UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

ANÁLISE MOLECULAR DE PACIENTES COM MUCOPOLISSACARIDOSE TIPO II

Ana Carolina Brusius Facchin

Porto Alegre,2012

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Orientadora: Dra. Sandra Leistner-Segal

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RESUMO

Introdução: Mucopolissacaridose tipo II é uma doença lisossômica causada pela deficiência da enzima idounato-2-sulfatase. A incidência de MPS II é muito baixa, geralmente menos de 1 caso para cada 1.000.000 recém-nascidos. Até o presente momento, cerca de 340 mutações foram identificadas no gene da idorunato-sulfatase. **Objetivo:** O presente trabalho teve como objetivo principal identificar as alterações moleculares presentes em 149 pacientes brasileiros e sul americanos com diagnóstico bioquímico de MPS II. **Métodos:** Após a realização do protocolo inicial proposto e que incluía o sequenciamento completo do gene IDS, alguns pacientes não apresentaram alterações e foram incluídos num protocolo adicional para casos “especais”. **Resultados:** Uma deleção de 178pb foi encontrada na região promotora do gene, em 2 pacientes que não apresentaram alterações em região codificante, bem como outros 2 pacientes apresentaram um polimorfismo na mesma região, que nunca havia sido relatado em pacientes com MPS II. A detecção de uma deleção em 3 pacientes através da técnica de PCR convencional,exon por exon, nos mostrou que a deleção se estendia da região proximal do gene *IDS* até a região dos genes *FRAXA* e *FRAXE*, contudo análises adicionais, através de *SNP-array*, confirmaram a deleção restrita a estas regiões em 2 pacientes e identificaram a deleção total retrita ao gene IDS em outro. Após a análise de 105 pacientes com MPS II, 30 novas mutações foram encontradas o que demonstra uma grande heterogeneidade genica. **Conclusão:** Tais análises são importantes para elucidar o defeito básico e assim poder identificar portadoras nas famílias, o que tem uma importância fundamental para o aconselhamento genético, diagnóstico pré-natal e prevenção de novos casos. Adicionalmente, a informação sobre o defeito genético-molecular é muito importante para a predição do fenótipo e consequentemente para a definição da melhor estratégia de tratamento.

Palavras-chaves:

Mucopolissacaridose, Síndrome de Hunter, Análise Molecular

ABSTRACT

Background: Mucopolysaccharidosis type II is a lysosomal disease caused by deficiency of the enzyme idounate-2-sulfatase. The incidence of MPS II is very low, usually less than 1 case per 1 million newborns. To date, about 340 mutations have been identified in the IDS gene. **Objective:** This study aimed to identify the molecular alterations present in 149 Brazilian and South American patients with biochemical diagnosis of MPS II. **Methods**: After molecular analysis using the initial protocol proposed, wich included complete sequencing of the IDS gene, someof the patients showed no DNA alterations of the gene coding region and were included an additional protocol for “special” cases. **Results:** A deletion of 178pb was found in the promoter region of the gene in two patients. An other 2 patients had a polymorphism in the same region, which had never been reported in patients with MPS II. A deletion was observed in 3 patients by conventional PCR, exon by exon, which extended from the proximal IDS gene until the fagile sites FRAXA and FRAXE. Additional analyzes using SNP-array confirmed the deletion of those regions in two patients and have identified the full deletion restricted to IDS in another. After analysis of 105 patients with MPS II, 30 new mutations were found which shows a broad genomic heterogeneity. **Conclusion:** Such analyzes are important to elucidate the basic defect and thus enable to identify carriers in families, which is of fundamental importance for genetic counseling, prenatal diagnosis and prevention of new cases. In addition, information about the molecular genetic defect is very important for the prediction of phenotype and thus for defining the best tstrategy for treatment.

Keywords:

Mucopolyssacharidosis, Hunter Syndrome, Molecular analysis

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Lista de Abreviaturas e Siglas

ANVISA- Agência Nacional de Vigilância Sanitária
ATP- Adenosina Trifosfato
cDNA- Àcido desoxiribonucleico complementar
DNA- Àcido desoxiribonucleico
DL- Doença lisossômica
DS- Dermatan sulfato
EIM- Erros Inatos do metabolismo
GAGs- Glicosaminoglicanos
gDNA- Àcido desoxiribonucleico genômico
GM2- Gangliosidose tipo 2
GM3- Gangliosidose tipo 3
HGMD- Human Genome Molecular Database
HS- Heparan sulfato
IDS- Iduronato sulfatase
IDSP1- Iduronato sulfatase pseudogene
KDa- Kilodalton
Kg- Kilograma
M6P- Manose 6 fosfato
mg- miligrama
MPS- Mucopolissacaridose
MPS I- Mucopolissacaridose tipo I
MPS II- Mucopolissacaridose tipo II
MPS III- Mucopolissacaridose tipo III
MPS IV- Mucopolissacaridose tipo IV
MPS V- Mucopolissacaridose tipo V
MPS VI- Mucopolissacaridose tipo VI
pb- pares de base
PCR- Reação em Cadeia da Polimerase (*Polymerase Chain Reaction*)
RE- Retículo endoplasmático
RN-Recém nascidos vivos
SNC- Sistema Nervoso Central
SNP- Polimorfismo de Nucleotídeo único *(Single Nucleotide Polymorfism)*
TCTH- Terapia de Células Tronco Hepatopoiéticas
TMO- Terapia de transplante de Medula Óssea
TRE- Terapia de Reposição Enzimática

Sumário

1. INTRODUÇÃO

As MPS são um grupo de doenças lisossômicas (DL) causadas pela deficiência de uma das onze enzimas lisossomais envolvidas na degradação dos glicosaminoglicanos (GAG) (Neufeld e Muenzer, 2001).

A MPS II (Síndrome de Hunter) é de herança recessiva ligada ao X (OMIM 309900), causada pela deficiência da L-iduronato-2-sulfato sulfatase (iduronato-sulfatase ou IDS). A IDS é uma das enzimas responsáveis pela degradação dos glicosaminoglicanos Heparan e Dermatan Sulfato.

As manifestações clínicas típicas da MPS II incluem graus variáveis de face característica, hepatoesplenomegalia, disostose múltipla, rigidez articular e envolvimento neurológico. Embora exista um espectro quase contínuo de gravidade das manifestações clínicas, duas formas são classicamente associadas à MPS II: forma grave e forma leve.

O gene que codifica a IDS foi mapeado no cromossomo Xq28.1, é composto por 9 éxons e 8 introns e tem um tamanho aproximado de 24 kb. Um pseudogene altamente homólogo aos éxons II e III e aos introns 2, 3 e 7 do gene IDS localiza-se 20 kb distal ao gene ativo *(*Bondeson *et al*., 1995*).* O promotor contém duas seqüências consenso do tipo GC Box, indicando que os níveis de transcrição são baixos e que este é um *housekeeping* gene.

Foram descritas, até o momento, 335 mutações patogênicas no gene IDS, a maioria mutações de ponto ou pequenas deleções (HGMD, 2012). Grandes deleções e rearranjos ocorrem em aproximadamente 20% dos pacientes.

Análises do gene IDS são importantes para elucidar o defeito básico e assim poder identificar portadoras nas famílias, o que tem uma importância fundamental para o aconselhamento genético, diagnóstico pré-natal e prevenção de novos casos. Adicionalmente, a informação sobre o defeito genético-molecular é muito importante para a predição do fenótipo e consequentemente para a definição da melhor estratégia de tratamento.

2.REVISÃO DE LITERATURA

2.1 Erros Inatos do Metabolismo

O conceito Erros Inatos do Metabolismo (EIM), foi proposto por Sir Archibald Garrod, em 1908, quando estudava pacientes portadores de quatro condições (alcaptonúria, pentosúria, albinismo e cistinúria), acreditando que essas eram causadas por defeitos no metabolismo intermediário de aminoácidos e monossacarídeos (Clarke,1996).

Os EIM são doenças determinadas geneticamente, causadas por alterações na estrutura e/ou função de moléculas protéicas importantes para reações bioquímicas, e conseqüente alteração do metabolismo humano (Saínz *et al.*,2002).

Atualmente incluem cerca de 500 enfermidades, e apresentam-se, em sua maioria, na infância (Neufeld e Muenzer, 2001). Apesar de terem uma incidência individual relativamente baixa (Hopwood e Morris 1990), os EIM são muito importantes do ponto de vista da sua magnitude como um problema de saúde, com mortes prematuras, transtornos neurológicos e baixa qualidade de vida (Saínz *et al.*,2002).

Tratando-se de alterações metabólicas bastante distintas, os EIM possuem diversas classificações, no entanto vale ressaltar a classificação de Saudubray e Charpentier (1995), por apresentar-se mais didática e de maior aplicação clínica.

De acordo com essa classificação os EIM dividem-se em duas categorias, a categoria 2, por apresentar grande variabilidade de alterações, é subdividida em 3 grupos de acordo com suas características fisiopatológicas e fenótipo clínico. A tabela 1 adaptado de Martins, 1999, apresenta uma classificação resumida, segundo os autores. Dentro do grupo 1 vale ressaltar a presença das doenças lisossômicas, causadas por distúrbios de síntese ou catabolismo de moléculas complexas

Tabela 1: Classificação clínica dos Erros inatos do metabolismo

Categoria 1	Envolvido no sistema funcional
Categoria 2	Afeta rotas metabólicas comuns
Grupo 1	Defeitos na síntese ou catabolismo de moléculas complexas
Grupo 2	Defeitos no metabolismo intermediário
Grupo 3	Deficiência na produção ou utilização de energia

(Adaptado de Martins, 1999)

2.2 Doenças Lisossômicas

A maioria das doenças que conhecemos como Doenças Lisossômicas (DL), foram descritas primeiramente no final do século 19, muito tempo após a descoberta do lisossomo por de Duve em 1955 (Wilcox 2004). Essas doenças são decorrentes da atividade deficiente de uma enzima lisossômica que estão envolvidas no processo de degradação de macromoléculas, que pode ser uma hidrolase, ou da deficiência de co-fatores envolvidos no processo de degradação de macromoléculas, ou ainda de transportadores. A maioria das enzimas são exohidrolases que agem em seqüência, sendo os substratos degradados em etapas sucessivas através da remoção de seus resíduos terminais. A deficiência de uma enzima lisossômica causa o bloqueio da via metabólica envolvida, levando a não remoção do substrato, o que torna inacessível a hidrólise por outras enzimas lisossômicas (Gieselmann,1995).

Na maioria das DL, mais de um composto é acumulado, por exemplo, na MPS II (OMIM 30900) os principais substratos acumulados são os glicosaminoglicanos (GAGs) dermatan e heparan sulfato, mas outras substâncias como ganglosídeos GM2 e GM3 e subunidade C do trifosfato de adenosina (ATP) da síntese mitocondrial também são acumulados principalmente no sistema nervoso central (Ballabio;Gieselmann, 2009).

As conseqüências celulares do acúmulo do substrato são determinadas por vários fatores: 1) tipo de material armazenado; 2) quantidade do acúmulo; 3) tipo

celular que contém o depósito; 4) processos celulares envolvidos, como tráfego intracelular, autofagia e a transdução de sinais (Ballabio;Gieselmann, 2009). (Figura 1) (Futerman e Meer, 2004).

Além disso, a maioria é herdada de maneira autossômica recessiva, com exceção da síndrome de Hunter (mucopolissacaridose tipo II ou MPS II) e da doença de Fabry, que são ligadas ao X (Wraith,2009;Walkley,2009).

A gravidade dessas doenças depende do tipo e quantidade de substratos acumulados, sendo a maioria progressiva. Os pacientes apresentam deformações esqueléticas e danos no cérebro, fígado, pulmões e outros órgãos internos.

As DL são doenças raras, com incidência individual muito baixa, mas apresentam uma incidência conjunta estimada de 1:7.000 recém-nascidos vivos (Meikle,1999; Poorthuis *et al.*,1999).

Conforme o tipo de substrato acumulado as DL podem ser classificadas em: esfingolipidoses, mucopolissacaridoses (MPS), glicoproteinoses e outras (Wilcox, 2004) (Tabela 1). No caso das mucopolissacaridoses os principais substratos acumulados são os glicosaminoglicanos (GAGs).

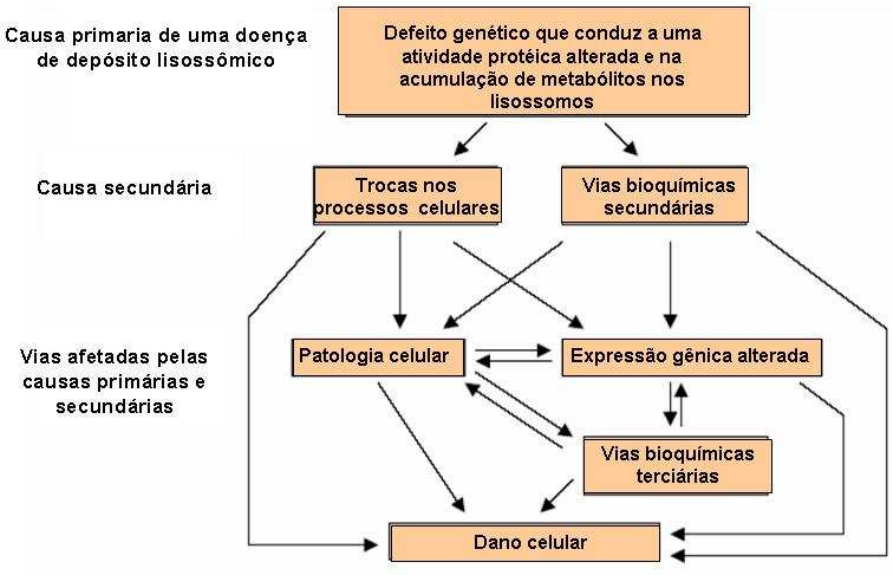

Figura 1: Esquema proposto da patologia das doenças de acúmulo lisossômico: A causa primária da doença é a acumulação intralisossômica de substratos não metabolizado, mas o amplo espectro de variedade de sintomas indica que deve haver vias bioquímicas e celulares secundárias ativas que provoquem a patologia celular, a alteração da expressão gênica e ativação das vias bioquímicas terciárias. (Adaptado de Futermann e Meer, 2004).

As DL são causadas por uma ou mais mutações em cerca de 40 genes diferentes que codificam proteínas de hidrólise e transporte de componentes celulares (Wenger *et al*., 2002).

Tabela 2 - Principais Doenças Lisossômicas

Doença	Proteína defeituosa	Principais materias armazenados
Esfingolipidoses		
Fabry	α-Galactosidase A	Globotriaosilceramida
Gaucher	β-Glicosidase	Glicosilceramida
Niemann-Pick A e B	Esfingomielinase	Esfingomielina
Gangliosidoses GM1	β-Galactosidase	Gangliosídeo GM1
Gangliosidoses GM2 (Tay-Sachs)	β-Hexosaminidase A	Gangliosídeo GM2 e glicolipídeos relacionados
Gangliosidoses GM2 (Sandhoff)	β-Hexosaminidase A e B	Gangliosídeo GM2 e glicolipídeos
Gangliosidoses GM2 (Deficiência do ativador de GM2)	Proteína ativadora de GM2	Gangliosídeo GM2 e glicolipídeos relacionados
Mucopolissacaridoses		
MPS I (Hurler, Scheie, Hurler/ Scheie)	α-Iduronidase	Dermatan Sulfato e Heparan Sulfato
MPSII (Hunter)	Iduronato2-Sulfatase	Dermatan Sulfato e Heparan Sulfato
MPS IIIA (Sanfilippo A)	Heparan N-Sulfatase	Heparan Sulfato
MPS IIIB (Sanfilippo B)	N-Acetil- α-glucosaminidase	Heparan Sulfato
MPS IIIC (Sanfilippo C)	Acetil-CoA: α-glucosamida N-acetiltransferase	Heparan Sulfato
MPS IIID (Sanfilippo D)	N-Acetilglucosamina-6-sulfatase	Heparan Sulfato
MPS IV (Morquio A)	N-Acelgalactosamina	Queratan Sulfato e Controitin Sulfato
MPS IV (Morquio B)	β-Galactosidase	Queratan Sulfato
MPS VI (Maroteaux-Lamy)	N-Acetilgalactosamina-4-sulfatase (Arilsulfatase B)	Dermatan Sulfato
MPS VII (Sly)	β-Glucuronidase	Heparan Sulfato, Dermatan Sulfato e Condroitin Sulfato
Oligosacaridoses e glicoproteinoses		
Pompe	α-Glicosidase	Glicogênio

Doenças causadas por defeitos em proteínas integrais da membrana		
Cistinoses	Cistinosina	Cistina
Doença de Danon	LAMP2	Restos citoplasmáticos e glicogênio
Mucolipidose (ML) IV	Mucolipina-1	Lipídios e mucopolisacarídeos ácidos
Niemann-Pick C (NPC)	NPC 1 e 2	Colesterol e esfingolipídeos
Outras		
Galactosialidoses	Catepsina A	Sialiloligosacarideos
Deficiência Múltipla de Sulfatases	Enzima geradora de α-formilglicina	Sulfatídeos
Lipofuscinose Ceroide Neuronal (NCL1)	Proteína palmitoiltioesterase-1	Tioésteres lipidados
Lipofuscinose Ceroide Neuronal (NCL2)	Tripeptidil Amino Peptidase-1	Subunidade c da ATP sintetase mitocondrial
Lipofuscinose Ceroide Neuronal (NCL3)	Transportador de Arginina	Subunidade c da ATP sintetase mitocondrial

(Adaptado de Futerman & van Meer, 2004)

As DL tem incidência estimada de 7-14:100.000 nascimentos, de modo geral variando conforme a procedência da amostra estudada: Meikle e colaboradores (1999), na Austrália, descreveram uma incidência de 13:1.000.000 nascimentos; Poorthuis e colaboradores (1999), na Holanda, uma incidência de 14:100.000 nascimentos; Applegarth e colaboradores (2000), no Canadá, uma incidência de 7,6:100.000 nascimentos; Tylhi-Szymanska e colaboradores (2001), na Polônia, uma incidência de 0,5 a 2:100.000 recém nascidos vivos. No Brasil, Coelho e colaboradores (1997), encontraram uma frequencia relativa de DL de 59,8% entre pacientes de risco.

2.3 Lisossomos e enzimas lisossômicas

Os lisossomos estão presentes em todas as células nucleadas, formam o sistema intracelular de reciclagem, estando envolvidos na degradação de grandes macromoléculas (Wraith, 2002). Contém numerosas hidrolases ácidas que catabolizam proteínas, ácidos nucléicos, lípideos, sulfatos, fosfatos e carboidratos complexos. Cada enzima lisossomal é parte de um complexo processo que reduz macromoléculas em pequenos componentes, os quais são reutilizados por células

ou eliminados, eventualmente, do corpo. A ausência de uma enzima causa um bloqueio do processo do catabolismo, causando o acúmulo de produtos do metabolismo intermediário. O acúmulo desses produtos causam o aumento do lisossomo, que ocupam um espaço intracelular maior, interferindo na função celular (Kornfeld e Mellman, 1989).

As enzimas lisossomais seguem uma rota intracelular comum. As formas precursoras são sintetizadas em polissomos ligados à membrana do retículo endoplásmático (RE). Proteínas nascentes são transportadas para o lúmen do RE, co-traducionalmente, por meio de uma sequencia específica, que se encontra na sua extremidade amino-terminal. No lúmen do RE, as formas precursoras são glicosiladas co e pós-traducionalmente em sítios específicos; a glicosilação é essencial para a formação da estrutura terciária da proteína. Após a translocação,ocorre clivagem do peptídeo sinalizador,as formas resultantes são transportadas para o aparelho de Golgi. No Golgi, inicia-se o processo de fosforilação de certos resíduos de manose da proteína, o qual culmina na criação dos sítios de reconhecimento do receptor de manose-6-fosfato (M6P). Após as proteínas ligam-se ao segmento luminal dos receptores de M6P transmembrana. Nos endossomos tardios, o pH ácido promove a dissoçiação das proteínas de seus receptores, os quais são reciclados e retornam ao aparelho de Golgi. Uma parte das enzimas lisossômicas que contém o M6P são secretadas antes de atingirem o lisossomo (Alberts,2004).

As várias etapas envolvidas na síntese das enzimas lisossomais são resumidas na figura 2. As etapas finais na maturação da enzima lisossômica incluem proteólise, dobramento e agregação (Vellodi, 2004).

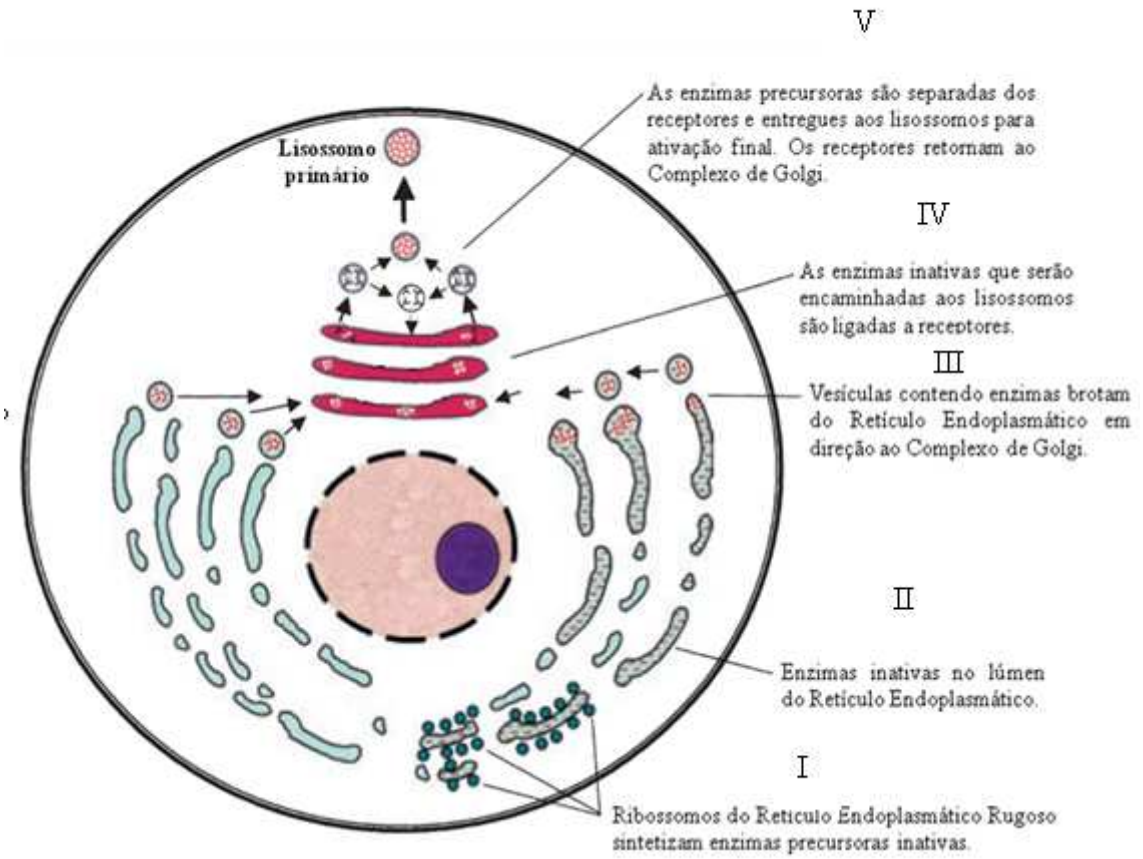

Figura 2: Síntese das enzimas lisossomais (Adaptado de Vellodi, 2004).

2.4 Glicosaminoglicanos e Mucopolissacaridoses

Os glicosaminoglicanos são cadeias polissacarídicas não ramificadas compostas de unidades dissacarídicas repetidas (Figura 3). São chamadaos GAGs porque um dos açúcares no dissacarídeo repetido é sempre um amino açúcar (*N*-acetilglucosamina ou *N*-acetilgalactosamina) o qual, na maioria das vezes, é sulfatado. O segundo açúcar é, normalmente, um ácido urônico (glucurônico ou idurônico) (Alberts *et al*., 2004).

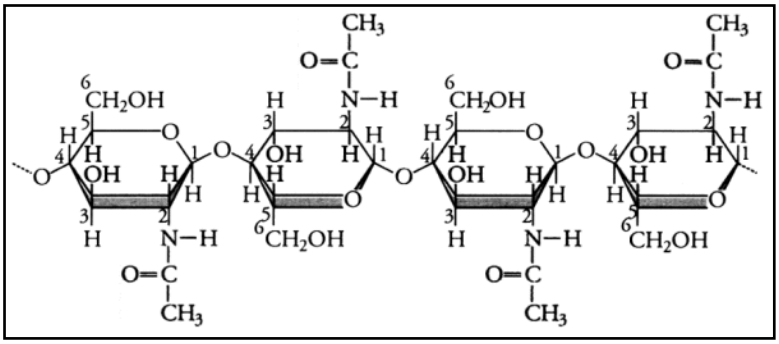

Figura 3: Cadeia polissacarídica

Quatro principais grupos de GAGs são distinguidos de acordo com seus açúcares, o tipo de ligação entre os açúcares, o número e localização dos grupos sulfato. São eles: (1) Ácido Hialurônico , (2) Condroitin Sulfato e Dermatan sulfato, (3) Heparan sulfato e (4) Queratan sulfato (Alberts *et al*., 2004).

Várias enzimas agem seqüencialmente na degradação desses GAGs, e a atividade deficiente de cada uma delas associa-se a um tipo específico de Mucopolissacaridose (MPS).

No caso das MPS a não degradação, ou degradação parcial dos GAGs ocorre nas células, tecidos e órgãos levando ao conjunto de sinais e sintomas que os pacientes apresentam. Esses substratos são excretados de forma aumentada na urina, e a classificação da doença se da através do tipo de substrato acumulado e da atividade enzimática deficiente. Ao todo existem onze deficiências enzimáticas para sete tipos e subtipos de MPS (Neufeld e Muenzer, 2001) (Tabela 2).

Tabela 3: Classificação dos tipos de MPS. (Adaptado de Neufeld e Muenzer,2001)

MPS	Enzima Deficiente	Sigla	Epônimo	GAGs na urina	Localização do gene
I	*α-L-iduronidase*	*IDUA*	Hurler,	DS+HS	4p16.3
II	*Iduronato-2-sulfatase*	*IDS*	Hunter	DS+HS	Xq28
IIIA	*Heparan-N-sulfatase*	*SGSH*	Sanfilipo A	HS	17q 25.3
IIIB	*α-N-acetilglicosaminidase*	*NAGLU*	Sanfilipo B	HS	17q21
III C	*AcCoA:α-glicosamina acetiltransferase*	*GNAT*	Sanfilipo C	HS	8q11-8p11
III D	*N-acetilglicosamina-6-sulfatase*	*G6S*	Sanfilipo D	HS	12q14
IV A	*Galactose-6-sulfatase*	*GALNS*	Morquio A	QS	16q24.3

IV B	*β-galactosidase*	*GLB1*	Morquio B	QS	3p21.33
VI	*N-acetilgalactosamina-4-sulfatase*	*ARSB*	Maroteaux-Lamy	DS	5q13-q14
VII	*β-glicuronidase*	*GUSB*	Sly	DS+HS	7q21.11
IX	*Hialuronidase*	*HYAL1*	Natowicz	H	3p21.1-p21.3

DS=dermatan sulfato; HS= heparan sulfato; QS= queratan sulfato; CS= condroitin sulfato; H= Hialuran

Estudos internacionais relatam uma incidência conjunta para todos os tipos de 1,9 a 4,5:100.000 em recém-nascidos vivos (Baehner F *et al.*, 2005; Nelson J *et al.*, 2003; Applegarth DA *et al.*, 2000; Meikle PJ *et al.*, 1999; Poorthuis BJ *et al.*, 1999 e Nelson J, *et al*, 2003).

A incidência das MPS no Brasil não é completamente conhecida. A Rede MPS Brasil, uma iniciativa para promoção do diagnóstico das MPS em nosso país, sediada no SGM/HCPA, tem um total de 983 pacientes brasileiros com MPS cadastrados desde 2004 até junho de 2012, sendo 194 com MPS I, 292 com MPS II, 134 com MPS III (MPS IIIA: 37; MPS IIIB: 68; MPS IIIC: 29; MPS IIID: 0), 113 com MPS IVA, 8 MPS IVB, 230 com MPS VI e 12 MPS VII.

As características clínicas das MPSs se dão pela presença de algumas caraterísticas fenotípicas como: face infiltrada, hepatoesplenomegalia, disostose múltipla, contraturas articulares, surdez, opacificação da córnea, cardiopatias e retardo mental (Neufeld e Muenzer, 2001). Os quadros clínicos são crônicos e progressivos, e o diagnóstico preciso para especificação de cada tipo de MPS, se torna difícil apenas pelo exame clínico.

Este trabalho tem ênfase na análise de pacientes com MPS do tipo II, na qual estaremos concentrando-nos próximos itens.

2.5 Síndrome de Hunter (MPS II)

Os primeiros pacientes com MPS II foram descritos por Charles Hunter em 1917, sendo os primeiros descritos na literatura internacional.

A MPS II é uma DL de herança recessiva ligada ao X (OMIM 309900), causada pela deficiência da L-iduronato-2-sulfato sulfatase (IDS). A IDS é uma das

enzimas responsáveis pela degradação dos glicosaminoglicanos (GAGs) dermatan (HS) e heparan sulfato (DS).

A incidência da MPS II varia entre 1:110.000 e 1:320.000 nascidos vivos (Meikle et al, 1999; Applegarth *et al.* 2000; Baehner *et al.*,2005). Em termos de freqüência relativa a MPS II parece ser o tipo mais freqüente em Taiwan e norte da Ásia (Lin *et al.*,2006). No Brasil não há dados epidemiológicos disponíveis a respeito da incidência da MPS II, mas segundo resultados da Rede MPS Brasil ela é a mais freqüentemente diagnosticada.

O dermatan sulfato é um polímero linear heterogêneo formado por uma sequência de 70 a 200 unidades sucessivas de *N*-acetil-D-galactosamina (D-GalNAc) unidas por ligações β (1, 3) a resíduos de ácido *L*-idurônico. O dermatan sulfato contém aproximadamente um grupo O-sulfato para cada unidade de dissacarídeo, encontrando-se normalmente na posição 4 da D-GalNAc, e em menor frequência na posição 6 da D-GalNAc e na posição 2 do ácido L-idurônico. O peso molecular do dermatan sulfato varia entre 12 e 45 KDa, com um peso molecular médio entre 20 e 30 KDa (Fernández, 2008) (Figura 4).

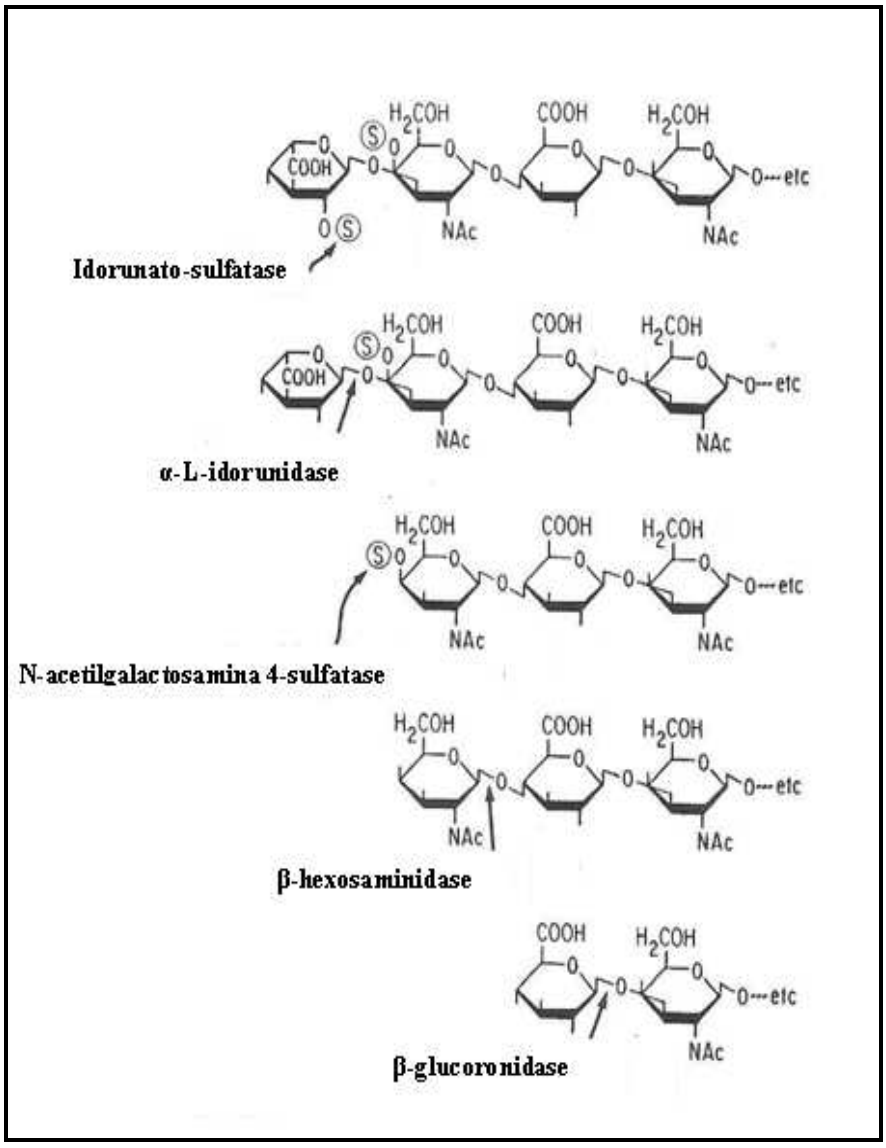

Figura 4: Rota de degradação do dermatan sulfato

O Heparan sulfato é um polímero polidisperso lineares constituído por unidades alternadas de α -D-glucosamina (GlcN) e ácido urónico, ou *β* -ácido D-glucurónico (GlcA) ou α -L-idurónico ácido (IdoA), unidas entre si por (1 →ligações 4) glicosídicas. A GlcN pode ser N-sulfatada, ou N-acetiladas. Está localizado de frente para o compartimento extracelular e, portanto, os seus papéis biológicos podem estar relacionados formação de matrizes extracelulares (Peretti et al. 2008), modulação da atividade de enzimas e / ou os seus inibidores (Raman et al. 2005,), fornecimento de um gradiente extracelular de factores de crescimento e quimiocinas (Grunert et al. 2008) entre outros (Figura 5).

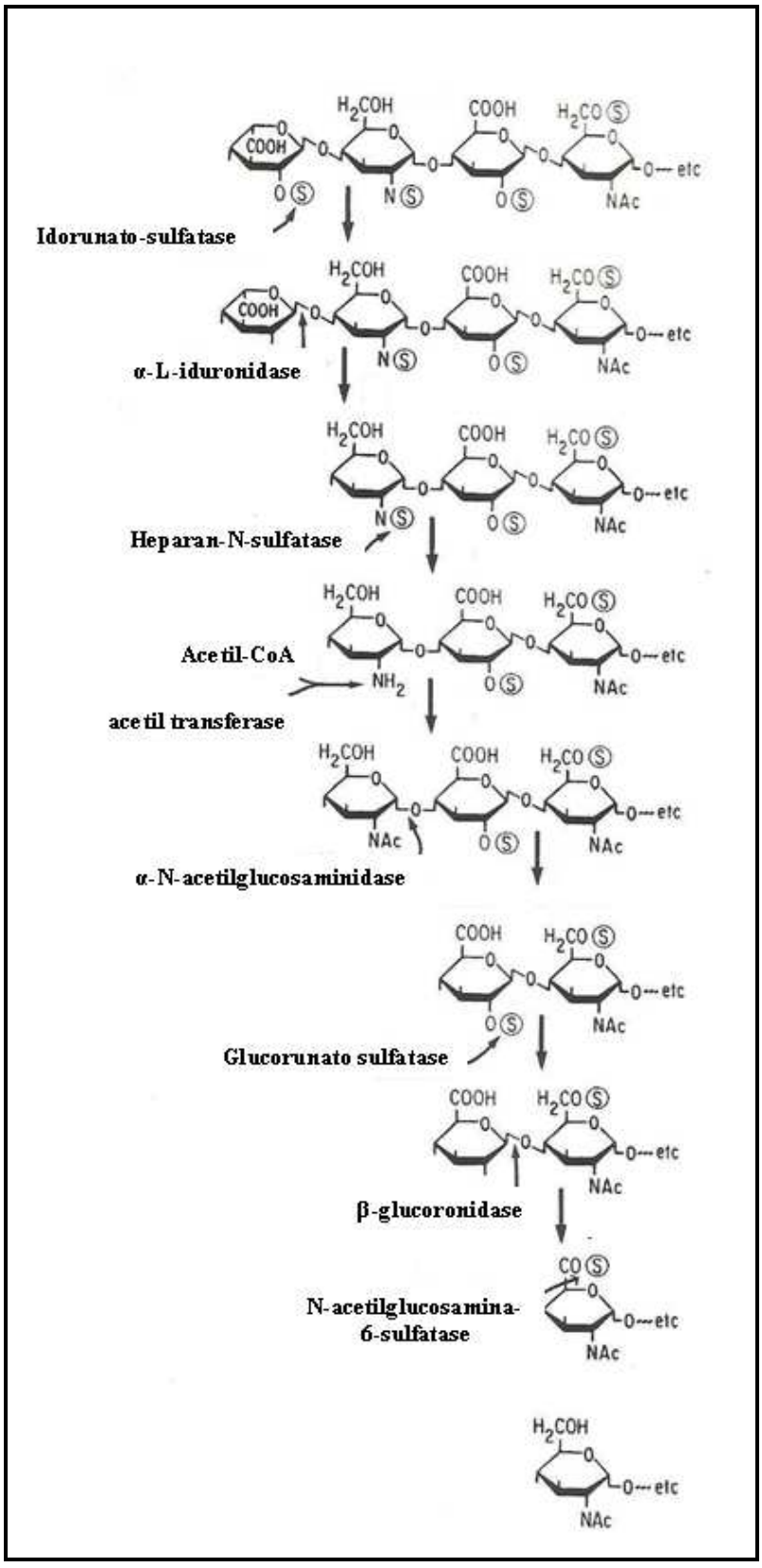

Figura 5: Rota de degradação do heparan sulfato

Na MPS II, estes GAGs parcialmente degradados acumulam-se no interior dos lisossomos e são excretados em quantidade aumentada na urina, levando as diferentes manifestações clínicas que os pacientes apresentam.

2.5.1 Manifestações Clínicas

A MPS II está associada à grande heterogeneidade clínica, já que os pacientes apresentam graus variáveis de envolvimento de vários órgãos e sistemas, com uma significativa variabilidade na idade de início dos sintomas e taxa de progressão (Neufeld e Muenzer, 2001).

Os pacientes são classificados em forma grave e forma atenuada:

a) forma grave: mais freqüentemente caracterizada por face infiltrada, baixa estatura, distose múltipla, surdez, hepatoesplenomegalia, deterioração mental progressiva.

b) forma atenuada: de início mais tardio, com leve ou nenhum retardo mental (Young and Harper 1982), as demais manifestações clínicas apresentam-se de modo variável entre os pacientes (Wraith et al, 2008).

O envolvimento do SNC, a característica mais significativa no grupo de crianças, muitas vezes identificadas com a doença "grave", manifesta-se principalmente pela deterioração cognitiva progressiva. Tal declínio cognitivo, combinado com a via aérea progressiva e doença cardíaca, normalmente resultam em morte na primeira ou segunda década de vida.

O fenótipo grave parece ser duas vezes mais frequente do que a forma atenuada da doença, embora as taxas de prevalência não estejam disponíveis. Oitenta e quatro por cento dos homens afetados têm algum tipo de envolvimento neurológico. Comprometimento cardiovascular foi relatado em 82% dos indivíduos afetados (Wraith et al 2008).

Acúmulo de GAG em praticamente todos os órgãos ocorre em MPS II, mas alguns sistemas específicos do corpo são mais afetados do que outros.

2.5.2- Aspectos Bioquímicos

Em 1957, quando foi identificada a presença de dermatan e heparan sulfatos na urina de pacientes com MPS I, as bases para um teste bioquímico de rotina para o diagnóstico da MPS foram estabelecidas (Matte, 1998). Há excreção aumentada na urina de tipos diferentes de GAGs, de acordo com a deficiência enzimática (Neufeltd & Muenzer, 2001). Por isso, em geral, faz-se primeiro o teste de triagem na urina para orientar a escolha do ensaio enzimático a ser realizado (Schwartz *et al*., 2001; Wraith, 1996; Beck, 2000).

A partir da suspeita clínica, o diagnóstico das MPS é estabelecido através da realização de exames bioquímicos específicos (Figura 7). Na maioria das vezes a suspeita diagnóstica não é específica para um tipo de MPS, com isso o primeiro exame realizado é a análise dos GAGs na urina através da dosagem e de cromatografia. Esses exames iniciais permitem orientar a direção dos estudos enzimáticos. A maioria das MPSs podem ser triadas por esses métodos, mas somente a medida da atividade da enzima específica permitirá o diagnóstico definitivo do tipo de MPS (Neufeld e Muenzer, 2001).

Os pacientes com MPS II excretam HS e DS em quantidade aumentada na urina. A mucopolissacaridúria anormal pode ser detectada por testes semiquantitativos, qualitativos (cromatografia em camada delgada de glicosaminoglicanos e eletroforese bi-dimensional) e quantitativos. Estes testes são rápidos, baratos e úteis para a avaliação laboratorial inicial (*screening)*, mas estão associados a resultados falso-positivos e falso-negativos (Barth *et al.*, 1990). O diagnóstico definitivo da MPS II depende da demonstração da deficiência da IDS em plasma, leucócitos ou fibroblastos.

O acúmulo de GAGs nos lisossomos das células produz uma excessiva vacuolinização que pode ser detectada, histologicamente, em diversos tipos celulares e tecidos, principalmente em fibroblastos, células endoteliais e parenquimais, macrófagos e leucócitos (Figura 6) (Fernández, 2008)

Tentativas de diferenciação entre as formas clínicas da MPS II através do estudo de parâmetros bioquímicos/imunológicos (atividade da IDS, imunoquantificação da IDS, dosagem de GAGs na urina foram somente parcialmente bem sucedidas (Neufeld e Muenzer, 2001).

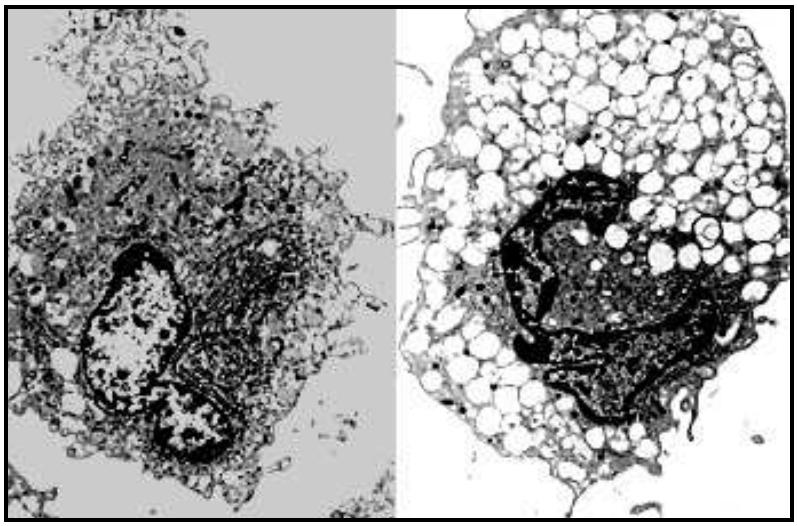

Figura 6: Representação de uma célula com degradação normal e anormal dos glicosaminoglicano.Célula da esquerda com degradação normal dos glicosaminoglicanos e célula da direita com degradação anormal dos glicosaminoglicanos, com consequente acúmulo dentro dos lisossomos, fazendo com que pareçam grandes, redondos e edemaciados ao microscópio (Adaptado de Neufeld & Muenzer, 2001).

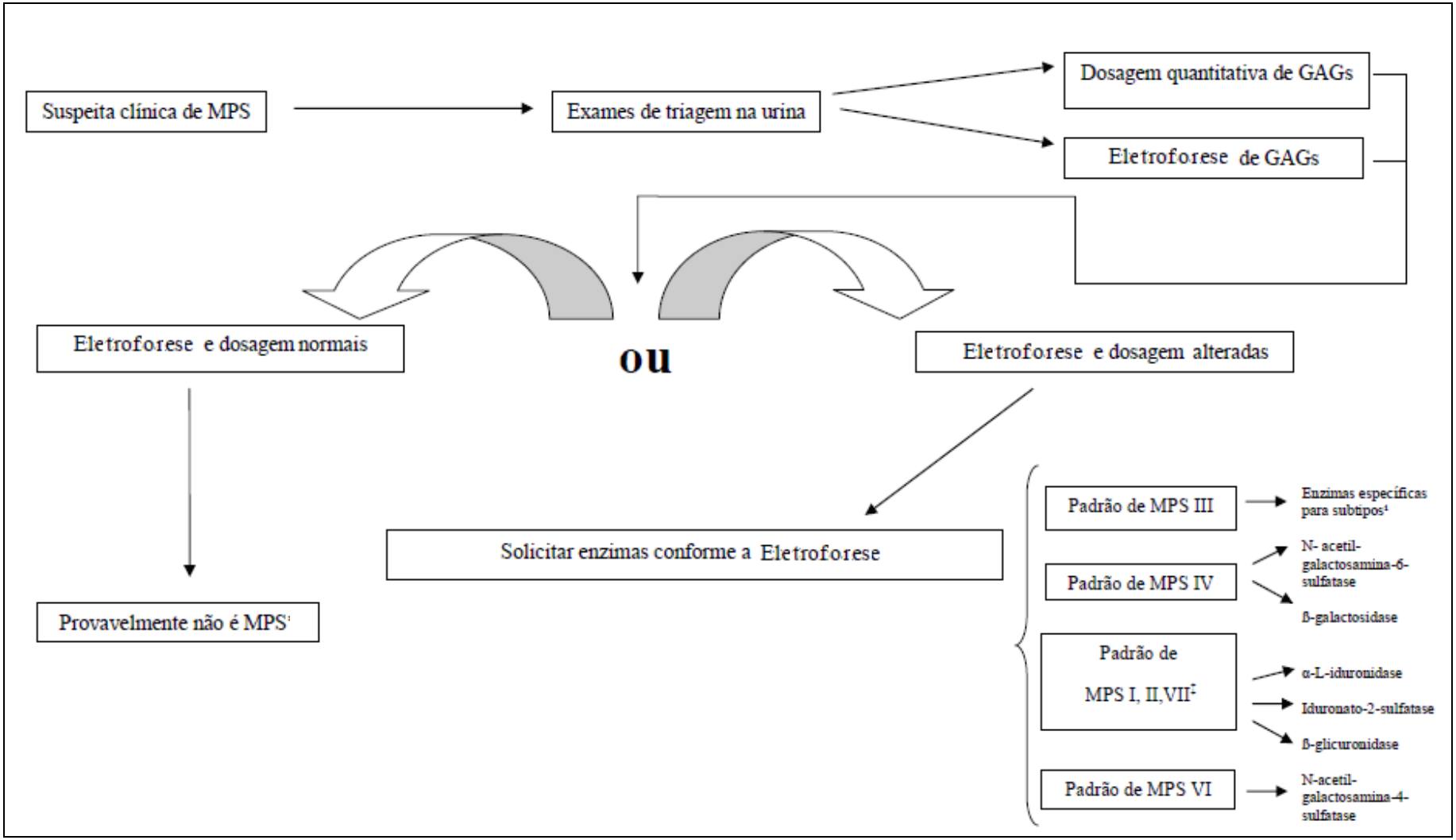

Figura 7: Fluxograma do diagnóstico bioquímico

2.5.3- Aspectos Genéticos

2.5.3.1 Sulfatases humanas e Idorunato-2-sulfatase

Constituem uma classe de enzimas altamente conservadas em nível de sequencia, estrutura e mecanismo catalítico tanto em eucariotos como em procariotos (Hanson *et al.*, 2004).

Ao todo se conhece 19 sulfatases humanas, sendo sua função, em doenças de depósito lisossômico, de grande importância, resultando no estudo de vários genes de sulfatases lisossômicas. O grau de homologia entre essas enzimas é de 20-30% para cada par analizado (Bond *et al*.,1997).

As sulfatases humanas são encontradas em vários locais subcelulares, onde desenvolvem papéis em processos biológicos importantes, incluindo síntese de hormônios, degradação de glicosaminoglicanos e glicolipídeos no lisossomo, e modulação de sinais entre célula e matriz extracelular. Algumas sulfatases humanas foram identificadas, mas suas funções biológicas continuam desconhecidas.

As sulfatases lisossômicas foram às primeiras associadas a uma função fisiológica, e são hoje, as melhores caracterizadas. Devido a sua localização subcelular, estas enzimas tem um pH ótimo na porção ácida (pH 5).

A enzima lisossômica idorunato-2-sulfatase (IDS) é uma glicoproteína monomérica composta por 550 aminoácidos (1650pb), incluindo um peptídeo sinalizador de 25 aminoácidos seguidos de 8 sítios de glicosilação que são removidos da proteína percursora durante o processo de maturação. As formas maduras da IDS possuem 45 e 55 kDa (Figura 8). A função principal dessa enzima é de remover o grupo sulfato da posição 2 do ácido L-idurônico no sulfato de dermatan e no sulfato de heparan (Bond et. al, 1997).

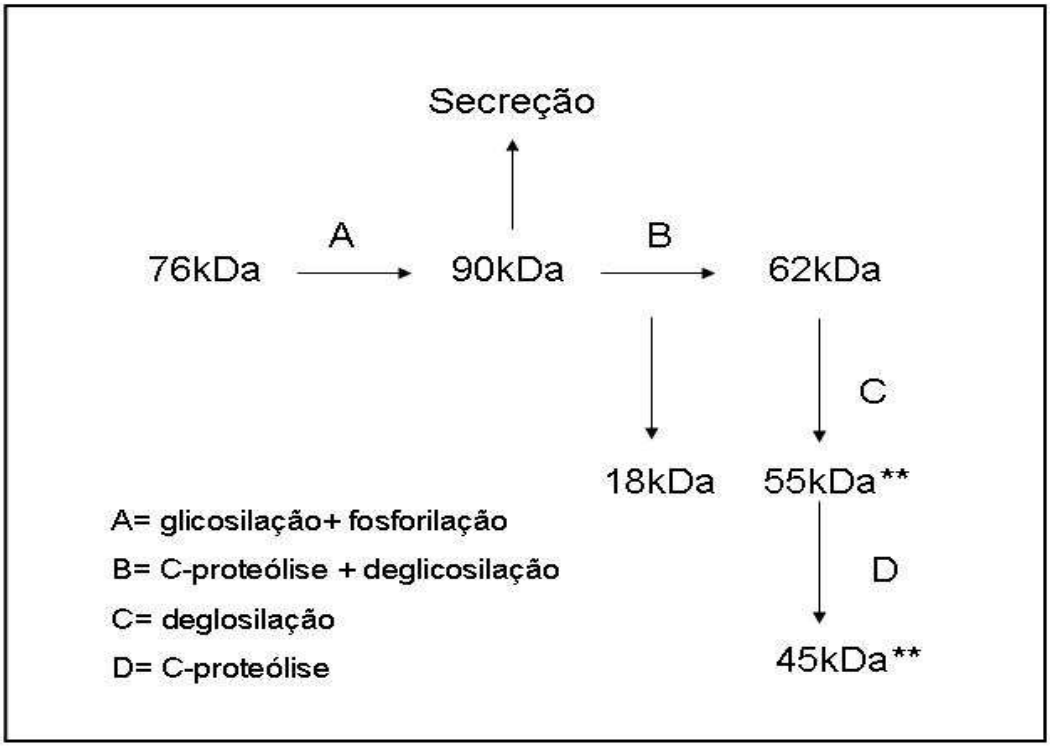

Figura 8: Processamento da idorunato-sulfatase em células COS (Adaptado de Millat *et al,*1997). ** formas maduras

2.5.3.2 Estrutura do gene

O gene que codifica a IDS foi mapeado no cromossomo Xq28.1, é composto por 9 exons e 8 introns e tem um tamanho aproximado de 24 kb (Wilson et. al,1990) (Figura 9). O cDNA de 2.3 Kb codifica um polipetídeo de 550 aminoácidos incluindo uma seqüência sinal de 25 aminoácidos (Wilson et al,1990). O promotor contém duas sequencias consenso do tipo GC Box, indicando que os níveis de transcrição são baixos e que este é um gene *housekeeping* (Wilson *et al.,*1993) (Figura 10).

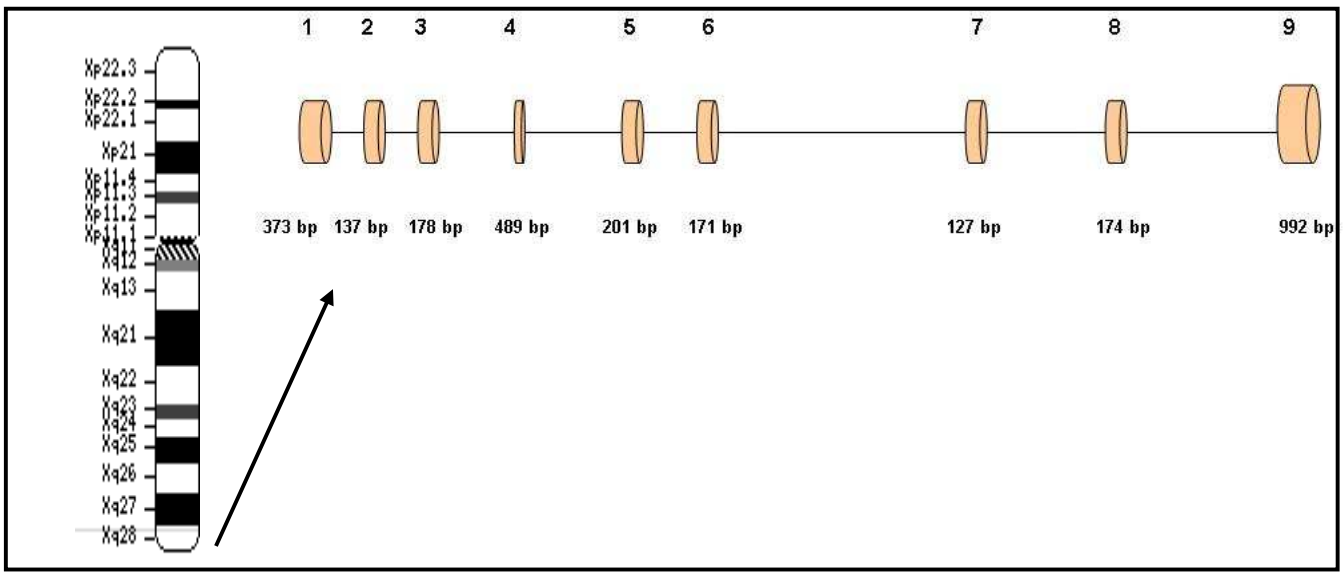

Figura 9: Representação da localização e tamanho do gene IDS.

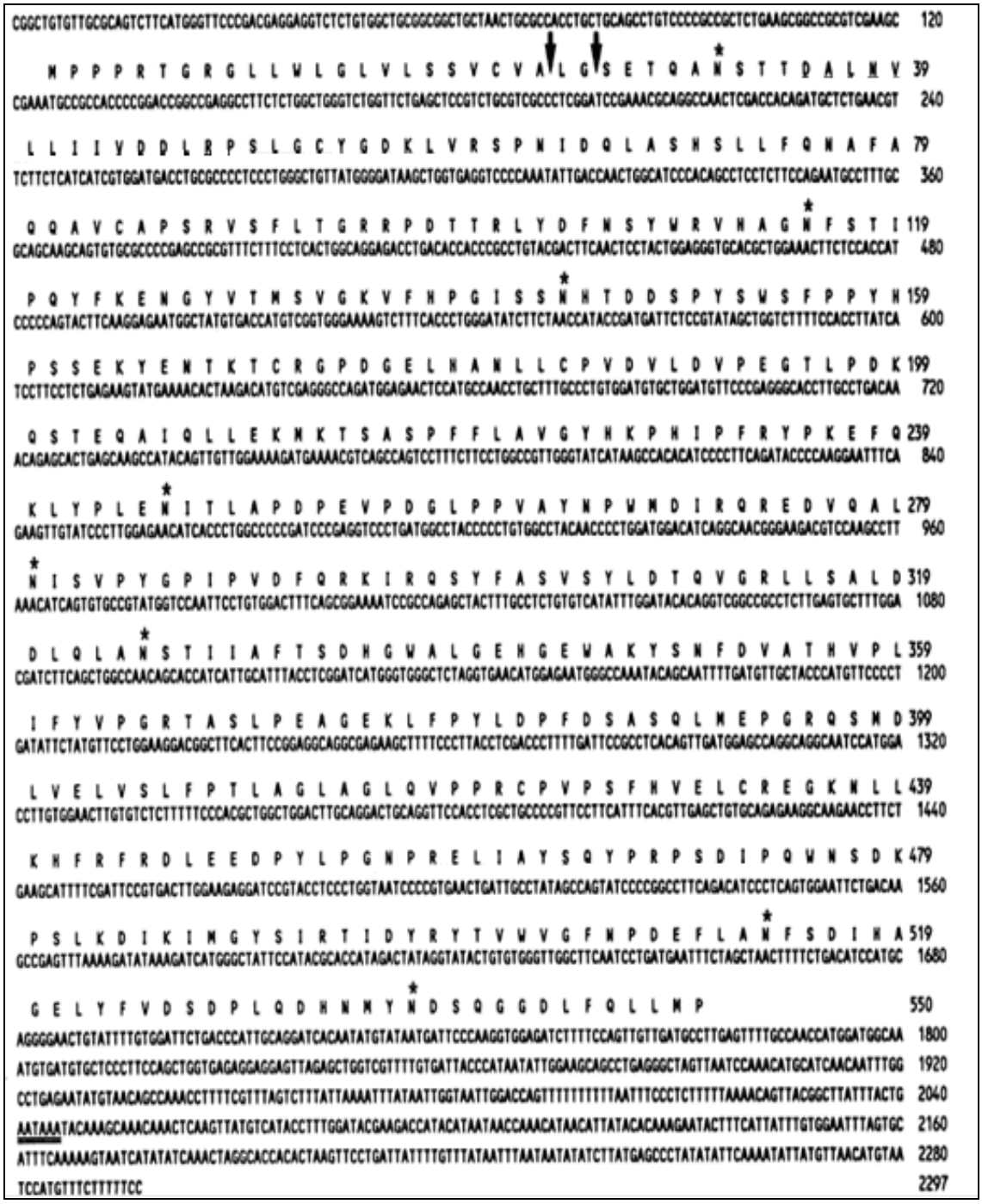

Figura 10: Sequência de nucleotídeos do gene IDS e dos respectivos aminoácidos da proteína *idorunato-2-sulfatase.* Possíveis sites de clivagem do peptídeo sinalizador estão indicados com setas. Potenciais sítios de glicosilação estão marcados com asterisco.

Foram descritas, até o momento, 346 mutações patogênicas no gene IDS, a maioria mutações de ponto (180/346) ou pequenas deleções (62/346) (HGMD, 2012) (Tabela 3). Grandes deleções e rearranjos ocorrem em aproximadamente 20% dos pacientes. A freqüência estimada de deleções completas é de 6 a 8%, podendo incluir outros genes e produzir fenótipos que resultam de síndromes de genes contíguos (Hopwood *et al.*, 1993).

Tabela 4: Tipo de mutações encontradas no gene IDS

Tipo de mutação	Número de mutações
Missense/nonsense	180
Splicing	35
Pequenas deleções	62
Pequenas inserções	25
Pequenas *indels*	5
Grandes deleções	28
Grandes duplicações/inserções	1
Rearranjos complexos	10
Total	**346**

(fonte www.hgmd.cf.ac.uk)

Um tipo específico de inversão do gene IDS, secundário à recombinação homóloga entre o gene ativo (intron 7) e o pseudogene (seqüência próxima ao exon 3), ocorre em aproximadamente 10% dos pacientes (Bondenson *et al.*, 1995; Timms *et al.*, 1997).

As mutações de ponto ocorrem mais freqüentemente nos exons III, VIII e IX e são geralmente privadas. Dinucleotídeos CpG estão envolvidos em 47% destas mutações e em praticamente todas as mutações de ponto recorrentes (Hopwood *et al.*, 1993; Rathmann *et al.*, 1996, Lin et al,2006).

Somente quatro mutações não patogênicas foram descritas na região codificadora do gene IDS, T146T(exon IV), R313C (exon VII), D319D (exon VII) e M488I (exon IX) (Li *et al.*,1996;Gort *et al.*, 1999; Vellodi *et al.*,1999,Ricci *et al.*,2003).

2.5.3.3 Recombinação entre gene e pseudogene

Um pseudogene (IDSP1) altamente homólogo aos exons II e III e aos introns 2, 3 e 7 do gene IDS localiza-se 20 kb distal ao gene ativo *(*Bondeson *et al.*, 1995) (Figura 11). O IDSP1 tem aproximadamente 3kb e apresenta a sequencia invertida em relação ao gene IDS, que é transcrito no sentido telômero-centrômero (Figura 11). As regiões do gene IDS e IDSP1 correspondentes ao exon II/intron 2 apresentam 96% de homologia, as correspondentes ao exon III apresentam 100% de homologia. As sequencias correspondentes ao intron 3 apresentam apenas homologia de 496pb após o início, sendo seguidas no IDSP1 pela sequencia correspondente ao intron 7, que apresenta uma homologia de 96% em relação ao intron 7 do gene IDS. (Bondenson *et al.*, 1995).

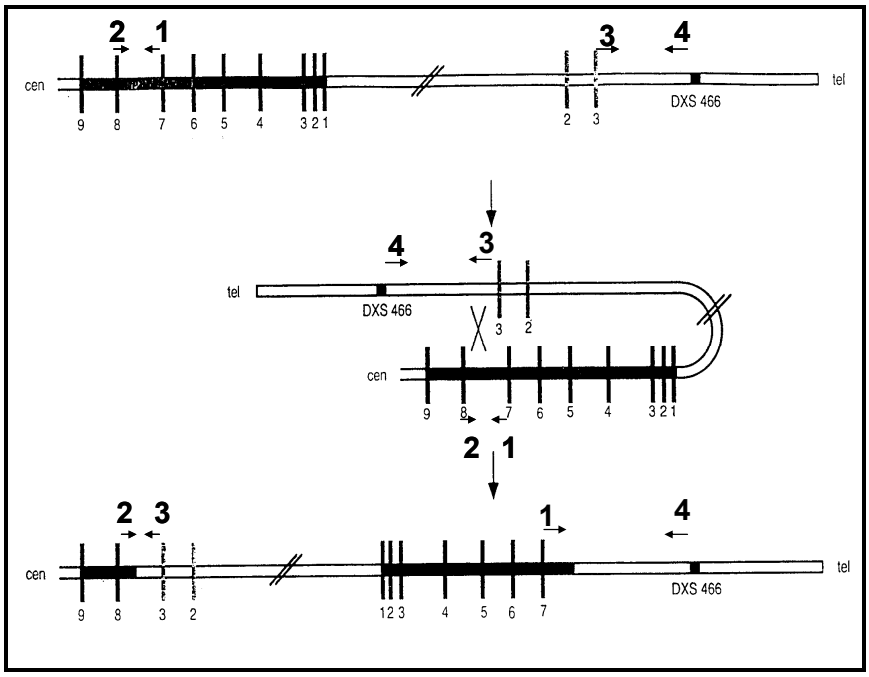

Figura 11: Organização do gene IDS e do pseudogene IDSP1 no cromossomo Xq28 e representação do evento de recombinação mais comum. (Adaptado de Bondenson *et al.*1995). As reações de amplificação utilizando os iniciadores 1 e 2, e os iniciadores 3 e 4, são positivas em controles normais para a inversão e negativas (não ocorre amplificação) em pacientes com MPS II que possuem a inversão. As reações de amplificação utilizando os iniciadores 2 e 3, e 1 e 4, são positivas em pacientes com MPS II portadores da inversão e negativas em controles normais.

A ocorrência de eventos de recombinação entre o gene IDS e IDSP1 faz com que a MPS II seja considerada uma doença genômica. Doenças genômicas são secundárias a rearranjos cromossômicos que causam deleções, duplicações, inversões ou formação de cromossomo marcador (Lupski,1998). Esses rearranjos são mediados por sequencias repetidas de DNA, denominadas duplicons, que funcionam como *hotspots* para quebras cromossômicas.

Um tipo de recombinação intracromossômica, que ocorre entre sequencias localizadas no intron 7 do gene IDS e sequencias locaizadas distalmente ao exon II do IDSP1, é encontrado em aproximadamente 10% dos pacientes com MPS II (Bondenson *et al.*,1995).

Evento semelhante ocorre na hemofilia tipo A, em que 45% das mutações são inversões paracêntricas que envolvem duas cópias do gene A, ambas com orientação invertida (Christian e Ledbetter, 2001).

A pesquisa para detecção de inversões do gene IDS pode ser realizada por meio de PCR, utilizando-se iniciadores que amplificam as regiões envolvidas no evento de recombinação.

2.5.3.4 Associação genótipo-fenótipo

Estudos de associação entre genótipo e fenótipo clínico mostram que:

- em 20-25% dos pacientes são encontradas deleções completas ou grandes rearranjos que estão associados à forma grave da MPS II; (Hopwood 1993;; Lin *et al.* 2006).

- deleções que também envolvem locos próximos ao gene IDS, como por exemplo, o gene *FMR2* estão associadas a fenótipos atípicos (ptose, síndrome da apnéia obstrutiva do sono, convulsões); (Timms *et. al.* 1997)

-mutações de ponto do tipo sem sentido estão associadas ao fenótipo grave; (Hopwood 1993, Froissart et al., 2007)

- deleções pequenas, mutações em sítios de junção e mutações de ponto do tipo sentido trocado podem associar-se a fenótipos clínicos diversos, que variam desde o

grave até o leve. Ainda não é possível o estabelecimento do fenótipo associado a cada tipo de mutação de ponto porque elas geralmente são privadas e, quando recorrentes, ocorrem em um pequeno número de pacientes.

- A mutação c.1122 C>T (G374G), no exon V está associada a um fenótipo atenuado (Froissart et al.,2007)

- pacientes com níveis enzimáticos extremamente reduzidos podem apresentar fenótipos mais leves, o que sugere que outros fatores genéticos ou epigenéticos possam contribuir para o fenótipo (Schwartz,2004).

A demonstração da patogenicidade destas mutações é geralmente feita através da constatação da sua ausência em um grupo de indivíduos controles normais ou da sua associação com a substituição de um aminoácido altamente conservado na proteína.

Estudos adicionais de associação genótipo-fenótipo são necessários para um melhor entendimento do prognóstico e da escolha das possíveis intervenções terapêuticas (Sukegawa *et. al*,2006).

2.5.3.5 Detecção de heterozigotas

A detecção de portadoras é fundamental para o aconselhamento genético, uma vez que o risco de recorrência para filhos do sexo masculino pode variar entre 50%, caso a paciente seja realmente portadora, até um risco muito baixo, caso não haja evidências de que seja portadora – mesmo diante do diagnóstico de não-portadora deve-se considerar a possibilidade de mosaicismo (Froissart *et al.*, 2007).

Assumindo ausência de seleção entre portadoras e não-portadoras e o fato de que a MPS II é um recessivo letal ligado ao X, espera-se que aproximadamente 1/3 dos casos sejam secundários a mutações novas (Haldane, 1935). No trabalho de Chase *et al.* (1986), 23% das mães de pacientes com MPS II foram identificadas como sendo não-portadoras, valor semelhante ao esperado (aproximadamente 33%). Machill *et al.* (1991), Rathmann *et al.* (1996), Froissart *et al.* (1997) e Bellows e cols. (2000), entretanto, encontraram uma proporção de mães portadoras (aproximadamente 90%)

superior àquela esperada (aproximamente 66%). Este achado é parcialmente explicado por um vício de amostragem (tendência para a seleção dos casos familiais); entretanto, o fato de que as mutações no gene IDS originam-se preferencialmente na espermatogênese (Rathmann *et al.*, 1996; Bellows *et al.* 2000) fornece uma base biológica para tanto.

Embora a atividade da IDS em heterozigotas seja teoricamente 50% inferior à média da atividade encontrada em indivíduos normais, a identificação das portadoras com base somente neste critério nem sempre é efetiva. Como a inativação do X pode ocorrer de forma não-randômica e como a IDS pode ser endocitada pelas células que produzem esta enzima em quantidade insuficiente (Adinolfi, 1993), resultados ambíguos podem ser encontrados em 10% dos casos (Zlogotora e Bach, 1984; Schröder *et al.*, 1993). A análise conjunta do heredograma, da atividade enzimática e do haplótipo (polimorfismos intragênicos e flanqueadores do gene IDS) aumenta a taxa de detecção das portadoras (Gal *et al.*, 1992). A identificação da mutação patogênica é considerada o padrão-áureo para a determinação do estado de portadora, mas para isso se faz necessária a identificação da mutação patogênica presente no probando, o que nem sempre é factível.

2.6 Diagnóstico Pré-Natal

O diagnóstico pré-natal se dá através da medida da atividade da enzima IDS, a partir da realização de biópsia de vilosidade coriônica ou em células do líquido amniótico (Neufeld e Muenzer, 2001). A análise qualitativa e quantitativa de GAGs no liquído amniótico também pode ser realizada (Keulemans *et al.*, 2002). Segundo Zlogotora e Bach (1986) a medida da atividade da IDS em gestantes de fetos masculinos com MPS II tem valores mais baixos do que mulheres gestantes da população em geral.

A dosagem bioquímica da IDS deve ser realizada somente em fetos masculinos, de acordo com as diretrizes de aconselhamento genético (Fryer, 2000).

Contudo, a realização de sexagem fetal, em casos de doenças de herança ligadas ao sexo, é indicado antes da dosagem enzimática e diagnóstico molecular (Keulemans *et al.*, 2002)

O diagnóstico bioquímico seguido da analise da mutação, previamente identificada no caso índice, no DNA fetal, serve para a confirmação ou exclusão do diagnóstico de MPS II

2.7 Tratamento

Embora muitos estudos tenham sido feitos no intuito de corrigir a deficiência da IDS com base em infusão plasmática, transplante de fibroblastos e transplante de células epiteliais amnióticas, nenhuma destas modalidades de tratamento mostrou-se efetiva na alteração do curso clínico da MPS II (Akle *et al.*, 1985; Neufeld e Muenzer, 2001).

O advento de terapias dirigidas ao tratamento das MPSs deu-se na década de 80, com o transplante de medula óssea (TMO) e transplante de células tronco hematopoiéticas (TCTH). Na década de 90 iniciaram-se os estudos de Terapia de Reposição Enzimática (TRE) que se tornou uma realidade aprovada para uso clínico das MPS I, II e VI, no início do século 21.

Os resultados do transplante de medula óssea (TMO) são controversos, mas os pacientes com a forma leve parecem ser os maiores beneficiados (Vellodi *et al.*, 1999; Coppa *et al.*, 1999).

O transplante de células tronco hematopoiéticas (TCTH) usando o sangue do cordão umbilical ou de medula óssea é um meio potencial de proporcionar atividade enzimática suficiente para retardar ou parar a progressão da doença, no entanto, a utilização de TCTH é controversa, devido ao risco associado elevado de morbidade e mortalidade. Além disso, não está claro se o tratamento no início da vida reduz significativamente a progressão da doença neurológica (Tanaka et. al,2012)

Estudos publicados até o momento não têm sido satisfatórios, ao contrário dos relatos de transplante de medula óssea (TMO) na síndrome de Hurler (MPS I), em que o tratamento precoce (idade ≤ 2 anos) mostrou-se benéfico (Tolar et al 2011)

A terapia de reposição enzimática (TRE) provavelmente é mais benéfica aos pacientes sem comprometimento neurológico, uma vez que a barreira sangue-cérebro limita o acesso da enzima ao sistema nervoso central. A identificação de fatores preditivos do fenótipo clínico, e que possam ser utilizados em idade precoce para a seleção dos potenciais candidatos ao TMO e TRE, é, portanto, fundamental.

A forma recombinante da iduronato-2-sulfatase humana que foi aprovada nos Estados Unidos e na União Europeia para o tratamento da MPS II, tendo o registro ocorrido na ANVISA em 2008 (Giugliani et al,2010)

A eficácia clínica da TRE foi demostrada em um estudo duplo-cego, placebo-controlado de 96 pacientes (Muenzer et al 2006). Após um ano de tratamento, os pacientes do grupo idursulfase semanal em comparação com o grupo placebo demonstraram uma melhoria estatisticamente significativa do desfecho primário. Com base na maior resposta clínica na semana em comparação com o grupo a cada duas semanas, idursulfase foi aprovado para o tratamento da MPS II em ambos nos EUA e União Europeia, numa dose de 0,5 mg / kg por semana.

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4. JUSTIFICATIVAS

1. O Serviço de Genética Médica do Hospital de Clínicas de Porto Alegre (SGM/HCPA) é conhecido como Centro de Referência Nacional e sul-americano para Doenças Lisossômicas de Déposito, principalmente para as Mucopolissacaridoses.

2. O SGM conta também com a Rede MPS Brasil uma parceria envolvendo diversos serviços que atendem pacientes com mucopolissacaridoses, tendo como objetivos principais facilitar o acesso ao diagnóstico, tratamento e prevenção dessas doenças, bem como apoiar o desenvolvimento de pesquisas na área.

3. A existência potencial de amostra a ser estudada (casos diagnosticados pelo Laboratório de Erros Inatos do Metabolismo-SGM/HCPA)

5. OBJETIVOS

1. Caracterizar o genótipo dos pacientes e portadoras de MPS II sul americanos;

2. Estabelecer novas estratégias para diagnóstico das alterações de pacientes que apresentam deleções totais do gene IDS;

3. Caracterizar os pacientes que não apresentam alterações, em região codificante do gene, após seqüenciamento de todos os exons.

6. ARTIGOS

Artigo 1

MUCOPOLYSACCHARIDOSIS TYPE II: IDENTIFICATION OF 30 NOVEL MUTATIONS AMONG SOUTH-AMERICAN PATIENTS AND SOME INSIGHTS ON THE GENOTYPE-PHENOTYPE CORRELATION

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Running title: MPS II mutations

ABSTRACT

In this study, 149 unrelated South-American patients with Mucopolysaccharidosis type II (MPS II) were investigated aiming at the identification of iduronate-2-sulfatase (*IDS*) gene mutations and the analysis of genotype-phenotype correlation. The strategy used for genotyping involved identification of the previously reported inversion/disruption of the *IDS* gene by PCR, and screening for other mutations by PCR/SSCP. The exons with altered mobility on SSCP were sequenced, as well as all the exons of patients with no SSCP alteration. By using this strategy, we were able to find the pathogenic mutation in 105 patients. In two cases no mutation was found after sequencing all coding exons and screening for the common gene-pseudogene recombination. For these cases, the promoter region of the *IDS* gene was analyzed suggesting the presence of a novel polymorphism. Alterations such as inversion/disruption and partial/total deletions of the *IDS* gene were found in 20/105 (19%) patients. Small insertions/deletions/indels (<22bp) and point mutations were identified in 83/105 (88%) patients, including 30 novel mutations; except for a higher frequency of small duplications in relation to small deletions, the frequencies of major and minor alterations found in our sample are in accordance to those described in the literature.

Key words: mucopolysaccharidoses, glycosaminoglycans, mucopolysaccharidosis type II, Hunter syndrome, iduronate-sulfatase, genotype-phenotype correlation

INTRODUCTION

Mucopolysaccharidosis type II (MPS II, McKusick 309900) is an X-linked recessively inherited lysosomal storage disorder (LSD), resulting from deficiency of iduronate-sulfatase activity (*IDS*, EC 3.1.6.13). *IDS* is involved in the degradation of glycosaminoglycans (GAGs) dermatan sulfate and heparan sulfate. Failure to hydrolase the terminal iduronate-2-sulfate esters in these GAGs results in progressive accumulation of undegraded substrates within the lysosomes and in the clinical manifestations associated to MPS II (Neufeld and Muenzer, 2001).

The *IDS* gene is located on the Xq27/28 boundary of the long arm of the X-chromosome, comprises 9 exons spanning approximately 24 kb (Wilson et al., 1990). A pseudogene, containing sequences homologous to exon II, intron 2, exon III and a chimerical intron 3-intron 7, is located approximately 20 kb far, telomeric to the active *IDS* gene, which makes this region prone to the occurrence of recombination events. Approximately 10-20% of MPS II patients present large gene alterations, including rearrangements and total *IDS* gene deletions, while 80-90% of them present small gene alterations (Filocamo et al., 2001; Froissart et al., 2002). According to the Human Gene Mutation Database (HGMD 2012), about 345 different mutations in the *IDS* gene have already been described, most of which being private point mutations (52%) or small deletions (17%).

MPS II shows not only wide allelic heterogeneity, but also wide phenotypical heterogeneity. Patients are usually classified as having the severe, intermediate, or attenuated forms, depending mainly on the degree of mental retardation present (Neufeld and Muenzer, 2001; Froissart et al., 2002; Schwartz et al.,2007). In general, it

seems that patients with the more severe forms are diagnosed earlier, have mental retardation that is evident by the age of 4-6 years, and are dead by the age of 10-15 years, while patients with the attenuated forms present normal intelligence (Young et al., 1982; Neufeld and Muenzer, 2001; Froissart et al., 2002). However, as there is no standardized scoring index of severity for MPS II, this classification is not always reliable, especially in the case of patients diagnosed during childhood (Froissart et al., 2002). Consequently, studies of the genotype-phenotype correlation are subject to various biases. As simple Mendelian disorders are in fact complex traits (Dipple and McCabe, 2000), it is likely that genotype-phenotype correlation does not exist for several *IDS* gene mutations (Froissart et al., 2002).

In the present study, we have investigated 105 unrelated South-American MPS II patients in order to characterize their genotypes, establish the relationship between *IDS* genotype and clinical phenotype and carrier detection.

MATERIAL AND METHODS

In this study 149 MPS II patients were referred by services from all over Brazil and some South-American countries. The diagnoses were based on clinical criteria, abnormal urinary GAGs excretion and deficient plasma and/or leukocyte *IDS* activity. In all cases, the possibility of multiple sulfatase deficiency was excluded by the measurement of the activity of another sulfatase. DNA samples were analised from all unrelated patients.Regarding the origin, 137/105 patients were from Brazil, 4/105 were from Chile, 2/105 were from Peru, 1/105 from Paraguay, 2/105 from Argentina, 1/105 from Cuba and 2/105 from Bolivia. DNA samples from 66 mothers and 22 affected

brothers/cousins of the probands were also analyzed. This study was approved by the Institutional Review Board and by the National Research Ethics Committee of Brazil (#100066).

Genotype analysis

Genomic DNA of patients/relatives was extracted from blood following the salt precipitation method (Miller et al., 1988), and preserved in a Tris-EDTA 0,1M solution (Tris-HCl 10mM pH 7.5 and EDTA 1mM pH 8.0). Samples were first analyzed through PCR for the presence of the inversion/disruption of the *IDS* gene caused by intrachromosomal recombination between intron 7 of the gene and its homologous region on the pseudogene. Samples with no evidence of the inversion/disruption were analyzed by PCR/SSCP throughout the 9 exons and intron-exon boundaries of the *IDS* gene.

The PCR amplifications were performed using 5µL of buffer concentrated 10X (200mM Tris HCl pH 8.0; 500mM KCl), 0.2mM dNTPs, 1.5 – 3.0mM $MgCl_2$, 0 - 6% of DMSO (Sigma), 1U of Taq DNA Polimerase (Invitrogen), and 20pm of each primer in a final reaction volume of 50µL. PCR conditions were as follows: 94^{o}C for 5 minutes; 34 cycles of 94^{o}C for 40 seconds, 50.5^{o}-61^{o}C for 40 seconds (based on each exon) and 72^{o}C for 40 seconds; and a final extension time of 72^{o}C for 10 minutes. The primer sequences used for amplification of all the *IDS* exons and for the identification of the inversion/disruption events have been previously described (Bunge et al. 1993; Goldenfum et al., 1996; Lagerstedt et al., 1997). Exon III was amplified using nested-PCR (amplification with primers 2A and 3B followed by amplification with primers 3A and

3B) (Goldenfum et al., 1996). This extra step prevents the amplification of the pseudogene sequence.

Two negative PCRs for one determined exon of the *IDS* gene were considered as indicative of deletion of this exon. In case of total deletions, DNA integrity was also tested by simultaneous amplification of the α-1-antitrypsin gene.

SSCP was used to screen samples, using mutation detection enhancement gels (BMA) ranging from 0.7 to 1X. The electrophoresis was carried out at 160V for 16-24h at room temperature. Exon IX was digested with *Dde*I restriction enzyme prior to SSCP due to its large fragment size.

The samples showing mobility shifts on SSCP were sequenced on ABI Prism^{TM} 3500 Genetic Analyzer (Applied Biosystem). Mutations were confirmed on a second PCR product by the sequencing of both strands using the same methodology. In case of novel missense mutations, 100 control alleles were analyzed in order to confirm the pathogenicity of the identified mutation. The samples of patients that did not show any alteration on SSCP were submitted to sequencing of the 9 IDS exons according to the previously described protocol.

After identification of mutation in the DNA sample from the index case, family members were tested either by restriction endonuclease analysis, PCR (mothers of patients presenting the inversion/disruption), SSCP or sequencing.

Clinical phenotype

Data on the clinical phenotype were obtained following a standard protocol and registered on a form developed especially for this study.

RESULTS

Detection of large gene alterations (n: 20/149)

The recombination between the *IDS* gene and pseudogene, which causes a disruption of the *IDS* gene and an inversion of the intervening region, was found in 13/149 patients (8%). Seven patients (5%) appear to have partial or total deletions of the *IDS* gene.Three of these patients showed a total deletion of the *IDS* gene including contigous genes (Brusius-Facchin AC et al.,2012). The remaning patients will be further characterized.All of these patients have neurological impairment.

Identification of small gene alterations (n: 85/105) (Tables 1and 2)

Amplification, SSCP analysis, and sequencing of exons I to IX of the *IDS* gene allowed for the identification of a small alteration (<22 bp) in 83/149 patients. The success rate found for SSCP screening was 72%. All the missense point mutations were considered to be causative for the disease since the mutation was not found in 100 alleles of unaffected and unrelated controls and involves a highly conserved gene sequence.

We have found 57 different small gene mutations in 83 patients. Data on the novel (n: 30) and recurrent mutations (n: 27) found in our sample are shown in Tables 1 and 2. Only 8 mutations were found in at least two unrelated patients: p.R88C, p.Y103X p.W109X, p.S333L, c.1122C>T, p.R443X, p.P467L and p.R468W. Intronic mutations

were found in 3/100 (3%) patients, all of which involving the consensus splice donor or acceptor site.

Regarding the type of the 57 different small alterations found, 2/57 (3,5%) were deletions, 2/57 (3,5%) were indels, 4/57 (7,0%) were duplications, and 49/57 (85%) were point mutations. Among point mutations, 7/49 (10,6%) were splice site, 10/49 (21.2%) were nonsense, and 32/49 (68%) were missense mutations.

In 23/105 (22%) patients, the mutation was located in exon IX. Considering only the patients that presented exonic point mutations (n: 75/83), exon IX was the exon with the highest frequency of mutations found (21/75 patients), followed by exons III, VIII and VII (17/75, 13/75, 12/75 patients each) and exons II, 8/75 patients (Tables 1and 2). Point mutations seemed to be rare in exons V (1 patient each), I and IV (2 patients each).

In 2/105 patients no mutation was found after sequencing all coding exons and also no gene-pseudogene recombination was present. We then decided to sequence the promoter region and a single nucleotide substitution, 818bp upstream of the *IDS* gene coding sequence (g.272678A>G) was found. The alteration was tested in 100 control alleles, after PCR digestion with restriction enzyme (*Dde*I) and it was detected in 10 alleles (10%). This single-nucleotide substitution was found in the dbSNPs (reference SNP: rs56844123) (http://www.ncbi.nlm.nih.gov/projects/SNP/), confirming that it is, as supposed, a polymorphism.

Family studies

The analysis performed in 66 mothers of MPS II patients showed that 55/66 (83%) were carriers. Seven of 10 mothers of patients presenting the inversion/disruption

were carriers. Also, all brothers/cousins with MPS II that were studied (n: 22) presented the same mutation identified in the proband.

DISCUSSION

Genotype

This is the largest sample of South-American MPS II patients reported to date. Our findings regarding the genotype analysis are, in general, in agreement with the literature. We have found great allelic heterogeneity among our patients (57 different mutations in 149 patients), and 30 novel mutations and 1 novel polymorphism. Only 8 point mutations were recurrent in two or more unrelated patients (p.R88C, p.Y103X p.W109X, in exon III, p.S333L in exon VII, c.1122 C>T in exon VIII and p.R443X, p.P467L and p.R468W in exon IX); of these, only p.P467L did not involve CpG sites.

Discordant with the literature is the relatively higher number of small duplications/insertions in relation to the small deletions that we found (7,0% versus 3,5%). According to the literature, the frequency for small duplications/insertions range from 1.5% to 6% and from 10 to 20% for small deletions (Isogai et al., 1998; Froissart et al., 2007; Sohn et al., 2012).

As expected, the exon with the highest frequency of point mutations was exon IX (n: 21 patients). It has been reported that, while the distribution of small rearrangements in the *IDS* gene seems random, point mutations tend to be more frequent in exons III, VIII, VII and IX (Rathmann et al., 1996). In our sample, the number of patients showing point mutations in exons I, IV, and V were almost the same (exons V: 1 patients; exons I and IV and VI: 2 patients each). We found a highest frequency of the mutation

c.1122C>T, in codon 374. This is a silent mutation that creates an alternative splice site with loss of 20 *IDS* aminoacids and that corresponds to around 45% of the mutations in exon VIII (Rathmann et al., 1996). We had expected to find a greater frequency of this mutation, given the ethnic background of our samples and the fact that this is the most frequent mutation of the *IDS* gene found in Spain and Portugal, where more than 25 patients have been reported. All of these patients have an attenuated phenotype, suggesting that the very low percentage of normal transcript was sufficient to avoid a severe phenotype (Gort et al., 1998; da Silva et al., 2001; Froissart et al., 2007). On its turn, the high frequency of point mutations in exon IX may be explained by the existence of mutational hotspots (codon 468, for example) and by the size of this exon (473 bp, approximately twice the size of the remaining exons). The higher frequency of mutations in exons II, III, VII, VIII and IX in relation to exons I, IV, V and VI can be explained by the fact that highly homologous regions of the *IDS* gene are numerous in the former (Sukegawa et al., 1995).

The polymorphism g.272678A>G was the only alteration found in two patients, after sequencing of the whole gene, as well as testing for the common homologous recombination event between the *IDS* gene and an adjacent unexpressed IDS pseudogene (*IDSP*) resulting in an inversion of the intervening DNA, described by Bondenson et.al, 1995 and the recombinants described by Lualdi et al, 2005.

Very few polymorphisms have been previously reported within the *IDS* gene, and no specific nucleotide variation changes can be found in the literature for the promoter region of the gene. In another X-linked lysosomal storage disorder, Fabry disease, the promoter region has a higher frequency of polymorphisms. Three variations were

reported by Davies et al, 1993; one of them is associated with decrease of the α-Gal enzyme expression (Oliveira et al. 2008).

Regarding the pathogenicity of the novel missense mutations described, it is important to point out that all occur in codons that are conserved among the human and the murine *IDS* (Daniele et al., 1993) and that they were not found in 100 control alleles. We also analyzed mothers and MPS II patients that are relatives of several index cases. As expected, about 83% of the mothers are carriers and the affected relatives were found to have the same mutation as the index-case.

Genotype-phenotype correlation

The genetic heterogeneity found in MPS II patients represents a major difficulty in estabilishing genotype-feenotype correlations. This problem is further complicated by the absence of a standardized classification of disease severity and the fact that patients are typically very young and difficult to assess (Froissart et al.,2007).

Although there are no uniform criteria to classify the clinical severity of patients with MPS II, most studies that analyze the correlation between genotype and phenotype in this disease classify patients into the severe (presence of severe or moderate mental retardation), intermediate (absence or presence of mild mental retardation and presence of severe somatic disease) or the attenuated form (absence of mental retardation and presence of attenuated somatic disease). However, these studies usually do not provide a more detailed clinical description of the patients. Young et al. (1982), when reporting the clinical study of the largest series of MPS II patients reported to date, supported the

classification of MPS II patients in only two groups: severe and attenuated. This classification is valid mainly from the age of 4-6 years and up, since these authors have demonstrated that, from this age onwards, all severe patients presented a global cognitive deficit and that attenuated patients presented relatively normal intelligence. The finding of neurological regression would be pathognomonic of the severe form.

The possibility of considering MPS II a biphenotypical disease, taking into account the intermediate forms as more severe expressions of the attenuated forms (or milder forms of the severe ones) and all the existing limitations of the studies of genotype-phenotype correlation led us to the paper published by Dipple and McCabe (2000). These authors tried to explain the genotype-phenotype correlation in a biphenotypical monogenic disease from five threshold models relating mutant-protein function to phenotype: 1) in the discrete-threshold model, there would be a discrete threshold for protein function, with mutations consistently leading to predictable functional consequences above or below the threshold; 2) in the two-threshold nondiscrete model, specific alleles would be associated consistently with either the attenuated or the severe phenotype, whereas others would be indeterminate. For the indeterminate mutations, protein function would be in an intermediate range, in which nonallelic genetic variations and/or environmental effects would influence the in vivo function and, therefore, the clinical phenotype; 3) in the single-threshold nondiscrete model (severe/indeterminate), some mutations would be associated only with the severe phenotype, and all other mutations would be indeterminate; 4) in the single-threshold nondiscrete model (attenuated/indeterminate), some mutations would be associated only with the attenuated phenotype, and all other mutations would be indeterminate; 5)

in the nonthreshold model, no mutation would correlate absolutely with the phenotype. Models 3, 4 and 5 could not be applied to MPS II, since some recurrent mutations in the *IDS* gene seem to show a good genotype-phenotype correlation (some with the severe phenotype, others with the attenuated phenotype). For instance, *IDS* gene total deletions and the p.S333L mutation are always reported as being associated with the severe phenotype (Rathmann et al., 1996; Gort el al., 1998; Isogai et al., 1998; Froissart et al., 2002; Zhang et al., 2011; Sohn et al., 2012), while the c.1122C>T mutation (codon 374) is always reported as being associated with normal intelligence (Froissart et al., 2007), two of our patients present a severe phenotype. Also, model 1 does not apply to this case, because patients with, for example, p.A85T, p.R443X, p.R468W and p.R468Q mutations, are reported as having the severe or the attenuated form of MPS II. Therefore, the model that best applies to MPS II, in the present state of knowledge, is model 2: some few mutations present correlation with the severe form (total deletions, p.S333L), other few present correlation with the attenuated form (c.1122C>T), while a greater number of mutations is correlated both with the severe form and the attenuated form (p.A85T, p.R443X, p.R468W, p.R468Q). It is important to point out that these generalizations can only be made in relation to the small number of recurrent mutations, since most mutations in the *IDS* gene are private or have been described in a limited number of patients.

Regarding frameshift mutations, that we found in three of our patients clearly appear to have the severe phenotype.

According to our findings, point mutations (including mutations in splice sites) seem to be associated with any of the clinical phenotypes. It is interesting to point out

that, differently from what was expected, the clinical phenotypes associated with the 7 nonsense mutations found range from the attenuated to the severe form of the disease. The mutations p.Y54X (exon II,) and p.Y536X (exon IX,) are clearly associated with the severe and attenuated phenotype, respectively. Mutation p.Y536X is the nearest to the carboxyl terminus of the *IDS* protein described to date. It may be compared with the recurrent mutation p.Q531X, which has already been described in two patients with the attenuated form of MPS II (Sukegawa et al., 1995; Froissart et al., 1998); both mutations lead to the synthesis of a protein missing the C-terminal amino ac*IDS* removed during *IDS* protein maturation, and should not prevent the *IDS* processing to the lysosome (Froissart et al., 2002). The phenotypes found in the patients that present the following recurrent mutations are in agreement with those reported in the literature: p.R8X (exon I, patients intermediate phenotype), p.Q389X (exon VIII, severe phenotype), p.R443X (exon IX, attenuated phenotype), and p.L482X (exon IX, intermediate phenotype). The p.R8X phenotype could be explained by the proximity of the mutation to the initiation codon, a context in which nonsense-mediated decay could be circumvented (Lualdi et al.,2010), and by the presence of the low- fidelity stop codon, which may result in limited natural read trough possibilities (Froissart et al.,2007). In patients carring the mutation p.R443X a complete spectrum of phenotypes from attenuated to severe have been described suggesting that the natural read-trough could vary between individuals and/or that other factors could influence the phenotype (Froissart et al.,2007). It seems that the severity of nonsense mutations, in the case of the *IDS* gene, shows a relation with its location within the gene, since mutations located in the amino and carboxy terminal ends tend to be associated with attenuated phenotypes. Interestingly, one of the novel

point mutations described in the carboxy terminal end (p.V503D,) is also associated with the attenuated phenotype.

As to the mutations that are being described in this study for the second time in the literature (p.S87N, c.709-2A>G, p.C422Y), there is total discordance as to the phenotype associated to p.S87N: the Brazilian patient clearly presents the severe form of MPS II, while the other patient described (Popowska et al., 1995) presents the attenuated form. In our sample, the phenotype associated with c.709-2A>G is the severe one; however, Lissens et al (1995) did not describe the phenotype of their patient. There is some agreement as to the phenotype associated with p.C422Y: the patient being described herein presents an extremely attenuated form of MPS II, while Gort et al. (1998) classified their patient as having the intermediate form. Moreover, we are describing two brothers with the c.708G>A, who apparently have no neurological involvement. Interestingly, this mutation was described in one patient with the intermediate form (Rathmann et al., 1996) as well as in other patient with the severe form of MPS II (Vafiadaki et al., 1998). The clinical phenotypes associated with the other recurrent mutations found (p.A85T, p.S333L, c.1122C>T, p.P467L, p.R468W and p.R468Q) are in agreement with the literature.

We believe that the methodology employed in this study is appropriate for both the diagnosis and the identification of mutations in patients with MPS II and possible carriers. The correlation between genotype and phenotype for MPS II exists for a small subset of mutations, being probably influenced by other factors that modulate the residual activity of the *IDS*. Therefore, in the present state of knowledge, the

identification of the mutation present in individuals with MPS II has, as main objective, the genetic counseling of families.

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Table 1 – Characterization of the novel* IDS *gene mutations described in this study

Insertions, duplications, deletions (n: 7)

Patient	Exon	Mutation (cDNA position)*	Conserved region among *IDS* murine protein (Daniele et al., 1993)	Protein alteration	Comments
H1	I	c.124_129dupATCATC	Yes	insertion of two aa (ile)	
H2	II	c.155delG	Yes	fs; 7 altered aa; truncated	GG preceding the deleted G; CM
H3	VI	c.805_808dupGACA	Yes	fs; 25 altered aa	
H4	Intron VII	IVS7-16_IVS7-9 del gcttttta ins GGTGGGCTCTAGG	Yes	splice site mutation	the sequence inserted is similar to a close sequence
H5	VIII	c.1134_1152dupCCCTTACCTC GACCCTTTT	Yes	fs; 5 altered aa; truncated	
H6	IX	c.1300_1306dupGAAGGCA	Not highly	fs; 12 altered	1 patient already described having a deletion in the same region:

			aa; truncated	GAGAAGGCA (Froissart et al., 1998);	
H7	IX	c.1349_1364delATCCGTACCTCCCTGG	No	fs; 5 alterated aa; truncated	

fs: frameshift; aa: amino acid

Point mutations (n: 27)

Patient	Exon	Mutation	cDNA position*	Sequence change	Conserved codon among human and murine *IDS* (Daniele et al.,1993)	PSIC profile scores for two amino ac*ids* variants (V.Ramensky, 2002)**	Comments
H8	II	p.D45V	134	GAT to GTT	Yes	2.980	D45N (Vafiadaki et al., 1998)
H9	II	p.D45G	134	GAT to GGT	Yes(catalytic site)	2.530	D45N (Vafiadaki et al., 1998)
H10	II	p.S61Y	180	TCC to TAC	Yes	2.245	no mutation described i this codon yet
H11	II	p.Q80R	239	CAG to CGG	Yes (near catalytic site)	2.306	splice site mutation; Q80X

							(Vafiadaki et al., 1998; Carrozo et al., 1996)
H12	II	p.Q81Y	240	CAA to TAA	Yes	2.475	no mutation described in this codon yet
H13	III	p.C84Y	250	TGC to TAC	Yes	3.681	C84X (Vafiadaki et al., 1998)
H14	III	p.W109X	325	TGG to TGA	Yes	-	W109K (Emre et al, 2002)
H15	III	p.W109X	325	TGG to TGA	Yes	-	Same as above
H16	III	p. R95S	409	AGG to AGC	Yes (catalytic site)	2..355	R95T, R95G (da Silva et al., 2001; Goldenfum et al., 1996)
H17	III	p.H138Y	411	CAC to TAC	Yes (catalytic site)	2.667	H138D (Froissart et al., 1998)
H18	III	c.417G>C	417	CCTGgt to CCTCgt	Yes (catalytic site)	-	splice site mutation
H19	IV	p.P160H	479	CCT to CAT	Yes	2.775	P160R (Flomen et al., 1992)
H20	Intron V	c.709-2^{A}>T	709-2	ccaagGAA to ccatggaa	Yes	-	splice site mutation; 709-2A>G (Lissens et al., 1997)
H21	VI	p.Q239X	715	CAG to TAG	Yes	-	no mutation described in this codon yet
H22	VI	p.N265K	793	AAc to AAG	Yes	1.817	N265I (Filocamo et al,2001)
H23	VII	p.L314H	941	CTC to CAC	Yes	2.446	L314P (Isogai et al.1998)

H24	VII	p.D308H	922	GAT to CAT	Yes	2.530	D308N, D308E (Gort et al., 1998; Isogai et al., 1998)
H25	VII	p.D334Y	1000	GAT to TAT	Yes	2.980	D334N, D334G (Froissart et al., 1998; Li et al., 1996)
H26	VII	p.D334V	1000	GAT to GTT	Yes	2.980	D334G,D334N (Li et al,1996, Froissart 1998)
H27	VIII	c.1007G>T	1007	tagGG to tagTG	Yes	-	splice site mutation; 1007G>A, 1006G>A (Lissens et al., 1997; Froissart et al., 1998; Li et al., 1999)
H28	VIII	p.H342P	1025	CAT to CTT	Yes	3.552	H342Y, H342Q (Vallance et al., 1999; Timms et al., 1998)
H29	VIII	p.E344K	1031	GAA to AAA	Yes	1.833	no mutation described in this codon yet
H30	IX	p.V503D	1508	GTT to GAT	Yes	2.038	same as above
H31	IX	p.Y536X	1608	TAT to TAA	Yes	-	same as above

cDNA numbering follows den Dunnen and Antonorakis (2001), beginning with the A of the initiation codon. Gene sequence follows GenBank entry M58342. Position specific independent counts (PSIC) using Polyphen program;* **probably damaging** (score>2), it is with high confidence supposed to affect protein function or structure, **possibly damaging** (score 1,5-20,) it is supposed to affect protein function or structure ;*aa: amino acid, fs: frameshift*

Table 2 – Recurrent *IDS* gene point mutations worldwide, including patients described in this work

Patients*	Mutation**	Origin	Clinical phenotype (literature)***
H32 a, b, c	R8X	Brazil	Intermediate: 1 patient (Filocamo et al., 2001); attenuated: 2 patients (Vafiadaki et al., 1998 ; Froissart et al. 2007).
H33	Y54X	Brazil	Severe 1 patient (Zhang et al.,2011)
H34	A77D	Brazil	Attenuatted (Kim et al.,*in press*)
H35	N63D	Brazil	Mild 1 patient (Goldenfum 1996)
H36	A85T	Brazil	Severe: 1 patient (Li et al., 1999); intermediate: 2 patients (Rathmann et al., 1996; Isogai et al., 1998); attenuated: 4 patients (Froissart et al., 1998; Gort et al, 1998 ; Tomatsu et al .,2006 ; Keeratichamraen et al., 2011).
H37	P86L	Brazil	(popowska 1995)
H38	S87N	Brazil	Attenuated: 1 patient (Popowska et al., 1995).
H39	R88C	Brazil	Severe 3 patients (Rathman 1996)
H40	R88C	Brazil	Same as above
H41	R88C	Brazil	Same as above
H42	R88H	Brazil	Severe 3 patient (Rathman et al.,1996 ; Zhang et al. ,2011)
H43	Y103X	Brazil	Intermediate :Karsten et al.,1998a
H44	Y103X	Brazil	

H45	T130I	Brazil	Mild 1 patient (Zhang et al., 2011)
H46	P231L	Brazil	Mild 1 patient (Gort 1998) Severe 1 patient (Sohn et al.2012)
H47a)	c.708G>A	Brazil	Severe: 1 patient (Vafiadaki et al., 1998); intermediate: 1 patient, (Rathmann et al., 1996).
H48	c.709-2A>G	Paraguay	Lissens et al.,1997 (no clinical description).
H49	S305P	Brazil	Attenuated 1 patient (Chang et al., 2007
H50,H52,H53, H54	S333L	Brazil	Severe: 5 (Vafiadaki et al., 1998; Sukegawa,1995; Froissart et al., 1998; Karsten et al., 1998b; Li et al., 1999 ; Zhang et al.., 2011 ; Sohn et al.,2012).
H51	S333L	Chile	Same as above.
H55	H335R	Brazil	Intermediate 2 patient (Froissart 1998. Sohn eta l.,2012)
H56	G336V	Brazil	Severe (Kato e Kato,2005)
H57,H59,H60, H61,H62,H63, H64	c.1122C>T	Brazil	Intermediate: 1 patient (Filocamo et al., 2001); attenuated: 21 patients (Bunge et al., 1993; Simon-Schiff et al., 1994; Popowska et al., 1995; Goldenfum et al., 1996; Rathmann et al., 1996; Vafiadaki et al.1998; Gort et al., 1998; Hartog et al., 1999; da Silva et al., 2001 ; Sohn et al.,2012).
H58	c.1122C>T	Bolivia	Same as above.
H65 a, b	Q389X	Brazil	Severe: 2 patients (Jonsson et al., 1995; Carrozo t al., 1996).
H66	C422Y	Chile	Intermediate: 1 patient (Gort et al., 1998).

H67,H68,H69, H71,H72	R443X	Brazil	Severe: 4 patients (Bunge et al., 1993; Froissart et al., 1998; da Silva et al., 2001 ; Keeratichamroen et al.,2011)); intermediate: 5 patients (Sukegawa et al., 1995; Rathmann et al., 1996; Froissart et al., 1998; Karsten et al., 1998b; Filocamo et al., 2001); attenuated: 6 patient (Gort et al., 1998 ; Froissart etal al., 2007 ;Sohn et al., 2012).
H70	R443X	Bolivia	Same as above.
H73	P467L	Chile	Severe: 2 patients (Froissart et al., 1998; da Silva et al., 2001).
H74 a,b, H75	P467L	Brazil	Same as above.
H76,H77,H78, H79	R468W	Brazil	Severe: 10 patients (Isogai et al., 1998; Hartog et al., 1999; da Silva et al., 2001; Goldenfum et al., 1996; Popowska et al., 1995; Froissart et al., 1998; Schröder et al., 1994 ; Lin et al.,2006 ; Sohn et al.,2012); attenuated: 2 patients (Crotty et al., 1992; Jonsson et al., 1995).
H80	R468W	Argentina	Same as above.
H81	R468Q	Argentina	Severe:30 patients (Whitley et al., 1993; Sukegawa et al.,1995; Rathmann et al., 1996; Goldenfum et al., 1996; Villani et al., 1997; Vafiadaki et al., 1998; Isogai et al., 1998; Froissart et al., 1998; Karsten et al., 1998a; Li et al., 1999; Filocamo et al., 2001; Keeratichanroen et al., 2011); intermediate: 1 patient (Goldenfum et al., 1996).
H82	D478G	Brazil	(Schroeder 1994)
H83	L482X	Brazil	Intermediate: 2 patients (Froissart et al., 2007; Li et al., 1999).
a, b, c			

*MDD: delay in motor development; LDD: delay in language development; MR: mental retardation; BP: behavioral problems; NR: neurological regression;NA: not available; * patients described with the same number and different letters (a, b, c,) are relatives; **cDNA numbering follows den Dunnen and Antonorakis (2001), beginning with the A of the initiation codon; ***cases when author has not characterized the clinical phenotype or*

when the clinical phenotype was unknown have not been included in this column. See specific reference for clarification of the criteria used for the classification of clinical severity in each study.

Artigo 2

Extension of the molecular analysis to the promoter region of iduronate-2-sulfatase gene reveals genomic alterations in mucopolysaccharidosis type II patients with normal coding sequence

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Abstract

Hunter disease or mucopolysaccharidosis type II is an X-linked recessive lysosomal disorder caused by the deficiency of iduronate-2-sulfatase, which is involved in the catabolism of the glycosaminoglycans heparan and dermatan sulfate. Our aim was to search for molecular defects in the promoter region of iduronate-2-sulfatase gene in patients with Hunter Syndrome who did not have mutations identified in the coding region. Findings would help to clarify the relation between genotype and phenotype and would allow the identification of female carriers, important for genetic counseling. Ten patients with previous biochemical diagnosis of mucopolysaccharidosis type II were included in this study after we sequenced the whole iduronate-2-sulfatase gene coding sequence and the exon/intron boundaries without finding any pathogenic mutation. After screening the promoter region of the iduronate-2-sulfatase gene, two of these 10 patients showed a 178bp deletion and a single nucleotide substitution 818bp upstream of the coding region were found in two other patients. The second variation found has not been described before in the literature and it was shown to be a polymorphism. We suggest that mucopolysaccharidosis type II patients with no mutations detected in the iduronate-2-sulfatase coding region should be screened for mutations in the promoter region of the gene.

Key words: Hunter disease, iduronate 2-sulfatase, mucopolysaccharidosis, mutation analysis, promoter

Introduction

Hunter disease or mucopolysaccharidosis type II (MPS II) (OMIM 309900) is an X-linked recessive lysosomal disorder caused by deficiency of the activity of iduronate-2-sulfatase (*IDS*; EC 3.1.6.13), which is involved in the catabolism of the glycosaminoglycans (GAGs) heparan and dermatan sulfate (1).Two main clinical forms (severe and attenuated) can be recognized based on the presence/absence of mental retardation or neurological deterioration (2).The clinical heterogeneity of the syndrome is presumed to reflect, at least partially, various mutations at the *IDS* locus affecting enzyme expression, stability or function.

The human *IDS* gene spans approximately 24 kb, contains 9 exons, and has been mapped to chromosome Xq28.1. The entire *IDS* gene has been sequenced, and a pseudogene located 20 kb telomeric to the active gene has been identified (3).The 1650-bp full-length cDNA encodes a 550 amino-acid polypeptide which shows a high degree of homology with sulfatase protein family. The frequency of large alterations (complete or partial gene deletions and large rearrangements) is approximately 20%. The majority (80%) of mutations identified, are small deletions or insertions or single base substitutions (missense mutations, nonsense mutations, and mutations affecting splicing) (4).

Many patients with previous biochemical diagnosis of MPS II were analyzed through molecular biology techniques in our laboratory, and we were able to detect a mutation in 100 of them (data not show). Surprisingly, even after sequencing the whole coding region and exclusion of recombination events, no pathological alterations were found in some patients, despite clinical and laboratory findings, including enzyme activity measurement, had confirmed the MPS II diagnosis.

Based on these observations, we decided to search for molecular defects in the promoter region of *IDS* gene investigating the disease-causing variants in these patients, in order to help and clarify the relationship between genotype and phenotype in this disease and also to allow the identification of female carriers, which is important for genetic counseling.

Material and Methods

Ten unrelated patients with previous biochemical diagnosis of MPS II in whom the pathogenic mutation could not be found even after sequencing the whole *IDS*

gene coding region and exon/intron boundaries were referred for molecular analysis of the promoter region.

All cases were diagnosed based on the finding of a deficient *IDS* activity in serum, leucocytes or fibroblasts (with a normal level of at least another sulfatase, thus excluding multiple sulfatase deficiency) and a clinical and laboratory picture compatible with MPS II. Detailed phenotypic and genealogical data were obtained whenever possible. Clinical phenotype was evaluated according to the criteria described by Schwartz et al 2007.

Peripheral blood was obtained from patients, relatives and controls. Genomic DNA was isolated from each specimen using standard procedures. The DNA was used as a template for PCR synthesis of *IDS* promoter region specific amplicons, which was performed to produce two overlapping fragments (A and B), with primers designed by us (Table 1). PCR products were purified and sequenced using the Big Dye Terminator kit version 3.1 in an ABI 310 automated sequencer (Applied Biosystems). The sequences obtained were aligned with the genomic reference sequence AF011889.1

Table 1: Sequence of primers used for the analysis of IDS promoter region in two overlapping fragments.

Region	*Primers sequence*	*Location (gDNA)*
RP1A	5' TGAGCAGTGCACCACAGCACC 3'	1-21
RP2B	5' ACACTTCTTGTAAGCCAGCC 3'	599-580
RP3A	5' GGAAAGGGAGTCCCTTTAGAT 3'	510-530
RP41B	5' GACGGAGCTCAGAACCAGACC 3'	1116-1096

A: sense primers; B: antisense primers

Fibroblasts of patients H1 e H2 were grown in DMEM medium containing 10% (v/v) of foetal calf serum, with penicillin (10 U/ml) and streptomycin (100 μg /ml).

For mRNA extraction the cells were centrifuged at 1800 rpm for 10 min and resuspended in 1ml of Trizol Reagent (Sigma). The mRNA was extracted according

to manufacturer's instructions using β-actin mRNA as control. Controls and patients' cDNA was obtained by RT-PCR amplification of total RNA (~1µg) using MuLV Reverse Transcriptase and Random Hexamers (Applied Biosystems), following the manufacturer's instructions. A set of exonic primers were designed in order to amplify three overlapping fragments (f1, f2, f3) of the transcriptional variant 1 cDNA of IDS gene (reference sequence NM_000202.5) (Table 2); moreover fragment f1 was divided in three shorter subfragments (a, b, c) amplified with appropriate primers (available on request). All fragments and subfragments were sequenced.

Table 2: Exonic primers were designed in order to amplify three overlapping fragments (f1, f2, f3) of the transcriptional variant 1 cDNA of IDS gene.

Primer name (position in NM_000202.5)	Sequence
f1 fw (94-114)	CTGTGTTGCGCAGTCTTCATG
f1 rv (903-922)	TTATGATACCCAACGGCCAGG
f2 fw (798-818)	CCTTGCCTGACAAACAGAGCA
f2 rv (1482-1502)	CAACGTGAAATGAAGGAACGG
f3 fw (1404-1425)	CCATGGACCTTGTGGAACTTG
f3 rv (1995-2014)	CAGGCCAAATTGTTGATGCA

Results

Patients H1 and H2 showed features of attenuated Hunter syndrome according to the classification of Schwartz *et al.*, 2007. In Patient H1 leucocytes IDS activity was 1,07 nmoles/4h/mg proteins (normal range: 25-95) while leukocytes arylsulfatase B activity was 187 nmoles/h/mg proteins (normal range: 72-176); urinary GAGs thin-layer chromatography detected the dermatan and heparan sulphate molecules while urinary GAGs level was 260 µg/mg creatinine (normal range: 13-45).

In patient H2 plasma IDS activity was 10 nmoles/h/mL (normal range: 110-370), the enzymatic activity in leukocytes was 5,9 nmoles/4h/mg proteins (normal range: 25-95) and was 1,3 nmoles/4h/mg protein (normal range: 31-110) in skin fibroblasts; the arylsulfatase B activity in leukocytes was 268 nmoles/h/mg prot (normal range: 72-176); urinary GAGs dermatan and heparan sulphate: 203µg/mg creatinine (normal range: 13-45)

In both patients the sequencing of the coding and flanking regions failed to detect any sequence variation, except for patient H2 in which we revealed the presence of the polymorphism c.438C>T (p.T146T) in exon 4.

The electrophoresis of the amplified fragments of the promoter region (fragment A and B) of two patients (H1 and H2) showed a faster running band on fragment B, which was sequenced and showed a 178bp deletion.The deletion (g.271996_272174del) is located upstream of the observed *IDS* transcription start site (5) and is flanked by highly GC-rich (92%) 13-bp direct repeats. Both H1 and H2 mothers were tested for the mutation and showed to be non-carriers.

In silico promoter prediction of the 3200 nucleotides region located upstream of exon 1 was performed; the analysis evidenced four candidate promoters which could be recognized by the RNA polymerase II in substitution of the deleted one. All the sequences predicted give rise to a transcript carrying a longer 5'UTR (from 425 nt to 2856 nt) than that carried by the normal transcript (table 3).

Table 3: In silico prediction of the promoter sequence: the 3200 nucleotides sequence upstream of exon 1 was run in the on line software Neural network (http://www.fruitfly.org/seq_tools/promoter.html).

	Sequence (5'-3')	Position in AF011889	5' UTR length (nt)	Score
P1	CCTTTCACC**T**GGAAGCCCTGCCCTTCCTTCTTTTTATAAAAGACACACTT	272277-272326	425	0.96
P2	CTTCAGAGC**T**CCCTGTGGGGTTAACTGAAGCCTTTTTTGAGACTGCCCAC	273133-273182	1281	0.98
P3	ACCAATGAA**G**TTCATTGATGAACACCTGGGGTTTATTATACTATTTTCTG	273624-273673	1772	0.86
P4	AAGATTGA**T**TGTCTCAGCTGGGATAGAAAATTATATATGCCACTTGGCAC	274438-274487	2586	0.81

The cDNA analysis on the transcriptional variant 1 revealed that patients H1 and H2 expressed a 5' truncated form of this variant. In fact the cDNA of both subjects amplified only fragments f2 and f3, while the most 5' fragment f1 didn't amplify. The segmentation of fragment f1 in 3 shorter subfragments (a, b, c) allowed us to define approximately the location of the 5' boundary of the transcript between nucleotide 258 and 369 of the reference sequence NM_000202.5. The anomalous transcript lacks certainly 41 nucleotides of exon 1 and probably part of exon 2 (figure 1).

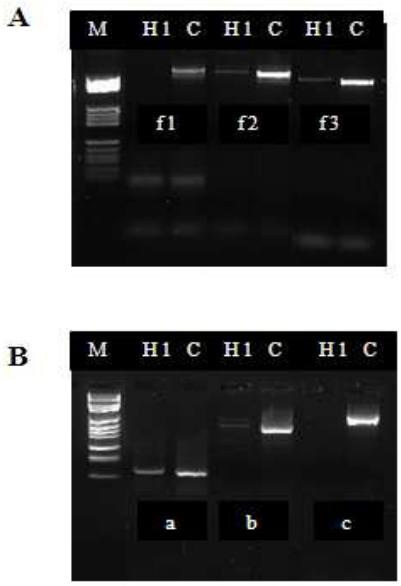

Figure 1: (A) Gel eletrophoresis of cDNA amplification products (f1, f2, f3) of the transcriptional variant 1 of hIDS gene in patient H1. (B) Gel eletrophoresis of cDNA PCR products of the subfragments (a, b, c) of fragment f1 in patient H1. Patient H1 = H1, control cDNA = C, molecular weight marker = M.

Two other patients (H3 and H4), after sequencing of fragment A, showed a single nucleotide substitution , 818bp upstream of the *IDS* gene coding sequence (g.272678T>C) .The alteration was tested in 100 control alleles, after PCR digestion with restriction enzyme (*Dde*I) and it was detected in 10 alleles (10%). This single-nucleotide substitution was found in the dbSNPs (reference SNP: rs56844123) (http://www.ncbi.nlm.nih.gov/projects/SNP/), confirming that it is, as supposed, a polymorphism, which, however was never described previously in literature. The mother of patient H3 and the sister of patient H4 were tested for the polymorphism and both showed to be heterozygotes.

In patient H4 plasma IDS activity was 3,4 nmoles/4h/mL (normal range: 110-370); the arylsulfatase B activity in leucocytes was 115 nmoles/h/mg prot (normal range: 72-176); B glucoronidase activity in plasma was 121 nmoles/h/mL (normal range: 30-300), patient H3 data non available.

In order to analyze the inheritance of the polymorphism, we tested the alteration in both carrier mothers and in mothers who are not carriers, but have affected siblings. Twenty heterozygotes were tested and three of them showed the

polymorphism. Amongst the non carrier mothers (n= 6) two showed this polymorphism.

We also tested for this polymorphism the remaining 8 patients who were under investigation in our lab for mutations in the promoter region, and it was absent.

Discussion

The 178bp deletion found in the IDS promoter region was described only once by Timms et al (7), in a patient with a moderate to severe phenotype. In this manuscript we described two Brazilian unrelated patients with mild phenotype, carrying the same deletion on the promoter region.

Mutations within promoter areas have been reported for other diseases. In Hemophilia type B the mutation n-6 G>A was identified as one of a variety of mutations in the promoter region of Factor IX and results in a severe or moderate phenotype reducing the transcriptional activity by 50% (6). Also, in the $^{A}\gamma$-globin gene a 4-bp deletion located upstream of the promoter region results in the deficient expression of this gene (8).

Through an in silico analysis of the region located upstream of exon 1 (about 3200 nucleotides), we evaluated the hypothesis that the RNA polymerase II could recognize and bind an alternative promoter region from which starting the translation. The software output revealed four candidate promoter sites predicting four transcripts with a 5' UTR longer than the normal one. Previously published papers (9,10) suggest that "longer 5' UTR gives the propensity to formation of stable secondary structures which may inhibit translation initiation by impeding the progress of the scanning ribosome". Thereby we hypothesized that the mRNA could be produced starting from one of the four possible promoters (more probably from the two nearest), thus presenting a longer 5'UTR sequence. In addition, differently from Timms and colleagues, which did not amplify any IDS transcripts, in subjects H1 and H2 we detected a 5' truncated transcript, presenting in both patients the same pattern of amplification and presumably the same 5' end.

Based on our findings, we could assume that a longer 5'UTR with its stable secondary structure, could impede the normal scanning of the ribosome and that a mRNA surveillance mechanism would be activated, detecting the stalled ribosomes

and endonucleolytically cleaving the mRNA near the stall site. This causes the release of the stopped ribosome and of the mRNA fragments, impeding the reclutation of translation factors on an anomalous transcript. Although observed only in *S.cerevisiae*, this mechanism, called no-go decay (11,12), could explain the presence of the same pattern of transcriptional cDNA fragments on both patients. We therefore hypothesize that the fragments detected in our investigation could be the products of degradation of one or more of the four faulty transcriptional variants predicted, presumably the longer ones, while the variant(s) carrying a shorter 5'UTR possibly by-pass this surveillance pathway, proceed through translation and permit the synthesis of a reduced amount of active protein. This could in turn explain the residual IDS enzyme activity detected and the attenuated phenotypes observed in the two patients examined.

The reason for the inability to detect any IDS transcript by Timms and coworkers could reside in the forward primer used to fish the cDNA which, according to the description of the authors, was positioned within exon 1, which we failed to amplify completely and surely is partly degraded.

The polymorphism g.272678T>C was the only alteration found in patients H3 and H4 after sequencing of the whole gene, as well as testing for the common homologous recombination event between the *IDS* gene and an adjacent unexpressed IDS pseudogene (*IDSP*) resulting in an inversion of the intervening DNA, described by Bondenson et.al (3), and the recombinants described by Lualdi et al (13).

Very few polymorphisms have been previously reported within the *IDS* gene, and no specific nucleotide variation changes can be found in literature for the promoter region of the gene. In another X-linked lysosomal storage disorder, Fabry disease, the promoter region has a higher frequency of polymorphisms. Three variations were reported by Davies et al (14); one of them is associated with decrease of the α-Gal enzyme expression (15)

The analysis of relatives who are non-carriers and present the new variant suggests that the pathogenic mutation is not being transmitted together with the polymorphism.

The remaining patients who do not present the variations described here will be tested for other possible alterations, such as inversions of the gene which could not be found in genomic DNA and requires additional investigations.

Data presented in this report suggests that in about 10 % of the patients with MPS II the sequence variation causing the disorder cannot be identify by analysis of the *IDS* coding region. In these subjects screening of the promoter region of the gene could help the identification of possible disease-causing mutations, thus also increasing the ability to correctly identify heterozygous subjects, potential carriers of the genomic alterations.

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Artigo 3

Severe phenotype in MPS II patients associated with a large deletion including contiguous genes

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ABSTRACT

Hunter disease or mucopolysaccharidosis type II (MPS II) is an X-linked recessive lysosomal disorder caused by the deficiency of iduronate-2-sulfatase, which is involved in the catabolism of the glycosaminoglycans (GAGs) heparan and dermatan sulphate. Our aim was to analyze three patients with severe Hunter syndrome that showed a total deletion of the IDS gene, after exon by exon PCR. DNA was used as a template for PCR synthesis of *IDS*, *FRAXA*, *FRAXE* and *DXS1113* specific amplicons. The DNA analysis for all three patients demonstrated a complete deletion of IDS*, FRAXA and FRAXE* contiguous genes. We further performed whole-genome array-Comparative Genomic Hybridization (Array-CGH) to delineate the deletion breakpoints and to characterize the deletion extension in the different patients. The results indicated a ~ 9,4 Mb deletion in patient 1, a ~3,9 Mb deletion of the Xq27.3-Xq28 and a ~ 3,1 Mb duplication of the X q28 region in patient 2 and a ~ 41,8 Kb deletion in patient 3. Array-CGH was shown to be important to map for deletion breakpoints. A comprehensive molecular analysis in patients with Hunter syndrome, especially in the ones presenting the severe form, is important to the understanding of the genetic determinants of the phenotype and for the genetic counseling to be provided to the families.

Key words: Mucopolyssacaridosis II, Hunter Syndrome, deletion, Array-CGH, mutation analysis.

INTRODUCTION

Mucopolysaccharidosis type II (MPS II) is a rare lysosomal storage disorder caused by deficient activity of the enzyme iduronate-2-sulphatase (*IDS*). This enzyme is responsible for the catabolism of two essential glycosaminoglycans (GAGs), dermatan sulfate and heparan sulfate. Lysosomal accumulation of these GAG molecules results in cell, tissue, and organ dysfunction. The skeletal-muscle system involvement is due to essential accumulated GAGs in joints and connective tissue. MPS II has many clinical features and includes two main phenotypes, mild and severe, that represent two ends of a wide spectrum of clinical severity [Neufeld and Muenzer 2001].

The *IDS* gene spans about 24kb in Xq28 and consists of nine exons. The majority of cases are caused by point mutations and small deletions or insertions, with 20% of cases being the result of major structural alterations, including large deletions and rearrangements [Froissart et al., 2002]. In approximately 6-8% of cases the disease results of a complete deletion of the *IDS* gene [Hopwood et al, 1993]. Some of those patients have additional symptoms not commonly associated with Hunter syndrome, including the occurrence of seizures. These variant phenotypes may be due to involvement of loci adjacent to the *IDS* [Hopwood et al, 1993].

Three patients with severe Hunter syndrome, and total deletion after IDS gene exon by exon PCR, were analyzed to determine whether the mutations in these individuals involved genes located in close proximity to the *IDS* gene

MATERIAL AND METHODS

Clinical Report

Patient 1.

The patient is a sixteen year-old boy who was born from an uneventful pregnancy with normal height, weight and cephalic measures. He is the first child from a healthy couple without family history of MPSII. Hypotonia was noticed at birth. At 6 months of age macrocephaly was observed (OFC 59 cm). At 2 years of age he was submitted to the first genetic evaluation due to severe mental and motor delay, seizures, coarse face, hepatosplenomegaly, umbilical hernia and thoracolumbar kyphosis. He was

suspected to have a lysosomal storage disorder, mainly a MPS, but due to the severity of his presentation a karyotype was performed. It showed a deletion on the Xq28 region, which was also present in his mother. Biochemical investigation showed iduronate-2-sulphatase activity in leukocytes of 2.5 nmol/h/mg (normal range: 110-320). Other tests showed the following: auditory evoked potential showed moderate hearing loss; brain MRI showed hydrocephaly and mega cisterna magna; electroencephalogram showed diffuse alterations; and echocardiogram showed thickening of the mitral and aortic valves.

Patient 2.

The patient is a five-year old boy who was born from an uneventful pregnancy with normal height, weight and cephalic measures. He is the second child from a non-consanguineous couple, without other MPS II suspected cases in the family. At 6 months of age he was referred for genetic evaluation to investigate motor and mental retardation (slow development, no regression noticed) associated with hydrocephalus. At the first evaluation, he was noticed to have severe hypotonia, coarse facies, macrocephaly and seizures. Initially, he was thought to have a dysmorphic syndrome. Laboratory investigations showed the following; normal karyotype; increased urinary glycosaminoglycans (960 mg creatinine (age-related normal range of 133-480), and undetectable iduronate-2-sulphatase activity in leukocytes. At 5 years of age, he has severe mental retardation and hypotonia. He lacks head control, cannot sit, is not visually attentive, and does not speak. His face was reported as coarse sened, his fingers have limitation to extension, and he has lumbar gibbus and macrocephaly (OFC 61cm). Other tests included: brain MRI performed at 2 years of age showed dilated perivascular spaces, ventriculomegaly, and prominence of subarachnoid space; electroencephalogram showed spikes on the posterior region; echocardiogram was normal; polisomnography demonstrated severe obstructive respiratory disorder (IAH: 25.8).

Patient 3.

The patient is a 7 year-old boy who was referred for genetic evaluation due to developmental delay and joint stiffness. He was born from an uneventful pregnancy

with normal height, weight and cephalic measures. He was the first child from a healthy couple. On the maternal side there are two other healthy half siblings. On physical examination, he had umbilical hernia, severe restriction of arms and legs joints mobility, and severe developmental delay. The laboratory investigation showed low *IDS* in plasma (3,6 nmoles/4h/ml protein (normal range of 110-370) and normal karyotype. He did not have a seizure disorder, but motor and cognitive development was very slow. He walked at 18 months of age and had never spoken. Other tests: brain MRI performed at 7 years old showed dilated ventriculomegaly, reduction of periventricular white matter, prominence of subarachnoid space and severe cortical atrophy; echocardiogram showed left ventricular hypertrophy. He died at the age of 10 years due to respiratory failure, secondary to pulmonary hypertension.

PCR Amplification of IDS and neighboring genes

MPS II patients from different regions of Brazil were referred to our center for biochemical and genetic analysis. All cases were diagnosed with deficient *IDS* activity in serum, leucocytes or fibroblast culture. Clinical phenotype was evaluated according to the criteria described by [Schwartz et al 2007], which differentiated the severe type from the mild one by the age of onset, presence of developmental delay and neurological involvement.

Peripheral blood was obtained from patients and genomic DNA was isolated using standard salting out procedure. The DNA was used as a template for PCR synthesis of *IDS*, *FRAXA*, *FRAXE and DXS 1113* specific amplicons. Exon-by-exon *IDS* PCR was routinely performed to generate *IDS* exon-specific amplicons using 9 primer pairs each flanking one of the 9 exons of the *IDS* gene. Oligonucleotide primers designed and used for the amplification of the *IDS* sequence were the same used by [Goldenfum et al 1996]. *FRAXA* and *FRAXE* amplicons were obtained with primers that flanked the trinucleotide repeat regions used for routine analysis of Fragile X Syndrome [Wang et al., 1995]. DXS 1113 is 250 Kb distal to IDS gene and amplicons were obtained using primers described by [Weber et al. 1993].

Whole-genome array-Comparative Genomic Hybridization (Array-CGH)

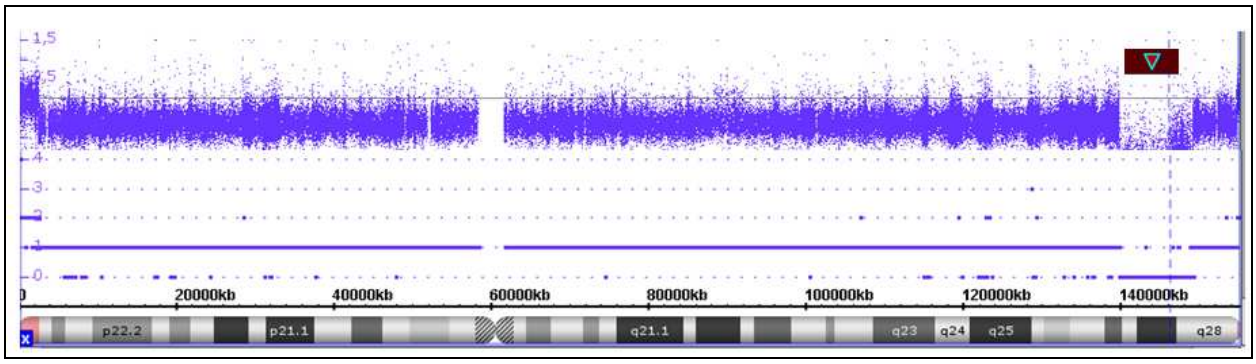
Microarray analysis was performed on DNA isolated from the patient´s peripheral blood leukocytes. The Array-CGH using the Affymetrix ® CytoScan^{TM} HD Array was applied in each of the patients in order to better delineate the deletion breakpoints and to detect differences in size of the deletion in the different patients. The array contains more than 2.4 million markers for copy number and approximately 750,000 single nucleotide polymorphisms (SNPs). The arrays were analyzed through the Affymetrix® Chromosome Analysis Suite (ChAS) software. Map position was based on the UCSC Genome Browser, February 2009, gh19 (NCBI Build 37 reference sequence).

RESULTS

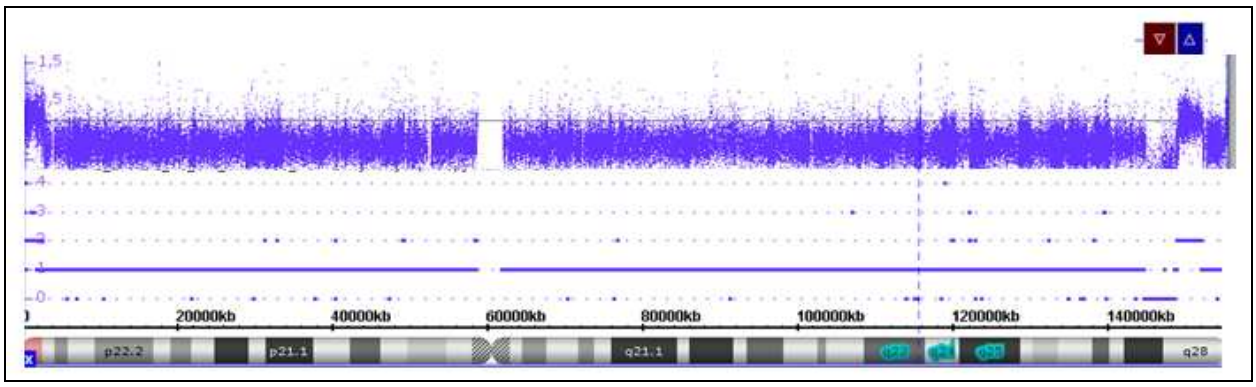
The PCR amplification DNA analysis for all 3 patients demonstrated complete deletion of the *IDS* gene. Two negative PCRs for one determined exon of the IDS gene were considered as indicative of deletion of this exon, in all PCRs control samples were used for demonstrating that a PCR failure did not occur. DNA integrity was also tested by simultaneous amplification of the *ARSB* gene. The *DXS 1113* marker in the neighboring region of the Xq28 chromosome was analyzed to check for the deletion extension and, at a minimum, the *FRAXA* and *FRAXE* genes were also deleted covering both sides of the IDS gene contiguous region.

In order to more precisely define the size of the deletion, additional chromosomal microarray analysis was performed in the 3 patients. The Array-CGH for patient 1 showed a copy loss of the long arm of chromosome X corresponding to band Xq27.1 to band Xq28: arr Xq27.1Xq28(139,990,405-149,404,134)x0. The results (Fig. 1) indicate that patient 1 has a ~ 9,4 Mb deletion of chromosome X. This deletion encompasses the following known disease genes: *FMR1*, *AFF2* and *IDS*.

A



B



C

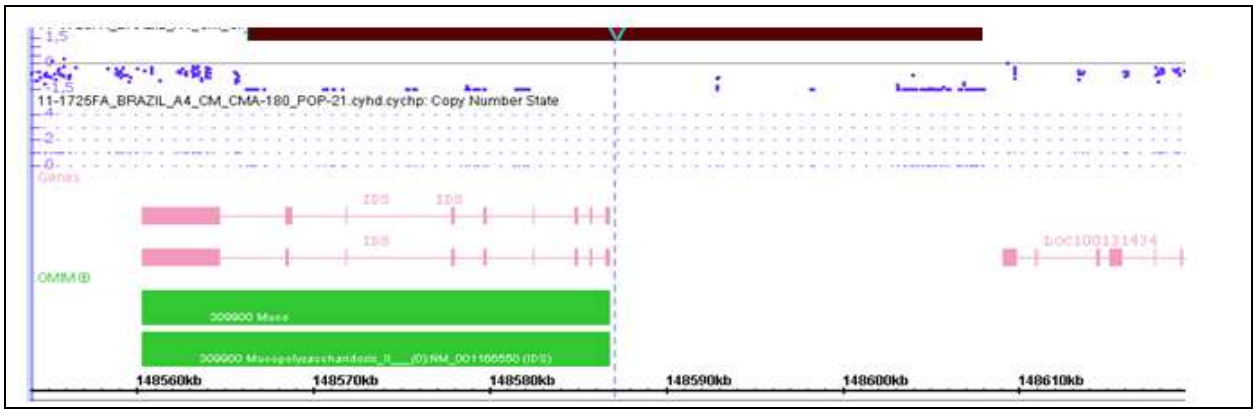


Figure 1. The results of array-CGH analysis using genomic DNA from the patients and a normal male as a test and a reference, respectively. The CytoScan™ HD Array (Affymetrix) test/reference ratio data for the X chromosome are shown. The ideogram of X chromosome shows the location of each probe. A: a ~9.4 Mb deletion at Xq27.1-Xq28 (red Arrow) in patient 1. B: a ~ 3,9 Mb deletions at Xq27.3-Xq28 (red arrow) and a ~3,1 Mb duplication at Xq28 (blue arrow) in patient 2. C: a high resolution view of the Xq28 deletion region in patient 3. A ~ 41,8 Kb deletion is shown (red bar).

Array-CGH for patient 2 showed a copy loss of the long arm of chromosome X corresponding to band Xq27.3 to band Xq28 and a copy gain of the long arm of

chromosome X corresponding to band Xq28: arr Xq27.3Xq28(144,726,761-148,623,869)x0 and arrXq28(148,884,728-151,975,281)x2, respectively. The results (Fig. 1) indicate that patient 2 has a ~3,9 Mb deletion of the Xq27.3-Xq28 and a ~ 3,1 Mb duplication of the Xq28 region. Additionally, the Array-CGH analysis revealed no copy number variation of the region between the deleted and duplicated region on Xq28: arr Xq28(148,623,958-148,875,827)x1, encompassing approximately 252 Kb.

Array-CGH for patient 3 showed a copy loss of the long arm of chromosome X corresponding to band Xq28: arr Xq28(148,566,217-148,608,042)x0. The results (Fig. 1) indicate that patient 3 has a ~ 41,8 Kb deletion of the chromosome Xq28. This deletion encompasses only the *IDS* disease gene.

Table I. Deleted and duplicated known disease genes (OMIM ®) of Chromosome X detected in our three patients.

Gene	Patient 1	Patient 2	Patient 3
FMR1	del	del	+
AFF2	del	del	+
IDS	del	del	del
MAMLD1	+	dup	+
MTM1	+	dup	+

del: deletion, dup:duplication, +:gene present.

DISCUSSION

Hunter syndrome, MPS II, is an inborn error of lysossomal catabolism with a wide range of clinical severity, at least partially related to the mutational heterogeneity of the *IDS* gene. It has been suggested that approximately 20% of MPS II patients result from a complete or partial gene deletion or major *IDS* gene rearrangements [Froissart et al., 2002], from these, 6-8% represents total deletions of the *IDS* gene [Hoopwood 1993].

Genomic analyses of sequences surrounding the *IDS* gene locus enabled us to carry out a comparison of the genotypes observed in patients with atypical

presentation of Hunter syndrome. Markers in the Xq28 region were used to map the deletions spanning the *IDS* gene in those patients.

The deletion showed in the 3 patients described here extended from the proximal *IDS* region towards *FRAXA* and *FRAXE*, by PCR amplification technique. Analysis by Array-CGH confirms the deletion on these sites, only for patients 1 and 2. This demonstrates the importance of using more sensitive techniques, such as Array-CGH to map for big deletions. Probably, the DNA sample obtained for patient 3 contained some which could have inhibited this specific PCR reaction.

All three patients reported here have severe motor and mental delay. Patients 1 and 2, showed respectively deletion of *FRAXA (FMR1)* and *FRAXE (FMR2)* genes, and patient 2 duplication of the *MAMLD1* and *MTM1* genes. Patient 3 presents only *IDS* gene deletion.

Fragile X Syndrome (caused by trinucleotide expansion in the *FMR1* gene) is characterized by moderate to severe mental retardation, macroorchidism, and distinct facial features [Devys et al., 1993]. Deletion can include a conserved locus that is tightly linked to *IDS*, suggesting that the deletion of nearby genes may contribute to the variable clinical severity noted in Hunter syndrome.

FRAXE is a folate sensitive fragile site, located at Xq28 within, or immediately adjacent to the 5' untranslated region (UTR) of the adjacent *AFF2(FMR2)* gene, the expansion of a $(CCG)^n$ repeat sequence is associated with mild/moderate mental retardation and decreased expression of this gene [Sutherland 1992;Hamel et al.1994; Mulley et al. 1995].

The X-linked mental retardation is caused by disruption of the FMR2 gene (AFF2), either by expansion of a CCG repeat in the 5-prime untranslated region or by deletion. [Timms et al.,1997] used genomic DNA sequencing to identify several new genes in the IDS region. DNA deletion patients with atypical symptoms were analyzed to determine whether these atypical symptoms could be due to involvement of these other loci. The occurrence of seizures in 2 individuals correlated with a deletion extending proximal to IDS, up to and including part of the FMR2 locus. Our results agree with these findings, since only patients 1 and 2 showing a deletion of FMR2 locus presented with seizures.

Clinically the phenotype of MPS II varies from severe to mild, depending on the different mutations and deletions at the *IDS* gene. Severe case, with mental retardation, was reported by [Karsten et al 1998] including a deletion of *IDS* only.

Myotubular myopathy-1(*MTM1*), also known as X-linked centronuclear myopathy (*CNMX*) is caused by mutation in the myotubularin gene. [Laporte et al. 2000] stated that 133 different mutations in the MTM1 gene had been identified as the cause of X-linked myotubular myopathy. They found that most truncating mutations caused a severe and early lethal phenotype, and that some missense mutations were associated with milder forms and prolonged survival, up to 54 years in the first reported family.

MAMLD1 has been shown to be a causative gene for hypospadias, congenital malformation in boys and is partly caused by genetic factors. Mutations in the gene have been reported in 99 sporadic hypospadias cases. Five non-synonymous mutations, one synonymous and one non-encoding mutation were found in [Chen et.al 2010] study.

Rearrangements including inversions, deletions or duplication of these two genes (*MTM1* and *MAMLD1)* have not been reported as the cause of clinical symptoms. Patient 2, who presents duplication of these regions, does not show any symptoms of Myotubular myopathy or hypospadia, suggesting that over expression of these genes are not associated with clinical phenotype.

In summary, a comprehensive molecular analysis in patients with Hunter syndrome, especially in the ones presenting the severe form, is important to the understanding of the genetic determinants of the phenotype and for the genetic counseling to be provided to the families.

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Artigo 4

Important aspects in the molecular diagnosis of mucopolysaccharidoses

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Recently, Pollard et al. (2012) reported the molecular characterization of 355 patients with mucopolysaccharidoses, in whom 104 novel mutations were found. We read this article with great interest and would like to share some insights and concerns regarding these results, which we think relevant as the authors propose that genetic diagnosis should be performed before or even without biochemical diagnosis. Enzyme diagnosis is presently considered the "gold standard" approach (Neufeld & Muenzer et al., 2001), usually followed by genetic diagnosis mainly to help the understanding of the genotype-phenotype correlation, to detect carriers, to improve prenatal diagnosis and to confirm biochemical diagnosis in the few doubtful cases.

We agree with these authors that information about ethnic background and clinical phenotype is of great importance once the ratio of heterogeneity could differ greatly amongst populations and mutation detection protocols based on local recurrent mutations could be helpful, depending on the population studied. In Brazil, several MPS clusters were identified, and mutation analysis of index cases was very useful to guide molecular testing customized for these areas. For instance, several mutations which are recurrent in our area, especially for MPS II, IVA and VI, were not detected among the patients reported here, which could reflect distinct genetic background.

Regarding the fact that no mutations were identified in a patient with MPS II even after sequencing the whole gene and performance of MLPA analysis and search of the common IDS/IDSP1 abnormality, the authors should consider the

analysis of the promoter region. In our sample 2/105 patients analyzed and confirmed by enzyme deficiency had a deletion (178bpdel) in this region as the disease-cause mutation. Also, other types of rearrangements, apart from the common inversion described, could be undetectable by the methods employed. Also, among patients with whole *IDS* gene deletions it is relevant to analyze the extension of the deletions in order to delineate the breakpoints as contiguous genes could also be deleted inferring genetic determinants of phenotype in different patients.

Other important aspect refers to the lower heterogeneity found in *GALNS* gene nevertheless considering a sample size of 6 patients (including 2 siblings) this could be easily predicted.

Taking into account the probability of uniparental disomy (UPD) and the presence of gene deletions in the *GALNS* gene, we suggest that in homozygous cases a genetic analysis should be performed in the parents as well to rule out this possibility, very important for genetic counseling purposes.

Regarding MPS VI and MPS IVA genotyping, it is important to acknowledge the high frequency of intragenic polymorphisms which could modify genotypes even within families, being also useful for haplotype analysis in the case of recurrent mutations within a specific group. This was partly stated by the authors in the discussion section, but the results for the patients genotyped were not shown.

We believe that molecular analysis is of great importance for this group of patients, especially for providing useful information for genetic counseling and prenatal diagnosis and for helping the phenotype prediction, which could be important to define the treatment plan in some cases. However, we stress that these results should be interpreted in conjunction with clinical findings, biochemical results and family history.

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7. PERSPECTIVAS

Nos últimos anos o Serviço de Genética Médica, deste hospital, Centro de Referência em Doenças Lisossômicas de Depósito, especialmente em Mucopolissacaridoses (MPS), vem desenvolvendo, junto ao Laboratório de Genética Molecular, diversos projetos na área de MPS tipo II, permitindo a caracterização genotípica da maioria dos pacientes, o que auxilia no entendimento desta patologia e permite novas perspectivas para um tratamento ou prevenção mais específicos.

O interesse do SGM e da comunidade médicanas MPS faz com que estudos científicos na área sejam cada vez mais realizados, tendo a partir da presente tese novos desdobramentos, tais como:

- Verificar a presença de algum tipo de rearranjo nos pacientes com análise molecular inconclusiva, através da análise de cDNA;
- Estabelecer o genótipo de mães, cujos filhos apresentam deleção parcial/total do gene, por qPCR e aCGH;
- Definir o status de metilação, por MS-HRM/PCR;
- Analise da proteína por TaqMan® protein assay e Western blot;
- Definir o padrão de citocinas através de determinação quantitativa utilizando o equipamento Luminex®;
- Realizar sequenciamento de Nova Geração (*Next Generation Sequencing)* através da plataforma Ion Torrent^{TM} para elucidação de casos complexos;
- Montar um repositório de material biológico com amostras de pacientes, familiares e controles estudados no presente projeto;
- Contribuir para orientações sobre manejo, tratamento, seguimento e aconselhamento genético, uma vez que a identificação molecular dos pacientes e seu seguimento sistemático produzirão dados sobre os efeitos das intervenções, que podem ser relevantes para a elaboração de linhas de cuidado para esta doença.

8. CONCLUSÕES

Objetivo 1: Caracterizar o genótipo dos pacientes e portadoras de MPS II.

Conclusões:

- Dos 149 pacientes analisados através de análise de mutações recorrentes e sequenciamento completo do gene, 105 tiveram a mutação definida por este protocolo;

- O gene IDS apresenta grande heterogeneidade alélica, 57 diferentes mutações em 105 pacientes;

- Implementação de fluxograma para análise de novos pacientes;

- Mutações de ponto apresentam maior frequencia, o que está de acordo com dados publicados em outras populações

- Grande número de mutações não descritas, corroborando o fato de que a maioria das mutações é específica para cada núcleo familiar

- Alta freqüência da mutação c.1222 C>T, correspondente a 45% das mutações encontradas no exon VIII;

- Exon IX é o que apresenta maior número de mutações, o que é explicado por seu tamanho e por incluir códons que apresentam altas taxas de mutação, como por exemplo o códon 468 que é um *hotspot*.

- Associação genótipo-fenótipo limitada, podendo ser associado ao fenótipo grave as mutações p.S333L, p.Y54X, p.Q389X e a inversão do gene *IDS*, e a mutação c.1222 C>T, p.Y536X,p.R443X associadas a um fenótipo moderado.

Objetivo 2: Estabelecer novas estratégias pra diagnóstico das alterações de pacientes que apresentam deleções totais do gene IDS.

Conclusões:

- Pacientes portadores de deleções totais do gene IDS estão associados a um fenótipo grave

- Deleções de genes próximos ao IDS podem contribuir para a variabilidade clínica e gravidade da doença, sendo importante a definição dos pontos de quebra para a determinação da extensão da deleção nos casos de deleção completa do gene.

- A determinação da associação genótipo-fenótipo para os pacientes é importante, bem como a determinação do estatus de portadora das familiares do sexo feminino de um paciente com deleção no gene IDS,, a fim de se realizar um aconselhamento genético mais específico

Objetivo 3*:* Caracterizar os pacientes que não apresentam alterações, em região codificante do gene, nos estudos até agora realizados.

Conclusões:

- A presença da deleção de 178pb na região promotora, descrita uma única vez em paciente moderado, ao contrário de nossos achados, mostra à grande variabilidade clínica da doença e reforça a importância da análise de regiões não codificadas.

9. ANEXOS

Anexo I: Termo de Consentimento Rede MPS

REDE MPS BRASIL

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

TÍTULO DO PROJETO: Mucopolissacaridoses no Brasil: um estudo clínico, epidemiológico, genético, bioquímico e genético molecular com impacto no diagnóstico, manejo e prevenção.

PESQUISADOR RESPONSÁVEL: Roberto Giugliani, Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Rua Ramiro Barcelos 2350, Porto Alegre, RS.
PESQUISADOR ASSOCIADO (informar nome do pesquisador e do centro participante que está avaliando o paciente): _____

_____

Nome do paciente: _____

Endereço: _____

Cidade: _____ Estado _____ CEP: _____ Fone: (___) _____

Email: _____

Responsável: _____ Idade: _____

RG: _____ Grau de parentesco: _____

Justificativa e objetivos do estudo

As mucopolissacaridoses são doenças genéticas de curso progressivo e muitas vezes de diagnóstico difícil. Com o objetivo de diagnosticar corretamente os pacientes afetados, permitindo que recebam as medidas de tratamento disponíveis em cada situação, foi montado um projeto para avaliar clínica e laboratorialmente os pacientes com suspeita de apresentar uma mucopolissacaridose. Os casos identificados serão referidos a centros regionais que possam realizar o manejo adequado e oferecer as medidas de prevenção de novos casos para a família. O estudo pretende identificar as mucopolissacaridoses mais freqüentes e as regiões de maior incidência, bem como incentivar a pesquisa sobre essas doenças no Brasil.

Procedimentos a que serão submetidos os pacientes

Os pacientes com suspeita de apresentar uma mucopolissacaridose serão submetidos a uma avaliação clínica. Caso a suspeita persista, serão coletadas amostras de urina para análise bioquímica e amostras de sangue para análises bioquímica e/ou molecular. A investigação molecular inclui a análise de DNA para identificação das mutações presentes nas mucopolissacaridoses. Em alguns casos será necessária a repetição dos exames e mesmo a coleta de uma biópsia de pele para o completo esclarecimento do caso. Alguns procedimentos adicionais, como exames de imagens, testes de função pulmonar, testes de mobilidade articular, testes de resistência, estudo do sono, entre outros, poderão ser indicados. Esses exames fazem parte da rotina de atendimento de pacientes com suspeita de mucopolissacaridose. Eventualmente, poderão ser solicitadas amostras de familiares de pacientes para o esclarecimento do caso, identificação de familiares portadores de mutações, aconselhamento genético e diagnóstico pré-natal. Os resultados das análises serão encaminhados ao médico assistente do paciente, ficando sob responsabilidade deste a informação do resultado ao paciente e/ou familiar.

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Armazenamento de dados e amostras
Os dados clínicos e laboratoriais serão registrados em um banco de dados e as amostras ficarão armazenadas em um banco de material biológico. Essas amostras poderão ser utilizadas em estudos sobre mucopolissacaridoses que estejam vinculados a este projeto.

Benefícios esperados
Com o diagnóstico do tipo de Mucopolissacaridose, o paciente poderá se beneficiar das medidas de tratamento eventualmente disponíveis, além de possibilitar aconselhamento genético, detecção de portadores e diagnóstico pré-natal.

Caso tenha alguma dúvida ou queira algum esclarecimento adicional, contactar _____no seguinte endereço e telefone:
_____
_____

Concordo em participar do projeto de pesquisa "Mucopolissacaridoses no Brasil: um estudo clínico, epidemiológico, genético, bioquímico e genético molecular com impacto no diagnóstico, manejo e prevenção" e autorizo a utilização das minhas amostras biológicas em estudos relacionados com este projeto.

Data: _____ / _____ / _____

Paciente ou responsável: _____

Responsável pelo estudo: _____

HCPA / GPPG
VERSÃO APROVADA
21 / 07 / 09
Nº 030666