UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE BIOCIÊNCIAS
DEPARTAMENTO DE GENÉTICA

CARACTERIZAÇÃO CLÍNICA, MOLECULAR E DE VIAS CELULARES DE PACIENTES COM NEUROFIBROMATOSE E ESCLEROSE TUBEROSANO BRASIL

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Sumário

Lista de Abreviaturas

aCGH: *array Comparative Genomic Hybridization*

AKT: homólogo do *V-akt murine thymoma viral oncogene*

CSRD: *Cystein and Serine Rich Domain*

DHPLC: *Denaturing High Performance Liquid Chromatography*

EGF: *Epidermal Growth Factor*

ERM: Ezrina, Radixina e Moesina

ET: Esclerose Tuberosa

FDA*: Food and Drug Administration*

FERM: Família Ezrina, Radixina e Moesina

FISH: *Fluorescent in situ Hybridization*

FKBP-12: *FK506 binding protein-12*

GAP:*GTPase Activating Protein*

GDP: Guanosina difosfato

Grb2: receptor de fator de crescimento

GSK3: *Glycogen Synthase Kinase*

GTP: Guanosina trifosfato

HGMD*: The Human Gene Mutation Database*

IGF: *Insulin Growth Factor*

LCR: *Low Copy Repeats*

MEK: MAPK-ERK quinase

MLPA: *Multiplex Ligation-dependent Probe Amplification*

mTOR: Mammalian Target of Rapamycin

NCBI: *National Center for Biotechnology Information*

NF1: Neurofibromatose tipo 1

NF1- GRD: *NF1-GAP related domain*

NF2: Neurofibromatose tipo 2

NGS: *Next generation Sequencing*

NIH: *National Institutes of Health*

NLS: *Nuclear Localization Sequence*

OMIM: *Online Mendelian Inheritance in Man*

PAK1 = *P21-Activated Kinase*

PI3K: Fosfatidilinositol-3 quinase

PTT: *Protein Truncation test*

Rac1 = *Ras-related C3 botulinum toxin substrate 1*

RAF: homólogo do *murine sarcoma viral oncogene*

RAS: homólogo do *rat sarcoma viral oncogene*

Rheb: *Ras Homologue Enriched in Brain*

RTKs: receptores de tirosina quinases

Sec14-PH: *Bipartite Lipid Binding Domain*

SOS: homólogo do gene *Drosophila son of Sevenless*

SSCP: *Single Strand Conformation Polymorphism*

TBD: *Tubulin Binding Domain*

TGGE: *Temperature-Gradient Gel Electrophoresis*

TNF-α: *Tumor necrosis factor alpha*

UTR: U*nstranslated Region*

VEGF: *Vascular Endothelial Growth Factor*

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Resumo

Introdução. As neurofibromatoses tipo 1 (NF1) e tipo 2 (NF2) e a esclerose tuberosa (ET) são as genodermatoses mais comuns, que possuem potencial de desenvolvimento de tumores benignos e malignos. Elas são síndromes hereditárias com padrão de herança autossômico dominante e expressividade altamente variável, causadas, respectivamente, por mutações nos genes supressores tumorais *NF1, NF2 e TSC1 ouTSC2,* todos com grande número de mutações diferentes descritas em bases de dados, envolvendo rearranjos e mutações de ponto. **Objetivos.** O objetivo deste trabalho foi caracterizar, do ponto de vista clínico e molecular, ospacientes e familiarescom diagnóstico deNF1 e NF2avaliados no Hospital de Clínicas de Porto Alegre e pacientes com diagnóstico de ETavaliados em centros da Rede Nacional de Câncer Familial, além de avaliar a expressão gênica global e os processos celulares em células com mutações em *TSC1* e *TSC2*. **Resultados.** Foram recrutados 93 pacientes com NF1 e 7 com NF2, no sul do Brasil, e 53 pacientes com ET oriundos de 4 regiões do Brasil. Na análise molecular, a frequência de grandes rearranjos e mutações de ponto foi de 4,3 e 79,0% em *NF1*; 14,3 e 29,0% em *NF2*; e 9,0 e 81,0% em *TSC1* e *TSC2*, sendo 33 variantes novas em *NF1*e 20 em *TSC1* e *TSC2*. Correlações genótipo-fenótipo específicas para a população brasileira não foram estabelecidas, mas algumas correlações previamente descritas também foram identificadas em nosso estudo. *Hotspots* para mutações não foram encontrados. Por fim, análise de processos celulares e expressão gênica com células mutadas e normais (com e sem rapamicina) mostrou que não há morte celular e mudança em fases de ciclo celular; entretanto, em pacientes com esclerose tuberosa, a indução de autofagia é muito maior do que em células controles após o tratamento com rapamicina (p=0,039). **Discussão e conclusões.** Este é o primeiro estudo do Brasil a realizar a caracterização molecular completa de pacientes com neurofibromatoses e esclerose tuberosa. A caracterização das variantes nesses pacientes é muito importante para confirmar o diagnóstico quando a clínica é duvidosa, para o acompanhamento da doença e a possível prevenção das características multissistêmicas. Além disso, foi demonstrado que a autofagia pode ser o mecanismo inicial que leva a formação de tumores em células com mutações em *TSC1* ou *TSC2*. O processo de autofagia precisa ser estudado com mais detalhe a fim de esclarecer o papel desse mecanismo na esclerose tuberosa, e, possivelemente, levar ao uso de terapia de alvo molecular direcionada à via de autofagia, em conjunto com inibidores de mTOR ou sozinha.

Abstract

Introduction. Neurofibromatosis type 1 (NF1) and type 2 (NF2) and tuberous sclerosis (TSC) are the most common genodermatoses, with the potential to develop benign and malignant tumors. These genodermatoses are hereditary syndromes with an autosomal dominant inheritance pattern and highly variable expressivity. They are caused, respectively, by mutations in the tumor suppressor genes *NF1*, *NF2* and *TSC1* or *TSC2*, all with a large number of different mutations described, including rearrangements and point mutations. **Objectives.** The aim of this study was to perform clinical and molecular characterization of NF1 and NF2 patients and their relatives, evaluated at Hospital de Clínicas of Porto Alegre and patients diagnosed with TSC evaluated in the Familial Cancer National Network. In addition, we aimed to evaluate the global gene expression and cellular processes in cells with mutations in *TSC1* and *TSC2*. **Results.** Ninety-three NF1 patients and 7 NF2 patients were recruited from south Brazil; 53 TSC patients were recruited from 4 Brazilian regions. In molecular analysis, the frequency of large rearrangements and point mutations was 4.3 and 79.0% in *NF1*; 14.3 and 29.0% in *NF2*; and 9.0 and 81.0% in *TSC1* and *TSC2*, with 33 new variants in *NF1* and 20 in *TSC1* and *TSC2*. Specific genotype-phenotype correlations in Brazilian population were not established, but some previously described correlations were also identified in our study. Hotspots for mutations were not found. Finally, analysis of cellular processes and gene expression in normal and mutated cells (with and without rapamycin) showed that there is no cell death and change in cell cycle phases; however, in patients with tuberous sclerosis, the induction of autophagy is much greater than in control cells after treatment with rapamycin (p=0.039). **Discussion and conclusion.** This is the first Brazilian study to perform the complete molecular characterization of patients with neurofibromatoses and tuberous sclerosis. Variant characterization in these patients is very important to confirm diagnosis when the clinic is doubtful, to follow disease progress and possibly prevent the occurrence of multisystemic characteristics. In addition, we showed that autophagy may be the initial mechanism leading to tumor formation in cells with *TSC1* or *TSC2* mutations. Autophagy needs to be studied in more detail to clarify the role of this mechanism in tuberous sclerosis, and possibly lead to the use of autophagy-targeted molecular therapy, alone or in conjunction with mTOR inhibitors.

Capítulo I

Introdução Geral

1. Predispoição Hereditária ao Câncer

As doenças neoplásicas em seres humanos podem ocorrer esporadicamente (sem uma predisposição familiar clara) ou menos comumente como doenças hereditárias, com uma predisposição familiar. Várias síndromes de predisposição ao câncer são conhecidas e a causa genética já foi identificada na maioria delas. Os tumores relacionados a essas síndromes podem ser tanto benignos quanto malignos e são, na maioria das vezes, fenotipicamente idênticos aos que ocorrem esporadicamente. Esses tumores também ocorrem geralmente em idade mais jovem e em maior número do que os que ocorrem esporadicamente (Neel e Kumar, 1993). A maioria das síndromes de predisposição ao câncer possui padrão de herança autossômico dominante e são causadas por mutações em genes supressores tumorais. Um número muito menor dessas síndromes é causado por mutações em proto-oncogenes, como, por exemplo, a neoplasia endócrina múltipla tipo 2, o câncer medular da tiróide familial, o carcinoma papilar renal hereditário e alguns casos de melanoma familiar. Além dos genes supressores tumorais e proto-oncogenes, outros genes envolvidos em síndromes de câncer hereditário são os genes de reparo de DNA (Neel e Kumar, 1993).

1.1 Oncogenes

Os proto-oncogenes são reguladores da proliferação celular e da morte celular programada (apoptose). Quando os proto-oncogenes sofrem algum tipo de mutação, passam a ser chamados de oncogenes. Os produtos dos oncogenes, também conhecidos como oncoproteínas, atuam como fatores de crescimento, receptores de fatores de crescimento, proteínas envolvidas na transdução de sinais intracelulares e proteínas envolvidas na apoptose(Neel e Kumar, 1993). Mutações emoncogenes são dominantes, resultando em ganho de função das oncoproteínas e perda da capacidade da célula de controlar a proliferação celular.O conceito de oncogene foi descrito após a descoberta de que a integração de genes retrovirais em genomas de animais hospedeiros poderia levar a formação de tumores (Varmus e Weinberg, 1993). Nos seres humanos, a oncogênese retroviral é rara; na maioria dos casos, os proto-oncogenes celulares são ativados por mutações e translocações.

1.2 Genes supressores tumorais

Genes supressores tumorais são genes celulares em que ambos os alelos precisam ser inativados para que a oncogênese ocorra. A evidência para a existência de genes supressores tumorais veio de experiências de fusão celular, onde a capacidade de formação de tumores foi perdida depois da fusão de células cancerígenas com células normais. O conceito de genes supressores de tumor foi desenvolvido por Knudson através da observação da epidemiologia de casos de retinoblastoma esporádicos (unilaterais) e familiares (bilaterais). Neste modelo, chamado de “hipótese de dois eventos”, uma mutação de linhagem germinativa em um gene supressor tumoral (primeiro evento) é seguida por uma mutação somática no outro alelo do gene em uma célula específica (segundo evento), resultando na falta do produto do gene funcional nesse tecido(Knudson, 1971).Por esse modelo, as mutações em genes supressores tumorais são de natureza recessiva para que a formação de tumores ocorra; porém, a presença da mutação em linhagem germinativa (apenas o primeiro evento) resulta em características fenotípicas hereditáriasnão tumorais que são dominantes. Apenas nos tecidos em que o segundo evento somático ocorre, podem surgir tumores. A ocorrência de mutações em genes supressores tumorais em homozigose é letal em embriões, levando a crer que esses genes têm um papel importante no desenvolvimento embrionário, além do seu papel importante na carcinogênese e progressão tumoral (Weinberg, 1989).

1.3 Genes de reparo do DNA

O DNA do genoma humano está constantemente ameaçado por fatores endógenos e exógenos, que podem causar mutações no DNA. Por isso, diversos sistemas de reparo de DNA se desenvolveram através de pressões evolutivas para manter a integridade do genoma. Se mutações de ganho de função em proto-oncogenes ou mutações de perda de função em genes supressores tumorais não forem corrigidos pelos mecanismos de reparo de DNA, o crescimento descontrolado e o câncer podem surgir nessas células. Os dois principais mecanismos de reparo de DNA são reparo por excisão de nucleotídeos e reparo de mal-pareamento de bases. Mutações germinativas em genes envolvidos no reparo por excisão de nucleotídeos levam a síndromes clínicas bem definidas, incluindo o xerodermapigmentoso. Mutações em genes envolvidos no reparo de mal-pareamento

de bases podem resultar em síndromes de herança dominante em que as neoplasias mais frequentes envolvem o cólon (por exemplo câncer colorretal não-poliposo hereditário e síndrome de Lynch).

2. Genodermatoses

Asgenodermatosessão um grande grupode doenças monogênicas hereditáriascom manifestaçõessomente cutâneas ou cutâneas e também em outros órgãos, divididas em vários subgruposde acordo com o tipo de herança ou pelas principais características clínicas. Instalam-se durante a concepção por mutações espontâneas ou herdadas, porém nem sempre se manifestam ao nascimento. Algumas genodermatoses são gravemente incapacitantes ou mesmo letais, representando um enorme impacto na qualidade de vida dos doentes e familiares, podendo causar exclusão social, dificuldade na inserção profissional, vulnerabilidade a nível psicológico e cultural, bem como redução da expectativa de vida.Como exemplos dessas alterações estão a epidermólise bolhosa, a ictiose,a hiperqueratose palmo-plantar, o xeroderma pigmentoso, asneurofibromatoses, a esclerose tuberosa, entre outros. Muitas dessas desordens são muito raras, e algumas apresentam potencial de desenvolvimento de tumores malignos, como o xeroderma pigmentoso. As neurofibromatoses tipo 1 e tipo 2 e a esclerose tuberosa são as genodermatoses mais comuns e conhecidas como síndromes neurocutâneas, que possuem potencial de desenvolvimento de tumores benignos e malignos, sendo portanto síndromes de predisposição ao câncer. Essas três genodermatoses possuem padrão de herança autossômico dominante e são o foco deste trabalho.

2.1 Neurofibromatose tipo 1

A neurofibromatose tipo 1 ou NF1 (anteriormente conhecida como neurofibromatosede von Recklinghausenouneurofibromatoseperiférica) foi descrita em detalhes por Von Recklinghausenem 1882, através de um relato de caso. A NF1 é a genodermatose mais comum, causada por mutações de perda de função em heterozigose no gene *NF1*, um gene supressor tumoral.

2.1.1 Epidemiologia

A NF1(OMIM #162200)é uma das doenças genéticas autossômicas dominantes mais comuns, com incidência mundial de 1/2500 nascidos vivos e prevalência de 1/4000 (Carey, 1986), independente de sexo e etnia. Dois tipos de estudo também têm avaliado a mortalidade e expectativa de vida em NF1: estudos de coorte e estudos de notificação de óbitos. Ambos os tipos de estudos mostraram uma diminuição de 8-15 anos na expectativa de vida em pacientes com NF1 (Upadhyaya e Cooper, 2012). No Brasil, não há dados sobre incidência, prevalência, mortalidade e expectativa de vida de pacientes com NF1.

2.1.2 Diagnóstico e Sintomatologia

O diagnóstico clínico de NF1 é baseado nos critérios de diagnóstico delineados pelo *National Institutes of Health* (*NIH*) em uma Conferência de Consenso em 1987 (Quadro 1) (Stumpf *et al*., 1988). A presença de dois ou mais dos sintomas apresentados no Quadro 1 é suficiente para o diagnóstico clínico de NF1. Os sintomas mais comuns são as manchas café-com-leite, neurofibromas, nódulos de Lisch e efélides axilares e inguinais. As manchas café-com-leite são máculas na pele caracterizadas por pigmentação aumentada dos queratinócitos basais e melanócitos. Elas possuem mais de 0,5 cm de diâmetro, de forma oval, e tem bordas bem definidas e aumentam em número e tamanho conforme o avanço da idade dos pacientes. Os nódulos de Lisch são lesões benignas assintomáticas da íris, que não causam problemas visuais. Neurofibromas cutâneos são lesões benignas, nodulares e não encapsuladas, compostas por elementos similares aos de um nervo periférico normal (Peltonen *et al*., 1988), mas com padrão desorganizado, que não sofrem transformação maligna. Já os neurofibromas plexiformes infiltram difusamente grandes nervos periféricos e possuem composição similar aos nervos normais, porém com uma quantidade desproporcional de matriz extracelular. Esses tumores podem atingir tamanhos muito grandes e podem sofrer transformação maligna. Sintomas adicionais da NF1 incluem déficit cognitivo, macrocefalia, lesões ósseas e baixa estatura. Dificuldades de aprendizado ocorrem em até 50% dos indivíduos com NF1 (Ferner, 2010). Várias complicações resultantes dos tumores e malformações ósseas podem surgir, incluindo escoliose, epilepsia e anormalidades endocrinológicas. Além disso, diferentes tumores malignos podem ocorrer. Essas complicações malignas são menos frequentes, mas potencialmente a

manifestação mais séria da doença. Em geral, os pacientes com NF1tem2,7 vezes mais risco de desenvolver câncer, com um risco cumulativo de 20% em pacientes afetados com mais de 50 anos de idade (Friedman e Birch, 1997). A incidência demalignidadevariaentre 4% e52% empacientes com NF1 (Walker *et al*., 2006). Essas complicações geralmente levam à morte prematura dos indivíduos e incluem sarcomas agressivos, como os tumores malignos da bainha de nervos periféricos (neurofibromas plexiformes transformados), que ocorrem em 10-15% dos pacientes (Upadhyaya, 2001), feocromocitomas, rabdomiossarcomas, osteosarcomas, leiomiossarcomas, leucemias, tumores cerebrais, gliomas óticos, tumores de estroma gastrointestinal, carcinomas de mama e carcinoma medular da tireóide (Jett e Friedman, 2010). Os tumores malignos não relacionados ao sistema nervoso central são mais raros.

Quadro 1. Critérios de diagnóstico clínicode NF1 segundo o *National Institutes of Health*.

1 - Seis ou mais manchas café-com-leite
> de 0,5 cm no maior diâmetro em indivíduos pré-puberais, ou
> de 1,5 cm no maior diâmetro em indivíduos pós-puberais
2 - Dois ou mais neurofibromas de qualquer tipo ou um neurofibromas plexiformes
3 - Efélides na região axilar ou inguinal
4 - Glioma óptico
5 - Dois ou mais nódulos de Lisch (hamartomas de íris)
6 - Lesão óssea distinta, como a displasia do esfenóide ou adelgaçamento do córtex dos ossos longos com ou sem pseudoartrose.
7 - Familiar de primeiro grau (pai/mãe, irmão/irmã, filho/filha) com NF1 diagnosticado pelos critérios acima.
NF1: presença de dois ou mais dos critérios acima.

Aproximadamente 95% dos pacientes com NF1 podem ser clinicamente diagnosticados com os parâmetros estabelecidos pelo *NIH* com oito anos de idade (DeBella *et al*., 2000). Entretanto, o diagnóstico de NF1 depende de um conjunto ligeiramente diferente de sinais nas diferentes faixas etárias. Em crianças com menos de seis anos, as manchas café-com-leite são o primeiro sinal mais comum de NF1, e elas

estão presentes em 95% dos pacientes com a idade de 1 ano (DeBella *et al*., 2000). O aparecimento precoce dessas manchas é muito importante para o diagnóstico. Manchas café-com-leite isoladas ocorrem em 10-15% da população em geral, e por isso são muitas vezes ignoradas como um primeiro sinal da síndrome. Outra característica importante a ser avaliada em crianças menores de seis anos é a presença de neurofibromas plexiformes. Geralmente, tumores plexiformes faciais que serão sintomáticos se manifestam durante os primeiros anos de vida (Ferner *et al*., 2007) e possuem risco de transformação maligna ainda na infância (Evans *et al.,* 2002). A displasia de ossos longos também é uma complicação de NF1 que ocorre em 3-5% dos pacientes (Friedman e Birch, 1997) e pode tornar-se evidente quando a criança aprende a se levantar e andar, como, por exemplo, nas pseudoartroses da tíbia. Além desses sintomas, 15% das crianças com NF1 possuem gliomas ópticos (Listernick*et al*., 1994). A maioria dos gliomas ópticos aparece nos primeiros anos de vida, com idade média de diagnóstico aos cinco anos de idade (Nicolin *et al*., 2009), podendo interferir na acuidade visual e causar puberdade precoce devido a interferência com a glândula hipófise. Outras características adicionais que devem ser levadas em conta em crianças com suspeita de NF1 são macrocefalia, déficit cognitivo, escoliose e epilepsia.

Em crianças maiores de seis anos, as efélides axilares e inguinais são visíveis em 80% dos pacientes com sete anos de idade, sendo muito úteis no diagnóstico, pois não ocorrem na população em geral. Os nódulos de Lisch também costumam aparecer na idade de seis anos ou mais, e precisam ser examinados por um oftalmologista.

Em adolescentes e adultos, inicia o aparecimento de neurofibromas cutâneos. Apesar de benignos, o número desses tumores pode variar de poucos a mais de mil, causando distúrbios estéticos e sociais. Neurofibromas subcutâneos (plexiformes) também crescem durante a puberdade. Como esses tumores podem causar dor e tem potencial de transformação maligna, deve ser feita uma avaliação cuidadosa para retirada cirúrgica. Estudos também mostraram que mulheres adultas (principalmente abaixo dos 50 anos) possuem risco aumentado de desenvolver câncer de mama (Madanikia *et al*., 2012). O acompanhamento clínico desses pacientes é fundamental.

Quando o diagnóstico clínico não é possível e para finalidades de aconselhamento genético, o teste molecular é a estratégia mais importante para definição diagnóstica. Por exemplo, o diagnóstico de crianças pequenas é um desafio porque cerca de metade dos casos de NF1 esporádicos não preenchem os critérios de diagnóstico do *NIH* no primeiro ano de vida (DeBella *et al*., 2000). Se crianças menores

de seis anos com apenas um critério de diagnóstico de NF1 (por exemplo, manchas café-com-leite) não tiverem os pais com a doença, o diagnóstico de NF1 não pode ser feito até as características clínicas adicionais aparecerem. Nesses casos, o diagnóstico molecular pode ser muito útil, tanto para eliminar a hipótese da doença ou para que o acompanhamento adequado tenha seguimento. Ainda, em alguns casos, os critérios de diagnóstico clínico do *NIH* podem levar a falsos diagnósticos de NF1 (por exemplo, as manchas café-com-leite estão associadas com várias outras síndromes além de NF1 e os neurofibromas subcutâneos podem ser confundidos com lipomas, que são muito comuns na população em geral). Os testes moleculares seriam muito úteis para confirmação desses casos. O teste genético também tem grande utilidade nos casos em que se observa mais de um fenótipo de síndrome genética de predisposição ao câncer na família (por exemplo, fenótipo de NF1 em pelo menos uma pessoa da família e história familiar importante de câncer de mama).Outro fator que complica o diagnóstico clínico de NF1 é a expressividade variável da doença. Embora a NF1 seja autossômica dominante e possua penetrância completa, a expressão fenotípica mostra um alto grau de variabilidade, até mesmo em membros da mesma família. Essa variabilidade é difícil de explicar e pode ocorrer até mesmo em gêmeos que herdam o mesmo alelo causador da doença (Patil e Chamberlain, 2012). Nesses casos, os testes moleculares também são muito úteis. A compreensão da biologia molecular da doença pode permitir que o diagnóstico e a terapêutica da doença sejam melhores no futuro.

Após o diagnóstico clínico ou molecular, todos pacientes com NF1, independente da idade, devem ser submetidos a uma série de avaliações por uma equipe multidisciplinar para acompanhamento da progressão e gravidade da doença, geralmente envolvendo psicólogos, neurologistas, geneticistas, dermatologistas, oftalmologistas e ortopedistas. Não hácura paraa NF1 e atualmente o tratamento é focado namelhoria dos sintomasclínicos.Algumas cirurgias podem ser necessárias para a remoção de neurofibromas, mas muitas vezes aremoção completa não é possível por causa do tamanho e características infiltrativas, sendo que muitos pacientes têm lesões nervosas e hemorragias durante as cirurgias (Prada *et al*., 2012). Como resultado dos avanços na compreensão da NF1, uma série de estudos recentes têm avaliado o potencial terapêutico de drogas de alvo molecular no controle das neoplasias associadas a essa doença. A maioria destes estudos está ainda em fase pré-clínica (estudos *in vitro* ou em modelos animais) embora alguns resultados de tratamento em séries de casos esteja disponível (Ardern-Holmes e North, 2011; Yap *et al*., 2014). Essas novas terapias

para tumores associados a NF1 podem ser agrupadas em terapias alvo das vias de sinalização afetadas ou terapias que tem como alvo o estromado microambiente tumoral. Mais adiante, quando a via de sinalização envolvida na NF1 for apresentada, algumas abordagens de tratamento envolvendo essa via serão brevemente discutidas.

2.1.2 O gene *NF1* e a proteína neurofibromina

O gene *NF1*é um gene supressor tumoral que foi identificado e clonado em 1990 (Cawthon *et al*., 1990), abrange 289.701pb e está localizado no cromossomo humano 17q11.2, sendo um dos maiores genes do genoma humano. A sequência genômica do NCBI (NG_009018.1) inicia 4951pb a montante do sítio de início da transcrição e 5334pb a montante do sítio de início de tradução no éxon 1, com uma região 5' não traduzida (*unstranslated region – UTR*) de 484pb. Essa região anterior ao sítio de início de transcrição e tradução compreende uma região promotora, que possui uma clássica ilha CpG que se estende do promotor até aproximadamente 100pb do éxon 1. Essas ilhas CpG devem estar envolvidas na regulação da expressão gênica de *NF1*. O promotor de *NF1*não possui regiões TATA ou CCAAT e ainda é uma região pouco estudada.

O gene *NF1* possui 57 éxons consitutivos (sequência do NCBI NM_000267.3), além de três pequenos éxons que sofrem processamento alternativo (9a, 23a e 48a, baseado na nomenclatura inicial; e, com base na nomenclatura atual, eles estão entre os éxons 11 e 12 (9a), éxon 31 (23a) e entre os éxons 56 e 57 (48a), respectivamente). O transcrito que contém o éxon 31 (NM_001042492.2) possui 12359pb que codificam uma proteína de 2818 aminoácidos, chamada neurofibromina.As duas principais isoformas da proteína neurofibromina são as isoformas I e II. A neurofibromina tipo I é codificada pelo transcrito que contém o éxon 31 e possui atividade reguladora de Ras mais fraca. Essa isoforma é expressa principalmente em células de Schwann, e é essencial para o aprendizado e memória em ratos (Costa *et al*., 2001). A neurofibromina tipo II não possui o éxon 31 (NM_000267.3), é expressa predominantemente no cérebro com alta atividade de regulação de Ras nas células (Andersen *et al*., 1993).A proporção entre a expressão das isoformas I e II parece variar em tecidos tumorais, sendo a expressão da isoforma I aumentada (Iyengar*et al*., 1990). O éxon 31 está dentro de um dos domínios funcionais da proteína, por isso sua adição muda o nível de atividade da proteína. A inclusão desse éxon é finamente regulada em diferentes tecidos.

Informações sobre outras isoformas de neurofibrina são limitadas. As neurofibrinas tipos III e IV, que contém o éxon 48a e ambos os éxons 23a e 48a, respectivamente, são expressas em músculos cardíacos e esqueléticos e parecem ser essenciais para o desenvolvimento muscular (Brannan*et al*., 1994; Gutmann *et al*., 1995). A adição do éxon alternativo entre os éxon 11 e 12 parece ser exclusiva do sistema nervoso central e não afeta a função da proteína (Danglot *et al*., 1995).

Ainda, existem 13 duplicações parciais de *NF1* em outros cromossomos (pseudogenes), com evidências de transcrição nos *loci* do cromossomo 2, 15 e 21 (Yu *et al*., 2005). Esses transcritos de pseudogenes podem interferir na análise molecular de *NF1*. Além disso, exitem três genes dentro do intron 27b (nomenclatura tradicional) do gene *NF1:EVI2B, EVI2A* (sítios de integração virais ecotrópicos) e *OMG* (glicoproteína oligodendrocítica-mielina), codificados na fita oposta de *NF1*. A Figura 1A mostra de forma esquemática os 58 éxons da isoforma mais longa de NF1 e os pequenos éxons que sofrem processamento alternativo. A região 3'UTR do gene compreende 3522pb dentro do éxon 58 e possui dois sítios de poliadenilação principais, que geram um transcrito maior e um menor. Essa região também não foi muito estudada, mas provavelmente possui muitas sequências de regulação da expressão de *NF1*. Até o momento, há evidência de apenas um códon de iniciação de tradução de NF1.

Na figura 1B estão representados os domínios funcionais da neurofibromina. Essa proteína possui massa molecular de aproximadamente 327kDa e é expressa constitutivamente e em níveis mais altos no sistema nervoso central, especialmente em neurônios, astrócitos, oligodendrócitos e células de Schwann (Nordlund *et al*., 1993). A neurofibromina parece controlar diversos processos intracelulares, sendo um deles a via RAS (*rat sarcoma)*-adenosina cíclica monofosfato, responsável pelo controle da proliferação celular e ativação dos genes supressores de tumor. Essa função é atribuídaprincipalmente a uma pequena região central da proteína que compreende 360 aminoácidos codificados pelos éxons 28 a 35 (20-27a na nomenclatura tradicional). Esta região possui alta homologia de sequência e estrutura com o domínio catalítico da *GTPase activating protein (GAP*) de mamíferos e com os produtos dos genes *IRA1* e *IRA 2* de leveduras (Xu *et al.,* 1990) e é chamada de domínio relacionado à *GAP* (NF1-GRD). A ligação do nucleotídeo guanina à proteína RAS medeia faz a mediação da transdução de sinal que regula o crescimento celular. A ligação de guanosina trifosfato (GTP) ativa a sinalização, enquanto a hidrólise para guanosina difosfato (GDP) cessa o sinal (Barbacid, 1987). A regulação negativa do oncogene *Ras* pela neurofibromina

impede a ativação das vias de MAPK e PI3K/Akt/mTOR, responsáveis pelo controle da proliferação celular, síntese de DNA, apoptose, migração, crescimento e diferenciação celulares, como demonstrado na Figura 2 (Martin *et al*., 1990; Ballester *et al*., 1990; Bollag *et al*., 1991). Como a conservação evolutiva de *NF1* não é confinada a esse domínio Ras-GAP, a função Ras-GAP da neurofibromina pode ser reforçada por outros domínios, como, por exemplo, pela fosforilação do domínio rico em cisteínas (CSRD) codificado pelos éxons 16 a 23 (Figura 1B). A neurofibromina também parece se ligar a caveolina-1, uma proteína de membrana que regula moléculas de sinalização como p21ras, proteína quinase C e receptores de fatores de crescimento. A formação de um complexo NF1-caveolina modula as vias de p21ras/MAPK e PI3K/Akt, controlando a proliferação e diferenciação celular (Boyanapalli *et al*., 2006).

Além dos mecanismos de supressão tumoral envolvendo Ras, há vários outros mecanismos propostos para a atividade de supressão tumoral da proteína. A neurofibromina também é uma reguladora positiva da enzima adenilato ciclase, que gera o AMP cíclico intracelular, o qual é muito importante para a memória e aprendizado, bem como possui atividade supressora tumoral (Tong *et al.*, 2002). O papel da neurofibromina na motilidade celular é importante não só para a função dos neurônios, mas também contribui para sua atividade de supressão tumoral, através da regulação da dinâmica e reorganização dos filamentos de actina pela via Rho-ROCK-LIMK2-cofillin. A falta de neurofibrina altera a organização do citoesqueleto de actina, promove a motilidade celular, capacidade de invasão e adesão célula-célula, resultando na formação de grandes agregados de células. Isto pode levar à formação dos múltiplos neurofibromas em pacientes com NF1, os quais consistem de agregados de vários tipos de células, incluindo as células de Schwann, fibroblastos, células endoteliais e mastócitos em excessiva matriz extracelular (Ozawa *et al*., 2005). Ainda, também há dados que sugerem que a neurofibromina esta envolvida na transição epitélio-mesenquimal, implicada na tumorigênese e mestástase.

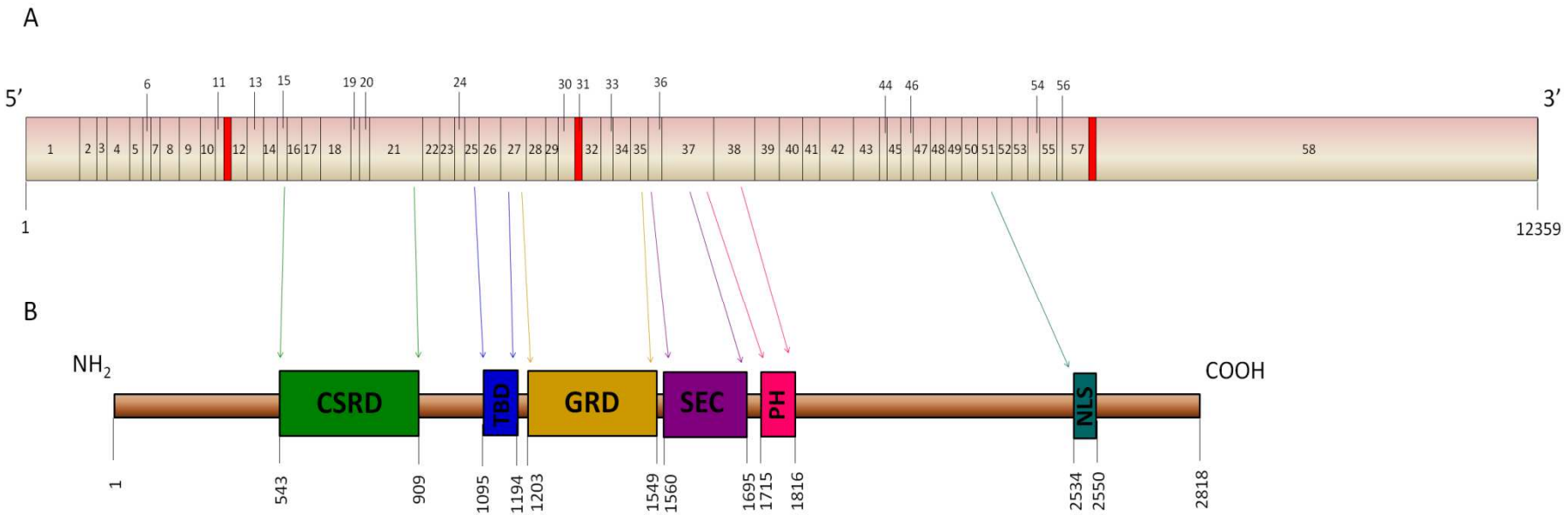

Figura 1. **Esquema do transcrito e proteína de NF1.A.** Esquema do transcrito maior de *NF1* (NM_001042492.2) que codifica a isoforma I de neurofibromina, com 12359pb e com todos os 57 éxons constitutivos em rosa e o éxon 31 que está incluído nesse transcrito em vermelho, representados em tamanho proporcional ao número de bases de cada éxon e nomeados de acordo com a nomenclatura atual. Os demais éxons representados em vermelho sofrem processamento alternativo e geram outras isoformas da proteína. **B.** Esquema representando a proteína neurofibromina com seus domínios funcionais e os respectivos éxons que codificam cada domínio.**Domínios da neurofibromina: domínio CSRD (*cystein and serine rich domain*); domínio TBD (*tubulin binding domain*); domínio GRD (domínio responsável pela interação com Ras e hidrólise de GTP); domínio Sec14-PH (*bipartite lipid binding domain*); NLS (*nuclear localization sequence*).**

O tratamento de NF1, como mencionado anteriormente, envolve a retirada cirúrgica de tumores e acompanhamento dos sintomas. As complicações associadas às cirurgias e a baixa qualidade de vida dos pacientes torna necessário o desenvolvimento de terapias de alvo molecular. Tendo como alvo o estroma dos tumores, alguns estudos clínicos em pacientes com NF1 já foram feitos utilizando talidomida, ácido cisretinóico e interferon α-2b e mostraram controlar de forma limitada a angiogênese e diferenciação em neurofibromas plexiformes (Gupta *et al*., 2003; Jakacki *et al*., 2011). Ensaios iniciais utilizando pirfenidona, uma droga antifibrótica que tem como alvo as contribuições do estroma, mostraram resultados similares em neurofibromas plexiformes (Babovic-Vuksanovic *et al*., 2007). A Figura 2 mostra alguns alvos terapêuticos possíveis na via de NF1, que foram explorados em alguns estudos pré-clínicos recentes. Um exemplo é a farnesilação de Ras, que é essencial para a translocação de Ras até a membrana. A atividade de tipifarnib, um inibidor da farnesil transferase, foi avaliada em um estudo de fase 1 com tumores plexiformes; não houve regressão dos tumores mas a doença ficou estável (Widemann *et al*., 2006). Da mesma forma, os resultados de um estudo de fase 2 em pacientes com neurofibroma plexiforme utilizando sirolimus, um inibidor de mTOR(*Mammalian target of rapamycin*), não mostrou regressão das lesões, mas estabilizou a progressão tumoral (Weiss *et al*., 2014). Estudos clínicos utilizando o everolimus, um inibidor de mTOR mais recente, estão em andamento (http:// www.clinicaltrials.gov). Estudos com sorafenib(um inibidor de quinase) não mostraram resposta antitumoral em estudos de fase1em crianças com tumores plexiformes (Kim *et al*., 2013).

A maioria dos estudos em NF1 ainda está em fase pré-clínica. Há dados que suportam a atividade de inibidores de MEK (PD0325901) em camundongos e inibidores da farnesilação de Ras, sirolimus, everolimus e inibidores de PI3K/Akt em linhagens celulares derivadas de tumores malignos de bainha de nervo periférico de pacientes com NF1 (Johansson *et al*., 2008; Zou *et al*., 2009; Endo *et al*., 2013). O uso de erlotinib, um inibidor do receptor de fator de crescimento epitelial (EGFR), junto com everolimus, inibiu o crescimento e induziu a apoptose em linhagens de tumores malignos da bainha de nervos periféricos derivados de NF1 (Johansson *et al*., 2008). Inibidores de PAK1 também mostraram eficácia em camundongos e diminuíram os tumores malignos da bainha de nervos periféricos e tumores de mama deficientes de NF1 (Hirokawa *et al*., 2006; Hirokawa *et al*., 2007). Combinações de drogas podem ser superiores a monoterapia.

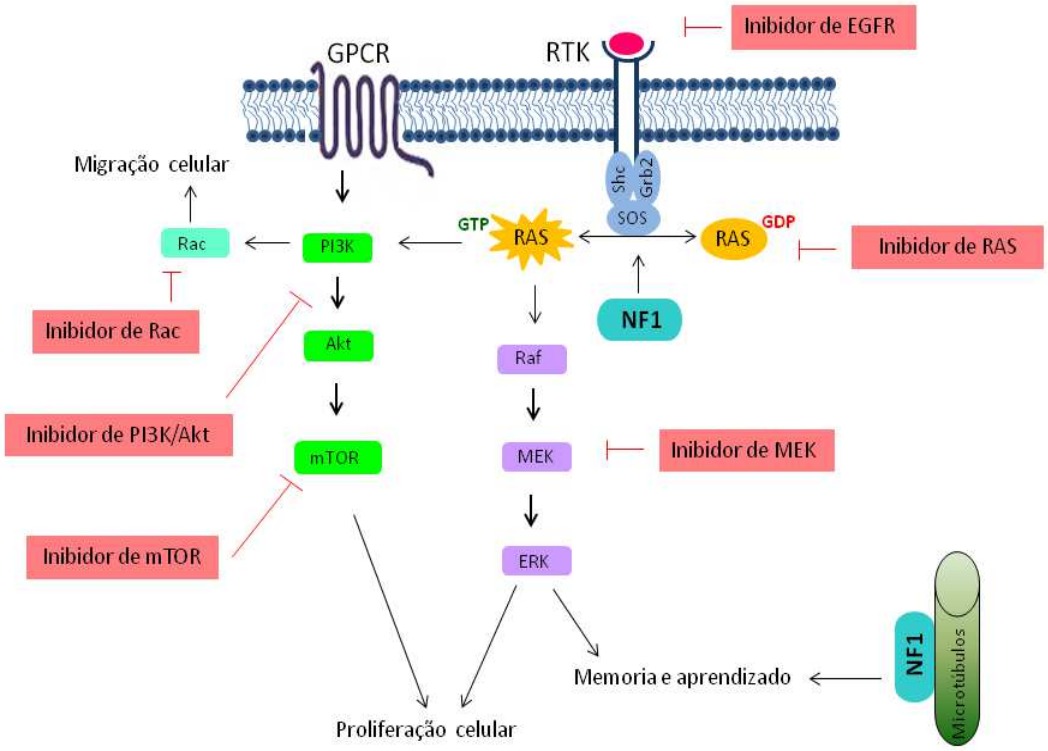

Figura 2. O papel da neurofibrina na via Ras. Receptores acoplados a proteína G (GPCR) e receptores de tirosina quinases (RTKs), quando ativados por seus ligantes, promovem adição de nucleotídeo a guanina e a formação do complexo ativado Ras-GTP. A neurofibromina inativa Ras através da aceleração da conversão de Ras-GTP ativo para Ras-GDP inativo, atividade realizada pelo domínio GRD da proteína que hidrolisa GTP. Consequentemente, a neurofibromina suprime a ativação de efetores da via Ras, incluindo PI3K, Akt, mTOR, S6 quinase e RAF, MEK, ERK e Rac. Todas essas vias regulam crescimento, proliferação e migração celular. A neurofibromina também interage com os microtúbulos através do domínio TBD, influenciando na memória e aprendizado. Também estão representados na figura os principais alvos terapêuticos que estão sendo estudados para tratamento de tumores associados a NF1. **RTKs = receptores de tirosina quinases. Grb2 = receptor de fator de crescimento. SOS = homólogo do gene *Drosophila son of Sevenless* em mamíferos. RAS = homólogo do *rat sarcoma viral oncogene*. GDP = guanosina difosfato. GTP= guanosina trifosfato. RAF = homólogo do *murine sarcoma viral oncogene*. MEK = MAPK-ERK quinase. PI3K = fosfatidilinositol-3 quinase. AKT = homólogo do *V-akt murine thymoma viral oncogene*. mTOR = *mammalian target of rapamycin*. Rac1 = *Ras-related C3 botulinum toxin substrate 1*. PAK1 = *P21-Activated Kinase.***

2.1.3 Mutações no gene NF1 e correlações genótipo-fenótipo

Como a NF1 é causada por mutações no gene supressor tumoral *NF1*, para que os tumores benignos ou malignos associados a doença apareçam é necessária a inativação dos dois alelos do *NF1*, levando à inativação funcional total da neurofribomina, seguindo o modelo de dois eventos descrito por Knudson (1971).O primeiro eventoé a mutação germinativa hereditária eo segundo evento é somático. Há múltiplos mecanismos possíveis para a inativação somática do alelo selvagem de *NF1*, incluindo perda de heterozigosidade, mutação e metilação do promotor. É possível que outros mecanismos ocorram, como o silenciamento epigenético mediado por micro-RNAs. Esses mecanismos também podem afetar outros genes do cromossomo 17, que incluem o gene supressor tumoral *TP53*, bem como os genes *HER2, TOP2A, STAT3* e *BRCA1*, o que ajuda na progressão de tumores.

O gene *NF1* possui uma alta taxa de mutação, estimada entre 1,4–2,6.10^{-4} e 3–5.10^{-5}, que é 10–100 vezes maior que para outros genes humanos associados a doenças.Já foram identificadas 2223 mutações diferentes no gene *NF1*, descritas no banco de dados *The Human Gene Mutation Database* (HGMD - http://www.hgmd.cf.ac.uk), que descreve tanto mutações somáticas quanto germinativas. Essas mutações são de vários tipos, envolvendo desde mutações de ponto até grandes deleções e rearranjos que envolvem milhares de bases (Tabela 1). A maioria das mutações de *NF1* são mutações pequenas (85-90%), como substituições de uma única base, pequenas inserções e pequenas deleções. Outras mutações são deleções ou duplicações de um ou mais éxons (2%) e microdeleções que englobam todo gene *NF1* e genes adjacentes (5-10%). Além disso, rearranjos complexos ocorrem em menor frequência (0.4%).

Genes supressores tumorais exibem uma grande quantidade de mutações sem sentido, o que também ocorre com o *NF1* (54% das mutações de substituição de única base na região codificadora de *NF1* são sem sentido) (Mort *et al*., 2008). Muitas dessas mutações sem sentido estão localizadas em nucleotídeos CpG, um *hotspot* para mutações devido a desaminação de citosinas. Esses nucleotídeos CpG estão distribuídos ao longo de todo gene *NF1* e por isso não há uma região específica do gene em que essas mutações ocorrem.

Tabela 1. Mutações descritas no gene *NF1* no banco de dados HGMD (*The Human Mutation Database*). Acesso em junho/2016.

Tipo de mutação	Número de mutações diferentes
Sentido trocado/ sem sentido	672
Sítios de processamento	395
Pequenas deleções	544
Pequenas inserções	269
Indel	41
Grandes deleções	248
Grandes inserções	38
Rearranjos complexos	16
Total	**2223**

O genoma humano possui várias sequências parálogas ao gene *NF1*(pseudogenes) que podem, a princípio, ser capazes de introduzir mutações em *NF1* via conversão gênica (Yu *et al*., 2005). Essas sequências divergiram do gene *NF1* parental em múltiplos locais e possuem um grande número de alterações genéticas que podem ser introduzidas por conversão gênica. Pelo menos nove mutações de sentido trocado conhecidas podem ter sido introduzidas no *NF1* através de conversão gênica (Casola *et al*., 2012).

As mutações em sítios de processamento de RNA aparecem em uma alta proporção no gene *NF1* (pelo menos 20%), o que parece ser consequência do grande tamanho do gene, com 60 introns. Além das mutações nos sítios de processamento, mutações mais distantes nos introns podem impactar o processamento do RNA mensageiro e causar a doença. Essas mutações podem causar impactos muito diferentes no fenótipo clínico. Fernández-Rodríguez e colegas (2011) reportaram uma mutação intrônica em *NF1* (c.3198-314G > A) associada a um fenótipo relativamente benigno, provavelmente devido à geração de alguns transcritos com processamento correto. Algumas mutações também podem alterar os reforçadores de sítios de processamento, que podem se localizar tanto nos introns como nos éxons, e essas alterações são pouco estudadas. É

difícil saber o efeito de mutações que podem levar a inclusão de um éxon críptico, criação de novos sítios de processamento e alteração nos reforçadores desses sítios.

Considerando as regiões regulatórias de *NF1* (promotor, 5'UTR e 3'UTR), por enquanto, ainda não há mutações descritas. Apesar de haver um grande número de elementos funcionais na região próxima ao promotor de *NF1,* nenhuma alteração genética patogênica envolvendo 987pb do promotor e da região 5' UTR do gene foi encontrada (Osborn *et al.*, 2000; Horan *et al.*, 2004). Entretanto, em 1993, Bernards e colegas compararam o cDNA de *NF1* de camundongos com o transcrito humano e encontraram que as regiões não traduzidas são altamente conservadas, assim como as regiões codificantes. Por isso, embora não existam mutações patogênicas descritas nessas regiões, é possível que elas estejam presentes e causem doença. Até mesmo polimorfismos existentes nessa região podem ser modificadores de fenótipo de NF1.

Pequenas deleções, pequenas inserções e indels correspondem a 31%, 15% e 2,5% das mutações no gene *NF1,* respectivamente. A maioria das pequenas deleções e inserções ocorrem dentro de intervalos de repetição de mononucleotídicos (Rodenhiser *et al*., 1997) e são potencialmente explicadas pelo deslizamento da polimerase durante a replicação, envolvendo a deleção ou adição de nucleotídeos na repetição. Mutações recorrentes em alguns códons de NF1 estão associadas a esse tipo de mutação (por exemplo, códons 76, 1303 e 1818).

Quanto às mutações maiores envolvendo *NF1,* apenas um exemplo de duplicação total do gene *NF1* e mais 12 genes adjacentes foi descrito até o momento por Grisart e colegas (2008). Outro exemplo de mutação grande em *NF1* é a inativação insercional envolvendoelementos *Alu* por retrotransposição, que abrangem 0,4% das mutações em *NF1*. Essas inserções podem ocorrer dentro de um éxon (Valero *et al.*, 2011) ou em um intron de *NF1* (Wallace *et al.,* 1991), preferencialmente em regiões ricas em A-T (Wallace *et al.,* 1991).Translocações que rompem o gene *NF1* também já foram descritas (Viskochil *et al.*, 1990; Kehrer-Sawatzki *et al*., 1997), principalmente envolvendo os cromossomos 17 e 22 t(17;22), bem como inversões que envolvem o gene *NF1* (Asamoah *et al*., 1995). Esses rearranjos complexos são as mutações menos frequentes em *NF1* (HGMD).

Um tipo de alteração gênica que merece destaque em NF1 são as microdeleções. O tipo mais comum de microdeleção recorrente em *NF1* é a microdeleção tipo 1, que abrange 1.4Mb. Essa microdeleção está associada com a perda de 14 genes que codificam proteínas, incluindo o *NF1,* e dois genes de microRNAs (*MIR365*-

*2*e*MIR193a*) (Figura 3). A maior parte dessas deleções é herdada da linhagem germinativa materna (Upadhyaya *et al.*, 1998), com pontos de quebra entre duas regiões de *Low Copy Repeats* (LCR) que são *hotspots* para a recombinação homóloga não-alélica meiótica, chamadas NF1-REPa e NF1-REPc (Dorschner *et al.*, 2000). Os estudos com grandes séries de casos de NF1 mostram que 70-80% das microdeleções causadoras da doença são do tipo 1 (Messiaen *et al*., 2011; Pasmant *et al*., 2010).

As microdeleções do tipo 2 englobam 1.2Mb e são associadas com a perda de 13 genes, incluindo *NF1*. Os seus pontos de quebra estão localizados dentro do gene *SUZ12* e dentro do pseudogene do *SUZ12*, respectivamente, que flanqueiam imediatamente as NF1-REPs A e C (Figura 3). Esse tipo de microdeleção é encontrada menos frequentemente que a do tipo 1, com apenas 10-20% das microdeleções sendo do tipo 2 (Messiaen *et al.*, 2011). Tanto as deleções do tipo 1 como as do tipo 2 são recorrentes porque os pontos de quebra dessas mutações em pacientes não relacionados estão localizados na mesma região. Um terceiro tipo de microdeleção recorrente também ocorre, a do tipo 3, que tem pontos de quebra caracterizados entre as regiões LCR NF1-REP B e NF1-REP C (Pasmant *et al*., 2010). Esse tipo de microdeleção abrange 1.0Mb e 9 genes codificantes (Figura 3) e é a menos frequente, contando com apenas 1.4–4 % de todas as microdeleções de *NF1* (Pasmant *et al.*, 2010). Além desses três tipos de microdeleções recorrentes, deleções atípicas de *NF1* têm sido descritas e não possuem pontos de quebra recorrentes, sendo, portanto, heterogêneas em termos de tamanho, posição dos pontos de quebra e número de genes localizados dentro da região deletada. Aproximadamente 8–10% das microdeleções de *NF1* são atípicas (Pasmant *et al*., 2010).

A recombinação homóloga não-alélica é o mecanismo de ocorrência das microdeleções recorrentes do tipo 1, 2 e 3. Esse mecanismo utiliza um molde não alélico, mas muito similar, para reparar quebras de fitas duplas no DNA (Sasaki *et al.*, 2010). Durante a meiose ou mitose, a recombinação homóloga é um mecanismo preciso de reparo de lesões de dupla fita no DNA; entretanto, regiões cromossômicas que possuem múltiplas sequências duplicadas, como a região do gene *NF1,* são propensas a sofrerem rearranjos mediados pela recombinação homóloga. Entre as sequências duplicadas localizadas perto do gene *NF1,* estão as regiões LCRs (NF1-REPs), que são regiões repetidas de várias subunidades de sequências parálogas (Figura 3). NF1-REPs A e C contêm sequências parálogas muito similares às localizadas no cromossomo 19p13.12, derivadas do gene *LPHN1* (Forbes *et al.*, 2004).Outro grupo de sequências

localizadas nas três NF1-REPs são sópias de pseudogenes do gene funcional *LRRC37B*, o qual está localizado dentro da NF1-REPc. Além disso, as NF1-REPs A e B possuem fragmentos de pseudogenes do gene *SMURF2* (Figura 3). A homologia entre algumas dessas sequências parálogas excede 95%, o que, em conjunto com a distância dessas sequências, permite o pareamento ectópico e recombinação homóloga não-alélica entre elas. Enquanto as microdeleções dos tipos 1 e 3 são, na maioria, de origem meiótica, a maioria das microdeleções do tipo 2 ocorrem durante o desenvolvimento embrionário (antes da gastrulação) e são mediadas pela recombinação homóloga não-alélica mitótica (Roehl *et al.*, 2012). Por isso, esse tipo de microdeleção pode ser associado com mosaicismo em NF1. Os mecanismos que levam a ocorrência das microdeleções atípicas são menos conhecidos. Poucas mutações desse tipo tiveram os pontos de quebra caracterizados a nível molecular. Parece que a junção de extremidades não-homóloga é o mecanismo principal de ocorrência dessas microdeleções.

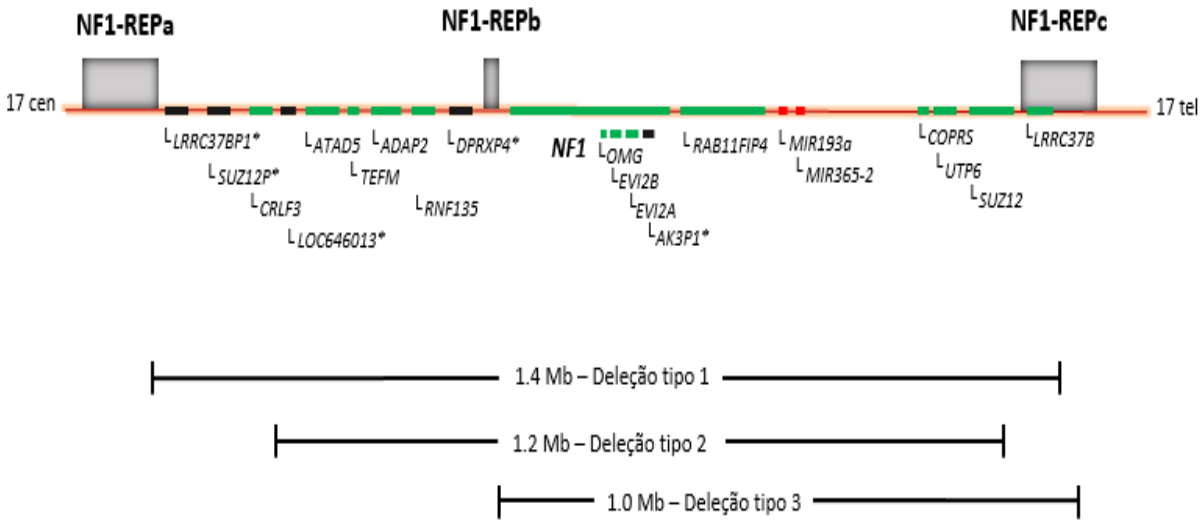

Figura 3. Representação esquemática da região do gene *NF1*. As posições relativas das três NF1-REPs estão indicadas, bem como os genes codificantes localizados na região (barras verdes) e os dois genes de micro-RNAs (MIR365-2 and MIR193a), mostrados em barras vermelhas. Os tamanhos relativos das microdeleções tipo 1, tipo 2 e tipo 3 também estão demonstrados. Adaptado de Upadhyaya e Cooper, 2012.

Todas as mutações descritas acima, com seus diferentes mecanismos de ocorrência, podem ser herdadas ou ocorrer esporádicamente em casos de NF1. Nos

casos de NF1 familiar, a mutação é herdada de um dos pais; entretanto, aproximadamente 50% dos casos de NF1 são esporádicos e causados por mutações *de novo* em *NF1* (Evans *et al.,* 2010). Uma porção dessas mutações*de novo* é somática, resultando em mosaicismo. O mosaicismo pode se manifestar de maneira localizada ou como NF1 segmentar, caracterizada por sinais de NF1 em apenas algumas áreas do corpo. O mosaicismo da linhagem germinativa parece ser raro em NF1, já que poucas famílias com mais de uma criança afetada que nasceram de pais não afetados foram descritas (Lázaro *et al*., 1994). Já na NF1 segmentar, o evento mutacional parece ocorrer em uma fase mais tardia do desenvolvimento embrionário, o que explicaria o fenótipo observado em apenas um segmento específico do corpo, como, por exemplo, em apenas metade do corpo. A NF1 segmentar é rara, com prevalência estimada 15 vezes menor que a de NF1 (Listernick *et al.* 2003). Os casos de NF1 segmentar necessitam de análise molecular, especialmente quando também há indícios de traços não associados a NF1.

A análise de mutações no gene *NF1* tem sido considerada muito complexa pelo grande tamanho do gene, presença de múltiplos pseudogenes com alta homologia de sequência, a falta de *hotspots* para mutações e o espectro mutacional complexo da doença, incluindo mutações não usuais em sítios de processamento de RNA mensageiro (p.ex. mutações que afetam os reforçadores de processamento). Até o momento, mutações em *NF1* têm sido detectadas com a utilização de combinações de técnicas que consomem tempo, são trabalhosas e caras. Com o surgimento de tecnologias de sequenciamento de nova geração, o diagnóstico molecular de NF1 pode se tornar mais fácil, inclusive na detecção de mosaicismos. Alguns grupos já iniciaram a validação de métodos de nova geração no diagnóstico molecular de NF1. A Tabela 2 mostra alguns estudos que avaliaram mutações germinativas em todo gene *NF1*em diferentes países.

Tabela 2. Tipo e frequência de mutações germinativas no gene *NF1* em alguns estudos de séries de casos em diferentes populações do mundo.

População	N	Mutações de ponto (missense/nonsense)	Pequenos rearranjos	Grandes rearranjos	Mutações em sítios de processamento	Pacientes sem mutação	Métodos utilizados	Referência
Europa								
França	565	NA	NA	22	NA	-	MLPA, aCGH	Imbard *et al*., 2015
Eslováquia	108	10/11	33	2	14	8	cDNA sequencing, MLPA	Nemethova *et al*., 2013
Hungria	7	3/1	0	NA	0	3	NGS - Ion Torrent	Balla *et al*., 2014
Reino Unido	169	16/30	30	18	15	60	FISH, DHPLC, MLPA	Griffiths *et al*., 2007
Bélgica	67	6/25	12	2	19	3	PTT, FISH, Southern blot	Messiaen *et al*., 2000
Espanha	56	3/17	27	6	0	3	RT-PCR, DHPLC, MLPA	Valero *et al*., 2011
Holanda	1985	115/346	423	62	232	807	FISH, SSCP/ Sanger, MLPA	van Minkelen *et al*., 2013
França	279	28/70	70	20	58	33	NGS - Ion Torrent	Pasmant *et al*., 2014
Alemanha	521	29/84	140	-	25	243	PTT, TGGE, Sanger	Fahsold *et al*., 2000
Ásia								
Japão	86	13/30	19	10	8	6	NGS - Illumina	Maruoka *et al*., 2014
China	100	12/21	28	13	15	11	MLPA, Sanger	Zhang *et al.*, 2015
Coréia do Sul	60	12/18	7	4	11	8	RT-PCR, MLPA, FISH	Min Ko *et al*., 2013
Taiwan	107	11/20	23	5	9	39	DHPLC, RT-PCR, Sanger	Lee *et al*., 2006

MLPA = Multiplex Ligation-dependent Probe Amplification; aCGH = array Comparative Genomic Hybridization; NGS = Next generation Sequencing; FISH = Fluorescent *in situ* Hybridization; PTT = Protein Truncation test; DHPLC = Denaturing High Performance Liquid Chromatography;SSCP = Single Strand Conformation Polymorphism; TGGE = Temperature-Gradient Gel Electrophoresis; NA = Não avaliado.

Além dos estudos que avaliaram a presença de mutações germinativas utilizando amostras de sangue dos pacientes, mostrados na Tabela 2, diversos estudos também avaliaram a presença de mutações somáticas em tumores de pacientes com NF1. Os estudos de séries de casos com ambos os tipos de materiais são realizados principalmente na Europa, como podemos observar. A detecção de mutações pelos diferentes grupos de estudo utiliza combinações de diferentes técnicas, para que diferentes tipos de mutações possam ser detectados. Três grupos já realizaram a validação do sequenciamento de nova geração para NF1, um utilizando a tecnologia Illumina e dois utilizando a tecnologia *Ion Torrent*. Por fim, podemos observar que todos os tipos de mutação são detectados nas diferentes populações, desde mutações de ponto até grandes rearranjos. Uma análise de um maior número de estudos (HGMD) mostra que essas mutações não ocorrem em um *hotspot,* como esperado. Todos os éxons de *NF1* possuem pelo menos uma alteração descrita.

Poucos estudos investigaram correlações genótipo-fenótipo em *NF1* até o momento e dados adicionais de séries de casos de diferentes países são necessários para verificar a utilidade do teste genético na predição do fenótipo. Apenas três correlações claras têm sido observadas entre alelos mutantes particulares e os fenótipos clínicos consistentes. Primeiro, pacientes que possuem microdeleções de*NF1*possuem sintomas mais graves do que os que possuem mutações intragênicas, incluindo grande número e aparecimento precoce de neurofibromas cutâneos, anormalidades cognitivas mais frequentes e mais graves do que a média, mãos e pés grandes, características faciais dismórficas, malformações cardiovasculares e maior incidência de tumores malignos da bainha dos nervos periféricos (Mautner *et al*., 2010; Pasmant *et al*., 2011). Esses sintomas podem estar associados aos outros genes deletados nesses casos, o que ainda é pouco estudado. As outras duas correlações descritas envolvem uma deleção de 3pb na extremidade do éxon 17 que confere menor risco de desenvolvimento de neurofibromas cutâneos ou plexiformes (Upadhyaya *et al.,* 2007) e os indivíduos com a duplicação de todo o locus *NF1,* que não têm o fenótipo NF1 clássico, mas podem ter deficiência mental e convulsões (Grisart *et al.,* 2008). Além disso, Alkindy e colegas (2012) encontraram uma possível quarta correlação genótipo-fenótipo, com pacientes que possuem mutações em sítios de processamento apresentando uma tendência a desenvolverem gliomas e tumores malignos da bainha de nervos periféricos. Sharif ecolegas (2011) também encontraram uma tendência dos pacientes que apresentam gliomas da via óptica possuírem mutações na primeira terça parte do gene *NF1*.A

extrema variabilidade clínica de NF1 sugere que os eventos aleatórios são também importantes na determinação do fenótipo dos indivíduos afetados.É provável que a variabilidade clínica de NF1 resulte de uma combinação de fatores genéticos, como alterações não patogênicas e polimorfismos no gene *NF1* ou em outros genes que possam influenciar no fenótipo, além de fatores ambientais. Tal complexidade torna a correlação genótipo-fenótipo difícil.

2.2 Neurofibromatose tipo 2

A neurofibromatose tipo 2 ouNF2 (anteriormente conhecida como neurofibromatosecentral) é o tipo de neurofibromatose menos comum, causado por mutações de perda de função em heterozigose no gene *NF2*, um gene que também atua como um supressor tumoral.

2.2.1 Epidemiologia

A NF2 (OMIM #101000) é em torno de 10 vezes mais rara que a NF1, afeta ambos os sexos e todas as etnias. Há apenas dois estudos epidemiológicos em NF2: um na Inglaterra (Evans *et al*., 1992a) e um na Finlândia (Antinheimo *et al*., 2000). A incidência de NF2 foi inicialmente reportada em 1:33-40.000 indivíduos em uma população de 4 milhões de ingleses. A prevalência da doença é bem menor (1:200.000), mas aumentou para 1:60.000 devido ao diagnóstico precoce e melhora no tratamento. Uma incidência de 1:87.410 foi reportada em uma população de 1,7 milhões de indivíduos da Finlândia. Os primeiros sinais da doença aparecemna segunda ou terceira década de vida, e a média de sobrevivência após o diagnóstico é de 15 anos. No Brasil, não há dados sobre incidência, prevalência, mortalidade e expectativa de vida de pacientes com NF2.

2.2.2 Sintomatologia e diagnóstico

O diagnóstico clínico de NF2 é baseado nos critérios de diagnóstico delineados pelo *NIH* na mesma Conferência de Consenso que definiu os critérios de diagnóstico clínico para NF1 em 1987 (Quadro 2) (Stumpf *et al*., 1988).Pacientes com NF2 têm predisposição a desenvolver múltiplos tumores benignos,incluindo schwannomas, meningiomas e ependimomas. Embora esses tumores sejam histologicamente benignos,

a sua localização anatômicatorna o manejo difícil e os pacientes sofremgrande morbidade emortalidade.Evans e colegas (1992a, 1992b e 1992c) descreveram duas formas de NF2. A forma mais grave é diagnosticada em idade mais jovem com múltiplos tumores que se tornam rapidamente agressivos; a forma mais leve, com diagnóstico em idade tardia é limitada ao aparecimento de schwannomas vestibulares.

Quadro 2. Critérios de diagnóstico clínico de NF2 segundo o *National Institutes of Health*.

1 – Massas bilaterais do oitavo nervo vistas com técnicas de imagem apropriadas (ex: tomografia ou ressonância magnética), ou

2 – Um familiar de primeiro grau com NF2 e ainda

1. Massa unilateral no oitavo nervo, ou
2. Dois dos seguintes:

- Neurofibroma
- Meningioma
- Glioma
- Schwannoma
- Opacidade lenticular subcapsular posterior juvenil

Os schwannomas são os tumores mais comuns na NF2; o desenvolvimento de schwannomas nos ramos vestibulares de ambos os oitavos nervos cranianos é um sinal clássico de NF2. A compressão do nervo vestíbulo-coclear pelo schwannoma pode resultar em zumbido no ouvido, além da perda de audição e equilíbrio. Além dos schwannomas vestibulares, schwannomas que ocorrem na medula espinhal e nervos periféricos também ocorrem. Embora esses tumores estejam localizados fora das fibras nervosas, eles produzem sintomas através da compressão do tecido nervoso adjacente. Assim, o crescimento progressivo dos tumores também pode causar convulsões ou déficit neurológico. As células de Schwann, a origem dos schwannomas relacionados à NF2, estão em contato direto com os axônios, sugerindo que os axônios também contribuam para a atividade tumorigênica das células de Schwann.

Os meningiomas são o segundo tipo mais frequente de tumor em NF2 e são encontrados em 45 a 58% dos pacientes (Asthagiri *et al*., 2009), sendo frequentemente múltiplos e com localização cranial (podendo se desenvolver inclusive sobre o nervo óptico) ou espinhal. Em torno de 8 a 10% dos meningiomas retirados de pacientes com NF2 são espinhais. Os meningiomas são tumores benignos que provavelmente se originam das células aracnoidais nas meninges. A presença dos meningiomas é um

marcador de gravidade de doença, sendo que o risco relativo de mortalidade é 2.5 vezes maior em pacientes com essas lesões do que em pacientes sem essas lesões. Baser e colegas (2002) analisaram fatores associados à mortalidade em 368 pacientes com NF2 do Reino Unido; a idade ao diagnóstico, presença de meningiomas intracranianos e especialização do centro do tratamento foram informativos na predição de risco de mortalidade. O risco relativo de mortalidade foi 2.51 vezes maior em pacientes com meningiomas comparados com os sem meningiomas. Nos pacientes pediátricos, os meningiomas são frequentemente o primeiro sinal de NF2 (Ruggieri *et al*., 2005).

Lesões de medula espinhal ocorrem em 90% dos pacientes com NF2 (Mautner *et al*., 1996) e incluem schwannomas, meningiomas e ependimomas. Ependimomas são tumores intramedulares de células gliais, mais frequentemente localizados na coluna cervical.Lesões não-tumorais de NF2 incluem a polineuropatia periférica (Drouet *et al*., 2005) e manifestações cutâneas e oftalmológicas. Pelo menos 70% dos pacientes com NF2 desenvolvem lesões de pele: as mais comuns são as lesões semelhantes a placas intracutâneas, que são pigmentadas, em relevo mais alto e frequentemente com pêlos. Em camadas mais profundas da pele, tumores nodulares geralmente correspondem a schwannomas (Evans *et al*., 1992). O exame oftalmológico pode mostrar catarata posterior subcapsular juvenil e membranas epiretinais, achados sugestivos de NF2 (McLaughlin *et al*., 2007). Os pacientes que sofrem da neuropatia periférica podem sofrer de hipoalgesia e hipestesia, além da perda de sensações de vibração. Em um estudo, a neuropatia periférica ocorreu em 47% dos pacientes investigados (Sperfeld *et al*., 2002).

Uma desordem genética e clinicamente distinta de NF2 é a schwannomatose, caracterizada por múltiplos schwannomas sem envolvimento do nervo vestibular (MacCollin *et al*., 2003). Alguns pacientes com essa desordem preenchem os critérios diagnósticos para NF2, o que pode causar confusão.O produto do gene *INI1/SMARCB1* é uma subunidade do complexo de remodelamento da cromatina SWI/SNF, que atua na trasncrição de genes e foi implicado na patogênese da schwannomatose em dois membros de uma família com shwannomatose familial (Hulsebos *et al*., 2007). O diagnóstico molecular envolvendo a análise dessa região, além do gene *NF2*, é útil para o estabelecimento do diagnóstico correto em pacientes com sintomas do espectro dessas doenças e para que o acompanhamento adequado tenha seguimento. Após o diagnóstico clínico ou molecular, todos pacientesdevem ser submetidos a uma série de avaliações por uma equipe multidisciplinar para acompanhamento da progressão e gravidade da

doença. Não hácura paraa NF2 eo tratamento também é focado namelhoria dos sintomasclínicos. As cirurgias permanecem sendo o principal tratamento para meningiomas que estão crescendo ou são sintomáticos. A radioterapia também pode ser indicada em casos de múltiplos tumores. Wentworth e colegas (2009) avaliaram o uso de radiação em uma série de 49 meningiomas em 12 pacientes com NF2. Entretanto, a rádiocirurgia e radioterapia podem induzir a formação de tumores malignos, e seu uso é controverso.Novas drogas promissoras têm sido identificadas como candidatas para tratamentos de meningiomas associados a NF2 (Evans *et al*., 2009) e têm sido avaliadas em estudos clínicos após a validação em modelos pré-clínicos (Kalamarides *et al*., 2002; 2008). Para os schwannomas vestibulares, o Bevacizumab, um anticorpo monoclonal anti-VEGF, é a primeira linha de tratamento. Plotkin e colegas (2009) reportaram uma redução no volume na maioria dos schwannomas vestibulares após tratamento com Bevacizumab em um grupo de 10 pacientes com NF2. Na maior série de casos estudada, 57% dos indivíduos apresentaram melhora auditiva e 55% tiveram diminuição do volume dos tumores após o tratamento (Plotkin *et al*., 2012).

2.2.3 O gene *NF2* e a proteína Merlina

O gene *NF2* é um gene supressor tumoral que foi identificado no cromossomo 22q12 através de clonagem posicional e abrange 95.045pb com 17 éxons (Rouleau *et al*., 1993). Poucas informações estão disponíveis sobre a região promotora do gene *NF2*. A região 5'UTR do gene possui 443pb e a 3'UTR possui 3858pb; essas regiões também são pouco estudadas.Várias isoformas resultantes de processamento alternativo foram descritas para esse gene (Arakawa *et al.*, 1994). A isoforma I, sem o éxon 16 (NCBI - NM_000268.3) e a isoforma II, contendo todos os éxons (NCBI - NM_016418.5) são as predominantes. A Figura 4A mostra de forma esquemática os 17 éxons do gene *NF2*, que codificam a isoforma mais comum, de 595 aminoácidos, chamada merlina (proteína semelhante à moesina-ezrina-radixina), pertencente à superfamília de proteínas banda 4.1. A isoforma I, sem o éxon 16, possui 590 aminoácidos. Todas as isoformas de merlina contêm um domínio N-terminal chamado FERM (resíduos19-314) seguido por uma região de α-hélice em espiral (314-492) e um domínio globular C-terminal (492-595) (Arakawa *et al.*, 1994). NaFigura 4B estão representados os domínios funcionais da merlina. Essa proteína possui massa molecular de aproximadamente 66kDa e é expressa constitutivamente em todos os tecidos durante todos os períodos de

desenvolvimento embrionário (Gutmann *et al*., 1995). A deleção em homozigose da merlina em camundongos leva a perda do embrião, ainda antes da gastrulação, indicando a importância da merlina durante os primeiros estágios do desenvolvimento. Em adultos, a expressão de merlina se mantém elevada no cérebro. Além disso, diversos estudos reportaram a expressão de merlina em diferentes tipos de células neuronais, tanto do sistema nervoso periférico quanto do sistema nervoso central. Através de diferentes técnicas de imagem, imunohistoquímica e hibridização *in situ*, a merlina tem sido detectada nos axônios do nervo ciático e em neurônios do trato intestinal no sistema nervoso periférico (Schulz *et al*., 2013) e em neurônios motores da medula espinhal, neurônios corticais, neurônios do hipocampo e nas células de Purkinje do cerebelo no sistema nervoso central (Schulz *et al*., 2010; Claudio *et al*., 1995). Em nível subcelular, a merlina neuronal é expressa em dendritos, axônios, citoplasma e em junções sinápticas. Alguns estudos sugerem que a localização subcelular da merlina é crítica para a sua função. Quanto às isoformas, as duas principais são expressas de forma equivalente, mas a isoforma II é um pouco mais prevalente (Chang *et al*., 2002). Entretanto, a isoforma II tem baixa expressão no oitavo nervo craniano, onde os principais schwannomas relacionados à NF2 ocorrem (Bianchi *et al.*, 1994).

Os membros da superfamília de proteínas banda 4.1, um grande grupo de proteínas citoplasmáticas associadas à membrana, incluem a proteína 4.1, a talina, a merlina, um grupo de três proteínas conhecidas como proteínas ERM (ezrina, radixina e moesina), diversas fosfatases e pelo menos duas miosinas não-musculares (McCartney and Fehon, 1997). Uma característica dessa superfamília é uma região conservada de 200-300 aminoácidos geralmente localizada na porção N-terminal da proteína. A homologia entre a merlina e as proteínas ERM é maior na região N-terminal (61%). Enquanto a porção N-terminal das proteínas ERM tem alta homologia, a região de α-hélice e a C-terminal são menos conservadas (Figura 4B). Os membros dessa superfamília de proteínas dividem algumas propriedades funcionais, incluindo a capacidade de heterodimerização (Gronholm *et al.*, 1999). A merlina possui capacidade de formar homodímeros, bem como heterodímeros com membros da família ERM. Entretanto, a merlina é o único membro que possui propriedades de inibição de crescimento celular (supressão tumoral). Como a região C-terminal é a menos conservada em relação às outras proteínas da família ERM, a diferença funcional da merlina pode ser determinada por essa região.

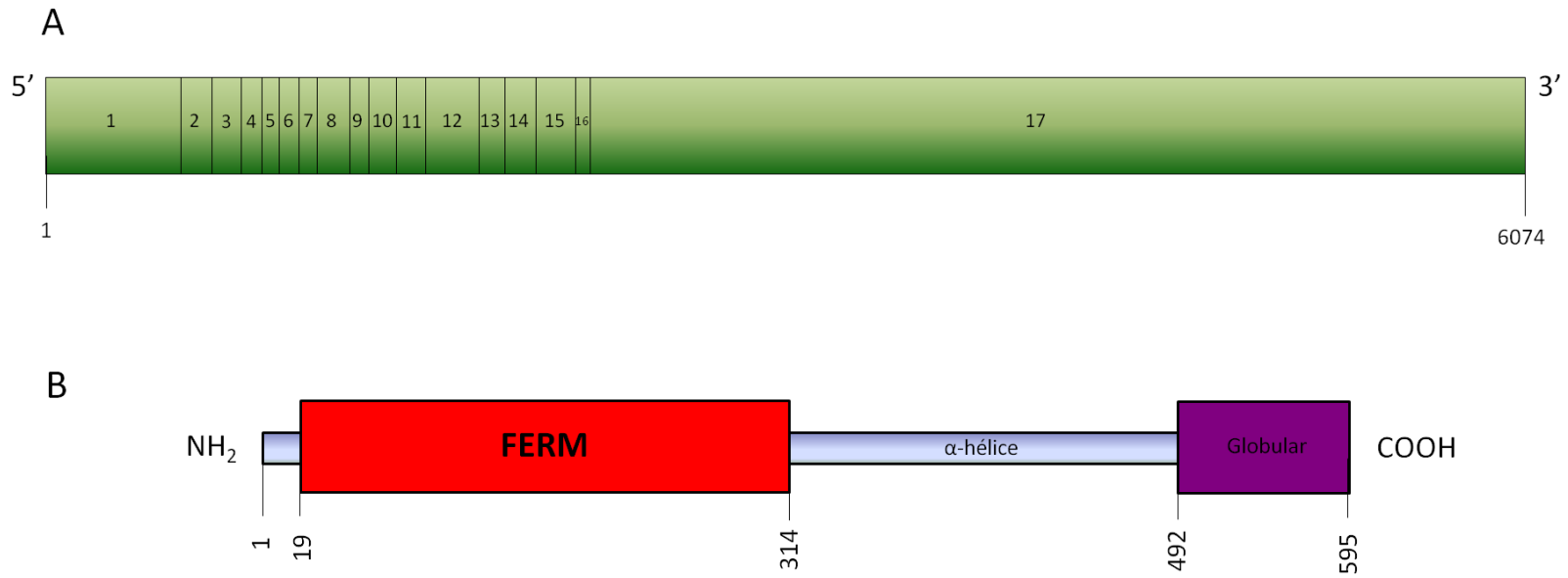

Figura 4. **Esquema do transcrito e proteína de NF2.A.** Esquema do transcrito maior do gene*NF2* (NM_016418.5) com 6074pb e com todos os 17 éxons codificantes em verde, representados em tamanho proporcional ao número de bases de cada éxon.**B.** Esquema representando a proteínamerlina com seus domínios funcionais. **Domínios da merlina: domínio FERM (conservado nas proteínas da superfamília de proteínas anda 4.1); região de α-hélice; domínio globular (região menos conservada).**

As proteínas ERM clássicas, que consistem em um domínio FERM N-terminal, um domínio enrolado e um segmento C-terminal que liga filamentos de actina, se intercalam entre a conformação fechada e a conformação aberta. A conformação aberta medeia aligação de receptores de adesão celular aos filamentos de actina de citoesqueleto. Entretanto, a merlina não possui um motivo canônico de ligação a actina exibido pela conformação aberta das proteínas ERM. Porém, tanto a merlina quanto as outras proteínas da família são capazes de formar interações intramoleculares pela ligação da extremidade N-terminal a C- terminal da própria proteína (Gronholm *et al.*, 1999), formando a conformação fechada. Esse dobramento intramolecular que parece controlar a atividade da merlina é regulado pela fosforilação da serina 518, catalisada pela quinase ativada p-21 (Kissil *et al*., 2002). A proteína desdobrada e fosforilada éincapaz de inibir o crescimento celular, enquanto a forma fechada e desfosforilada é a forma supressora tumoral ativa (Morrison *et al*., 2001). Há uma diferença na capacidade de formação do dobramento intramolecular nas duas isoformas principais da merlina: a isoforma II possui a porção C-terminal hidrofílica e carregada positivamente, enquanto a isoforma I é menos hidrofílica e não possui carga. Como consequência, a extremidade C-terminal da isoforma I se liga fortemente a extremidade N-terminal do domínio FERM, enquanto a isoforma II apresenta apenas uma ligação fraca. Por essa razão, parece que somente a isoforma I possui atividade de supressão tumoral (Sherman *et al*., 1997), pois consegue manter a conformação fechada. Ainda, devido a essa diferença, as duas isoformas se ligam a diferentes proteínas; até o momento, somente a sistenina, uma proteína adaptadora envolvida no tráfego intracelular de receptores, parece interagir especificamente com a porção C-terminal da isoforma I (Jannatipour *et al*., 2001). Embora a merlina seja implicada nesse tráfego de receptores, um papel funcional específico da porção C-terminal da isoforma I ainda não foi descrito. Apesar das diferenças na estrutura C-terminal, ambas as isoformas têm funções sobrepostas, pois a porção N-terminal é envolvida na regulação de vias celulares. Por fim, além da serina 518, outros três sítios de fosforilação da merlina foram descritos: serina 10, treonina 230 e serina 315. Em resposta ao EGF (*epidermal growth factor*) ou IGF (*insulin growth factor*), a proteína Akt fosforila a merlina nas posições treonina 230 e serina315, o que direciona a merlina para a degradação proteossômica (Tang *et al*., 2007).

Tradicionalmente, as proteínas da superfamília de proteínas 4.1 processam sinais oriundos da matriz extracelular e transmitem esses sinais para dentro da célula. Assim, três mecanismos principais para inibir o crescimento celular são mediados pela merlina:

inibição de crescimento dependente de contato, diminuição da proliferação e aumento da apoptose.A merlina medeia a inibição por contato da proliferação de múltiplos tipos celulares, incluindo as células de Schwann (Morrison *et al*., 2001) e tem como alvos alguns componentes de vias de sinalização que restringem a proliferação. Além disso, a atividade de supressão tumoral da merlina acontece em diferentes compartimentos sub-celulares, incluindo o núcleo da célula (Muranen *et al*., 2005), a membrana plasmática (Morrison *et al*., 2001), os endossomos (Scoles *et al*., 2000) e até mesmo em associação com o fuso mitótico durante a mitose (Muranen *et al*., 2007). A Figura 5 mostra a via de sinalização envolvida no mecanismo de inibição de crescimento por contato da merlina. Quando muitas células estão presentes, a adesão celular mediada pela caderina diminui o recrutamento de Rac a membrana plasmática einibe PAK, causando um acúmulo da forma desfosforilada (fechada) e inibidora de crescimento da merlina(Okada *et al*., 2005). Por outro lado, quando poucas células estão presentes, a adesão celular dependente de integrinas ativa PAK, causando a inativação da merlina, o que remove o bloqueio de ciclo celular. A inativação da merlina induz a saída da inibição por contato (Okada *et al*., 2005) e acelera a progressão do ciclo celular através de G1. Esses estudos mostram que a merlina integra sinais opostos de caderinas e integrinas para regular a progressão do ciclo celular. Essa integração de sinais opostos regula, por sua vez, diversas GTPases, como a familia de GTPases Rho e da via Hippo (Yin *et al*., 2013). Através do rearranjo dos filamentos de actina, a família de GTPases Rho é essencial para o desenvolvimento de células altamente polarizadas, como os neurônios (Luo *et al*., 2000). Portanto, os reguladores dessas GTPases, como a merlina, tem interesse especial no campo da neuromorfogênese. A via Hippo, primeiramente descrita em drosófilas, regula o tamanho dos órgãos através do balanço do crescimento e morte celular. Informações dessa via são transmitidas para o núcleo da célula. Em células inibidas pelo contato, a via Hippo é ativada, enquanto em células espaçadas, a via está inibida. Alguns tipos de tumores são associados com a desregulação dessa via. Além disso, a ativação da merlina inibe mTORC1, um complexo que regula a tradução de proteínas na célula, de forma independente de Akt, bem como inibe as vias Ras-ERK, PI3K-Akt e FAK-Src (Ammoun *et al*., 2008; Jin *et al*., 2006a; Poulikakos *et al*., 2006; Rong *et al*., 2004). Por fim, a forma fechada da merlinapode se deslocar diretamente para o núcleo da célula, se ligar àubiquitinaligaseE3CRL4^{DCAF1}e suprimira sua capacidade deubiquitinarproteínas alvo. A inibição dessa ubiquitina ligase é essencial

para induzir uma parada no crescimento celular e suprimir a formação de tumores (Li *et al*., 2010).

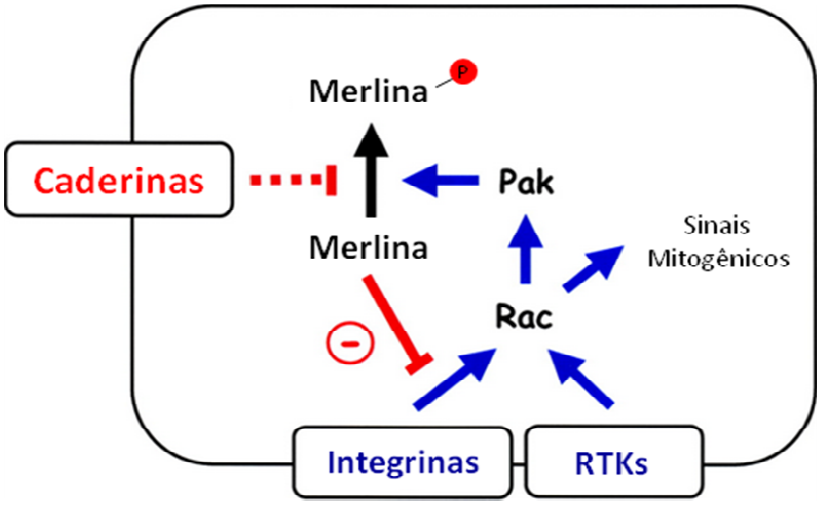

Figura 5. Modelo hipotético da função da merlina.A adesão celular iniciada pelas caderinas inibe a ativação de Pak em células inibidas pelo contato e causa acúmulo de merlina desfosforilada (forma fechada). Essa forma fechada suprime o recrutamento de Rac mediado por integrinas, e, consequentemente, inibe sinais mitogênicos. Após o fim da inibição por contato, Pak fosforila e inativa a merlina, permitindo o recrutamento de Rac para a membrana, ativando sinais mitogênicos que antes estavam inibidos. Adaptado de Okada *et al*., 2005.

Além dos mecanismos de inibição por contato, Kim e colegas (2004) mostraram que a merlina pode induzir a apoptose através da superexpressão da proteína supressora tumoral p53. Outros estudos mostraram que a merlina pode inibir a progressão do ciclo celular através da supressão da expressão de ciclina D1 (Xiao *et al*., 2005). A merlina também pode reduzir a proliferação através da interação com CD44. Entretanto, a contribuição de cada uma das vias da merlina na tumorigênese ainda não foi definida. Outros fatores que podem afetar as vias de sinalização que envolvem a merlina são fatores que podem alterar a expressão da própria proteína, como as alterações epigenéticas, estabilidade do transcrito e modificações pós-traducionais. A metilação do promotor é a única alteração epigenética que tem sido associada com alterações na expressão da proteína. Evidências são inconclusivas quanto ao papel da estabilidade de RNA mensageiro na progressão tumoral e quais mecanismos são importantes para a estabilidade do transcrito (microRNAs ou eventos mutacionais).

2.2.4 Mutações no gene *NF2* e correlações genótipo-fenótipo

A NF2 é causada por mutações no gene supressor tumoral*NF2*.Assim como na NF1, para que os tumores benignos ou malignos associados a doença apareçam é necessária a inativação dos dois alelos de*NF2*, levando à inativação funcional total da merlina, seguindo o modelo de dois eventos descrito por Knudson (1971).

A penetrância estimada da NF2 é maior que 95%, chegando a 100% aos 60 anos de idade. A taxa de mutação do gene *NF2* é estimada em 6.5×10^{-6},uma taxa bem menor do que a estimada para o gene *NF1*(Evans *et al*., 1994). Já foram identificadas 406 mutações diferentes no gene *NF2*, descritas no banco de dados *The Human Gene Mutation Database* (HGMD - http://www.hgmd.cf.ac.uk). Essas mutações são de vários tipos, envolvendo desde mutações de ponto até grandes deleções e rearranjos que envolvem milhares de bases (Tabela 3). A maioria das mutações de *NF2* são mutações pequenas (80%), como substituições de uma única base, pequenas inserções e pequenas deleções. Outras mutações são deleções ou duplicações de um ou mais éxons (18%) e rearranjos complexos, que ocorrem em menor frequência (1,2%).O mecanismo de ocorrência de grandes rearranjos parece ser a recombinação, promovida pela instabilidade da região genômica de *NF2*. Já foram descritas a recombinação homóloga não-alélica promovida por sequências *Alu* e a junção de pontas não-homólogas no gene.

Tabela 3. Mutações descritas no gene *NF2* no banco de dados HGMD (*The Human Mutation Database*). Acesso em junho/2016.

Tipo de mutação	Número de mutações diferentes
Sentido trocado/ sem sentido	93
Sítios de processamento	86
Pequenas deleções	106
Pequenas duplicações	35
Indel	8
Grandes deleções	65
Grandes inserções	8
Rearranjos complexos	5
Total	**406**

Como pode ser observado na tabela acima, diferentes tipos de mutações têm sido descritos. A frequência de mutações em sítios de processamento é alta em *NF2*, o que reforça a necessidade do estudo de regiões intrônicas em pacientes com esta doença. As diferentes mutações de *NF2* podem ser herdadas ou esporádicas. Nos casos de NF2 familiar, a mutação é herdada de um dos pais; entretanto, aproximadamente 50% dos casos de NF2 são esporádicos e causados por mutações *de novo* em *NF2* (Evans *et al*., 1992a). Estudos prévios têm demonstrado que pelo menos 25-33% dos casos esporádicos são mosaicos com mutações detectadas apenas nos tumores e não no DNA extraído de sangue periférico, com uma alta estimativa de ocorrência em pacientes com schwannoma vestibular unilateral (Evans *et al*., 2007). Até o momento, mutações germinativas em *NF2* têm sido detectadas com a utilização de combinações de técnicas que consomem tempo, são trabalhosas e caras. Os mosaicismos são mais difíceis de detectar nas análises moleculares. Com o surgimento de tecnologias de sequenciamento de nova geração, o diagnóstico molecular de NF2 pode se tornar mais fácil, inclusive nos mosaicos, que podem ser detectados utilizando uma alta cobertura nas metodologias de nova geração, mesmo em DNA genômico. A Tabela 4 mostra alguns estudos que avaliaram mutações germinativas em todo gene *NF2* em quatro países.

Tabela 4. Tipo e frequência de mutações germinativas no gene *NF2* em alguns estudos de séries de casos em diferentes populações do mundo.

População	N	Mutações de ponto (missense/nonsense)	Pequenos rearranjos	Grandes rearranjos	Mutações em sítios de processamento	Pacientes sem mutação	Métodos utilizados	Referência
Europa								
Polônia	12	2/0	3	NA	0	7	CE-SSCP, Sanger	Laniewski-Wollk e*t al*., 2008
Reino Unido	125	6/22	15	5	6	71	PCR-SSCP	Evans *et al*., 1998
América								
EUA	32	0/10	2	1	7	11	PCR-SSCP	Parry*et al*., 1996
Ásia								
Coréia do Sul	7	1/0	1	0	2	3	Sanger, MLPA	Seong *et al*., 2010

NA=Não avaliado; **CE-SSCP**=Capillary Electrophoresis-Single Strand Conformation Polymorphism; **SSCP**=Single Strand Conformation Polymorphism; **MLPA**=Multiplex Ligation-dependent Probe Amplification.

Além dos estudos mostrados na tabela 4, outros trabalhos de rastreamento de mutações no gene *NF2* foram realizados na Europa, Ásia e América (HGMD). As populações estudadas são menores que as de NF1, devido à raridade da NF2, além de haver um número menor de estudos disponível. Tanto os estudos recentes quanto os mais novos possuem baixa taxa de detecção de mutações em DNA de sangue periférico, provavelmente devido aos mosaicismos. Os estudos mais antigos também possuem as limitações técnicas; os grandes rearranjos gênicos, por exemplo, não eram facilmente detectados e muitas vezes não eram pesquisados. As regiões regulatórias de *NF2* também são pouco exploradas e não há mutações no promotor e regiões não traduzidas descritas. A análise dessas regiões também pode aumentar a taxa de detecção de mutações no gene. Entre as regiões que já foram analisadas, alterações patogênicas têm sido descritas apenas nos éxons 1-15 e nenhuma alteração foi descrita nos éxons que sofrem processamento alternativo (éxons 16 e 17). Entretanto, nenhuma mutação patogênica que afete especificamente uma das isoformas foi descrita; essas mutações sempre inativam ambas as isoformas. Nos éxons 1-15, nenhum local propenso ao surgimento de mutações foi encontrado, e a maioria das mutações germinativas observadas são mutações de perda de sentido, troca de fase de leitura ou que alteram um sítio de processamento. Além disso, a maioria das mutações de sentido trocado descritas, que ocorrem em menor número de pacientes, parecem romper a conformação fechada da merlina, sendo consideradas patogênicas.

Além dos estudos que avaliaram a presença de mutações germinativas utilizando amostras de sangue dos pacientes, mostrados na Tabela 4, diversos estudos também avaliaram a presença de mutações somáticas em tumores de pacientes com NF2. A detecção de mutações germinativas e somáticas pelos diferentes grupos de estudo utiliza combinações de diferentes técnicas, para que diferentes tipos de mutações possam ser detectados. Quando a combinação de sequencimaneto e estratégias de detecção de grandes alterações são realizadas, a taxa dedetecção de mutaçõesse aproxima de72% nos casos esporádicos e ultrapassa92% emcasos familiares(Kluwe *et al.,* 2005).

Desde os primeiros relatosdemutações germinativas em*NF2*, tem havido tentativas de estabelecerumaligação entrea mutaçãoespecífica e o fenótipo da dença. A princípio, parece que mutações que produzem uma proteína truncada (mutações sem sentido ou que mudam a fase de leitura da proteína) resultam em um fenótipo mais grave (início precoce da doença e maior número de tumores), enquanto que mutações

em sítios de processamento, de sentido trocado e grandes e pequenas deleções foram associadas com a doença mais leve (Bourn *et al*., 1994). Ainda é questionável se proteínas muito curtas retêm alguma função biológica. Entretanto, essas correlações não são perfeitas e algumas mutações são relacionadas tanto com a froma grave quanto a leve da doença (Kluwe *et al.*, 1995). Além disso, há variabilidade de fenótipos clínicos entre pessoas de uma mesma família, embora a variabilidade seja maior ainda entre indivíduos de famílias diferentes. Dentro de uma mesma família, um fator que pode ser responsável pela variabilidade fenotípica é o mosaicismo na primeira pessoa afetada da família. Em todos os pacientes, alguns marcadores parecem refletir a gravidade de NF2. Um deles é a idade de aparecimento dos sintomas da doença, que parece ser um preditor do curso da doença em estudos longitudinais. A idade do aparecimento dos sintomas e idade ao diagnóstico são os dois preditores mais importantes do crescimento de schwannomas vestibulares em NF2 (Ruttledge *et al*., 1996; Mautner *et al*., 2002) e também do risco de morte em NF2 (Otsuka *et al.*, 2003). Pacientes com mutações que resultam em uma proteína truncada, além do início da doença ser mais precoce, possuem maior prevalência de meningiomas, tumores de medula espinhal, tumores fora do oitavo nervo craniano, lesões cutâneas, zumbido e perda auditiva (Selvanathan *et al*., 2009). Assim, o diagnóstico molecular se torna uma ferramenta muito importante para que esses pacientes tenham acompanhamento e aconselhamento genético apropriados. O espectro de mutações em uma população pode ser muito útil no estabelecimento de uma estratégia mais rápida e barata de diagnóstico genético para a doença. Alternativamente, genes modificadores podem causar variações de fenótipo nos pacientes, e a variabilidade fenotípica também decorre de variáveis estocásticas, epigenéticas e ambientais.

2.3 Esclerose Tuberosa

A esclerose tuberosa ou ET foi inicialmente descrita por von Recklinghausen em 1862, eé uma genodermatose causada por mutações de perda de função em heterozigose nos genes supressores tumorais *TSC1*(OMIM #191100) ou *TSC2*(OMIM # 613254).

2.3.1 Epidemiologia

A esclerose tuberosa pode ser identificada em todos os grupos étnicos eé igualmente identificada em ambos os sexos. Ela é uma das causas genéticas mais comuns de epilepsia. Estudos populacionais têm estimado a prevalência da doença em 1 em 6.000-9.000 indivíduos. O número de americanos afetados com ET é estimado em 40.000, e pelo menos 2 milhões de pessoas são afetadas com ET no mundo (Hyman e Whittemore, 2000). No Brasil, não há dados sobre incidência, prevalência, mortalidade e expectativa de vida de pacientes com a doença.

2.3.2 Sintomatologia e diagnóstico

Em 1908, Heinrich Vogt estabeleceu os critérios de diagnóstico de ET baseados em uma tríade: epilepsia, atraso mental e adenoma sebáceo. Como nenhum desses sinais é patognomônico de ET, os critérios de diagnóstico foram revisados por um consórcio em 1998 (Roach *et al*., 1998), que propôs três categorias de diagnóstico (definitivo, provável e possível ET) dependendo da presença de critérios principais e secundários. O Quadro 3 mostra os critérios de diagnóstico clínico revisados e atualizados para ET, estabelecidos pelo mesmo consórcio em 2012 (Northrup *et al*.,2013).Um diagnóstico clínico definitivo é feito quando duas características principais ou uma característica principal e duas menos frequentes estão presentes. A maioria das características principais está localizada na pele e sistema nervoso central. Além disso, é preciso considerar que as manifestações clínicas da ET podem aparecer em momentos distintos do desenvolvimento e em diferentes idades; portanto, uma pessoa com suspeita de ET pode precisar de múltiplas avaliações sequenciais antes de um diagnóstico clínico definitivo.

A ET é carcterizada pelo desenvolvimento de massas semelhantes a tumores (hamartomas) em uma variedade de órgãos. O envolvimento do cérebro causa manisfestações clínicas como epilepsia, retardo mental, autismo e déficit de atenção e hiperatividade. Outrossistemas comumente e sigificativamente envolvidos incluem a pele, o coraçãoe os rins. As lesõessão frequentemente patognomônicas deET,e incluem angiofibromas faciais, fibroma subungual, rabdomiomas cardíacos e angiomiolipomas e cistos renais.

Quadro 3. Critérios de diagnóstico clínico para esclerose tuberosa (2012).

Principais características
Angiofibromas faciais
Fibroma não-traumático ungueal ou periungueal
Máculas hipomelanóticas (mais de três)
Tubérculos corticais
Nódulo subependimal
Astrocitoma de células gigantes subependimal
Múltiplos hamartomas retinais nodulares
Rabdomioma cardíaco, um ou mais
Linfangiomiomatose
Angiomiolipoma renal
Características menos frequentes
Manchas múltiplas distribuídas pelo esmalte dental
Pólipos retais
Cistos ósseos
Tratos de migração da susbstância branca cerebral
Fibromas gengivais
Hamartoma não-renal
Lesões de pele em “confeti”
Múltiplos cistos renais
ET definitivo: Dois critérios principais ou um principal com dois menos frequentes
Provável ET: Um critério principal e um menos frequente
Possível ET: Um critério princiapal ou dois menos frequentes

Lesões de pele são detectadas em 70% dos pacientes com ET e incluem máculas hipomelanóticas, lesões em confeti, placas fibrosas na testa, angiofibromas faciais e fibromas ungueais e periungueais (Schwartz *et al*., 2007). Dependendo da população estudada, até 100% dos pacientes com ET apresentam máculas hipomelanóticas (Jozwiak *et al*., 1998). Um agregado de pápulas vermelhas que aparecem no nariz e bochechas em formato de borboleta é um sinal clássico de ET. Os angiofibromas faciais (conhecidos como adenomas sebáceos) são lesões de tecido conjuntivo da pele que crescem e formam hamartomas com alta vascularização, e podem resultar em

diminuição da qualidade de vida quando afetam a aparência ou causam sangramentos que podem levar a infecções. Os fibromas ungueais e periungueais são pequenos tumores que crescem em torno e sob as unhas dos pés ou mãos. A idade média de aparecimento é 14,9 anos (Sun *et al*., 2005) e sua prevalência em pacientes mais velhos (acima de 30 anos) é de cerca de 90%. As placas fibrosas na testa aparecemem média aos 14 anos de idade(Jozwiak *et al*., 1998), com idade média de aparecimento sendo 2,6 anos (Sun *et al*., 2005). Histopatologicamente, essas placas contêm tecido fibrótico com menos elementos vasculares do que os angiofibromas faciais.

As complicações neurológicas em ET são tão comuns quando as complicações que envolvem a pele. As três principais lesões intracranianas associadas à ET são os tubérculos corticais, nódulos subependimais e tumores subependimais de células gigantes (Sherpherd *et al.,* 1991).Tubérculos corticais estão presentes em mais de 88% das crianças com ET (Cuccia *et al.,* 2003) e o número médio de tubérculos por paciente varia de 5 a 50, levando à perda da citoarquitetura de seis camadas do córtex cerebral e podendo causar epilepsia. A segunda lesão neurológica mais frequente em ET são os nódulos subependimais, que são pequenos hamartomas localizados nas paredes laterais dos ventrículos. Não há evidências de que eles causem problemas neurológicos; somente os nódulos localizados perto do forâmen de Monro têm potencial de crescer e se tranformar em tumores subependimais de células gigantes, o que acontece em 5-20% dos pacientes. A lesão neurológica menos frequente em ET são os tumores subependimais de células gigantes, que afetam em média 10% das crianças com ET. Esses tumores são benignos e não-infiltrativos, de crescimento lento e com várias células gliais e neuronais, incluindo células gigantes. A proliferação de astrócitos nesses tumores é associada com o crescimento progressivo e compressão do tecido adjacente. As apresentações clínicas mais comuns dos pacientes com esses tumores incluem convulsões, retardo mental, déficit cognitivo, distúrbios visuais, dores de cabeça e vômitos. Alguns tumores de células gigantes podem se tornar malignos e podem infiltrar o tálamo, hipotálamo e gânglios basais, produzindo edema significativo ao redor da lesão. Além disso, devido à sua localização e potencial de crescimento, podem levar a hipertensão intracraniana, hidrocefalia obstrutiva, déficit neurológico focal e até morte súbita (Byard *et al.*,2003). Os tumores de células gigantes ocorrem quase exclusivamente no ventrículo lateral próximo ao forâmen de Monro e raramente em outros sítios, como o quarto ventrículo e terceiro ventrículo (Franz *et al.*, 2012) e são responsáveis por 25% da mortalidade atribuída a ET (Nabbout *et al.,* 1999).

Aproximadamente 90% dos pacientes com ET apresentam epilepsia (Webb *et al.,* 1991) e 50% apresentam déficit cognitivo, autismo ou outro problema de comportamento.

Depois das manifestações no sistema nervoso central e pele, manifestações renais são as mais comuns. Elas incluem carcinoma de células renais, oncocitomas, angiomiolipomas (80% dos pacientes) e doença policística renal (50% dos pacientes) (Dixon *et al*., 2011) e iniciam na infância, com piora com o aumento da idade. Angiomiolipomas podem ser múltiplos e bilaterais, sendo uma das causas de morte em ET, pois destroem o parênquima renal.

O envolvimento pulmonar, especialmente a linfangioleiomiomatose, é a terceira causa mais comum de mortalidade em ET, ocorrendo em 35% das pacientes com ET (envolvimento exclusivo feminino). Essas lesões são causadas pela proliferação atípica de células de músculo liso nos tecidos peribronquial, perivascular e perilinfático do pulmão (Kumasaka *et al.,* 2004). Além disso, a ET também é associada com achados oftalmológicos retinais e não-retinais, sendo os hamartomas retinais o achado oftalmológico mais comum, ocorrendo em aproximadamente 40-50% dos indivíduos. Esses tumores raramente comprometem a visão (Robertson *et al*., 1999).Múltiplos rabdomiomas cardíacos ocorrem em aproximadamente 30% dos pacientes com ET. Esses rabdomiomas geralmente não causam sintomas ou comprometimento hemodinâmico e a história natural dessas lesões é regressão espontânea na grande maioria dos casos. Por fim, hamartomas também podem ocorrer em órgãos do sistema endócrino em pacientes com ET. Alguns detalhes adicionais sobre a sintomatologia na esclerose tuberosa estão descritos no Capítulo IV desta tese. Toda essa variabilidade de fenótipos que podem ocorrer na doença e o provável início tardio de alguns sintomas tornam o diagnóstico clínico muito complicado. Ainda, para todos os critériosclínicos, há chance de que os pacientescomdoençasubclínicanão sejam diagnosticados. Os testes genéticos podemdesempenharum papel fundamentalna confirmação dessesdiagnósticos, e é recomendadoem todos os indivíduosque apresentam suspeitade manifestar esclerose tuberosa. Para isso, uma abordagem para testes genéticos é necessária nas populações afetadas.

2.3.3 Genética da Esclerose Tuberosa

Os genes *TSC1* e *TSC2*estão localizadosnos cromossomos9q34e16p13.3, respectivamente. O gene *TSC1* (NCBI - NM_000368.4) abrangecerca de 53kb deDNA

genômico,com 23éxonsque codificam a hamartina,uma proteína hidrofílica de 1164 aminoácidos e 130kDa. Essa proteína não exibe homologia com nenhuma outra proteína de vertebrados. O gene *TSC2* (NCBI - NM_000548.3) compreendecerca de 40kb deDNA genômico e possui 42 éxons que codificam atuberina, uma proteína de 1807 aminoácidos e 200kDa. Essa proteína contém um domínio N-terminal relativamente hidrofílico (Maheshwar*et al*., 1996) e uma região conservada de 163 aminoácidos codificada pelos éxons 34-38, próxima à porção C-terminal, que possui homologia com as proteínas da superfamília ras GTPases rap1GAP e mSpa1 (Maheshwar*et al*., 1997). Essa região conservada possui função GTPase, semelhante ao domínio GRD da neurofibromina, e regula a atividade de outras proteínas através da clivagem de GTP. Outras homologias entre a tuberina e outras proteínas não foram identificadas e abordagens alternativas serão necessárias para elucidar a função de outros domínios da proteína.

A tuberina e a hamartina interagem fisicamente no aparelho de Golgi e atuam em conjunto como um único complexo, o que explica porque mutações em dois genes diferentes causam a mesma doença.Esse complexo é expresso em vários tecidos adultos e regula negativamente o crescimento celular e proliferação (van Slegtenhorst *et al.*, 1998) e tambémmodula a sinalização dependente de PI3K através de mTOR (Tee *et al*., 2002). A hamartina parece estabilizar o complexo, (a tuberina sozinha é ubiquitinada e direcionada para a degradação) (Chong-Kopera *et al*., 2006), enquanto que a tuberina possui a função de hidrólise de GTP. Ambas as proteínas têm um papel central em vários processos que são cruciais para o desenvolvimento normal do cérebro, incluindo a regulação do tamanho, dendritogênese, formação de axônios, proliferação de astrócitos e laminação cortical. Além disso, já que essas proteínas são altamente expressas no cérebro maduro, elas devem ter funções regulatórias importantes em neurônios na vida adulta. Esse papel importante se deve a integração de sinais que controlam a homeostase celular, os níveis de oxigênio, a presença de nutrientes, e a estimulação por fatores de crescimento. Todos esses sinais modificam a atividade do complexo TSC1-TSC2, que naturalmente inibe Rheb (*Ras homologue enriched in brain*), a molécula responsável pela ativação da quinase mTOR. mTOR, por sua vez, regula a tradução de uma proporção significativa de proteínas celulares, incluindo as responsáveis pelo controle do crescimento e proliferação celular (Kwiatkowski, 2003).

mTOR é uma proteína quinase evolutivamente conservada e reguladora essencial de inúmeras funções que se associa com a proteína Raptor, formando um

complexo chamado mTORC1. Diversos mecanismos de ativação e inibição de mTORC1 já foram descritos. Fatores de crescimento, citocinas e outros fatores estimulam mTORC1 através de PI3K, que induz a fosforilação de Akt na treonina 308. Akt fosforila TSC2 nas serinas 939 e 981 e treonina 1462. A fosforilação desses locais facilita a ligação de TSC2 à proteína de ancoramento 14-3-3, rompendo a formação do complexo TSC1-TSC2 e liberando TSC2 de Rheb. Sem a interação com TSC1, TSC2 é ubiquitinado e degradado. Assim, TSC2 não pode mais inibir a conversão de GTP ligado à Rheb, e ocorre um acúmulo de Rheb-GTP, que vai ativar diretamente mTORC1. Outra via de inativação do complexo TSC1-TSC2 é a Wnt: a inativação de Wnt inibe a GSK3 (*glycogen synthase kinase 3*) que fosforila TSC2 em duas serinas, 1341 e 1337. Estes eventos de fosforilação em TSC2 inativam o complexo TSC1-TSC2 e ativam mTORC1. Quando os níveis de aminoácidos estão altos na célula, mTORC1 reside no citoplasma. Aminoácidos, especialmente a leucina, ativam as proteínas *Rag guanosinetriphosphatases* (Rag GTPases).As proteínas Rag A/B interagem com pequenos complexos protéicos chamados Reguladores (Sancak *et al*., 2010), e essa associação promove a translocação de mTORC1 para a superfície dos lisossomos, onde Rheb-GTP está localizado (Sancak *et al*., 2008). Fatores de crescimento estimulam PI3K e produzem PIP3 na membrana plasmática, o que por sua vez ativa PDK1 e Akt. Akt fosforila e inibe o complexo TSC, possivelmente nos lisossomos e peroxissomos. Com isso, a atividade reduzida deGTPase do complexo TSC leva a um aumento nas quantidades de Rheb ligado a GTP. Rheb-GTP, na superfície lisossomal se liga e ativa mTORC1. Algumas citocinas como TNF-α (*tumor necrosis factor alpha*) também podem induzir a atividade de mTOR através da quinase ikkapa beta (IKK-b), que causa dissociação do complexo TSC1-TSC2 através da fosforilação de TSC1 nas serinas 487 e 511 (Salminen *et al*., 2012). Desse modo,mTORC1 regula positivamente os processos anabólicos, como a síntese de proteínas, biogênese ribossomal, transcrição, síntese lipídica, biossíntese de nucleotídeos e entrada de nutrientes (Shimobayashi e Hall, 2014). Outra atividade celular regulada por mTORC1 é a autofagia. A autofagia é um processo de auto-degradação da célula, conservado desde as leveduras, que mantém a homeostase celular em condições normais e de estresse (Boya *et al*., 2013). Para controlar a autofagia, mais de 30 proteínas específicas são necessárias para regular o processo de modo hierárquico depois da privação de nutrientes. O iniciador dessa cascata em mamíferos é o complexo Ulk1, o qual por sua vez é diretamente regulado por mTORC1. A privação de nutrientes em longo prazo leva a reativação de

mTORC1pelos nutrientes gerados na autofagia, resultando na inibição da autofagia. Portanto, uma inibição prolongada da autofagia resulta no acúmulo de agregados protéicos e organelas danificadas, causando desordens patológicas, como doenças neurodegenerativas e miodegenerativas. Assim, a autofagia interfere em várias doenças, e está intimamente relacionada à mTOR e o complexo TSC-TSC2. Outros detalhes da estrutura dos genes *TSC1* e *TSC2*, seus éxons codificantes e domínios importantes da hamartina e tuberina está disponível no Capítulo IV desta tese. A via em que o complexo hamartina-tuberina age também está representada no Capítulo IV.

Não hácura para a ET e o tratamento é focado namelhoria dos sintomasclínicos. Tanto a quimioterapia quanto a radioterapia demonstraram ser ineficazes para os tumores subependimais de células gigantes, e, por conseguinte, o tratamento padrão tem sido a ressecção cirúrgica, que pode apresentar diversas complicações, incluindo hemorragia intraventricular, hidrocefalia, e déficit cognitivo. Além disso, a recorrência dos tumores tem sido inevitável se a ressecção total dos tumores não for possível (Moavero *et al.*, 2011). Recentemente, como uma alternativa para as cirurgias, o tratamento com inibidores seletivos de mTORC1, da via de mTOR, tem sido proposto. Um desses inibidores é a rapamicina, que foi identificada em 1975 como um antibiótico produzido pelo fungo *Streptomycin hygroscopicus*, isolado do solo da ilha Rapa Nui. Ela é um antibiótico da classe dos macrolídeos e possui propriedades imunosupressoras. Tanto a Rapamicina quanto os seus análogos se ligam a proteína chamada *immunophilin FK506 binding protein-12* (FKBP-12). O complexo da droga ligada a essa proteína se liga diretamente àmTOR e impede sua ligação ao Raptor, que é essencial para a formação do complexo mTORC1. Assim, a atividade quinase de mTORC1 fica inibida.Em 2007 e 2009, dois análogos da rapamicina, temsirolimus e everolimus, foram independentemente aprovados pelo *Food and Drug Administration* (FDA) para o tratamento de carcinoma de células renais avançado. Em 2011, o everolimus também foi aprovado para o tratamento de tumores neuroendócrinos do pâncreas. O primeiro estudo com rapamicina (sirolimus) em quatro pacientes com ET etumores de células gigantes foi publicado em 2006 e demonstrou uma redução significativa do volume dos tumores, com 46-63% de redução do volume tumoral em 2-5 meses em doses comumente utilizadas em medicina de transplantes (Franz *et al.*, 2006). Sua eficácia nesses tumores foi subsequentemente confirmada em estudos posteriores. No estudo EXIST-1 (*Examining everolimus in a Study of tuberous sclerosis complex*), que foi um teste de fase III internacional, multicêntrico, duplo cego, randomizado e placebo-controlado do

everolimus, a taxa de resposta (definida como redução de 50% no volume de astrocitomas subependimais de células gigantes) foi 35% (P < 0.0001) (Franz *et al.*, 2012). A epilepsia pode ser refratária a terapias convencionais, com taxas de recaída de até 25% em pacientes que atingiram remissão (Holmes & Stafstrom, 2007). Muitas crianças refratárias à terapia antiepiléptica são consideradas para cirurgia. A prevenção do desenvolvimento da encefalopatia epiléptica é crucial para melhorar a cognição e comportamento e alguns estudos mostram diminuição da frequência de crises epilépticas com uso de rapamicina. Estudos também mostraram a eficácia do everolimus no tratamento de angiomiolipomas renais na ET, bem como de tumores ungueais e angiofibromas faciais, apresentando sucesso no tratamento desses dois últimos sintomas com rapamicina tópica, melhorando a dor e comprometimento facial, respectivamente, associados a essas patologias (John *et al*., 2014; Dill *et al*. 2014). Além de todos esses sintomas possíveis de serem tratados com inibidores de mTOR, tratamentos para crianças com ET e autismo, que não diferem dos tratamentos disponíveis para crianças sem ET, podem ser desenvolvidos. Rabdomiomas cardíacos também podem representar uma nova opção terapêutica para inibidores de mTOR.

2.3.4 Mutações nos genes *TSC1* e *TSC2* e correlações genótipo-fenótipo

O desenvolvimento de hamartomas na ET se encaixa no mecanismo de dois eventos que foi primeiramente reportado por Knudson em 1970 para explicar a oncogênese em retinoblastoma. O primeiro evento corresponde à mutação germinativa (espontânea ou herdada), que inativa um dos alelos de *TSC1* ou de *TSC2*, e o segundo evento, chamado perda de heterozigosidade, pode ser uma mutação somática que inativa o segundo alelo. Esse modelo se aplica a maioria dos hamartomas em ET.

A penetrância estimada deET é maior que 95%. Já foram identificadas 303 mutações diferentes no gene *TSC1* e 967 mutações diferentes no gene *TSC2*, descritas no banco de dados do HGMD, totalizando 1.270 mutações diferentes associadas com ET. Assim como nos genes *NF1* e *NF2*, essas mutações são de vários tipos, envolvendo desde mutações de ponto até grandes deleções e rearranjos que envolvem milhares de bases (Tabela 5). A maioria das mutações em ambos os genes são mutações pequenas (85%), como substituições de uma única base, pequenas inserções e pequenas deleções. Outras mutações são deleções ou duplicações de um ou mais éxons (14,5%) e rearranjos complexos, que ocorrem em menor frequência (0,5%).

Tabela 5. Diferentes mutações descritas nos genes *TSC1* e *TSC2* no banco de dados HGMD (*The Human Mutation Database*). Acesso em junho/2016.

Tipo de mutação	**Número de mutações em *TSC1***	**Número de mutações em *TSC2***
Sentido trocado/ sem sentido	97	353
Sítios de processamento	35	129
Pequenas deleções	99	205
Pequenas duplicações	44	109
Indel	5	11
Grandes deleções	18	137
Grandes inserções	2	11
Rearranjos complexos	3	12
Total	**303**	**967**

Apesar das numerosas mutações descritas nos genes *TSC1* e *TSC2* na literatura, aproximadamente 15% dosindivíduos afetados não apresentam mutações identificadas em nenhum dos dois genes (Kozlowski*etal.*,2007). Além disso, cerca de doisterços dos casos de ET são esporádicos, o que dificulta a análise molecular em DNA genômico e diminui a taxa de detecção de mutações nos diferentes estudos moleculares de ET. Apesar disso, os testes moleculares são de extrema importância. Para todos os critériosclínicos, há chance de que os pacientescomdoençasubclínicanão sejam diagnosticados. Os testes genéticos podemdesempenharum papel fundamentalna confirmação dessesdiagnósticos, e é recomendadoem todos os indivíduosque apresentam suspeitade manifestar esclerose tuberosa. Para isso, uma abordagem para testes genéticos é necessária nas populações afetadas. Uma revisão completa dos estudos que pesquisaram mutações nos genes *TSC1* e *TSC2*, a frequência das mutações em diferentes populações do mundo e as correlações genótipo-fenótipo estabelecidas até o momento foi publicada por nosso grupo (Capítulo IV).

3. Justificativa

O ambulatório de Oncogenética do Hospital de Clínicas de Porto Alegre iniciou as atividades em 2006, tendo o maior número de atendimentos de pacientes com diversas síndromes de predisposição ao câncer iniciado em 2008. Em 2013, 40% das consultas realizadas foram primeiras consultas, sendo metade dessas primeiras consultas pacientes com genodermatoses. Com o aumento significativo no atendimento de pacientes com essas patologias, o grupo de Oncogenética iniciou as pesquisas nesta área, recentemente, para melhorar o atendimento e acompanhamento desses pacientes.Ainda não hácoleta sistemática de dadosao nível nacional ou regional paraentender as característicasclínicas e moleculares deindivíduose famílias afetadascom genodermatoses, incluindo as mais comuns, NF1, NF2 e ET.As principais medidas em relação às genodermatoses, em países europeus, por exemplo, têm centrado fundamentalmente na investigação genética, além dos pacientes serem acompanhados de forma multidisciplinar por dermatologistas, pediatras, geneticistas, neurologistas, médicos de família e outros especialistas, de acordo com as principais manifestações. Em muitos locais, a falta de conhecimentos científicos e médicos pode impedir o correto diagnóstico dessas doenças, bem como falta de investimento tanto nos testes moleculares quanto nos testes de fármacos não proporcionar o tratamento adequado para os pacientes.

O estabelecimento de banco de dados com informações clínicas e moleculares dos pacientes com as genodermatoses mais comuns pode contribuir para o melhor entendimento da história natural e características destas doenças. O espectro de mutações que ocorrem nesses pacientes ainda pode ser útil para a compreensão da função dos genes envolvidos, para o aconselhamento genético, para determinar se há diferença significativa na taxa de detecção entre casos familiares e esporádicos, se há correlações genótipo-fenótipo claras nessa população, além de possibilitar a predição de alterações na estrutura tridimensional das proteínas implicadas na patogênese da doença. Os resultados desta caracterização poderão ajudar na identificação de grupos de pacientes de maior risco que se beneficiariam com programas de prevenção e detecção precoce de neoplasias, bem como as possíveis correlações genótipo-fenótipo estabelecidas podem ajudar a prever os tipos de tumores que serão desenvolvidos e as possíveis terapias que podem ser utilizadas em cada paciente.

4. Objetivos

4.1 Objetivo Geral

Caracterizar do ponto de vista clínico e molecular ospacientes e familiarescom diagnóstico deneurofibromatose tipo1 eneurofibromatosetipo 2avaliados no Hospital de Clínicas de Porto Alegre, Rio Grande do Sul e pacientes e familiarescom diagnóstico de esclerose tuberosaavaliados em centros da Rede Nacional de Câncer Familial.

4.2 Objetivos Específicos

a) Descrever os dados demográficose história natural(idade ao diagnóstico, co-morbidades, complicações) de pacientes com NF1,NF2e ET;

b) Caracterizar ofenótipo clínicode pacientes com NF1,NF2e ETatendidos no programa de oncogenética do Hospital de Clínicas de Porto Alegre e Rede Nacional de Câncer Familial;

c) Caracterizar mutações germinativas nos genes *NF1, NF2, TSC1, TSC2* e *INI1* em pacientes com NF1,NF2e ET, de acordo com o fenótipo clínico;

d) Realizar análise *in silico* do provável impacto das diferentes mutações encontradas nos genes *NF1, NF2, TSC1*, *TSC2* e *INI1* nas respectivas proteínas;

e) Tentar estabelecer correlações genótipo-fenótipo específicas, quando da identificação de mutações recorrentes e/ou avaliar se mutações em diferentes regiões dos genes estudados estão associadas com um determinado fenótipo;

f) Correlacionar os achados clínicos e moleculares dos pacientes estudados com demais familiares em risco e/ou afetados pela doença sempre que possível;

g) Estabelecer culturas primárias de fibroblastos de pacientes com diagnóstico clínico de esclerose tuberosa e com mutações diferentes para avaliar o fenótipo celular de expressão de genes da via mTOR e de outras vias;

h)Tratar as células de pacientes com ET com rapamicina (inibidor de mTOR) e avaliar se o fenótipo original é reestabelecido;

i) Descrever o efeito de atuação dos inibidores de mTOR nos processos celulares (mecanismo de ação) e avaliar a toxicidade dessas drogas.

Capítulo II

Manuscrito I

Manuscrito em preparo, a ser submetido para a revista Familial Cancer e formatado conforme as regras da mesma

Large gene rearrangements in Brazilian patients with Neurofibromatosis type 1 and type 2: frequency and molecular characterization

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Conflicts of Interest

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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ABSTRACT

The neurofibromatoses are a group of autosomal dominant cancer predisposition syndromes with variable expressivity. They result from dominant loss-of-function mutations mainly in the *NF1* and *NF2* genes. These genes show significant molecular heterogeneity and in addition to point mutations and small insertions/deletions a high frequency of large gene rearrangementshas been described in affected patients. The aim of this study was to characterize, for the first time in a Brazilian population, the frequency and type of large gene rearrangements in patients with neurofibromatosis type 1 (NF1) and type 2 (NF2),and the associated clinical features of the disease in affected individuals. A total of 93 unrelated NF1 and 7 unrelated NF2 probands who met the NIH diagnostic criteria were recruited and the presence of large rearrangements was assessed by Multiplex Ligation-dependent Probe Amplification (MLPA) and confirmed using a chromossome microarray assay (CytoScan HD). Single exon deletions were confirmed by Sanger Sequencing. The overall frequency of large rearrangements was 4.3% in NF1 and 14.3% in NF2 probands. Four *NF1* microdeletions and a previously described *NF2* exon 1 deletion were found. As our results demonstrate, MLPA technique is a suitable diagnostic tool for identification of germline *NF1*and *NF2* mutations. We were not able to show clear genotype-phenotype correlations in this small patient series,however we confirmed the existence of a contiguous gene syndrome associated with specific clinical signs in patients carrying a *NF1* microdeletion.

Keywords: genotype-phenotype correlation; large gene rearrangements; microdeletions; neurofibromatosis type 1; neurofibromatosis type 2.

INTRODUCTION

The neurofibromatoses are a group of autosomal dominant tumor predisposition syndromesaffecting all ethnic groups and both sexes. Neurofibromatosis type 1 (NF1, OMIM #162200) is the most common, with an estimated incidence of 1/3000 and a range of prevalence of clinically diagnosed cases from 1/2000 to 1/5000 in most population-based studies [1]. Neurofibromatosis type 2 (NF2, OMIM#101000) is more rare, with an estimated incidence of 1/33000-40000 live births [2]. NF1 and NF2 result from dominant loss-of-function mutations mainly occuring in the *NF1* and *NF2*genes, respectively.

The clinical diagnosis of both neurofibromatoses is based on criteria established by the National Institutes of Health Consensus Development Conference convened in the USAin 1988 [3]. The NF1 and NF2 phenotypes are highly variable and the clinical features develop over time.One possible explanation for this variation is the large number of different mutations in different regions ofthe *NF1*and *NF2* genes[4]. Single or multiexon deletions or duplications represent about 20% of *NF1* mutations; 5% to 10% of these *NF1* deletions are microdeletions encompassing *NF1* and its neighboring genes [5]. The most common *NF1* microdeletion, type 1, is a 1.4Mb deletion mediated by low copy repeats (LCRs) NF1-REPa and NF1-REPc and is hypothesized to preferentially arise during meiotic non-allelic homologous recombination (NAHR), leading to the loss of 14 functional genes and two microRNA genes [6]. The less common type-2 NF1 microdeletion spans 1.2 Mb and has predominantly been observed as a result of mitotic NAHR, with breakpoints within *SUZ12* and its pseudogene *SUZ12P* adjacent to NF1-REPc and NF1-REPa, respectively. They result in the loss of 13 functional genes and are often associated with mosaicism [7]. The rare 1.0-Mb type 3 *NF1* microdeletionencompasses 9 protein-coding genes, originated by mitotic NAHR and mediated by LCRs NF1-REPb and c [8]. Atypical *NF1* deletions are of variable size and are characterized by non-recurrent breakpoints [9]. Patients with large *NF1* deletions have been reported to have an increased risk of malignant peripheral nerve sheath tumors, lower average intelligence,connective tissue dysplasia, skeletal malformations, dysmorphic facial features, cardiovascular malformations, higher burden of cutaneous neurofibromas and earlier onset of benign neurofibromas [10-14]. Some authors have speculated that increased malignancy may be explained by variations in the expression of tumor suppressor genes located in co-deleted regions [15, 16]. Based

on all these correlations, the detection of single and multiexon deletions as well as microdeletions in the *NF1* gene is of great importance in the clinic. Moreover, precise molecular analyses can confirm a clinical diagnosis of NF1 in about 95% of patients [18], mainly because of the clinical overlap between NF1 and other Rasopathies. Molecular analysis is also important in NF2. Genotype-phenotype correlation studies in NF2 have been based on relatively few patients because of the rarity of the disease. In general, truncating *NF2*mutations are linked to earlier onset of symptomsand increasedtumor burden in comparison tolarge deletions, missense mutations and in-frame deletions [19].

Detecting*NF1* and *NF2* variantshas proved challenging, and current protocols are based on combinations of complementary techniques. Recently, Multiplex Ligation-dependent Probe Amplification (MLPA) has been extensively used to detect large gene rearrangements associated with various diseases, but it needs to be validated for use in different laboratories.Numerous studies aimed at the clinical and molecular characterization of the neurofibromatosishave been performed in many populations worldwide. However, none of these studies have involved a Brazilian background.The aims of the present study were (i) to evaluate the usefulness and accuracy of MLPA as first approach for screening large *NF1* and *NF2* rearrangements; (ii) to determine the frequency and exact breakpoints of large gene rearrangements in NF1 and NF2 patients from the admixed population of Southern Brazil population; and (iii) to describe the clinical features and attempt to establish genotype-phenotype correlations in probands carrying these type of genetic alterations.

MATERIALS AND METHODS

Patients

Ninety-three unrelated NF1 and 7 unrelated NF2 probands who met the NIH diagnostic criteria [3] were recruited at the Oncogenetics clinics of Hospital de Clínicas de Porto Alegre, located in the state of Rio Grande do Sul, southern Brazil. Recruitment occurred betweenAugust /2013 and December/ 2015. The study was approved by the institutional review board (CEP-HCPA) and all participants or legal representatives provided informed consent.Detailed information about the clinical symptoms of the patients was obtained during the clinical visits by a clinical geneticist and from medical

records. The clinical severity and visibility of NF1 for each patient were evaluated using the instruments described by Riccardi and Ablon, respectively [20, 21].

DNA Extraction

Genomic DNA was extracted from peripheral blood lymphocytes using a commercial kit (FlexiGene DNA kit, Qiagen, USA). Two separated blood samples (4ml) in EDTA were obtained for each patient.

Multiplex Ligation-dependent Probe Amplification analysis

NF1 single- and multi-exon deletion/duplication screening was performed by Multiplex Ligation-dependent Probe Amplification (MLPA) using first the SALSA MLPA kits P081/P082-C1 as recommended by the manufacturer (MRC Holland, Amsterdam, The Netherlands). When a complete deletion of *NF1* gene was identified, the SALSA MLPA P122-C1 kit was used to investigate de presence of microdeletions, as recommended by the manufacturer (MRC Holland, Amsterdam, The Netherlands). NF2 patients were screened using the SALSA MLPA kit P044-B2 (MRC Holland, Amsterdam, The Netherlands). For all analyses, commercial male DNA was used in triplicates as inter sample control. Normalization of peak areas was performed using the Coffalyser.net software. Ratios <0.7 were considered deletions and ratios >1.4 were considered duplications.

Chromosomal Microarray

The chromosomal microarray assay CytoScan HD (Affymetrix, USA) was used to determine the breakpoint junctions and the type of *NF1* microdeletions previously detected by MLPA and to corroborate and validate MLPA analysis for use in molecular diagnosis routine. The high-density, whole-genome CytoScan Array includes 750,000 bi-allelic SNP probes and 1.9 million non-polymorphic markers for copy number analysis, with an average spacing of approximately 880 bases apart in genic regions and 1700 bases apart in non-genic regions.Large rearrangements identified by MLPA were confirmed by CytoScan HD, as recommended in the manufacturer's protocol.Chromosome Analysis Suite software (ChAS software 3.1) was used to analyze microarray data. The software includes a built-in reference with more than 400 samples.The signal intensity of the hybridized DNA from the patient sample was compared to this reference DNA. The ratio of patient sample to reference intensity is

expressed as a $\log^2$ ratio, and represents the relative intensity for each marker. This technique is not recommended to detect single exon deletions and therefore, was not used in these cases.

Sanger Sequencing

Single exon deletions identified by MLPA were confirmed by Sanger sequencing. Deleted exons were amplified by PCR using specific primer pairs, designed using the Primer Blast tool. PCR products were purified using the Exo-Sap kit (Amersham Biosciences, Les Ulis, France) and sequencing performed on an ABI 3500 Genetic Analyzer (Applied Biosystems, California, USA); the CodonCode aligner softwarewas used for sequence alignment.

Sequences and mutation nomenclature

DNA mutation numbering was based on cDNA sequences NM_001042492.2 (https://www.ncbi.nlm.nih.gov/pubmed) and LRG 214 (http://www.lrg-sequence.org/LRG) for *NF1* and NM_000268.3 and LRG 511 for *NF2*, with nucleotide +1 corresponding to the A of the ATG translation initiation codon of the reference sequence. DNA and protein changes are reported according to international recommendations for the description of sequence variants of the Human Genome Variation Society (HGVS; http://www.HGVS.org). Mutation calling and interpretation were based on the American College of Medical Genetics guidelines. The databases ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), dbSNP (https://www.ncbi.nlm.nih.gov/SNP/), Leiden Open Variation Database – LOVD (http://www.lovd.nl/3.0/home), The Human Gene Mutation Database - HGMD (http://www.hgmd.cf.ac.uk/ac/index.php) and current literature were searched for mutation information and interpretation.

RESULTS

Multiplex Ligation-dependent Probe Amplification

The clinical features of all recruited patients are shown in Table 1. First, all the patients were tested for single and multiexon deletions/duplications by MLPA. Among the 96 NF1 probands, this analysis identified 5 heterozygous deletions (5.4%) one exon 13 deletion (0.9%) and four complete*NF1*deletions (4.3%). Among the 7 NF2 probands,

only one *NF2*heterozygous deletion (14.3%) was identified. Duplications were not observed. Complete *NF1* deletions were corroborated with the SALSA MLPA P122-C1 *NF1* Area assay, revealing that one patient had a type I deletion, determined to be a deletion extending from the binding sites of the *SUZ12P* pseudogene to the *LRRC37B* gene. Two patients carried a smaller deletion, with a normal copy number for the three MLPA probes in the *SUZ12P* pseudogene and also for the MLPA probe in exon 10 of the *SUZ12* gene. Since the classical breakpoints for type II deletions are within *SUZ12P* and *SUZ12*, we could not determine the exact type of *NF1* microdeletion in these two patients by MLPA.An atypical *NF1* microdeletion in another patient was detected, extending from the *SUZ12P* to the *NF1* gene. In this initial analysis, the breakpoints seemed to be near NF1-REPa and downstream of the *NF1* gene. The classical LCR breakpoints in *NF1* and MLPA P122 results are summarized in Figures 1A and 1B, respectively.

Chromosomal Microarray

All MLPA results were confirmed using the high-resolution chromosomal microarray technique CytoScan HD, allowing better characterization of the deletion boundaries. The deletions detected by CytoScan with the revised names and positions of each co-deleted genes are presented in Figure 1C. Patient one has the classical 1.4Mb type 1 microdeletion, spanning from *SUZ12P1* to *LRRC37B*, including 22 genes (16 functional). The breakpoints corroborate the MLPA P122 result, which suggested breakpoints within NF1-REPa (downstream of *LRRC37BP1* and upstream of*SUZ12P1*) and within NF1-REPc (3.8kb downstream of the *LRRC37B*gene). Patients two and three have a similar deletion, but their breakpoints differ slightly. Patient two has breakpoints near NF1-REPa (within *CRLF3* intron 1) and NF1-REPc (within *SUZ12*intron 5), including 19 genes (13 functional) and spanning 1.15Mb; patient three also has breakpoints near NF1-REPa and NF1-REPc, but within *CRLF3* intron 4 and upstream of the *SUZ12* gene (between *UTP6* and *SUZ12*), including 18 genes (12 functional) and spanning 1.13Mb. MLPA P122 probes did not identify the deletions in *SUZ12* the corresponging MLPA probe is located upstream of the deletion. Considering that the classical type 2 microdeletion (1.2Mb) breakpoints are within *SUZ12* and its pseudogene *SUZ12P*, patients two and three have atypical *NF1* microdeletions since their breakpoints are adjacent to the classical type 2, but not the same. Patient four also has an atypical microdeletion, with breakpoints between *SUZ12P1* and*LRRC37BP*, near

NF1-REPa, and between *MIR193A* and *MIR365B*. This deletion spans 890kb and 15 genes (12 functional). MLPA P122 probes did not detect three deleted genes in this patient (*RAB11FIP4,MIR193A* and *MIR365B*).

Sanger Sequencing

The only single exon deletion identified by MLPA in the *NF1* gene (exon 13) was not confirmed by Sanger sequencing, which showed a point mutation, c.1466A>G within the MLPA probe hybridization region. This mutation is classified as pathogenic in the ClinVar database (http://www.ncbi.nlm.nih.gov/clinvar/). The single exon deletion found in the *NF2* gene (exon 1) was not submitted to Sanger sequencing since the *NF2* MLPA P044 kit includes three probes for this exon which all showed a decreased signal.

Genotype-phenotype correlations

Table 2 describes in more detail the clinical signs and symptoms of each proband carrying a large deletion. As the number of patients with large deletions is small, the statistical comparison between this group and non-deleted probands is not possible. However, we observed that the most commonly affected systems were the same in both groups including the skin (café-au-lait spots, axillary and inguinal freckling), peripheral nervous system (cutaneous and plexiform neurofibromas) and eyes (Lisch nodules) although the age of NF1 diagnosis is apparently earlier in the probands carrying a deletion (3.2 vs 7.0 years). Furthermore, patient one harboring the type I *NF1* microdeletion has several of the previously described symptoms for individuals with such rearrangement: malignant peripheral nerve sheath tumors, lower average IQ, skeletal malformations and higher burden of cutaneous neurofibromas.This mutation occured*de novo* in this patient. Patients two and three (Table 2) have similar phenotypes and both cases are familial. The Riccardi scale scored equally for them, but patient two has the worst visibility (Ablon). The occurrence of these deletions in two unrelated patients reinforces that NF1REPa and NF1REPc are recurrent breakpoints.Patient four's phenptypeis similar to that observed in patients two and three and the mutation occurred *de novo* (Figure 1). The genes between *NF1* and *SUZ12*, when deleted, do not seem to be relevant for NF1 phenotype in these patients, since patients 2, 3 and 4 have very similar phenotypes. Finally, patient five is a sporadic case with an exon 1 *NF2* deletion. This mutation has already been described in a patient with

late NF2 onset [22]. The deleted exon is part of the FERM domain of the *NF2* protein, called merlin. Merlin is a membrane-cytoskeleton scaffolding protein and the FERM domain is responsible for the cytoskeletal-membrane organizing. We did not identify clear differences between phenotypes of the patient with the *NF2* deletion and the other non-deleted NF2 probands.

DISCUSSION

In this study, we performed the analysis of large *NF1*rearrangements in 93probandswith the clinical diagnosis of NF1 and of large *NF2*rearrangements in 7 probands with the clinical diagnosis of NF2 in Southern Brazil.

The overall frequency of large rearrangements in NF1 patients worldwide is 5-10% [4], similar to the overall frequency observed in our study (4.3%). One of the NF1 probands had a well described *NF1* microdeletion, type I, which has been associated with a specific phenotype. This is the most common type of recurrent *NF1* microdeletion, associated with the loss of 14 protein coding genes including *NF1* and two microRNA genes (*MIR365-2* and *MIR193a*). The majority of type 1 NF1 microdeletions are maternally inherited germline deletions [23] whose breakpoints are frequently located within two hotspot regions, NF1REPa and NF1REPc,during interchromosomal meiotic nonallelic homologous recombination.It has been estimated, from thestudy of large cohorts of NF1 patients, that 70-80 % of all NF1 microdeletions are type I [24]. In our study, this specific rearrangement accounted for only 25% of the microdeletions identified, but our sample size is relatively small.Atypical *NF1*deletions have been described which do not have recurrent breakpoints.An estimated 8–10 % of all *NF1* microdeletions are atypical [7]. We found three patients with this type of microdeletion, accounting for most (75%) of the microdeletions identified in our cohort. All three atypical deletions have one breakpoint at a NF1 LCR and another outsidethese regions and are not among those previously described in other populations [25]. The mutational mechanisms underlying atypical NF1 microdeletions are less well characterized than those mediated by NAHR. It isreasonable to assume that non-homologous end joining can mediate these atypical NF1 deletions.However, since only a few *NF1* microdeletions with non-recurrent breakpoints haveso far been analyzed, other mechanisms cannot be excluded. The detailed analysis of the breakpoints of a

large series of atypical NF1microdeletions will be required to elucidate the role of replication-based errors as the putative mechanism underlying these deletions.*Alu* sequences that are present within the *NF1* gene in large numbers could be one of the underlying mechanisms to explain the occurrence of these rearrangements.

Unfortunately, attempts to correlate the many and varied germline *NF1* gene mutations with specific clinical features of NF1 have been largely unsuccessful, mainly due to the marked inter and intrafamilial variability in disease expression and the extent of the allelic heterogeneity underlying the disease. Given the marked clinical phenotypic variation associated with NF1 even in individuals carrying the same *NF1* mutation, it has been suggested that otherprotein-coding or microRNA genes which are unlinked to the *NF1* locus itself, epigenetic alterations orenvironmental factors may also contribute to such variable expression in NF1. Moreover, variations in the NF1 phenotype could be determined by a single modifier gene locus, or by interaction between several modifier genes. Age is also a confounding factor in familial NF1 studies, with many disease features being more prevalent in older patients [26]. In microdeleted patients, the co-deleted genes may also influence phenotypic expression. One gene in the region between NF1-REPa and NF1-REPb, which is variably included in microdeletions and is deleted in four of our patients, is ring finger protein 135 (*RNF135*). *RNF135* loss-of-function mutations, as well as an NF1-REPa to NF1-REPb deletion including this gene, have been implicated in an overgrowth syndrome which includes tall stature, macrocephaly, dysmorphic features, and variable additional features, including learning disability [17]. One of our patients (Patient 2, Table 2) has an NF1 deletion including NF1-REPa to NF1-REPb and *RNF135* and has several dysmorphic features as well as tall stature. Other genes in the region may also contribute to the phenotype in microdeleted patients. *SUZ12* (also known as *JJAZ1*) is critical in embryonic development [27] and *OMG* is an important inhibitor of neurite overgrowth [28]. One could hypothesize that either or both of these genes contribute to phenotypes seen in *NF1* microdeletions, but this is yet to be explored in detail. Haploinsufficiency *OMG* gene has been proposed to be associated with learning disability. *SUZ12* and *ADAP2* are highly expressed in cardiac tissues and may be related to cardiac malformations. Haploinsufficiency of *UTP6* seems to reduce cellular apoptosis, increasing the risk of tumor development [29-31]. MicroRNAs have an important role in the regulation of gene expression, and the deletion of three *MIR* genes in the NF1 microdeletion region may also influence disease phenotype. Microdeletions occur in heterozygosis; *NF1*

deletion has a dominant effect, but the otherdeleted genes need further exploration to determine the effect of heterozygous mutations on their function. *In silico* analyses and functional studies could help to clarify the function of these co-deleted genes, and in the future, they could possibly be included in the *NF1* molecular diagnostic workup if associated with significant phenotype variation.

On the other hand, NF2 studies conducted worldwide have identified large rearrangements in 10 to 30% of the patients, a frequency found in our study (14.3%). We found a well described singleexon 1 *NF2* deletion in one patient. The mechanisms associated with this deletion are not known and recurrent breakpoints are not described for the *NF2* gene. Also, genotype-phenotype correlation studies in NF2 have been scarce and based on relatively few patients, as in our study, because of the rarity of the disease.

We were not able to show clear genotype-phenotype correlations in our patient series, since the number of individuals carrying deletions is too small to reachrobust conclusions. However, we confirmed the existence of a contiguous gene syndrome associated with specific clinical signs in *NF1* microdeletion patients. Although it is reasonable to suggest that patients with large deletions of the *NF1* locus have a greater probability of showing learning disabilities and facial dysmorphism, it is still impossible to predict the presence of an*NF1* microdeletion based solely on the patient's phenotype. As our results demonstrate, MLPA is a straightforward and sensitive screening methodology for identifying germline *NF1*and *NF2* rearrangements. However, patients with complete *NF1* deletion generally have co-deleted genes and the number of co-deleted genes and exact extension of the deletion cannot be determined by MLPA and has to be complemented by other methods. Continued research on the mutational breakpoints and co-deleted genes in patients diagnosed with NF1 and NF2 may help elucidate the role of the *NF1, NF2* and neighboring genes in the development of disease. Finally, mutational spectrum has been shown to vary among different populations, underscoring the need to expand our knowledge in specific, less studied populations.

CONCLUSION

This study shows the large rearrangement characterization of NF1 and NF2 patients for the first time in Brazil. We showed that the overall frequency of large

rearrangements in NF1 and NF2 patients from south Brazil is similar to other populations worldwide (4.3 and 14.3%, respectively). The methodologies used were able to unambiguously differentiate between the types of microdeletions and the frequency of atypical NF1 deletions was high in our patient series. We showed a clear genotype-phenotype correlation in the type one NF1 microdeletion patient, but atypical deletions did not show a clear correlation. Studies with larger series of patients from Brazil are needed to stablish clear genotype-phenotype correlations.Inclusion of co-deleted genes in molecular diagnosis of NF1 would be helpful to predict more severe or specific characteristics that may occur in these patients. A precise molecular diagnosis would be relevant for genetic counseling, diagnosis and cancer prevention.

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Table 1. Characterization of the NF1 and NF2 patients recruited in this study from August/2013 to December/2015.

	Average age in years (range)	**Ethnicity (Euro/Afrodescendant)**	**Gender (M/F)**	**Average age at onset (years)**	**Family History (Y/N)**
NF1	31 (1-79)	86 /10	36/ 60	7.0	67/ 29
NF2	23 (11-44)	7 / 0	6 / 1	13	3 / 4

Table 2. Clinical signs and symptoms of NF1 and NF2 patients with large deletions from this study.

Patient	Age at onset/gender	Age	Familial	Mutation	Riccardi/Ablon	Main symptoms	Other pathologies/characteristics
1	At birth/female	26	No	Type I *NF1* microdeletion	4/moderate	CALM, cutaneous and malignant peripheral nerve sheath tumour, axillary and inguinal freckling, Lisch nodules, escoliosis, cognitive deficit	No
2	4 years/male	57	Yes	Atypical *NF1* microdeletion	2/moderate	CALM, cutaneous neurofibromas, axillary and inguinal freckling	Hemocromatosis, hipogonadism
3	4 years/male	46	Yes	Atypical *NF1* microdeletion	2/mild	CALM, cutaneous and plexiform neurofibromas, axillary and inguinal freckling, macrocephaly	No
4	4 years/male	14	No	Atypical *NF1* microdeletion	2/moderate	CALM, cutaneous neurofibromas, axillary and inguinal freckling, macrocephaly	Dysmorphic features, oblique palpebral fissures, protruding ears and tall stature
5	16 years/male	17	No	*NF2* exon 1 deletion	NA	Spinal ependimoma, 5 cranial meningiomas	No

CALM = Café-au-lait macules; NA = not applicable

Fig1A. Schematic representation of the *NF1* gene region. The relative positions of the three NF1-REPs that are common deletion breakpoints are indicated, together with the protein-coding genes located within these regions (blue bars), the two microRNA genes (red bars) and the pseudogenes (orange bars). The relative extents of the type 1, type 2 and type 3 *NF1* microdeletions are also shown. **B.** MLPA P122 results of the four microdeleted patients. Probes with ratios ≤0.7 are considered deletions and between 0.7 and 1.4 are considered normal. The deleted probes are represented in the graphics.**C.** CytoScan HD results of the four microdeleted patients. The figure is showing only the chromosome 17. The relative microdeletions sizes and positions between patients are shown. The 1.4Mb deletion has the same size and position of the microdeletion type 1 represented on Figure 1A.

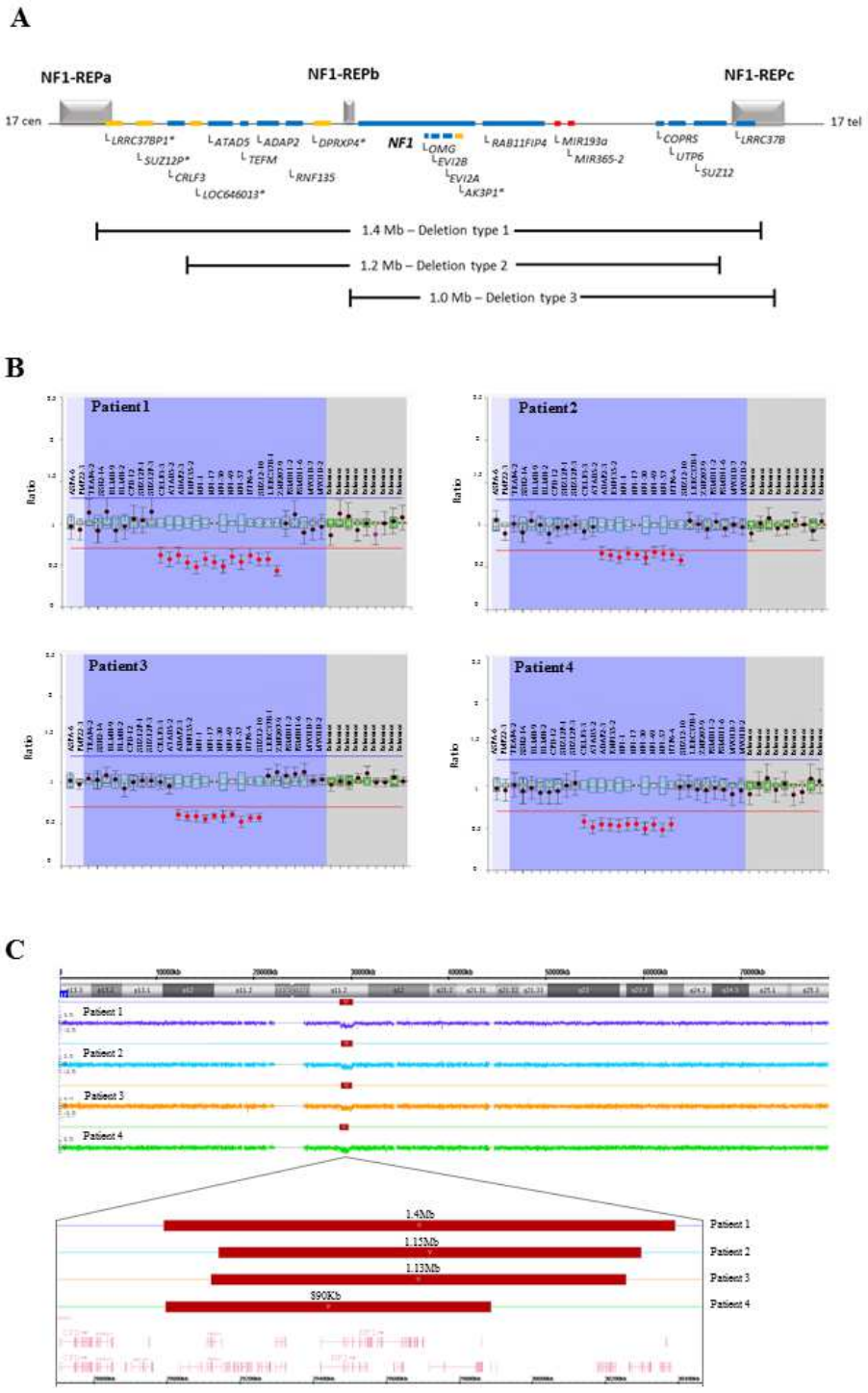
A
NF1-REPa
NF1-REPb
NF1-REPc
17 cen
17 tel
LRRC37BP1*
SUZ12P*
CRLF3
LOC646013*
ATAD5
ADAP2
TEFM
RNF135
DPRXP4*
NF1
OMG
EVI2B
EVI2A
AK3P1*
RAB11FIP4
MIR193a
MIR365-2
COPRS
UTP6
SUZ12
LRRC37B
1.4 Mb – Deletion type 1
1.2 Mb – Deletion type 2
1.0 Mb – Deletion type 3
B
Patient1
Patient2
Patient3
Patient4
Ratio
C
Patient 1
Patient 2
Patient 3
Patient 4
1.4Mb
1.15Mb
1.13Mb
890Kb

Figure 1

Capítulo I

Manuscrito II

Manuscrito concluído, a ser submetido para a revista Neurogenetics e formatado conforme as regras da mesma

Clinical and Molecular characterization of Neurofibromatosis in Southern Brazil: detection of point mutations by Next Generation Sequencing.

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Keywords: Genodermatoses; genotype-phenotype correlations; neurofibromatosis type 1; neurofibromatosis type 2; Next-generation sequencing.

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Abstract

The neurofibromatoses (type 1: NF1 and type 2: NF2) are a group of autosomal dominant tumour predisposition syndromes with variable expressivity, affecting multiple organ systems. In both syndromes, anincreased lifetime risk for developing several malignancies is observed. These are mostly caused by loss-of-function mutations of the tumor suppressor genes*NF1* and *NF2*, respectively, in which a large number of mutations have been described. The clinical diagnosis of NF1 and NF2 can be complicated and therefore, genotyping is an important diagnostic tool in these diseases. We recruited 93 unrelated probands with clinically diagnosed or suspected NF1 and 7 unrelated patients suspected of NF2 from a single oncogenetics reference center in Southern Brazil, and customized two next generation sequencing panels to identifygermline mutations in this cohort. The NF1 panel included the *NF1,RNF135, and SUZ12*genes and the NF2 panel included the *NF2*and *INI1*genes. All variants were, confirmed by Sanger sequencing.Overall, 69 distinct heterozygous *NF1* variants were identified in 73 (79%) of the 93 probands and 2 distinct heterozygous *NF2* variants were identified in two (29%) of the seven probands. Of the variants identified, 14 were pathogenic, 34 were likely pathogenic, two were likely benign, 21 were variants of uncertain significance and 33 were novel according to the ACMG variant classification system. Severity and visibility of NF1 patients was evaluated according to the type and location of *NF1* mutations and no significant and/or novel genotype-phenotype correlations were observed. Further studies with larger scales are required to identify potential genotype-phenotype correlations in brazilian patients with NF1 and NF2. Also, functional analyses of each VUS may clarify the mechanisms of pathogenesis associated with NF1 and NF2.

Introduction

The neurofibromatoses are a group of autosomal dominant tumour predisposition syndromes with variable expressivity.Neurofibromatosis type 1 (NF1; OMIM 162200) is the most common genodermatosis and accounts for about 90% of all cases [1], with an estimated birth incidence of 1 in 2,500.[2]. The distribution is equal between sexes and incidence does not vary significantly among different populations [3].Neurofibromatosis type 2 (NF2; OMIM 101000) occurs less frequently and is usually diagnosed in early adulthood, with incidences ranging from 1 in 25,000 to 1 in 40,000 [4].

The phenotypic hallmarks of NF1 are café-au-lait spots, peripheral neurofibromas (cutaneous and/or plexiform), freckling of the axillary and/or inguinal regions and Lisch nodules of the iris. Also, a greater risk than the general population for developing several malignancies is observed; the most common tumors arising in individuals with NF1 are neurofibromas, malignant peripheral nerve sheath tumours, and gliomas [5].An increased risk of premenopausal breast cancer has been reported in affected women. Individuals with NF2 are prone to the formation of schwannomas of the eighth cranial nerve and spinal cord, meningiomas, and ependymomas, with associated symptoms of tinnitus, hearing loss, and balance dysfunction [5]. The diagnosis of NF1 and NF2 is established using diagnostic criteria that were originally formulated by the National Institutes of Health Consensus Development panel in 1987 [6] and were revised for NF2 in 2002 [7].

NF1 and NF2 are caused by loss-of-function mutations of the tumor suppressor genes*NF1* and *NF2*, respectively. Over half of individuals diagnosed with NF1 and NF2 carry *de novo* mutations [4]. To date, more than 1,500 different *NF1* mutations and 300 different *NF2* mutations have been reported in the Human Gene Mutation Database (HGMD, Institute of Medical Genetics, Cardiff, http://www.hgmd.org/) and in the Leiden Open Variation Database (LOVD: www.lovd.nl/NF1). The *NF1* gene maps to chromosome 17q11.2 and spans approximately 350 kb of genomic DNA, with the longest isoform containing 58 exons (NM_001042492.2; LRG_214) and exhibiting one of the highest mutation rates [8].The protein product of the *NF1* gene (neurofibromin) is a cytoplasmic protein with 2,818 amino acids. Neurofibromin inhibits the activity of Ras GTPase proteins. Loss of neurofibromin results in deregulated Ras activity, leading to activation of several important downstream signaling intermediates [9].

Neurofibromin also functions to positively regulate cyclic adenosine monophosphate levels, interfering in multiple mitogenic signaling pathways [10]. The *NF2* gene maps to chromosome 22q12.2. The most common *NF2*isoforms are I, without exon 16 (NM_000268.3; LRG_511) and II, with 17 exons (NM_016418.5). The latter codifies the 595 amino acid protein merlin [11], which mediates contact inhibition of proliferation in multiple cell types, including Schwann cells [12] and is reported to target many signaling components to restrict proliferation [13].

The clinical diagnosis of NF1 and NF2 can be complicated in sporadic cases and when clinical criteria are not fulfilled. Therefore, genetic testing is an important diagnostic tool for these diseases. Identification of mutations in the *NF1*gene is challenging due to its size and complexity, significant molecular heterogeneity, absence of mutational hotspots and presence of several pseudogenes. Approximately one-third of pathologenic *NF1* mutations influence mRNA splicing and 30% of all such mutations lie outside the classical consensus splice-regulatory sequences [14], which are difficult to detect. The *NF2* gene also lacks hot-spots for mutations and has significant molecular heterogeneity. Numerous studies have performed comprehensive *NF1* and *NF2* genotyping worldwide, but none in Brazil. The aim of the present study is to characterize a wide spectrum of mutations in NF1 and NF2 patients from Southern Brazil and attempt to establish potential genotype-phenotype correlations.

Patients and Methods

Patients and DNA samples

Ninety-three unrelated probands who were clinically diagnosed or suspected of NF1 and seven unrelated patients suspected of NF2 were included in this study.The patients were recruited at the Oncogenetics service from Hospital de Clinicas de Porto Alegre, Rio Grande do Sul, Brazil, between August/2013 and May/2016. The patients were evaluated by clinical geneticists and the clinical diagnosis established, whenever possible, according to the National Institutes of Health criteria [6]. The study protocol was reviewed and approved by the Institutional Review Board (GPPG 13-0260), and written informed consent was obtained from all subjects or legal representatives. Genomic DNA was obtained from peripheral blood using a commercial DNA extraction kit (Flexigene Blood Kit, Qiagen, USA). Clinical severity and visibility of NF1 were

assessed using the Riccardi and Ablon's scales, respectively [15, 16]. Clinical and demographic information was obtained by patient interview or chart review.

Next Generation Sequencing (NGS)

Two NGS panels were designed: the first panel, used for genotyping of NF1 probands includes the *NF1* (NM_001042492.2), *RNF135* (NM_032322.3) and *SUZ12* (NM_015355.2) genes and the second panel, used for genotyping of NF2 probands includedthe *NF2* (NM_000268.3) and *INI1* (NM_003073.3) genes. Primers were designed using the AmpliSeq Designer software (Thermo Fisher Scientific, CA, USA), targeting 99.32% of the coding sequence, exon-intron junctions and 5' and 3' untranslated regions of *NF1,* 90.09%of *RNF135* and100% of *SUZ12*, resulting in 119 amplicons in the first panel, and 93,86% of *NF2* and 10% of *INI1* resulting in a total of 46 amplicons in the second panel. Amplicon library was prepared using the Ion AmpliSeq Library Kit 2.0 (Thermo Fisher Scientific, CA, USA) and NGS performed using 20 ng of genomic DNA and an Ion 316 sequencing chip on an Ion Personal Genome Machine and the 200 Sequencing kit (Thermo Fisher Scientific, CA, USA) with 500 flows.Data from the Ion Torrent runs were analyzed using the platform-specific pipeline software Torrent Suite v3.2.1 for base calling, trim adapter and primer sequences and filtering out poor quality reads. The sequences were aligned to the hg19 human reference genome. For variant calling, the sequence runs were imported to the Ion Reporter software v5.0. Presence of *NF1* pseudogenes showing a high sequence identity with the functional *NF1* gene was taken into account in the bioinformatics workflow. During the sequence alignment with the Torrent Suite (Life Technologies), reads mapping at two different positions in the genome were discarded (single hits). Sequence alignment was performed against the whole genome to avoid forcing alignment of pseudogene amplicons on the functional NF1 sequence.Variants were also reviewed and annotated using this software. Integrative genomics viewer (IGV-http://software.broadinstitute.org/software/igv/)) was used for visualization of the mapped reads.

NGS validation by Sanger Sequencing

All pathogenic and likely pathogenicvariants identified were confirmed by Sanger sequencing. Specific primers for the corresponding exons were designed using Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and the same reference

sequences were used for direct capillary sequencing. Coding regions with low coverage on the NGS assay were also Sanger sequenced. The 3'end of the *NF1* primers were designed not to match the genomic sequences of the highly homologous pseudogene sequences.Forward and reverse primers (available upon request) were used to sequence the purified PCR products, using the BigDye Terminator v3.1 Cycle Sequencing Kit using an ABI 3500 instrument (Thermo Fisher Scientific, CA, USA). Sequences were aligned to the reference sequences used to design PCR primers using CodonCode Aligner software implemented in MEGA 5.04. When additional family members of a mutation carrier accepted to participate in this study, the direct sequencing of the correponding exon was performed for segregation analysis.

Bioinformatics analysis

To predict whether the missense and small indel changes that have not been reported as pathogenic in the literature were likely pathogenic, we employedfive algorithms that are commonly used to predict the impact of a given mutation on protein structure and function: PolyPhen-2; Mutation Assessor; PHANTER; SIFT (Sorting Intolerant From Tolerant); and Mutation Taster [17-22]. Splice site mutations were analyzed using the BDGP software (Berkeley Drosophila Genome Project) [23]. Nonsense and frameshift mutations were not evaluated by *in silico* analyses. Moreover, all variants were searched in the ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/), COSMIC (Catalogue of Somatic Mutations in Cancer - http://cancer.sanger.ac.uk/cosmic), HGMD (The Human Gene Mutation Database - http://www.hgmd.cf.ac.uk/ac/index.php), LOVD (Leiden Open Variation Database - http://www.lovd.nl/3.0/home), 1000 Genomes Project (http://www.1000genomes.org/) Exome Aggregation Consortium (http://exac.broadinstitute.org/), and dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) databases.

Mutation nomenclature

DNA mutation numbering was based on the cDNA sequences described above, with nucleotide +1 corresponding to the A of the ATG translation initiation codon of the reference sequence. *NF1* exons were named according to the NCBI nomenclature (exons numbered 1–58). DNA and protein changes were reported according to international recommendations for the description of sequence variants of the Human

Genome Variation Society (HGVS; http://www.HGVS.org). Mutation calling and interpretation were based on the American College of Medical Genetics guidelines [24].

Statistical analysis

We compared the frequency of each clinical finding to the type and location of mutations. *NF1*mutation carriers were arbitrarily divided into a group with mutations in the N-terminal region of the gene (exons 1-26), a group with mutations in the central region of the gene (exons 27-38), where the most important functional domains are localized, and a group with mutations in the C-terminal region of the gene (exons 39-58). Statistical comparisons were performed by conventional chi-square or Fisher's exact test using the SPSS software (version 19.0).

Results

We recruited 93 unrelated NF1 patients; most were born in the State of Rio Grande do Sul (97.8%) and only 2 were born in two different cities from the neighboring state of Santa Catarina (Figure 1A). All seven NF2probandswere from Rio Grande do Sul. Demographic and family history data are summarized in Table 1.Eighty-nine NF1 patients(95,7%) and all NF2 patients fulfilled the diagnostic criteria established by the National Institutes of Health (NIH).

Detailed clinical features and results of the severity and visibility scales for NF1 probands are shown in Tables 2 and 3, respectively. Most NF1 probands (69,9%) were classified at the intermediate levels (2 and 3) by the Riccardi severity scale. Using Ablon's visibility scale, most probands (49,5%) were classified in the mild category. Clinical features of the NF2 patients are shown in Table 4; the presence of NF2 associated tumors were evaluated by image tests. We did not observe a significant difference in frequency of symptoms in when comparingprobands with and without a family history.

Overall, 69 distinctheterozygous *NF1*variants were identified in 73 (79%) of the 93 patients and 2 distinct heterozygous *NF2* variants were identified in two (29%) of the seven patients (Table S1). Five NF1 probands had other clinically affected relatives tested, and all were found to be carriers. No sequence variants were identified in the *RNF135*, *SUZ12* and *INI1* genes.

Of all variants identified, 14 were pathogenic (all in *NF1*), 34 were likely pathogenic (33 in *NF1* and 1 in *NF2*), 2 were likely benign (*NF1*) and 21 were variants

of uncertain significance (VUS – 20 in *NF1* and 1 in *NF2*). Seven variants were identified in more than one proband and all other had single occurrence (Table S1). Four probands presented two variants; in three, one variant was pathogenic or probably pathogenic and the other variant was a VUS; in one proband two VUS were found. Eight-five percent of NF1 familial cases had a pathogenic or likely pathogenic variant (n=54), while only 73% of sporadic cases had a mutation (n=22). Thirty-three alterations had not been previously reported in the analyzed databases and were also not described in the 1000 genomes and Exome Aggregation Consortium projects. Overall, classification of mutation-positive probands by type of mutation identified was as follows: 19 (28%) nonsense, 17 (25%) splice site mutations, 13 (19%) frameshift deletions, 12 (17%) missense, 4 (6%) in frame deletions, three (4%) frameshift insertions and one (1%) frameshift duplication for *NF1* gene; one splice site mutation (50%) and one nonsense mutation (50%) for *NF2* gene. All frameshift insertions and deletions were predicted to cause premature stop codons in their respective protein products. Frequencies of the different types of mutation identified in *NF1*are shown in Figure 1B and the predicted effect of the novel/VUS missense mutations is shown in Table S2.The distribution of small mutations within the *NF1* gene is shown in Figure 2. We calculated the mean number of small mutations (including splice site changes) per nucleotide for each *NF1*exon. Exons 3, 13, 17, 18, 21, 22, 37, 38, 46 and 49 had the highest mutation frequencies, with more than one mutation per 50 base pairs. Finally,we identified ten synonymous polymorphisms in *NF1* gene. These polymorphisms were previously described in 1000 genomes project and could not be related to disease.

Results of the analysis of NF1 severity and visibility according to type and location of *NF1* mutations are showb in Table 5. Eight variants were inside the *NF1* GAP-Related Domain (GRD), the most important domain of neurofibromin (exons 27-35). However, comparisons of severity and visibility of NF1 measured by Riccardi and Ablon scales between patients with mutations in different *NF1* positions did not show statistically significant differences. Visibility categories according to Ablon's scale and severity categories according to Riccardi scale did not show statiscally different distributions when considering patients with mutations in either C-terminal, central and N-terminal regions of the gene (p=0.082) and (p=0.241), respectively. The different types of mutations also did not occur more frequently in a specific *NF1* position. Considering patients with a GRD mutation, no clear genotype-phenotype correlation or a more severe symptomathology seems to occur. Clinical features of NF1 patients

withno mutations detected are summarized in Table S3. All of them had at least two major diagnostic features of NF1, however, bone alterations were not present in this group.

Discussion

Although NF1 is one of the most common autosomal dominant genetic syndromes described to date, genotyping is often not done, since in most suspected cases the phenotype that allows clinical diagnosis develops in the first years of life. NF2, although much less frequent, also has a very typical presentation and phenotype which allow clinical definition of the diagnosis. Thus, molecular testing is usually reserved for the atypical cases and for purposes of genetic counseling and prenatal/preimplantation diagnosis. In Brazil, a country where still resources are limited in the field of clinical genetics and molecular diagnosis, genotyping of NF1 and NF2 patients is rarely done in the clinical setting. This is perhaps the reason for the existence of only a small number of reports involving molecular screening of *NF1* and/or *NF2*.To our knowledge, only one previous study evaluated the clinical symptoms and mutations of NF1 patients in Brazil, but molecular analysis included only the GAP domain of the *NF1* geneand used a screening method with reduced sensitivity (single-strand conformation polymorphism) [25]. Interestingly, it was conducted in a different Brazilian region and thefour germline mutations described were not present in our cohort from southern Brazil. Also, their series of patients seems to have been enriched for NF1 patients with severe diseasesince they describe and unusually high frequency of plexiform neurofibromas, cognitive deficiency and scoliosis, in their cohort, different from our cohort in which frequency of clinical findings is more similar to what has been described internationally [26]. Although only a minority of the NF1 probands described in the present article have severe disease, our series has a higher frequency of familial NF1 cases than described in the literature (68% vs 50%) [4], while in NF2, familial cases accounted for 42% of the probands, which is within the range observed internationally [4]. The point mutation detection rate in our series was 79% and 29% for the*NF1*and*NF2*genes, respectively. Overall *NF1* mutation detection rate is comparable to other studies (80 and 78%) [27, 28], higher than reported by Fahsold *et al.* (53%) [29] and lower than described by Mattocks *et al.* (89%) [30]; *NF2*mutation detection rate was lower than previously described in other populations [31]. Only 17% of the NF1 patients had no mutation detected; many explanations for disease occurrence in these

patients can be listed. A small percentage of deep intronic mutations and *Alu*insertions in *NF1* will be missed when a gDNA testing approach is used. A number of other studies have employed a RNA based analysis to identify *NF1* mutations [32-35] with increased sensitivity and variant detection rate. A mosaic *NF1* mutation could also be missed in gDNA mutation screening obtained from peripheral blood; however, NGS technology may overcome this problem when high coverage sequencing is achieved. In our approach, the median coverage in NGS was 700x per amplicon, ranging from 100 to 2400x, which usually sufficesto indicate the presence of mosaicism. Finally, there is also a possibility of a mutation in another gene or regulatory element associated with an NF1 or NF1-like phenotype. Recently, mutations in the *SPRED1* gene have been described in patients with a phenotype resembling NF1 [36].

Most of the mutations described here were nonsense, followed by splice site mutations. We described 33 novel *NF1* variants in Brazil, all with single occurrence, which is likely due to the small number of mutation reports from NF1 patients in Brazil.Twenty-one variants were classified as VUS (20 in *NF1* and 1 in *NF2*). Determining pathogenicity of these variants is challenging without further functional and/or segregation studies.*In silico* prediction tools are often contradictory in their predictions for the same variant and it is still not possible to predict phenotypic effects from the location and type of mutation. Moreover, the high mutation rate of the gene results in a large number of novel mutations that need to be tested. In our series, we were able to classify only two of the VUS in *NF1* based on databases and previously published functional studies. For most of them, bioinformatics algorithms showed contradictory results (Table S2). However, with the exception of p.Ile1679Val and p.His2414Pro mutations, missense variants seem to be disease causing by this *in silico* analysis. It remains to be determined how these variants contribute to neurofibromin function. Functional analysis may help to assign the pathogenicity of unclassified variants.

Regarding mutation localization within the gene, we identified more than one variant in five of the*NF1* domains. Mutations in the *NF1*GAP-related domain were shown, many years ago, to disable GAP activity [37], and this provided direct evidence that failure of neurofibromin GAP function is the critical element in NF1 pathogenesis. In our study, six mutations (4 nonsense and 2 small deletions) occur in this domain and are, thus, predicted to severely decrease neurofibromin function. In addition, Mangoura and colleagues showed that neurofibromin GAP activity is regulated by protein kinase

C (PKC) dependent phosphorylation of the cysteine- and serine-rich domain (CSRD) of *NF1*, and, thus, mutations in this domain may affect this function. In our cohort, we identified nine mutations affecting CSRD. Other domains of the NF1 protein are the caveolin-1 binding domains. These domains are believed to be involved in the formation of a complex between neurofibromin and caveolin-1, and the complex is potentially involved in the regulation of several important signalling molecules including PKC and p21ras [39,40]. We did not find mutations in TBD and NLS domains (Figure 2). No mutations were detected in exon 31, the alternatively spliced exon inside NF1 GAP-related domain; most of the mutations were outside NF1 GAP-related domain, as described in other studies [30].

The fact that neurofibromin contains several functional domains makes it conceivable that different mutations occurring at different positions of the gene might lead to different phenotypes. However, only one well documented genotype-phenotype correlation was established to date when considering *NF1*point mutations: a 3-bp in-frame deletion c.2970_2972delAAT, which is associated with absence of neurofibromas [41]. This mutation was found in two unrelated familial probands in our study, both with café-au-lait macules, axillary and inguinal freckling and absence of neurofibromas. We were unable to identify other potential genotype-phenotype correlations. Several factors can have influenced this: most NF1 mutations are unique there is often significantly variable expressivity even among individuals with the same mutation, and modifier genes could contribute to explain this intra- and inter-familial variability. A few genes, inclusing *CDKN2B-AS1 GDNF*, *TLF*, and *MSH2*have been proposed as potential modifiers of the NF1 phenotype [42-45].

Finally, we did not find significant differences when comparing severity and visbility of NF1 patients according to the type and location of their mutations. Probands with mutations inside the GAP-related domain also did not show a more severe phenotype. Also, we found in a previous study conducted by our group (data not published) that *RNF135*and *SUZ12* genes could influence NF1 phenotype, mainly in microdeletions syndromes. These genes are located near *NF1* and are often involved in microdeletions. We did not found point mutations in *RNF135* and *SUZ12* in our series and could not confirm our previous hyphotesis.

Conclusion

The description of mutations detected in unrelated patients with NF1 and NF2 is very important for the follow-up of this multisystemic disease with high expression variability. The clinical diagnosis is complicated by a high degree of phenotypic variability. Thus, confirmation of a clinical diagnosis by identifying the pathogenic mutation can result in a better treatment of patients and better counseling for their family members. We designed a molecular diagnosis strategy to detect point mutations which showed point mutation detection rate of 79% in *NF1* and 29% in *NF2*. This is the first report where NGS technology was used to determine the complete known genetic background of neurofibromatosis type 1 in Brazil. Because of the numerous pseudogenes, in the AmpliSeq technique could achieve better efficacy and showed similar detection rates as Sanger sequencing. No specific genotype-phenotype correlations were established for Brazilian population, but other correlations described in literature were also observed by us. Further studies with larger scales are required to identify potential genotype-phenotype correlations in brazilian patients with NF1 and NF2. Also, functional analyses of each VUS may clarify the mechanisms of pathogenesis associated with NF1 and NF2.

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Table 1. Characteristics of the NF1 and NF2 patients included in this study.

Region	N	Average age in years (range)	Skin color* White/Black/Admixed/NI	Gender M/F	Average age at onset (years)	Family history (Y/N)
NF1	93	31.7 (1-64)	83/8/2/0	35/58	6.9	63/30
NF2	7	22.8 (11-44)	7/0/0/0	6/1	12.4	3/4

*Assessed by self-declaration and visual inspection;NI = Not informed.

Table 2. Clinical features of NF1 patients included in this study.

Symptom	P	N	%	% NE
Six or more café au lait macules	89	93	95.7	0
Two or more cutaneous/subcutaneous neurofibromas	79	91	86.8	2.1
Axillary freckling	74	89	83.1	4.3
Inguinal freckling	71	89	79.8	4.3
Plexiform neurofibroma	40	74	54.0	17.2
Two or more Lisch nodules	34	60	56.7	35.5
Optic pathway glioma	7	59	11.9	36.6
Scoliosis	17	53	32.0	43.0
Sphenoid wing dysplasia	3	48	6.2	48.4
Cognitive deficit	19	24	79.2	74.2
Macrocephaly	3	16	18.7	82.8
Breast Cancer	5	-	5.4	-
Gastrointestinal stromal tumor	1	-	1.1	-
Malignant *triton tumor*	1	-	1.1	-

P = presence (number of patients with the feature); **N** = total number of patients examined for this feature; **%** = frequency of each clinical feature according to the number of patients analyzed for this feature; % NE = percent number of the total patients that were not evaluated for this feature.

Table 3. Severity and visibility of NF1 patients evaluated by Riccardi and Ablon scales, respectively.

	Classification	Number of patients
Severity (Riccardi scale)	1	14
	2	29
	3	36
	4	9
	Not informed	5
Visibility (Ablon scale)	Mild	46
	Moderate	23
	Severe	19
	Not informed	5

Table 4. Clinical features of NF2 patients included in this study.

Symptom	P/N	%	% NE
Schwannoma	6/7	85.8	0
Bilateral vestibular schwannomas	3	42.9	0
Unilateral vestibular schwannoma	3	42.9	0
Plexiform neurofibroma	1/1	100.0	85.7
Meningioma	3/5	60.0	28.6
Hearing loss	4/5	80.0	28.6
Spinal cord tumor	2/4	50.0	42.8
Tinnitus	2/4	50.0	42.8
Ependimoma	1/4	25.0	42.8

P = presence (number of patients with the feature); **N** = total number of patients examined for this feature; **%** = frequency of each clinical feature according to the number of patients analyzed for this feature.

% NE = percent number of the total patients that were not evaluated for this feature.

Table 5. Severity and visibility of NF1 patients according to the type and location of mutations in *NF1* gene.

Type	Position	Riccardi					Ablon		
		1	**2**	**3**	**4**	**Total**	**Mild**	**Moderate**	**Severe**
Small deletion	Exon 1-26	2	1	4	1	**8**	3	3	2
Splice site	Exon 1-26	1	2	7	1	**11**	4	3	6
Nonsense	Exon 1-26	0	2	5	1	**8**	4	2	2
Missense	Exon 1-26	0	1	2	1	**4**	3	0	1
Small insertion	Exon 1-26	0	3	0	0	**3**	2	0	1
TOTAL		**3**	**9**	**18**	**4**		**16**	**8**	**12**
Small deletion	Exons 27-38	0	2	2	2	**6**	2	3	1
Splice site	Exons 27-38	1	1	1	0	**3**	2	1	0
Nonsense	Exons 27-38	2	4	3	0	**9**	4	6	2
Missense	Exons 27-38	3	2	0	0	**5**	4	1	0
Small duplication	Exons 27-38	0	0	1	0	**1**	1	0	0
TOTAL		**6**	**9**	**7**	**2**		**13**	**11**	**3**
Small deletion	Exon 39-58	1	3	1	0	**5**	2	0	3
Splice site	Exon 39-58	0	0	1	0	**1**	1	0	0
Nonsense	Exon 39-58	2	1	1	2	**6**	3	1	1
Missense	Exon 39-58	0	2	0	1	**3**	2	0	1
TOTAL		**3**	**6**	**3**	**3**		**8**	**1**	**5**

Table S1.*NF1* and *NF2*mutations detected in this study.

N	Inheritance	Gene	Position	Coding change	Amino acid change	Mutation type	Reported*/ MAF*	Pathogenicity
4	2 Familial/ 2 Sporadic	*NF1*	*NF1*	c.(?_-1)_(*1_?)del	-	Complete deletion	Yes	Pathogenic
1	Familial	*NF1*	Intron 1	c.61-13C>T	-	Splice site	No/ND	VUS
1	Familial	*NF1*	Intron 1	c.61-2 A>G	-	Splice site	Yes/ND	VUS
1	Familial	*NF1*	Intron 1	c.61-1 G>T	-	Splice site	No/ND	Likely Pathogenic
1	Familial	*NF1*	Exon 2	c.79C>T	p.Gln27Ter	Nonsense	No/ND	Likely Pathogenic
1	Familial	*NF1*	Exon 2	c.169G>A	p.Gly57Ser	Missense	Yes/0.00003	VUS
1	Sporadic	*NF1*	Exon 3	c.233delT	p.Asn78Ilefs*7	Frameshift deletion	Yes/ND	Likely Pathogenic
1	Familial	*NF1*	Intron 3	c.288+1G>C	-	Splice site	Yes/ND	Likely Pathogenic
1	Familial	*NF1*	Intron 8	c.889-21 C>T	-	Splice site	No/ND	VUS
1	Familial	*NF1*	Exon 9	c.980T>C	p.Leu327Pro	Missense	Yes/ND	VUS
1	Familial	*NF1*	Exon 9	c.999C>G	p.Tyr333Ter	Nonsense	No/ND	Likely Pathogenic
2	Familial	*NF1*	Exon 11	c.1246C>T	p.Arg416Ter	Nonsense	Yes/ND	Likely Pathogenic
1	Sporadic	*NF1*	Exon 13	c.1459A>T	p.Arg487Ter	Nonsense	No/ND	Likely Pathogenic
1	Familial	*NF1*	Exon 13	c.1466A>G	p.Tyr489Cys	Missense	Yes/0.00002	Pathogenic
1	Familial	*NF1*	Intron 13	c.1527+1_1527+4del	-	Splice site	No/ND	Likely Pathogenic
1	Sporadic	*NF1*	Intron 13	c.1527+5 G>C	-	Splice site	Yes/ND	VUS
1	Familial	*NF1*	Exon 14	c.1541_1542del	p.Gln514Argfs*43	Frameshift deletion	Yes/ND	Pathogenic
1	Sporadic	*NF1*	Exon 15	c.1721G>A	p.Ser574Asn	Missense	Yes/ND	VUS
1	Sporadic	*NF1*	Intron 15	c.1722-1G>A	-	Splice site	Yes/ND	Likely Pathogenic
1	Sporadic	*NF1*	Exon 16	c.1754_1757del	p.Thr586Valfs*18	Frameshift deletion	No/ND	Likely Pathogenic

Table S1.*Cont.*

N	Inheritance	Gene	Position	Coding change	Amino acid change	Mutation type	Reported*/MAF	Pathogenicity
1	Familial	*NF1*	Exon 17	c.1895_1896insTGAT	p.Ile634Ter	Frameshift insertion	No/ND	Likely Pathogenic
1	Familial	*NF1*	Exon 17	c.1901del	p.Pro635Leufs*53	Frameshift deletion	No/ND	Likely Pathogenic
1	Familial	*NF1*	Exon 17	c.1924C>T	p.Gln642Ter	Nonsense	No/ND	Likely Pathogenic
2	Familial	*NF1*	Exon 18	c.2041C>T	p.Arg681Ter	Nonsense	Yes/0.000008	Pathogenic
1	Familial	*NF1*	Exon 18	c.2160_2161insT	p.Cys721Leufs*5	Frameshift insertion	No/ND	Likely Pathogenic
1	Sporadic	*NF1*	Intron 18	c.2251+3 A>T	-	Splice site	Yes/ND	VUS
1	Familial	*NF1*	Exon 21	c.2585_2586insC	p.Tyr863Leufs*2	Frameshift insertion	No/ND	Likely Pathogenic
1	Familial	*NF1*	Exon 21	c.2693T>C	p.Leu898Pro	Missense	Yes/ND	Likely pathogenic
2	Familial	*NF1*	Exon 22	c.2970_2972del	p.Met991del	Deletion	Yes/0.000008	Pathogenic
1	Familial	*NF1*	Intron 22	c.2991-1 G>A	-	Splice site	Yes/ND	Likely Pathogenic
1	Familial	*NF1*	Intron 23	c.3113+5 G>T	-	Splice site	Yes/ND	VUS
1	Familial	*NF1*	Exon 24	c.3156_3157del	p.Ser1053Lysfs*6	Frameshift deletion	No/ND	Likely Pathogenic
1	Sporadic	*NF1*	Exon 24	c.3193_3194delAC	p.Thr1065Lysfs*23	Frameshift deletion	No/ND	Likely Pathogenic
1	Familial	*NF1*	Intron 24	c.3198-4T>C	-	Splice site	Yes/0.0004	Likely benign
1	Familial	*NF1*	Exon 27	c.3687_3701del	p.Val1230_Ser1234del	Deletion	No/ND	VUS
1	Familial	*NF1*	Exon 28	c.3737_3740del	p.Phe1247Ilefs*18	Frameshift deletion	No/ND	Likely Pathogenic
1	Familial	*NF1*	Exon 28	c.3826C>T	p.Arg1276Ter	Nonsense	Yes/0.000008	Pathogenic
1	Familial	*NF1*	Exon 29	c.3876T>G	p.Tyr1292Ter	Nonsense	No/ND	Likely Pathogenic
1	Sporadic	*NF1*	Exon 30	c.4077delT	p.Gln1360Asnfs*18	Frameshift deletion	Yes/ND	Likely Pathogenic
3	Sporadic/ 2 Familial	*NF1*	Exon 30	c.4084C>T	p.Arg1362Ter	Nonsense	Yes/0.000008	Pathogenic

Table S1.*Cont.*

N	Inheritance	Gene	Position	Coding change	Amino acid change	Mutation type	Reported*/ MAF*	Pathogenicity
1	Familial	*NF1*	Exon 32	c.4288A>T	p.Lys1430Ter	Nonsense	No/ND	Likely Pathogenic
1	Familial	*NF1*	Exon 35	c.4600C>T	p.Arg1534Ter	Nonsense	Yes/0.000008	Pathogenic
1	Sporadic	*NF1*	Exon 36	c.4743_4747del	p.Glu1582Ilefs*38	Frameshift deletion	No/ND	Likely Pathogenic
1	Familial	*NF1*	Exon 36	c.4783C>T	p.Gln1595Ter	Nonsense	No/ND	Likely Pathogenic
1	Familial	*NF1*	Exon 37	c.4928T>A	p.Val1643Glu	Missense	No/ND	VUS
2	Familial	*NF1*	Exon 37	c.5035A>G	p.Ile1679Val	Missense	Yes/ND	Likely pathogenic
1	Sporadic	*NF1*	Exon 37	c.5036_5041del	p.Tyr1678_Ile1679del	Deletion	No/ND	VUS
1	Familial	*NF1*	Intron 37	c.5269-9A>G	-	Splice site	Yes/0.0002	Likely benign
1	Familial	*NF1*	Intron 37	c.5269-1 G>C	-	Splice site	No/ND	Likely Pathogenic
1	Familial	*NF1*	Exon 38	c.5305C>T	p.Arg1769Ter	Nonsense	Yes/ND	Pathogenic
1	Sporadic	*NF1*	Exon 38	c.5345delT	p.Tyr1783Ilefs*11	Frameshift deletion	No/ND	Likely Pathogenic
1	Sporadic	*NF1*	Exon 38	c.5464C>T	p.Gln1822Ter	Nonsense	Yes/ND	Likely Pathogenic
1	Sporadic	*NF1*	Exon 38	c.5514_5515dupT	p.Ile1839Tyrfs*23	Frameshift dup	No/ND	Likely Pathogenic
1	Sporadic	*NF1*	Exon 38	c.5561T>G	p.Leu1854Arg	Missense	Yes/ND	VUS
1	Familial	*NF1*	Exon 38	c.5609G>A	p.Arg1870Gln	Missense	Yes/ND	Pathogenic
1	Familial	*NF1*	Intron 38	c.5609+5G>A	-	Splice site	Yes/ND	VUS
1	Familial	*NF1*	Exon 39	c.5788T>C	p.Cys1930Arg	Missense	Yes/ND	VUS
1	Familial	*NF1*	Exon 40	c.5907_5908del	p.Arg1970Serfs*6	Frameshift deletion	Yes/ND	Pathogenic
1	Sporadic	*NF1*	Exon 40	c.5991G>A	p.Trp1997Ter	Nonsense	Yes/ND	Pathogenic
1	Familial	*NF1*	Exon 43	c.6515C>A	p.Ser2172Ter	Nonsense	No/ND	Likely Pathogenic
1	Familial	*NF1*	Intron 45	c.6819+3 A>G	-	Splice site	Yes/ND	VUS

Table S1.*Cont.*

N	Inheritance	Gene	Position	Coding change	Amino acid change	Mutation type	Reported*/ MAF*	Pathogenicity
2	Familial/ Sporadic	*NF1*	Exon 45	c.6772C>T	p.Arg2258Ter	Nonsense	Yes/ND	Pathogenic
1	Sporadic	*NF1*	Exon 46	c.6850_6853del	p.Tyr2285Thrfs*5	Frameshift deletion	Yes/ND	Pathogenic
2	Familial	*NF1*	Exon 46	c.6855C>G	p.Tyr2285Ter	Nonsense	No/ND	Pathogenic
1	Familial	*NF1*	Exon 47	c.6989T>A	p.Leu2330Ter	Nonsense	No/ND	Likely Pathogenic
1	Familial	*NF1*	Exon 48	c.7188del	p.Tyr2398Thrfs*20	Frameshift deletion	No/ND	Likely Pathogenic
1	Familial	*NF1*	Intron 48	c.7190-33_7190-22del	-	Splice site	Yes/0.0120	VUS
1	Familial	*NF1*	Exon 49	c.7241A>C	p.His2414Pro	Missense	No/ND	VUS
1	Sporadic	*NF1*	Exon 49	c.7311_7313del	p.Tyr2438del	Deletion	No/ND	VUS
1	Familial	*NF1*	Exon 49	c.7321G>A	p.Ala2441Thr	Missense	No/ND	VUS
1	Sporadic	*NF2*	Exon 1	c.(?_-1)_(114+1_115-1)del	-	Exon 1 deletion	Yes/ND	Likely pathogenic
1	Sporadic	*NF2*	Intron 3	c.363+1G>A	-	Splice site	Yes/ND	VUS
1	Familial	*NF2*	Exon 12	c.1228C>T	p.Gln410Ter	Nonsense	Yes/ND	Likely Pathogenic

N = number of patients with the mutation; *****Reported in at least one of the databases analyzed; **MAF =** Minor Allele Frequency described in 1000 Genomes Project; **ND** = Not Described; **VUS** = Variant of Uncertain Significance.

Table S2. Predicted effect of the detected novel/VUS single aminoacid substitution variants on neurofibromin and merlin proteins.

Variant	Gene	PolyPhen-2	Mutation Assessor	Phanter	SIFT	Mutation Taster
p.Gly57Ser	*NF1*	Possibly damaging	Medium	NI	Damaging	Disease causing
p.Leu327Pro	*NF1*	Probably damaging	Medium	NI	Damaging	Disease causing
p.Ser574Asn	*NF1*	Probably damaging	Low	NI	Damaging	Disease causing
p.Leu898Pro	*NF1*	Probably damaging	Medium	NI	Damaging	Disease causing
p.Val1643Glu	*NF1*	Possibly damaging	Medium	NI	Damaging	Disease causing
p.Ile1679Val	*NF1*	Benign	Low	NI	Tolerated	Disease causing
p.Leu1854Arg	*NF1*	Probably damaging	Medium	NI	Damaging	Disease causing
p.Cys1930Arg	*NF1*	Possibly damaging	Medium	NI	Damaging	Disease causing
p.His2414Pro	*NF1*	Benign	Neutral	NI	Tolerated	Disease causing
p.Ala2441Thr	*NF1*	Probably damaging	Medium	NI	Damaging	Disease causing

SIFT = Sorting Intolerant From Tolerant; **NI** = Not Informed.

Table S3. Clinical phenotypes of NF1 patients with synonymous/without *NF1* mutations.

Inheritance	Gender	Skin					Eyes		Bones		Cognitive deficit	Breast cancer
		CALM	CN	AF	IF	PN	LN	OG	Sc	SD		
Sporadic	F	+	+	-	-	-	-	-	-	-	-	-
Sporadic	F	-	+	-	-	+	-	-	-	-	-	-
Sporadic	M	+	+	+	+	-	+	-	-	-	+	-
Sporadic	F	+	+	-	-	-	-	-	-	-	-	-
Sporadic	F	+	+	+	+	+	+	+	-	-	-	-
Sporadic	F	+	+	-	-	-	-	-	-	-	-	-
Sporadic	F	+	+	+	+	-	+	-	-	-	+	-
Sporadic	F	+	-	-	-	-	+	+	-	-	-	-
Familial	F	+	-	-	-	-	+	-	-	-	+	-
Familial	F	+	+	+	+	-	-	-	-	-	-	-
Familial	M	+	-	-	-	-	-	-	-	-	-	-
Familial	F	+	+	-	-	-	-	-	-	-	-	-
Familial	M	+	+	-	-	-	-	-	-	-	-	-
Familial	M	+	+	+	+	+	+	-	-	-	-	-
Familial	F	+	+	+	+	+	-	-	-	-	-	+
Familial	F	+	+	+	+	+	+	-	-	-	-	+

*__CALM__ = Café-au-lait macules; __CN__ = Cutaneous neurofibroma; __AF__ = Axillary freckling; __IF__ = Inguinal freckling; __PN__ = Plexiform neurofibroma; __LN__ = Lisch nodules; __OG__=Optic pathway glioma; __Sc__ = Scoliosis; __SD__ = Sphenoid wing dysplasia.

Figure 1. A. Distribution of the NF1 patients from the State of Rio Grande do Sul according to place of birth. **B.**Distribution of all*NF1*mutations identified by mutation type and respective frequency.

Figure 2. Distribution of *NF1* sequencing variants using NM_001042492.2, with 58 exons (including exon 31) as reference sequence.Neurofibromin domains are indicated: blue, CSRD (cystein and serine rich domain); pink, TBD (tubulin binding domain); green, GRD (GAP-related domain – interaction with Ras and GTP hydrolyisis); red, Sec14-PH (bipartite lipid binding domain); orange, NLS (nuclear localization sequence).

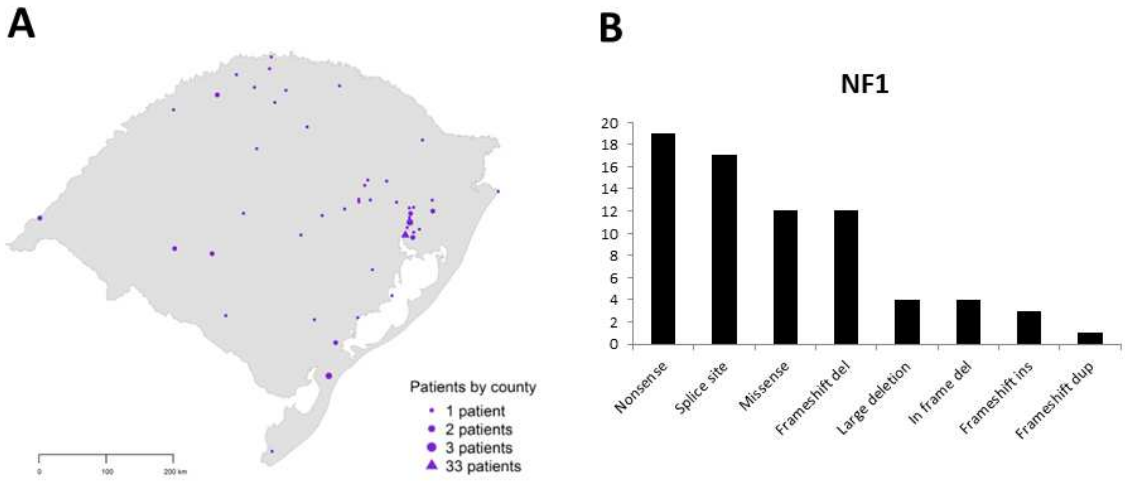
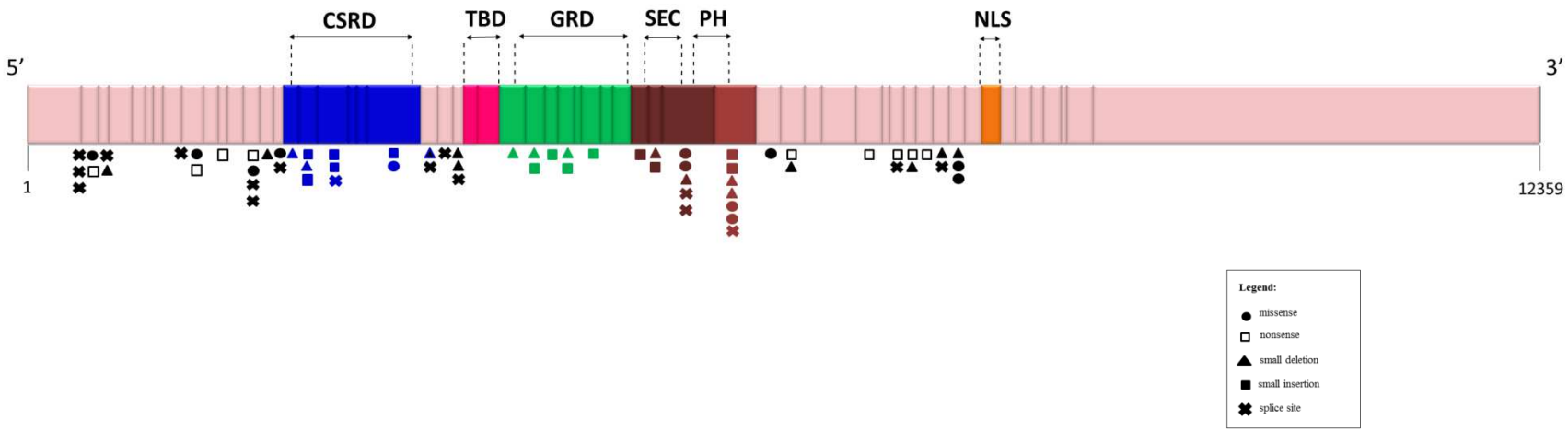

A
Patients by county
1 patient
2 patients
3 patients
33 patients
B
NF1
20
18
16
14
12
10
8
6
4
2
0
Nonsense
Splice site
Missense
Frameshift del
Large deletion
In frame del
Frameshift ins
Frameshift dup

Figure 1


CSRD
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Legend:
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small deletion
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splice site

Figure 2

Capítulo IV

Manuscrito III

Manuscrito publicado na revista Genetics and Molecular Biology

TSC1 and *TSC2* gene mutations and their implications for treatment in Tuberous Sclerosis Complex: a review

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Running title:*TSC* mutations: a review

Keywords: Tuberous sclerosis complex; *TSC* mutations; genotype-phenotype correlations; *TSC1*; *TSC2*.

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Clévia Rosset participated on literature review, reading and selecting studies to include in the paper, manuscript design, conceptualization and writing and analysis of the data; Cristina Brinckmann Oliveira Netto and Patricia Ashton-Prolla participated onliterature review, manuscript design, conceptualization, writing and revising for intellectual content.

Disclosures

All the authors report no disclosures.

Abstract

Tuberous sclerosis complex is an autosomal dominant disorder characterized by skin manifestations and formation of multiple tumors in different organs, mainly in the central nervous system. Tuberous sclerosis is caused by the mutation of one of two tumor suppressor genes, *TSC1* or *TSC2*. Currently, the development of novel techniques and great advances in high-throughput genetic analysis made mutation screening of the *TSC1* and *TSC2* genes more widely available. Extensive studies of the *TSC1* and *TSC2* genes in patients with TSC worldwide have revealed a wide spectrum of mutations. Consequently, the discovery of the underlying genetic defects in *TSC* has furthered our understanding of this complex genetic disorder and genotype-phenotype correlations are becoming possible, although there are still only a few clearly established correlations. This review focuses on the main symptoms and genetic alterations described in TSC patients from thirteen countries in three continents as well as genotype-phenotype correlations established to date. The determination of genotype-phenotype correlations may contribute to the establishment of successful personalized treatment for TSC.

1. Tuberous Sclerosis Complex

Tuberous sclerosis complex (TSC) is an autosomal dominant neurocutaneous andprogressive disorder, frequently characterized by the occurrence of multiple tumors in different organs. Penetrance reaches 95% and is variable; expressivity also varies greatly even within a given family(Northrup*et al*., 1993).The incidence of TSC is 1/10,000 births and its prevalence in the general population of Europe has been estimated to be 8.8/100,000(Orphanet: Tuberous Sclerosis, 2015), affecting multiple ethnic groups (Joinson *et al*., 2003).

2. Diagnosis and symptomatology

Tuberous sclerosis has been initially described by von Recklinghausen in 1862. In 1908, Heinrich Vogt established the diagnostic criteria for TSC as the so-called triad: epilepsy, mental retardation and adenoma sebaceum. As none of these clinical signs were pathognomonic for TSC, clinical diagnostic criteria were revised by a consortium in 1998 (Roach*et al*., 1998), which proposed three diagnostic categories (definite, probable or possible TSC) based on the presence of major and/or minor features of the disease. Table 1 shows the revised and updated diagnostic criteria for TSC, established by the same consortium in 2012 (Northrup *et al*., 2013). A definite clinical diagnosis is made when two major features or one major feature plus two minor features are present. Importantly, most major features are localized to the skin and central nervous system. Also, one must consider that the clinical manifestations of TSC appear at distinctdevelopmental points and a person with suspected TSC may need multiple sequential evaluations before a definite clinical diagnosis can be made.

After skin and CNS findings, renal manifestations are the most common abnormalities associated with TSC. These include renal cell carcinoma, oncocytomas, angiomyolipomas (in 80% of patients) and renal cystic disease (in 50% of the patients) (Dixon*et al*., 2011). Typically, renal manifestations in children with TSC are first seenin infancy and increase with age. Angiomyolipomas, one of the leading causes of death in TSC patients, are multiple and often bilateral. The associated mortality is due to complications when these lesions become very large. Another consequence of angiomyolipomas is destruction of the normal renal parenchyma resulting in renal failure and end-stage renal disease (Shepherd*et al*., 1991). Patients with clinically detectable renal cystic disease usually have a severe very early-onset polycystic phenotype (about 2% of TSC patients) (Sampson*et al*., 1997).

Pulmonary involvement, specifically lymphangioleiomyomatosis (LAM), is the third most common cause of TSC-associated morbidity, occurring in approximately 35% of female TSC patients. LAM is caused by proliferation of atypical smooth muscle cells in the peribronchial, perivascular, and perilymphatic tissues of the lung (Kumasaka*et al*., 2004).LAM occurs almost exclusively in young women, typically presenting between 30 to 35 years of age.Symptoms have been reported to begin or worsen during pregnancy, suggesting that LAM may be hormonally influenced (Castro*et al*., 1995).

Skin lesions are detected in 70% of patients with TSC and include hypomelanotic macules, shagreen patches, confetti-like lesions, forehead fibrous plaque, facial angiofibromas, and periungual and ungual fibromas (Schwartz*et al*., 2007). Depending on studied population, even as many as 100% of TSC patients younger than 5 years may present with hypopigmented macules. An aggregation of reddish papules, appearing on the nose and cheeks in a characteristic butterfly distribution, belongs to the Vogt triad of signs. Although usually symmetrical, occasionally they may be found

unilaterally (Jozwiak*et al*., 1998). Facial angiofibromas (adenoma sebaceum) are formed by hamartomatous growth of dermal connective tissue with rich vasculature and can result in decreased quality of life since they affect appearance, may cause disfigurement, and are prone to bleeding, which increases the possibility of infection (Yates, 2006). Shagreen Patches are areas of thick, irregularly shaped, and elevated skin, usually found on the lower back. Mean age of appearance is about 8.1 years (Sun *et al*., 2005). Ungual and subungual fibromas are small tumors that grow around and under toenails or fingernails. Their mean age of appearance is 14.9 years (Sun *et al*., 2005) and their prevalence in older patients (above 30 years) is close to 90%. Forehead plaques appear under the age of 14 years (Jozwiak*et al*., 1998), with mean age of appearance being 2.6 years (Sun *et al*., 2005).

TSC is also associated with both retinal and nonretinal ocular findings (Rowley*et al*., 2001). Hamartomas are the most common retinal manifestation of TSC and are identified in approximately 40 to 50% of individuals. Fortunately, they rarely compromise vision, although severe decreases in visual acuity and blindness has been reported in some cases due to hamartoma enlargement, macular involvement, retinal detachment, and vitreous hemorrhage (Robertson *et al*., 1999).

Multiple cardiac rhabdomyomas are cardiac tumors most frequently encountered during infancy and childhood and they occur in approximately 30% of TSC patients. On the other side, nearly 100% of fetuses with multiple rhabdomyomas have TSC. Cardiac rhabdomyomas usually do not cause symptoms or hemodynamic compromise and the natural history for these lesions is spontaneous regression in the vast majority of cases. However, the minority of the cases may become symptomatic shortly after birth or in the first year of life. Finally, hamartomas may also occur in organs of the

endocrine system and rare case reportsexist of angiomyolipomas or fibroadenomas in the pituitary gland, pancreas, or gonads (O'Callaghan*et al*., 2010).

3. Neurological involvement

Neurologic complications are the most common and often the most impairing aspect of TSC. Structural neurological abnormalities include cortical tubers, subependymal nodules (SENs) and subependymal giant cell tumors (SGCTs). Brain tumors in TSC are rare (2 to 10 % of patients with TSC and 1.1–1.4 % of all pediatric brain tumors) (Frèrebeau*et al*., 1985).Cortical tubers are developmental abnormalities present in more than 88% of children with TSC (Cuccia*et al.,* 2003) and the average number of tubers per patient ranges from 5 to50 in different studies. Tubers lead to loss of the classical six-layered cyto-architecture of the cerebral cortex and are thought to be responsible for more than 75% of the epileptic disorders in patients with TSC (Orphanet: Tuberous Sclerosis, 2015). The second more frequent structural neurologic lesions in children with TSC are SENs, which are small hamartomas that occur in the walls of the lateral ventricles. Only SENs located in the region of the Monro foramina may have the potentiality to grow and to transform into SGCTs (5%-20% of patients). The last but not least important type of encephalic lesion is SGCT, affecting an average of 10% of children with TSC. SGCTs are benign, slow-growing tumors of mixed glioneuronal cells including giant cells. They are typically located near the foramen of Monro, hence they can cause increased intracranial pressure, obstructive hydrocephalus, focal neurologic deficits and death (Orphanet: Tuberous Sclerosis, 2015).Approximately 90% of TSC patients experience seizures and about 50% have documented cognitive impairment, autism, or other behavioral disorders. Epilepsy is likely the most prevalent

and challenging clinical manifestation of TSC, and virtually all subtypes of seizure have been reported. At least one third of patients develop refractory epilepsy; attention deficit-hyperactivity disorder and psychiatric comorbidities such as mood disorders, anxiety, obsessive compulsive behavior and alcoholism are also frequently present. Among the different sites of tumor development, the brain remains undoubtedly the most problematic in terms of therapeutic management and screening.Brain tumors are the cause of more than 50% of deaths among children with TSC (Webb*et al*., 1996). Intellectual disability has a prevalence of 40%-50% in TSC; 30% are severely affected with IQs in the very low range, and 70% have IQs in the normal, yet slightly left-shifted range (Joinson*et al*., 2003).

4. Molecular Genetics of TSC: the *TSC1* and *TSC2* genes

Tuberous sclerosis is caused by the mutation of one of two tumor suppressor genes: *TSC1* (9q34) or *TSC2* (16p13.3). The*TSC1*gene spansabout53kbof genomicDNAwith 23exonscoding forhamartin, a hydrophilicproteinwith 1164 amino acidsand130kDa. Hamartin is expressedin several adult tissues and plays a key role in theregulation of cell adhesion. This proteinshowsnohomologywith any other vertebrate protein.The *TSC2*gene comprisesapproximately43kbof genomicDNA with 41exonsencoding a 5.5 kb transcript and a 1807amino acid protein,tuberin, with 198kDa. This proteincontains a hydrophilic N-terminal domain and a conserved163amino acidregion encoded by exons 34-38,near theC-terminal portion, which has homologywiththe RassuperfamilyGTPases proteins rap1GAPandmSpa1(Maheshwar*et al*., 1997).Therefore, tuberin is a GTPase activatingprotein that regulates the GTP binding and hydrolysing activity of the Ras superfamily of proteins and help to regulate cell

growth, proliferation and differentiation. The other domains of tuberin are less conserved, and additional homologies between tuberin and other proteins have not been identified. Serfontein and colleagues used bioinformatics tools to examine the presence of conserved elements of *TSC1* and *TSC2* across different organisms (Serfontein *et al.*, 2011). The analyzed organisms showed a wide range in the degree to which residues implicated in signalling are conserved (or present at all) in comparison to the human *TSC1* and *TSC2* sequences. Not surprisingly, the other mammalian proteins (*Rattus norvegicus* and *Mus musculus*) shared the largest number of residues with the human proteins.

Figure 1A schematically shows the structure of *TSC1* and *TSC2* genes, their coding exons and the main domains of hamartin and tuberin. These proteins bind each other via their respective coiled-coil domains to form an intracellular complex that integrates signals to control cellular homeostasis, oxygen levels, presence of nutrients, energy pool and stimulation by growth factors. Such signals regulate Rheb (a Ras homologue enriched in brain), responsible for the activation of mTOR (mammalian target of rapamycin) kinase. mTOR, in turn, regulates translation of a significant proportion of cellular proteins, including those responsible for the control of cell growth and proliferation (Kwiatkowski, 2003). Figure 2 shows the role of TSC2:TSC1 complex in mTOR pathway. Loss of function mutations in *TSC1* or *TSC2* lead to deregulated expression patterns in this pathway, abnormal production of the end products and ultimately promote tumorigenesis. To date, specific mechanisms by which these loss of function mutations cause disease are not established. It is suggested that tumor formation is initiated as a consequence of at least two hits (Knudson, 1971): as *TSC1* and *TSC2* are tumor suppressor genes, the inactivation of both *TSC1* or both *TSC2*alleles is necessary for benign and malignant tumor formation. The first hit is an

inherited germline mutation in *TSC1* or *TSC2*, which can be detected in approximately 85% of patients with the clinical features of TSC, and the second hit is somatic. There are multiple possible mechanisms for somatic inactivation of the wild-type alleles of *TSC1* and *TSC2*, including loss of heterozygosity, mutation and promoter methylation. It is possible that epigenetic silencing mediated by micro-RNAs also occurs. Moreover, binding of TSC1 to TSC2 appears to stabilize intracellular TSC2 levels since uncomplexed TSC2 is subject to ubiquitin mediated degradation (Chong-Kopera *et al*., 2006). Thus, TSC1 has a role in stabilizing the complex, while TSC2 has the GTPase activity. For this reason, inactivating mutations in either gene give rise to the same clinical disorder. Clearly, both proteins play pivotal roles in several processes that are crucial for normal braindevelopment. In addition, because they are widely expressed throughout the mature brain, these proteins likely have important homeostatic regulatory functions in neurons during adult life.

Although several TSC families exhibit an autosomal dominant pattern ofinheritance, 70% of cases result from *de novo* germline mutations. Linkage studies initially suggested that therewould be equivalent numbers of families with mutations in each *TSC* gene (Benvenuto*et al*., 2000). However, the frequencyof mutations reported in *TSC2* is consistently higher than in *TSC1; TSC1* mutations accountfor only 10% to 30% of the families identified with TSC. In sporadic TSC, there is aneven greater excess of mutations in *TSC2.* Nonetheless, identification of *TSC1* mutations appears tobe twice as likely in familial cases as in sporadic cases. The disparity in mutational frequency mayreflect an increased rate of germline and somatic mutations in *TSC2* as compared with *TSC1*, as wellas an ascertainment bias, since mutations in *TSC2* are associated with more severe disease (Dabora*et al*., 2001; Jansen*et al*., 2008; Kothare*et al*., 2014). In patients with the TSC phenotype and no identifiable mutations in either

*TSC1*or *TSC2*(15% to 20%),the disease is usually milder (Dabora*et al*., 2001). A milder phenotype has also been described in rare individuals with mosaicism for mutations in *TSC1* or*TSC2*. Caignec and colleagues (2009) reported a unique family with three independentpathogenic mutations in *TSC2* mapping to distincthaplotypes. The three mutations were most likely *de novo*, asparents of the affected patients did not present any featuresof TSC. In addition, findings consistent with gonadalmosaicism were seen in one branch of the family.

5. Molecular diagnosis in TSC

The development of novel techniques and great advances in high-throughput genetic analysis in the last few years made mutation screening of the *TSC1* and *TSC2* genes feasible. Recent massively parallel sequencing technologies (Next-Generation Sequencing, NGS) and copy number variation testing (Multiplex Ligation-dependent Probe Amplification – MLPA and array-Comparative Genomic Hybridization - aCGH) have been validated for clinical use in many disorders including TSC, rendering the analysis much faster and more cost-effective.

Extensive studies of the *TSC1* and *TSC2* genes in patients with TSC have revealed a wide spectrum of mutations. We searched the PubMeddatabase to retrieve available published literature in English from 1998 to 2014 that described mutations at *TSC1* and *TSC2* genes and established genotype-phenotype correlations for tuberous sclerosis disease. The following keywords were used: *TSC1* mutations; *TSC2* mutations; tuberous sclerosis complex; *TSC* mutations; *TSC* molecular analysis; genotype-phenotype correlation on tuberous sclerosis. Twenty-seven studies were included in the final analysis. Table 2summarizes the results obtained in the main studies performed

with unrelatedTSC patients worldwide; many of the changes listed were found for the first time in the investigated population. The most frequent mutation type is point mutations. Large gene rearrangements are less frequently reported, both because of their true prevalence in TSC and also because several studies did not use methodologies that are directed to the identification of such mutations.As expected, the observed mutation detection rate is not always complete. In this group, a mutation could exist in an intronic region distant from the exon-intron boundaries, which could have an effect on the splicing process or gene regulation, causing a reduction of normal mRNA transcript. Although a third gene for TSC may exist and explain this lack of mutation at *TSC1* and *TSC2* genes in some patients, there is currently no concrete evidence for this. Also, somatic and germ line mosaicism is a credible explanation for the failure to detect mutations in some patients and specialized methods can be used to enhance detection of thesespecific situations. Most studies in TSC patients were conducted in Europe and Asia. The largest cohorts are from the Netherlands and Poland/USA. As expected through observed mutation frequencies, in all populations described, the germline mutation rate at the *TSC2* locus was higher than that at the *TSC1* locus. Also, the frequency of small rearrangements (small insertions/deletions) is higher than missense, nonsense and splice site mutations in all populations.

The exponential discovery rate of novel genomic alterations that cause TSC stimulated the creation and storage of genetic information in mutation databases. In the Human Genome Mutation Database (HGMD, 2014) for instance, 30 unique missense and 59 nonsense mutations in *TSC1* had been described by 2014, as well as 91 small deletions, 41 small insertions, 31 splice site mutations and 21 large rearrangements. In this database, TSC1 mutations correspond to 93% of the mutations, with the largest number of them occurring in exon 15, which is the largest in basepairs (559). Proportionally, it

corresponds to a mutation frequency of 9.5% (determined as the percentage of mutations per base pairs, considering the size of eachexon) and the highest mutation frequency is in exon 13 (14.3%). Considering all exons, the average frequency of observed mutations is 5.9 %. Seven of the 23 exons have higher values (above 9%). Small deletions are responsible for 41% of the disease and small insertions, for 18.5%. Large rearrangements are responsible for 7% of mutations in TSC1 at this database. The predicted coiled-coil domain of hamartin corresponds to exons 17-23, where 21.9% of the mutations are localized. Exons 17-18 are responsible for interaction with tuberin, and they account for 15.9% of the point mutations described. Another database, Leiden Open Variation Database (LOVD, 2014), have reported 690 unique DNA variants on *TSC1* gene.

Considering the *TSC2* gene, 183 unique missense and 125 nonsense mutations gene were described in HGMD database, as well as 189 small deletions, 99 small insertions, 120 splice site mutations and 148 large rearrangements. Point mutations correspond for 82.6% of the mutations, with the largest number of them occurring in exon 33, which is the largest in basepairs (488). Proportionally, this exon corresponds to a mutation frequency of 15.45%, and the highest mutation frequency is in exon 37 (22.9%) and exon 38 (22.8%). Considering all exons, average frequency of observed mutations is 11.0 %, a higher number than mutation frequency at *TSC1* gene. Twenty-seven of the 41 exons have mutation frequency values above 9% and an overall mutation number and mutation frequency higher than in *TSC1* gene. Small deletions are responsible for 32% of the disease vs 41% in *TSC1* gene, and the small insertions for 17% vs 18.5% in *TSC1* gene. Large rearrangements are not shown in the table, and are responsible for 17.4% of mutations in *TSC2*, a higher number than the frequency of largerearrangements in *TSC1* gene. The predicted coiled-coil domain of tuberin

corresponds to exon 10 of the *TSC2* gene, where only 1.8% of small mutations are localized. Exons 34-38 encode the GAP-domain, responsible for the essential GTPase activaty, and they account for 18.1% of the point mutations described at this gene, with a high mutation frequency (95.1%). Exons 37 and 38 have shown the highest mutation frequency in *TSC2* gene, and these mutations can have a damage effect on the protein since GAP domain can be disrupted. In Leiden Open Variation Database, 1925 unique DNA variants on *TSC2* gene have been reported. Figure 1A illustrates the distribution of point mutations among all exons and domains of *TSC1* and *TSC2* genes described in these different studies and Figure 1B graphically represents the occurrence of point mutations in each *TSC1* and *TSC2* exons (percentage of the total number of described mutations in HGMD database that occurs in each exon). This percentage is not related to exon size, but larger exons contain more mutations than smaller exons.

Because TSC can be a devastating disease, family members of affected individuals are often eager to know whether they are carriers of *TSC* mutations. Actually, with the adventure of next generation sequencing platforms, it became possible to analyze point mutations in both *TSC1* and *TSC2* genes at the same time for a lower cost; if no mutations are detected, the search for large deletions and duplications should proceed. Prenatal and preimplantation genetic tests are also becoming more widely available. The mutation status of family members has great implications on genetic counseling. Furthermore, for all clinical diagnostic criteria, patients with subclinical TSC may not be correctly diagnosed, and genetic testing is also very important for these cases.

The second International Tuberous Sclerosis Complex Consensus Conference met seventy-nine experts from 14 countries to finalize diagnostic, surveillance, and management recommendations for patients with TSC (Northrup*et al*., 2013). At this meeting, the most significant change recommended was the incorporation of genetic

testing to the diagnostic criteria. Molecular testing of the *TSC1* and *TSC2* genes yields a positive mutation result for 75-90% of TSC-affected individuals categorized as having definite Clinical Diagnostic Criteria. The recommendation of the Genetics Panel was to make the identification of a pathogenic mutation in *TSC1* or *TSC2* an independent diagnostic criterion, regardless of the clinical findings. This will facilitate the diagnosis of TSC in some, particularly young individuals, allowing earlier implementation of surveillance and treatment with a potential for better clinical outcomes. *TSC1* and *TSC2* genetic variants whose functional effect is not definitely pathogenic would not be considered a major diagnostic criterion. Finally, a normal result from *TSC1* and *TSC2* testing does not exclude TSC, since a fraction of TSC patients have no mutation identified by conventional genetic testing. Nonetheless, if the mutation in an affected relative is known, testing for that mutation has very high predictive value for family members.

6. Genotype-phenotype correlations in TSC

The discovery of the underlying genetic defects in *TSC* has furthered our understanding of this complex genetic disorder and genotype-phenotype correlations are becoming possible. In a retrospective study, Kothare and colleagues (2014) analyzed a series of 919 TSC patients and found that carriage of a germline *TSC2* mutation was associated with SENs and SGCTs. Occurrence of tubers, however, did not differ between carriers of *TSC1*or*TSC2* mutations. In general, patients with *TSC2* mutation presented with symptoms at a younger age. Dabora and colleagues (2001) analyzed 224 TSC patients and found that seizures, average cortical tuber number and SEN are more frequent or severe in patients with *de novoTSC2* mutations than those with *TSC1* mutations. Jansen

and colleagues (2008) also reported a more severe neurologic phenotype including an earlier age of seizure onset, lower cognition index and more tubers in patients with a *TSC2* mutation as compared to those with a *TSC1* mutation. Another important correlation involves a subgroup of large genomic deletions at *TSC2* that also affect the adjacent *PKD1* gene, causing early-onset polycystic kidney disease (Osborne *et al*., 1991). Table 3 shows a compilation of the main genotype-phenotype correlations described to date. As expected, most *TSC2* mutations are generally associated with a more severe phenotype. Only one *TSC2* mutation, R905Q, was associated with milder disease. This mutation was found in 25 individuals from the same family, with a phenotype characterized by the complete absence of disfiguring skin lesions, intractable epilepsy, mental retardation, and severe organ involvement. So, the type and location of mutations in both *TSC1* and *TSC2* genes also have an influence in the phenotype. Hamartin and tuberin are known to bind to at least 40 additional proteins, and thus there are numerous potential and yet undefined effects of *TSC* gene mutations. Futhermore, it is likely that other events such as mosaicism, the nature and frequency of the second event of inactivation of the second allele and the modifying genes and environmental effects may interfere with the phenotype, which makes it more difficult to establish clear genotype-phenotype correlations. Moreover, polymorphic and non-pathogenic variants in *TSC1* and *TSC2* genes can act as phenotype modifiers in tuberous sclerosis, and they need to be further explored. To date, little is known about non-pathogenic variants in these genes, and phenotype modifiers in tuberous sclerosis have not been identified so far.

In light of emerging human genetic and molecular knowledge, molecular diagnosis of TSC and determination of genotyope-phenotype correlations might help in the establishment of personalized treatment for TSC patients and improve quality of life

among these patients. Continuous studies in this area can guide future directions in this line.

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Table 1. Revised Diagnostic Criteria for Tuberous Sclerosis Complex *.

Major Features

1. Facial angiofibromas or forehead plaque
2. Non-traumatic ungual or periungual fibroma
3. Hypomelanotic macules (more than three)
4. Shagreen patch (connective tissue nevus)
5. Multiple retinal nodular hamartomas
6. Cortical tuber[a]
7. Subependymal nodule
8. Subependymal giant cell astrocytoma
9. Cardiac rhabdomyoma, single or multiple
10. Lymphangiomyomatosis[b]
11. Renal angiomyolipoma[b]

Minor Features

1. Multiple randomly distributed pits in dental enamel
2. Hamartomatous rectal polyps[c]
3. Bone cysts[d]
4. Cerebral white matter migration lines [a,d,e]
5. Gingival fibromas
6. Non-renal hamartoma[c]
7. Retinal achromic patch
8. "Confetti" skin lesions
9. Multiple renal cysts[c]

Definite TSC: Either 2 major features or 1 major feature with 2 minor features

Probable TSC: One major feature and one minor feature

Possible TSC: Either 1 major feature or 2 or more minor features

[*] Revised Diagnostic Criteria for Tuberous Sclerosis Complex established by a consortium in 2012 (see reference number 5).

[a] When cerebral cortical dysplasia and cerebral white matter migration tracts occur together, they should be counted as one rather than two features of TSC.

[b] When both lymphangiomyomatosis and renal angiomyolipomas are present, other features of TSC should be present before a definitive diagnosis is assigned.

[c] Histologic confirmation is suggested.

[d] Radiographic confirmation is sufficient.

[e] One panel member recommended three or more radial migration lines constitute a major feature.

Table 2. Type and frequency of mutations found in *TSC* genes in patients from different studies in the world and diagnostic strategy (1998-2014).

Population	N	Noncoding/ polymorphic alterations	No mutation detected (%)	*TSC1*				*TSC2*				Mutation detection methods (Reference of the study)
				Point mutations (missense/ nonsense)	Rearrangements		Splice site mutations	Point mutations (missense/ nonsense)	Rearrangements		Splice site mutations	
					Small	Large			Small	Large		
Europe												
Germany	37	9	3 (8,1)	3/4	3	NA	1	10/3	8	NA	2	SSCP/Sequencing (Gumbinger *et al*., 2009)
Turkey	33	9	27 (81)	NA	NA	NA	NA	3/0	2	NA	1	SSCP/Sequencing (Apak *et al*., 2003)
Poland/USA	224	NA	38 (17)	0/11	15	0	2	31/37	43	20	27	DHPLC/Sequencing; long-range PCR/qPCR (Dabora *et al*., 2001)
Netherlands	490	76	128 (26)	0/37	38	0	7	56/67	94	20	43	SSCP/Sequencing/ Southern blot/ FISH(Sancak *et al*., 2005)
Germany	68	14	37 (54)	0/1	1	0	0	12/4	11	2	4	SSCP/Sequencing/ Southern blot/ FISH (Langkau *et al*., 2002)
Denmark	65	24	11 (17)	0/4	6	0	1	11/9	13	4	6	DGGE/ Sequencing; lomg range PCR/MLPA (Rendtorff *et al*., 2005)
United Kingdom	150	30	30 (20)	NI	NI	NI	NI	22/20	26	22	8	SSCP/ heteroduplex analysis/pulse field gel electrophoresis/ Southern blot/ long range PCR (Jones *et al*., 1999)
SUBTOTAL	1067	162	274 (26)	3/57	63	0	11	145/140	197	68	91	-
Asia												
India	24	10	12 (50)	0/0	1	NA	0	3/1	5	NA	2	SSCP/Sequencing (Ali *et al*., 2005)

Table 2. Cont.

Population	N	Noncoding/ polymorphic alterations	No mutation detected (%)	*TSC1*				*TSC2*				Mutation detection method (s)
				Point mutations (missense/ nonsense)	Rearrangements		Splice site mutations	Point mutations (missense/ nonsense)	Rearrangements		Splice site mutations	
					Small	Large			Small	Large		
China	2	NA	0	0/0	0	NA	0	0/0	1	NA	1	Sequencing (Mi *et al*., 2014)
Korea	44	NA	31 (70)	2/0	0	NA	0	0/4	6	NA	1	DHPLC/Sequencing (Choi *et al*., 2006)
Japan	21	2	0	0/1	8	NA	0	2/0	4	NA	3	DHPLC/Sequencing (Sasongko *et al*., 2008)
Japan	8	3	0	0/1	1	NA	0	4/0	0	NA	3	SSCP/Sequencing (Yamamotoa *et al.*, 2002)
China	6	2	3 (50)	0/0	3	NA	0	NA	NA	NA	NA	Sequencing (Tian *et al*., 2013)
Malaysia	2	-	0	-	-	-	-	-	-	2	-	MLPA (Ismail *et al*., 2014)
Korea	11	NA	2 (18)	1/3	0	1	0	2/1	0	1	2	Sequencing/MLPA (Jang *et al*., 2012)
Taiwan	84	21	20 (24)	0/5	4	NA	0	12/15	21	NA	7	DHPLC/Sequencing (Hung *et al*., 2006)
China	6	3	3 (50)	0/0	0	NA	0	2/1	0	NA	0	Sequencing (You*et al*., 2013)
SUBTOTAL	184	31	59 (32)	3/11	16	1	0	22/21	32	3	17	-
America												
USA	21	11	0	0/5	12	0	0	NA	NA	NA	NA	Southern blot/ heteroduplex/SSCP (Kwiatkowska *et al*., 1998)

Table 2. Cont.

Population	N	Noncoding/ polymorphic alterations	No mutation detected (%)	*TSC1*				*TSC2*				Mutation detection method (s)
				Point mutations (missense/ nonsense)	Rearrangements		Splice site mutations	Point mutations (missense/ nonsense)	Rearrangements		Splice site mutations	
					Small	Large			Small	Large		
USA	126	47	52 (41)	0/7	7	NA	2	13/14	23	NA	8	SSCP/Sequencing (Niida *et al*., 1999)
USA	36	NA	7 (19)	0/1	1	0	2	4/6	6	4	2	Sequencing/MLPA (Qin *et al*., 2010)
SUBTOTAL	183	58	59 (32)	0/13	20	0	4	17/20	29	4	10	-
TOTAL	1434	251	392 (27)	6/81	99	1	15	184/181	258	75	118	-

N= number of patients included in the study; SSCP=Single Strand Conformation Polymorphism; DHPLC=Denaturing High-Performance Chromatography; FISH= Fluorescent *In Situ* Hybridization; DGGE=Denaturing Gradient Gel Electrophoresis; MLPA=Multiplex Ligation-Dependent Probe Amplification; NA=not analyzed in the study; NI=Not informed

Table 3. Genotype-phenotype correlations established for TSC patients.

Population	N	Locus of DNA alteration	Amino acid change	Type of alteration	Main associated symptoms	Reference
EUA	1039	*TSC2*	-	Any type on *TSC2*	Mutations in the *TSC2* gene were more frequent than *TSC1* gene in patients with retinal findings	(Aronow *et al*., 2012)
Poland	170	*TSC2* c.5238-5255del 18pb	-	Frameshift	Epilepsy	(Rok *et al*., 2005)
USA	220	Contiguous deletion *TSC2-PKD1*	-	Large rearrangement	Arachnoid cysts and polycystic kidney disease	(Boronat *et al*., 2014)
Poland/USA	224	*TSC2*	-	Any type on *TSC2*	Seizures, mental retardation, average tuber count, subependymal nodules, renal angiomyolipomas, angiofibromas and fibrous forehead plaques were more common and severe in *TSC2* patients	(Dabora *et al*., 2001)
Netherlands	490	*TSC1*	-	Any type on *TSC1*	Shagreen patches are more frequent in patients with *TSC1* mutation	(Sancak *et al*., 2005)
Netherlands	490	*TSC2*	-	Any type on *TSC2*	Mental retardation is more frequent in patients with *TSC2* mutation	(Sancak *et al*., 2005)
Netherlands	490	*TSC2*	-	Nonsense and frameshift	Shagreen patches, forehead plaques, facial angiofibromas, ungual fibromas, renal angiomyolipomas and renal cysts	(Sancak *et al*., 2005)
Netherlands	490	*TSC2*	-	Mutations in the GAP domain	Mental retardation, seizures and subependymal nodules	(Sancak *et al*., 2005)
Korea	11	*TSC2*	-	Mutations in exons 33-41	Cardiac rhabdomyomas	(Jang *et al*., 2012)

Table 3. Cont.

Population	N	Locus of DNA alteration	Amino acid change	Type of alteration	Main associated symptoms	Reference
USA	65	*TSC2*	-	Any type on *TSC2*	Higher number of cysts than *TSC1* woman with pulmonary lymphangioleiomyomatosis	(Muzykewicz *et al.*, 2009)
Canada	19 families	*TSC2*	R905Q	Missense	Milder disease severity	(Jansen *et al.*, 2006)
USA	478	*TSC2* proximal region (exons 1-22) and distal region (exons 34-41)	-	Missense mutations and small in-frame deletions or insertions	Proximal and distal *TSC2* mutations showed a significantly higher risk of infantile spasms compared with mutations in the central region of the gene	(van Eeghena *et al.*, 2013)
USA and Belgium	919	*TSC2*	-	Any type on *TSC2*	More frequent occurrence of several kinds of seizures/epilepsy subtypes: partial epilepsy, complex partial seizures, infantile spasms, SENs, SGCTs and cognitive impairment.	(Kothare *et al*., 2014)
United Kingdom	One case report	*TSC1* intron 10 (c.1030-3 C>G)	-	Splice site mutation	Mild phenotype (seizures and small number of hypomelanotic macules)	(Blyth *et al*., 2010)

N= number of patients included in the study
NI=Not informed

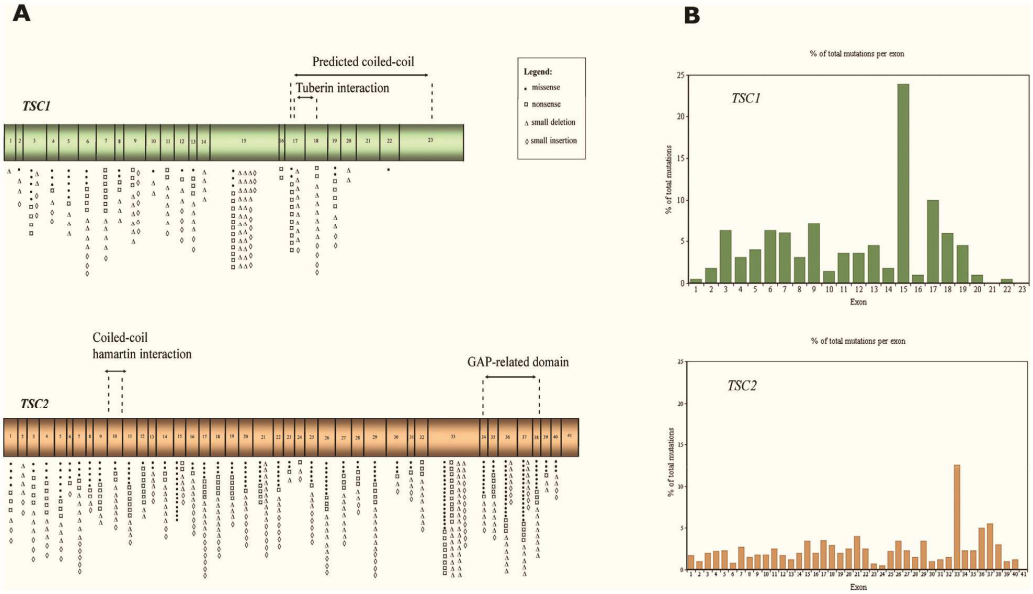

Figure 1 - *TSC1* and T*SC2* gene structure, domains and distribution of point mutations. (A) Schematic representation of *TSC1* and *TSC2* exons and the domains of hamartin and tuberin, respectively, codified by them. The symbols represent the number of different mutations described at each exon. (B) The graph shows the percentage of the total number of described mutations that occur at each *TSC1* and *TSC2* exon.

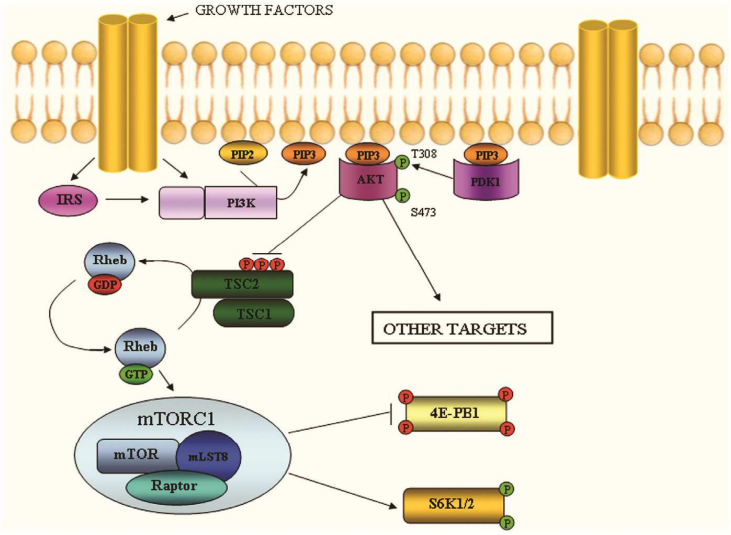

Figure 2 - The role of the TSC2:TSC1 complex in the mTOR pathway. PI3K is activated by growth factors through direct interaction with receptors or through interaction with scaffolding adaptors, such as the IRS proteins. These interactions recruit PI3K to its substrate PtdIns(4,5)*P*2 (PIP2), allowing generation of the lipid second messenger PtdIns(3,4,5)*P*3 (PIP3). Akt and PDK1 are recruited to the cell plasma membrane through association with PIP3. This allows Akt to be activated through phosphorylation on Thr308 by PDK1 and Ser473 by mTORC2 (not shown). Once active, Akt phosphorylates many downstream targets, including multiple sites on TSC2. Phosphorylation of TSC2 impairs the GTPase activity of the TSC2:TSC1 complex, allowing Rheb-GTP to accumulate. Rheb-GTP in excess activates high levels of mTORC1, which in turn phosphorylates and inhibits 4E-BP1 and activates S6K1 and S6K2. By this way, mTORC1 influences on cell growth, translation factors activation and cell nutrition.

Capítulo V

Manuscrito IV

Manuscrito submetido para a revista Plos One e formatado conforme as regras da mesma

Molecular analysis of *TSC1* and *TSC2* genes and phenotypic correlationsin Brazilian families with Tuberous Sclerosis

Short title: Molecular analysis of *TSC1* and *TSC2* genes in Brazil

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Abstract

Tuberous sclerosis complex is an autosomal dominant multisystem disorder characterized by the development of multiple hamartomas in many organs and tissues. It occurs due to inactivating mutations in either of the two genes, *TSC1* and *TSC2*, following a two hit tumor suppressor molecular pathogenesis in most hamartomas. Comprehensive screens for mutations in both the *TSC1* and *TSC2* loci have been performed in several cohorts of patients and a broad spectrum of pathogenic mutations have been described. In Brazil, there is no data regarding incidence and prevalence of tuberous sclerosis and mutations in *TSC1* and *TSC2*.We analyzed both genes in 53 patients diagnosed with or with high suspicion of tuberous sclerosis from different regions of Brazil, using multiplex-ligation dependent probe amplification and a customized next generation sequencing panel. Confirmation of all variants was done by the Sanger method. We identified 50 distinct variants in 47 (89%) of the patients. Five were large rearrangements and 45 were point mutations. The types and severity of mutations were not different across Brazilian regions. The symptoms presented by our series of patients were not different between male and female individuals, except for the more common occurrence of shagreen patch in women (p=0.028). In our series, consistent with other studies,*TSC2* mutations were associated with a more severe phenotypic spectrum than *TSC1* mutations. This is the first study that sought to characterize the molecular spectrum of Brazilian tuberous sclerosis patients.

Keywords: Tuberous sclerosis complex; *TSC* mutations; genotype-phenotype correlations; *TSC1*; *TSC2*.

Introduction

Tuberous sclerosis complex (TSC - OMIM 191100) is an autosomal dominant multisystem disorder that occurs in all ethnic groups and both sexes. Population studies have estimated the prevalence of the disease in 1 in 6000 to 9000 individuals and at least 2 million people are affected worldwide [1]. The clinical findings and severity of TSC are highly variable; for this reason, clinical diagnostic criteria were established by a consortium in 1998 [2], and revised and updated by the same group in 2012 [3]. Most TSC patients have hamartomas in the brain, skin, kidneys and heart. Involvement of the lung, gastrointestinal tract, bones, retina and/or gingiva is also common [4].

TSC occurs due to inactivating mutations in either of two genes, *TSC1* in chromosome 9q34 or *TSC2* in chromosome 16p13, and follows the two hit tumor suppressor model of pathogenesis in most hamartomas [5]. *TSC1* is composed of 23 exons and encodes for hamartin, a ubiquitously expressed 1164 amino acid protein [6] while *TSC2* consists of 42 exons and encodes for tuberin, a ubiquitously expressed 1807 amino acid protein [7]. Both proteins form a complex that regulates cell growth and tumorigenesis [8]. About one third of the patients with TSC have a familial form, in which the disorder follows a clearly dominant inheritance, whilst the other two-thirds are sporadic cases resulting from *de novo* germline mutations in one of the *TSC* genes [9, 10].

Comprehensive *TSC1* and *TSC2* mutation screening results have been reported in several cohorts of patients with TSC, as described in the Human Gene Mutation Database (HGMD) [11], and a broad spectrum of pathogenic mutations has been described. Among individuals who meet the clinical criteria, 75-90% have an identifiable mutation in either *TSC1* or *TSC2*, and the majority of mutation-positive

TSC patients have a mutation in *TSC2*. In sporadic cases, *TSC2* mutations are 2-10 times more common than *TSC1* mutations. In contrast, in multi-generation families segregating TSC, approximately half show linkage to each of the genes. About 80-95% of the *TSC1* and *TSC2* mutations are small mutations (missense, nonsense, small deletions, small insertions and splicing site mutations), and 5-20% are large duplications, large deletions or complex rearrangements. The high variability in type and position of mutations render molecular diagnosis of TSC challenging. This variability may explain, at least in part, the wide range of clinical symptoms observed in TSC patients, although timing and location of the second hit event is more likely to contribute to the variability of clinical symptoms. Only a few studies described a possible genotype-phenotype correlations for TSC [10, 12-18]. Although, *TSC1*-relateddisease is usually less severe than *TSC2*-relateddisease.

In Brazil, there is no data regarding TSC incidence or *TSC1* and *TSC2* mutation prevalence among affected individuals. The molecular diagnosis is particularly important for patients with suspected TSC who do not fulfill clinical diagnostic criteria, and for the purpose of genetic counseling since the disease can have intra-familial variable expressivity. Therefore, the aims of this study were to describe demographics and clinical phenotype of patients with TSC from different Brazilian regions and characterize the germline *TSC1* and *TSC2* mutations observed in a group of individuals with clinical diagnosis of TSC.

Methods

Patients and DNA samples

Twenty-two male and 31 female individuals with clinically diagnosed or highly suspicion of TSC were recruited at eight Oncogenetics services from four different

Brazilian regions, between August/2013 and May/2016. All patients were unrelated probands, including 17 familial and 36 sporadic cases. The study was approved by the institutional review board, Comitê de Ética em Pesquisa do Hospital de Clínicas de Porto Alegre (CEP-HCPA), under registration numbers GPPG 13-0260 and GPPG 15-0049. All individuals or legal representatives signed a written informed consent. Germline DNA samples were obtained from peripheral blood using a commercial kit (Flexigene Blood Kit, Qiagen, USA). Standardized clinical information was collected retrospectively by clinicians from each center after reviewing the medical records.

Large deletion/duplication analysis

All 53 unrelated individuals were screened for large *TSC1* and *TSC2* deletions and duplications by SALSA Multiplex ligation-dependent probe amplification (MLPA) analysis. Commercial SALSA MLPA kits P124-C1 and P046-C1 (MRC-Holland, Amsterdam, The Netherlands) were used for *TSC1* and *TSC2* analysis, respectively, according to manufacturer's instructions. The P124-C1 and P046-C1 probe mixes contain probes for each of the *TSC1* and *TSC2* exons and 9 and 8 reference probes detecting different autosomal chromosomal locations, respectively. In addition, P046-C1 contains one probe for the *PKD1* gene, adjacent to *TSC2*, which is associated with polycystic kidney disease. DNA samples from healthy individuals were used as normal copy number controls. MLPA amplification products were separated on an ABI3500 capillary sequencer (Applied Biosystems, Foster City, CA, USA) and the results were analyzed using Coffalyser.net. Ratios <0.7 were considered deletions and ratios >1.4 were considered duplications. The chromosomal microarray technique CytoScan HD (Affymetrix, USA) was used to confirm MLPA analysis when a deletion/duplication larger than 300kb was identified by MLPA, as recommended by the manufacturer. The

high-density, whole-genome CytoScan Array includes 2.69 million markers for copy number analysis. Chromosome Analysis Suite software (ChAS software 3.1) was used to analyze and visualize microarray data as well as for comparison of results with built-in reference of more than 400 samples.

Next Generation Sequencing (NGS)

TSC1 (NM_000368.4) and *TSC2* (NM_000548.3) amplicons were designed using the AmpliSeq Designer software (Thermo Fisher Scientific, CA, USA), targeting the complete coding sequence, 50 bp exon-intron junctions and 5' and 3' untranslated regions of *TSC1* and 99,83% of the coding sequence, 50 bp exon-intron junctions and 5' and 3' untranslated regions of *TSC2* gene, resulting in a total of 112 amplicons. A region of 17 base pairs of *TSC2* exon 29 remained uncovered. Amplicon library was prepared using the Ion AmpliSeq Library Kit 2.0 (Thermo Fisher Scientific, CA, USA) and NGS performed using 20 ng of genomic DNA and an Ion 316 sequencing chip on an Ion Personal Genome Machine and the 200 Sequencing kit (Thermo Fisher Scientific, CA, USA), with 500 flows. Data from the Ion Torrent runs were analyzed using the platform-specific pipeline software Torrent Suite v3.2.1 for base calling, trim adapter and primer sequences and filtering out poor quality reads. The sequences were aligned to the hg19 human reference genome and for variant calling, the sequence runs were imported to the Ion Reporter software v5.0. Allele call frequency cutoff of 10% was used to investigate mosaic and non-mosaic germ-line variants. Phred score >500 was considered to filter variants.Variants were also reviewed and annotated using this software. Integrative genomics viewer was used for visualization of the mapped reads [19].

Bioinformatics analysis

All variants identified by NGS were sought in the following databases: HGMD, ClinVar, Catalogue of Somatic Mutations in Cancer (COSMIC), Leiden Open Variation Database (LOVD), Tuberous Sclerosis Project database (TSP), and Genome Aggregation Database (gnomAD) [11, 20-24]. To predict the pathogenicity of missense variants and small indels we used two comprehensive *in silico* prediction tools: Mendelian Clinically Applicable Pathogenicity (M-CAP), and PredictSNP [25,26]. Splice site mutations were analyzed using BDGP software (Berkeley Drosophila Genome Project) and Mutation Taster [27, 28].

NGS Validation by Sanger Sequencing

For every sample with a variant of interest in one of the *TSC* genes, specific primers for the corresponding exon(s) were designed using Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and the reference sequences NM_000368.4 – *TSC1* and NM_000548.3 – *TSC2*. In addition, a specific primer for *TSC2* exon 29 (not covered in the NGS panel) was designed and DNA from all individuals was sequenced by the Sanger method for this exon. Primers were also designed for *TSC1* and *TSC2* promoter regions for variant screening in individuals with no identifiable pathogenic or probably pathogenic variants detected by MLPA or NGS. Primer sequences are available upon request. Forward and reverse primers were used to sequence the purified PCR products, using the BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI 3500 Genetic Analyzer (Thermo Fisher Scientific, CA, USA). Sequences were aligned to their reference using CodonCode Aligner software implemented in MEGA 5.04. Variant calling and interpretation were based on the American College of Medical Genetics most recent guidelines [29]. Points are attributed

to each variant according to these criteria, and they are classified as pathogenic, likely pathogenic, variant of uncertain significance (VUS), or likely benign.

Statistical analysis

We compared the frequency of each clinical finding between male and female individuals. The total number of Statistical comparisons were performed by conventional chi-squared or Fisher's exact test using SPSS software (version 19.0).

Results

We recruited 53 individuals with TSC from four different Brazilian regions (only the North region was not represented). Region of birth of the patients studied is summarized in Supplementary figure 1A, frequencies of the different types of mutation are in Supplementary figure 1B and individuals' characteristics are shown in Supplementary table 1. Median age at recruitment was 14 years (range: 6 months to 50 years) and average age at onset/recognition of the first symptoms was 3.3 years. Average age at TSC diagnosis was 7.1 years in familial cases and 2.6 years in sporadic cases. Fifty-two patients fulfilled the definitive TSC criteria established by the 1999 Tuberous Sclerosis Consensus Conference [2].

A phenotypic comparison between genders for each clinical feature is shown in Table 1. Only one feature (shagreen patch) was more frequently observed in females ($p = 0.028$). This difference may occur due to random chance, since multiple comparisons were performed. Lymphangioleiomyomatosis (LAM) was only detected in one female patient. There was no difference in the frequency of any symptom when we compared familial and sporadic cases.

Table 1. Phenotypic comparison between male and female individuals with TSC.

	Male (n = 22)		Female (n = 31)		
	P/N	**%**	**P/N**	**%**	**p-values**
Median age (years) / interaquartile range	10 /17		15 /16		
Hypomelanotic macules	18/22	82	24/28	86	0.738
Facial angiofibromas	16/22	73	21/29	72	0.889
Confetti lesions	5/22	22	5/28	18	0.732
Shagreen patch	3/22	14	11/28	40	0.028*
Ungual fibromas	2/22	9	7/28	25	0.439
Renal angiomyolipoma	10/22	45	19/27	70	0.071
Multiple renal cysts	5/17	29	5/20	25	0.717
Cortical tubers	17/21	81	22/27	81	0.264
Seizures	12/15	80	10/15	67	0.682
Mental retardation	10/17	58	11/18	61	0.890
Subependymal nodules	7/21	33	15/26	58	0.161
Astrocytomas	3/21	14	7/26	27	0.731
Cardiac rhabdomyomas	7/21	33	9/27	33	0.927
Retinal hamartomas	3/20	15	2/27	7	0.638
Gingival fibromas	2/22	9	3/28	11	1.000
Dental pits	2/22	9	5/28	18	0.444
Hepatic angiomyolipoma	1/22	4	6/25	24	0.194
Lymphangiomyomatosis	0/22	0	1/28	5	0.246
Rectal polyp	0/20	0	2/28	8	0.504

P = presence (number of patients with the feature); **N** = total number of patients examined;

% = frequency of each clinical feature in each group. *****Indicates statistically significant values.

Overall, 50 distinct *TSC1* and *TSC2* variants were identified in 47 (89%) out of the 53 patients. MLPA analysis identified five (9%) patients who were heterozygous for large rearrangements in *TSC2*: four large deletions (7%) and one large duplication (2%). complete *TSC2* deletion observed in one family was confirmed by CytoScan HD as a heterozygous deletion of 2.0Mb (108 genes including *TSC2* and *PKD1*). Two single exon deletions (exon 8 in *TSC1* and exon 19 in *TSC2*) were detected by MLPA. NGS and Sanger sequencing of these probands revealed point mutations at the hybridization probe sites of the specific exons, thus excluding the occurrence of these single-exon deletions. A *PKD1* deletion was found in one patient, since the MLPA kit P046 contains a probe for this gene. In addition, we identified 13 distinct heterozygous small variants in the coding region of *TSC1* and 32 in *TSC2*. We did not observe evidence of mosaicism (considering allele proportions between 10-50%). Read depth achieved in Ion Torrent analysis per amplicon of *TSC1* and *TSC2* in each subject is shown on supplementary table 2. A summary of the pathogenic and likely pathogenic variants is in table 2. Families with a likely benign or a VUS are shown in table 3.

Table 2. Families with a pathogenic or likely pathogenic variant in *TSC1* and *TSC2* (classified according to ACMG criteria).

Family	Inheritance	Gene	Position	Coding change	Amino acid change	ClinVar/HGMD/ LOVD/TSP (classification)	gnomAD MAF (homozygous)	M-CAP	Predict SNP1	ACMG*	Pathogenicity
1	Familial	*TSC1*	Exon 5	c.338T>A	p.(Leu113*)	No	NR	ND	ND	PVS1+PP1+PM2+PP4	Pathogenic
2	Familial	*TSC1*	Exon 8	c.682C>T	p.(Arg228*)	Yes (P)	NR	ND	ND	PVS1+PS4+PP1+PM2+PP4	Pathogenic
3	Sporadic	*TSC1*	Exon 8	c.733C>T	p.(Arg245*)	Yes (P)	NR	ND	ND	PVS1+PS4+PM2+PM6+PP4	Pathogenic
4	Sporadic	*TSC1*	Exon 9	c.801dup	p.(Glu268Argfs*5)	No	NR	ND	ND	PVS1+PM2+PM6+PP4	Pathogenic
5	Sporadic	*TSC1*	Exon 10	c.988del	p.(Leu330*)	No	NR	ND	ND	PVS1+PM2+PM6+PP4	Pathogenic
6	Familial	*TSC1*	Exon 10	c.989dupT	p.(Ser331Glufs*10)	Yes (P)	NR	ND	ND	PVS1+PS4+PP1+PM2+PP4	Pathogenic
7	Familial	*TSC1*	Intron 14	c.1439-2T>C	p.?	No	NR	ND	ND	PVS1+PP1+PM2+PP4	Pathogenic
8	Sporadic	*TSC1*	Exon 15	c.1888_1891del	p.(Lys630Glnfs*22)	Yes (P)	NR	ND	ND	PVS1+PS4+PM2+PM6+PP4	Pathogenic
9	Familial	*TSC1*	Exon 17	c.2071_2074dup	p.(Arg692Profs*15)	No	NR	ND	ND	PVS1+PP1+PM2+PP4	Pathogenic
10	Sporadic	*TSC1*	Exon 17	c.2090T>A	p.(Leu697*)	No	NR	ND	ND	PVS1+PM2+PM6+PP4	Pathogenic
11	Familial	*TSC1*	Exon 18	c.2287C>T	p.(Gln763*)	No	NR	ND	ND	PVS1+PP1+PM2+PP4	Pathogenic
12	Sporadic	*TSC2*	Exons 1-10	*TSC2*del e1-e10	p.?	Yes (P)	NR	ND	ND	PVS1+PS4+PM2+PM6+PP4	Pathogenic
13	Sporadic	*TSC2*	Exons 3-6	*TSC2*del e3-e6	p.?	No	NR	ND	ND	PVS1+PM2+PM6+PP4	Pathogenic
14	Sporadic	*TSC2*	Exon 3	c.169del	p.(Arg57Alafs*4)	No	NR	ND	ND	PVS1+PM2+PM6+PP4	Pathogenic
15	Familial	*TSC2*	Exon 8	c.724delinsTCCT	p.(Thr242delinsSerSer)	No	NR	ND	ND	PP1+PM2+PM4+PP4	Likely Pathogenic
16	Familial	*TSC2*	Exon 10	c.911G>A	p.(Trp304*)	Yes (NP)	NR	ND	ND	PVS1+PP1+PM2+PP4	Pathogenic
17	Sporadic	*TSC2*	Intron 10	c.975 + 1G>T	p.?	Yes (P)	NR	ND	ND	PVS1+PS4+PM2+PM6+PP4	Pathogenic
18	Sporadic	*TSC2*	Intron 10	c.976-15G>A	p.?	Yes (P)	NR	ND	ND	PS4+PM2+PM6+PP3+PP4	Pathogenic
19	Sporadic	*TSC2*	Exon 11	c.1008T>G	p.(Tyr336*)	Yes (NP)	NR	ND	ND	PVS1+PM2+PM6+PP4	Pathogenic
20	Familial	*TSC2*	Exon 11	c.1019T>C	p.(Leu340Pro)	Yes (P)	NR	P	D	PS4+PP1+PM2+PP3+PP4	Pathogenic
21	Sporadic	*TSC2*	Exon 12	c.1239_1240ins15	p.(Arg413_Cys414ins ValGlnPro*)	No	NR	ND	ND	PVS1+PM2+PM6+PP4	Pathogenic
22	Sporadic	*TSC2*	Exon 13	c.1323G>A	p.(Trp441*)	No	NR	ND	ND	PVS1+PM2+PM6+PP4	Pathogenic
23	Sporadic	*TSC2*	Exon 15	c.1513C>T	p.(Arg505*)	Yes (P)	NR	ND	ND	PVS1+PS4+PM2+PM6+PP4	Pathogenic

Table 2. Cont.

Family	Inheritance	Gene	Position	Coding change	Amino acid change	ClinVar/HGMD/ LOVD/TSP (classification)	gnomAD MAF (homozygous)	M-CAP	Predict SNP1	ACMG*	Pathogenicity
24	Familial	*TSC2*	Exon 16	c.1693del	p.(Leu565Trpfs*133)	Yes (NP)	NR	ND	ND	PVS1+PP1+PM2+PP4	Pathogenic
25	Sporadic	*TSC2*	Exons 17-33	*TSC2* dup e17-e33	p.?	No	NR	ND	ND	PVS1+PM2+PM6+PP4	Pathogenic
26	Familial	*TSC2*	Exon 19	c.1976_1977insA	p.(Ser660Glnfs*43)	No	NR	ND	ND	PVS1+PP1+PM2+PP4	Pathogenic
27	Familial	*TSC2*	Exon 19	c.2071del	p.(Arg691Alafs*7)	Yes (P)	NR	ND	ND	PVS1+PS4+PP1+PM2+PP4	Pathogenic
28	Sporadic	*TSC2*	Exon 20	c.2194C>T	p.(Gln732*)	Yes (P)	NR	ND	ND	PVS1+PS4+PM2+PM6+PP4	Pathogenic
29	Sporadic	*TSC2*	Intron 21	c.2355+1_2355+4del	p.?	Yes (P)	NR	ND	ND	PVS1+PS4+PM2+PM6+PP4	Pathogenic
30	Sporadic	*TSC2*	Exon 23	c.2551_2570dup	p.(Tyr857*)	No	NR	ND	ND	PVS1+PM2+PM6+PP4	Pathogenic
31	Sporadic	*TSC2*	Exon 27	c.2974C>T	p.(Gln992*)	Yes (P)	NR	ND	ND	PVS1+PS4+PM2+PM6+PP4	Pathogenic
32	Familial	*TSC2*	Exon 31	c.3685C>T	p.(Gln1229*)	Yes (P)	NR	ND	ND	PVS1+PS4+PP1+PM2+PP4	Pathogenic
33	Familial	*TSC2*	Exon 31	c.3772_3778del	p.(Ala1258Argfs*65)	No	NR	ND	ND	PVS1+PS4+PP1+PM2+PP4	Pathogenic
34	Sporadic	*TSC2*	Exon 34	c.4235_4236del	p.(Pro1412Argfs*3)	No	NR	ND	ND	PVS1+PS4+PM2+PM6+PP4	Pathogenic
35	Familial	*TSC2*	Exon 34	c.4375C>T	p.(Arg1459*)	Yes (P)	NR	ND	ND	PVS1+PS4+PP1+PM2+PP4	Pathogenic
36	Sporadic	*TSC2*	Exon 37	c.4685T>C	p.(Leu1562Pro)	Yes (NP)	NR	P	D	PM2+PM6+PP3+PP4	Likely Pathogenic
37	Familial	*TSC2*	Exon 40	5138_5149del	p.(Arg1713_Ala1716del)	Yes (LP)	NR	ND	ND	PP1+PM2+PM4+PP4+PP5	Pathogenic
38	Sporadic	*TSC2*	Exon 40	c.5106_5107insCACA	p.(Val1703Thrfs*4)	No	NR	ND	ND	PVS1+PM2+PM6+PP4	Pathogenic
39	Sporadic	*TSC2*	Exon 40	c.5126C>T	p.(Pro1709Leu)	Yes (P)	NR	P	D	PS4+PM2+PM6+PP3+PP4+ BP1	Pathogenic
40	Sporadic	*TSC2/ PKD1*	Exons 41-42/*PKD1*	*TSC2*del e41-e42/PKD1	p.?	Yes (P)	NR	ND	ND	PVS1+PS4+PM2+PM6+PP4	Pathogenic
41	Sporadic	*TSC2*	Exon 41	c.5238_5255del	p.(His1746_Arg1751del)	Yes (P)	NR	ND	ND	PS4+PP1+PM2+PM4+PP4	Pathogenic
42	Sporadic	*TSC2/ PKD1*	*TSC2/PKD1*	*TSC2/PKD1*del	p.?	Yes (P)	NR	ND	ND	PVS1+PS4+PM2+PM6+PP4	Pathogenic

HGMD: Human Gene Mutation Database; LOVD: Leiden Open Variation Database; TSP: Tuberous Sclerosis Project; MAF: Minor allele frequency; B: Benign; LB: Likely benign; VUS: Variant of uncertain significance; LP: Likely pathogenic; P: Pathogenic; NP: Not provided; NR: Not reported; ND: Not determined; N: Neutral; D: Deleterious. *ACMG 2015 pathogenicity criteria: PVS1: very strong; PS1-4: strong; PM1-6: moderate; PP1-5: supporting. ACMG 2015 benign criteria: BA1: stand-alone; BS1-4: strong; BP1-6: supporting.

Table 3. Families with a likely benign or uncertain significance variant in *TSC1* and *TSC2* (classified according to ACMG criteria).

Family	Inheritance	Gene	Position	Coding change	Amino acid change	ClinVar/HGMD/ LOVD/TSP (classification)	gnomAD MAF (homozygous)	M-CAP	Predict SNP1	ACMG*	Pathogenicity
43	Sporadic	*TSC1*	Intron 7	c.664-10A>C	p.?	No	NR	ND	ND	PM2+PM6+PP4	VUS
44	Sporadic	*TSC1*	Exon 23	c.3387C>T	p.(=)	Yes (B; LB; VUS)	233/277142 (1)	ND	ND	PP4+BP4+BP6+BP7+BS1	Likely benign
45	Familial	*TSC2*	Intron 10	c.975+8G>A	p.?	Yes (LB)	13/262922 (0)	ND	ND	PP1+PP4+BP6	VUS
40	Sporadic	*TSC2*	Exon 19	c.2011G>T	p.(Gly671Cys)	No	NR	P	N	PM2+PM6+PP4+BP1	VUS
6, 46	Sporadic	*TSC2*	Exon 33	c.3986G>A	p.(Arg1329His)	Yes (B;LB)	1797/271710 (36)	ND	ND	PP4+BP1+BP4+BP6+BS1	Likely benign
37	Familial	*TSC2*	Exon 34	c.4397C>T	p.(Ser1466Leu)	Yes (LB; VUS)	4/266180 (0)	P	N	PP1+PP3+PP4+BP6	VUS
35	Familial	*TSC2*	Exon 35	c.4527_4529del	p.(Phe1510del)	Yes (B; LB)	1409/276714 (10)	ND	ND	PP1+PP4+BP4+BP6+BS1	Likely benign

HGMD: Human Gene Mutation Database; LOVD: Leiden Open Variation Database; TSP: Tuberous Sclerosis Project; MAF: Minor allele frequency; B: Benign; LB: Likely benign; VUS: Variant of uncertain significance; LP: Likely pathogenic; P: Pathogenic; NP: Not provided; NR: Not reported; ND: Not determined; N: Neutral; D: Deleterious. *ACMG 2015 pathogenicity criteria: PVS1: very strong; PS1-4: strong; PM1-6: moderate; PP1-5: supporting. ACMG 2015 benign criteria: BA1: stand-alone; BS1-4: strong; BP1-6: supporting.

The distribution of small mutations within the *TSC1* and *TSC2* genes is shown in Figure 1. The tuberin domain that interacts with hamartin was recently solved and is shown accordingly in the figure 1 [30]. We calculated the mean number of small mutations (including splice site changes) per nucleotide for each exon of both genes. The overall mutation frequency was higher at the *TSC2* locus (0.006 mutations per nucleotide) when compared to *TSC1* (0.003 mutations per nucleotide). Exons 11, 19, 34 and 40 of the *TSC2* gene had the highest frequency of mutations.

Figure 1. Distribution of *TSC1* and *TSC2* sequencing variants. The reference sequences used were NM_000368.4 for *TSC1* and NM_000548.4 for *TSC2*. The 3'unstranslated region of *TSC1* is not represented due to its large size. Synonymous variants are not shown. The alterations represented in green are variants of uncertain significance (VUS) and the other were considered pathogenic/likely pathogenic.

Considering clinical data, the most commonly observed signs and skin/mucosal findings were hypopigmented macules, facial angiofibromas, Shagreen patches and ungueal fibromas. Regarding central nervous system symptoms, the most common findings were cortical tubers, subependymal nodules, cognitive deficiency and seizures. Subependymal giant astrocytomas occurred in 23% of the patients. Other common findings were renal angiomyolipomas, multiple renal cysts and cardiac rhabdomyomas. We examined the clinical manifestations of patients with different types of mutations in different domains of the *TSC1* and *TSC2* genes to assess whether there was any correlation between mutation type and position with specific clinical features. Comparing the total number of individuals with a *TSC2* mutation and seizures with the total number of individuals with *TSC1* mutation and seizures, individuals with *TSC2*

mutation had a higher frequency of this symptom (p = 0.008). The same occurred when considering astrocytomas (p = 0.0038). The total number of patients with *TSC2* mutations and central nervous system, renal and cardiac symptoms is also higher than patients with *TSC1* mutations that presented these symptoms, but did not reach statistical difference. We did not observe a difference in symptoms between patients with mutations in the start of *TSC1* and in the coiled-coil domain. However, patients with a nonsense mutation in either region of *TSC1* had cognitive deficiency and seizures, while patients with other types of mutation did not present these symptoms. Regarding *TSC2* mutations, the symptoms were similar in patients with mutations in the start, middle or GAP-related domain of the gene. The different types of mutations also did not result in specific phenotypes in this gene; even the patient with an entire *TSC2* deletion had a phenotype similar to that of patients with point mutations. These differences may occur due to random chance, since multiple comparisons were performed.

Clinical data for patients with a synonymous or no identified mutations is summarized in S3 table. All of these patients had at least two major diagnostic criterion of TSC and were sporadic cases. Cognitive deficiency, subependymal giant cell astrocytomas and retinal hamartomas did not occur in the group without an identifiable mutation. Other symptoms were also observed less frequently in this group, although the differences were not statistically significant.

Discussion

This study sought to characterize the clinical and molecular profile of Brazilian individuals with tuberous sclerosis. Although many *TSC1* and *TSC2* gene mutations causing the disease have been identified in other populations, no studies on Brazilian patients have been undertaken. Brazil is a country with continental dimensions; migrations of different populations to different regions of the country occurred during its history (e.g. there is a predominance of European migration to the southern region). For this reason, we analyzed patient characteristics and mutations according to their birth region and investigated if a certain type of mutation or a specific mutation occurred at a higher frequency in a region. In our set of patients, no predominance of skin color or differences in frequencies and type of mutations were found between regions. The overall mutation detection rate (89%) was within the expected in our study. Approximately two thirds of TSC probands worldwide are simplex cases [8]. Presence of a family history of the disease directly correlates with presence of a deleterious mutation either in *TSC1* or *TSC2* [9, 31]. In our series, the majority of probands (68%), as in the literature, had no family history of the disease; of these cases, 63% had a variant in *TSC1* or *TSC2* identified. In the familial cases, 82% had an identifiable variant. In addition, distribution of mutations was also similar to the rates observed in other studies, which show a predominance of *TSC2* rearrangements and point mutations over *TSC1* variants [32]. However, while *TSC2* mutations are 4-5 times more common than *TSC1* mutations in the literature, in our study *TSC2* mutations were only 2.5 times more common than *TSC1* mutations [9, 31]. The reason for this higher frequency of *TSC2* mutations is currently unknown. The coding region of *TSC2* is about 50% larger than *TSC1,* the number of exons is nearly doubled and the number of nonsense

mutations and small indels in the two genes are roughly proportional to this size difference. In addition, *TSC2* has a much higher GC content than *TSC1* (60% vs. 43%), which could favor point mutation occurrence. On the other side, *TSC1* contains more repeat elements than *TSC2* (32% vs. 25% total sequence), which could favor the occurrence of gene rearrangements. However, *TSC2* rearrangements were seen in our cohort, while *TSC1* rearrangements were not. Considering the regions inside *TSC1* and *TSC2*,mutations were distributed throughout their whole sequences, with the exception of *TSC2* 3' portion. Fifteen variants occur in a hamartin or tuberin functional domains and all frameshift and nonsense alterations outside these domains create a stop codon that produces an incomplete protein with partial or no functional domains. The different types of mutations also occur throughout the whole sequences, with a high occurrence of splice site mutations at the end of *TSC2* exon 10 and no preference of one type of mutation in others regions.

Using a combined approach of NGS and rearrangement analysis with MLPA, we were able to identify 20 novel variants (8 in *TSC1* and 12 in *TSC2*) and 30 previously reported variants. Three deletions had already been described in the literature, including a deletion of all *TSC2* and *PKD1* regions analyzed, which was identified in one proband. All 20 novel variants had single occurrence. One possible explanation for the occurrence of a disease phenotype with no identifiable germline *TSC1* or *TSC2* mutation in six probands (11%), could be related to intronic mutations distant from the exon-intron boundaries, with effects on the splicing process or gene regulation, causing a reduction of normal mRNA transcript. In addition, somatic mosaicism could account for some of these cases, as described before [9], although this possibility was initially eliminated by NGS. Finally, a third genetic locus related to TSC could exist.

Results from several studies over the past few years have provided new insights into how tuberin and hamartin might affect cell proliferation, growth, adhesion, migration, or protein trafficking. It has been demonstrated that tuberin and hamartin interact directly with each other, forming a cytoplasmic protein complex [33]. The C-terminal putative coiled-coil domain of hamartin is necessary for interaction with tuberin HEAT repeat domain. Additionally, tuberin is phosphorylated at serine and tyrosine residues in response to growth factors, which affects the interaction between hamartin and tuberin [34]. The GAP-related domain (Figure 2) of tuberin is responsible for the inhibition of cell division by indirect modulation of mammalian target of rapamycin (mTOR), a central regulator of translation [35]. Considering the importance of these domains, mutations in the coding regions of the interaction domains or GAP-related domain, as well as in phosphorylated residues in tuberin or frameshift and nonsense mutations that exclude these domains from the proteins are likely to be pathogenic. Missense and splice site mutations may also affect directly these domains or interfere in protein correct folding, charge and hydrophobicity. Although we did not find mutations in tuberin phosphorylation sites, we identified mutations that affect hamartin or tuberin functional domains. Furthermore, all nonsense mutations observed cause a premature stop codon that excludes an important domain. Nonsense-mediated decay (NMD) could also explain the loss of function effect of nonsense and frameshift variants in *TSC1* and *TSC2*. Canonical splice site changes were classified as potentially pathogenic by bioinformatics algorithms and have functional tests already described in literature that proved their pathogenicity (they exclude the corresponding exons of the genes): c.975 + 1G>T and c.976-15G>A in *TSC2* and c.1439-2T>C in *TSC1* [36-38]. The mutation c.2355+1_2355+4del in *TSC2* that may also affect splice site is proved to

be pathogenic by functional studies [39]. The other splice site variants found in this study did not change the splice site scores predicted by the softwares used.

Regarding missense variants, it is often difficult to predict their effect on protein function. Analysis of familial segregation may help, but the main problem with this approach is the progressively small size of families, lack of information within families and in the specific case of TSC, the predominance of simplex cases. We chose to use two *in silico* prediction tools that combine several pathogenicity scores to achieve a consensus classification and try to reduce misclassification of the variants. M-CAP uses existing pathogenicity likelihood scores and direct measures of evolutionary conservation to achieve a misclassification rate of the pathogenic variants of less than 5%. PredictSNP[1] is a consensus classifier that combines six tools and provides significant improvement in prediction performance over the individual tools and over other consensus classifiers, such as CONDEL and Meta-SNP [40, 41]. We were able to classify as pathogenic or likely pathogenic three of the six missense variants found in our probands: the *TSC2* exon 11 (c.1019T>C) variant has functional studies indicating significant lower *TSC2* expression [42]; the mutations p.Leu1562Pro and p.Pro1709Leu are localized inside the GAP related domain of tuberin. Prolines are known to have a very rigid structure, sometimes forcing the backbone in a specific conformation. In the first mutation, a proline is gained and this might disturb the GAP domain conformation; in the second mutation, the mutant residue is bigger and this might lead to bumps in this important domain. The other three missense variants (p.Gly671Cys -VUS, p.Arg1329His - likely benign and p.Ser1466Leu -VUS) are outside tuberin functional domains and occur concomitantly with other pathogenic mutations in *TSC2*. Both p.Arg1329His and p.Ser1466Leu variants are already described with low frequencies in

1000genomes database. VUS detected in the present study would be good candidates for functional studies which could help to establish their pathogenicity.

Clinical presentation did not differ significantly in male and female individuals and signs and symptoms of TSC were most commonly observed in adults than in children. Dermatologic and central nervous system lesions and renal findings are described as the most common clinical features of TSC, observed in 80-90% and 60-80% of the patients, while cardiac rhabdomyomas are present in 50%, and lymphangioleiomyomatosis in 40% of the female patients [43]. The frequencies of the most common symptoms in our patient series were similar to those previously described, with exception of lymphangioleiomyomatosis, which we observed only in one female patient. When we analyzed phenotype and variant location, we observed that *TSC2* variants were associated with a more severe phenotypic spectrum when compared to *TSC1* variants.This is consistent with other observations that *TSC1* patients have milder overall disease severity [9, 31]. All statistical results found by us may occur due to random chance, since multiple comparisons were performed. Finally, all patients with *PKD1* alterations had renal polycystic disease, as expected for a *TSC2-PKD1* contiguous gene deletion syndrome or even isolated PKD1 deletions, which is characterized by severe juvenile polycystic disease, combined with variable phenotypic expression of tuberous sclerosis. A previous study described two cases with *TSC2-PKD1* contiguous gene deletion with severe renal manifestations and skin alterations of tuberous sclerosis [44]. We did not observe more severe renal symptoms in patients with *PKD1* deletion. We were unable to establish any additional meaningful genotype-phenotype correlations in this series, what could be due to the extensive molecular heterogeneity observed in this first series of Brazilian patients with TSC. Several limitations must be considered when analyzing the results of our study: patients were

classified as sporadic cases when no relatives presented symptoms of TSC; recruitment of patients' relatives to make a complete mutation segregation analysis depends on their availability to perform genetic tests. This is particularly difficult in sporadic cases, when relatives do not have symptoms of TSC and need to be submitted to genetic tests. Also, clinical data collection was performed carefully, but some characteristics were not evaluated in all patients, as shown in Table 1. This occur when evaluations are requested but not performed by the patients, or when the data is not available in the medical records. Although, these limitations probably did not interfere in variant classification and genotype-phenotype correlation assessment.In 23% of the patients no pathogenic or likely pathogenic *TSC1* or *TSC2* germline variant was identified. The molecular cause of the TSC phenotype of these patients remains elusive.

Conclusion

Genetic testing is currently part of the TSC diagnostic criteria [3]. In individuals with suspected TSC, clinical diagnosis is complicated by a high degree of phenotypic variability and the potential for a late onset of certain features of the disease. Thus, genetic testing can play an important role in diagnostic confirmation, enabling genetic counseling to families, and providing additional understanding towards the etiology of the disorder. We designed a molecular diagnosis strategy for TSC that showed an overall variant detection rate of 89%; 69% of the patients had a pathogenic or likely pathogenic variant. No specific genotype-phenotype correlations were established in this specific cohort, but other correlations described in the literature were also observed by us. Early molecular characterization of TSC patients will become even more important as better therapeutic interventions become available.

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S1 Figure. Origin of TSC patients and mutation frequencies in *TSC1* and *TSC2*. A. Places of birth of the patients studied are represented as green spots in the Brazilian map. **B.** Overall *TSC1* and *TSC2* mutation frequencies (above) and *TSC1* and *TSC2* mutation frequencies according to the region of birth (below).

S1 Table. Characteristics of the TSC patients included in this study according to their birth regions in Brazil.

S2 Table. Read depth of Ion Torrent analysis *per* amplicon of *TSC1* and *TSC2*.

S3 Table. Clinical phenotypes of TSC patients with a synonymous or without *TSC1* or *TSC2* mutations.

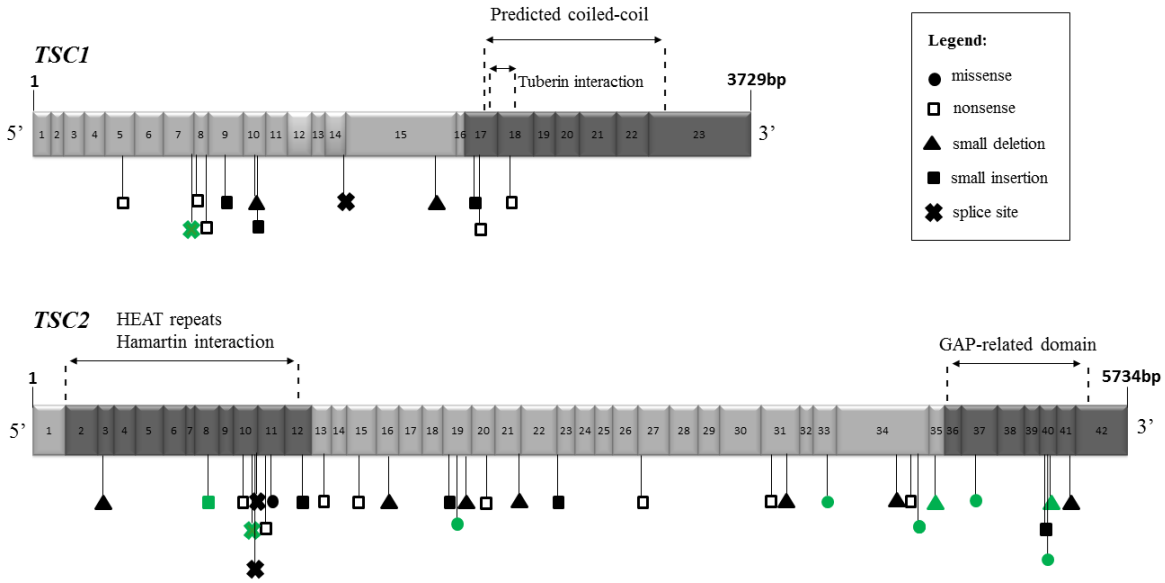
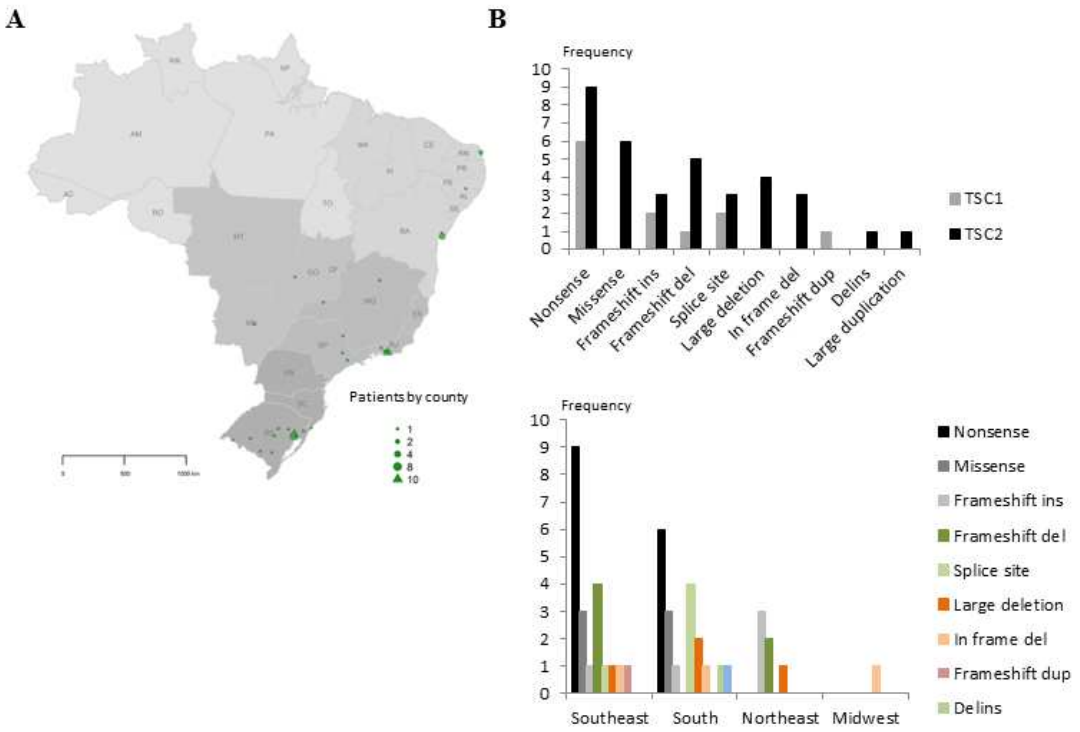


Figure 1



S1 Figure

Capítulo VI

Manuscrito V

Manuscrito em preparo

Para este manuscrito, a técnica de western blot será realizada para confirmar as diferenças de expressão gênica a nível proteico(GATSL2 and PRR5L), confirmar as alterações de autofagia em células tratadas (p62, LC3), avaliar a expressão de proteínas específicas (TSC1, TSC2, mTOR), avaliar se proteínas específicas estão superativadas através da fosforilação, o que não poderia ser visto pela técnica de microaaranjo de expressão gênica (phopho TSC2, phosphor mTOR) e avaliar se mTOR está superativado (phospho-4E-BP1 and phospho-p70 S6 Kinase - efetores de mTOR).

Effects and mechanism of Rapamicin treatment on Tuberous Sclerosis Complex fibroblasts

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Keywords:mTOR inhibitors; Rapamycin; Tuberous Sclerosis Complex.

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Abstract

Tuberous sclerosis complex (TSC) is a autosomal dominant disorder caused by mutations in either *TSC1* or *TSC2*genes. The corresponding proteins form a heterodimeric complex that acts in the suppression of mammalian target of rapamycin (mTOR) in mTORC1 complex, a master regulator of cell growth.TSC is characterized by the development of multiple hamartomas in many organs; mTOR inhibitors are specific inhibitors of mTORC1 and are currently being investigated for a number of indications in TSC. The mechanism by which mTOR inhibitors stop hamartoma growth is not completely clear. Also, it is not known if the different types of mutations that can occur in *TSC1* and *TSC2* result in diffrent responses to mTOR inhibitors. The aim of this study was to try to evaluate the effect of mTOR inhibition in normal and mutated TSC cells, with different mutations. Biopsies of normal appearing skin were obtained from five adult female patients, diagnosed with TSC and with an identified mutation and from two wild-type individuals. Skin biopsies were used to establish primary fibroblast cultures. Cell viability, cell cycle progression and autophagy were evaluated by flow cytometry. Whole transcriptome analysis was performed using Affymetrix Gene Chip Human Transcriptome Array 2.0. We did not observe differences in cell viability between mutated and wild-type cells after treatment with rapamycin; rapamycin also did not cause cellcycle arrest in any particular stage. Autophagy was reduced in mutated cells; after treatment,the increase in the number of autophagic cells was more significative in mutated cells ($p=0.039$). No correlation between mutation profile and response to rapamycin was observed. We found a nutrient sensor gene (*GATSL2*) differentially expressed due to mutation in *TSC1* or *TSC2* and a pro-apoptotic gene (*PRR5L*) altered due to treatment with rapamycin. We showed that autophagy alterations could be the primary mechanism that leads to tumor formation in TSC cells. Although rapamycin has some side effects, the preventive use of this drug should be evaluated, since autophagy could be altered in normal appearing cells. Autophagy could also be a target therapy. Future studies using normal and tumoral cells could be useful to confirm these findings.

Introduction

Tuberous sclerosis complex (TSC) is a multisystem autosomal dominant disorder caused by mutations in either of two genes, *TSC1* or *TSC2* [1], that code for hamartin and tuberin proteins, respectively. The birth incidence of TSC is estimated to be 1 in 6000 [2] with an equal male/female distribution. The two proteins form a heterodimeric complex that acts in the suppression of mammalian target of rapamycin (mTOR) in mTORC1 complex and in cell cycle regulation (G_1 phase) [3]. mTOR, through its effector proteins, 4E-BP1, p70 ribosomal S6 kinase 1 (S6K1), and eukaryotic initiation factor 4 (eIF4E), is a master regulator of cell growth, proliferation, survival, angiogenesis, autophagy, cellular senescence and immune reactions [4]. Hamartin acts as a chaperone for tuberin; tuberin contains a GAP (GTPase-activating protein) domain that stimulates the intrinsic GTPase activity of the small G-protein Rheb, thereby enhancing the conversion of Rheb into its GDP-bound inactive state [5]. GTP-bound Rheb is a potent activator of mTORC1. To control this GTPase activity in response to growth factors, Akt phosphorylates tuberin directly on four or five distinct residues [6]. Mutations in either *TSC1* or *TSC2* can reduce tuberin GTPase activity, leading to chronic activation of mTORC1and cell overgrowth, which in turn cause TSC.

TSC is characterized by the development of multiple hamartomas in virtually any organ system, mainly in the brain, kidneys, heart, and lungs, with some manifestations more prevalent than others across the lifespan [7]. Most hamartomas develop according to Knudson two hit hypothesis [8], but there are evidences showing that hamartomas in lung and brain may arise without the second event [9, 10]. TSC also includes a vast range of neuropsychiatricdisorders, typically neurodevelopmental, behaviouraland psychiatric difficulties. Significant phenotypicvariability in the number and severity ofphysical features and neuropsychiatric manifestations are seen [11, 12]. In the central nervous system, three main lesions associated with TSC are cortical tubers, subependimal nodules and subependimal giant cell astrocytomas (SEGAs) [13]. The most common clinical presentations in patients with SEGA are epilepsy, mental retardation, cognitive deficit, headache, vomit and visual disturbing. They are generally benign, but their spatial location and growth potential can lead to several complications [14], including malignant transformation [15]. Both quimiotherapy and radiotherapy are ineffective for SEGAs; surgical resection has been the standard treatment, which can show a variety of severe adverse effects and tumor recurrence [16]. As an alternative for surgeries, treatment with mTOR inhibitors has been proposed. The mTOR inhibitors

rapamycin (sirolimus), CCI-779 (temsirolimus) and RAD001 (everolimus) are specific inhibitors of mTORC1 and are currently being investigated for a number of indications, including SEGAs and epilepsy associated to TSC [17-19]. They are also potentially promising for the treatment of renal angiomyolipomas, lymphangioleiomyomatosis, subungual fibromasand skin lesions [20-22], showing great results in all indications. The mechanism by which mTOR inhibitors stop hamartoma growth is not completely clear (through cell cycle regulation, inhibition of translation, autophagy, or other). Also, it is not known if the different types of mutations that occur in *TSC1* and *TSC2* genes result in different responses to mTOR inhibitors. In this line, the aim of this study was to evaluate cell cycle progression, autophagy and whole transcriptome expression levels in cells from patients diagnosed with TSC and with different mutations, treated with the mTOR inhibitor rapamycin. This can help to describe mechanism of action and toxicity of this drug *in vitro,* which could be useful to provide appropriate managementand care for TSC patients.

Methodology

Samples.Five adult female patients, diagnosed with TSCaccording to clinical criteria [23] and with an identified mutation in *TSC1* or *TSC2* were enrolled. Two wild-type individuals without *TSC* mutations (mutation in *TSC* was not detected by Sanger sequencing) were also included. Biopsies (6mm) of normal appearing skin were obtained from all participants. The study was approved by the institutional reviewboard (CEP-HCPA; GPPG 15-0049).

Cell culture.Skin biopsies were used to establish primary fibroblast cultures. Cells were grown in 25cm^2 flasks in a humidified incubator at 5% CO_2 and 37 °C and maintained in HAM-F10 medium with 1% penicillin-streptomycin and 15% fetal bovine serum (Gibco Laboratories, USA). Cells were trypsinized and expanded until five flasks wih 80% confluence were obtained (passages 3-7). Subsequently, cells were transferred to a 12 and a 6-well culture plate for each sample. Twenty-four hours after seeding, 7 wells were treated with 10nM of the mTOR inhibitor rapamycin (R0395, Sigma Aldrich, Rehovot, Israel), the dose that corresponds to target therapeutic level (10ng/ml) [24], 7 were not treated (vehicle DMSO was added), and 4 were supplemented with HAM-F10

medium (not treated and no vehicle). Fibroblasts from each of the 18 wells were collected after 48 h (rapamycin half-life in plasma) and directly counted using trypan blue in Neubauerchamber. All procedures were performed in triplicates.

Cell viability. One treated, one untreated and one no vehicle well of each sample was centrifugated (7 minutes; 2100rpm) and washed with PBS 1x three times. Pellets were resuspended in PBS and propidium iodide (50 μg/ml; Sigma, Rehovot, Israel), then analyzed in Attune Flow Cytometer (ThermoFisher Scientific, USA) in triplicates.

Cell cycle evaluation.One treated, one untreated and one no vehicle well of each sample was centrifugated (7 minutes; 2100rpm) and washed with PBS 1x three times. Cells were lysated and stained in hypotonic stain buffer (sodium citrate 3.6mM, propidium iodide 50ug/ml, Triton X-100 0.1% and water). Cell cycle distribution was analyzed in Attune Flow Cytometer (ThermoFisher Scientific, USA) in triplicates and student-t test was performed for statistical analysis.

Autophagy evaluation by flow cytometry.One treated, one untreated and one no vehicle well of each sample was ressupended in 500ul HAM-F10 medium and incubated for 15 minutes with acridine orange solution (1ug/mL). The percentage of autophagic cells was measured in Attune Flow Cytometer (ThermoFisher Scientific, USA) in triplicates.Student-t test was performed for statistical analysis.

Gene expression array.One tretated and one untreated well of each sample was centrifugated and washed once with PBS 1x. Total RNA was extracted from pellets using PureLinkRNA Mini Kit (ThermoFisher Scientific, USA). Then, total RNA was analyzed using Affymetrix Gene Chip Human Transcriptome Array 2.0 (a single microarray for each condition), as recommended in the manufacturer's protocol.Gene level SST-RMA sketch algorithm (Affymetrix Expression Console, USA) was used to generate raw data. Raw data were imported to Transcriptome Analysis Console software (Affymetrix Expression Console, USA) to make comparisons between untreated and treated cells; cells with mutations in *TSC1* or *TSC2;* cells with *TSC* mutations and without *TSC* mutations. One-Way Analysis of Variance (ANOVA) for each pair of condition groups was performed. Genes were filtered and analyzed in each comparison using linear fold change (< -1.5 or > 1.5) and ANOVA p-value ($p<0.001$) cutoffs.

Pseudogenes, microRNAs and uncharacterized *loci* (*LOC*) were also filtered out. To create expression array heatmap we used *heatmap.2* function of *gplots* R-package, performed in R 3.3.2 statistical software.To visualize the number of over and underexpressed genes we created Venn diagrams using *VennDiagram* R-package.In order to search protein-protein interaction networks we used String 10.0 software [25]. Differentially expressed genes (DEGs) from array analysis were used as input data, with the following parameters: 0.40 medium confidence score; textmining exclusion; no more than 20 and 5 interactions at the first and second shells.The resulting interaction data was further analyzed using Cytoscape version 3.4.0 [27]. Functional enrichment analysis was perfomed using BiNGO 3.0.3 plugin [26], implemented in Cytoscape, to access biological processes of DEGs.

Expression array validation. Three downregulated genes (*GATSL2*, *GBP2*, *MMP12*), three upregulated genes (*MSH2*, *RPS6KA5*, *PRR5L*) and one gene with no differential expression (*TSC1*) found by expression array analysis were selected for validation. Total RNA of three patients and two healthy controls was reverse transcribed and subjected to quantitative PCR using SYBR green real time PCR master mix (ThermoFisher Scientific, USA). Primers were designed in exon-exon junctions to guarantee that possible DNA contaminations would not affect results. Primer sequences are available on request. The relative RNA expression of the target genes was normalized to theexpression of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH)reference gene.

Western Blot.To confirm gene expression differences at protein level and to evaluate expression of specific proteins, western blot was performed. Three untreated and three treated wells of each sample were centrifugated (7 minutes; 2100rpm) and washed with PBS 1x three times. Pellets were used for total protein extraction using Radio-Immunoprecipitation Assay (RIPA) lysis buffer, quantified with a Bradford assay. For blotting, a total of 15 µg of protein was separated by SDS-PAGE and electroblotted to PVDF membranes (Amersham Pharmacia Biotech, Piscataway), which were incubated overnight at 4 °C with primary rabbit antibodies. The following primary antibodies were used: p62, LC3B, Hamartin/TSC1, Tuberin/TSC2, Phospho-Tuberin/TSC2 (Ser939), mTOR, Phospho-mTOR (Ser2448), Phospho-p70 S6 Kinase (Thr389) and Phospho-4E-BP1 (Thr37/46) (all at 1:1000 and from Cell Signaling Technologies, Danvers, USA)

and GATSL2 and PRR5L (at 1:1000 from Sanbio, Uden, The Netherlands). Anti-rabbit horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technologies, Danvers, USA) was used. Immobilon Western HRP Substrate (Millipore, USA) was used for chemiluminescent detection in ImageQuant LAS 500 imager (GE Health Sciences, USA). Band density was analyzed using the ImageJ software (https://imagej.net). All procedures were performed in duplicates for each sample.

Results

TSC patients included in this study had *TSC1* and *TSC2* genes previously analyzed by a customized Next Generation Sequencing panel and Multiplex Ligation Probe-dependent Amplification (MLPA). Wild-type controls were also evaluated for the presence of *TSC1* and *TSC2* mutations. Table 1 shows mutation analysis results.We did not observe differences in fibroblast growth rate and morphology between patients' cells with and without mutations in plates, as well as in treated and non-treated cells.

In order to show the mechanism of cell growth inhibition by rapamycin, we evaluated cell viability, cell cycle distribution and authophagy in treated and untreated cells by flow cytometry. Figure 1A shows the percentage of viable cells in mutated and wild-type cells, treated with vehicle or rapamycin. We did not observe differences in cell viability between these groups. This was also strengthened bycell counting experiment with Trypan blue (data not shown). Cell cycle distribution of different groups is shown in Figure 1B. There was no significant difference inthe percentage of subG1 population in both mutated and wild-type groups after treatment with rapamycin, which means that this drug do not induce cell death. Also, no statistical differences were observed in the average percentage of other cellcycle populations between groups, indicating that rapamycin did not cause cellcycle arrest in any particular stage. The percentage of acridine orange positive cells (autophagic cells) and acridine orange negative cells of mutated and wild-type wells, treated with vehicle or rapamycin are shown in representative plots in Figure 1C. The ratio of autophagic cells in wells treated and untreated with rapamycin for each case is shown in the right graphic of Figure 1C. Autophagy was reduced in mutated cells; after treatment,the increase in the number of autophagic cells was more significant in mutated cells than in the wild-type cells. Cell viability, cycle distribution and autophagy were not different in cells with and without

vehicle (DMSO). No correlation between mutation profile and response to rapamycin was observed in flow cytometry analyses.

Next, we performed gene array analyses with mutated and wild-type cells treated or not-treated (control) with rapamycin, in order to compare the effects of mutation profile and treatment on whole gene expression. Supplementary Figure 1 shows the box-plot of the normalized relative gene expression of all samples. Transcriptome analysis was performed for each of the condition pairs specified in Table 2. The number of differentially expressed genes between each condition pairs is also shown in Table 2. Figure 2A is a representative hierarchical clustering of gene array probes for each of the mutated and wild-type samples, treated or not-treated. Treated and not-treated cells of each sample were grouped side by side, showing that individual expression is different before and after treatment, and different *TSC* mutations result in different expression patterns. We then analyzed the DEGs in each condition pair: group 1 (treated vs control) showed 14 DEGs, including *GATSL2*(log_2FC -1.52); when we excluded wild-type cells and analyzed only mutated cells (treated vs control; group 2), only *GATSL2* gene was differentially expressed (downregulated in mutated cells, log_2FC -1.62). This gene could be altered due to mutation in *TSC1* or *TSC2*. When considering groups 3, 4 and 5 (Figure 2B, left), *RPS6KA5* gene was altered in both groups 3 and 4 and presented experimental protein-protein interaction with mTOR in STRING (Figure 2C). We focused on DEGs that were present in group 3 (treated wild-type vs treated TSC) and were not present in group 4 (non-treated wild-type vs non-treated TSC). Among these 102 genes (Figure 2B), *PRR5L* was directly connected to PI3K-mTOR pathway in STRING (Figure 2C) and was further analyzed (upregulated in wild-type cells; log_2FC 1,59). The 32 genes that are altered only in group 5 (treated wild-type vs non-treated wild-type) could be related to drug toxicity in cells. Finally, groups 6 and 7 are comparisons between *TSC1* and *TSC2* mutations, treated or not (Figure 2B, right). Since these groups are too small to make statistical comparisons, we did not make further analysis with these groups. We used Bingo plugin to analyze the most enriched biological processes in groups 3 and 4 and the results are shown in Tables 3 and 4, respectively. Processes with a corrected p-value smaller than 5E-2 are shown.

To validate the results of gene array, the first three patients in Table 1 and two wild-type controls were chosen. Results for *GATSL2*(downregulated in mutated: treated vs control array analysis), *GBP2*(downregulated in wild-type: treated vs control array analysis), *MMP12*(downregulated in treated: wild-type vs mutated array analysis),

MSH2, RPS6KA5, PRR5L(all upregulated in treated: wild-type vs mutated array analysis) and one gene with no differential expression (*TSC1*) are shown in Figure 3. We found that theqRT-PCR results indeed correlated with gene array results. Other significant and not significant results also correlated between both techniques.

Discussion

To investigate the molecular basis of TSC and cell response to treatment with rapamycin, we recruited five TSC patients and two wild-type controls for biopsy of normal appearing skin. A previous study found that fibroblasts from a healthy portion of skin from a TSC patient show activation of S6K1, a downstream effector of mTOR often used to visualize mTOR activation in TSC associated tumors. No upregulation of Erk or Akt kinases or other respective kinases participating in their pathways was detected [28]. Thus, an additional kinase, for example Akt or Erk, is necessary for the development of tumors. For this reason, initial studies with normal TSC skin are useful, since they already have mTOR overactivation and are easier to collect.

mTOR is a highly conserved serine/threonine kinase that plays a significant role in controlling cell growth and metabolism. In humans, mTOR is constitutively activated in the presence of growth factor and nutrients and acts as a master switch of cellular catabolism and anabolism [29]. When nutrients are available, growth-factor-activated kinases phosphorylate TSC2, leading to decreased GAP activity towards Rheb, which remains GTP-bound and activates mTORC1, leading to increased cell growth through phosphorylation of several different translation proteins, mainly 4E-BP1, S6K1 and eIF4E [30]. In contrast, when growth factor or nutrients are lacking, AMPK phosphorylates and activates TSC1/2 leading to conversion of Rheb-GTP into Rheb-GDP and mTORC1 inhibition. In this case, 4E-BP1 becomes hypophosphorylated, which increases its binding with eIF4E and prevents initiation of translation. As a consequence, catabolic processes such as fatty acid oxidation or autophagy are induced to provide a constant supply of nutrients and maintain ATP production [31].

There is great interest in early and preventive treatment of individuals with TSC with mTOR inhibitors. Several studies showed a significantly increased response ratein pediatric patients with TSC treated with mTOR inhibitor therapy compared with those treated with non-mTOR inhibitor therapy, as reviewed by Yang and colleagues [32]. However, these studies show that tumors exhibit growth inhibition but little shrinkage[20-22]. This is consistent with our results in experiments with cell viability,

which do not show cell death. Culture of tumoral cells could be useful to confirm these data.

mTOR inhibition also results in an increase in the turnover of cyclin D1 and a decrease in the elimination of the cyclin dependant kinase inhibitor P27 [33]. The pharmacological inhibition of mTOR was found to decrease G1 transit in the cell cycle [34]. In another study, silencing *TSC2* in Rat1 fibroblasts shortened the G1 phase of the cell cycle, favouring cell cycle entry [35]. However, the mechanism that may underlie the effects of mTOR on the cell cycle is not clear. In a study with *TSC2* expressing and *TSC2* null cell lines, no differences in percentage of cells in cell cycle subsets were found after treatment with rapamycin [36], as our study with normal appearing skin also found.

Autophagy is increasingly recognized to play a critical role in tumor development and cancer therapy [37]. mTORC1 is an important negative regulator of autophagy. It induces autophagy in response to reduced growth factor signalling, starvation, and other metabolic and genotoxic stresses [38] which leads to the formation of phagophores, inside which the lysosomal hydrolases degrade organelles and intracellular proteins [39]. During physiological conditions, the phagophore formation is inhibited by mTORC1, since it directly interacts with and phosphorylates the Ulk1 kinase complex (Ulk1-Atg13-FIP200-Atg101) which is required for the initiation of autophagy [40]. In situations of bioenergetics stress, the Ulk1 complex is released from mTOR, thereby allowing it to associate with the membranes from which phagophores are formed [41]. In this way, autophagy promotes the survival of established tumors by supplying metabolic precursors during nutrient deprivation; however, excessive autophagy has been associated with cell death [42]. In other situations, inhibition of autophagy promotes tumorigenesis [43]. Parkhitko and colleagues, using genetic and pharmacologic approaches, found that autophagy and the autophagy substrate p62/sequestosome 1 (SQSTM1) are critical components of TSC driven tumorigenesis and that tuberin-null cells had less LC3-II and more p62/SQSTM1, along with fewer and smaller autophagosomes, indicating decreased autophagy levels [44]. Paradoxically, accumulation of the autophagy substrate p62/SQSTM1 promotes tumorigenesis via activation of NF-κB [45] and Nrf2 [46]. In concordance with these findings, we observed a reduction in the number of acridine orange positive cells in flow cytometry analyses of mutated TSC cells in comparison with wild-type cells. After treatment with rapamycin, the increase in the number of autophagic cells is more significative in

mutated cells than in the wild-type cells (p=0.039), indicating that autophagy is restablished by mTORC1 inhibition. As our results were obtained in normal appearing skin, autophagic alterations may be present in non-tumoral cells of TSC patients, and could be the initial mechanism that leads to tumor formation in these cells. Also, autophagy could be a target therapy to TSC tumors, either alone or in combination with mTORC1 inhibition, since mTORC1 inhibitor discontinuation leads to tumor regrowth [20].

Human transcriptome analysis of group 1 (treated vs control) showed 14 DEGs, including *GATSL2*(downregulated, log_2FC -1.52); considering only mutated cells (treated vs control; group 2), only *GATSL2* gene was downregulated (log_2FC -1.62). Thus, this gene could be downregulated due to mutation in *TSC1* or *TSC2*. In protein-protein interaction network analysis using STRING, we found a connection between mTOR pathway and *GATSL2*(Figure 2C). This gene codifies a poorly characterized protein, CASTOR 2, which forms a heterodimer with CASTOR 1. The complex is an arginine sensor that interacts with GATOR2 to negatively regulate mTORC1 activity. Arginine disrupts this interaction by binding directly to CASTOR1. CASTOR2 is defective in amino acid binding but is important in complex formation to inhibit mTORC1 signaling [47]. CASTOR 2 (*GATSL2*) was downregulated in treated cells with *TSC* mutations. A possible explanation is that in mutated cells without rapamycin treatment, mTORC1 is overactivated and CASTOR 2 is increased in order to try to regulate mTORC1 activity through its inhibition activity. In treated cells, the drug directly binds and inhibits mTORC1, and, as a consequence,*GATSL2* levels are reduced. However, further studies regarding CASTOR2 function and characterization of other amino acid sensors are needed to clarify how nutrients and sensors regulate the mTORC1 pathway.

PRR5L (Proline rich protein 5 like) was upregulated in group 3 (treated - wild-type vs TSC) and was not in group 4 (control wild-type vs TSC). Thus,*PRR5L* must be altered due to treatment with rapamycin. In network analysis using STRING, we verified an interaction between mTOR pathway and *PRR5L* (Figure 2C). PRR5-Like protein is an uncharacterized protein. Thedieck and colleagues found that PRR5L is phosphorylated by both mTORC1 and mTORC2. They also found that PRR5L knock down cells were less apoptotic compared to control cells, at various time points after TNFa/cycloheximide treatment, suggesting that PRR5L is pro-apoptotic. We did not analyze apoptosis in our experiments because we did not found marks of cell death in

culture plates. Treatment with rapamycin could enhance apoptosis in TSC cells. However, the exact significance of PRR5L phosphorylation by mTOR, in particular by mTORC1, and its role in apoptosis remains to be determined. Genes involved in autophagy were not differentially expressed in array analyses, but they could be in different levels of activation or have protein accumulation instead of RNA accumulation.

Makovski and colleagues showed that inhibition of the Rheb/mTORC1 pathway results in a reduction in IRF7 nuclear localization, which in turn decreases the transcription of IFN-stimulated genes [36]. The 32 genes that are altered only in group 5 in our study (treated wild-type vs non-treated wild-type) seem to interact with IRF7 in STRING analysis, indicating that normal cells treated with rapamycin alter the expression of these immune system genes. Makovski and colleagues also analyzed the most enriched processes in *TSC2* expressing and *TSC2* null cells treated with rapamycin; most of the processes involved were of immune system regulation, cell migration, death and proliferation. In our study, treated and not treated cells have similar processes enriched: cell division, nutrient metabolization genes (Table 3) and immune system genes (data not shown).

Conclusion

Although the present study has several limitations such as the small number of cell cultures tested, we showed that autophagy alterations could be the primary mechanism that leads to tumor formation in TSC cells. Also, nutrient sensors and pro-apoptotic genes need to be furthered studied to elucidate the role of rapamycin in gene expression. The alterations foundedin healthy portions of TSC skin provides an explanation to such a variety of lesions observed in this condition. Although rapamycin has some side effects, the preventive use of this drug should be evaluated. Autophagy could also be a target therapy, either alone or in combination with mTORC1 inhibition.Although each patient showed different expression patterns before and after treatment with rapamycin, we were not able to establish correlations between mutation profile and response to rapamycin. Future studies using normal and tumoral cells could be useful to confirm expression array and autophagy findings.

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Figure 1.Cell viability, cell cycle and autophagy flow cytometry analyses. Mutated cells (TSC) and wild-type (WT) cells were seeded and grown in the absence (control) and in the presence of rapamycin. (A) Cells were stained with propidium iodide and submitted to Attune Cytometer analysis. Viable cells (%) of a representative mutated and WT samples are shown in the left panel. The average percentage of viable cells in mutated and wild-type groups is shown in the right panel. (B) Cells were lysated, stained in hypotonic stain buffer and submitted to Attune Cytometer cell cycle analysis. The quantification of the different cell cycle phases in a representative mutated and WT samples is shown in the left panel. The average percentage of cells in each cell cycle phase in mutated and WT cells is shown on the right. No significant differences were observed in (A) and (B) analyses. (C) Cells were incubated with acridine orange solution and submitted to Attune Cytometer analysis. Percentage of acridine orange positive (AO+) and acridine orange negative (AO-) cells of representative samples is show on the left. The ratio of autophagic cells in wells treated and untreated with rapamycin for each case in shown in the right panel (cases 1-5 are mutated and cases 6-7 are wild type). The increase in the number of autophagic cells after rapamycin treatment is more significative in mutated cells ($p=0.039$).

Figure 2. Comparative gene array analysis of wild-type and mutated cells treated with rapamycin. (A) Hierarchical clustering of gene array probes (pseudogenes, uncharacterized *loci* and microRNA probes were excluded), depicted in a dendrogram. Red, high relative expression; green, low relative expression. Genes are shown in rows; samples are shown in columns. (B) Venn diagram of the number of differentially expressed genes in groups 3, 4 and 5 (left) and 6, 7 (right). Common and uncommon genes were evaluated. (C) Differentially expressed genes from array analysis were used as input data in STRING; the resulting interaction data was further analyzed using Cytoscape and results from group 3 (treated: wild-type vs mutated) are represented in the figure. Pink nodes represent genes with increased expression values; purple nodes characterize genes with decreased expression; gray nodes represent interactors with no difference in expression level.

Figure 3.Validation of representative genes from the gene array using qRT-PCR. Three downregulated genes (*GATSL2*, *GBP2*, *MMP12*), three upregulated genes (*MSH2*, *RPS6KA5*, *PRR5L*) and one gene with no differential expression (*TSC1*) found by expression array analysis were selected for validation. The relative RNA expression of the target genes was normalized to the expression of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) reference gene. Results are shown according to analysis groups that were significant in array analysis. All results correlate with the gene array analysis (mean – SD is shown; positive error is not shown for better visualization).

Supplementary Figure 1. Normalized relative gene expression. Human transcriptome array results were normalized using SST-RMA algorithm in Affymetrix Expression Console. Normalized relative expression for each sample (14 treated with rapamycin and 14 treated with vehicle) is shown in the box blot.

Table 1. Mutation analysis of TSC patients included in this study.

Sample	Inheritance	Coding change	Amino acid change	Gene	Position	Mutation Type	Classification
Patient 1*	Familial	c.338T>A	p.Leu113Ter	*TSC1*	Exon 5	Nonsense	Likely Pathogenic
Patient 2*	Familial	c.2074_2075insCTCC	p.Arg692fs*15	*TSC1*	Exon 17	Frameshift insertion	Likely Pathogenic
Patient 3*	Sporadic	c.1008T>G	p.Tyr336Ter	*TSC2*	Exon 11	Nonsense	Likely Pathogenic
Patient 4	Familial	c.724delinsTCCT	p.Thr242Ser_Ser243del	*TSC2*	Exon 8	In frame delins	VUS
Patient 5	Familial	c.4375C>T	p.Arg1459Ter	*TSC2*	Exon 34	Nonsense	Pathogenic

VUS = Variant of Uncertain Significance. * Patients 1, 2 and 3 were selected for expression array validation by qPCR.

Table 2. Groups used to analyze differential gene expression by gene array.

Group	Condition pair	Differentially expressed genes *
1	Treated vs non-treated	14
2	Treated TSC vs non-treated TSC	1
3	Treated wild-type vs treated TSC	168
4	Non-treated wild-type vs non-treated TSC	106
5	Treated wild-type vs non-treated wild-type	33
6	Non-treated *TSC1* vs non-treated *TSC2*	320
7	Treated *TSC1* vs treated *TSC2*	307

*Number of genes filtered according to fold change(< -1.5 or > 1.5)and ANOVA p-value ($p<0.001$).

Table 3. Biological processes most enriched in the comparison between mutated and wild-type cells treated with rapamycin.

GO-ID	Process	Count	%	Corr p-value
7059	chromosome segregation	12	16.2	1,80E-07
22402	cell cycle process	20	27.0	2,92E-05
70	mitotic sister chromatid segregation	8	10.8	2,92E-05
280	nuclear division	14	18.9	2,92E-05
7067	mitosis	14	18.9	2,92E-05
6996	organelle organization	29	39.2	2,92E-05
819	sister chromatid segregation	8	10.8	2,92E-05
87	M phase of mitotic cell cycle	14	18.9	3,62E-05
48285	organelle fission	14	18.9	3,62E-05
7049	cell cycle	22	29.7	7,19E-05
16043	cellular component organization	38	51.3	9,89E-05
279	M phase	15	20.3	3,68E-04
22403	cell cycle phase	16	21.6	7,18E-04
51301	cell division	14	18.9	7,74E-04
51276	chromosome organization	17	22.9	1,17E-03
7076	mitotic chromosome condensation	5	6.7	2,66E-03
278	mitotic cell cycle	14	18.9	7,46E-03
6323	DNA packaging	9	12.2	8,69E-03
45944	positive regulation of transcription from RNA polymerase II promoter	14	18.9	8,69E-03
45935	positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic	17	22.9	2,39E-02
45941	positive regulation of transcription	16	21.6	2,39E-02
51254	positive regulation of RNA metabolic process	15	20.3	2,88E-02
71103	DNA conformation change	9	12.2	2,88E-02
10557	positive regulation of macromolecule biosynthetic process	17	22.9	3,09E-02
51173	positive regulation of nitrogen compound metabolic process	17	22.9	3,09E-02
10628	positive regulation of gene expression	16	21.6	3,73E-02

Table 4. Biological processes most enriched in the comparison between mutated and wild-type cells without treatment.

GO-ID	Process	Count	%	Corr p-value
8543	fibroblast growth factor receptor signaling pathway	9	19.1	9,20E-09
6364	rRNA processing	9	19.1	1,41E-04
16072	rRNA metabolic process	9	19.1	1,41E-04
7169	transmembrane receptor protein tyrosine kinase signaling pathway	11	23.4	2,89E-04
8284	positive regulation of cell proliferation	14	29.8	3,34E-04
42254	ribosome biogenesis	9	19.1	7,43E-04
34470	ncRNA processing	9	19.1	1,67E-02
7167	enzyme linked receptor protein signaling pathway	11	23.4	1,67E-02
22613	ribonucleoprotein complex biogenesis	9	19.1	1,67E-02

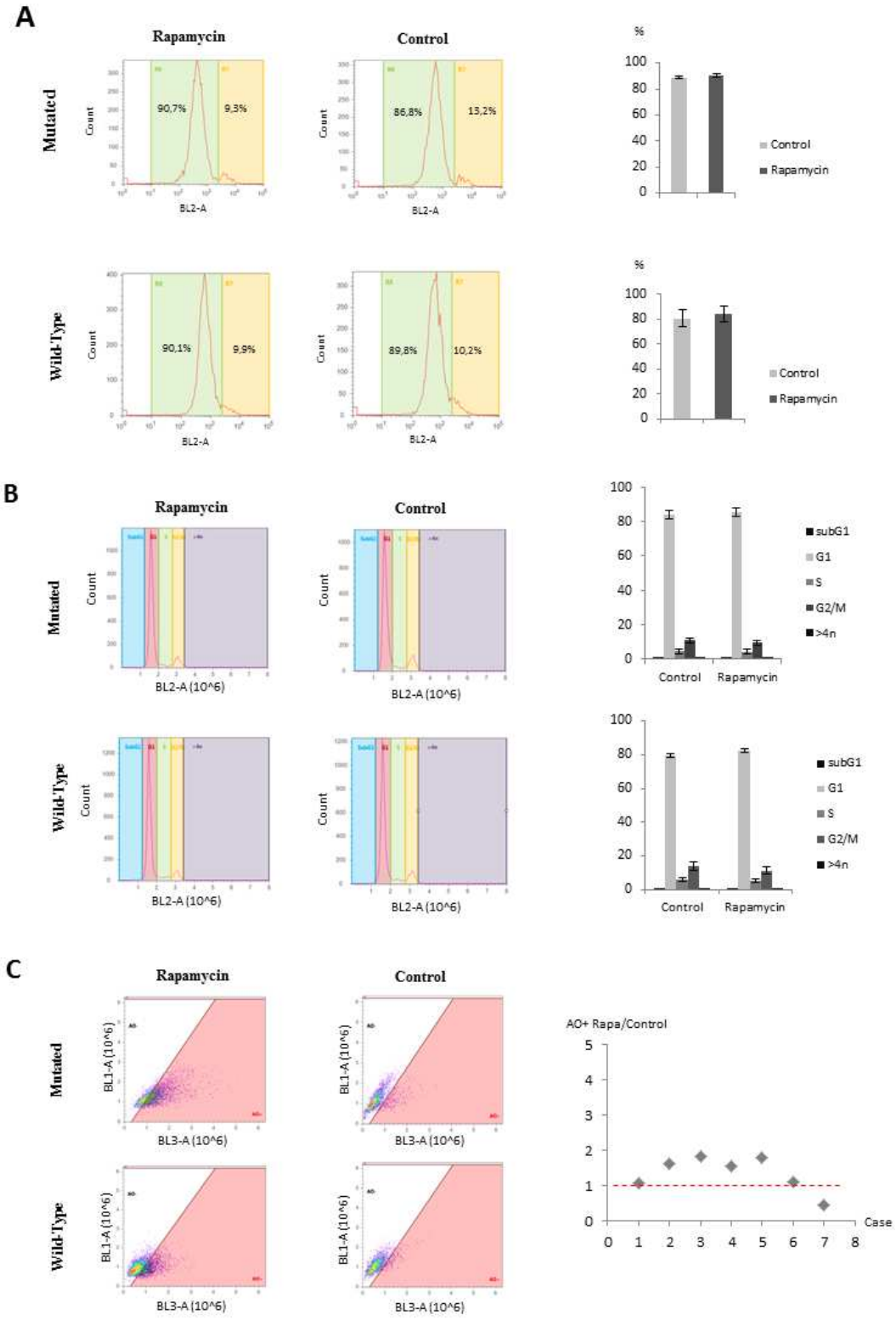
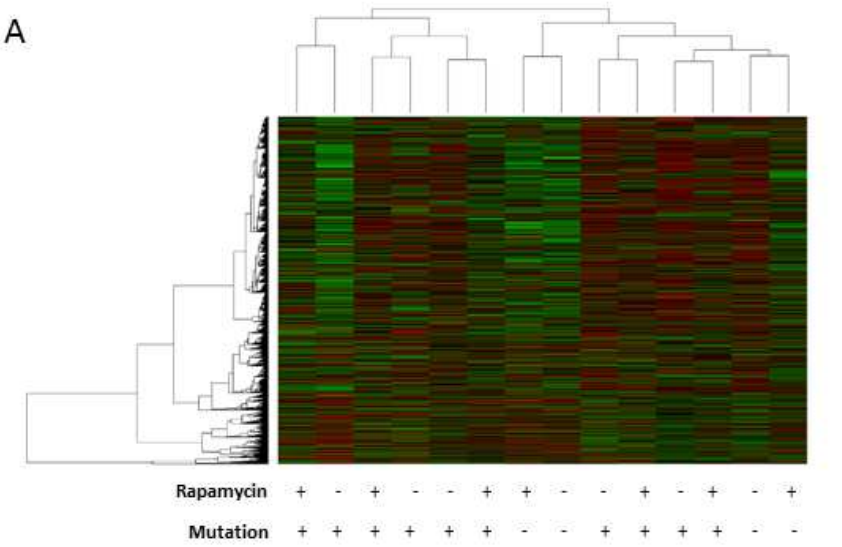

A
Rapamycin
Control
Mutated
Wild-Type
90,7%
9,3%
86,8%
13,2%
90,1%
9,9%
89,8%
10,2%
Count
BL2-A
%
Control
Rapamycin
B
BL2-A (10^6)
subG1
G1
S
G2/M
>4n
C
BL1-A (10^6)
BL3-A (10^6)
AO+ Rapa/Control
Case

Figure 1


A
Rapamycin + - + - - + + - - + - + - +
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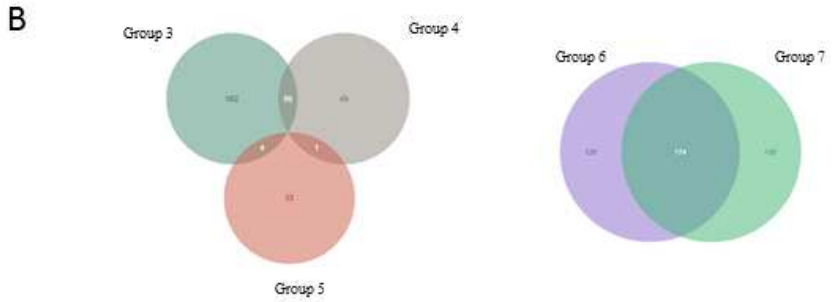

B
Group 3
Group 4
Group 5
Group 6
Group 7

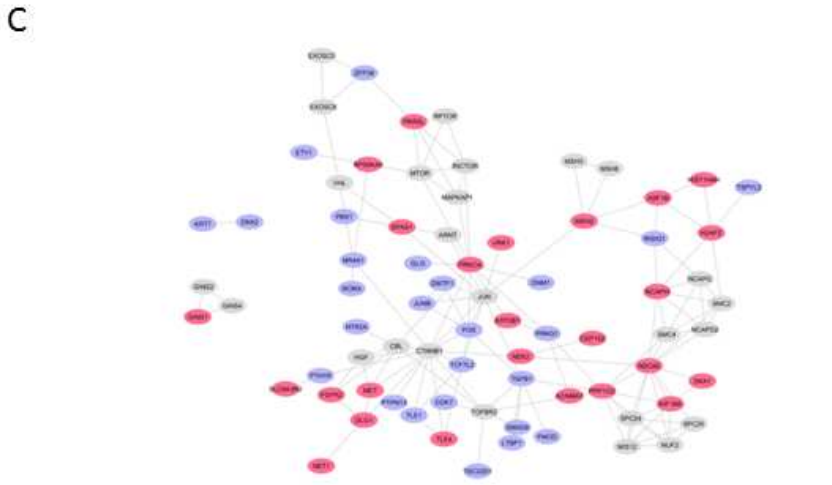

C

Figure 2

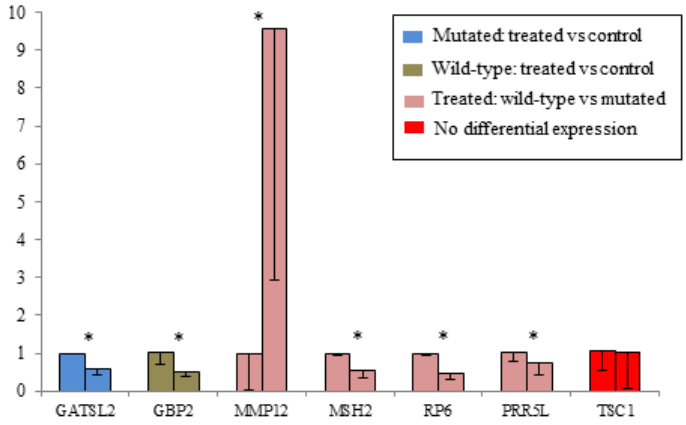
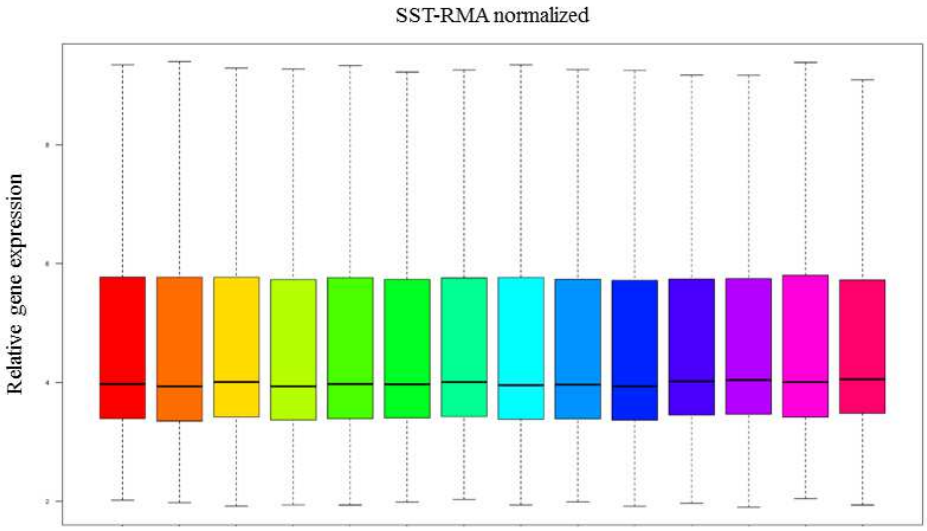


Figure 3



Supplementary Figure 1

Capítulo VII

Discussão geral

Embora exista um grande número de trabalhos publicados que descrevem mutações em NF1, NF2 e esclerose tuberosa, realizados em diferentes populações do mundo, a identificação das mesmas permanece sendo muito importante, pois possibilita uma melhor compreensão da genética molecular da doença na população estudada. Além disso, um grande número de variantes novas é descrito em cada estudo, devido à alta taxa de mutação desses genes. A disponibilidade do diagnóstico molecular possibilita a caracterização das doenças com um grau de detalhe muito aprofundado, o que ainda não era realizado no sul do Brasil. O acesso a esse tipo de diagnóstico é restrito a rede privada, o que dificulta a caracterização de diversos pacientes que não possuem esse acesso. Nesse trabalho, a caracterização molecular completa foi realizada em 93 pacientes oriundos do Rio Grande do Sul, não relacionados, com NF1. A caracterização clínica foi realizada da maneira mais completa possível, sendo que algumas limitações foram observadas pela não adesão dos pacientes a alguns exames solicitados. A frequência geral de grandes rearranjos em NF1 foi de 4,3%, semelhante à descrita na literatura. Foi encontrada a microdeleção tipo 1 em um dos pacientes, associada previamente com um fenótipo mais grave de NF1, o que foi corroborado em nosso estudo. A caracterização molecular de pacientes com suspeita ou diagnóstico clínico de NF1 pode mostrar a presença dessa microdelação, o que pode ser usado para prever o fenótipo e a gravidade da doença nesses pacientes. Outras três microdeleções atípicas (com ponto de quebra fora das regiões de repetição) foram encontradas. Para essas microdelções, a predição do fenótipo fica mais difícil. Os genes co-deletados nos casos de microdeleções também podem influenciar no fenótipo; o gene *RNF135*, por exemplo, um gene próximo ao*NF1*, foi associado com alta estatura, macrocefalia e características dismórficas quando deletado; o gene *SUZ12* pode estar associado a mal-formações cardíacas. Em nosso estudo, um dos pacientes que possui microdeleção atípica, com deleção de *NF1*, *RNF135* e mais 12 genes funcionais, possui alta estatura, macrocefalia e características dismórficas. Já que os genes vizinhos, quando deletados, podem influenciar o fenótipo de NF1, mutações pontuais nesses genes também poderiam resultar em alteração do fenótipo. Por esse motivo, dois dos genes mais importantes e mais relacionados com fenótipos alterados foram incluídos em um painel de sequenciamento de nova geração para triagem de alterações de ponto na série de casos recrutada. Na análise de mutações de ponto, a taxa de detecção de alterações foi similar à descrita em outros estudos (79%), distribuídas ao longo dos éxons 1-49 de *NF1* e sem nenhum *hotspot* claro para mutações. Nenhuma variante em *RNF135* e *SUZ12* foi encontrada. Trinta e três variantes novas foram descritas na região sul do Brasil. A análise de segregação foi possível de ser realizada em apenas 5 famílias, onde a variante encontrada foi claramente

segregada e deve ser a causadora da doença. Considerando a detecção de mutações de ponto e grandes rearranjos, 83% dos pacientes tiveram alguma variante detectada. Os pacientes restantes podem ter alguma variante localizada em introns, o que poderia afetar os sítios de processamento de RNA. O mosaicismo também pode ser uma causa para a não detecção, mas a alta cobertura atingida nos painéis de sequenciamento de nova geração pode mostrar esse tipo de alteração. Nenhuma correlação genótipo-fenótipo nova foi estabelecida na análise de mutações de ponto, apenas uma correlação previamente existente na literatura foi encontrada também: a deleção de 3 pares de bases (c.2970_2972delAAT) em *NF1*, que foi associada com a ausência de neurofibromas, foi encontrada em dois pacientes do nosso estudo, ambos com ausência de neurofibromas.

A caracterização clínica e molecular também foi realizada em sete pacientes com NF2, oriundos do Rio grande do Sul. Devido à raridade da doença, o recrutamento de pacientes é mais difícil. O pequeno número de probandos analisados torna improvável que uma correlação genótipo-fenótipo seja encontrada. A taxa de grandes rearranjos encontrada em nosso estudo foi de 14,3%, similar a outros estudos da literatura. Apenas uma grande deleção do éxon 1, já descrita, foi encontrada em um paciente. Duas alterações de ponto foram encontradas em *NF2* (taxa de detecção de 29%) e nenhuma no gene *INI1*, gene previamente associado à schwanomatose, incluído no painel de sequenciamento de nova geração.

A caracterização molecular completa de 53 pacientes com esclerose tuberosa, oriundos de quatro regiões brasileiras diferentes e não relacionados, também foi realizada. Nessa série de casos, a maioria dos probandos (68%) não tem história familiar da doença, e a taxa geral de detecção de mutações foi de 89%, uma taxa alta e similar a outros estudos com metodologias complementares para detecção de mutações de ponto e grandes rearranjos. Através do MLPA, quatro grandes deleções e uma grande duplicação foram encontradas, totalizando uma frequência de grandes rearranjos de 9%. Um painel de sequenciamento de nova geração customizado abrangendo os genes *TSC1* e *TSC2* detectou alteraçõs em 81% dos pacientes. Um número maior de alterações foi encontrado em *TSC2*, com uma alta ocorrência de alterações em sítios de processamento no exon 10 desse gene. Além desse ponto, nenhum *hotspot* para mutações foi encontrado, e a análise molecular completa continua sendo necessária para o rastreamento de alterações em pacientes com esclerose tuberosa. Vinte variantes novas foram identificadas nesses dois genes. Um paciente com a síndrome contígua (deleção de parte de *TSC2* e de *PKD1*, um gene adjacente), além de um paciente com deleção apenas de *PKD1*, foram encontradas. Pacientes com deleção de *PKD1* possuem maior chance de desenvolver cistos renais (ambos apresentaram doença policística renal) e a análise

molecular pode ser muito útil para a prevenção de complicações mais graves em pacientes que possam ter essa deleção. A caracterização clínica dos pacientes com esclerose tuberosa foi mais facilmente acessada, e não houve diferenças entre sintomas em homens e mulheres. As correlações genótipo-fenótipo também não foram encontradas em ET, já que as mutações não foram recorrentes nos pacientes. Apenas uma tendência dos pacientes com mutação em *TSC2* serem mais graves foi encontrada.

Este é o primeiro estudo a fazer o rastreamento molecular completo em pacientes com NF1, NF2 e esclerose tuberosa no Brasil. Todas as alterações detectadas por sequenciamento de nova geração foram corroboradas por sequenciamento de Sanger.A patogenicidade das alterações novas, tanto em *NF1* quanto em *TSC1* e *TSC2* é muito difícil de determinar. As ferramentas de bioinformática podem mostrar variação considerável, mas servem para mostrar um primeiro indício de patogenicidade. O estudo funcional é difícil e de alto custo na maioria dos casos. As variantes já descritas foram classificadas de acordo com as regras estabelecidas pela ACMG, com 14 variantes patogênicas em *NF1* e 11 em *TSC1* e *TSC2*. Em geral, nenhuma correlação genótipo-fenótipo nova foi estabelecida. O estabelecimento de correlações genótipo-fenótipo nos genes estudados aqui é difícil, devido à complexidade dos mesmos, falta de *hotspots*, presença de pseudogenes e tamanhos grandes. O grande espectro de mutações, incluindo alta taxa de mutações *de novo*, faz com que variantes recorrentes sejam detectadas raramente, o que torna a análise mais difícil devido ao baixo número de pacientes com a mesma mutação.

Por fim, para tentar elucidar o mecanismo de inibição de crescimento de tumores associados à esclerose tuberosa pelo inibidor de mTOR rapamicina, análises com citometria de fluxo e expressão gênica global foram realizadas. As análises foram realizadas com tecidos de aparência normal (pele), tanto de pacientes com mutações em *TSC1* ou *TSC2* como em indivíduos saudáveis, sem mutação. A análise de tecido normal pode elucidar os processos que estão alterados nas células antes da formação de um tumor, processo que pode ser o inicial na tumorigênese dessas células. Ainda, alterações na via de mTOR já foram descritas em células normais de pacientes com ET (Jozwiak *et al*., 2009). A análise de ciclo celular mostrou que não há parada em nenhum estágio específico do ciclo celular e nem indução de morte celular em células mutadas, antes e após o tratamento com rapamicina. Não houve alteração na viabilidade celular também. Já nas análises de autofagia, a razão de células autofágicas aumenta significativamente em células com mutação. A autofagia tem papel fundamental no desenvolvimento de tumores (Mizushima *et al*, 2008). O complexo mTORC1, no qual mTOR tem um papel fundamental, induz a autofagia na ausência de fatores de

crescimento e falta de nutrientes; quando as condições fisiológicas estão normais, mTORC1 inibe a iniciação da autofagia. Em algumas situações, a autofagia promove a sobrevivência de tumores; em outras situações, ela foi associada com morte celular (Rabinowitz *et al*., 2010). Em pacientes com esclerose tuberosa, a indução de autofagia é muito maior do que em células controles após o tratamento com rapamicina (p=0,039). Como esses resultados foram obtidos em células não tumorais, alterações de autofagia devem estar presentes antes mesmo da formação de tumores, em células com mutação em heterozigose em *TSC1* ou *TSC2*. A perda de heterozigosidade nem sempre ocorre na formação de tumores em ET, e a alateração de autofagia pode ser um dos mecanismos iniciais de desregulação do crescimento celular em células com mutação. Assim, a autofagia pode ser uma terapia alvo para a esclerose tuberosa, em combinação com inibidor de mTOR ou sozinha.

Na análise de expressão gênica global, foram analisados diferentes grupos, considerando as células com e sem mutação, tratadas ou não. Entre os genes diferencialmente expressos, foram encontrados dois genes principais, que interagem com mTOR no STRING: um sensor de nutrientes (*GATSL2*), alterado devido à mutações nas células e um gene pró-apoptótico (*PRR5L*), alterado devido ao tratamento com rapamicina. Esse genes podem ser estudados com mais detalhe para esclarecer a participação na via de mTOR e seu papel nas células tratadas. Outros genes alterados foram os de via de proliferação e crescimento celular, divisão celular e sistema imune. Análises complementares de expressão em nível de proteína (western blot) serão realizadas para confirmação e esclarecimento dos resultados.

Capítulo VIII

Conclusões

Em conclusão, nossa estratégia de caracterização molecular identificou,pela primeira vez em uma população do Brasil, alterações em 83% dos 93 pacientes com NF1, em 43% dos sete pacientes com NF2 e em 89% dos 53 pacientes com esclerose tuberosa. O estabelecimento da nova metodologia de sequenciamento de nova geração poderá ser utilizado futuramente como alternativa ao sequenciamento direto para o diagnóstico molecular desses pacientes. A caracterização das variantes nesses pacientes é muito importante para o acompanhamento da doença e a possível prevenção das características multissistêmicas. O diagnóstico clínico das genodermatoses pode ser complicado, pelo alto grau de variabilidade fenotípica, início tardio de alguns sintomas e muitas vezes pelo não preenchimento dos critérios clínicos. Assim, a confirmação do diagnóstico clínico através do diagnóstico molecular pode resultar em melhorias no tratamento e aconselhamento genético das famílias, além de auxiliar na compreesão da etiologia dessas doenças. O estabelecimento de um diagnóstico definitivo, o mais cedo possível, é muito importante à medida que intervenções terapêuticas estão se tornando disponíveis para esses pacientes. Correlações genótipo-fenótipo específicas para a população brasileira não foram estabelecidas, apenas algumas previamente descritas foram identificadas também em nosso estudo. Estudos adicionais com séries de casos maiores podem ser úteis na identificação dessas correlações. Além disso, foi demonstrado que a autofagia pode ser o mecanismo inicial que leva a formação de tumores em células com mutações em *TSC1* ou *TSC2*. O processo de autofagia precisa ser estudado com mais detalhes a fim de esclarecer esse mecanismo, e, possivelemente, levar ao uso de terapia de alvo molecular direcionada à via de autofagia, em conjunto com inibidores de mTOR ou sozinha. O uso preventivo de terapias de alvo molecular poderia ser avaliado, levando em consideração os efeitos colaterais que podem causar. A expressão gênica global parece variar de acordo com cada paciente, e não de acordo com o tratamento. Assim, o tipo de mutação pode influenciar na expressão gênica, tanto antes quanto depois do tratamento com rapamicina. Apesar disso, não foi possível estabelecer correlações entre os perfis de mutações e a resposta à rapamicina. Estudos futuros utilizando células normais e tumorais podem ser úteis para confirmar os achados em nível de expressão e alterações de autofagia, que podem ser muito importantes na definição de terapias de alvo molecular.

Capítulo IX

Perspectivas

Como perspectivas da continuidade desse trabalho, pode-se mencionar:

- O uso de novas abordagens a fim de esclarecer a causa da doença em pacientes sem mutações detectadas nas regiões analisadas, como, por exemplo, a análise de cDNA;
- O estudo funcional das variantes novas encontradas;
- A análise do RNAm de pacientes com novas variantes em sítios de processamento;
- Análise de mais familiares para avaliar a segregação de mutações, quando estes estiverem disponíveis;
- Análise de alterações somáticas em tumores de NF1, NF2 e ET, quando a mutação germinativa já estiver identificada, para verificar se há perda de heterozigosidade em tumores e o espectro de alterações nos mesmos;
- Realizar análises de autofagia por citometria de fluxo, western blot e microscopia de fluorescência em células com mutação em *TSC1* ou *TSC2*, células normais e células de linhagens de diferentes tumores associados à esclerose tuberosa, com diferentes dosagens e tempos de ação das drogas inibidoras de mTOR e inibidoras de autofagia.

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Anexo

Artigo publicado durante o período de Doutorado relacionado com o tema.

Vitamin D status and VDR genotype in NF1 patients: a case-control study from Southern Brazil

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Abstract

Neurofibromatosis type 1 (NF1) patients are more likely to have vitamin D deficiency when compared to the general population. This study aimed to determine the levels of 25-OH-vitamin D [25(OH)D] in individuals with NF1 and disease-unaffected controls and analyze *FokI and Bsm*I *VDR* gene polymorphisms in a case and in a control group. Vitamin D levels were compared between a group of 45 NF1 patients from Southern Brazil and 45 healthy controls matched by sex, skin type and age. Genotypic and allelic frequencies of *VDR* gene polymorphisms were obtained from the same NF1 patients and 150 healthy controls. 25(OH)D deficiency or insufficiency were not more frequent in NF1 patients than in controls (p=0.074). We also did not observe an association between *FokI and Bsm*I *VDR* gene polymorphisms and vitamin D levels in NF1 patients, suggesting that theirdeficient or insufficient biochemical phenotypes are not associated with these genetic variants. The differences between the groups in genotypic and allelic frequencies for *FokI* and *Bsm*I *VDR* gene polymorphisms were small and did not reach statistical significance. These polymorphisms are in partial linkage disequilibrium and the haplotype frequencies also did not differ in a significant way in the twogroups (p=0.613).

Introduction

Neurofibromatosis type 1 (NF1) is an autosomal dominant disease caused by mutations in the *NF1* gene, mapped at chromosome 17q11.2, which produces an ubiquous protein called neurofibromin. NF1 is a cancer predisposition disease with variable expressivity. The main features involve the skin, bone and central nervous system. Approximately one-half of the cases are familiar and the remainder is caused by de novo mutations in the *NF1* gene. The estimated incidence of the disease is 1 in 2.500-3.500 live births, independent of gender and ethnic background [1-5]. The diagnosis of NF1 is usually clinical and most of the affected individuals are identified in infancy or childhood. The clinical diagnosis is made when at least two of the National Institute of Health (NIH) Diagnostic Criteria for NF1are met [6]. One of these criteria is skeletal lesions, such as sphenoid dysplasia or thinning of the long bone cortex with or without pseudoarthrosis. In addition to the classical signs and symptoms involving skeleton, NF1 patients are prone to osteomalacia, osteopenia, and osteoporosis of unknown etiology [7-12]. Neurofibrominfunctions as a GTPase in mesothelial-derived tissues including blood cells, fibroblasts, and osteoprogenitor cells, leading to deregulation of osteoblast and osteoclast activity [13]. However, the pathogenesis of lower bone mass depends of the density and also mineral content. Therefore, metabolic abnormalities may also contribute to a predilection for bony defects in NF1, like bone-regulating hormones (i.e. vitamin D). A few studies have suggested that NF1 patients are more commonly diagnosed with hypovitaminosis D when compared to the general population [9-12, 14, 15].

Vitamin D plays a pivotal role in the homeostasis of body calcium. It increases the absorption of calcium from the small intestine and promotes its reabsorption back into bones, an essential process for proper bone metabolism. Ultraviolet B light photoisomerizes provitamin D to vitamin D in the skin, which is transported to the liver and hydrolysed to 25-

hydroxy-vitamin-D [25(OH)D]. Further hydroxylation of 25(OH)D to 1.25-dihydroxy-vitamin D [1.25$(OH)_2$D], the physiologically active form of vitamin D, occurs mainly in the kidney. In the clinic, 25(OH)D levels are used to assess vitamin D status since 1.25$(OH)_2$D usually reflects serum calcium better than total vitamin D content. Several factors interfere with serum vitamin D levels such as age, sun exposure, skin type [16] and disorders that interfere with vitamin D metabolism (hepatic, kidney and intestinal disease). Vitamin D insufficiency is associated with osteoporosis, bone fractures, decreased immune function, bone pain, muscle weakness and possibly with propensity to cancer and cardiovascular disease [17-20].1.25$(OH)_2$D exerts its biological effects through binding to the vitamin D receptor (VDR), a nuclear receptor that acts as a transcription factor. Calcium absorptionoccurs primarily in the duodenum where the VDR is expressed in the highest concentration, so the regulation of *VDR* gene is most important in high efficiency of calcium absorption [21].Vitamin D receptor's genotypes have been associated with the development of several bone diseases as well as multiple sclerosis (MS), osteoporosis, and vitamin D-dependent rickets type II and other complex maladies [22].

The gene encoding the VDR is mapped on the long arm of chromosome 12 (12q12–14) and is composed of 9 exons, with an alternatively spliced promoter region [23]. A series of polymorphisms in the *VDR* gene were reported to be linked to various biological processes [24]. FokI restriction enzyme can identify a variable site in exon 2 of the gene. This alteration is characterized by a C/T transition located inside a start codon (ATG), and when the C variant is present, an alternative start site is used, leading to the expression of a shorter VDR protein (424aa), which demonstrates increased biological activity than the longer one (427aa) [25]. BsmI polymorphism apparently does not change the translated protein [24]. This G/A polymorphism is located on intron 8 and is linked in a haplotype with variable-length poly A sequence within the 3' untranslated region, altering VDR mRNA stability [26]. Therefore,

both FokI and BsmI polymorphisms can decrease VDR receptor expression. We hypothesize that since VDR receptor mediates the effects of $1.25(OH)_2D$, its reduced expression may also reduce $1.25(OH)_2D$ activity, even when normal vitamin D levels are present. This mechanism would affect vitamin D activity. Low vitamin D levels or decreased vitamin D activity could impair calcium absorption in duodenum and consequently, the lack of calcium could decrease bone turnover. This alteration in bone metabolism may not be sufficient to cause the classical signs and symptoms involving skeleton in NF1 patients, but may have an association that influences their occurrence, acting together with deregulation of osteoblast and osteoclast activity. Differences in VDR allele frequencies for FokI and BsmI polymorphisms between NF1 and general population or differences in vitamin D levels between groups could help to clarify this possible association.

The aim of this study was to assess and compare 25(OH)D levels ina group of 45 patients with the clinical diagnosis of NF1 with a 45 sex-, skin type- and age-matched control group. We sought to correlate clinical features of NF1 with serum vitamin D levels and to investigate wether *Fok*I and *Bsm*I polymorphisms in the *VDR* gene were associated with hypovitaminosis D and the NF1 phenotype. Secondly, we compared genotypic and allelic frequencies of*Fok*I and *Bsm*I polymorphisms in the *VDR* gene between a NF1 group and a control group.

Materials and Methods

Vitamin D Status

Patients and controls. A consecutive series of NF1 patients seen at the genetics outpatient clinics of Hospital de Clínicas de Porto Alegre (HCPA), Southern Brazil (30° 2' 0" South. 51° 12' 0" West) from November 18th to December 20th, 2009 were invited to participate in this

study and enrolled after signature of informed consent. The study was approved by the Institutional Research and Ethics Committee of HCPA. The minimum number of patients and controls to be enrolled was estimated at 22 in each group and was calculated using Winpepi version 9.2 based on the findings of Lammert *et al.*[14] with a power of 90% and an alpha=0.05. Considering the possibility of differences in sun exposure between individuals from this study (recruited in the spring in Southern Brazil) and those of Lammert *et al.*[14] (recruited in Germany during the winter, spring or summer) and in order to have sufficient patients to allow clinical correlations, we set the group sizes at 45 individuals each. The group of cases consisted of adult individuals (above age 18 years) diagnosed with NF1 according to the Criteria of the Consensus Development Conference [6]. Controls were recruited from the companions of patients seen in the same genetics clinics and were matched to cases by sex, type of skin and age (allowing a difference of ± 5 years at the most). Exclusion criteria for both groups were age < 18 years, incapacity to provide informed consent, vitamin D supplementation within the last 6 months, diagnosis of gastrointestinal, skin, liver, kidney or parathyroid disease, use of medication that could interfere with the vitamin D metabolism, known vitamin D deficiency and hospitalization in the previous 2 months. In addition, we clinically excluded NF1 patients who met criteria for other genetic disorders such as Noonan Syndrome and segmental NF1 and controls with 1st, 2nd or 3rd degree family history of NF1.

Clinical evaluation: Data on clinical presentation was obtained from chart review and full physical examination was performed on all participants. To model NF1 phenotype, the presence of eight mayor NF1 characteristics were evaluated: café-au-lait spots, cutaneous neurofibromas, plexiform neurofibromas, axillary and inguinal freckling, optic pathway glioma, Lisch nodules, sphenoid wing dysplasia and tibial pseudoarthrosis. The number of café-au-lait spots and cutaneous neurofibromas was also obtained. Family history of all

participants was assessed and registered in pedigrees.The clinical assessment was performed before vitamin D testing by the same clinical geneticist.

Vitamin D dosage: In order to limit the effect of seasonal fluctuations of vitamin D photosynthesis, patients were recruited in the spring between the dates previously described. Fasting (minimum 4 hours) peripheral blood samples were collected in EDTA and the plasma isolated by centrifugation was frozen within one hour of collection at -80°C for posterior analysis. All samples were analyzed simultaneously after a storage period of five months. Plasma 25(OH)D levels were measured by chemiluminescence using the LIAISON commercial kit (DiaSorin Inc. Stillwater/MN.CV 6% intra-assay). Samples were scored as Vitamin D deficient when 25(OH)D results were <20ng/ml. The normal cutoff for 25(OH)D levels was defined at >30ng/ml. Plasma levels between 20 and 30 ng/ml were classified at the insufficiency status.

VDR Genotyping

To determine *VDR* genotype, genomic DNA from 45 NF1 patients and a healthy control group of 150 patients was extracted from leukocytes by conventional salting-out methods. Analysis of the*Fok*I (rs2228570; T and C alleles) and*Bsm*I (rs1544410; A and G alleles) polymorphisms in the *VDR* gene was performed by PCR-RFLP in duplicates as described by Monticielo *et al.*[27] and was blinded for vitamin D status and clinical phenotype. The control group, constituted of 150 healthy individuals,was recruited from Porto Alegre and previously tested for the *Fok*I and *Bsm*I polymorphisms with the same methodology as described aboveand tested in the same laboratory as the NF1 samples.

Statistical analyses

All analyses were done using the statistical package SPSS version 18.0. For categorical variables the Chi-Square and Fisher's Exact Test were used and for quantitative variables the Student's t-test was used. A p-Value < 0.05 was considered statistically significant.

Results

Clinical and demographic features of the patients and controls used to determine vitamin D status are summarized in Table 1 and 2. There was no significant difference between groups in age at assessment, sex, skin type (according to the Fitzpatrick classification, avoidance of sun exposure), habit of smoking or use of alcohol. As expected, patients with NF1 had an increased frequency of short stature and had been more often diagnosed with cancer when compared to controls. There was also no significant difference between the mean and median 25(OH)D levels, respectively, between groups: 25.25ng/mL and 25.10ng/mL (±8.46) in patients and 22.79ng/mL and 21.90ng/mL (±6.28) in controls. In the NF1 group, 29 (64.4%) of the 45 individuals studied had levels of 25(OH)D below 30ng/mL: vitamin D deficiency was observed in 11 (24.4%) and vitamin D insufficiency in 18 (40.0%) subjects.The minimum 25(OH)D level detected in this group was 5.27ng/ml and maximum level was 41.3ng/ml. In the control group, 39 (86.6%) of the 45 individuals studied had levels of 25(OH)D below 30ng/mL: vitamin D deficiency was observed in 17 (37.7%) and vitamin D insufficiency in 22 (48.8%) subjects.The minimum 25(OH)D level detected in this group was 14.1ng/ml and maximum level was 44.3ng/ml.When we categorized 25(OH)D using a cutoff of 30 ng/ml, NF1 patients had more frequently normal 25(OH)D levels than controls. Although this difference did not reach statistical significance, distinct distribution can be further observed in the 25(OH)D levels (ng/ml) histograms depicted in Figure 1. We

did not observe a more severe phenotype in NF1 patients with lower 25(OH)D levels (data not shown).

VDR genotyping results of the NF1 patients are depicted in Table 3. Genotypic frequencies of the *Fok*I and *Bsm*I polymorphisms were in Hardy-Weinberg equilibrium. When compared to a subset of 150 healthy, NF1 unaffected individuals recruited at the same hospital as the NF1 patients (as described by Monticielo *et al.* [27]), allelic and genotypic frequencies encountered in the patients did not differ significantly. These polymorphisms are in partial linkage disequilibrium and the haplotype frequencies also do not differ in a significant way between the two groups (p=0.613). Additionally, we compared 25(OH)D levels obtained from NF1 patients with their different *Fok*I and *Bsm*Igenotypes (Table 4), and did not find any association.

Discussion

So far, seven studies assessed 25(OH)D levels in patients diagnosed with NF1 (Table 5). Among these, six were case-control studies and one a descriptive study, all undertaken in the Northern Hemisphere (USA and Europe). Although biologically plausible, the association of NF1 with vitamin D deficiency remains controversial and has not been clearly demonstrated in all studies, corroborating our findings in a Southern Brazilian population. Hypovitaminosis D might indeed be involved in the pathogenesis of bone, neurological and skin disorders of NF1, since it has a significant role in calcium homeostasis and bone metabolism but it is also involved in the regulation of cell proliferation, differentiation, apoptosis and angiogenesis. In this line, there is consistent evidence in favor of a role for Vitamin D in the expression of genes related to decreased cell proliferation for both normal and cancer cells and induction of terminal cell differentiation [18-20, 28]. However, only one group [14] described an inverse association between increased number of neurofibromas and

low plasma 25(OH)D levels, suggesting an effect of the vitamin levels on disease expression. Against this hypothesis, Stevenson and colleagues [15], found no association between levels of 25(OH)D and the occurrence of optic gliomas or neurofibromas in NF1 patients.Hockett and colleagues [29] described in United Kingdom a case-control study in which the overall mean of 25(OH)D levels in control group was within deficient range, and showed no statistically significant difference with NF1 group. This deficient 25(OH)D value found in control population also occurs in our control group and may reflect poor sun exposure of these populations.

In the 90s, Nakayama and colleagues suggested an improvement of two cardinal signs of NF1, neurofibromas (NF) and cafe-au-lait spots (CLS) in patients treated with vitamin D [30-31]. In addition, Yoshida *et al.* [32], published a paper in which eight patients with the clinical diagnosis of NF1 were treated with intense lightradio frequency combined with topical vitamin D, with improvement of the phenotype. Such findings could be explained by the potent antiproliferative effect of vitamin D by inhibiting the transcription specific genes (i.e. c-fos oncogene, as observed in experimental studies with mice). Finally, Lammert *et al.* [14] suggested that the lower vitamin D levels observed in NF1 patients relative to controls could be related to less exposure to sunlight in patients with greater visibility of the disease.

The frequency of vitamin D deficiency in the Nordic countries is higher than expected both by the low sun exposure and the low dietary intake of vitamin D precursors [33].This can easily be explained by geographic and cultural aspects of those countries. In Brazil, a country with tropical and sub-tropical climates (depending on the geographic region), adequate 25(OH)D levels have been reported in the general population of the city of Recife (8°S) in the Northeastern region. In the Southeast part of the country, studies are controversial, showing normal 25(OH)D levels in the population of the State of São Paulo (21°S) but hypovitaminosis D in 42.4% in the population of Minas Gerais (19°S). In the southernmost

State of Rio Grande do Sul (30°S), probably due to its climatic conditions and the risk profile of most of the individuals studied to date (hospitalized patients), a high prevalence of hypovitaminosis D has been observed [34-40]. In a cross-sectional study with resident physicians of Hospital de Clínicas de Porto Alegre (the same hospital from which the patients in this study derive), the mean serum level of 25(OH)D was 17.9+/-8.0 ng/mL and 57.4% of them presented 25(OH)D below 20ng/mL [38]. The high overall frequency of vitamin D deficiency andinsufficiency observed in this study corroborates with previous reports that studied populations from Southern Brazil. The reasons why the overall frequency of hypovitaminosis D is so high in this study remain elusive and the lack of an observed difference between NF1 patients and controls may be related to the deficient and insufficient status of a significant proportion of individuals in the community. We can not exclude certain ascertainment biases such as the period of study (collection during the summer could definitively exclude lack of sun exposure as a factor) and sample size. However, the lower 25(OH)D levels consistently observed in controls, both in terms of mean values and distribution of individual 25(OH)D measurements, is against the hypothesis of an association of hypovitaminosis D and NF1. Finally, although functional data have been inconclusive for Bsm1 *VDR* gene polymorphism, several small studies evaluating this polymorphism have reported significant associations with osteoporosis. Some studies have shown a relationship between *VDR* polymorphisms and bone mineral density, serum 25(OH)D levels, as well as neoplastic and immune diseases [41-45]. Based on these articles, 65.9% of studies reported a significant correlation between BsmI and osteoporosis risk. Likewise, 60.0% of studies reported a significant correlation between FokI *VDR* gene polymorphism and osteoporosis risk. As expected, in our study, *VDR* gene polymorphisms *Fok*I and *Bsm*I were not more common in vitamin D deficient or insufficient NF1 patients, suggesting that these biochemical phenotypes are not related to these genetic variants. As we hypothesized,

*VDR*gene polymorphisms *Fok*I and *Bsm*I could interfere in vitamin D activity, even when normal levels are present. The effects of *VDR* gene polymorphisms are in connection with each other, but the different haplotypes between the studied groups also did not reach statistical significance. The reasons for the heterogeneous results found in many association studies are numerous and varied. Sample sizes, ascertainment differences, population and trait genetic heterogeneities may be mentioned. In addition, in quantitative characteristics, most factors account for only a small proportion of the total genetic risk.

In our patient series, the differences in vitamin D levels between cases and controls are not statistically significant; however, the lower vitamin D levels are found in NF1 patients (5 cases below 15ng/ml). Curiously, the two patients with the lower vitamin D levels (5.24 and 8.45 ng/ml) have the greatest number of cutaneous neurofibromas (50-100 neurofibromas), although we did not find statistical evidence for a more severe phenotype in NF1 patients with lower 25(OH)D levels.

Conclusion

In conclusion, there is no evidence of vitamin D lower levels in NF1 patients and no association between *VDR* gene polymorphisms and the occurrence of the disease in a population from Southern Brazil. Further studies could definitively exclude or show a role for VDR polymorphisms and vitamin D levels on skeleton signs and symptoms of NF1. Possible associations would be of great importance to support future directions for treatment of NF1 patients with abnormal bone metabolism caused by vitamin D deficiency.

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The authors stated that they had no interests which might be perceiving as posing a conflict or bias.

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Table 1. Clinical and demographic features of NF1 patients and controls included in the study.

Features	NF1 Patients (n=45) N (%)	NF1 Patients (n=45) Media/range (years)	Controls (n=45) N (%)	Controls (n=45) Media/range (years)	p-value
Gender Female	31 (68.9)		33 (73.3)		0.646[3]
Age		38.6 /18 to 72		36.7 /18.6 to 58.6	0.212[5]
Skintype (Fitzpatrick)					0.129[4]
1	1 (2.2)		0		
2	8 (17.8)		12 (26.7)		
3	13 (28.9)		19 (42.2)		
4	5 (11.1)		7 (15.6)		
5	14 (31.1)		5 (11.1)		
6	4 (8.9)		2 (4.4)		
Habit of smoking	6 (13.3)		8 (17.8)		0.722[3]
Use of alcohol [1]	26 (57.8)		21 (46.7)		0.297[3]
No sun avoidance	39 (86.7)		35 (77.8)		0.275[3]
Previous cancer diagnosis	5 (11.1)		0 (0.0)		0.021[3]
Short stature [2]	11 (25.5)		3 (6.8)		0.011[3]

[1] socially; [2]according to the World Health Organization (p<3) for controls and to Neurofibromatosis 1 Growth Charts forthe cases, n=40 among cases and n=44 among controls; [3]Fisher's Exact Test; [4]Chi-Square Test; [5]Student's t-test.

Table 2. Clinical profile of patients with clinical diagnosis of Neurofibromatosis 1 in this study.

Neurofibromatosis 1 Diagnostic Criteria[1]	Presence of the changes / evaluated	%
Cafe-au-lait spots (>1,5 cm)	33/45	73.3
Two or more cutaneos neurofibromas	37/45	82.2
Plexiform Neurofibroma[2]	17/45	37.8
Axillary freckling or freckling in inguinal regions	43/45	95.5
Optic Pathway Gliomas	1/45	2.2
Two or more Lisch nodules	20/23[3]	87.0
Sphenoid wing dysplasia	2/30[4]	6.7
Pseudoarthrosis	1/45	2.2
First-degree relative with Neurofibromatosis 1	33/44[5]	75.0

[1] National Institute of Health Consensus development Conference Statement: Neurofibromatosis Bethesda, 1988

[2] Only cases confirmed by biopsy

[3] twenty-two patients did not attend the appointment with the ophthalmologist for personal reasons

[4] Fifteen patients did not attend the performance of the RX for personal reasons, but none had evidence of sphenoid bone dysplasia

[5] One person was adopted and unaware of this information

Table 3.*Bsm*I (A/G) and *Fok*I (C/T) genotypic and allelic frequencies in Neurofibromatosis 1 patients and in unaffected controls.

	Patients(%) N=45	Controls(%) N=150	p value
*Bsm*I			0.284[1]
AA	4(8.9)	23(15.3)	
AG	27(60.0)	71(47.3)	
GG	14(31.1)	56(37.3)	
*Bsm*I			>0.999[2]
Allele A	35(38.9)	117(39.0)	
Allele G	55(61.1)	183(61.0)	
*Fok*I			0.430[1]
CC	14(31.1)	63(42.0)	
CT	26(57.8)	73(48.7)	
TT	5(11.1)	14(9.3)	
*Fok*I			0.314[2]
Allele C	54(60.0)	199(66.3)	
Allele T	36(40.0)	101(33.7)	

[1]Chi-Square Test; [2]Fisher's Exact Test

Table 4.*VDR* polymorphisms and vitamin D levels in NF 1 patients.

Genotype	25(OH)D (ng/ml)		p value
	<30	≥ 30	
BsmI			0.8875[1]
AA (n=4)	3 (9.7)	1 (7.1)	
AG (n=27)	19 (61.3)	8 (57.1)	
GG (n=14)	9 (29.0)	5 (35.7)	
FokI			>0.999[1]
CC (n=14)	10 (32.3)	4 (28.6)	
CT (n=26)	18 (58.1)	8 (57.1)	
TT (n=5)	3 (9.7)	2 (14.3)	

[1]Fisher's Exact Test based on 10000 sampled tables with starting seed 2000000

25(OH)D – 25-hydroxy-vitamin D

Table 5. Studies that assessed 25(OH)D levels in patients diagnosed with NF1.

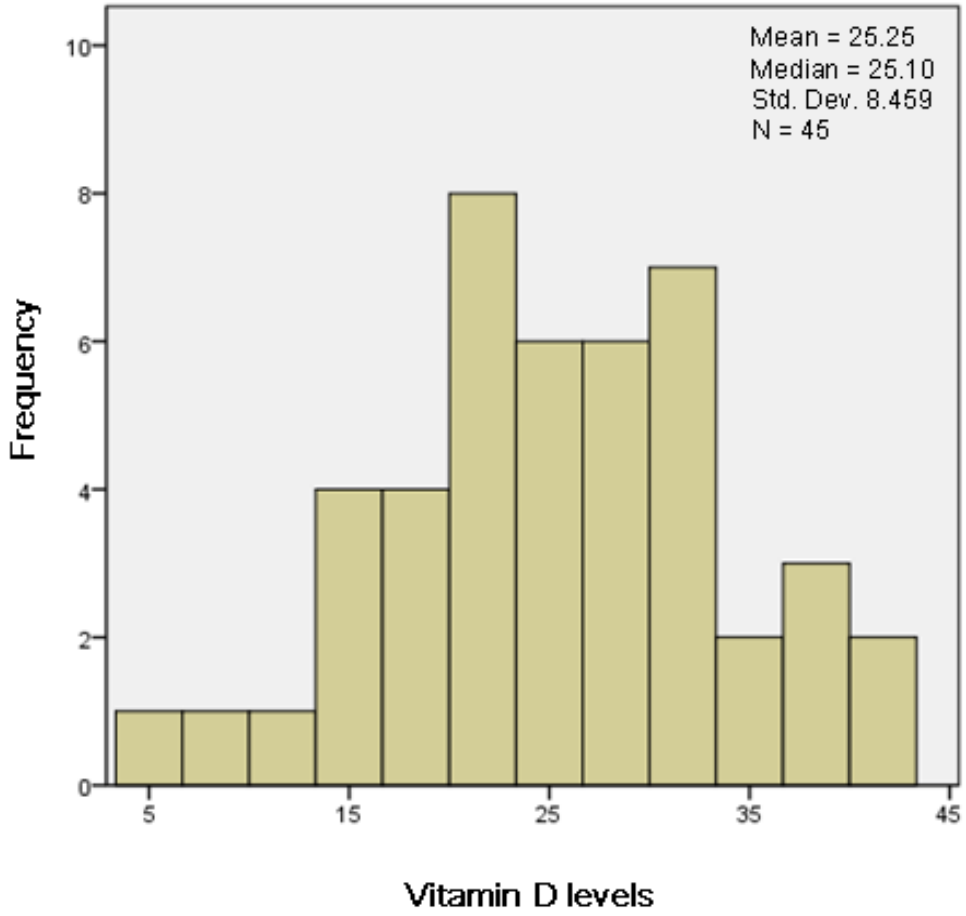
Author	Country	Method for dosing 25(OH)D levels *	Study design	Mean Age years (cases: controls)	Number, (female/male rate)	25(OH)D levels	Results (p-value)
Lammert *et al*., 2006 [14]	Germany	CLBPA; autumn or winter	Case-control	40.3:36	55 cases (33:22) 58 controls (38:20)	Mean: 15.7ng/mL(cases): 35.5ng/mL (controls)	Lower 25(OH)D levels in NF1 patients ($p<0.0001$) and inverse correlation with the number of neurofibromas ($p<0.0003$).
Brunetti-Pierri *et al*., 2008 [9]	USA	Chromatography	Descriptive	13.5 (cases)	16 cases with osteoporosis or osteopenia	Mean: 20.6ng/mL (cases)	____
Tucker *et al*., 2009 [10]	Germany	Technical unreported; winter and summer	Case-control	Men 43.4and Women 42.1 (cases)	72 cases (43:29)	<20ng/mL in 56% (cases)	Lower 25(OH)D levels in NF1 patients ($p<0.001$)
Seitz *et al*., 2010 [11]	Germany	RIA; autumn, winter and spring	Case-control	44.36: 46.97	14 cases (9:5) 42 controls (27:15)	Range: 5-23ng/mL (cases): 13-46ng/mL (controls)	Lower 25(OH)D levels in NF1 patients (<0.05)
Stevenson *et al.*, 2011 [15]	USA	CLIA	Case-control	9.3 (controls)	109 cases (50:59) 218 controls	Mean: 31.76ng/mL (cases): 33.79ng/mL (contros).	Lower 25(OH)D levels in NF1 patients (0.0129)
Petramala *et al.*, 2011 [12]	Italy	RIA; autumn and winter	Case-control	41.1:44.3	70 cases 60 controls	Mean: 21.8ng/mL (cases): 32.9ng/mL (controls)	Lower 25(OH)D levels in NF1 patients (<0.01)
Hockett *et al*., 2013 [29]	United Kingdom	Chromatography	Case-control	11.8:11.5	15 cases (10:5) 15 controls (8:7)	Mean: 15.6 (cases) 16.6 (controls)	25(OH)D levels were not significantly different between groups; overall mean in total population was within deficient range

NF: Neurofibromas; 25(OH)D: 25-hydroxy-vitamin D; CLBPA: chemiluminescence binding protein assay; RIA: radioimmunoassay; CLIA: chemiluminescence intra-assay.

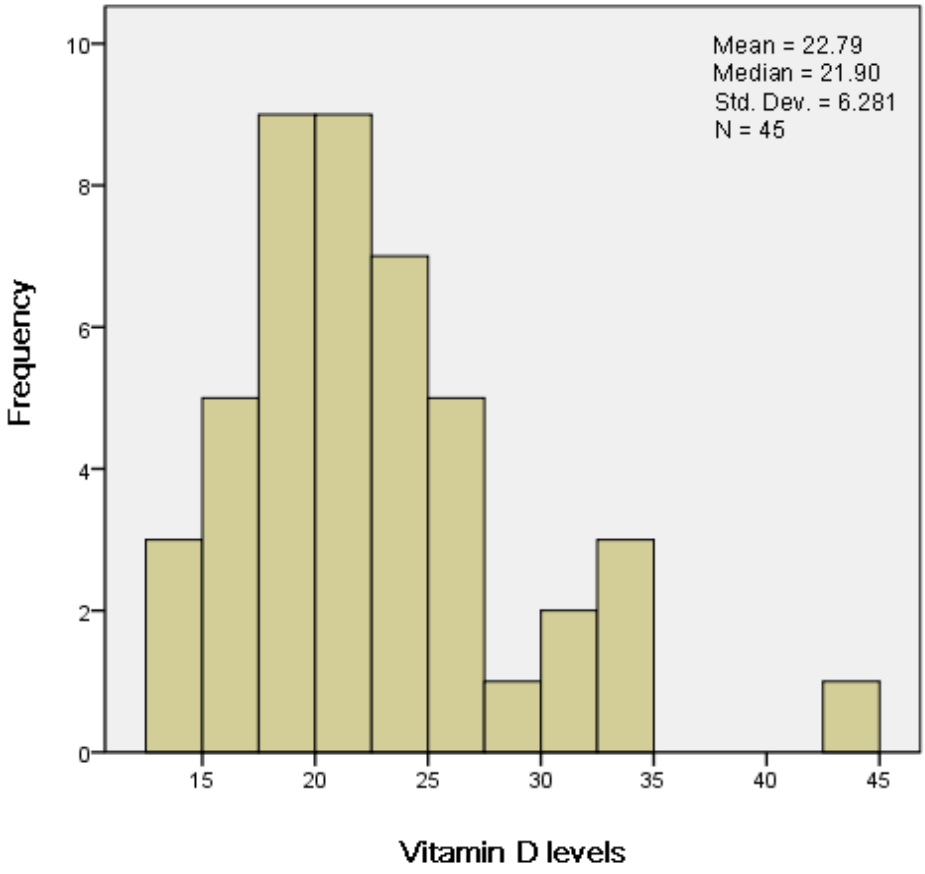
* Levels may vary according to the method used in the dosage [19].

Figure 1. Histograms showing the distribution of plasma 25(OH)D levels (ng/mL) in NF1 patients (A) and controls (B).

A



B


Mean = 22.79
Median = 21.90
Std. Dev. = 6.281
N = 45
Frequency
0
2
4
6
8
10
15
20
25
30
35
40
45
Vitamin D levels