Lúcia Inês Macedo de Souza

**Investigação genética de duas novas doenças
neurodegenerativas: síndrome Spoan (*Spastic Paraglegia
with Optic Atrophy and Neuropathy*) e SPG34**

São Paulo

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neurodegenerativas: síndrome Spoon (*Spastic Paraplegia with
Optic Atrophy and Neuropathy*) e SPG34**

Tese apresentada ao Instituto de
Biociências da Universidade de São
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Orientador(a): Profa. Dra. Mayana
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Investigação genética de duas novas doenças neurodegenerativas:
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SPG34

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I. Universidade de São Paulo. Instituto de Biociências. Departamento de
Genética e Biologia Evolutiva.

Comissão Julgadora:

Prof(a). Dr(a).

Prof(a). Dr(a).

Prof(a). Dr(a).

Prof(a). Dr(a).

Prof(a). Dr(a). Mayana Zatz

Orientador(a)

Dedicatória

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razão da minha busca pelo conhecimento.

Epígrafe

“O sucesso nasce do querer, da determinação e persistência em se chegar a um objetivo. Mesmo não atingindo o alvo, quem busca e vence obstáculos, no mínimo fará coisas admiráveis”

José de Alencar

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Nota da Autora

A proposição geral deste trabalho é contribuir para a compreensão dos mecanismos genéticos envolvidos na manifestação de doenças neurodegenerativas, em particular, das paraparesias espásticas associadas ou não a outros sinais clínicos. Para tanto, foram estudadas duas grandes famílias. Uma delas é originária do alto oeste do estado do Rio Grande do Norte e a outra da região de São José do Rio Preto, em São Paulo. Organizamos esta tese no formato de capítulos, sendo o primeiro deles composto por uma revisão geral, metodologia e objetivos. Os seguintes são as publicações originais dos estudos clínico-genéticos das doenças que acometem essas duas famílias.

A síndrome Spoan (*Spastic Paraplegia, Optic Atrophy, and Neuropathy*) é uma doença neurodegenerativa de herança autossômica recessiva, desconhecida na literatura até que nosso grupo de pesquisadores a descrevesse, em 2005. Até o presente momento, foram identificados 68 indivíduos afetados por essa síndrome, distribuídos em dez municípios de três estados brasileiros. Estimamos que um em cada sete moradores do município de Serrinha dos Pintos (RN) e um em cada 14 de São Miguel (RN) sejam portadores da mutação associada à doença em estado heterozigoto. A síndrome Spoan é a doença genética prevalente nesses municípios e certamente contribui para que eles estejam entre os 50 municípios brasileiros com maior índice de indivíduos portadores de deficiência (IBGE, censo 2000). Nos capítulos 2 e 3, apresentamos as publicações com a descrição clínica e o mapeamento genético da síndrome Spoan.

No capítulo 4, apresentamos os resultados da revisão do mapeamento genético realizado pela equipe da Dra. Mayana Zatz há alguns anos, por meio do qual demonstramos a existência de um novo loco gênico associado à paraparesia espástica simples no cromossomo X, por nós nomeado SPG34. Em Metodologia Complementar, no capítulo 1, estão descritos os protocolos que não foram abordados nas publicações. O mesmo ocorre em Bibliografia, ou seja, apenas as que não foram citadas nas publicações são referidas. Por fim,

encontram-se nos anexos outros resultados envolvendo colaboração com grupo no exterior, além de figura e tabelas referentes aos resultados do refinamento do mapeamento genético da síndrome Spoan.

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Capítulo 1

I – INTRODUÇÃO

1.1. DOENÇAS NEURODEGENERATIVAS HEREDITÁRIAS

As doenças neuromusculares constituem um grupo bastante diversificado de distúrbios que acometem o sistema muscular em associação ou não com o sistema nervoso e, portanto, incluem as distrofias musculares (DM) e as doenças do neurônio motor (DNM), por exemplo. As DNMs, por sua vez, são assim denominadas por acometer, especialmente, os neurônios efetores, tendo a degeneração muscular como efeito secundário, com variabilidade clínica de acordo com o grupo de células nervosas acometidas. Quando os neurônios motores inferiores estão afetados, tem-se um quadro característico de atrofia espinhal progressiva (AEP) ou de atrofia muscular espinobulbar (doença de Kennedy). Nos casos em que o problema se encontra nos neurônios motores superiores, pode-se ter um dos diversos tipos de paraplegias espásticas ou a esclerose lateral primária, entre outras doenças. Por fim, quando ambos os grupos de células nervosas encontram-se envolvidos, pode ser observado um quadro de esclerose lateral amiotrófica (ELA), por exemplo.

As doenças neurodegenerativas geneticamente determinadas podem ter herança autossômica recessiva, autossômica dominante, ligada ao sexo ou serem de transmissão mitocondrial. Elas afetam de forma preferencial uma determinada região ou via do sistema nervoso e, desta forma, determinam sintomas relacionados a esse comprometimento. Podem ocorrer, por exemplo, distúrbios sensitivos, da coordenação, alteração do tônus muscular, ou comprometimento da visão. A partir da avaliação clínica e de exames complementares como a ressonância magnética e a eletroneuromiografia, as doenças neurodegenerativas são denominadas, por exemplo, neuropatia periférica, amiotrofia espinal, ataxia espinocerebelar, paraplegia espástica, e distonia. São conhecidas formas *não complicadas* ou *puras* de

neurodegenerações, quando há comprometimento de uma única via ou região do sistema nervoso, e formas *complicadas*, quando há mais de uma via ou região afetada.

Nós sugerimos que os distúrbios descritos no presente trabalho representam duas novas condições neurodegenerativas hereditárias; a primeira, síndrome Sp oan, transmitida como uma característica autossômica recessiva e a segunda, SPG34, como padrão recessivo ligado ao cromossomo X.

1.2. CARACTERIZAÇÃO CLÍNICA E GENÉTICA DA SÍNDROME SPOAN E SPG34

1.2.1. Síndrome Sp oan (Spastic Paraplegia, Optic Atrophy, and Neuropathy)

Em 2002, a probanda ZDQ, 26 anos de idade, natural de Serrinha dos Pintos, RN, foi avaliada no Centro de Estudo do Genoma Humano (IB-USP) com história de baixa acuidade visual, percebida já nos primeiros meses de vida, em decorrência de dificuldade na fixação do olhar e pela presença de abalos oculares. A seguir, apresentou atraso nas aquisições motoras, tendo demorado para engatinhar, ficar em pé e caminhar, o que passou a fazer com dificuldade, na ponta dos pés, aos 2 anos de idade. Andou sem apoio por três anos, quando se agravaram as dificuldades de locomoção. O quadro motor foi se deteriorando e, com cerca de 15 anos, ficou restrita à cadeira de rodas. A força e mobilidade dos membros superiores foram inicialmente normal, mas, a partir da adolescência, começou a ter fraqueza nas mãos e perdeu a habilidade de executar atividades domésticas. O desenvolvimento da fala e a capacidade de comunicação não foram afetadas, embora tenha ficado com a voz baixa e a fala levemente disártrica. Também não se observou declínio das aptidões intelectuais.

Ao exame neurológico, ZDQ demonstrava bom contato, linguagem preservada e boa orientação no tempo e no espaço. Apresentava tetraparesia, com ausência de movimentos espontâneos em membros inferiores (MMII) e diminuição distal da força muscular em membros superiores (MMSS).

Observava-se ainda acentuada espasticidade em MMII, com abolição de reflexos miotáticos e ausência de reflexo cutâneo plantar; amiotrofia distal das mãos; incapacidade em perceber estímulos táteis e vibratórios nas mãos e abaixo dos joelhos e anartrestesia dos háluces; hiperidrose de mãos e pés e hipotermia de extremidades; cifoescoliose e retração tendínea em tornozelos e joelhos. Apresentava baixa acuidade visual, com atrofia óptica bilateralmente e nistagmo discreto. Finalmente, tinha sobressaltos à estimulação sonora. O levantamento da genealogia revelou a existência de outros 20 indivíduos aparentemente com o mesmo quadro clínico.

Em revisão da literatura, não encontramos descrição de condição clínica que apresentasse o mesmo conjunto de sinais e de sintomas, com a mesma cronologia de aparecimento. O estudo de ligação incluindo cinco afetados e sete parentes normais, com o uso de marcadores moleculares ao longo do genoma, permitiu mapear uma região onde não havia nenhuma doença descrita com as mesmas características. Este resultado sugeriu se tratar de uma nova doença, por nós designada síndrome Sp oan (*Spastic Paraplegia, Optic Atrophy, and Neuropathy*).

1.2.2. SPG34

O probando AD, 31 anos de idade, natural de São José do Rio Preto, SP, foi avaliado em 1976, no Instituto de Biociências (IB-USP). Apresentava dificuldade para caminhar desde os 16 anos de idade, que foi progressivamente se acentuando. Esse era o seu único sintoma, e pelo histórico familiar, apresentava padrão de herança recessivo ligado ao cromossomo X. Outros 23 homens afetados da mesma família foram estudados naquela ocasião (Zatz e cols., 1976). Em 2002, Starling e cols. investigaram um ramo desta família e, com o uso de marcadores moleculares, mapearam o loco para essa condição em Xq22. Na ocasião, investigaram o gene *PLP1*, responsável pela SPG2, mas nenhuma alteração foi encontrada.

No entanto, em 2006, com a identificação de outro paciente com o mesmo quadro clínico, cuja família era proveniente da mesma região de São Paulo, o estudo dessa família com paraplegia espástica foi retomado. O estudo detalhado

da genealogia da família demonstrou que ele era relacionado ao núcleo originalmente descrito em 1976 e que não havia sido investigado em 2002. Com uso de marcadores moleculares, redefinimos a região que era compartilhada entre os afetados e com isso determinamos o loco da SPG34, uma nova forma de HSP de herança ligada ao cromossomo X

1.3. DOENÇAS DE INTERESSE NESTE ESTUDO

1.3.1. Paraplegias Espásticas Hereditárias

As paraplegias espásticas hereditárias (HSP - *Hereditary Spastic Paraplegias*) são incluídas no grupo de doenças do neurônio motor superior caracterizadas por fraqueza muscular, rigidez progressiva e exaltação dos reflexos dos membros inferiores. Clinicamente, podem ser classificadas como: 1. *não complicadas, ou puras*, quando há deficiência neurológica progressiva, fraqueza muscular, espasticidade e leve diminuição da sensibilidade nos membros inferiores, dificuldade para urinar, e, ocasionalmente, anartrestesia, ou 2. *complicadas*, neste caso associadas a outras manifestações clínicas tais como demência, epilepsia, retinopatia, alteração da via extrapiramidal, amiotrofia, surdez, neuropatia periférica ou atrofia óptica (Gundersen e cols., 1986; Lino e cols., 2000, Fink, 2008). O padrão de herança das HSPs pode ser autossômico dominante, autossômico recessivo, ou ligado ao cromossomo X.

O diagnóstico é estabelecido pela ocorrência de fraqueza muscular progressiva nos membros inferiores, aumento do tônus muscular com espasticidade, exaltação dos reflexos miopáticos, e presença de sinal de Babinski à pesquisa do reflexo cutâneo plantar. Pode ainda ocorrer diminuição da sensibilidade vibratória nas extremidades distais. Os estudos de imagem por ressonância magnética do encéfalo e da medula espinhal são, geralmente, normais.

A neuropatologia se caracteriza, nas HSPs puras, por degeneração axonal distal do trato corticoespinhal e, em menor intensidade, do funículo posterior. Além da desmielinização também pode ocorrer perda moderada de células do corno anterior da medula.

Até o momento, já foram identificados 32 locos, com 11 genes já descritos (Fink, 2008). Esses locos são genericamente denominados SPG (*Spastic Gait*). As HSPs de padrão autossômico dominante são 13: SPG3A, SPG4, SPG6, SPG8, SPG9, SPG10, SPG12, SPG13, SPG17, SPG19, SPG29, SPG31 e SPG33, nove das quais com genes identificados. As de padrão autossômico recessivo são 15: SPG5A, SPG7, SPG11, SPG14, SPG15, SPG20, SPG21, SPG23, SPG24, SPG25, SPG26, SPG27, SPG28, SPG30, e a síndrome Spoan. Apenas quatro tiveram seus genes identificados. Duas formas ligadas ao cromossomo X têm genes conhecidos: SPG1, em Xq28, conhecido por *L1CAM*; SPG2, em Xq22.2, conhecido por *PLP1*. Ambas são formas complicadas. As evidências para um terceiro loco de herança ligada ao X, SPG16, em Xq11.2-q23, não são fortes e baseiam-se em dois estudos com famílias únicas com poucos indivíduos afetados (Steinmuller e cols., 1997; Tamagaki e cols., 2000). A SPG34, estudada neste projeto, é uma forma de HSP pura, cujo loco está situado em região cromossômica distinta das demais.

As HSPs de padrão autossômico recessivo apresentam-se majoritariamente como uma forma complicada. Dillmann e cols. (1997) relataram dois afetados em uma família consangüínea em que há associação de espasticidade progressiva de início na infância, neuropatia periférica causada por comprometimento axonal e atrofia óptica progressiva de início na adolescência. Essa família, cuja doença apresentava padrão de herança autossômico recessivo, difere da estudada neste projeto (Síndrome Spoan) pelo início mais tardio da atrofia óptica e pela velocidade de progressão da espasticidade, significativamente mais lenta.

Outros casos de HSP complicados e semelhantes à Spoan foram relatados, mas nenhum com o mesmo conjunto ou cronologia de sintomas. MacDermot e Walker (1987) relataram uma família consangüínea em Gujerati, Índia, com três afetados por condição neurodegenerativa que cursa com neuropatia periférica progressiva, paraplegia espástica, atrofia óptica e retardo mental profundo. Miyama e cols. (2000) descreveram condição similar em uma menina de 8 anos, japonesa, filha de pais não consangüíneos. O estudo de neuroimagem dessa paciente revelou afilamento do corpo caloso. Uma condição

bastante similar foi descrita no Brasil por Teive e cols. em 2001, que relataram o caso de dois pacientes com paraplegia espástica, associada a corpo caloso, e perda cognitiva na segunda década de vida.

1.3.2. Neuropatias Periféricas Hereditárias

As neuropatias periféricas hereditárias (HMSN - *hereditary motor and sensory neuropathy*) são doenças de evolução crônica caracterizadas pelo comprometimento dos nervos periféricos de modo simétrico, com atrofia progressiva e secundária dos músculos e conseqüente perda de movimentos. Podem ocorrer distúrbios sensitivos distais. Coletivamente, esse grupo de moléstias é conhecido como doença de Charcot-Marie-Tooth (CMT). Originalmente, as HMSNs foram divididas em sete tipos (Dyck e cols., 1993), que têm em comum atrofia e fraqueza distal leve e graus variáveis de alterações sensoriais. Dois tipos mais comuns foram reconhecidos: HMSN I, que primeiramente afeta a bainha de mielina, e HMSN II, com degeneração axonal.

Atualmente, HMSN I é conhecido como CMT1, quando herdada como traço autossômico dominante; CMT4, quando transmitida como uma condição recessiva, e CMTX, quando ligado ao cromossomo X. HMSN II é atualmente chamado de CMT2. Numa grande família consangüínea com CMT2 e leve sinal piramidal, Barhoumi e cols. (2001) encontraram ligação em 8q21.3, nomeando-a CMT2H.

HMSN III é conhecido como CMT3, ou doença de Déjérine-Sottas, com padrão de herança autossômico dominante ou recessivo (Pearce e cols., 2006), uma grave neuropatia desmielinizante com início na infância e associada à hiperproteínoorraquia. As HMSN V, VI e VII cursam com, respectivamente, espasticidade e sinais piramidais, atrofia óptica com comprometimento axonal e, por fim, retinite pigmentar. A base genética do HMSN V é, em grande parte, desconhecida. Mais comumente tem padrão de herança autossômico dominante (Harding e cols., 1984; Vucic e cols., 2003) e freqüentemente há sinais de envolvimento axonal dos nervos periféricos (Vucic e cols., 2003; Barhoumi e cols., 2001). A HMSN VI, que associa paraplegia espástica e atrofia óptica, foi relatada em 1889 (Chalmers e cols., 1997; Voo e cols., 2003) e pode ter padrão

de herança autossômico dominante ou recessivo. Suas bases genéticas também permanecem em grande parte desconhecidas, mas a mutação no gene *MFN2* pode estar relacionada a esta HMSN (Züchner e cols., 2006). A idade de início é geralmente antes da terceira década de vida.

Existem, contudo, alguns relatos de HMSN com características que não se encaixam na classificação original de Dyck, quer devido a uma combinação de características (Dillmann e cols., 1997; Züchner e Vance, 2006) ou a associação de sintomas como retardo mental (Voo e cols., 2003; MacDermot e cols., 1987), corpo caloso afilado (Shibasaki e cols., 2000) e glaucoma (Arruda e cols., 1999; Azzedine e cols., 2003).

1.3.3. Atrofia Óptica

A atrofia óptica também pode ser um distúrbio *simples*, em que apenas a visão fica comprometida, como é o caso da neuropatia óptica de Leber (LHON) (Man e cols., 2002), ou *complicada*, quando é acompanhada de comprometimento de outros sistemas. O padrão de herança pode ser mitocondrial, autossômico dominante ou recessivo. Uma forma complicada de padrão autossômico recessivo é OPA3, ou 3-methylglutaconic aciduria tipo III, um erro inato de metabolismo caracterizada por atrofia óptica congênita, sinais piramidais e extrapiramidais e retardo mental (Anikster e cols., 2001). Outra forma complicada que também se aproxima a Spoan foi descrita por Züchner e cols. (2006), em que os pacientes apresentavam CMT2A e atrofia óptica, provocada por mutação em *MFN2*. O produto protéico de *MFN2*, *mitofusion2*, atua na membrana das mitocôndrias e apresenta um domínio comum ao gene OPA1 (Alexander e cols., 2000), que, quando alterado, condiciona atrofia óptica simples. Ambas têm padrão autossômico dominante.

II – OBJETIVOS

Os objetivos específicos deste trabalho são:

- mapear os locos gênicos associados a duas doenças neurodegenerativas, permitindo a descrição clínica e genética dessas condições e seu diagnóstico;

- tentar identificar os genes e suas mutações que estão associadas ao quadro clínico de duas doenças neurodegenerativas, testando genes candidatos;
- desenvolver testes genéticos que permitam a identificação de indivíduos portadores das mutações associadas a essas doenças em estado heterozigoto para fins de aconselhamento genético.

III – METODOLOGIA COMPLEMENTAR

Os métodos utilizados para cumprir com os objetivos foram descritos nos artigos publicados, apresentados nos capítulos seguintes. Informações adicionais que não foram tratadas nas publicações são descritas a seguir.

3.1. Extração de DNA a partir de linfócitos

A extração de DNA a partir de linfócitos foi feita de acordo com o protocolo já utilizado no laboratório, descrito por Miller e cols (1988), no qual 10 mL de sangue é coletado em um tubo contendo EDTA 5 % como anti-coagulante. As hemácias são rompidas e separadas dos leucócitos através de centrifugação diferencial. Os leucócitos são então lisados, as proteínas são removidas com soluções salinas e o DNA é precipitado.

Para saber a concentração do DNA, foi utilizado o espectrofotômetro Ultrospec 3000pro (Pharmacia Biotech.), que se baseia no fato de a absorção da luz UV, em 260 nm, pelos anéis aromáticos dos nucleotídeos ser freqüentemente usada para se estimar a concentração de DNA em extratos solúveis.

3.2. Extração de RNA a partir de cultura de células e linfócitos

A cultura e coleta das células segue o protocolo descrito no item anterior. Ao extrato obtido devem-se acrescentar 250 µl de trizol e incubar durante cinco minutos a 30°C, em seguida, agitar e incubar por mais dois minutos. Centrifugar a 12000rpm durante 15 minutos a 4°C, em seguida, transfere-se a fase aquosa para um eppendorf e adicionam-se 250 µl de álcool isopropílico. A mistura deve

ser incubada por dez minutos, centrifugada a 12000 rpm por dez minutos e o sobrenadante descartado. Ao pellet acrescentam-se 500 μ L de etanol 70% e novamente centrifugado a 7500 rpm por 15 minutos. Após descartar o sobrenadante, secar o pellet e ressuspendê-lo em água DEPC.

3.3. Estudo de Ligação:

O estudo de ligação é uma ferramenta bastante utilizada no mapeamento de genes, pois se baseia no fato de que seqüências, perto da região contendo o gene da doença, são herdadas junto com a doença dentro de uma família. Isto é resultado da baixa probabilidade de recombinação entre o gene da doença e marcadores genéticos vizinhos, devido à sua grande proximidade. Assim, dentro de uma família, pessoas que têm uma mesma doença compartilharão alelos para marcadores bem próximos ao gene da doença. Os alelos herdados junto com uma doença freqüentemente diferem entre as famílias, devido à heterogeneidade alélica ou eventos de recombinação genética em um ancestral.

O resultado de um estudo de ligação é dado em *lod* (*Logarithm of Odds*) score, que representa a probabilidade de um marcador genético segregar com a doença em relação aos mesmos segregarem independentemente. Um *lod score* (Z) de pelo menos +3.0 é considerado uma evidência de ligação, $Z < -2$ exclui a ligação da doença com a região, já um Z entre -2 e +3 é considerado inconclusivo (Strachan e Read, 1999). A partir dos genótipos obtidos e do programa de computador M.Link do “FASTLINK package version 5.1” (Lathrop e Lalouel, 1984; Lathrop e cols., 1984; Cottingham e cols., 1993), são estimados os cálculos de *lod score*.

Para mapear o loco foi feito o estudo de ligação das famílias estudadas utilizando marcadores de microssatélite. Os microssatélites são seqüências de pequenas repetições (frequentemente de 1 a 4 pb) encontradas ao longo do genoma, e que, por serem abundantes, fáceis de tipar e muitas vezes informativas, são bastante utilizadas no mapeamento de genes. Para analisar o genoma, foram utilizados os marcadores fluorescentes, que distam, em média,

10 cM um do outro, do kit “ABI Prism™ Linkage Mapping Set Version 2”, além de marcadores selecionados no *Gene Bank* e marcados com fluorescência.

Para uma reação, foi adicionado 3 μL de DNA (20 ng/ μL), 1 μL de tampão, 1 μL de dNTP (2,5 mM), 0,3 μL de MgCl_2 , 0,6 μL dos *primers* (R/F), 0,06 μL da enzima *Taq DNA Polimerase*, 4,04 μL de água deionizada, totalizando um volume final de 10 μL . Em seguida, a reação de PCR (*Polymerase Chain Reaction*) foi realizada no termociclador, utilizando o seguinte programa:

Temperatura (°C)	Tempo
94	5'
94	15''
55	15''
72	30''
72	15'
10	∞

} 30 x

O produto da PCR é então diluído em água (FAM 1:20, HEX e NED 1:10) e 2 μL desta diluição é misturada a 0,3 μL size standard e 2,7 μL tween 20 0,1%. Os produtos da amplificação foram separados por meio de eletroforese em capilar, sistema com 96 capilares, no aparelho “MegaBACE™ 1000 DNA Sequencers” (Amersham Biosciences, Little Chalfont, UK) juntamente com o padrão de peso molecular “MegaBACE™ ET 550-R Size Standard”, que acompanha o aparelho. A análise dos marcadores moleculares fluorescentes é realizada utilizando o *software* “Genetic Profiler versão 1.5”, que também acompanha o aparelho MegaBACE™ 1000.

Para restringir ainda mais a região, foram escolhidos outros marcadores de microssatélite, porém, como estes não eram fluorescentes, foi necessário correr os PCR's, feitos com radioativo ($[\alpha\text{-}^{32}\text{P}]\text{dCTP}$), em um gel feito com 6,5 % acrilamida e 40 % uréia, que age como agente desnaturante, evitando a

formação dos “hairpin loops”, grandes responsáveis pela alteração na mobilidade da molécula do DNA, APS (persulfato de amônio) e TEMED (N,N,N',N'-tetrametiletenodiamina). As amostras correram a 2100 V, 60 mA e 90 W, o que mantém o gel aquecido até aproximadamente 65 °C, evitando assim o pareamento entre as bases (*hairpin loops*), durante aproximadamente 2 horas, utilizando como tampão de corrida 1 x TBE, que possui uma grande capacidade para agüentar a alta voltagem da corrida. Em seguida, o gel é retirado do vidro com ajuda de um papel-de-filtro e colocado para secar a 80 °C durante 30 minutos em um aparato ligado a uma bomba a vácuo. Então, o gel é colocado dentro de um cassete junto com um filme de raio-X para ser revelado, após um dia a -70°C, utilizando a solução reveladora e fixadora (Kodak).

Uma outra forma de estudo com uso de microssatélites não fluorescente foi a aplicação dos produtos da PCR em um gel semelhante ao anterior (feito com 6,5 % acrilamida e 40 % uréia). As amostras foram submetidas a 2100 V, 60 mA e 90 W, durante aproximadamente 2 horas. A visualização das bandas foi feita após coloração por impregnação por nitrato de prata, que consiste de uma fixação do gel em solução de 10% de etanol e 0,5% de ácido acético por cerca de 10 minutos, exposição a uma solução de 0,17% de AgNO₃ por 10 minutos sob agitação, retirada do excesso de nitrato de prata por lavagem com água milli-Q, revelação com solução de NaOH a 3% e formaldeído a 0,1% e, por fim, nova fixação na primeira solução (Santos e cols., 1993).

3.4. Seqüenciamento:

Para seqüenciar o gene candidato, os primers foram desenhados no “Primer 3” e para fazer a PCR, foram colocados, para cada reação: 2,5 µL de tampão; 2,5 µL de dNTP (2,5mM); 0,45µL de MgCl₂; 0,5 µL do primer F (50 µM); 0,5 µL do primer R (50 µM); 0,2 µL de Taq DNA Polimerase; 17,8 µL de água deionizada e 1 µL de DNA (~200ng), totalizando um volume de 25 µL. Em geral, foi utilizado no termociclador o programa *Touch Down*:

Temperatura (°C)	Tempo	
94	4'	
94	30''	
69	40''	} 14 x reduzindo 0,5 °C por ciclo
72	1'	
94	30''	
62	40''	
72	1'	} 23 x
72	10'	
10	∞	

Em seguida, uma pequena quantidade do produto de PCR ($\pm 2\mu\text{L}$) é corrida em um gel de agarose, junto com um tampão (azul de bromofenol), para verificar se houve amplificação do produto, cujo tamanho é conhecido. Se houver, 10 μL do produto são então purificados, adicionando 5 U de EXO (Exonuclease I, *E.coli*) e 1 U de SAP (Shrimp Alkaline Phosphatase), ficando, em seguida, 1 hora a 37 °C e 20 minutos a 80 °C. Depois é feita a reação de seqüência, na qual é adicionada uma quantidade do produto purificado dependendo da intensidade da banda no “check gel” realizado, 4 μL de Pré-Mix (Amersham), 1 μL do primer F ou R (5 μM) e água deionizada para totalizar 10 μL , no termociclador: 95°C por 20 segundos e 60°C por 90 minutos com 25 ciclos de repetição. A precipitação é feita utilizando acetato de amônio (7,5 M) , etanol e, no final, o “Loading Solution” (kit do MegaBace – Amersham).

Os produtos da amplificação foram separados por meio de eletroforese em capilar no aparelho MegaBACE™ 1000 (sistema com 96 capilares) da Amersham Biosciences juntamente com o padrão de peso molecular *Dynamic*

ET dye terminator kit MegaBACE que acompanha o aparelho. A análise das seqüências foi realizada utilizando o *software* “Sequencher versão 4.2”.

3.5. Investigação de Deleção por *Southern Blotting*:

Quando o fragmento investigado do DNA do indivíduo afetado não amplificou na PCR, digerimos aproximadamente 5 µg de seu DNA e do DNA controle com a enzima *HindIII*. Em seguida as digestões foram submetidas à eletroforese em gel de agarose 0,5% horizontal durante aproximadamente 24 h a 40 V, junto com os marcadores de alto peso molecular e λ *HindIII*.

Após a corrida foi realizado o *Southern Blotting*, que consiste na transferência do DNA digerido, do gel para uma membrana de nylon por capilaridade. Para isso, o gel é lavado duas vezes com uma solução de desnaturação (0,4 M de NaOH e 0,6 M de NaCl) durante 20 minutos cada lavagem. Em um recipiente contendo a solução de transferência (desnaturação), são colocadas folhas de papel saturadas com a solução sobre uma plataforma, e, em seguida, o gel, a membrana de nylon (Hybond-N+ - GE Healthcare) e sobre esta uma pilha de folhas de papel toalha (Figura 1). No dia seguinte, o *blot* é desmontado, a membrana de nylon é lavada durante 7 minutos com uma solução de neutralização (2 x SSC; 0,2 M Tris-HCl pH 7,5) e colocada na estufa a 80 °C por duas horas para fixar o DNA.

Para hibridizar a membrana, esta é colocada em um saco plástico e adicionam-se 10 mL da solução de pré-hibridização junto com 120 µL de DNA de esperma de salmão desnaturado. Incuba-se a membrana na estufa a 42 °C por pelo menos 1 h. A sonda, que consiste no fragmento da PCR a partir do DNA controle, é então marcada com radioativo [α -³²P] dCTP, de acordo com o protocolo do kit *Random Primers DNA Labeling System* (Invitrogen). Aproximadamente 70 ng da sonda é dissolvida em água para um volume final de 10 µL, desnatura-se a mistura durante 5 minutos a 100 °C, que é imediatamente colocada no gelo. Adicionam-se 2 µL de cada solução de dATP, dGTP, dTTP; 15 µL de *Random Primers Buffer Mixture*, 1 µL de fragmento Klenow e 3,2 µL de [α -³²P] dCTP. Incuba-se a mistura por 1 h a 25 °C. Adiciona-se 5 µL do *Stop Mix* e 2 µL do DNA controle marcado com radioativo. A mistura é fervida por 5 minutos

e adicionam-se 5 mL de solução de hibridização. Retira-se a solução de pré-hibridização da membrana e coloca-se a solução de hibridização com a sonda marcada, deixando na estufa a 42 °C durante 1 *overnight*. No dia seguinte, lava-se a membrana duas vezes com a solução 2 x SSC, 0,1% SDS durante aproximadamente 10 minutos. Coloca-se a membrana em um cassete junto com um filme de raio-X (Kodak) na câmara-escura. Após cerca de 3 dias a -70 °C, revela-se o filme utilizando a solução reveladora (Kodak) e fixadora (Kodak).

Figura 1: Representação da transferência de *Southern Blotting*

3.6. Investigação de produto protéico por *Western Blotting*:

A partir de fragmentos de biópsia, realizadas para fins de diagnóstico no Centro de Estudos do Genoma Humano, foram estabelecidas linhagens de fibroblastos controle e paciente (Figura 2). As células foram cultivadas conforme Nunes e cols. (2005).

Figura 2: cultura de fibroblastos obtidos a partir da biópsia da paciente. Aumento de 5X.

A coleta das células foi feita retirando-se totalmente o meio de cultura presente na garrafa e acrescentando-se 2 ou 3 ml de PBS (ou HBSS) para lavagem das células sem Ca^{+2} e sem Mg^{+2} . Após a retirada do PBS, acrescenta-se 1 ml de tripsina (0,25%) ao frasco contendo as células. Quando a maior parte das células já estiver descolada (aproximadamente 90%), estas são então transferidas para um tubo Falcon estéril de 15 ml, seguido de centrifugação a 1700 rpm. Ao *pellet*, transferido para um eppendorf estéril, devem-se acrescentar inibidores de proteases.

O extrato obtido foi então centrifugado a 12000 rpm por 10 minutos (a 4°C) e o sobrenadante aplicado num gel de poliacrilamida 6%, com *stacking* gel de 4% e submetido a uma corrida com *Reservoir Buffer* a 100V por uma hora.

Após a corrida, foi realizado o *Western Blotting*, que consiste na transferência das proteínas do gel para uma membrana de nitrocelulose. A transferência foi feita por eletroforese, uma hora a 300 mA. Terminada a corrida, a membrana é secada na estufa a 37°C *overnight*.

A hibridização com anticorpo é precedida pela coloração com Ponceau, corante específico para proteínas. Em seguida, a membrana é lavada e nela aplica-se leite em pó a 5% por uma hora. Removido o leite, a membrana recebe o anticorpo primário, sendo encubada a 4°C *overnight*. A revelação consiste na reação com um anticorpo secundário conjugado com fosfatase alcalina (Figura3).

Figura 3: *Blot* contendo extrato de proteínas de um controle normal (C) e paciente (P), ambos hibridizados com o anticorpo anti-*Scyl1* e pré-corados com *Ponceau*.

Capítulo 2

SÍNDROME SPOAN

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Abstract

We report an autosomal recessive neurodegenerative disorder in 25 caucasian members from a large inbred Brazilian family, 22 of whom were evaluated clinically. This condition is characterized by (1) subnormal vision secondary to apparently nonprogressive congenital optic atrophy; (2) onset of progressive spastic paraplegia in infancy; (3) onset of progressive motor and sensory axonal neuropathy in late childhood/early adolescence; (4) dysarthria starting in the third decade of life; (5) exacerbated acoustic startle response; and (6) progressive joint contractures and spine deformities. Motor handicap was severe, and all patients were wheelchair bound after 15 years old. We performed a genome-wide screen including 25 affected individuals and 49 of their unaffected relatives. Linkage was detected at 11q13 region with a maximum logarithm of odds score of +14.43, obtained with marker D11S1883. The candidate region, which lies between D11S1908 and D11S1889, encompasses 4.8Mb and has more than 100 genes and expressed sequences. We propose the acronym SPOAN (spastic paraplegia, optic atrophy, and neuropathy) for this complex syndrome.

Resumo

Estudamos uma extensa família com tradição de casamentos consangüíneos que apresenta 25 indivíduos afetados pela síndrome Spoan (*Spastic Paraplegia, Optic Atrophy, Neuropathy*). A síndrome Spoan é uma doença neurodegenerativa de herança autossômica recessiva, caracterizada por atrofia óptica congênita, espasticidade, polineuropatia periférica axonal sensitivo-motora, sobressaltos à estimulação sonora, deformidades articulares e da coluna e disartria. Essa família é proveniente de região remota conhecida como

Serrinha dos Pintos localizada no Rio Grande do Norte que apresentava no ano 2000 4.295 habitantes. Foram avaliados clinicamente 22 indivíduos afetados entre 9 e 63 anos de idade. Os primeiros sintomas foram falta de fixação do olhar e abalos oculares. Foram coletadas amostras de sangue de 70 indivíduos, incluindo 25 afetados. A varredura inicial, com marcadores moleculares do tipo microssatélites permitiu a exclusão dos cromossomos 10, 12, 13, 14 e 15 como regiões candidatas associadas à síndrome Spoan. Para o marcador D11S1883 localizado em 11q13.1 foi obtido um lod score máximo de 14,43 para fração de recombinação igual a zero. O estudo de mais marcadores moleculares nessa região cromossômica permitiu delimitar uma região candidata a conter o gene responsável pela síndrome Spoan com cerca de 4.8Mb entre os marcadores D11S1908 e D11S1889.

Spastic Paraplegia, Optic Atrophy, and Neuropathy Is Linked to Chromosome 11q13

Lucia I. Macedo-Souza, BSc,¹ Fernando Kok, MD, PhD,^{1,2} Silvana Santos, PhD,¹ Simone C. Amorim, MD,² Alessandra Starling, PhD,¹ Agnes Nishimura, BSc,¹ Karina Lezirovitz, BSc,¹ Angelina M. M. Lino, MD, PhD,³ and Mayana Zatz, PhD¹

We report an autosomal recessive neurodegenerative disorder in 25 white members from a large inbred Brazilian family, 22 of whom were evaluated clinically. This condition is characterized by (1) subnormal vision secondary to apparently nonprogressive congenital optic atrophy; (2) onset of progressive spastic paraplegia in infancy; (3) onset of progressive motor and sensory axonal neuropathy in late childhood/early adolescence; (4) dysarthria starting in the third decade of life; (5) exacerbated acoustic startle response; and (6) progressive joint contractures and spine deformities. Motor handicap was severe, and all patients were wheelchair bound after 15 years old. We performed a genome-wide screen including 25 affected individuals and 49 of their unaffected relatives. Linkage was detected at 11q13 region with a maximum logarithm of odds score of +14.43, obtained with marker D11S1883. The candidate region, which lies between D11S1908 and D11S1889, encompasses ~4.8Mb and has more than 100 genes and expressed sequences. We propose the acronym SPOAN (*s*pastic *p*araplegia, *o*ptic *a*trophy, and *n*europathy) for this complex syndrome.

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In this article, we describe a large inbred family with 25 affected individuals, of whom 22 were evaluated clinically, with a previously unrecognized neurological disorder. Major manifestations include congenital optic atrophy, early-onset progressive spastic paraplegia, distal axonal motor and sensory peripheral neuropathy, and acoustic startle. A genetic analysis for this condition was able to find a linkage with marker D11S1883 on 11q13.

Subjects and Methods

The index case (Patient VII-34; Fig 1) is a 26-year-old woman who was born after uncomplicated pregnancy and labor to healthy second-cousin parents. Early in life, she had jerky eye movements and poor vision. Her motor development was slightly delayed, but she was able to tiptoe walk alone at 15 months old. Nevertheless, the motor disability progressed, and she kept an independent gait up to aged 6 years; however, by 12 years old, she was wheelchair bound. At this age, a distal weakness that had a progressive course was also recognized.

Physical examination disclosed scoliosis, club foot deformity, and limited knee extension. Hands and feet were hyperhidrotic. Her neurological examination demonstrated

spastic tetraparesis (MRC grade 0 in lower limbs, grade 3 in upper limbs) with absent plantar response, brisk patellar and upper limb reflexes, and absence of ankle reflexes. Hands amyotrophy and distal tactile, vibratory, and positional sensory insensitivity were also detected. Her speech was slightly dysarthric, with low-tone voice. Wrist cogwheel sign was present. She had spontaneous fixation nystagmus and low visual acuity, being able to count fingers at a distance up to 2m. Fundus oculi showed bilateral optic disk pallor. A startle response to unexpected sounds was constantly present. No ataxia, incontinence, dystonia, or cognitive impairment was detected.

Further family history analysis disclosed several additional affected relatives of Caucasian Ibero-European background who settled more than a century ago in an isolated area in Northeastern Brazil (Serrinha dos Pintos, Rio Grande do Norte State, population 4,295). Two authors (F.K., S.C.A.) were able to evaluate 18 affected individuals in this community and three more in nearby villages (see Fig 1). Four other patients were recognized later but were not evaluated clinically; nevertheless, three of them were included in the linkage analysis. In total, 19 consanguineous mates were parents of the 25 studied individuals. It was also known that, in the last 20 years, at least 4 more individuals (VI-18, VI-31, VI-50 and VIII-4) affected by a similar condition have died.

From the ¹Department of Biology, Institute of Biological Sciences and Center for Study of Human Genome, University of São Paulo; ²Child Neurology Service and ³Clinical Neurology Service, Hospital das Clínicas, University of São Paulo School of Medicine, São Paulo, Brazil.

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Address correspondence to Dr Kok, Child Neurology Service, Hospital das Clínicas, University of São Paulo School of Medicine. Av. Dr. Enéas de Carvalho Aguiar, 255 S 5011–CEP 05403-000 São Paulo, SP, Brazil. E-mail: fernando.kok@fleury.com.br

Fig 1. SPOAN family pedigree. Haplotypes for markers from the SPOAN critical region are given, and haplotypes segregating with the disease are boxed. The arrow indicates the proband. The individuals with a little bar over their symbols were clinically evaluated.

This syndrome was already recognized in this region by the end of 19th century and at least six more individuals were reportedly affected. Interestingly, the concept that this condition is genetically determined was not present in the community, and several other reasons were evoked to explain this phenomenon, most of them related to syphilis.

Twenty-two patients (15 female and 7 male patients), with ages ranging from 9 to 63 years, were evaluated clinically (Table 1). We collected, after obtaining informed consent for participation and publication of results, as well as institutional review board approval, blood samples from 25 affected subjects and 49 healthy family members.

Mapping Strategy

Genomic DNA was obtained following standard techniques. A genome-wide search was undertaken using 400 microsatellite markers that comprise an approximate 10cM human index map from ABI PRISM linkage-mapping set version 2.5 (Applied Biosystems, Foster City, CA). Those markers were amplified by polymerase chain reaction using standard protocols. The polymerase chain reaction products were sequenced in MegaBace 1000 DNA Sequencer (Amersham Biosciences, Little Chalfont, United Kingdom) and were analyzed with Genetic Profiler software (Amersham Biosciences). Required additional microsatellite markers primer sequences, as well as distances, were obtained from National Center for Biotechnology Information databases. These markers were amplified by polymerase chain reaction using ^{32}P deoxycytidine triphosphate in a 10 μl reaction volume. The amplification products were visualized by autoradiography after application to a standard sequencing gel.

Linkage Analysis

The disease was analyzed as an autosomal recessive trait with an allele frequency of 0.0001. Two-point logarithm of odds (LOD) scores were calculated under the assumption of equal allele frequencies and using the computer MLINK program from FASTLINK package version 5.1. (<http://linkage.rockefeller.edu/soft/>)^{1,2} Recombination frequencies were assumed to be equal in male and female subjects. Allele frequencies were assumed to be 1/N (N = number of different alleles observed on the pedigree). For linkage analysis, many loops of consanguinity were broken because the linkage software allows only eight loops. Therefore, the LOD score values were underestimated, because we used just 8 loops instead of the 19 present in this family.

Results

Clinical Features

Table 1 presents the main clinical features of the affected individuals. As already mentioned, cognitive decline, mental retardation, ataxia, or deafness were not detected in any patient. Symptoms intensity could vary among patients, but disease was fully penetrant.

CONGENITAL AND NONPROGRESSIVE OPTIC ATROPHY. Symptoms related to optic atrophy were recognized early in life and apparently were not progressive. Fixation nystagmus was observed in 18 patients (82%) and was caused by subnormal vision, which was seen in 21 of 22 patients (95.5%) who had pale optic disks. Patients were able to count fingers at a distance of 2m.

Table 1. Clinical Data Summary

Characteristic	No. (%)
Sex	
Male	7 (31.8%)
Female	15 (68.2%)
Age at ascertainment (yr)	
<10	1 (4.5%)
10 to <20	2 (9.1%)
20 to <30	4 (18.2%)
30 to <40	7 (31.8%)
40 to <50	5 (22.7%)
>50	3 (13.6%)
Age of onset of motor symptoms (yr)	
<1	17 (77.3%)
1 to <2	1 (4.5%)
Not known	4 (18.2%)
Maintenance of independent gait (for patients older than 15 yr)	
Never achieved	3 (14.3%)
<6 yr	5 (23.8%)
6 to <10 yr	11 (52.4%)
Not known	2 (9.5%)
Maintenance of any kind of independent locomotion (for patients older than 15 yr)	
Up to 10 y	1 (4.8%)
10 to <15 yr	15 (71.4%)
15 to <20 yr	2 (9.5%)
Not known	3 (14.3%)
Fixation nystagmus	
Absent	5 (22.7%)
Present	17 (77.3%)
Optic atrophy	
Absent	1 (4.5%)
Present	21 (95.5%)
Joint retractions	
Absent	5 (22.7%)
Present	17 (77.3%)
Proximal reflexes	
Absent	3 (13.6%)
Brisk	19 (86.3%)
Distal reflexes	
Absent	14 (63.6%)
Reduced/Present	6 (27.3%)
Brisk	2 (9.1%)
Plantar response	
Absent	20 (90.9%)
Babinski sign	2 (9.1%)
Distal wasting	
Absent	3/3, younger than 20 yr
Present	19/19, older than 20 yr
Acoustic startle	
Present	22 (100%)
Spine deformity	
Scoliosis and kyphosis	14 (63.6%)
Absent	8 (36.3%)
Tactile, vibratory, and arthresthetic sense reduction (for patients older than 15 yr)	
Lower limbs only	6 (28.6%)
Upper & lower limbs	14 (66.7%)
Not known	1 (4.8%)
Dysarthria	
Absent	3/3, younger than 20 yr
Present	19/19, older than 20 yr
Extrapyramidal signs (dystonia, etc.)	
Absent	17 (77.3%)
Present	5 (22.7%)
Hyperhydrosis	
Absent	6 (27.3%)
Present	16 (72.75%)

Only one affected woman (VI-49; 63 years old) did not report visual problems and had a normal fundus oculi.

PROGRESSIVE, INFANCY-ONSET SPASTIC MOTOR DEFICIENCY. Early motor signs presented as motor development delay and tiptoe walk; in three patients, independent gait was never achieved; all other affected subjects lost it by 10 years old. All types of independent locomotion expression, including walking with support or crawling, were lost before 20 years old. Interestingly, spontaneous but not provoked ankle clonus was present in eight patients. Babinski sign was seen in only two patients; all other patients had no response. Triple-flexion response also was detected in eight patients, all of whom had severe spastic paraplegia and no voluntary movements. Lower limb involvement was always more premature and intense than in upper limbs. In only a severely affected, 47-year-old woman (VI-46) was spasticity not detected, even though it was present in her disease history.

MOTOR AND SENSORY NEUROPATHY WITH ONSET IN LATE CHILDHOOD/ADOLESCENCE. Distal amyotrophy was seen in all patients older than 20 years; hyperhydrosis, tactile insensitivity, and lack of distal vibratory and positional senses were seen in 20 affected subjects. Pain and temperature sensory perception was not affected even later in life, and patients never reported spontaneous pain. We could not obtain reliable information on sensory abnormality in two patients (a 9-year-old child [VIII-8] and a severely dysarthric 47-year-old woman [VI-46]). Severe distal muscle wasting was seen in all patients older than 20 years. No fasciculation was observed. Because of a combination of pyramidal and peripheral signs, proximal reflexes were usually more easily obtained than distal ones. Bicipital, adductor, and knee reflexes were absent in 3 patients and were brisk in the remaining 19 patients; stylo-radial reflexes were absent in 14, present in 6, and brisk in 2 patients; ankle reflexes were abolished in 18 and present in 4 patients.

DYSARTHRIA AND EXTRAPYRAMIDAL SIGNS. Dysarthria, associated with low-tone voice, was present in all 19 patients older than 20 years of age. In a markedly affected, 47-year-old woman (VI-46), dysarthria and dysphonia were so intense her speech was barely understandable. Wrist cogwheel sign was present in four patients, and neck dystonia was present in one. One patient had myoclonic thumb movements.

STARTLE ACOUSTIC RESPONSE. Exacerbated startle acoustic response was detected in all patients, being recalled since early infancy, observed even late in adulthood, and easily elicited including in severely handicapped patients. Paradoxically, patients with total

absence of lower limb spontaneous movement had involuntary muscle contractions precipitated by unexpected noise.

SPINE AND JOINT DEFORMITIES. Deformities and limited mobility were present in ankle, knee, wrist, and elbow joints in 21 of the 22 patients, with a variable degree of range limitation. Cervicothoracic kyphosis was seen in two patients and scoliosis in 12 and; it was so severe in five individuals that they were unable to sit independently. No clinical evidence of connective tissue disease was present.

Diagnostic Tests

Brain magnetic resonance imaging performed in three patients (VIII-8, VII-23, and VII-34) showed no conspicuous alterations. Spinal cord magnetic resonance imaging was suggestive of mild atrophy in Patient VIII-8. Electromyography, performed in Patients VIII-8 and VII-34 at 8 and 26 years old, respectively, was suggestive of axonal motor and sensory neuropathy, with normal motor nerve conduction velocity. An electroretinogram, performed in Patient VII-34, and cerebrospinal fluid study, done in Patient VII-38, were normal. In Patient VII-34, radiographs of the hands were normal, but spine radiographs disclosed scoliosis. Sural nerve biopsy was performed according to standard protocol³ in Patient VII-34 and was processed for light and electron microscopy (JEOL 100CX; JEOL, Peabody, MA). The densities of myelinated and unmyelinated fibers were determined. Morphometric analyses were performed by stereology-based measurements, and the results were expressed as median per square millimeter of fascicular area.⁴ Median densities of 2,570 myelinated fibers/mm² and 10,630 unmyelinated fibers/mm² were found (our reference age-matched median values for sural nerve were 10,479 myelinated fibers/mm² and 32,560 unmyelinated fibers/mm²).⁵ The most striking pathological finding was several fibers with bizarre shape (Fig 2) that appear to have a disproportionately thin myelin sheath. Fibers with features of Wallerian degeneration were rare, and regenerative clusters were uncommon. There were few

typical onion bulb formations around single axons, and some regenerative clusters were surrounded by a concentrically arranged Schwann cell process forming small onion bulbs (Fig 3). Some axons showed axoplasm vacuolization (Fig 4). We observed many Büngner's bands and normal periodicity of myelin lamellae (data not shown). No specific abnormalities of axonal organelles, cytoplasmic inclusions in Schwann cell (SC), or focal myelin enlargements were found.

Linkage

A genome-wide screening was first performed using 12 members of this pedigree, 5 affected subjects and 7 unaffected relatives. Linkage was detected with marker D11S987 located at 11q13. Analysis of 25 affected subjects showed a maximum LOD score of +13.56 ($\theta = 0.01$) with D11S987. Additional microsatellite markers closely linked to D11S987 between markers D11S4191 and D11S1314 were typed. The greatest two-point LOD score, $Z_{\max}$ 14.43 at $\theta = 0.0$, was obtained with marker D11S1883 (Table 2). Haplotype reconstruction and analysis of the recombination events between markers and the disease locus in affected subjects (see Fig 1) placed the locus in a 4.79Mb region flanked by markers D11S1908 and D11S1889.

Discussion

The combination of inherited spastic paraplegia, axonal neuropathy, dysarthria, acoustic startle, and congenital optical atrophy as seen in the 22 patients described here has not been reported previously. This condition has clinical features that overlap with complicated forms of other neurodegenerative disorders, as hereditary motor and sensory neuropathies (HMSNs), currently better known as Charcot-Marie-Tooth (CMT) disease⁶; hereditary spastic paraplegias (HSPs)^{7,8}; and hereditary optic atrophies.

Originally, HMSNs were divided into seven types⁹ that have in common distal weakness with atrophy and mild and variable degrees of sensory disturbance. Two more common types were recognized: I, which primarily affects myelin sheaths, and II, which shows axonal degeneration. Currently, HMSN type I is known as

Fig 2. (A–C) Transverse electron micrographs of sural nerve (Patient VII-34), showing fibers with bizarre shapes, probably caused by myelin outfolding (arrows). $\times 4,000$ original magnification.

Fig 3. Electron micrograph of sural nerve (A). Typical onion bulb formation around a single axon. $\times 8,000$ original magnification. (B). Regenerative cluster surrounded by concentric Schwann cell in a small onion bulb. $\times 8,000$ original magnification.

CMT1 when inherited as an autosomal dominant trait, CMT4 when transmitted as a recessive condition, and CMTX, when X-linked. HMSN type II currently is called CMT2, and HMSN III is known as CMT3, or Déjérine-Sottas disease, a severe demyelinating neuropathy with onset at infancy.^{3,6} Dick⁹ further recognizes HMSN types V, which is associated with pyramidal signs, and VI, which is associated with optic atrophy.

The genetic basis of HMSN type V is largely unknown, but it is probably a heterogeneous condition, more commonly dominantly inherited,¹⁰⁻¹² with axonal involvement being commonly described.^{12,13} In a large inbred family with CMT2 and mild pyramidal signs, Barhoumi and colleagues¹³ report linkage to 8q21.3, naming this disorder CMT2H.

Although description of combined optic atrophy and neuropathy (HMSN type VI) dates back from 1889,¹⁴ with several reports in recent years,¹⁴⁻¹⁷ its genetic basis remains largely unknown. Both autosomal domi-

nant and recessive inheritance have been postulated, age at onset is usually before the third decade of life, and axonal involvement is the rule.

There exist, however, some reports of HMSN with features that do not fit Dick's original classification,⁹ either because of a combination of manifestations¹⁸ or the association of symptoms as mental retardation,^{17,19} thin corpus callosum,²⁰ and glaucoma.^{21,22}

MacDermot and Walker¹⁹ describe three adults from an inbred family affected by axonal neuropathy, mental retardation, pyramidal signs, and congenital optic atrophy. All patients remained ambulatory, and peripheral signs were more prominent than pyramidal signs.

Dillmann and colleagues¹⁸ report two siblings born to consanguineous parents, with a slowly progressive, infancy-onset spastic paraplegia followed by adolescence-onset axonal neuropathy and optic atrophy. No dysarthria or startle response, as seen in our series, was described.

HSPs are characterized by progressive and often severe lower limb spasticity.^{7,8} When this sign occurs alone, it is known as pure HSP. When accompanied by other deficits, including impaired position sense, bladder disturbances, extrapyramidal symptoms, neuropathy, mental retardation, dementia, or optic atrophy, it is known as complicated HSP.^{7,8} Inheritance of HSP can be autosomal dominant, autosomal recessive, or X-linked recessive. Pure forms of HSP are more common than complicated ones. Twenty-three loci of HSPs have been assigned so far, and nine genes have been identified.^{7,8}

Among the currently recognized complicated forms of HSP, seven can be inherited as a recessive trait: SPG7,²³ SPG11,²⁰ SPG14,²⁴ SPG15,²⁵ SPG20,²⁶ SPG21,²⁷ and SPG23,²⁸ as shown in Table 3. Patients in our series do not have clinical features that fit the characteristics of any of these conditions.

Fig 4. Electron micrograph of sural nerve. $\times 8,000$ original magnification. Presence of axoplasm vacuolar degeneration.

Table 2. Two-Point LOD Scores for Linkage of Microsatellite Markers on Chromosome 11

Marker	LOD Score at θ						
	0.000	0.010	0.050	0.100	0.200	0.300	0.400
D11S4191	4.95	9.46	10.20	9.42	6.99	4.37	1.97
D11S1908	— ∞	4.50	6.46	6.45	5.06	3.23	1.44
D11S1883	14.43	14.07	12.66	10.90	7.56	4.56	2.03
D11S1889	12.48	12.16	10.91	9.37	6.41	3.79	1.63
D11S987	— ∞	13.56	13.45	12.14	8.86	5.45	2.37
D11S1314	— ∞	5.20	7.93	7.91	6.05	3.62	1.42

LOD = logarithm of odds.

The genetic basis of hereditary optic atrophy is less diverse than that of CMT or HSP, but a complicated form of autosomal recessive optic atrophy also has been recognized: OPA3, or 3-methylglutaconic aciduria type III, is an inborn error of metabolism characterized by congenital optic atrophy, pyramidal and extrapyramidal signs, and mental retardation.²⁹

Startle response to unexpected noises is a peculiar sign that has been reported in GM2 gangliosidosis (Tay–Sachs disease)³⁰ and in hyperekplexia,^{31,32} a condition characterized by exaggerated response to both tactile and acoustic stimuli.

Sural nerve pathological findings suggested an axonal process with loss of myelinated and unmyelinated fibers. Axonal degeneration was characterized by axoplasm vacuolization, abundant Büngner's bands that represent vestige of a degenerated nerves, and fibers with disproportionately thin myelin sheath, probably secondary to axonal atrophy.³ Although onion bulbs are characteristic of a demyelinating process, they are also found in axonal neuropathies.^{3,33,34} Schwann cells hypertrophy, seen in axonal neuropathy, might suggest its concomitant involvement or might represent cycles of axonal degeneration and repair.⁹ The abundance of fibers with bizarre shapes, also described in inherited neuropathies with myelin outfolding, probably result from profound abnormalities in axonal cytoskeleton or in its interaction with Schwann cells.^{33,35–37}

We suggest that the disorder described in this article represents a new neurodegenerative condition inherited as an autosomal recessive trait, and we propose the ac-

ronym SPOAN (for spastic paraplegia, optic atrophy, and neuropathy) for this complex syndrome. The burden of this condition in Serrinha dos Pintos is overwhelming, with estimates that 1 in every 250 of its inhabitants is affected by SPOAN syndrome, and that 1 of 9 individuals in this village are heterozygous carriers of the responsible mutated gene. Interestingly, according to the Brazilian 2000 census of 5,507 Brazilian municipalities, Serrinha dos Pintos is ranked 38th among the 50 communities with the most disabilities (see http://www.fgv.br/cps/deficiencia_br/Ranking/PPD for more detailed information). SPOAN syndrome might be partially responsible for this unfortunate position.

The candidate region for SPOAN syndrome lies in chromosome 11q13 flanked by markers D11S1908 and D11S1889 and is approximately 4.79Mb. No inherited neuropathy optic atrophy maps in this interval. Nevertheless, this region was previously assigned for a complicated dominant HSP, known as Silver syndrome, or SPG17,³⁸ a condition characterized by lower limb spasticity associated with hands and feet weakness and amyotrophy. Patel and colleagues³⁸ report linkage of Silver syndrome to chromosome 11q12–q14 in a region of ~13cM flanked by markers D11S1883 and D11S4136, with a 2cM overlap with the SPOAN syndrome candidate region. However, Silver syndrome has clinical features and mode of inheritance different from the disease described here. Some rare genes, such as *lamin A/C*,³⁹ have been associated with both autosomal dominant and recessive inheritance. Therefore, we

Table 3. Complicated Spastic Paraplegia with Autosomal Recessive Inheritance

Type	Location	Gene Product	Accompanying Features	Reference
SPG7	16q24.3	Paraplegin	Optic, cortical cerebellar atrophy	23
SPG11	15q13–14	Unknown	MR, thin corpus callosum, PN	20
SPG14	3q27–28	Unknown	MR, PN	24
SPG15	14q	Unknown	MR, dementia, PN	25
SPG21	15q22.31	Masparidin	Dementia, extrapyramidal and cerebellar signs	26
SPG20	13q12.3	Spartin	Dysarthria, distal wasting	27
SPG23	1q24–32	Unknown	Pigmentary skin defects	29

MR = mental retardation; PN = peripheral neuropathy.

could not rule out the possibility that SPOAN and Silver syndromes are allelic. However, it is more likely that these two disorders are caused by different genes because there are at least 143 genes and expressed sequences located in the SPOAN candidate region and many more transcripts in the Silver syndrome critical region. Of the 143 transcripts in the SPOAN candidate region, 96 have a significant level of expression in the nervous system and should be seen as bona fide candidates for this syndrome.

Identification of the gene responsible for SPOAN syndrome might help the genetic counseling of individuals belonging to this at-risk population and also might help to increase our understanding about the pathophysiology of hereditary optic atrophies, spastic paraplegias, and axonal neuropathies.

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Capítulo 3

EXCLUSÃO DE GENES E REDUÇÃO DA REGIÃO CANDIDATA DA SÍNDROME SPOAN

Submission being processed.

Abstract

For the study of the SPOAN syndrome we selected 23 candidate genes that had all their exons sequenced, but no mutation was observed. RNA expression analysis from fibroblasts and lymphocytes of the patients revealed that transcripts for *DNAJC4*, *MARK2*, *RTN3*, *SCYL1*, *SNX15*, *STIP1*, *STX5* e *VEGF β* genes were present. In addition, during the development of this study 13 microsatellites and 50 single nucleotide polymorphisms (SNP) in many of these genes were investigated. We identified six SNPs in heterozygosity, four of which were in the genes: *ASRGL1*, *CFL1*, *SCYL1* and *VEGF β* , this allowed us to restrict the candidate region. As we suspected the possibility of a deletion in the gene *NRXN2*, we investigated the region by Southern Blotting, which was not confirmed. We also evaluated the expression of the gene *SCYL1* by Western Blotting, in fibroblasts and lymphocytes. The results of this collaborative study were published in a manuscript in the EMBO reports vol 8, n 7, 2007. The identification of 44 new cases of SPOAN located in São Miguel, Pilões, Pau dos Ferros, Serrinha dos Pintos, all in RN, and Ererê in CE, in Brazil, allowed us to have a comprehensive view about SPOAN clinical phenotype and to reduce the mapped region from 4.8 to 2.3Mb, between the SNP rs1939212 and microsatellite D11S987, in 11q13. The samples of 149 individuals were genotyped, 65 affected and their relatives, for six microsatellite markers. All patients are homozygous only at D11S1889, which two-point lod score with a Z_{max} of 27 at $\theta=0.0$ was obtained. The results of this study are being submitted.

Resumo

Durante o estudo da síndrome Spooan selecionamos 23 genes que tiveram todos os exons seqüenciados. Nenhuma mutação foi observada. Além disso achamos interessante estudar a expressão de alguns desses genes, como o *BSCL2*. Para esta finalidade foi extraído RNA de fibroblastos e linfócitos dos pacientes, a partir do qual foi obtido o cDNA, que codifica para as proteínas de interesse. Verificamos que o RNA dos genes *DNAJC4*, *MARK2*, *RTN3*, *SCYL1*, *SNX15*, *STIP1*, *STX5* e *VEGF β* estavam presentes nestes tipos celulares estudados. Além disso, durante o desenvolvimento do trabalho 13 microssatélites e 50 SNPs foram estudados, muitos destes presentes nos genes investigados. Seis SNPs em heterozigose foram identificados, dos quais quatro nos genes *ASRGL1*, *CFL1*, *SCYL1* e *VEGF β* , o que nos permitiu, além da exclusão dos mesmos, restringir a região candidata. Como houve suspeita de deleção no gene *NRXN2*, investigamos a região por Southern Blotting, que não foi confirmada. A presença da proteína codificada pelo gene *SCYL1* foi investigada em fibroblastos e linfócitos por Western Blotting. Os resultados desse estudo colaborativo permitiram a redação de um manuscrito publicado na EMBO reports vol 8, n 7, 2007. Contudo, a identificação de 44 casos novos de Spooan localizados nos municípios de São Miguel, Pilões, Pau dos ferros, Serrinha dos Pintos, todos no RN, e Ererê no CE, permitiu reduzir a região de 4,8 para 2.3Mb, entre o SNP rs1939212 e o microssatélite D11S987, em 11q13, bem como a compreender melhor a clínica da síndrome SPOAN. Amostras de 65 afetados e seus parentes foram estudados para seis marcadores de microssatélite, totalizando 149 indivíduos genotipados. Para o marcador D11S1889, com alelos em homozigose para todos os pacientes, foi obtido um *lod score* máximo de 27, para fração de recombinação igual a zero ($\theta=0.0$). Os resultados deste estudo se encontram em fase de submissão.

Dados Complementares

Segue em anexo o paper EMBO reports vol 8, n 7, 2007 (ANEXO 1); a genotipagem dos 65 afetados e seus 83 parentes não afetados (ANEXO 2); a lista dos marcadores moleculares utilizados (ANEXO 3); e o mapa do cromossomo 11 com os 23 genes seqüenciados (ANEXO 4).

New observations and linkage refining in spastic paraplegia, optic atrophy, and neuropathy

Lúcia Inês Macedo-Souza*¹; Fernando Kok*^{1,2}; Silvana Santos*¹; Luciana Licinio¹; Karina Lezirovitz¹; Natale Cavaçana¹; Clarissa Bueno²; Simone Amorim²; André Pessoa²; Zodja Graciani²; Áurea Ferreira²; Abdísio Prazeres³; Áurea Nogueira de Melo⁴; Alessandra A. Cavalcanti-Souza⁵; Paulo A. Otto¹; Mayana Zatz¹

*These authors contributed equally to this study.

1. Human Genome Research Center, Institute of Biosciences, University of São Paulo,(USP) São Paulo, SP; 2. Child Neurology Service, USP School of Medicine, São Paulo, SP; 3. Ophthalmology Department, USP School of Medicine, São Paulo, SP; 4. Department of Pediatrics, Federal University of Rio Grande do Norte, Natal, RN; 5. Children's Rehabilitation Center, Natal, RN, Brazil.

Corresponding author:

Fernando Kok, MD, PhD

Child Neurology Service, Department of Neurology, University of São Paulo School of Medicine

Av. Dr. Enéas de Carvalho Aguiar, 255 S 5011 CEP 05403-000 São Paulo, SP, Brazil

Phone: 55-11-3069-7877

Fax: 55-11-3069-7878

E-mail: fernando.kok@fleury.com.br

Key words: spastic paraplegia, optic atrophy, neuropathy, SPOAN, genetic epidemiology.

ABSTRACT

Spastic paraplegia, optic atrophy, and neuropathy (SPOAN) is an autosomal recessive neurodegenerative disorder which was recently characterized by our group in a large inbred Brazilian family with 25 affected individuals. This condition, initially found in individuals living in a small village in Northeastern Brazil, is clinically defined by: 1. subnormal vision secondary to apparently non-progressive congenital optic atrophy; 2. progressive spastic paraplegia with onset in infancy; and 3. progressive motor and sensory axonal neuropathy, clinically apparent in late childhood or early adolescence. In order to detect additional individuals with SPOAN in the region, we conducted a survey in five villages of the region (39,054 inhabitants in 2007) which are among the top 50 in Brazil's Deficiency Ranking. We were able to diagnose 41 patients, with a prevalence of 1 SPOAN patient for every 953 inhabitants, with an estimated heterozygosity frequency of about 1 in every 15 inhabitants. Furthermore, we diagnosed 18 SPOAN patients living in the neighboring region (population: 200,462) and 8 other affected individuals native from this area but living elsewhere in the country. Overall, we are aware of 68 SPOAN patients (45 females and 23 males, with age ranging from 5 to 72 years), 44 of which are new observations. Those 68 patients belonged to 43 families, 40 of which were known to be consanguineous. Sixty patients have been fully clinically evaluated and 64 included in the genetic investigation. All analyzed patients are homozygotes for D11S1889 at 11q13. With the identification of those additional patients, we were able to reduce the critical region for the SPOAN gene from 4.8 to 2.3 Mb, with a maximum two point lod score of 33.2 with marker D11S987 and of 27.0 with marker D11S1889. Three genes located in this newly defined critical region were sequenced, but no mutation was detected. The gene responsible for SPOAN remains elusive.

INTRODUCTION

Hereditary spastic paraplegias (HSP) are a heterogeneous group of genetic disorders that can be inherited either as an X-linked recessive or as an autosomal dominant or recessive trait. Two distinct phenotypes for HSP can be recognized: pure, with isolated spastic paraplegia of lower limbs, and complicated, associated with other symptoms as dementia, optic atrophy, epilepsy, neuropathy, retinopathy, and mental retardation^{1, 2}. In 2005, our group reported³ an autosomal recessive complicated form of spastic paraplegia in a large Brazilian inbred family with a previously undescribed phenotype characterized by: 1. subnormal vision secondary to apparently non-progressive congenital optic atrophy; 2. spastic paraplegia with onset in infancy; and 3. progressive motor and sensory axonal neuropathy clinically apparent in late childhood or early adolescence. Additional common observations were dysarthria, spine deformity, and joint retractions, as well as startle response following unexpected noises; less common abnormalities were extrapyramidal signs, such as dystonia and parkinsonism. This condition was named SPOAN (for spastic paraplegia, optic atrophy, and neuropathy) and linkage study, performed with 25 affected individuals, resulted in a two point lod score of +14.43 at D11S1883.

Herein, we expanded the epidemiological, clinical and genetic investigation of SPOAN, and we were able to confirm the diagnosis in 68 individuals, 64 of which were included in the genetic study and 60 fully clinically evaluated. This investigation allowed us to have a comprehensive view about SPOAN clinical phenotype and to reduce the candidate region for this gene to 2.3 Mb.

CASUISTIC AND METHODS

Epidemiological study and patient recruitment

An epidemiological survey regarding deficiency and consanguinity was conducted in five villages (population in 2007: 39,054) of the Southwestern region of Rio Grande do Norte State, Brazil (Supplemental Figure 1), selected because they ranked among the top 50 in Brazil's Deficiency Ranking (IBGE, 2000 Census, which investigated all 5,507 Brazilian towns). Those villages are located in an impoverished area of the country, with an average human development index (HDI) of 0.629 (Brazil's

2007 HDI: 0,800). One of them, Serrinha dos Pintos, was already known as the town where SPOAN was originally described. Community Health agents from Family Health Program collected information about consanguinity and deficiencies in all families of the villages. Individuals with disability were interviewed and subjected to a preliminary evaluation by a geneticist (SS).

Additionally, we were also informed about affected individuals with SPOAN living in other villages of the region (200,462 inhabitants), as well about affected individuals living elsewhere in the country but native from this area.

Clinical study and diagnostic tests

Individuals in which the diagnosis of SPOAN was suspected were fully evaluated by a team of experienced neurologists, clinical geneticists and ophthalmologist and a detailed family history was also collected. In a small number of individuals, brain MRI, nerve conduction velocity, electroretinogram, nerve biopsy, CSF analysis and urine organic acid determination were also performed.

Mapping and linkage refining

Genomic DNA was obtained following standard techniques. A search was performed using six informative microsatellites (D11S4191, D11S4076, D11S1883, D11S1889, D11S987, and D11S1314), located at 11q12-13, inside and flanking the 4.8 Mb critical region for SPOAN. These markers were amplified by polymerase chain reaction (PCR) using standard protocols. The PCR products were subjected to electrophoresis in the MegaBace 1000 DNA Sequencer (Amersham Biosciences, Little Chalfont, UK) and subsequently analyzed with Genetic Profiler software (Amersham Biosciences, Little Chalfont, UK). Fifty single nucleotide polymorphisms (SNPs), located in the critical region, some of them in candidate genes, were also investigated (list available under request).

Parametric two-point linkage analysis for each marker was performed using the MLINK software from the FASTLINK (version 5.1) software package⁴. The disease was analyzed as an autosomal recessive trait with a disease allele frequency of 0.0001. Marker allele frequencies were assumed to be $1/N$, N being the number of different alleles observed on the pedigree. Although assuming allele frequencies equal to $1/N$ could lead to an overestimation of the lod score values, we believed it was the best

approach since there was not enough genotypic data from founder individuals or individuals from unaffected branches of the inbred “SPOAN family”.

Mutational Analysis

We sequenced 23 transcripts located in the original critical interval for SPOAN, using primers and amplification conditions, which are available under request. All exons and their flanking intronic boundaries were directly sequenced using a MegaBACE 1000 DNA Sequencer and results were analyzed with Sequencher software support (<http://www.genecodes.com>). Of these 23 sequenced transcripts, only three remained in the newly defined candidate region for SPOAN: *LRFN4* (leucine-rich repeat and fibronectin III domain-containing)⁵, which is a membrane spanning glycoprotein which plays a role in the development of the CNS; *KLC2* (kinesin light chain 2)⁶, which is an ATP-dependent molecular motor involved in vesicle and organelle traffic along microtubules; and *CCS* (copper chaperone for SOD1)⁷, whose product is responsible for intracellular copper loading of superoxide dismutase 1 (SOD1).

RESULTS

Epidemiological study and patient recruitment

Forty-one patients with SPOAN were diagnosed in two out of the five surveyed villages: 23 in São Miguel (22,579 inhabitants, with a frequency of consanguineous marriages of 19%) and 18 in Serrinha dos Pintos (4,360 inhabitants, and a consanguineous unions rate of 32%). Those 41 individuals belonged to 27 families, 26 of which (96.3%) were highly inbred.

Additionally, 18 patients affected by this disorder living in the same geographical area and 9 living elsewhere but native from this region were evaluated. In total, we recognized 68 individuals with SPOAN, 44 of which were new observations. Those 68 patients belonged to 43 families, 40 of which (93%) were known to be consanguineous. It is believed that all individuals affected by SPOAN in this region were close or distantly related.

Clinical study and diagnostic tests

Of the 68 known cases of SPOAN, 60 were fully clinically evaluated. Clinical information regarding 22 patients have been already published³, but one of them, a 63-

year-old woman assigned previously as affected, was reevaluated and excluded because she had no optic atrophy or clinically evident peripheral neuropathy. Clinical data regarding 21 previously reported SPOAN patients and 39 new observations are summarized in Table 1.

Brain MRI, performed in five patients, showed a normal result. Four limbs nerve conduction velocity was performed in four individuals 5, 14, 26, and 59 years old. The youngest patient was the only one with normal results; in all others, a severe motor and sensory neuropathy with axonal pattern was detected. Sural nerve biopsy, performed in a single patient, was abnormal and had already been reported³. Electroretinogram was performed in two patients, CSF analysis in four, and urine organic acid in one individual, all with normal results.

Mapping and linkage refining

The identification of additional cases of SPOAN allowed us to recalculate the lod score and to restrict the candidate region. We analyzed six microsatellites located in 11q12-13, from 64 patients and 85 unaffected relatives, and obtained with marker D11S987 the highest two-point lod score with a Zmax of 33.2 at $\theta=0.0$, followed by marker D11S1889 with a Zmax of 27.00 at $\theta=0.0$. Markers D11S987 and D11S1889 are only 580Kb apart, and D11S987 is more polymorphic than D11S1889, which explains why the former yielded a higher lod score, even though all patients are homozygous only at D11S1889. We further analyzed 50 SNPs and found an informative polymorphism, allowing us to reduce the candidate region from approximately 4.8 to 2.3 Mb, now flanked by SNP rs1939212 and by microsatellite D11S987, at 11q13.

Mutational Analysis

No mutation was found in the coding region or intron-exon boundaries of genes *KLC2*, *CCS2* and *LRFN4*, located in the newly defined critical region for SPOAN.

DISCUSSION

In the five surveyed villages located in Southwestern region of the state of Rio Grande do Norte, SPOAN prevalence was of 1 per 952 inhabitants, with an estimated heterozygosity frequency of about 1/15. Moreover, in Serrinha dos Pintos, SPOAN prevalence was of 1/242, with an estimated heterozygous frequency of about 1/8.

Overall the minimum prevalence of SPOAN in Southwestern region of Rio Grande do Norte state was of 1:4,060 with a heterozygote frequency of about 1/64. While the frequency of consanguineous marriages is about 1% in the State of São Paulo, the lowest in Southeastern Brazil, rates as high as 6 to 10% have been registered in rural areas of Northeastern Brazil^{8,9}; in most surveyed villages here reported the inbreeding rates were far more higher, ranging from 9% to 32.5% (see Supplemental Table 1). Burden imposed by disability related to genetic disorders in this impoverished region, with an average HDI of 0.629, is not negligible.

These results confirm our previous report regarding SPOAN clinical phenotype, with some minor variations. Six patients (10%) never walked without support. Onset of motor symptoms might be even more delayed than the reported in our first publication³, and for six patients (10%), it ranged from 6 to 8 years of age. The severity of spastic paraplegia and neuropathy increases with age; motor deficiency was always more severe in lower than in upper limbs, and spasticity might be masked by neuropathy progression. As a rule, Achillean reflexes were abolished and plantar responses were either extensor or absent. An apparently non-progressive optic atrophy was usually suspected at infancy, and was confirmed in all individuals, except in three older patients with cataracts but with previous history of poor vision. Fixation nystagmus, commonly seen in congenital visual deficiency, was present in 46 patients and absent in 14. Startle to sudden noise was reported in 49 subjects. Distal amyotrophy and spine deformities as kyphosis and scoliosis, seen in 61.7 % of patients, and joint retraction, present in 83%, were very common manifestations, probably secondary to motor and sensory imbalance. Progressive dysarthria was commonly seen after 20 years of age and might have impaired communication ability, especially after the fifth decade of life. Extrapyrmidal signs as tremor, parkinsonism and dystonia were infrequent, occurring in 8.3% of individuals with SPOAN.

Interestingly, among the 68 ascertained affected individuals here reported, 45 are females and 23 are males, that is, the sex ratio among affected individuals is close to 2f:1m. These proportions differ grossly from the usual ratio 1:1 expected in autosomal recessive diseases ($\chi^2 = 242/34 = 7.11$; d.f.=1; $P < 0.01$). We found no clear explanation for this finding, since the disease seems to exhibit the same expressivity

pattern among individuals from both sexes and no obvious ascertainment biases favoring the inclusion of female patients appear to exist, since family history as well as pedigree construction were checked with many different family members.

The refined candidate region for SPOAN at 11q12 spans from markers rs1939212 to D11S987 and its size was now narrowed down from 4.8 to 2.3 Mb. Silver syndrome, or SPG17, an autosomal dominant condition characterized by lower limb spasticity associated with hand and feet weakness and amyotrophy, is caused by mutation in *BSCL2* gene, located at 11q13, which was right inside the previous SPOAN candidate area. The present linkage refining, allowed to exclude this gene from the SPOAN critical region where no other transcript has been associated with any form of spastic paraplegia, optic atrophy or peripheral neuropathy. Overall, 72 genes and *bona fide* transcripts have been assigned to the 2.3 Mb current critical region, three of which, *LRFN4*, *KLC2*, and *CCS*, have been sequenced; however, no pathogenic mutation was detected. Therefore, the gene responsible for SPOAN remains elusive.

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Table 1: Clinical Data Summary on 60 patients with SPOAN

Characteristic	Macedo-Souza, 2005 n=21	Present study n=39	Total n=60
Sex			
Male	7	12	19
Female	14	27	41
Age at ascertainment (y)			
<10	1	1	2
10 to <20	2	3	5
20 to <30	4	11	15
30 to <40	7	11	18
40 to < 50	5	6	11
> 50	2	7	9
Age of onset of motor symptoms (y)			
<1	17	5	23
1<2	1	13	14
2<6	0	11	11
6<8	0	6	6
Not known	3	4	4
Fixation nistagmus			
Absent	4	11	15
Present	17	28	45
Optic atrophy			
Present	21	36	57
Leukocoria	0	3	3
Joint retractions			
Present	4	0	4
Absent	17	39	56
Proximal reflexes			

Absent	3	1	4
Present	0	8	8
Brisk	18	30	48
Distal reflexes			
Absent	13	35	48
Reduced/Present	6	2	8
Brisk	2	2	4
Plantar response			
Flexor	0	1	1
Absent	19	29	48
Babinski sign	2	9	11
Distal wasting			
Absent (all < 20 years)	3	3	6
Present	18	36	54
Acoustic startle			
Absent	0	11	11
Present	21	28	49
Spine deformity			
Scoliosis and/or kyphosis	14	23	37
Absent	7	16	23
Tactile, vibratory and arthrestesic sense reduction			
Lower limbs only	6	0	6
Upper & lower limbs	14	32	46
Not known	1	7	8
Dysarthria			
Absent	7	13	20
Present	14	26	40
Extrapyramidal signs (dystonia, etc).			
Absent	16	37	53
Present	5	2	7

Supplementary data (Online)

Supplemental Table 1: Surveyed villages in Southwestern region of Rio Grande do Norte State, Brazil

Village (Human Development Index)	Position in Brazil's deficiency ranking (2000) (n = 5,507 villages)	Population (2007)*	Consanguineous marriages (%)	Patients with SPOAN (n)
São Miguel (HDI: 0.615)	8 th	22,579	19	23
Olho D'Água dos Borges (HDI: 0.631)	42 nd	4,442	9	0
Serrinha dos Pintos (HDI: 0.637)	38 th	4,360	32.5	18
Riacho de Santana (HDI: 0.621)	28 th	4,292	19	0
Pilões (HDI: 0.643)	2 nd	3,321	12	0
Total		39,054		41

* Source: <http://www.ibge.gov.br/home/estatistica/populacao/contagem2007/>

Supplemental Figure 1. Location of surveyed villages (Serrinha dos Pintos, São Miguel, Olho D'Água do Borges, Pilões e Riacho de Santana) in Northwestern region of Rio Grande do Norte State.

Capítulo 4

SPG34

Neurogenet *on line* em 08/05/2008

Abstract

Hereditary spastic paraplegias (HSP) are a heterogeneous group of genetic neurodegenerative disorders characterized by progressive spasticity of lower limbs; 32 loci and 11 genes for HSP have been so far identified, three of them (SPG1, SPG2, and SPG16) X-linked. Both pure and complicated forms of HSP have been described. We have identified a fourth loci for a pure form X-linked HSP based on linkage analysis of a large Brazilian multigenerational family. Twenty-four individuals, 12 of which men with pure spastic paraplegia, were included in this linkage analysis and allowed us to map the putative gene at Xq25, within a 14 cM interval with a maximum lod score of 4.13. This new locus for HSP, named SPG34, is located in a chromosomal region where no HSP have been previously mapped and defines the fourth X-linked form of HSP. The results of this study were published in a manuscript in the **Neurogenet** on line on may.08.2008

Resumo

As paraplegias espásticas familiares são um grupo heterogêneo de doenças de herança dominante ou recessiva. Já foram identificados 32 locos até o momento (Fink, 2008), três dos quais ligados ao cromossomo X: SPG2 (gene *PLP1* em Xq22.2), SPG1 (gene *L1CAM* em Xq28) e SPG16 (gene não identificado, Xq11.2-q23). Zatz e cols (1976) descreveram uma extensa família com 24 homens afetados por um tipo de paraplegia espástica pura, com padrão de herança recessiva ligada ao cromossomo X. Em 2002, Starling e cols, estudando um ramo dessa família encontraram ligação com a região Xq22.2, mas não identificaram alteração patogênica ao seqüenciar o gene *PLP1*. No

presente trabalho, investigamos 12 indivíduos afetados e 12 não afetados de diferentes ramos dessa mesma família, procedente da região de São José do Rio Preto, SP. Dentre estes, sete, com idade entre 30 e 60 anos, foram clinicamente avaliados. A idade de início foi a partir da terceira década de vida e a doença mostrou comportamento muito uniforme em todos os afetados, sendo a paraplegia espástica o único sintoma. A varredura inicial do cromossomo X com marcadores moleculares do tipo microssatélites em 24 indivíduos permitiu a exclusão dos locos conhecidos e o mapeamento na região Xq24-q26.3, para a qual não existe nenhuma forma de paraplegia espástica descrita até o momento. Para o marcador DXS8057 localizado em Xq25 foi obtido um *lod score* máximo de 4.13 para fração de recombinação igual a zero. O estudo de marcadores moleculares nessa região cromossômica permitiu delimitar uma região candidata entre os marcadores DXS1001 e DXS8033 de cerca de 14Mb. Os resultados deste estudo estão publicados no **Neurogenet** *on line* em 08/05/2008

Reevaluation of a large family defines a new *locus* for X-linked recessive pure spastic paraplegia (SPG34) on chromosome Xq25

Lúcia Inês Macedo-Souza · Fernando Kok ·
Silvana Santos · Luciana Licinio · Karina Lezirovitz ·
Rafaella M. P. Nascimento · Clarissa Bueno ·
Marcília Martyn · Emília K. E. A. Leão · Mayana Zatz

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Herein, we report a new *locus*, named SPG34, for a pure form of X-linked hereditary spastic paraplegia (HSP) in a large Brazilian family followed by our group since 1976 [1]. In 2002, a study of seven patients suggested linkage to Xq22.2 [2]. We now were able to perform a more comprehensive clinical and molecular evaluation, including five patients not previously ascertained, and reassigned the putative locus to Xq25.

After Institutional Review Board approval, we genotyped 12 affected individuals (aged 24 to 79 years), one unaffected 60-year-old man, and 11 women (aged 40 to 81), seven of which were obligate carriers (Fig. 1). Neurological examination was performed in 11 of the 12 affected men and in all obligate women carriers. Age of onset varied from 12 to 25 years but was sometimes difficult to be determined. The clinical phenotype was stereotyped, and shuffling gait was

the first recognized clinical sign. The disease was invariably progressive, and after two decades of onset, patients usually need support to walk; after three to four decades, they are usually wheelchair-bound. In upper limbs, strength was never affected, even late in life, but tendon reflexes were brisk. Lower limbs spasticity was progressive and debilitating. Babinski sign, ankle clonus, and brisk reflexes were frequently present. Lower limb vibratory sensibility was commonly reduced after the sixth decade of life; spontaneous lower limb pain was also a common complaint. No urinary or bowel sphincter dysfunction were ever reported.

The SPG34 *locus* encompasses a 14 cM region at Xq24–q25, in which 69 genes and *bona fide* transcripts have been assigned. Using a candidate gene approach to try to identify the causative sequence abnormality of SPG34, we fully sequenced *AIFM1*, which encodes a mitochondrial flavo-protein essential for apoptotic nuclear disassembly [3], but no mutation was detected. Therefore, the molecular basis for SPG34 remains unknown and we will sequence additional candidate genes that are highly expressed in the central nervous system.

The calculated logarithm of the odds score of 4.13 at marker DXS8057 was much higher than in the previous study [2], and additional markers definitely excluded Xq22.2. No other X-linked HSP have been so far assigned to this region, which defines the fourth *locus* for X-linked HSP. Differently from the other two well-characterized X-linked HSP, SPG1 and SPG2 [4, 5], SPG34 is not associated to mental retardation. Evidences for the SPG16 [6] *locus*, located in a large region at Xq11.2–q23 overlapping SPG2 *locus*, are weaker and based only in two small families.

In short, we identified in a large multigenerational family with pure spastic paraplegia a new *locus* (named SPG34) at Xq25 for an X-linked pure form of HSP, with onset in the

Lúcia Inês Macedo-Souza and Fernando Kok contributed equally to this study.

L. I. Macedo-Souza · F. Kok · S. Santos · L. Licinio ·
K. Lezirovitz · R. M. P. Nascimento · M. Zatz
Center for Studies of the Human Genome,
University of São Paulo Institute of Biosciences,
São Paulo, São Paulo, Brazil

F. Kok · C. Bueno · M. Martyn · E. K. E. A. Leão
Child Neurology Service,
University of São Paulo School of Medicine,
São Paulo, São Paulo, Brazil

F. Kok (✉)
Department of Neurology,
University of São Paulo School of Medicine,
Av. Dr. Enéas de Carvalho Aguiar,
255/5131 São Paulo, São Paulo, Brazil
e-mail: fernando.kok@fleury.com.br

Fig. 1 SPG 34 family pedigree. Haplotypes for markers from the SPG 34 critical region are given, and haplotypes segregating with the disease are boxed. The arrow indicates the proband. The individuals with a little bar over their symbols were clinically evaluated

second or third decade of life and a slowly progressive course. This is the fourth *locus* for X-linked recessive HSP recognized so far.

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Capítulo 5

DISCUSSÃO GERAL E CONCLUSÕES

Entender os mecanismos patológicos responsáveis pelas doenças neurodegenerativas é de grande interesse científico. O estudo de famílias brasileiras com múltiplos afetados tem sido de extrema importância para o mapeamento de novos locos gênicos, a identificação de mutações associadas aos quadros clínicos e a melhor compreensão do genoma humano funcional. A oportunidade de se associar investigação genética sistemática a criterioso estudo clínico permite a descrição de novas síndromes geneticamente determinadas. Além disso, estudos como este podem contribuir para a melhor compreensão dos mecanismos biológicos envolvidos na manifestação dessas doenças, abrindo perspectivas para seu tratamento.

Neste contexto, contribuímos com a descrição de uma nova condição genética, a síndrome Spoan, que afeta 68 indivíduos de uma mesma família, na região de Serrinha dos Pintos, 38ª comunidade no ranking de deficientes físicos do Brasil (IBGE, censo 2000). Nenhuma outra doença apresenta a mesma cronologia de aparecimento do conjunto de sinais e sintomas, a saber: paraplegia espástica hereditária, neuropatia axonal, disartria, sobressaltos à estimulação sonora, e atrofia óptica congênita.

A identificação do gene responsável pela síndrome Spoan permitirá a melhor compreensão sobre a fisiopatologia da atrofia óptica hereditária, das paraplegias espásticas e das neuropatias axonais. Em nossa primeira publicação, com a descrição clínica e genética da síndrome Spoan, localizamos a região candidata em 11q12-13, flanqueada pelos marcadores D11S1908 e D11S1889, com aproximadamente 4.8Mb. Nenhuma neuropatia periférica hereditária ou atrofia óptica eram conhecidas neste intervalo, apenas uma HSP complicada, a SPG17 (Patel e cols., 2002), conhecida como Silver syndrome, hoje atribuída a uma mutação no gene *BSCL2*. Posteriormente, a região candidata foi reduzida para 2.3Mb com o uso de 63 marcadores moleculares, a exclusão de 23 genes candidatos e a identificação de mais indivíduos afetados.

Nessa região, existem 72 transcritos, cerca de 40 destes com significativa expressão no sistema nervoso.

No estudo da SPG34, contribuímos com a descrição de um novo loco para uma forma de HSP ligada ao cromossomo X. Todos os 11 pacientes avaliados apresentavam paraplegia espástica pura com início na segunda ou terceira década de vida, e com ligeira perda de sensibilidade vibratória distal dos membros inferiores. A doença é compatível com a vida e não afeta a capacidade reprodutora. Sendo assim, como esperado para uma condição recessiva ligada ao cromossomo X, portadoras obrigatórias são assintomáticas e transmitem a mutação ao longo das gerações.

O loco da SPG34 de 14Mb corresponde à região Xq24-q25 e está flanqueada pelos marcadores DXS1001 e DXS8033. Esta é a quarta SPG reconhecida ligada ao cromossomo X e, nesta região, não existem outras formas sobrepostas, como acontece com a SPG16. Diferentemente de outras paraparesias espásticas ligadas ao cromossomo X, SPG1 e SPG2, SPG34 não está associada a retardo mental. Neste estudo, em que foram incluídos maior número de membros da família em relação ao estudo anterior (Starling e cols., 2002), o *lod score* obtido foi de 4,13 para o marcador DXS8057. Isso nos permitiu excluir a região anteriormente mapeada, em que tinha sido estimado um *lod score* de 2,41 para DXS1210. Na região candidata, existem 69 genes, entre os quais *AIFM1*, que codifica uma flavoproteína localizada no espaço entre as membranas interna e externa da mitocôndria, sendo essencial para apoptose nuclear (Krantic e cols., 2007). Esse gene foi seqüenciado, mas nenhuma mutação foi detectada. Portanto, a base molecular para SPG34 continua em investigação. A identificação do gene responsável pela SPG34 poderá ajudar no aconselhamento genético dos indivíduos pertencentes a esta família em risco e na investigação de outras famílias com SPG ligada ao cromossomo X.

Resumo

Estudamos duas grandes famílias com manifestações de doenças neurodegenerativas. Uma delas é originária do alto oeste do estado do Rio Grande do Norte e a outra, da região de São José do Rio Preto, SP. A primeira, uma extensa família com tradição de casamentos consangüíneos, apresenta 68 indivíduos afetados pela síndrome a qual nomeamos Spoan (*Spastic Paraplegia, Optic Atrophy, Neuropathy*). A mesma é uma doença neurodegenerativa de herança autossômica recessiva, caracterizada por atrofia óptica congênita, espasticidade, polineuropatia periférica axonal sensitivo-motora, sobressaltos à estimulação sonora, deformidades articulares e da coluna e disartria. Estes resultados foram publicados em 2005 no **Ann Neurol.** **57(5):**730-7. Dando continuidade ao estudo, selecionamos 23 genes que tiveram todos os exons seqüenciados. Nenhuma mutação foi observada. Amostras de 65 afetados e seus parentes foram estudados para seis marcadores de microsatélite, totalizando 149 indivíduos genotipados. Cinquenta SNPs foram investigados, o que nos permitiu reduzir a região candidata de 4.8 para 2.3Mb em 11q13, entre o SNP rs1939212 e o microsatélite D11S987. Para o marcador D11S1889, com alelos em homozigose para todo os pacientes, foi obtido um *lod score* máximo de 27 em $\theta=0.0$. Os resultados deste estudo se encontram em fase de submissão. A segunda família foi estudada pela equipe da Dra. Mayana Zatz há alguns anos. Nela, investigamos 12 indivíduos afetados e 12 normais. Dentre estes, sete, com idades entre 30 e 60 anos, foram clinicamente avaliados. A idade de início foi a partir da terceira década de vida, sendo a paraplegia espástica o único sintoma. Para o marcador DXS8057 localizado em Xq25 foi obtido um *lod score* máximo de 4.13 em $\theta=0.0$. Com o estudo de marcadores moleculares, delimitamos uma região candidata entre os marcadores DXS1001 e DXS8033, de cerca de 14Mb, e demonstramos a existência de um novo loco gênico no cromossomo X, por nós denominado SPG34. Os resultados deste estudo estão publicados no **Neurogenet on line** em 08/05/2008.

Abstract

We studied two large families with expressions of neurodegenerative diseases. One is from the high west of the state of Rio Grande do Norte and the other from São José do Rio Preto region, in São Paulo. The first, an extended family with a tradition of consanguineous marriages, has 68 individuals affected by the syndrome named by us Spoan (Spastic Paraplegia, Optic Atrophy, Neuropathy). The Spoan syndrome is a neurodegenerative disease, autosomal recessive, characterized by congenital optic atrophy, spasticity, axonal polyneuropathy peripheral sensory-motor, shocks to the sound stimuli, joint and spine deformities, and dysarthria. These results were published in 2005 in *Ann Neurol.* 57 (5):730-7. Latter we analyzed 23 genes that were entirely sequenced. No mutation was observed. Samples of 65 affected and their relatives were studied for six microsatellite markers, totaling 149 individuals genotyped. Fifty single nucleotide polymorphisms (SNPs), located in the critical region, were also investigated, which allowed us to reduce the region for the SPOAN gene from 4.8 to 2.3 Mb, between the SNP rs1939212 and microsatellite D11S987 in 11q13. All patients are homozygous only at D11S1889, which two-point lod score with a Z_{max} of 27 at $\theta=0.0$ was obtained. The results of this study are being submitted. The second family was studied by Dr. Mayana Zatz group a few years ago. We investigated 12 affected and 12 normal relatives. Among these, seven patients, aged between 30 and 60 years, were clinically evaluated. The age of onset was from the third decade of life and disease showed behaviour very uniform, all affected showed spastic paraplegia as the only symptom. For the marker DXS8057, in Xq25, was obtained a maximum lod score of 4.13 at $\theta=0.0$. The candidate region was mapped between the markers DXS1001 and DXS8033, about 14Mb and demonstrate the existence of a new gene locus on chromosome X, named by us SPG34. The results of this study were published in **Neurogenet** on line on may.08.2008.

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Anexo1

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Mutation in the Scyl1 gene encoding amino-terminal kinase-like protein causes a recessive form of spinocerebellar neurodegeneration.

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Mutation in the *Scyl1* gene encoding amino-terminal kinase-like protein causes a recessive form of spinocerebellar neurodegeneration

Wolfgang M. Schmidt^{1,2}, Cornelia Kraus³, Harald Höger⁴, Sonja Hochmeister⁵, Felicitas Oberndorfer¹, Manuela Branka¹, Sonja Bingemann¹, Hans Lassmann⁵, Markus Müller², Lúcia Inês Macedo-Souza⁶, Mariz Vainzof⁶, Mayana Zatz⁶, André Reis³ & Reginald E. Bittner¹⁺

¹Neuromuscular Research Department, Center of Anatomy & Cell Biology, Medical University of Vienna, Vienna, Austria,

²Department of Clinical Pharmacology, Section of Cardiovascular Medicine, Medical University of Vienna, Vienna, Austria, ³Institute of Human Genetics, Friedrich-Alexander-University Erlangen-Nuremberg, Erlangen, Germany, ⁴Division for Laboratory Animal Science and Genetics, Medical University of Vienna, Himmelpfortgasse, Austria, ⁵Center for Brain Research, Division of Neuroimmunology, Medical University of Vienna, Vienna, Austria, and ⁶Department of Biology, Institute of Biological Sciences and Center for Study of Human Genome, University of São Paulo, São Paulo, Brazil

Here, we show that the murine neurodegenerative disease *mdf* (autosomal recessive mouse mutant 'muscle deficient') is caused by a loss-of-function mutation in *Scyl1*, disrupting the expression of N-terminal kinase-like protein, an evolutionarily conserved putative component of the nucleocytoplasmic transport machinery. *Scyl1* is prominently expressed in neurons, and enriched at central nervous system synapses and neuromuscular junctions. We show that the pathology of *mdf* comprises cerebellar atrophy, Purkinje cell loss and optic nerve atrophy, and therefore defines a new animal model for neurodegenerative diseases with cerebellar involvement in humans.

Keywords: muscle deficient (*mdf*); motor neuron disease Purkinje cells; SCY1-like 1 (*S. cerevisiae*); spinocerebellar ataxia

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INTRODUCTION

So far, several molecular mechanisms associated with specific neurodegenerative disorders have been identified; however, a vast majority of diseases characterized by neuronal degeneration and the resultant disabilities such as muscular atrophy, motor dysfunction and paralysis are still poorly understood. Most of these diseases are fatal and no therapeutic strategies are available. Therefore, identification of new molecules involved in cellular pathways, which are indispensable for neuronal and neuromuscular functional integrity and that could be targeted therapeutically is crucial.

To this end, we investigated the molecular defect underlying the autosomal recessive mouse mutant 'muscle deficient' (*mdf*; Womack *et al*, 1980; Sweet, 1983). Although several clinical and pathological changes such as progressive neuromuscular atrophy and hindlimb paralysis are compatible with motor neuron disease (Blot *et al*, 1995), the *mdf* mouse also shows phenotypes indicating cerebellar involvement such as gait ataxia, abnormal hindlimb posture and tremor (supplementary Fig 1 and video 1 online). Therefore, we proposed that *mdf* can be linked to both motor neuron disease and spinocerebellar ataxia (SCA), thus representing an important model for recessively inherited neurodegenerative disorders in humans.

RESULTS AND DISCUSSION

By using intercross breeding strategies, we confined the *mdf* locus that had been previously mapped to mouse chromosome 19 A (Sweet, 1983; Poirier *et al*, 1998). Among the 48 candidate genes within the approximately 0.9 Mb *mdf* region, we identified *Scyl1* (RefSeq DNA NM_023912.1) as the disease causing gene after we detected an inserted thymidine in exon 8 (Fig 1A). This mutation

¹Neuromuscular Research Department, Center of Anatomy & Cell Biology, Medical University of Vienna, Währinger Strasse 13, A-1090 Vienna, Austria

²Department of Clinical Pharmacology, Section of Cardiovascular Medicine, Medical University of Vienna, Währinger Gürtel 18-20, A-1090 Vienna, Austria

³Institute of Human Genetics, Friedrich-Alexander-University Erlangen-Nuremberg, Schwabachanlage 10, D-91054 Erlangen, Germany

⁴Division for Laboratory Animal Science and Genetics, Medical University of Vienna, Brauhausgasse 34, A-2325 Himmelpfort, Austria

⁵Center for Brain Research, Division of Neuroimmunology, Medical University of Vienna, Spitalgasse 4, A-1090 Vienna, Austria

⁶Department of Biology, Institute of Biological Sciences and Center for Study of Human Genome, University of São Paulo, Rua do Matão, 277 Cidade Universitária, São Paulo, Brazil

⁺Corresponding author. Tel: +43 664 80016 37514; Fax: +43 1 4277 61198; E-mail: reginald.bittner@meduniwien.ac.at

Fig 1 | A single thymidine-nucleotide insertion causes disruption of *Scyl1* expression in the *mdf*-mouse. (A) Mapping of sequence tagged sites located *mdf* to an approximately 0.9 Mb chromosomal region between D19Mit59 and D19Mit109. Location of *Scyl1* is indicated and the exon-intron structure depicted (the exons are numbered). The *mdf* mutation is indicated by an arrow (inset: representative sequence traces showing the nucleotide insertion at position c.1169_1170insT). (B) Schematic 'in silico' annotation of *Scyl1* protein domains (numbers indicate amino-acid (aa) residues): serine-threonine-tyrosine protein kinase, HEAT repeats, secretory carrier membrane protein domain (KOG3088). Positions of peptides for raising antibodies (Ab) are shown. The premature stop codon created by the *mdf* mutation is indicated (arrowhead). (C) Quantitative reverse transcription-PCR of total RNA isolated from skeletal muscle (Skm) or brain (Br) showing a marked reduction of *Scyl1* messenger RNA in *Scyl1^{mdf/mdf}*. Error bars indicate standard deviation ($n=4$). (D) Western blot loaded with extracts from brain (Br), cerebellum (Cb), spinal cord (Sc) and skeletal muscle (Skm) of wild-type *Scyl1^{+/+}* and *Scyl1^{mdf/mdf}* mice probed with polyclonal antibody raised against the amino terminus of *Scyl1*. In wild-type tissues, the antibody detected an approximately 105 kDa band (arrowhead) that was completely absent in corresponding tissues from *Scyl1^{mdf/mdf}* mice. A second, minor reactive band of approximately 70 kDa is also indicated. MW, molecular weight standard (~70, ~100, ~130, ~150 kDa). (E) Detection of *Scyl1*-mRNA by *in situ* hybridization (ISH) in a wild-type mouse brain section showing high expression in the granular and Purkinje cell layers of the cerebellum (gcl), the hippocampus (hc) and the gyrus dentatus (gd). Right panel is a magnified view showing abundant *Scyl1* expression in the synaptic region of the cerebellar granular layer. Left panel: control experiments showing specificity of the antisense ISH probe as opposed to the sense control probe.

fully segregated with the disease phenotype on different genetic backgrounds (supplementary video 2 online) and all parental breeders, as well as approximately 50% of their offspring, were

heterozygous carriers. We also found the mutation in an *mdf/mdf* DNA sample obtained directly from the Jackson Laboratories (Bar Harbor, ME, USA), where the mutant originally arose (Womack

et al, 1980). We suggest renaming this spontaneous mouse mutant *Scyl1^{mdf}*. As the insertion within *Scyl1* exon 8 (c.1169_1170insT) causes a frame shift and creates a premature stop codon (p.H392PfsX30), we wanted to confirm the predictably deleterious consequences for messenger RNA and protein expression, respectively. By using quantitative mRNA assays, we found that *Scyl1* transcripts were markedly reduced in nervous tissues and to a lesser extent in skeletal muscle from *Scyl1^{mdf/mdf}* mice, which is indicative of nonsense-mediated mRNA decay (Fig 1C).

Scyl1 is highly conserved among eukaryotes and contains an amino-terminal serine–threonine kinase-like domain (Liu *et al*, 2000; Kato *et al*, 2002). Further domains, detected by *in silico* annotation, comprise four HEAT repeats and a secretory carrier membrane protein domain (Fig 1B), indicating that *Scyl1* interacts with other proteins and is involved in intracellular transport processes. To investigate *Scyl1* expression and distribution, we raised antibodies against N- and carboxy-terminal peptides. On western blots, both antibodies detected a major immunoreactive band with a molecular weight of approximately 105 kDa, which is in accordance with previously reported migration properties (Liu *et al*, 2000), in all probed murine and human tissue extracts. By contrast, the antibodies failed to detect a corresponding band in all tissue extracts from mutant *Scyl1^{mdf/mdf}* mice, indicating that the mutation causes a complete loss of full-length *Scyl1* protein (supplementary Fig 2A online). No lower-molecular-weight bands were detected by the antibody against the N terminus in *Scyl1^{mdf/mdf}* tissues, indicating the absence of truncated protein products (Fig 1D). When we probed brain sections using *in situ* hybridization, we found prominent *Scyl1* expression confined to the neuronal perikarya—most pronounced in the Purkinje cells in the cerebella of normal mice (Fig 1E)—which is fully compatible with expression patterns shown in the Allen Brain Atlas (Lein *et al*, 2007). *Scyl1*-specific antibodies intensely labelled all central nervous system (CNS) neurons in sections from wild-type mice and humans, whereas no such staining was observed in mutant *Scyl1^{mdf/mdf}* CNS tissues (Fig 2A,B). We found prominent *Scyl1* expression in all CNS neurons, including cortical neurons (Fig 2C), brain-stem neurons (Fig 2D) and anterior horn spinal cord motor neurons (Fig 2E).

In addition to *Scyl1* expression throughout CNS neurons and neuronal dendrites (Fig 2C–F), the protein was also detected in the axons of peripheral nerves (Fig 2H), indicating axonal transport of *Scyl1*. In agreement with this hypothesis, we detected *Scyl1* accumulation in axonal spheroids (Fig 2G): these age-related, neuropathological structures represent sites where axonally transported proteins usually accumulate. Furthermore, we found *Scyl1* to be enriched at synapses in the CNS and in the periphery at the neuromuscular junction (Fig 2I), indicating further that *Scyl1* is subjected to axonal transport.

As we detected prominent *Scyl1* expression in Purkinje cells of the cerebella of wild-type mice and humans (Fig 3A,B), we investigated this cell type more closely in *Scyl1*-deficient mice and found that many Purkinje cells showed various pathological changes indicative of neuronal degeneration in *mdf* cerebella (Fig 3C). Our observation of basket formations devoid of Purkinje cells ('empty baskets'; inset in Fig 3C) indicates that pre-existing Purkinje cells have disappeared in *Scyl1*-deficient cerebella. Consistent with this observation, we found that the overall

number of Purkinje cells in all areas of *Scyl1^{mdf/mdf}* cerebella was markedly reduced (Fig 3D). As this reduction in Purkinje cells was already pronounced in young mutants (45 days), we propose that loss of Purkinje cells represents an early, pre-clinical pathology in *Scyl1*-deficient mice. Purkinje cell-related pathologies ranging from loss of Purkinje cells to multiple degenerative abnormalities became most clearly visible with Purkinje-cell-specific Calbindin staining (Fig 3E,F). Dendritic arborization of Purkinje cells was found to be markedly reduced—characterized by paucity of ramification and axonal swellings (inset in Fig 3F)—which is compatible with ongoing neurodegeneration. Whole median sagittal cross-sections showed a marked atrophy of the cerebellar vermis in *mdf* mice (Fig 3G,H).

Our findings on the cerebellar neuropathology in *Scyl1*-deficient mice suggest that the loss of Purkinje cells in *mdf* is an early-onset and progressive degenerative condition associated with atrophy rather than a developmental defect in the formation of the Purkinje cell layer. We have therefore unraveled a series of clinical and pathological findings in *mdf*, which are characteristic of SCA in humans and corresponding mouse models. This prompted us to investigate the optic nerve of *mdf* animals more closely, because optic atrophy represents a pathology common to several types of SCAs. Indeed, we found that *Scyl1^{mdf/mdf}* optic nerve cross-sections from comparable retrobulbar regions had visibly smaller diameters when compared with wild-type samples (supplementary Fig 3A online). Morphometric analysis based on electron micrographs of ultrathin cross-sections from optic nerves showed a significantly increased proportion of small-diameter myelinated fibres and thinner myelin sheaths in 2-year-old *Scyl1^{mdf/mdf}* mice compared with wild-type animals, compatible with hypomyelination and axonal degeneration (supplementary Fig 3B online). Recently, an autosomal recessive disorder, characterized by spastic paraplegia, optic atrophy and neuropathy (SPOAN), has been mapped to human chromosome 11q13, a region encompassing the human *Scyl1* orthologue (Macedo-Souza *et al*, 2005). To test whether SPOAN represents the human *mdf* disease counterpart, we sequenced the entire *SCYL1* gene of two representative patients of two SPOAN families. However, we did not find any mutation, excluding SPOAN as the human disease counterpart.

In our study, *Scyl1* emerged as a new protein essential for neuronal and, particularly, Purkinje cell survival, the disruption of which causes an early-onset and progressive neurodegenerative disorder. Intriguingly, recent evidence from a protein–protein interactome for human inherited diseases characterized by degeneration of Purkinje cells, developed by Lim *et al* (2006), readily supports the relevance of *Scyl1* in the context of human neurodegenerative disorders: the human *SCYL1*-encoded protein was found to interact with the Coilin protein (COIL), which is also a binding partner for ataxin 1 (which causes SCA1), puratrophin 1 (implicated in degeneration of Purkinje cells) and Survival motor neuron protein (SMN; the loss of which causes spinal muscular atrophy). This established *SCYL1* as a member of the 'ataxia-ome'—the protein–protein interaction network for human ataxias and disorders of Purkinje cell degeneration (Humbert & Saudou, 2006; Lim *et al*, 2006). Together with our findings, this classifies *SCYL1* as a putative candidate gene for human neurodegenerative diseases involving the cerebellum.

As for many proteins associated with disease, the precise cellular function of *Scyl1* and its role in pathogenesis of neuronal

Fig 2 | Scyl1 protein is a neuronal protein expressed in the axoplasm and concentrated at synapses. (A,B) Immunodetection of Scyl1 on semicoronal brain sections from (A) wild type *Scyl1*^{+/+}, but a lack of reactivity in (B) *Scyl1*^{mdf/mdf}. Note the strong Scyl1-specific immunosignal of the neuropil (A, white stars) and of the choroid plexus (A, arrow) in wild type. The neuropil in mutant brain does not yield comparable immunosignals (B, white stars). ccl, corpus callosum. (C) Prominent Scyl1 staining of cortical neurons (arrows) and (D) brain-stem neurons (arrows) of wild-type mice. (E) Prominent Scyl1 reactivity of anterior horn motor neurons (arrows) in wild-type spinal cord. (F) Confocal immunofluorescence images showing intense Scyl1 staining in the cytoplasm (arrowheads) and axons (arrows) of murine cerebral cortex neurons (white star, nonreactive nucleus). (G) Section from aged human brain showing SCYL1-specific immunosignal accumulation within axonal spheroids (arrows). (H) Strong axoplasmic Scyl1-specific staining of an intramuscular nerve branch in wild-type skeletal muscle (skm; arrowheads in inset). Scyl1 reactivity of a neuromuscular junction (NMJ) is indicated (arrow). (I) Scyl1 distribution at a wild-type NMJ: pronounced labelling of presynaptic terminal axons (arrows) and weaker reactivity of the post-synaptic sarcoplasm (arrowhead). α -Bungarotoxin (BgTx, arrowhead) was used as a postsynaptic marker. Confocal overlay of Scyl1 immunosignals (green) with BgTx-specific red fluorescence shows predominant presynaptic Scyl1 expression, which was not detectable at NMJs in *Scyl1*^{mdf/mdf} mice (lower panel). All immunostainings shown were performed using the antibody raised against the Scyl1 C terminus.

degeneration remains unclear. However, a very recently published study on the yeast orthologue Cex1p (Yor112wp), suggests a possible function in intracellular transport: Cex1p is a component of the nuclear aminoacylation-dependent

transfer RNA export machinery and collects cargo from export receptors at the cytoplasmic side of the nuclear pore complex (McGuire & Mangroo, 2007). The idea of an evolutionarily conserved role of Scyl1 in nucleocytoplasmic transport

Fig 3 | Purkinje cell degeneration and cerebellar atrophy in *mdf*. (A) Immunodetection of SCYL1 in Purkinje cell (PC) somata (arrows) in human cerebellum with fine granular immunosignal accumulation in the synaptic region of the granular layer (white stars). (B) Scyl1-immunolocalization (carboxy-terminal antibody) in wild-type (*Scyl1^{+/+}*) mouse (255 days) cerebellum showing strong labelling within the PC layer. Arrows indicate the periodic continuity of PCs in wild-type cerebellum. (C) By contrast, PCs (arrows) in *Scyl1^{mdf/mdf}*-cerebellum (age-matched) were frequently abnormally shaped and displayed basophilic, condensed cytoplasm (H&E-staining). Arrowheads indicate discontinuity of the PC layer owing to the loss of single cells. Inset: two remaining PCs neighboured by persisting basket formations devoid of PCs (arrowheads), as shown by Bielschowsky staining. (D) Quantification of PC loss in *mdf*. PC counts in various areas of wild-type and *Scyl1^{mdf/mdf}* cerebella show an overall loss of 42% in young mice ($P = 5.638e-05$, Kruskal-Wallis rank sum test) and 48% in adult mice ($P = 1.539e-07$), indicating early-onset and slowly progressive PC loss. Notches indicate confidence intervals of medians. (E) Immunostaining of calbindin D-28k in the cerebella of 274-day-old wild-type and (F) *Scyl1^{mdf/mdf}* mice, showing pronounced loss of PCs (arrowheads). Insets: commensurate close-up views of representative PCs showing regular and complex dendritic arbors in wild-type-cerebella (inset in E), whereas multiple swellings (inset in F, arrowheads) and paucity of arborization are hallmarks in *mdf*. (G,H) H&E-stained median-sagittal sections of cerebella from (G) age-matched wild-type and (H) *Scyl1^{mdf/mdf}* mice showing atrophy of *mdf* cerebellum compared with wild-type. Scale bars, 1 mm. H&E, haematoxylin and eosin.

is supported further by reported interactions with the nuclear pore protein NUP98 and the nuclear export receptor exportin-7 (XPO7). Therefore, we propose that Scyl1 deficiency in neurons might cause degeneration owing to a defect in the nucleocytoplasmic transport machinery, a new pathway involved in neurodegeneration.

METHODS

Animals used in this study. We obtained *mdf* mice (B6C3Fe a/a-*mdf*/J) from the Jackson Laboratory (Bar Harbor, ME, USA), where *mdf* originally emerged as a spontaneous autosomal recessive mutant in a C57BL/6J-*ln fz* stock (Womack et al, 1980). Mice stocks were maintained at the Division of Laboratory Animal Science and Genetics (Medical University Vienna, Himberg, Lower Austria) following institutionally approved protocols for the humane treatment of animals. Animals were killed by cervical dislocation.

Genetic mapping and high-throughput sequencing. We generated an intercross by breeding B6C3Fe-*mdf*-mice with *SJL* mice. Fine mapping was carried out by PCR for markers D19Mit32, D19Mit68, D19Mit59, D19Mit109 and D19Mit126. Corresponding PCR fragments were generated from 123 *mdf/mdf* mice (correlating to 246 meioses) by standard PCR, using fluorescence-labelled primers. PCR products were separated in an ABI3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA) and subsequently analysed with the GenScan and Genotyper Software (Applied Biosystems).

Primers for all coding exons of the 48 candidate genes located in the critical interval were designed using Exon Locator and eXtractor (http://variation.swmed.edu/ex_lax/elxrdp_query.html), allowing amplification of all fragments under the same PCR conditions. PCR products were purified using 96-well filter plates (Millipore, Billerica, MA, USA) on a Miniprep robot (TECAN, Crailsheim, Germany). The fragments were sequenced using BigDye Version 3.1 (Applied Biosystems), according to the manufacturer's recommendations. Sequences were purified using the 96-well filter plates (Millipore), using the Miniprep robot and separated in an ABI3730 sequencer (Applied Biosystems). Sequence traces were then analysed using the SeqMan software (DNASTAR, Madison, WI, USA).

Scyl1 genotyping. After identification of the *Scyl1* mutation, the single-nucleotide insertion was genotyped by a PCR assay using allele-specific primers. After subjecting mouse tail-tip DNA to PCR amplification, the respective genotypes were deduced from banding patterns after electrophoretic separation of reaction products on polyacrylamide gels and visualization by fluorescence scanning (Typhoon; GE Healthcare, Chalfont St Giles, UK).

In situ hybridization. Cryosections (10 µm) of wild-type mouse brains were probed for Scyl1-specific probes. A 475 bp complementary DNA reverse transcription-PCR (RT-PCR) product was generated with primers 5'-AGCCAGCTGAGAAGCAGAAG-3', and 5'-ATAGGACCCTGGTCGTCCTT-3', and subsequently inserted into the pCR II TOPO Vector (Invitrogen, Carlsbad, CA, USA). Templates for antisense and sense probes were generated by digesting the plasmid with *Apal* and *KpnI*, respectively. RNA probes were generated using the RNA labelling Kit (Roche Diagnostics, Mannheim, Germany), in the presence of digoxigenin (DIG)-labelled UTP. Slides were fixed, dehydrated, hybridized overnight at 37 °C, thoroughly washed, dehydrated and stained with DIG antibody (Roche) and NBT-BCIP

(nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt).

Quantitative real-time PCR. Total RNA was extracted from muscle and brain tissue of *Scyl1*^{+/+} and *Scyl1*^{mdf/mdf} mice, respectively, and transcribed into cDNA by SuperScript II Reverse Transcriptase (Invitrogen) using random hexamer oligonucleotides. To quantify the amount of *Scyl1* mRNA, we used a real-time RT-PCR assay based on TaqMan technology, involving separate amplification of *Scyl1* and an internal reference, β-2-microglobulin (*B2m*), using commercial Taqman probes (Applied Biosystems; assays-on-demand: *Scyl1*-Mm00452459_m1; *B2m*-Mm00437762_m1). Quadruple measurement of each cDNA was calculated. The amount of *Scyl1* mRNA was calculated using the comparative C_t method (ΔΔC_t) as described previously (Thiel et al, 2003).

Generation of polyclonal antibodies. Two polyclonal antibodies were directed against the unique mouse amino-acid sequences MWFFARDPVRDFPFEL or KGPMKLGARKLD, present either at the N terminus (aa 1–16, Scyl1-N) or at the C terminus (aa 795–806; Scyl1-C) of Scyl1. Peptides were synthesized with terminal cysteine residues, conjugated to the carrier protein keyhole limpet haemocyanin and purified by a commercial laboratory (Gramsch Laboratories, Schwabhausen, Germany). Antibodies from rabbit antisera were purified by affinity chromatography using the corresponding peptides coupled to thiopropyl sepharose 6B (GE Healthcare). Antibody specificity was established by pre-adsorption of the antibodies with their cognate peptide antigens, which completely abolished immunoreactivity (supplementary Fig 2B,C online). Owing to high homology, both antibodies recognize human SCYL1 and are predicted to crossreact with the rat homologue of Scyl1.

SDS-polyacrylamide gel electrophoresis and western blots. Tissue extracts were prepared by disruption in lysis buffer (120 mM Tris (pH 6.8), 200 mM dithiothreitol, 4% SDS, 20% glycerol) and separated on 6–12% polyacrylamide Tris-glycine SDS gels, and electrotransferred onto polyvinylidene difluoride or nitrocellulose membranes (Hybond, GE Healthcare). Equal loading of total protein was ascertained by electrophoresis of extracts on separate gels stained with Coomassie blue and densitometric analysis using the ImageJ software package. Detection was carried out with AP-conjugated secondary antibodies (1:1,000) and NBT-BCIP. Scyl1 antibodies were used at 1:800 dilution (Scyl1-C) or 1:200 (Scyl1-N).

Histology and immunohistochemistry. After dissection, tissues were snap-frozen in dry ice-cooled 2-methylbutane. Tissue samples were stored at –80 °C and cut on a cryostat. Cryosections were stained with haematoxylin and eosin (H&E). For paraffin-embedded histology, tissues were fixed in 3.7% paraformaldehyde, embedded in paraffin and subsequently processed for respective histological analyses.

Purkinje cell counts were taken by two independent, blinded investigators on the basis of H&E-stained cerebellar sections from age-matched wild-type and *mdf*-mutant mice. To account for variant Purkinje cell densities in different folia, a series of representative images were obtained (*n* ≥ 25). Images were analysed using ImageJ and Purkinje cells were counted on segmented lines and adjusted to an arbitrary length unit. Statistical analysis using the Kruskal–Wallis rank sum test and boxplots were prepared with the statistical software package R (<http://www.r-project.org>).

Bielschowsky's silver impregnation method was carried out by treating formalin-fixed paraffin-embedded sections with silver nitrate, followed by reduction to visible metallic silver.

For immunohistochemistry, 10 µm cryosections were fixed using 3.7% paraformaldehyde (room temperature, 20 min), rinsed in PBS and subsequently incubated with polyclonal rabbit Scyl1-C-antibody (1:500) or goat Calbindin D-28K antibody (Sigma, St Louis, MI, USA). Secondary antibodies were conjugated to Alexa Fluor 488, Alexa Fluor 594 (Molecular Probes and Invitrogen, Eugene, OR, USA) or to horseradish peroxidase, respectively. Neuromuscular junctions were detected by α -Bungarotoxin conjugated with Alexa Fluor 594 (Molecular Probes). Immunostained sections were analysed using a confocal laser scanning microscope (Fluoview; Olympus, Tokyo).

Electron microscopy and morphometric analysis. Animals were transcardially perfused with 4% paraformaldehyde/1% cacodylate buffer and dissected brain tissue was fixed in cacodylate-buffered glutaraldehyde, embedded in epoxy resin and processed for transmission electron microscopy. Optic nerve images were analysed with ImageJ and myelin sheath thickness and g-ratios using outer and inner diameters were calculated from feret diameters.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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Anexo2

GENOTIPAGEM DOS 65 INDIVÍDUOS AFETADOS E SEUS 83 PARENTES NORMAIS

Em azul, o registro dos indivíduos afetados; em preto, seus parentes normais

Reg.	D11S4191	D11S4076	D11S1883	D11S1889	D11S987	D11S1314
19027	17/6	7/10	22/22	16/16	20/20	14/11
19028	6/17	10/10	20/22	16/16	18/20	13/14
19331	17/17	7/10	20/22	16/16	15/18	6/13
19332	6/17	7/10	20/22	16/16	20/18	11/13
19333	6/6	7/7	22/22	16/16	15/20	6/11
19386	17/6	7/10	22/22	16/16	20/20	11/11
19387	6/6	7/10	21/22	6/16	20/20	9/11
19388	6/17	10/11	20/22	8/16	14/20	6/11
19389	17/17	10/10	22/22	16/16	20/20	11/11
19390	17/17	7/10	22/23	6/16	20/20	11/11
19391	17/14	10/11	20/22	16/16	20/20	11/6
19392	14/17	10/11	20/22	16/16	20/20	6/11
19393	17/17	10/10	22/22	16/16	20/20	14/11
19394	17/17	10/10	22/22	16/16	20/20	14/11
19395	17/10	10/11	21/22	16/16	20/19	11/13
19396	17/10	10/11	21/22	15/16	20/19	14/13
19397	6/17	10/10	22/22	16/16	20/20	6/14
19398	10/17	10/12	20/22	16/17	22/20	6/14
19399	6/7	10/13	22/28	16/17	20/18	6/14
19400	17/17	10/10	22/22	16/16	20/20	6/6
19401	17/10	10/11	22/28	16/18	20/21	6/6
19402	17/6	10/10	22/23	14/16	20/18	6/6
19403	17/10	10/11	22/28	16/18	20/21	6/6
19404	7/7	10/10	22/22	16/16	20/20	14/14
19405	7/7	10/10	22/22	16/16	20/20	14/14
19406	7/14	10/11	20/22	16/16	20/20	14/6
19407	6/7	10/10	22/22	16/16	20/20	13/14
19408	7/14	10/11	20/22	16/16	20/20	14/6
19955	17/17	10/10	22/22	16/16	20/20	11/14
19956	8/17	7/10	22/23	6/16	20/20	6/11
19957	8/17	7/10	22/23	6/13	20/20	6/9
19958	14/17	10/11	22/22	13/16	20/20	9/14
19959	17/17	10/10	22/22	16/16	20/20	11/14
19960	8/17	7/10	20/23	6/16	18/20	11/11
19961	7/17	10/10	22/22	16/16	16/20	11/14
19962	9/17	7/10	20/22	16/17	18/16	11/6

19963	8/17	?	22/23	6/16	18/20	11/11
19964	17/17	10/10	22/22	16/16	15/20	11/11
19965	8/17	10/10	20/22	16/17	5/20	9/11
19966	8/8	10/10	20/22	16/17	15/5	6/9
19967	6/17	10/10	21/22	6/16	20/20	9/11
19968	6/6	10/11	20/21	6/8	14/20	6/9
19970	17/7	10/10	22/22	16/16	20/20	14/6
19971	17/7	10/10	22/22	16/16	20/20	14/6
19972	6/7	7/10	22/22	16/16	20/20	14/14
19973	6/7	7/10	22/22	16/16	20/20	14/6
19974	10/6	7/10	20/22	16/16	20/20	13/14
19975	7/10	10/10	20/22	16/16	20/20	14/13
19976	10/6	7/10	22/22	15/16	20/18	14/10
19977	10/10	7/10	22/22	15/16	18/20	10/14
19978	10/10	7/10	22/22	15/16	18/20	10/14
19979	7/17	10/10	22/22	16/16	20/20	14/14
19980	17/17	10/11	22/22	16/16	20/23	14/11
19981	7/14	10/10	22/23	16/16	20/19	14/14
19982	14/17	10/10	22/23	16/16	19/20	14/14
19983	6/17	?	?	16/16	20/20	6/11
19984	7/10	9/10	20/21	16/16	16/19	14/14
19985	7/17	9/10	20/22	16/16	16/20	14/14
19986	10/17	10/10	21/22	16/16	19/20	14/14
19987	17/17	10/10	22/22	16/16	20/20	14/14
19988	17/17	10/10	22/22	16/16	20/20	11/11
19989	7/17	10/12	22/22	9/16	19/20	11/11
19990	7/17	10/11	22/26	16/16	17/20	14/11
19991	6/17	10/10	22/22	16/16	20/20	13/14
19992	9/17	7/10	20/22	16/17	18/20	11/14
19993	9/6	9/10	20/22	16/16	16/20	6/13
19994	9/9	7/9	20/20	16/17	16/18	6/11
19995	9/9	7/9	20/20	16/17	16/18	6/11
19996	9/9	7/9	20/22	16/17	18/20	11/13
19997	17/17	10/10	22/22	16/16	20/20	11/14
19998	8/17	7/10	22/23	6/16	20/20	6/11
20018	6/17	10/11	20/22	16/16	20/16	6/14
20215	17/17	10/11	20/22	16/16	16/20	6/11
20216	10/17	10/10	21/22	16/16	19/20	11/14
20217	17/17	10/10	22/22	16/16	20/20	11/11
20218	17/17	10/10	22/22	16/16	20/20	11/11
20219	17/17	10/10	22/22	16/16	20/20	11/11
20656	14/14	10/10	22/22	16/16	20/20	15/15
20657	11/14	10/11	20/22	15/16	18/20	4/15
20658	11/14	10/11	21/22	6/16	17/20	15/2
20659	11/11	11/11	20/21	6/15	18/20	4/2
22334	10/9	?	20/22	15/16	18/16	10/6

22335	6/10	7/10	22/22	15/16	18/19	10/13
K037	17/17	10/10	22/22	16/16	20/20	11/14
K038	17/17	10/10	22/22	16/16	20/20	11/14
K039	8/17	10/10	22/22	16/16	17/20	9/11
K040	6/17	10/13	22/22	16/16	20/20	6/14
K041	14/14	10/10	22/22	16/16	20/20	12/14
K042	6/14	10/11	22/29	16/16	18/20	14/15
K043	6/17	10/10	22/22	16/16	20/20	14/14
K044	6/17	10/10	22/22	16/16	20/20	13/14
K045	15/17	7/10	22/26	16/16	18/20	13/14
K046	10/16	10/10	22/22	16/16	20/20	13/14
K047	10/16	10/10	22/22	16/16	20/20	13/14
K048	9/10	7/10	20/22	16/17	18/20	11/14
K049	17/17	10/10	22/22	16/16	20/20	11/14
K050	17/17	10/10	22/22	16/16	20/20	14/14
K051	10/17	10/10	22/22	16/16	20/20	14/14
K052	10/18	10/11	21/22	16/16	20/20	11/11
K053	7/18	7/11	20/22	16/16	20/20	11/14
K054	7/10	7/10	20/21	16/17	18/20	11/14
K055	9/14	7/10	20/22	16/16	20/20	14/14
K056	9/17	?	20/22	16/17	18/20	11/14
K076	8/14	7/10	22/25	16/16	20/21	6/14
K077	6/8	7/10	20/22	16/16	15/20	6/13
K078	6/6	4/7	?	16/16	15/16	6/6
K079	9/9	7/9	20/20	16/17	16/18	6/6
K080	9/14	7/10	20/23	16/17	18/19	6/11
K081	6/9	4/7	20/22	16/17	16/18	6/11
K082	6/18	9/11	20/21	16/16	16/19	6/13
K083	6/17	10/10	20/22	15/16	18/19	8/13
K084	6/18	10/11	20/20	15/16	16/18	8/13
K091	9/9	9/9	20/20	16/16	6/20	6/14
K092	9/18	9/11	20/20	13/16	19/20	13/14
K093	8/15	10/10	20/20	16/17	2/18	8/14
K094	17/17	10/10	22/22	16/16	20/20	8/11
K100	7/16	10/10	22/22	16/16	20/20	14/14
K101	6/17	10/10	22/24	16/16	20/20	10/14
K102	6/17	10/10	22/24	16/16	20/20	11/14
K103	6/6	10/11	23/24	8/16	18/20	6/14
K104	6/16	10/11	23/23	16/16	20/20	13/13
K105	6/16	10/11	23/23	16/16	20/20	13/13
K106	9/16	7/11	20/23	16/17	18/20	13/13
K109	16/17	10/10	22/22	16/16	20/20	11/14
K110	?	10/10	22/22	16/16	20/20	11/14
K111	6/17	10/11	22/22	16/16	16/20	11/14
K112	7/9	7/10	20/22	8/17	18/19	11/13
K113	17/17	10/10	22/22	16/16	20/20	14/14

K114	17/17	10/10	22/22	16/16	20/20	14/14
K115	17/17	10/10	22/22	16/16	20/20	8/13
K116	6/17	10/10	22/22	16/16	20/20	8/13
K117	17/17	10/10	22/22	16/16	20/20	8/13
K118	7/17	10/11	22/22	14/15	17/19	9/13
K119	9/17	10/10	22/22	16/16	20/20	14/14
K120	9/17	10/10	?	16/16	20/20	14/14
K121	6/9	10/10	22/22	16/16	20/20	8/14
K123	17/17	10/10	22/22	16/16	20/20	11/14
K125	17/17	10/10	22/22	16/16	20/20	11/14
K126	8/14	7/10	22/25	16/16	20/21	6/14
K127	9/17	10/10	22/22	16/16	20/20	14/14
K128	17/17	10/10	22/22	16/16	20/20	14/14
K136	6/10	10/11	21/22	16/16	19/23	11/13
K154	9/14	?	?	16/16	16/19	6/6
K155	17/17	?	?	16/16	20/20	11/14
K156	10/10	?	?	?	?	10/14
K160	14/17	?	?	16/16	20/20	13/14
K161	14/17	?	?	16/16	20/20	13/14
K162	14/17	?	?	16/16	20/20	13/14

Anexo3

LISTA DOS MARCADORES MOLECULARES UTILIZADOS

Marcadores	pb
D11S4191	59.765.293
D11S1765	60.535.120
D11S4076	61.119.711
rs174548 (FADS1_G/C)	61.327.674
rs174549 (FADS1_A/G)	61.327.708
rs174550 (FADS1_C/T)	61.327.804
rs149698 (VMD2_A/G)	61.486.362
rs17854139 (VMD2_A/G)	61.486.482
rs1800008 (VMD2_C/T)	61.486.509
rs1800009 (VMD2_C/T)	61.486.560
rs1800010 (VMD2_A/T)	61.486.685
rs12281503 (INCENP_exon3 A/G)	61.653.624
rs1675133 (INCENP_exon3 C/T)	61.653.685
rs34441559 (INCENP_exon3 C/T)	61.653.735
rs3410125 (INCENP_exon3 A/G)	61.654.044
rs4432028 (ASRGL1_A/G)	61.914.987
rs7104831 (ASRGL1_A/C)	61.915.169
rs2958531 (ASRGL1_G/T)	61.915.212
rs2958532 (ASRGL1_C/T)	61.915.250
rs1801144 (ROM1_C/G)	62.138.134
rs17850301 (ROM1_C/T)	62.138.141
D11S1908	62.184.668
rs722508 (SNP1_C/T)	62.792.962
rs513338 (SNP2_G/T)	62.823.714
rs510713 (SNP2B_A/C)	62.971.648
D11S480	62.823.672
D11S4205	62.939.549
D11S1883	63.130.309
rs12366035 (VEGF β _exon5 C/T)	63.761.268
rs553587 (SNP2C_A/G)	63.958.504
rs606063 (SNP3_C/T)	63.959.305
rs7932437 (SNP3B_C/T)	64.130.080
rs10501396 (SNP4_A/C)	64.642.021
rs507005 (SNP4B_C/T)	64.802.191
rs1784222 (SCYL1)	65.056.789
rs1151488 (SNP5_A/G)	65.128.180

rs603645 (SNP6_A/C)	65.289.576
rs1939212 (CFL1_exon1_A/G)	65.383.277
rs528736 (SNP7_A/G)	65.461.684
rs3782080 (ACTN3_intron16 C/T)	66.084.870
rs618838 (ACTN3_intron16 C/T)	66.085.045
rs2290463 (ACTN3_intron16 C/G)	66.085.178
rs607736 (ACTN3_intron16 C/T)	66.085.203
rs2229456 (ACTN3_intron16 A/C)	66.085.317
rs34153934 (PC_intron9 -/C)	66.390.762
rs7948839 (PC_intron9 C/T)	66.390.723
rs35550425 (FBXL11_intron5 -/A)	66.723.914
rs660960 (FBLX11_intron5 A/T)	66.723.923
rs11227732 (FBLX11_intron5 C/T)	66.724.047
rs28364247 (POLD4_exon4 A/T)	66.874.993
rs28364248 (POLD4_exon4 C/T)	66.874.927
rs2514258 (POLD4_exon4 A/G)	66.874.855
rs28364249 (POLD4_exon4 C/T)	66.874.851
rs1131725 (ALDH3B2_intron2 A/G)	67.191.411
rs3741179 (ALDH3B2_intron2 C/T)	67.191.372
rs3825021 (ALDH3B2_intron2 C/T)	67.191.262
rs2126716 (ALDH3B2_intron2 C/G)	67.191.247
D11S1889	67.069.719
D11S987	67.649.916
D11S4113	68.522.210
D11S4136	69.324.892
D11S4139	70.181.917
D11S1314	72.000.840

Região Candidata da Síndrome Spoan

Região em homozigose

Região em heterozigose

Genes seqüenciados

Marcadores moleculares