendo em vista que verificou-se o correto transporte ao Golgi da proteína p.T1223del, as alterações conformacionais induzidas por esta mutação podem afetar o reconhecimento das hidrolases lisossomais.

Mutações sem sentido ou que alterem a fase de leitura são ligadas a um fenótipo mais grave das MLs, normalmente ML II alfa/beta. Neste sentido, os dados referentes as mutações p.R587X e p.E757KfsX1 são totalmente compatíveis à literatura por não serem corretamente direcionados ao Golgi e consequentemente, não serem proteoliticamente clivados e causarem a perda da atividade da GlcNAc-1-fosfotransferase (BRAULKE; RAAS-ROTHSCHILD; KORNFELD, 2013; DE PACE et al., 2014).

Os dados que chamam mais a atenção, no entanto, são os obtidos de mutações com sentido trocado presentes na região luminal da enzima e da mutação p.Y937_M972del. As mutações p.C505Y e p.G575R tiveram seu transporte ao Golgi prejudicado mesmo sem alterações presentes nos domínios de sinalização ao transporte RE-Golgi. Por serem parcialmente clivadas, exibem uma atividade residual da GlcNAc-1-fosfotransferase de 3% e 7%, respectivamente. Acredita-se que a substituição de um único resíduo pode afetar o centro catalítico da enzima e assim, ser tão impactante na atividade.

Já a mutação p.I403T, confirmou através do ensaio radioativo para medir a atividade enzimáticada GlcNAc-1-fosfotransferase (~4%) que a ausência de transporte e clivagem proteolítica nas subunidades α- e β- maduras é pré-requisito para a atividade enzimática o que já havia sido relatado para a mutação p.S399F (DE PACE et al., 2014). Na tentativa de entender a importância da região luminal S399- I403, mutações aleatórias foram realizadas dentre os aminoácidos I346- Y421. O que se percebe é que alguns resíduos que compreendem está porção da enzima podem interagir com uma proteína ainda desconhecido no lúmen do RE necessária para sua saída, incorporação de carga

nas vesículas nascentes, ou ainda, transporte ao Golgi o que explicaria os dados obtidos com as mutações p.I403T, p.C505Y e p.G575R. Indo ao encontro desses achados, várias proteínas são conhecidos por funcionarem como subunidades ou chaperones luminais no tráfego de proteínas transportadoras ou de fatores de transcrição do RE para outros organelas (DAS et al., 2009; LANGE et al., 2006; PAULUSMA et al., 2008).

De particular interesse, o mutante p.Y937_M972del (c.2808A>G) que apresente a deleção de 36 aminoácidos da subunidade β, foi parcialmente transportado ao aparelho de Golgi e anormalmente clivado. O local de clivagem do precursor das subunidades α e βestá localizado no motivo de reconhecimento consenso de S1P (R/K) X (hidrofóbico)↓Z, onde X representa qualquer aminoácido e Z, glicina ou treonina preferencialmente (MARSCHNER et al., 2011). Entretanto pelo menos 20 resíduos próximosao sítio de clivagem são necessários para permitir a interação da S1P com a proteína precursora. E, embora não haja outras sequências consenso para o reconhecimento da S1P na subunidade β, os experimentos em células SRD-12B-S1P (deficientes em S1P) demonstraram que a S1P é a protease responsável pela clivagem anormal das subunidade α e β neste constructo. Mesmo que clivada, as subunidades α e β formadas por este mutante não apresentaram qualquer atividade enzimática.

A partir dos resultados conclui-se que as mutações na proteína precursora das subunidades α/β codificada pelo gene *GNPTAB* podem prejudicar as diferentes etapas de um processo que inclui: i) o transporte da proteína precursora das subunidades α/β recém-sintetizada a partir do RE para o aparelho de Golgi, ii) ativação proteolítica pela protease S1P, e iii) a capacidade de transferir resíduos de GlcNAc-1-fosfato sobre os resíduos de manose de oligossacarídeos. Para todas as mutações investigadas as três possibilidades foram analisadas e discutidas.

Pacientes com ML III gama são caracterizados clinicamente como pacientes com um fenótipo leve quando comparados aos ML alfa/beta (BRAULKE; RAAS-ROTHSCHILD; KORNFELD, 2013; CATHEY et al., 2008, 2010). Isto pode ser confirmado neste trabalho por uma completa avaliação clínica para competências e funcionalidades além da análise de *GNPTAB* e *GNPTG* em três pacientes com diagnóstico clínico de ML III. As técnicas

moleculares empregadas aqui, permitiram a padronização de um protocolo para a análise molecular de *GNPTG* além da identificação deduas novas mutações patogênicase de uma outra variação gênica. A mutação nova p.E110X foi encontrada em homozigose em dois irmãos e em heterozigose em uma outra paciente com ML III gama. Em *cis* a mutação p.E110X, a variante c.-112C>G foi confirmada nos três pacientes e seus pais. A outra mutação patogênica identificada, p.F83X, interessantemente não foi confirmada nos pais da paciente. A mutação p.E110X foi confirmada no pai e a p.F83X não foi identificada da mãe da probando mesmo após análise de DNA extraído de saliva e sangue total. Neste caso, a mutação p.F83X foi atribuída como um evento *de novo* que ocorreu em um único óvulo ou de um mosaicismo germinativo no óvulo materno após confirmação de paternidade no trio. Eventos como mutações *de novo* e inserções correspondem a 74 e 3 novos casos por geração, respectivamente (EYRE-WALKER; KEIGHTLEY, 2007). Já em doenças autossômicas recessivas foi identificada em um paciente com Ataxia-telangiectasia, 2 pacientes com Gaucher (SARANJAM et al., 2013) e agora, em uma paciente com ML III gama (VELHO et al., 2014). Neste trabalho, a análise dos pais mostrou ser uma interessante ferramenta tanto para confirmação das mutações, aconselhamento genético, como para a descoberta de eventos interessantes como o caso da mutação *de novo.*

Ao investigar o potencial efeito das mutações sem sentido através de mRNA, resultados divergentes aos encontrados em DNA genômico foram obtidos.Não foi possível identificar a mutação c.328G>T (p.E110X), no lugar desta, todos os pacientes apresentaram a sequência selvagem de *GNPTG*, evento denominado edição de RNA (c.328G@T - PENG et al., 2012; RAMASWAMI; LI, 2014; RAMASWAMI et al., 2013). Com o advento da bioinformática e dos sequenciamentos de nova geração, mais casos como esse são apresentados em indivíduos de todos os reinos. Ao analisar os bancos de dados de edição de RNA, é possível identificar muitos eventos de edição de RNA em *GNPTAB* e *GNPTG* porém, nenhum relacionado às ML II e III, sendo esse, o primeiro caso (FARAJOLLAHI; MAAS, 2010; KNOOP, 2011b; RAMASWAMI; LI, 2014).

Numa primeira análise pode-se pensar que, ocorrendo edição de RNA, os pacientes com ML III gama seriam heterozigotos ou homozigotos normais, no caso, não afetados. Porém, o nível de expressão de *GNPTG* deve ser levado em consideração. Níveis de mRNA muito próximos a zero foram obtidos o que confirma a patogenicidade das mutações em *GNPTG* bem como o diagnóstico dos pacientes.Processos como a degradação de RNA mediado por mutações sem sentido (NMD) ou a presença da mutação c.-112C>G em homozigose na região 5'UTR são completamente capazes de explicar os baixíssimos níveis de mRNA o que já foi demonstrado em outras patologias e até em outros pacientes com ML III gama (ALBERS et al., 2012; ALMEIDA et al., 2012; PERSICHETTI et al., 2009).

Ao analisar a relação entre os genes *GNPTAB* e *GNPTG* diferentes respostas surgem. Em pacientes MLII e III alfa/beta e em MLIII gama, a redução dos níveis de mRNA de *GNPTAB* e *GNPTG*, respectivamente, pode ser facilmente explicada pela natureza das mutações identificadas nos pacientes. Mutações que geram PTC, identificadas em homozigose ou heterozigose, são previsivelmente alvo do mecanismo de NMD, porém, o que intriga é a expressão do gene não mutado. A expressão de *GNPTG* observada em sangue de pacientes MLII e III alfa/beta pode ser relacionada com a existência de um mecanismo de *feedback* já descrito entre os genes que codificam a enzima GlcNAc-1-fosfotransferase (ENCARNAÇÃO et al., 2009; HO et al., 2007). Neste caso, o gene não alterado, teria sua expressão diminuída em resposta à baixa expressão do gene mutado.

Ainda de acordo com os resultados obtidos com as amostras de sangue de pacientes MLII e III alfa/beta, foipossível distinguir os pacientes de acordo com o fenótipo. Pacientes que apresentam uma baixa expressão de *GNPTG* são classificados como MLII alpha/beta, já o paciente que mostrou um aumento nos níveis de mRNA de *GNPTG* é caracterizado como MLIII alfa/beta.

Em contra partida, em fibroblastos de pacientes com o genótipo p.L1168QfsX5;L1168QfsX5, houve um aumento dos níveis de mRNA de *GNPTG* comparados aos valores médios dos controles. Este resultado sugere um mecanismo de compensação entre *GNPTG*/*GNPTAB*, onde a subexpressão de *GNPTAB* induziu a superexpressão de *GNPTG* (POHL et al., 2009). Neste

mesmo sentido, verifica-se o aumento significativo porém de diferente magnitude, nos níveis de mRNA de *GNPTAB* em sangue e fibroblasto de pacientes com MLIII gama onde o mecanismo compensatório entre as subunidades α/β e γ também estaria sendo verificado (ENCARNAÇÃO et al., 2009; POHL et al., 2009).

Este é o primeiro estudo utilizando amostras de sangue para analisar a expressão dos genes que codificam a GlcNAc-1-fosfotransferase. No sangue, neutrófilos, eosinófilos, basófilos, linfócitos, monócitos e macrófagos compõem a amostra utilizada para a extração de RNA; diferentes tipos celulares em comparação a um único tipo, os fibroblastos. A divergência entre os resultados encontrados pode estar relacionadaao tipo de amostra, sabendo-se que as MLII e MLIII são tecido-específicas (KOLLMANN et al., 2010).

Apesar dos inúmeros esforços e avanços realizados no tratamento de doenças lisossômicas, muitas doenças permanecem sem uma opção efetiva de tratamento ou mesmo, sem tratamento algum. E, neste cenário as ML II e III são incluídas. Terapias experimentais têm sido desenvolvidas e testadas como a terapia de redução de substrato, o uso de chaperonas, tradução alternativa. Neste caminho, o uso de antibióticos com ação sobre a tradução foram testados em fibroblastos de pacientes com MLII alfa/beta e MLIII gama.

Para a realização deste trabalho, três fibroblastos de pacientes com MLIII gama homozigotos e heterozigotos (p.F83X;E110X) para a mutação p.E110X, foram utilizados como modelos de estudo. As respostas *in vitro* dos fibroblastos tratados com geneticina e cloranfenicol foram avaliadas.

Diversos fatores influenciam a resposta à terapia de tradução alternativa como a identidade do códon de terminação e a sequência contexto, particularmente o quarto nucleotídeo. Neste contexto, a eficiência da terapia de tradução alternativa ou *read through* seria inversamente proporcional a eficiência da terminação da tradução (DIOP; CHAUVIN; JEAN-JEAN, 2007a; KIMURA et al., 2005). O códon de terminação com menor eficiência de terminação e consequentemente, a maior eficiência de *read though* é UGA, exatamente o códon criado pelas mutações c.244_247dupGAGT (p.F83X), seguido pelo UAG (DIOP; CHAUVIN; JEAN-JEAN, 2007; KIMURA et al., 2005), códon de terminação criado pela alteração c.328G>T (p.E110X).

Outro fator que deve ser levado em consideração é o decaimento do mRNA em decorrência de códons de parada prematuros (NMD). Este mecanismo é responsável pela acurácia da regulação da expressão gênica (BHUVANAGIRI et al., 2010). Vale ressaltar que a posição do PTC é apenas um entre os fatores que determinam a eficiência NMD. Pacientes com o mesmo genótipo, porém diferentes níveis NMD já foram descritos(LINDE; KEREM, 2008). Neste contexto, a análise dos mRNAs antes e depois do tratamento é um bom indicativo do potencial de cada paciente em responder ao tratamento. Conjuntamente, esses fatores podem explicar melhor os resultados encontrados de expressão gênica e atividade enzimática.

Até o presente momento, a terapia disponível para pacientes com Mucolipidoses II e III restringe-se ao tratamento dos sintomas, representando apenas soluções paliativas. Sendo assim, a continua busca de novas terapias para doenças genéticas, incluindo as MLII e III deve ser incentivada. Apesar de apresentarem grande heterogeneidade genética, a existência de grupos de mutações como as de alterações troca de sentido, de mesmo sentido, que alterem a fase de leitura, ou ainda, mutações sem sentido corroboram para a hipótese de patogênese molecular específica para grupos de mutações. Neste sentido, uma estratégia desenvolvida para um grupo de mutações poderia ser também efetiva, para alterações do mesmo grupo, mesmo que em genes distintos(HU; GATTI, 2008). Seguindo esta linha é que o tratamento deste trabalho foi desenvolvido porém sem comprovação de eficácia.

Estudos de caracterização genética, clínica e populacional sempre contribuirão para a elucidação dos mecanismos da doença e concomitantemente, melhor atendimento ao paciente. Com isto, é de grande importância que esforços e recursos sejam destinados a pesquisas nesta área para o completo entendimento das Mucolipidoses II e III e dos processos biológicos envolvidos.

6 CONCLUSÕES

As conclusões da presente tese estão abaixo relacionadas, de acordo com os objetivos inicialmente propostos.

Objetivos Gerais

I. Verificar a patogenicidade, por meio de ensaios *in-vitro*, das seguintes mutações em *GNPTAB* previamente identificadas em pacientes brasileiros com ML II e III alfa/beta: c.1208T>C, c.1514G>A, c.1723G>A, c.1759C>T, c.1931_1932CA>TG, c.2269_2273delGAAAC, c.2808A>G e c.3668_3670delCTA;

Objetivos Específicos:

i. Inserir as mutações c.1208T>C, c.1514G>A, c.1723G>A, c.1759C>T, c.1931_1932CA>TG, c.2269_2273delGAAAC, c.2808A>G e c.3668_3670delCTA em sequência selvagem do vetor de expressão GNPTAB-pcDNA6/V5-His através da técnica de mutagênese sítio dirigida;
ii. Expressar as sequências mutantes de *GNPTAB* obtidas em linhagem de células HEK e HeLa;
iii. Identificar os efeitos das mutações em nível de mRNA e proteína por expressão e análises de localização;
iv. Avaliar o impacto de cada mutação sobre a atividade da GlcNAc-1-fosfotransferase;

Conclusões:

Mutações sem sentido e que alteram a fase de leitura da enzima GlcNAc-1-fosfotransferase não são corretamente transportados do RE ao aparelho de Golgi, devido a interrupção, a falta dos sinais de exportação localizados nas porções N- e C- terminais da proteína. O não transporte ao Golgi e consequentemente, a ausência da clivagem proteolítica da proteína precursora das subunidades α/β, são responsáveis pela falta de atividade enzimática. Além dessas, mutações com sentido trocado na porção luminal da enzima GlcNAc-1-fosfotransferase também podemter o transporte ao Golgi prejudicado, o que

sugere a presença de um local de contato para uma proteína necessária para a exportação eficiente do RE. O estabelecimento de um ensaio radioativo para medir a atividade enzimática da GlcNAc-1-fosfotransferase confirmou que o transporte para o Golgi e a clivagem proteolítica nas subunidades α- e β- maduras é um pré-requisito para a atividade enzimática. A análise dos mutantes que são corretamente transportados e clivados no aparelho de Golgi, mas carecem de atividade enzimática, sugerem que o centro catalítico é constituído por uma estrutura complexa que inclui os aminoácidos 431-848 da subunidade α e os resíduos 937-986 na subunidade β.

Os presentes dados fornecem uma nova visão sobre requisitos estruturais para localização e atividade da GlcNAc-1-phosphotransferase que podem ajudar a explicar a variabilidade nos fenótipos clínicos de pacientes com MLII e MLIII.

Objetivos Gerais

II. Caracterizar o perfil de mutações de *GNPTG* presente em uma amostra de pacientes com ML III gamma;

Objetivos Específicos

v. Avaliar a relação entre os genes *GNPTG* e *GNPTAB* em pacientes com ML II e III alfa/beta e gama;
vi. Identificar possíveis moduladores do fenótipo clínico de pacientes com ML II e III;

Conclusões:

As técnicas moleculares empregadas neste trabalho, permitiram a identificação de cinco novas mutações patogênicas e de uma outra variação nos genes que codificam a GlcNAc-1-fosfotransferase. As mutações p.F83X, p.E110X, c.233+5G>C e c.-112C>G caracterizam o gene *GNPTG* de quatro pacientes com MLIII gama. Após incesante investigação do trio cuja mutação causal p.F83X foi encontrada, a mesma foi atribuída como um evento *de novo* que ocorreu em um único óvulo ou de um mosaicismo germinativo no óvulo materno. Neste trabalho, a análise dos pais mostrou ser uma interessante ferramenta tanto para confirmação das mutações, aconselhamento genético,

como para a descoberta de eventos interessantes como o caso da mutação *de novo.*

Ao investigar o potencial efeito das mutações sem sentido através de mRNA, não foi possível identificar a mutação c.328G>T (p.E110X), no lugar desta, todos os pacientes apresentaram a sequência selvagem de *GNPTG*, evento denominado edição de RNA (c.328G@T). Porém, níveis de mRNA muito próximos a zero foram obtidos o que confirma a patogenicidade das mutações em *GNPTG* bem como o diagnóstico dos pacientes.

Em pacientes MLII e III alfa/beta e em MLIII gama, a redução dos níveis de mRNA de *GNPTAB* e *GNPTG*, respectivamente, pode ser facilmente explicada pela natureza das mutações identificadas nos pacientes. O que intriga, porém, é a expressão do gene não mutado. Em amostras de sangue, o gene não alterado, teria sua expressão diminuída em resposta à baixa expressão do gene mutado. Em contra partida, os resultados em fibroblastos sugerem um mecanismo de compensação entre *GNPTG*/*GNPTAB*, onde a subexpressão de um, induz a superexpressão do outro. A divergência entre os resultados encontrados pode estar relacionada ao tipo de amostra, sabendo-se que as MLII e MLIII são tecido-específicas.

Objetivo Gerais:

III. Analisar o efeito de gentamicina e cloranfenicol sobre a atividade das enzimas α-manosidase, β-glicuronidase e β-galactosidase em fibroblastos de pacientes com ML III gama heterozigotos ou homozigotos para mutações sem sentido em *GNPTG.*

Objetivos Específicos:

vii. Quantificar a atividade de hidrolases lisossomais em fibroblastos de pacientes antes e após o tratamento com os antibióticos;

viii. Analisar os níveis de mRNA de *GNPTG* em fibroblastos de pacientes antes e após o tratamento com gentamicina e cloranfenicol.

Conclusões:

Fibroblastos de três de pacientes com MLIII gama foram tratados com geneticina e cloranfenicol. Ambos os tratamentos apresentaram efeitos positivos sobre a expressão gênica e atividade enzimática na maioria dos pacientes, porém, a resposta ao tratamento, mais que genótipo-específico, foi paciente-específico.

Este estudo de conceitos não demonstrou a possibilidade e eficâcia de um tratamento ser desenvolvido baseado na utilização de compostos não antibióticos atuarem sobre a tradução alternativa ou *read through* em pacientes com Mucolipidoses II e III.

7 PERSPECTIVAS

A realização deste projeto permitiu compreender a importância do desenvolvimento, por parte do nosso grupo de pesquisa, de novos projetos sobre ML II e III que tenham como objetivos:

- Implementar a técnica radioativa para avaliação da atividade da enzima GlcNAc-1-fosfotransferase;
- Utilizar novos compostos sem atividade antimicrobiana com ação sobre códons de terminação prematura em fibroblastos de pacientes brasileiros com ML II e III;
- Avaliar o efeito concomitante de compostos que inibam o NMD e de drogas que induzam o *read through;*
- Analisar a possível existência de uma proteína citoplasmática que auxilia o transporte da enzima GlcNAc-1-fosfotransferase do retículo endoplasmático ao complexo de Golgi.

8 CONSIDERAÇÕES ÉTICAS

Este projeto é englobado por um estudo maior intitulado “Estudo abrangente sobre as Mucolipidoses II e III no Brasil: uma oportunidade para a compreensão dos processos genéticos que controlam o tráfego intracelular de proteínas” e aprovado pela Comissão Científica e de Pesquisa e Ética em Saúde do Grupo de Pesquisa e Pós Graduação do HCPA (projeto GPPG/ HCPA 07-0244). Além deste, projetos complementares como “Análise do gene *GNPTG e*m pacientes brasileiros com Mucolipidoses II e III” (projeto GPPG/ HCPA 11-0477), “Estudo do gene *GNPTG* e sua relação com *GNPTAB* em pacientes brasileiros com Mucolipidoses II e III” (projeto GPPG/ HCPA 12-0018) e “Uso de gentamicina e cloranfenicol *in vitro* como estratégia de tratamento para as Mucolipidoses II e III” (projeto GPPG/ HCPA 12-0019) foram elaborados e aprovados pela Comissão Científica e de Pesquisa e Ética em Saúde do Grupo de Pesquisa e Pós Graduação do HCPA durante esta tese para os estudos relacionados mais diretamente aos objetivos propostos.

Em relação ao armazenamento de materiais ou uso de materiais armazenados em pesquisas anteriores, este estudo seguirá todas as normas das Resoluções CNS 196/96 e CNS 441/2011, sendo que a responsável pela guarda e pela autorização de uso de material será a Dra. Ida Vanessa D. Schwartz.

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ANEXO I

Mucolipidosis II and III alpha/beta in Brazil: Analysis of the *GNPTAB* gene

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Short communication

Mucolipidosis II and III alpha/beta in Brazil: Analysis of the *GNPTAB* gene

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ABSTRACT

Mucolipidosis II and III (MLII and MLIII) alpha/beta are rare autosomal recessive lysosomal storage diseases (LSDs) caused by pathogenic variations in the *GNPTAB* gene. *GNPTAB* gene codes for the α and β subunits of phosphotransferase, the enzyme responsible for synthesis of the mannose-6-phosphate (M_6P) marker that directs lysosomal enzymes to the lysosome.

Objectives: The objective of this study is to identify sequence variations of the *GNPTAB* gene in Brazilian patients with MLII and MLIII alpha/beta.

Method: Sequencing of the *GNPTAB* gene was performed in samples of gDNA extracted from the peripheral blood of patients with MLII/III diagnosed at a national reference center for LSDs.

Results: Twelve unrelated patients, from several regions of Brazil, were included in this study. Only one was born of consanguineous parents. All patients were found to carry at least one nonpathogenic variation. Nine causal sequence variations were found: c.242G>T (p.W81L); c.1123C>T (p.R375X); c.1196C>T (p.S399F); c.1208T>C (p.I403T); c.1514G>A (p.C505Y); c.1759C>T (p.R587X); c.2808A>G (p.Y937_M972del, novel mutation); c. 2269_2273delGAAAC (p.E757KfsX2, novel mutation); and c.3503_3504delTC (p.L1168QfsX5). Both pathogenic variations were identified in 8 of 12 patients; in four patients, only one pathogenic variation was identified. Mutation c.3503_3504delTC, located in exon 19, was the most frequent pathogenic variation found (n = 11/24 alleles). The deleterious effect of the c.2808A>C mutation on splicing was confirmed by cDNA analysis.

Discussion/conclusions: Our findings confirm that the *GNPTAB* gene presents broad allelic heterogeneity and suggests that, in Brazilian ML II and III patients, screening for mutations should begin at exon 19 of the *GNPTAB* gene. Further analyses will be conducted on patients in whom both pathogenic mutations have not been found in this study.

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Abbreviations: A, adenine; ARSA, arylsulfatase A; CAA, mRNA codon for amino acid glutamine; CAG, mRNA codon for amino acid glutamic acid; cDNA, DNA complementary to RNA; CNPq, Conselho Nacional de Pesquisa e Desenvolvimento; CUA, mRNA codon for amino acid leucine; CUG, mRNA codon for amino acid leucine; DBS, dried blood spot; dNTPs, deoxyribonucleoside triphosphate; EC, enzyme code; ELISA, enzyme-linked immunosorbent assay; FAPERGS, Fundação de Amparo à Pesquisa do estado do Rio Grande do Sul; FIPE, Fundo de Incentivo à Pesquisa e Eventos do Hospital de Clínicas de Porto Alegre; GAGs, glycosaminoglycans; gDNA, genomic deoxyribonucleic acid; GlcNAc-PT, UDP-N-acetylglucosamine:lysosomal hydrolase N-acetyl_1-phosphotransferase; *GNPTAB*, official symbol for the gene "N-acetylglucosamine-1-phosphate transferase, alpha and beta subunits"; *GNPTG*, official symbol for the gene "N-acetylglucosamine-1-phosphate transferase, gamma subunit"; HCPA, Hospital de Clínicas de Porto Alegre; IDS, iduronate-sulfatase; IDUA, α-L-iduronidase; IEM, inborn errors of metabolism; kDa, kilodaltons; LC, liquid chromatografy; MS, mass spectrometry; LREIM-HCPA, Laboratório de Erros Inatos do Metabolismo Hospital de Clínicas de Porto Alegre; LSD, lysosomal storage disease; $MgCl_2$, magnesium chloride; ML II, mucolipidosis type II; ML III, mucolipidosis type III; mM, millimolar; mRNA, messenger RNA; M_6P, mannose-6-phosphate; PCR, polymerase chain reaction; Pmol, picomol; PolyPhen, polymorphism phenotyping; PRONEX, Programa de Apoio a Núcleos de Excelência; RNA, ribonucleic acid; SIFT, sorting intolerant from tolerant; *taq* DNA polymerase, thermostable DNA polymerase.

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1. Introduction

Mucolipidosis type II alpha/beta (MLII; MIM#252500) and type III alpha/beta (MLIII; MIM#252600) are inherited autosomal recessive diseases caused by deficient activity of UDP-N-acetylglucosamine: lysosomal hydrolase N-acetyl-1-phosphotransferase (UDP-GlcNAc-phosphotransferase, GlcNAc-PT or phosphotransferase; EC 2.7.8.17) (Brooks et al., 2007; Tiede et al., 2005; Zarghooni and Dittakavi, 2009). Phosphotransferase plays a role in the synthesis of mannose-6-phosphate (M_6P), a marker molecule responsible for directing lysosomal hydrolases to the lysosome (Braulke et al., 2008; Cathey et al., 2008; Tiede et al., 2005). In the absence of M_6P residues, correct targeting of lysosomal hydrolases is impaired, which results in massive secretion of these enzymes in both the extracellular space and body fluids, as well as a decrease of their activity in cells such as fibroblasts (Braulke et al., 2008; Cathey et al., 2008; Encarnação et al., 2009). MLII is the most severe form of the disease, and is often apparent at birth; progression is rapid, leading to death as early as the first decade of life due to cardiorespiratory complications. Conversely, MLIII follows a slower clinical course, and patients have been known to survive until the eighth decade of life (Cathey et al., 2010; Encarnação et al., 2009; Kornfeld and Sly, 2001). MLII and III correspond to the extreme phenotypes associated with phosphotransferase, deficiency, and patients with intermediate clinical manifestations have also been described (Cathey et al., 2010).

Phosphotransferase is a hexameric protein composed of two α subunits, two β subunits, and two γ subunits (Bao et al., 1996; Tiede et al., 2005), where the α and β subunits are encoded by the *GNPTAB* gene, located in chromosome 12q23.3, and the γ subunits encoded by the *GNPTG* gene, located in chromosome 16p13.3 (Cathey et al., 2008; Encarnação et al., 2009; Kudo et al., 2005; Zarghooni and Dittakavi, 2009). Patients who are homozygous or compound heterozygous for pathogenic mutations in *GNPTAB* (ML alpha/beta) exhibit a phenotype consistent with MLII or MLIII, whereas patients who are homozygous or heterozygous for pathogenic mutations in *GNPTG* (ML gamma) exhibit a phenotype compatible with MLIII (Bargal et al., 2006; Cathey et al., 2010; Encarnação et al., 2009; Persichetti et al., 2009; Tappino et al., 2009).

The main objective of the present study was to conduct an analysis of the *GNPTAB* gene in Brazilian patients with MLII and III alpha/beta.

2. Materials and methods

Twelve unrelated patients with a biochemical diagnosis of MLII or MLIII were included in the study. They were recruited from the cohort of patients (approximately 40,000) investigated for inborn errors of metabolism (IEM) at the Reference Laboratory for IEM at Hospital de Clínicas de Porto Alegre, Brazil (LREIM-HCPA), from 1983 to 2011. The LREIM-HCPA is a national reference laboratory for the diagnosis of lysosomal diseases, and its database of diagnoses probably includes most cases of MLII/III diagnosed in Brazil. Biochemical diagnosis of MLII/III at LREIM-HCPA includes analysis of arylsulfatase A (ARSA; EC 3.1.6.8) and several other lysosomal hydrolases, including α-L-iduronidase (IDUA; EC 3.2.1.76), iduronate-sulfatase (IDS; EC 3.1.6.12), β-glucuronidase (GUSB; EC 3.2.1.31), and β-hexosaminidase (EC 3.2.1.30), in plasma. Measurement of the activity of these enzymes in fibroblasts, as well as analysis of glycosaminoglycans (GAGs) and sialyloligosaccharides in urine, are also performed whenever samples are available. If only filter paper samples are available, the activity of IDS, GUSB, and hexosaminidases is analyzed.

Whole blood samples were collected for gDNA and RNA extraction. Determination of clinical severity (ML II or III) took into account the criteria usually reported in the literature, such as age at diagnosis, survival, and extent of skeletal involvement (and, consequently, patient height) (Cathey et al., 2010). The patients included in this study were under the care of different physicians, but a summary of their clinical records was always sent alongside each patient's blood sample, so that the classification assigned by the attending physician was always reviewed and confirmed by the investigators. Whenever possible, a sample of gDNA from the parents was also obtained for confirmation of the presence of the mutations found in the patient.

Genomic DNA was extracted from peripheral blood leukocytes with the DNeasy Blood and Tissue Kit (Qiagen, Germany). The 21 exons that comprise the *GNPTAB* gene, as well as the intron-exon boundaries and part of the 5′ and 3′ untranslated regions, were amplified from the specific sequences of oligonucleotides projected for this study. Amplification was performed by the polymerase chain reaction (PCR) using 50 ng of gDNA, 16 pmol of each oligonucleotide, 0.6 mM of dNTPs, 2.4 mM of $MgCl_2$, 1× reaction buffer, and 1 unit of *taq* DNA polymerase. The annealing temperatures and oligonucleotide sequences are available as supplementary data.

Sample sequencing was performed using the automatic ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems). The reference sequence of the *GNPTAB* gene was GenBank accession nº. NM_024312.3. Each mutation found was confirmed by sequencing performed with a new amplicon and the oligonucleotide inverse to that used in the first stage.

In silico analysis of the potential effect of missense mutations was done by means of PolyPhen2 (Polymorphism Phenotyping)

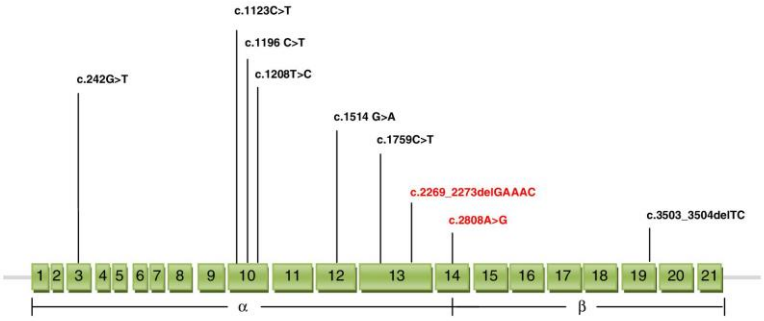

Fig. 1. *GNPTAB* gene: localization of pathogenic mutations found in the present study (modified from Tappino et al. (2009)). In red: mutations described for the first time in the present study. In black: mutations previously described in the literature. The α- and β-subunits are represented.

(http://genetics.bwh.harvard.edu.pph2/) and SIFT (Sorting Intolerant From Tolerant) (http://sift.jcvi.org/www.SIFT_seq_submit2.html). The MaxEntScan software was used to evaluate the effect of point mutations on generation or exclusion of splice sites (http://http://genes.mit.edu/burgellab/maxent/Xmaxentscan_scoreseq.html). The frequency of synonymous codon usage was evaluated according to the database of codon usage in *Homo sapiens* (http://www.kasuza.or.jp/codon/cgi-bin/showcodon.cgi?species=9606). To evaluate the pathogenicity of the novel exonic point mutation found (c.2808A>C), a hundred control alleles were analyzed using 0.3 U of *Hyp 188I* restriction enzyme (New England Biolabs, USA), 2 μl of buffer 4 (supplied with the restriction enzyme) and 17.7 μl of amplicon; samples were incubated for 3 h at 37 °C. Total RNA extraction was performed on a whole blood sample from the patient presenting this mutation using the Paxgene blood RNA Kit (Qiagen, Germany), and conversion to cDNA was made using the Superscript II conversion kit (Invitrogen, USA), according to manufacturer instructions. cDNA was subsequently sequenced as described above.

This study was approved by the Hospital de Clínicas de Porto Alegre (HCPA) Research Ethics Committee.

3. Results

Of the patients included, eight were classified as having MLII. Only one (patient 12) was born of consanguineous parents. Regarding biochemical investigation, fibroblast samples were unavailable for three patients (patients 6, 7 and 12); patient 6 was investigated only through a dried blood spot (DBS) sample. Urinary GAG measurements were normal in 10 of 10 patients, thin-layer chromatography of GAGs was abnormal in 4 of 10 patients (patient 4, dermatan/keratan sulfate; patients 5 and 7, dermatan sulfate; patient 9, keratan sulfate), while thin-layer chromatography of sialyloligosaccharides was abnormal in 1 of 3 patients (patient 10). Detailed data on the clinical and biochemical findings presented by the patients are available as supplementary material.

Nine causal sequence variations were found, two of which were novel: c.2808A>G (p.Y937_M972del) and c. 2269_2273delGAAAC (p.E757KfsX2) respectively (Fig. 1, Table 1). Both pathogenic mutations were identified in 8 of 12 patients, and only one pathogenic mutation was identified in four patients (Table 1). Mutation c.3503_3504delTC, located in exon 19, was the most frequent pathogenic mutation (n = 11/24 alleles; Table 1).

At least one nonpathogenic variant was found in each patient. The mutations c.365+145C>T in intron 4 (n = 17/24) and c.-41_-39delGGC in the 5′UTR (n = 16/24) were the most frequent nonpathogenic variants found, and the nonpathogenic mutation c.323+20delT (intron 3) has not been reported elsewhere. Additional information on nonpathogenic mutations is available as supplementary material.

3.1. The c.2808A>G mutation

As the c.2808A>G mutation (exon 14) was apparently not associated with an amino acid change, we decided to investigate its effect on splicing. According to MaxEntScan software (http://http://genes.mit.edu/burgellab/maxent/Xmaxentscan_scoreseq.html) results, this mutation would strongly interfere with *GNPTAB* mRNA splicing (Fig. 2). In fact, this mutation creates a similar sequence to the canonical donor splice site. cDNA analysis for this patient showed the presence of an abnormal transcript in which the final 108 bp of exon 14 is absent (Fig. 3). We did not perform expression studies, but this isoform is predicted to generate a truncated protein (p.Y937_M972del), with deletion of 36 amino acids of the phosphotransferase β-subunit. This mutation was not found in any of the 100 control alleles analyzed (Fig. 4).

Table 1
Pathogenic mutations found in the *GNPTAB* gene of 12 unrelated Brazilian patients with Mucolipidosis II and III alpha/beta.

Patients	Genotype (cDNA)	Genotype (protein)	Phenotype	In silico analysis of the effect in the protein*			Parental genotype**
				Polyphen2	SIFT	MaxEntScan	
1	c.[1123C>T]+[?]	p.[R375X]+[?]	ML II	NA	NA	NI	Mother: c.[1123C>T]+[N]
2	c.[1514G>A]+[1759C>T]	p.[C505Y]+[R587X]	ML III	Probably deleterious	Intolerable	NI	Mother: c.[1514G>A]+[N] Father: c.[1759C>T]+[N]
3	c.[1208T>C]+[3503_3504delTC]	p.[I403T]+[L1168QfsX5]	ML III	Probably deleterious	Intolerable	NI	Mother: c.[1208T>C]+[N] Father: c.[3503_3504delTC]+[N]
4	c.[3503_3504delTC]+[?]	p.[L1168QfsX5]+[?]	ML II	NA	NA	NA	Mother: c.[N]+[N] Father: NA
5	c.[242G>T]+[?]	p.[W81L]+[?]	ML II	Probably deleterious	Intolerable	NI	NA
6	c.[2269_2273delGAAAC]+[2269_2273delGAAAC]	p.[E757KfsX2]+[E757KfsX2]	ML II	NA	NA	NA	Mother: c.[2269_2273delGAAAC]+[N]
7	c.[2808A>G]+[3503_3504delTC]	p.[Y937_M972del]+[L1168QfsX5]	ML II	NA	NA	Probably splice site interference (exon 14)	Mother: c.[3503_3504delTC]+[N] Father: [c.2808A>G]+[N]
8	c.[1196C>T]+[3503_3504delTC]	p.[S399F]+[L1168QfsX5]	ML III	Probably deleterious	Intolerable	NI	Mother: c.[1196C>T]+[N] Father: c.[3503_3504delTC]+[N]
9	c.[3503_3504delTC]+[?]	p.[L1168QfsX5]+[?]	ML III	NA	NA	NA	NA
10	c.[3503_3504delTC]+[3503_3504delTC]	p.[L1168QfsX5]+[L1168QfsX5]	ML II	NA	NA	NA	NA
11	c.[3503_3504delTC]+[3503_3504delTC]	p.[L1168QfsX5]+[L1168QfsX5]	ML II	NA	NA	NA	Mother: c.[3503_3504delTC]+[N] Father: c.[3503_3504delTC]+[N]
12	c.[3503_3504delTC]+[3503_3504delTC]	p.[L1168QfsX5]+[L1168QfsX5]	ML II	NA	NA	NA	Mother: c.[3503_3504delTC]+[N] Father: c.[3503_3504delTC]+[N]

?: Unidentified mutation; NA: not analyzed; NI: no interference; N: normal allele. Polyphen2 is available at http://genetics.bwh.harvard.edu/pph2 and classifies variations as benign/possibly deleterious/or probably deleterious. SIFT is available at http://sift.jcvi.org/www/SIFT_seq_submit2.htm and classifies variation as tolerable or intolerable. MaxEntScan is available at http://genes.mit.edu/burgeldo/maxent/Xmaxentscan_scoreseq.html.
* Conducted only in case of missense mutations.
** Parents were analyzed only in relation to the mutations found in the proband.

4. Discussion

This is the first study to describe the pathogenic/nonpathogenic mutation spectrum of the *GNPTAB* gene in Brazilian patients with MLII and III alpha/beta. As in other populations, this analysis confirmed that *GNPTAB* exhibits great allelic heterogeneity, that there are few recurrent mutations, and that c.3503_3504delTC is the most prevalent pathogenic mutation (Bargal et al., 2006; Cathey et al., 2010; Kudo et al., 2006; Paik et al., 2005; Tiede et al., 2005). Furthermore, three novel sequence variations (two pathogenic) are described herein.

The number of patients included in this study (n = 12) is significant, considering that all patients were Brazilian and that ML is quite rare and severe (http://www.geneclinics.org). Unfortunately, not all patients had undergone biochemical investigation in plasma and/or fibroblasts. It bears stressing, however, that all patients exhibited a clinical phenotype consistent with MLII or MLIII. Patient 6 was investigated using filter paper only, as described by Chamoles et al. (2001); as this patient was later confirmed as having ML II alpha/beta through DNA analysis, this finding corroborates the hypothesis that DBS samples can also be used for MLII/III screening. Interestingly, four patients exhibited an abnormal pattern on urinary thin-layer chromatography of GAGs, although their levels of total urinary GAGs were normal. Abnormal catabolism of GAGs is expected to occur in patients with MLII/III, as lysosomal enzymes involved in the catabolism of GAGs are deficient in these disorders. Levels of keratan sulfate (determined by ELISA), as well as dermatan sulfate and heparan sulfate (determined by LC/MS/MS), were found to be increased in plasma of some patients with MLII/II (Tomatsu et al., 2005, 2010). However, secretion of abnormal GAGs is usually too low in MLII/III patients to be detected by thin-layer chromatography.

The methodology used in the present study was able to identify 20 of 24 pathogenic alleles (roughly 83%). This rate is not very different from that reported in the literature (approximately 95%) (http://www.geneclinics.org). The most frequently found pathogenic mutation was c.3503_3504delTC, which was present in homozygosity in three MLII patients (patients 10, 11 and 12) and in heterozygosity in another two MLII patients (patients 4 and 7) and three MLIII patients (patients 3, 8 and 9); the prevalence found for this mutation in our study (45%; 11 of 24 alleles) is similar to that described in the literature. Bargal et al. (2006) found this same mutation in 13 of 24 MLII patients, most of Arab-Muslim origin (11 homozygous; 2 compound heterozygous; 10 born to consanguineous couples). Encarnação et al. (2009) found this microdeletion in five patients with MLII (n = 9 of 18 alleles); four of these patients were homozygous for the mutation. Tappino et al. (2009) identified this mutation in 47 of 92 alleles; it was found in homozygosity in 14 patients with MLII, most of whom were Italian and born to non-consanguineous couples. Mutation c.3503_3504delTC was the only pathogenic mutation identified in the sample studied by Plante et al. (2008).

4.1. Effect on phenotype

Our data suggest that nonsense and frameshift mutations are associated with the severe phenotype (MLII alpha/beta), whereas missense mutations are associated with the attenuated phenotype (MLIII alpha/beta). These findings are in agreement with those previously described in the literature (Bargal et al., 2006; Encarnação et al., 2009; Tiede et al., 2005).

Thus far, c.3503_3504delTC, located at the region that codes for the β subunit of phosphotransferase, has been associated with the severe phenotype when found in homozygosity or when found in heterozygosity with nonsense mutations or frameshift mutations. Three of our patients with MLII alpha/beta presented this deletion in homozygosity (patients 10, 11 and 12). However, two patients with MLIII alpha/beta (patients 3 and 8, respectively) presented this deletion in heterozygosity with the missense mutations c.1208T>C (p.I403T) and c.1196C>T (p.S399F), both located at the region that codes for the α subunit of phosphotransferase. Other authors have also reported cases of compound heterozygosity for mutations in the α and β subunits (Cathey et al., 2010; Tappino et al., 2009), and Bargal et al. (2006) suggest that there is no intragenic complementation between these subunits. The genotype c.[1196C>T]+[3503_3504delTC] (patient 8) was also reported in a MLIII French patient by Bargal et al. (2006). In that same study, the authors reported that this patient exhibited a severe MLIII phenotype and that fibroblast testing showed cytoplasmic inclusions typical of patients with MLII. Our patient may also be considered a patient with severe MLIII alpha/beta, given the severity of his skeletal compromise, which is reflected by his present height. The c.[1208T>C]+[3503_3504delTC] genotype, on the other hand, is being described for the first time herein (patient 3, MLIII alpha/beta). Mutation c.1208T>C (p.I403T) has already been described by Tappino et al. (2009) in homozygosity in an Italian patient with MLIII, as well as by Encarnação et al. (2009) in a Portuguese patient. Expression studies of the mutant p.I403T protein in COS cells conducted by Tappino et al. (2009) showed that this mutation had an expected molecular mass of 170 kDa, and the authors presumed that the resulting protein would be partially dysfunctional, which could explain the attenuated phenotype exhibited by these patients.

Genotype c.[1514G>A]+[1759C>T] was found in one patient with MLIII alpha/beta (patient 2). The first is a missense mutation (p.C505Y), while the second is a nonsense mutation (p.R587X). This

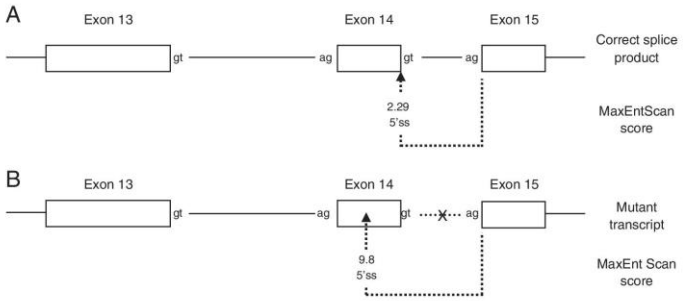

Fig. 2. Schematic representation of exons and introns 13, 14 and 15 of the *GNPTAB* gene and scores obtained with the MaxEntScan program for wild (A) and mutated (B) forms obtained in the presence of c.2808A>G. Dotted line: new splicing. X: splicing replaced.

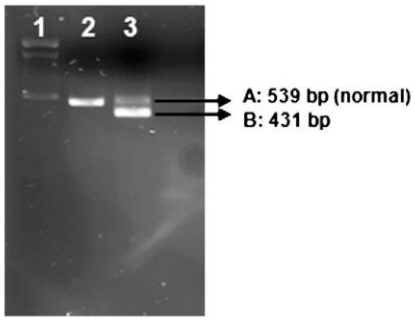

Fig. 3. Electrophoresis of RT-PCR for the c.2808A>G mutation (patient 7) showing the normal transcript (A) and the alternative transcript (B). 1: Standard molecular weight; 2: negative control for c.2808A>G; 3: patient sample.

genotype is being reported herein for the first time, but the mutations have already been reported by Cathey et al. (2010), in compound heterozygosity, in a patient with MLII (p.R587X) and in one patient with MLIII (p.C505Y).

Genotype c.[242G>T]+[?] was found in a patient with ML II alpha/beta (patient 5). Encarnação et al. (2009) described this mutation, in homozygosity, in one of their Portuguese patients with MLIII. One hypothesis that might justify the phenotype of patient 5, with only one pathogenic mutation identified, would be the presence of two silent mutations in exon 1 of the second allele: c.18G>A and c.27G>A. The first induces a change from the sixth CUG codon (frequency of codon usage per thousand = 39.6) to CUA (frequency of codon usage per thousand = 7.2). The second mutation changes the ninth CAG codon (frequency of codon usage per thousand = 34.2) to CAA (frequency of codon usage per thousand = 12.3) (http://www.kasuza.or.jp/codon/cgi-bin/showcodon.cgi?species=9606). This could cause a greater change in the translation kinetics of the protein, generating a protein with an altered conformation (Komar, 2007; Sauna and Kimchi-Sarfaty, 2011). Unfortunately, parental DNA and patient fibroblasts were unavailable, and, therefore, we are not able to confirm whether these mutations are in *cis* or in *trans*. Additional studies will be conducted to confirm this hypothesis.

5. Conclusions

This was the first DNA analysis-based study conducted in Brazilian patients with ML alpha/beta. Its findings suggest that analysis of *GNPTAB* in these patients should begin by exon 19, thus optimizing the investigation and reducing costs. The results obtained herein emphasize the need for further studies, such as application of other techniques that will enable completion of genotyping of the patients whose genotype has not been fully characterized (e.g. sequencing of promoter and intronic regions, exclusion of large deletions/rearrangements), and for determination of the frequency of recurrent variants in healthy Brazilian individuals.

Acknowledgments

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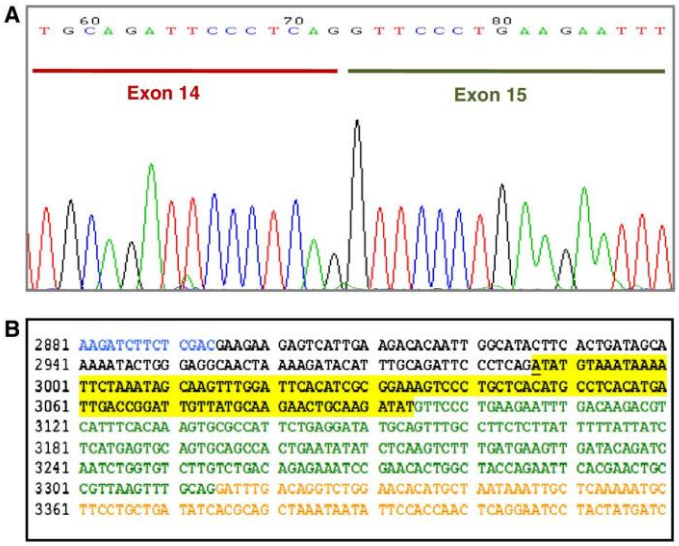

Fig. 4. A) Patient 7 (genotype: c.2808A>G; p.Y937_M972del): Sequencing of the abnormal fragment amplified by RT-PCR. B) Normal cDNA sequence of the *GNPTAB* gene. In blue, part of exon 13; in black, exon 14; in green, exon 15; in orange, exon 16; underlined, mutation site; in yellow, 108 bp lost due to the c.2808A>G mutation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2013.03.105.

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ANEXO II

Exome sequencing for mucolipidosis III: Detection of a novel GNPTAB gene mutation in a patient with a very mild phenotype

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Exome sequencing for mucolipidosis III: Detection of a novel *GNPTAB* gene mutation in a patient with a very mild phenotype

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ABSTRACT

Mucolipidosis II and III alpha/beta (ML II/III alpha/beta) are rare autosomal recessive lysosomal storage diseases that are caused by a deficiency of UDP-GlcNAc:lysosomal enzyme N-acetylglucosamine-1-phosphotransferase, the enzyme responsible for the synthesis of the mannose 6-phosphate targeting signal on lysosomal hydrolases. A Brazilian patient suspected of having a very mild ML III was investigated using whole next-generation sequencing (NGS). Two mutations in the *GNPTAB* gene were detected and confirmed to be *in trans* status by parental analysis: c.1208T>C (p.Ile403Thr), previously reported as being pathogenic, and the novel mutation c.1723G>A (p.Gly575Arg). This study demonstrates the effectiveness of using whole NGS for the molecular diagnosis of very mild ML III alpha/beta patients.

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1. Introduction

In recent years, the use of massive parallel sequencing, or next-generation sequencing (NGS), is revolutionizing genetic investigation as well as clinical practice, mostly when dealing with rare diseases. It is particularly useful when the same phenotype can be caused by mutations in different genes. As cost of sequencing is progressively coming down, it is expected that in the near future comprehensive molecular diagnosis will become a standard of care. NGS can be used in several ways: as a panel targeting selected genes, as a test where the whole exome (i.e., the ~2% coding regions of the genome) is captured and sequenced, or as an exam where the whole genome, with its coding and non-coding regions, is sequenced. Even though sequencing a limited number of genes is apparently more rationale than all genes, developing several panels can be expensive and burdensome. Additionally, having a single test (as whole exome or genome sequencing) for thousands of different genetic disorders is practical and gives opportunities for new discoveries.

Mucolipidosis II and III (ML II and III) are autosomal recessive lysosomal disorders (LSD) in which the essential mannose 6-phosphate (Man-6-P) recognition marker system is deficient. ML II and III are caused by mutations in the *GNPTAB* or *GNPTG* genes, which encode the subunits α and β (*GNPTAB*) or γ (*GNPTG*) of the N-acetylglucosamine-1-phosphotransferase (phosphotransferase, EC 2.7.8.17). Phosphotransferase is a hexameric enzyme (2 α, 2 β, and 2 γ subunits) that mediates the first step of the synthesis of Man-6-P, but there are other genes involved in this pathway, such as the *NAGPA* gene which encodes "uncovering enzyme" (EC 3.1.4.45), (reviewed in [1]) and the *MBTPS* gene that encodes the S1P enzyme. S1P or "site-1 protease" (EC 3.4.21.112) acts post-translationally to cleave the inactive α/β precursor to generate the α and β subunits of mature phosphotransferase [2]. At least theoretically, allelic mutations in the *NAGPA* or *MBTPS* genes could cause ML II/III, even though they have never been reported.

ML is classified as II or III according to the clinical manifestations. Although there is a phenotypic spectrum, ML II (MIM# 252500) is more severe, clinically evident at birth, and usually fatal during childhood. ML III (MIM# 252600 and 252605) has a later onset of symptoms and slower progression. Clinically, skeletal alterations similar to *dysostosis multiplex*, claw hands, failure to thrive, and coarse facial features are observed. Milder cases may have a normal or near normal survival and absence of cognitive involvement, but still present bone involvement

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and claw hands. Patients with ML II and most patients with ML III have been found to be homozygous or compound heterozygous for mutations in *GNPTAB* and, consequently, show alterations in subunits alpha and beta (being called ML II or III alpha/beta patients). In addition, a second group of ML III patients is homozygous or compound heterozygous for mutations in *GNPTG*, present abnormal gamma subunit (thus being ML III gamma patients) (reviewed in [3]). As the catalytic domain of phosphotransferase is located in the α/β3 region, patients with ML III gamma are believed to present the milder phenotypes. However, very few patients are described with ML III gamma, which prevent us from making any kind of generalization.

Schrader et al. [4] were the first to demonstrate the effectiveness of molecular diagnosis for ML III using NGS. They were able to show a 6-bp deletion in the *GNPTG* gene in a family with retinitis pigmentosa and skeletal abnormalities, patients who were not previously known to have ML III. In turn, using targeted NGS, Yang et al. [5] identified two homozygous nonsense mutations in the *GNPTAB* gene in two Chinese families, patients who had previously been diagnosed through biochemical assays as having ML II.

In this work, we evaluated the reliability and feasibility of molecular diagnosis by whole exome sequencing for ML II/III.

2. Materials and methods

2.1. Cation-independent Man-6-P receptor (CI-MPR) affinity chromatography

25 μL of the patient's plasma or 200 μL plasma from 2 healthy individuals was diluted in 50 mM imidazole, 150 mM NaCl, 0.05% Triton X-100, pH = 6.5 (Buffer A) in a total volume of 1 mL and loaded on a CI-MPR affinity column [6]. The column was washed with 7 mL buffer A, 10 mL buffer A containing 5 mM glucose 6-phosphate and eluted with 5 mL buffer A containing 10 mM Man-6-P. The lysosomal enzyme activities were measured in the plasma and the different fractions as described [6]. All enzymatic reactions were performed in 50 mM citrate buffer containing 0.5% Triton X-100, pH = 4.6.

2.2. Whole next-generation sequencing

Peripheral blood was extracted using Easy DNA kit (Invitrogen). Whole genome sequencing was performed with Nextera Exome Capture System, followed by NGS with Illumina HiSeq 2500 (Mendelics Genomic Analysis). The study was approved by the Hospital de Clínicas de Porto Alegre (HCPA) Research Ethics Committee.

3. Results

3.1. Case report

The first child born to a non-consanguineous couple, this male patient had normal development (e.g., walked at 10 months). At the age of 4, claudication was first noted. There was no history of respiratory infections or other significant comorbidity. Since the age of 8, his weight had been p>95, and his height, between p10 and 25 for his age. At the age of 11, after being followed by an orthopedics service, he was referred to our medical genetics service for suspected multiple epiphyseal dysplasia. His skeletal radiographs, however, were suggestive of a lysosomal disorder (e.g., *dysostosis multiplex* was present) and showed the following: a decrease in intervertebral spaces especially in the thoracolumbar transition, a decrease in the T10 and T11 vertebrae, oval-shaped L1/L2 vertebrae, flattening of the head of the humerus with an increase in the humerus-acromion distance, as well as bilateral alterations in the femoral head suggestive of Legg–Calvé–Perthes disease. His echocardiogram, complete ophthalmologic exam, and abdominal echography were normal, as well as serum levels of calcium (8.9 mg/dL; NRV = 8.9–10.7) and alkaline phosphatase (290 U/L; NRV = 98–317). On physical exam, he presented normal head circumference, atypical facies, varus knees, restricted range of motion of the shoulders, wrists, and knees and absence of corneal clouding, claw hands, and hepatosplenomegaly (Fig. 1). At the age of 14, his height was 149 cm (p< 5) and at 18, 152 cm (p< 5). He attended regular school and did not exhibit cognitive delay.

3.2. Biochemical analysis

Based on the clinical findings, the initial diagnosis raised was mucopolysaccharidosis type IV A or Morquio A syndrome (MPS IV-A, MIM# 253000), which was ruled out due to the normal activity of N-acetylgalactosamine-6-sulfatase (EC 3.1.6.4) in leukocytes (Table 1). As the patient exhibited *dysostosis multiplex* by X-ray, a biochemical investigation for lysosomal storage disorders was requested, which was suggestive of ML II/III (presence of an increased activity of lysosomal hydrolases in plasma and normal activity in leukocytes). Since there were no fibroblasts available for biochemical analysis, the presence of Man-6-P-containing lysosomal acid hydrolases in the plasma was investigated using CI-MPR affinity chromatography. As shown in Table 2, this analysis showed a striking decrease in phosphorylated acid hydrolases in the patient's plasma compared to normal, consistent with the diagnosis of ML II/III.

Although the investigation performed in this patient is considered enough for confirming ML II/III, due to the very mild clinical picture, especially the absence of claw hands – which is an unexpected finding even for ML III gamma – it was decided that whole NGS would be performed, also aiming to exclude the presence of pathogenic mutation in other genes involved in M6P biosynthesis (such as *NAGPA* and *MBTPS*).

3.3. Whole next-generation sequencing

Whole NGS generated 64,363,166 sequences, each target base was read on average 92× and 94% of the target bases were read at least 10×. Two heterozygous variants in *GNPTAB* were identified: c.1208T>C (p.Ile403Thr), previously reported as deleterious [7], and the novel variant c.1723G>A (p.Gly575Arg), which was not present in more than 8000 normal controls (Exome Server Variant and 1000 Genomes), including 1000 Brazilians (processed as controls at the NGS laboratory). No pathogenic variants were found in the *GNPTG*, *NAGPA*, and *MBTPS* genes. This result was confirmed by Sanger sequencing, and a segregation study demonstrated that the father was a carrier of the variant p.Ile403Thr, and the mother, of the transversion p.Gly575Arg.

Polyphen-2 predicted p.Gly575Arg as probably damaging (score of 0.974). Sift v.1.1.3 prediction indicated the variant p.Gly575Arg as tolerated/neutral. The MutPred software interpreted the pathogenicity as an actionable hypothesis, with a probability score of deleterious mutation of 0.382, supposedly causing the following: gain of solvent accessibility (p = 0.0171), gain of helix (p = 0.0425), gain of relative solvent accessibility (p = 0.0479), loss of loop (p = 0.0512), and loss of methylation at K573 (p = 0.0536).

4. Discussion

The aim of this work was to evaluate the molecular diagnosis of very mild ML III using whole NGS, and our results demonstrate the utility of this new technology.

The confirmation of the clinical diagnosis of ML II/III faces several challenges in regard to the performance of biochemical assays, since these assays are not widely available (nor easily done) and depend on the performance of fibroblast biopsy. The phosphotransferase assay, for instance, requires a radioactive substrate, is difficult to implement, and few groups in the world perform this assay.

Currently, biochemical diagnosis of ML II/III is usually performed indirectly, through the measurement of lysosomal hydrolases both in plasma (their activity should be high) and in fibroblasts (their activity

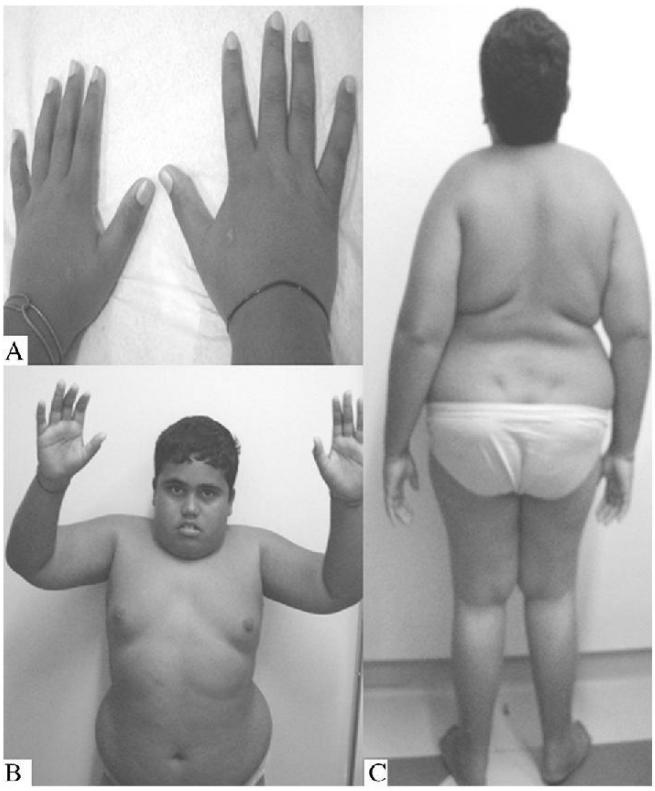

Fig. 1. Male patient with mucolipidosis type III, aged 14 years old, presenting a milder phenotype. A — Absence of hand joint contractures. B — Joint contractures in shoulders, hindering arm elevation above the head; atypical facies. C — Whole body posterior image. The obesity is evident.

Table 1
Biochemical characterization of patient.

Biochemical investigation	Sample	Patient	Reference Values
α-L-Iduronidase (EC 3.2.1.76)	Plasma	119	6.8–13.7
β-Glucuronidase (EC 3.2.1.31)	Plasma	906	30–300
α-N-acetylglucosaminidase (EC 3.2.1.50)	Plasma	875	34–162
α-Mannosidase (EC 3.2.1.24)	Plasma	2026	17–56
β-Hexosaminidases A (EC 3.2.1.30)	Plasma	11,747	550–1675
β-Hexosaminidases B (EC 3.2.1.30)	Plasma	27,535	265–1219
β-Hexosaminidases, total (EC 3.2.1.30)	Plasma	39,282	1000–2857
Chitotriosidase (EC 3.2.1.14)	Plasma	98	8.8–132
Iduronate 2-sulfatase (EC 3.1.6.12)	Plasma	1716	122–463
β-Galactosidase (EC 3.2.1.23)	Leukocyte	162	78–280
α-L-Iduronidase (EC 3.2.1.76)	Leukocyte	72	32–56
Arylsulfatase B (EC 3.1.6.12)	Leukocyte	–	72–176
β-Glucosidase (EC 3.2.1.21)	Leukocyte	19	10–45
α-N-acetylglucosaminidase (EC 3.2.1.50)	Leukocyte	64	68–352
Iduronate 2-sulfatase (EC 3.1.6.12)	Leukocyte	95	31–110
β-Glucuronidase (EC 3.2.1.31)	Leukocyte	175	23–151
Sphingomyelinase (EC 3.1.4.12)	Leukocyte	1.9	0.74–4.9
Total β-hexosaminidases (EC 3.2.1.30)	Leukocyte	7384	552–16,662
β-Hexosaminidase A	Leukocyte	53	150–390
N-acetylgalactosamine-6-sulfatase	Leukocyte	29	14–81
Dosage of GAGs	Urine	128 (79–256)	<9 years: 44–106 mg/L
Thin-layer chromatography of GAGs	Urine	DS + HS + CS/HS	Normal
Thin-layer chromatography of sialoligosaccharide	Urine	Normal	Normal

GAGs: Glycosaminoglycans; –: not available; DS: dermatan sulfate; HS: heparan sulfate; CS: chondroitin sulfate.

Table 2
CI-MPR affinity chromatography: presence of phosphorylated acid hydrolases in plasma.

% enzyme bound to CI-MPR column			
Enzyme	Control 1	Control 2	Patient
α-Mannosidase	12.5	11.2	0.5
β-Glucuronidase	5.9	14.7	0.1
β-Hexosaminidase	7.7	14.3	0
β-Mannosidase	13.5	19.7	0.4

should be low). When fibroblasts are not available, other tests are needed (such as the analysis of phosphorylated residues, which is also performed on a research basis, or DNA analysis only). Moreover, as occurred in the present case, the clinical picture can be so mild, sometimes limited to bone disease, that even experienced physicians miss its diagnosis.

Regarding DNA analysis, there is no consensus about which gene (*GNPTAB* or *GNPTG*) should be first analyzed when facing a case with clinical picture compatible with ML III. If the case is very mild, an initial analysis of *GNPTG* may be suggested, but, as reported herein, mutations in *GNPTAB* might also cause very mild phenotypes. Among the available technologies for DNA analysis, exome sequencing is one of the most modern tools available. It is important to point out that ML III presents locus heterogeneity and that Sanger sequencing of whole coding *GNPTG* and *GNPTAB* is made by amplification and sequencing of the 11 and 21 exons of each gene, respectively, a situation that requires time and investment and can delay the diagnosis. In this regard, exome sequencing is a reliable alternative for confirmation of the diagnosis of ML III.

However, different sequencing platforms vary in their ability to identify variants, even when sequencing the same genome. Besides that, prediction of the pathogenicity of the novel missense variants found is still a problem. For instance, the patient reported herein is a compound heterozygote for two missense mutations in the *GNPTAB* gene, p.Ile403Thr (a previously reported pathogenic mutation) and p.Gly575Arg (a novel mutation which presents divergent results regarding its pathogenicity by bioinformatics analysis). Up to date, more than 125 different mutations of *GNPTAB*, encoding the α/β-subunit precursor of the phosphotransferase, have been described to cause ML II or III alpha/beta (HGMD, 2014). In general ML II alpha/beta patients have nonsense, frameshift or splice-site mutations in *GNPTAB*, whereas ML III alpha/beta patients carry missense mutations [8].

Franke, Braulke, & Storch [9] showed that for efficient transport of the α/β-subunit precursor protein from the ER to the Golgi apparatus, a nonexchangeable dileucine (Leu 5-Leu 6) and the dibasic motif (Arg1253-Ile1254-Arg1255) are required in its cytosolic N- and C-terminal domains, respectively. After, the S1P-mediated cleavage α/β-subunit precursor protein occurs in the Golgi apparatus, a prerequisite for the catalytic activity of phosphotransferase [10].

The mature human α-subunit is a type II membrane protein with an N-terminal tail, a transmembrane region and a subsequent luminal domain, each one comprising 19, 22, and 886 amino acids respectively [11,12]. The luminal domain contains 17 potential N-glycosylation sites, and mutations associated to ML II and ML III are described in this domain [12–14].

The missense mutation p.Gly575Arg changes a polar amino acid (glycine, GGA) for an apolar (Arginine, AGA). It is located in the luminal domain of the α-subunit, in a region with no homolog domains, but next to an N-glycosylation site (N580). This residue and amino acid (Gly575) is highly conserved in mammals, fish, amphibians, birds, but not in zebrafish and frogs, and might be a binding site for luminal ER proteins required for ER exit of α/β-subunit precursor. Alternatively, p.Gly575Arg can cause misfolding of the mutant protein and retention in the ER [15]. Both alternatives could explain the pathogenicity of p.Gly575Arg and the patient's mild phenotype. There is no other mutation described in the 575 residue of phosphotransferase — the closest one is p.Arg587Pro (c.1760G>C) which is also associated to ML III [16].

The following evidence also suggests p.Gly575Arg is pathogenic: 1) p.Ile403Thr is described in ML III patients [7,8,17] and is presumed to be a mild mutation by expression studies [7]. The very mild phenotype of the patient could be explained by the fact he is compound heterozygous for two mild mutations (p.Ile403Thr and p.Gly575Arg); 2) no other mutations causing the phenotype were found in the *GNPTAB*, *GNPTG*, *NAGPA*, and *MBTPS* genes; 3) both mutations found are *in trans* and inherited; 4) p.Gly575Arg was not found in a high number of controls from the same population as the patient.

We agree with Schrader et al. [4] and reinforce the idea that the costs of NGS will gradually come down. As soon as this happens, this technique will become the most direct approach for the diagnosis of Mendelian disorders that are phenotypically and genetically heterogeneous, such as ML II/III. In the meantime, Sanger sequencing of *GNPTAB* and *GNPTG* remains the first strategy for DNA analysis of patients with ML II/III.

Acknowledgments

We thank the family who participated in this study for their outstanding collaboration. This project was supported by FIPE–HCPA, FAPERGS, CAPES, CNPq (Brazil), the Postgraduate Program in Genetics and Molecular Biology of UFRGS, and National Institutes of Health grant CA-008759.

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APÊNDICE I

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Projeto: **ESTUDO ABRANGENTE SOBRE AS MUCOLIPIDOSES II E III NO BRASIL: UMA OPORTUNIDADE PARA A COMPREENSÃO DOS PROCESSOS GENÉTICOS QUE CONTROLAM O TRÁFEGO INTRACELULAR DE PROTEÍNAS**

Investigador Responsável: Dra. Ida Vanessa D. Schwartz. Departamento de Genética, Universidade Federal do Rio Grande do Sul, Avenida Bento Gonçalves 9500, Porto AlegreRS. Tel: Tel: (51) 3308-6722

Paciente: _____

Prezado paciente ou responsável, Esta pesquisa tem por objetivo a obtenção de informações relativas aos sintomas de pessoas com Mucolipidose II ou Mucolipidose III, às alterações presentes em suas enzimas e às alterações presentes no gene que ocasiona estas doenças. Estas informações serão obtidas por meio de entrevistas com você e/ou com os seus médicos, e mediante consulta a seu prontuário. Caso a dosagem de todas as enzimas que podem estar alteradas na Mucolipidose II ou na Mucolipidose III não tenha sido ainda realizada em você, será necessário que você (paciente) seja submetido à coleta de sangue (10 mL para a análise das enzimas que podem estar alteradas em sangue) e realização de biópsia de pele (para a análise das enzimas que podem estar alteradas na pele). Caso a análise dos genes envolvidos na Mucolipidose II e na Mucolipidose III não tenha ainda sido realizada em você (paciente), também será necessária a coleta adicional de 05 mL de sangue. Você pode concordar ou não com a realização destes exames. Os riscos e desconfortos causados pela coleta de sangue são semelhantes aos riscos envolvidos na coleta de sangue para exames laboratoriais de rotina (manchas roxas e dor no local da coleta). A biópsia de pele deverá ser realizada em condições higiênicas e sob anestesia local; o fragmento a ser retirado é pequeno (em torno de 0,3 cm) e superficial, não sendo, portanto, necessária a realização de pontos. O desconforto e os riscos associados a estas avaliações serão minimizados pela realização da coleta por profissional treinado. Se você permitir, o material coletado, e que restar após a realização dos exames previstos neste estudo, será armazenado e utilizado em estudos futuros. Em relação a estas coletas, você declara que autorizou a coleta de (marcar com um X):

( ) 5 mL de sangue para a análise do gene envolvido nas Mucolipidoses II e III
( ) 10 mL de sangue para a análise das enzimas
( ) biópsia de pele para análise das enzimas
( ) eu não autorizei nenhuma das coletas acima relacionadas

Em relação ao armazenamento e utilização de algum material (sangue ou pele) que tenha restado após a realização dos exames previstos neste estudo, você declara que autorizou (marcar com um X):

( ) que este material poderá ser armazenado e poderá vir a ser utilizado em estudos futuros, desde que você revise e assine o termo de consentimento de tais estudos futuros.
( ) que este material não poderá ser armazenado e não poderá vir a ser utilizado em estudos futuros. O material coletado deverá ser utilizado somente neste estudo, e o material que sobrar não deverá ser armazenado.

Cabe salientar que as Mucolipidoses II e III não possuem, ainda, tratamento eficaz, e que esta pesquisa não tem como finalidade imediata a garantia de uma melhora para os pacientes. Pode, entretanto, contribuir para um melhor entendimento destas doenças, o qual, no futuro, pode levar ao desenvolvimento de uma terapia mais efetiva. A identificação das mutações (alterações do DNA) presentes no gene que ocasiona estas doenças poderá ser importante para o aconselhamento genético da sua família e para o diagnóstico pré-natal. Não existe um prazo exato ou estipulado para que você receba os resultados dos exames realizados nesta pesquisa, mas estes lhes serão informados assim que estiverem disponíveis. Você pode optar por não saber o resultado dos testes quando estes estiverem disponíveis.

DÚVIDAS

Se você tiver alguma dúvida em relação à pesquisa, deve contatar a Dra. Ida Vanessa D. Schwartz, no Departamento de Genética da Universidade Federal do Rio Grande do Sul.

AUTORIZAÇÃO PARA PERMITIR PESQUISA DOS REGISTROS MÉDICOS

Você tem direito à privacidade. Os resultados deste estudo poderão ser publicados, mas o seu nome não será revelado e todo esforço será feito que a sua identidade não seja revelada.

Por meio deste termo, você autoriza que os pesquisadores envolvidos neste estudo pesquisem os seus registros médicos a fim de obter as informações necessárias para a realização desta pesquisa.

RECUSA OU DESCONTINUAÇÃO NA PARTICIPAÇÃO DO ESTUDO

Sua participação no estudo é voluntária. Se você decidir não participar do estudo, isto não afetará em nada o seu tratamento no seu hospital. A sua participação pode também ser interrompida a qualquer momento por você mesmo (a). Em qualquer caso, você não será penalizado (a).

Pelo presente termo, você declara que foi informado (a), de forma clara e detalhada, sobre a presente pesquisa, e que teve suas dúvidas esclarecidas por _____. Declara ter sido esclarecido que não receberá nenhuma remuneração financeira pela participação no estudo. Declara que foi informado da garantia de receber resposta ou esclarecimento sobre a pesquisa a ser realizada, bem como da liberdade de não participar do estudo e da possibilidade de desistir, em qualquer momento, da participação. Além disso, declara que recebeu cópia deste termo de consentimento.

Data: ___/___/_____
Paciente: _____
Responsável legal: _____

Eu expliquei a _____os objetivos, riscos, benefícios e procedimentos necessários para esta pesquisa, e entreguei cópia deste termo de consentimento para o mesmo.

Data: ____/_____/_____
Nome:_____

HCPA - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE
Grupo de Pesquisa e Pós-Graduação
COMISSÃO CIENTÍFICA E COMISSÃO DE PESQUISA E ÉTICA EM SAÚDE

A Comissão Científica e a Comissão de Pesquisa e Ética em Saúde, que é reconhecida pela Comissão Nacional de Ética em Pesquisa (CONEP)/MS como Comitê de Ética em Pesquisa do HCPA e pelo Office For Human Research Protections (OHRP)/USDHHS, como Institucional Review Board (IRB0000921) analisaram o projeto:

Projeto: 07-244 **Versão do Projeto:** 03/09/2007 **Versão do TCLE:** 03/09/2007

Pesquisadores:
IDA VANESSA DOEDERLEIN SCHWARTZ
ROBERTO GIUGLIANI
MARCIO SCHNEIDER MEDEIROS
MAIRA GRAEFF BURIN
JANICE CARNEIRO COELHO
ANDRESSA FEDERHEN

Título: ESTUDO ABRANGENTE SOBRE AS MUCOLIPIDOSES II E III NO BRASIL: UMA OPORTUNIDADE PARA A COMPREENSÃO DOS PROCESSOS GENÉTICOS QUE CONTROLAM O TRÁFEGO INTRACELULAR DE PROTEÍNAS

- Este projeto foi Aprovado em seus aspectos éticos e metodológicos, inclusive quanto ao seu Termo de Consentimento Livre e Esclarecido, de acordo com as Diretrizes e Normas Internacionais e Nacionais, especialmente as Resoluções 196/96 e complementares do Conselho Nacional de Saúde. Os membros do CEP/HCPA não participaram do processo de avaliação dos projetos onde constam como pesquisadores. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente ao CEP/HCPA. Somente poderão ser utilizados os Termos de Consentimento onde conste a aprovação do GPPG/HCPA.

- De acordo com a regulamentação da Resolução 340/2004 do CNS/MS o CEP/HCPA foi credenciado, através da Carta Circular Nº 037 CONEP/CNS/MS de 11 de agosto de 2004, para dar aprovação final para este projeto.

Porto Alegre, 14 de setembro de 2007.

Profª Nadine Clausell
Coordenadora do GPPG e CEP-HCPA

APÊNDICE II

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Projeto: **ANÁLISE DO GENE *GNPTG* EM PACIENTES BRASILEIROS COM MUCOLIPIDOSES II E III**
Investigador Responsável: Dra. Ida Vanessa D. Schwartz. Serviço de Genética Médica do HCPA. Rua Ramiro Barcelos, 2350 – Porto Alegre-RS. Tel.: (51) 3359-8011.
Paciente: _____

Prezado paciente ou responsável,

Mucolipidoses II e III são doenças que ocorrem devido a um defeito genético nos genes *GNPTAB* ou *GNPTG.* Por este motivo, esta pesquisa tem por objetivo a obtenção de informações relativas às alterações presentes no gene *GNPTG* e aos sintomas de pessoas com estas doenças. Estas informações serão obtidas por meio de entrevistas com você e/ou com os seus médicos, mediante consulta a seu prontuário e mediante análise do gene *GNPTG*.

Será necessária a coleta de 5 mL de sangue. Os riscos e desconfortos causados pela coleta de sangue são semelhantes aos riscos envolvidos na coleta de sangue para exames laboratoriais de rotina (manchas roxas e dor no local da coleta). Se você já participou do estudo que envolveu a análise do *GNPTAB* (projeto GPPG-HCPA 07-244), e tem DNA armazenado em nosso laboratório, não será necessária a coleta de mais sangue. Neste caso, você (marcar com um X):
( ) autoriza a utilização do material que restou após a análise do gene *GNPTAB* para a análise do gene *GNPTG.*
( ) não autoriza a utilização do material que restou após a análise do gene *GNPTAB.*

Se você permitir, o material coletado, e que restar após a realização dos exames previstos neste estudo, será armazenado e utilizado em estudos futuros. Em relação ao armazenamento e utilização do material (sangue) que tenha restado, você declara que autorizou:
( ) que este material poderá ser armazenado e poderá vir a ser utilizado em estudos futuros, desde que você revise e assine o termo de consentimento de tais estudos futuros.
( ) que este material não poderá ser armazenado e não poderá vir a ser utilizado em estudos futuros. O material coletado deverá ser utilizado somente neste estudo, e o material que sobrar não deverá ser armazenado.

Cabe salientar que as Mucolipidoses II e III não possuem, ainda, tratamento eficaz, e que esta pesquisa não tem como finalidade imediata a garantia de uma melhora para os pacientes. Pode, entretanto, contribuir para um melhor entendimento destas doenças, o qual, no futuro, pode levar ao desenvolvimento de um tratamento mais efetivo. Por outro lado, a identificação das alterações do DNA que ocasionam estas doenças poderá ser importante para o entendimento e cálculo da possibilidade de recorrência dessas doenças na família. Não existe um prazo exato ou estipulado para que você receba os resultados dos exames realizados nesta pesquisa, mas estes lhes serão informados assim que estiverem disponíveis. Você pode optar por não saber o resultado dos testes quando estes estiverem disponíveis. Neste caso, você:
( ) deseja receber os resultados dos exames realizados nesta pesquisa.
( ) não deseja receber os resultados dos exames realizados nesta pesquisa.

DÚVIDAS

Se você tiver alguma dúvida em relação à pesquisa, deve contatar a Dra. Ida Vanessa D. Schwartz, no Serviço de Genética Médica do HCPA ou o Comitê de Ética em Pesquisa do HCPA através do telefone (51) 3359-8304.

AUTORIZAÇÃO PARA PERMITIR PESQUISA DOS REGISTROS MÉDICOS

Você tem direito à privacidade. Os resultados deste estudo poderão ser publicados, mas o seu nome não será revelado e todo esforço será feito que a sua identidade não seja revelada. Por meio deste termo, você autoriza que os pesquisadores envolvidos neste estudo pesquisem os seus registros médicos a fim de obter as informações necessárias para a realização desta pesquisa.

RECUSA OU DESCONTINUAÇÃO NA PARTICIPAÇÃO DO ESTUDO

Sua participação no estudo é voluntária. Se você decidir não participar do estudo, isto não afetará em nada o seu tratamento no seu hospital. A sua participação pode também ser interrompida a qualquer momento por você mesmo (a). Em qualquer caso, você não será penalizado (a).

Pelo presente termo, você declara que foi informado (a), de forma clara e detalhada, sobre a presente pesquisa, e que teve suas dúvidas esclarecidas por _____. Declara ter sido esclarecido que não receberá nenhuma remuneração financeira pela participação no estudo, bem como, que não terá custos em participar. Declara que foi informado da garantia de receber resposta ou esclarecimento sobre a pesquisa a ser realizada, bem como da liberdade de não participar do estudo e da possibilidade de desistir, em qualquer momento, da participação. Além disso, declara que recebeu cópia deste termo de consentimento.

Data: ___/___/_____
Paciente: _____
Responsável legal: _____
Assinatura: _____

Eu expliquei a _____os objetivos, riscos, benefícios e procedimentos necessários para esta pesquisa, e entreguei cópia deste termo de consentimento para o mesmo.

Data: ____/_____/_____
Nome:_____
Assinatura: _____

HCPA - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE
GRUPO DE PESQUISA E PÓS-GRADUAÇÃO

COMISSÃO CIENTÍFICA E COMITÊ DE ÉTICA EM PESQUISA

A Comissão Científica e o Comitê de Ética em Pesquisa do Hospital de Clínicas de Porto Alegre (CEP/HCPA), que é reconhecido pela Comissão Nacional de Ética em Pesquisa (CONEP)/MS e pelo Office For Human Research Protections (OHRP)/USDHHS, como Institutional Review Board (IRB00000921) analisaram o projeto:

Projeto: 110477
Data da Versão do Projeto: 01/12/2011
Data da Versão do TCLE: 21/10/2011

Pesquisadores:
RENATA VOLTOLINI VELHO
IDA VANESSA DOEDERLEIN SCHWARTZ

Título: ANÁLISE DO GENE GNPTG EM PACIENTES BRASILEIROS COM MUCOLIPIDOSES II E III

Este projeto foi APROVADO em seus aspectos éticos e metodológicos, bem como o seu respectivo Termo de Consentimento Livre e Esclarecido, de acordo com as diretrizes e normas nacionais e internacionais de pesquisa clínica, especialmente as Resoluções 196/96 e complementares do Conselho Nacional de Saúde.

- Os membros da Comissão Científica e do Comitê de Ética em Pesquisa não participaram do processo de avaliação dos projetos nos quais constam como pesquisadores.
- Toda e qualquer alteração do projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente ao CEP/HCPA.
- O pesquisador deverá apresentar relatórios semestrais de acompanhamento e relatório final ao CEP/HCPA.
- Somente poderá ser utilizado o Termo de Consentimento Livre e Esclarecido no qual conste o carimbo de aprovação do CEP/HCPA.

Porto Alegre, 12 de dezembro de 2011.

Profª Nadine Clausell
Coordenadora GPPG e CEP/HCPA

APÊNDICE III

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Projeto: **ESTUDO DO GENE *GNPTG* E SUA RELAÇÃO COM *GNPTAB* EM PACIENTES BRASILEIROS COM MUCOLIPIDOSES II E III**
Investigador Responsável: Dra. Ida Vanessa D. Schwartz. Serviço de Genética Médica do HCPA. Rua Ramiro Barcelos, 2350 – Porto Alegre-RS. Tel.: (51) 3359-8011.
Paciente: _____

Prezado paciente ou responsável,

Mucolipidoses II e III são doenças que ocorrem devido a um defeito genético nos genes *GNPTAB* ou *GNPTG.* Por este motivo, esta pesquisa tem por objetivo entender a relação destes genes e obter informações relativas aos sintomas de pessoas com Mucolipidoses II e III. Estas informações serão obtidas por meio de entrevistas com você e/ou com os seus médicos, mediante consulta a seu prontuário e mediante análise da expressão dos genes *GNPTG* e *GNPTAB*.

Será necessária a coleta de 5 mL de sangue. Os riscos e desconfortos causados pela coleta de sangue são semelhantes aos riscos envolvidos na coleta de sangue para exames laboratoriais de rotina (manchas roxas e dor no local da coleta).

Se você permitir, o material coletado, e que restar após a realização dos exames previstos neste estudo, será armazenado e utilizado em estudos futuros. Em relação ao armazenamento e utilização do material (sangue) que tenha restado, você declara que autorizou:
( ) que este material poderá ser armazenado e poderá vir a ser utilizado em estudos futuros, desde que você revise e assine o termo de consentimento de tais estudos futuros.
( ) que este material não poderá ser armazenado e não poderá vir a ser utilizado em estudos futuros. O material coletado deverá ser utilizado somente neste estudo, e o material que sobrar não deverá ser armazenado.

Cabe salientar que as Mucolipidoses II e III não possuem, ainda, tratamento eficaz, e que esta pesquisa não tem como finalidade imediata a garantia de uma melhora para os pacientes. Pode, entretanto, contribuir para um melhor entendimento destas doenças, o qual, no futuro, pode levar ao desenvolvimento de um tratamento mais efetivo. Não existe um prazo exato ou estipulado para que você receba os resultados dos exames realizados nesta pesquisa, mas estes lhes serão informados assim que estiverem disponíveis. Você pode optar por não saber o resultado dos testes quando estes estiverem disponíveis. Neste caso, você:
( ) deseja receber os resultados dos exames realizados nesta pesquisa.
( ) não deseja receber os resultados dos exames realizados nesta pesquisa.

DÚVIDAS

Se você tiver alguma dúvida em relação à pesquisa, deve contatar a Dra. Ida Vanessa D. Schwartz, no Serviço de Genética Médica do HCPA ou o Comitê de Ética em Pesquisa do HCPA através do telefone (51) 3359-8304.

AUTORIZAÇÃO PARA PERMITIR PESQUISA DOS REGISTROS MÉDICOS

Você tem direito à privacidade. Os resultados deste estudo poderão ser publicados, mas o seu nome não será revelado e todo esforço será feito que a sua identidade não seja revelada. Por meio deste termo, você autoriza que os pesquisadores envolvidos neste estudo pesquisem os seus registros médicos a fim de obter as informações necessárias para a realização desta pesquisa.

RECUSA OU DESCONTINUAÇÃO NA PARTICIPAÇÃO DO ESTUDO

Sua participação no estudo é voluntária. Se você decidir não participar do estudo, isto não afetará em nada o seu tratamento no seu hospital. A sua participação pode também ser interrompida a qualquer momento por você mesmo (a). Em qualquer caso, você não será penalizado (a).

Pelo presente termo, você declara que foi informado (a), de forma clara e detalhada, sobre a presente pesquisa, e que teve suas dúvidas esclarecidas por _____. Declara ter sido esclarecido que não receberá nenhuma remuneração financeira pela participação no estudo, bem como, que não terá custos em participar. Declara que foi informado da garantia de receber resposta ou esclarecimento sobre a pesquisa a ser realizada, bem como da liberdade de não participar do estudo e da possibilidade de desistir, em qualquer momento, da participação. Além disso, declara que recebeu cópia deste termo de consentimento.

Data: ___/___/_____
Paciente: _____
Responsável legal: _____
Assinatura: _____

Eu expliquei a _____os objetivos, riscos, benefícios e procedimentos necessários para esta pesquisa, e entreguei cópia deste termo de consentimento para o mesmo.

Data: ____/_____/_____
Nome:_____
Assinatura: _____

HCPA - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE
GRUPO DE PESQUISA E PÓS-GRADUAÇÃO

COMISSÃO CIENTÍFICA E COMITÊ DE ÉTICA EM PESQUISA

A Comissão Científica e o Comitê de Ética em Pesquisa do Hospital de Clínicas de Porto Alegre (CEP/HCPA), que é reconhecido pela Comissão Nacional de Ética em Pesquisa (CONEP)/MS e pelo Office For Human Research Protections (OHRP)/USDHHS, como Institutional Review Board (IRB00000921) analisaram o projeto:

Projeto: 120018
Data da Versão do Projeto: 13/01/2012
Data da Versão do TCLE: 13/01/2012

Pesquisadores:
RENATA VOLTOLINI VELHO
IDA VANESSA DOEDERLEIN SCHWARTZ

Título: ESTUDO DO GENE GNPTG E SUA RELAÇÃO COM GNPTAB EM PACIENTES BRASILEIROS COM MUCOLIPIDOSES II E III

Este projeto foi APROVADO em seus aspectos éticos e metodológicos, bem como o seu respectivo Termo de Consentimento Livre e Esclarecido, de acordo com as diretrizes e normas nacionais e internacionais de pesquisa clínica, especialmente as Resoluções 196/96 e complementares do Conselho Nacional de Saúde.

- Os membros da Comissão Científica e do Comitê de Ética em Pesquisa não participaram do processo de avaliação dos projetos nos quais constam como pesquisadores.
- Toda e qualquer alteração do projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente ao CEP/HCPA.
- O pesquisador deverá apresentar relatórios semestrais de acompanhamento e relatório final ao CEP/HCPA.
- Somente poderá ser utilizado o Termo de Consentimento Livre e Esclarecido no qual conste o carimbo de aprovação do CEP/HCPA.

Porto Alegre, 21 de março de 2012.

Profª Nadine Clausell
Coordenadora GPPG e CEP/HCPA

APÊNDICE IV

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO PARA PACIENTES

Projeto: **USO DE GENTAMICINA E CLORANFENICOL *IN VITRO* COMO ESTRATÉGIA DE TRATAMENTO PARA AS MUCOLIPIDOSES II E III**
Investigador Responsável: Dra. Ida Vanessa D. Schwartz. Serviço de Genética Médica do HCPA. Rua Ramiro Barcelos, 2350 – Porto Alegre-RS. Tel.: (51) 3359-8011.
Paciente: _____

Prezado paciente ou responsável,

O lisossomo é uma estrutura celular responsável pela limpeza e reciclagem de componentes da célula. Ele apresenta inúmeras enzimas que desempenham este papel. Mucolipidoses II e III são doenças genéticas em que a atividade da enzima lisossomal GlcNAc-1-fosfotransferase apresenta-se reduzida, o que causa os sintomas da doença. Há relatos na literatura de que os antibióticos, gentamicina e cloranfenicol, aumentam a atividade deste mesmo tipo de enzima em outras doenças genéticas. Por este motivo, esta pesquisa objetiva analisar o efeito destes medicamentos sobre células de pacientes com Mucolipidoses II e III. Para isso, é necessário que você autorize a utilização de seus fibroblastos, que foram necessários para seu diagnóstico bioquímico, e que estão armazenados no Serviço de Genética Médica do Hospital de Clínicas de Porto Alegre - SGM/HCPA. Você tem total liberdade de concordar ou não com a participação desta pesquisa.

Com relação à amostra de fibroblastos, você declara que (marque com um X):
( ) autoriza a utilização dos mesmos.
( ) não autoriza a utilização dos mesmos.

Cabe salientar que as Mucolipidoses II e III não possuem, ainda, tratamento eficaz, e que esta pesquisa não tem como finalidade imediata a garantia de uma melhora para os pacientes. Pode, entretanto, contribuir para um melhor entendimento destas doenças, o qual, no futuro, pode levar ao desenvolvimento de um tratamento mais efetivo.

Não existe um prazo exato ou estipulado para que você receba os resultados obtidos nesta pesquisa, mas estes lhes serão informados assim que estiverem disponíveis. Você pode optar por não saber o resultado dos testes quando estes estiverem disponíveis. Neste caso, você:
( ) deseja receber os resultados dos testes realizados nesta pesquisa.
( ) não deseja receber os resultados dos testes realizados nesta pesquisa.

DÚVIDAS

Se você tiver alguma dúvida em relação à pesquisa, deve contatar a Dra. Ida Vanessa D. Schwartz, no Serviço de Genética Médica do HCPA ou o Comitê de Ética em Pesquisa do HCPA através do telefone (51) 3359-8304.

AUTORIZAÇÃO PARA PERMITIR PESQUISA DOS REGISTROS MÉDICOS

Você tem direito à privacidade. Os resultados deste estudo poderão ser publicados, mas o seu nome não será revelado e todo esforço será feito que a sua identidade não seja revelada. Por meio deste termo, você autoriza que os pesquisadores envolvidos neste estudo pesquisem os seus registros médicos a fim de obter as informações necessárias para a realização desta pesquisa.

RECUSA OU DESCONTINUAÇÃO NA PARTICIPAÇÃO DO ESTUDO

Sua participação no estudo é voluntária. Se você decidir não participar do estudo, isto não afetará em nada o seu tratamento no seu hospital. A sua participação pode também ser interrompida a qualquer momento por você mesmo (a). Em qualquer caso, você não será penalizado (a).

Pelo presente termo, você declara que foi informado (a), de forma clara e detalhada, sobre a presente pesquisa, e que teve suas dúvidas esclarecidas por _____. Declara ter sido esclarecido que não receberá nenhuma remuneração financeira pela participação no estudo, bem como, que não terá custos em participar. Declara que foi informado da garantia de receber resposta ou esclarecimento sobre a pesquisa a ser realizada, bem como da liberdade de não participar do estudo e da possibilidade de desistir, em qualquer momento, da participação. Além disso, declara que recebeu cópia deste termo de consentimento.

Data: ___/___/_____
Paciente: _____
Responsável legal: _____
Assinatura: _____

Eu expliquei a _____os objetivos, riscos, benefícios e procedimentos necessários para esta pesquisa, e entreguei cópia deste termo de consentimento para o mesmo.

Data: ____/_____/_____
Nome:_____
Assinatura: _____

HCPA - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE
GRUPO DE PESQUISA E PÓS-GRADUAÇÃO

COMITÊ DE ÉTICA EM PESQUISA

O Comitê de Ética em Pesquisa do Hospital de Clínicas de Porto Alegre (CEP/HCPA), que é reconhecido pela Comissão Nacional de Ética em Pesquisa (CONEP)/MS e pelo Office For Human Research Protections (OHRP)/USDHHS, como Institutional Review Board (IRB00000921) analisou o projeto:

Projeto: 120019
Data da Versão do Projeto: 02/06/2012
Data da Versão do TCLE: 13/01/2012

Pesquisadores:
IDA VANESSA DOEDERLEIN SCHWARTZ
URSULA DA SILVEIRA MATTE
TACIANE ALEGRA
FERNANDA SPERB LUDWIG
GABRIELA KAMPF CURY
RENATA VOLTOLINI VELHO

Título: USO DE GENTAMICINA E CLORANFENICOL IN VITRO COMO ESTRATÉGIA DE TRATAMENTO PARA AS MUCOLIPIDOSES II E III

Este projeto foi APROVADO em seus aspectos éticos e metodológicos, bem como o seu respectivo Termo de Consentimento Livre e Esclarecido, de acordo com as diretrizes e normas nacionais e internacionais de pesquisa clínica, especialmente as Resoluções 196/96 e complementares do Conselho Nacional de Saúde.

- Os membros do Comitê de Ética em Pesquisa não participaram do processo de avaliação dos projetos nos quais constam como pesquisadores.
- Toda e qualquer alteração do projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente ao CEP/HCPA.
- O pesquisador deverá apresentar relatórios semestrais de acompanhamento e relatório final ao CEP/HCPA.
- Somente poderá ser utilizado o Termo de Consentimento Livre e Esclarecido no qual conste o carimbo de aprovação do CEP/HCPA.

Porto Alegre, 11 de junho de 2012.

Profª Nadine Clausell
Coordenadora GPPG e CEP/HCPA