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

LARISSA BRUSSA REIS

INVESTIGAÇÃO DE GRANDES REARRANJOS E MECANISMO DE DOENÇA NAS GENODERMATOSES MAIS COMUNS: NEUROFIBROMATOSE TIPO 1 E ESCLEROSE TUBEROSA

TESE DE DOUTORADO

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UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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Tese apresentada ao Programa de Pós-Graduação em Genética e Biologia Molecular da UFRGS como requisito parcial para a obtenção do grau de Doutora em Genética e Biologia Molecular.

LARISSA BRUSSA REIS

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CO-ORIENTADORA: Dra. Clévia Rosset

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Dedico essa tese de doutorado, assim como todas as minhas conquistas, às pessoas que foram o alicerce da minha trajetória e que hoje, infelizmente, não se encontram presentes fisicamente para acompanhar a minha maior superação diária que é viver sem eles.

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“É necessário olhar para a frente da colheita, não importa o quão distante isso seja, quando uma fruta for colhida, algo bom aconteceu”.

Charles Darwin 1809 - 1882

Sumário

RESUMO

Introdução: As doenças monogênicas neurofibromatose tipo 1 (NF1) e esclerose Tuberosa (ET) são consideradas síndromes genéticas raras com malignidade associada. Trata-se das duas genodermatoses mais frequentes, que apresentam como sintomas em comum as lesões cutâneas, ocorrência de tumores no Sistema Nervoso Central, deficiência cognitiva e/ou de desenvolvimento. Com uma gama variada de alterações genéticas envolvidas e sintomas em diferentes graus de comprometimento, caracterizar essas doenças, bem como investigar as alterações genéticas encontradas em subgrupos clínicos é um desafio. **Objetivos:** O objetivo dos trabalhos reunidos nesta tese foi caracterizar, do ponto de vista evolutivo, clínico e molecular, pacientes com NF1 e ET diagnosticados com rearranjos gênicos, realizar caracterização molecular adicional por análise de transcritos em pacientes com diagnóstico clínico de ET, bem como explorar o mecanismo de autofagia envolvido nesta doença. **Resultados:** Através de uma abordagem evolutiva foram avaliadas microdeleções encontradas em pacientes com NF1 envolvendo quatorze genes co-deletados. Destes, dez genes mostraram seleção purificadora e quatro genes mostraram seleção positiva, sendo um deles, *RNF135*, apresentando também seleção positiva de aminoácidos específicos, reforçando sua importância e contribuição para a doença e possível correlação da sua deleção com o fenótipo mais grave desses pacientes. Adicionalmente, foi apresentado relato de caso de um paciente com ET e rearranjo gênico, descrevendo manifestações clínicas não habitualmente relacionadas com a doença e sugerindo, através de revisão de todas as evidências existentes, a inclusão destas manifestações no fenótipo da síndrome. Como resultados preliminares, apresentamos o recrutamento de pacientes com ET e variantes de significado incerto ou ausência de variante patogênica na região codificadora dos genes *TSC1* e *TSC2*, bem como o desenho de primers que serão utilizados para a investigação de *exon-skipping* no RNA mensageiro desses pacientes. Por fim, uma revisão bibliográfica apresenta o paradoxo da via de autofagia e potenciais estratégias terapêuticas envolvidas com essa via para pacientes acometidos com ET e resultados preliminares apresentam o estabelecimento de fibroblastos de

paciente ET com mutação em *TSC2* e de fibroblastos de indivíduo sem ET, a fim de realizar avaliação do impacto de moduladores de autofagia no fluxo autofágico e nos mecanismos de mitofagia, apoptose e necrose celular. **Discussão e conclusões:** A NF1 e a ET são genodermatoses bem caracterizadas do ponto de vista clínico e molecular. No entanto, ainda existem lacunas importantes acerca destas síndromes, como o entendimento completo de relações genótipo-fenótipo (em especial de rearranjos gênicos), da ausência de identificação da lesão molecular em todos os casos com diagnóstico clínico, e dos mecanismos relacionados à patogênese destas doenças. Essa tese foi desenvolvida para contribuir no entendimento de alguns destes aspectos. Esforços voltados para uma melhor compreensão destas lacunas poderão resultar na inclusão dos achados desta tese no diagnóstico molecular dessas doenças e no aprimoramento do manejo dos pacientes e familiares, com impactos tanto no aconselhamento genético quanto para desenvolvimento de potenciais alternativas terapêuticas.

Palavras-chave: Genodermatoses; Esclerose Tuberosa, Neurofibromatose tipo 1.

ABSTRACT

Introduction: Neurofibromatosis type 1 (NF1) and tuberous sclerosis complex (TSC) are the most common genodermatoses. They are monogenic, autosomal dominant disorders associated with skin abnormalities, increased risk for developing certain tumors, mainly of the peripheral and central nervous system, cognitive and/or developmental disabilities and other findings. The disorders are characterized by complete penetrance but variable expressivity and significant molecular heterogeneity, which result in challenges in terms of the clinical and molecular diagnosis of the affected patients. **Objectives:** The main goal of this thesis was to characterize, from an evolutionary, clinical, and molecular point of view, patients with NF1 and TSC diagnosed with gene rearrangements, perform additional molecular investigations in patients with the clinical diagnosis of ET, as well as exploring the autophagy mechanism involved in this disease. **Results:** Through an evolutionary approach, microdeletions identified in NF1 patients and involving fourteen co-deleted genes were characterized. Of these, 10 genes showed purifying selection and 4 genes showed positive selection, with one of them, *RNF135*, also presenting positive selection of specific amino acids. These findings related to *RNF135* suggest its importance and contribution to the disease and a possible correlation of its deletion with a more severe phenotype in affected patients. Additionally, a case report of a patient with TSC and a gene rearrangement is presented. It describes clinical manifestations not usually observed in TSC and suggests, through a review of all the existing evidence, the inclusion of these manifestations in the syndrome's phenotype. In addition, the thesis contains preliminary results of the additional molecular investigation, focused on mRNA/exon skipping analyses, of TSC patients with variants of uncertain significance (VUS) or absence of pathogenic variants in the coding region of the *TSC1* or *TSC2* genes. Finally, a comprehensive review on the paradox of autophagy in TS and of potential novel therapeutic strategies related to autophagy for TS patients is presented as well as preliminary results on an investigation of autophagy modulators in fibroblast of TSC patients. **Discussion and conclusions:** NF1 and TSC are well characterized genodermatoses from a

clinical and molecular point of view. However, there are still important gaps about these syndromes, such as an incomplete understanding of genotype-phenotype relationships (especially related to gene rearrangements), absence of a specific disease-causing molecular lesion in some patients, and uncertainties related to the exact mechanisms that lead to these disorders and their complications. This thesis aims to contribute to the understanding of some of these aspects, ultimately leading to improved diagnosis and management of patients and their family members, with impacts both on genetic counseling and on development of potential novel therapeutic interventions.

Keywords: Genodermatoses; Neurofibromatosis type 1, Tuberous Sclerosis Complex.

LISTA DE ABREVIATURAS

aCGH: *array Comparative Genomic Hybridization*

ACMG: *American College of Medical Genetics and Genomics*

AMP: Monofosfato de Adenosina

AMPK: Proteína Quinase Ativada por Monofosfato de Adenosina

ATG: *Autophagy-related genes*

CNV: *Copy Number Variation*

DEPTOR: proteína de interação mTOR contendo domínio DEP

ERK: *Extracellular signal-regulated Kinases*

ET: Esclerose tuberosa

GAP*: GTPase activating protein*

GDP: Guanina di-fosfatada

GTEx: *Genotype-Tissue Expression*

GTP Guanina tri-fosfatada

HGMD: *The Human Gene Mutation Database*

IGFs: *Insulin-like Growth Factor*

IRS: *Insulin Receptor Substrate*

kDa: kiloDaltons

LAM: Linfangioleiomiomatoses

LCR: *Low Copy Repeats*

LOVD: *Leiden Open Variation Database*

MAPK: *Mitogen-Activated Kinase*

MEFs: *Mouse Embryonic Fibroblasts*

MH: Máculas hipopigmentadas

MLPA: *Multiplex Ligation-dependent Probe Amplification*

MLST8: *Target of Rapamycin Complex Subunit LST8*

MPNST: Tumores malignos da bainha de nervo periférico

mTOR: *Mammalian target of rapamycin*

NF1: Neurofibromatose tipo 1 ou Proteína neurofibromina 1

NGS: *New Generation Sequence*

NIH: *National Institutes of Health*

NMI*: No mutation identified*

OMIM: *Online Mendelian Inheritance in Man*

P/LP: variantes germinativas patogênicas/provavelmente patogênicas

PI3K: *Phosphatidylinositide 3-kinase*

PIP2: Fosfatidilinositol-4,5-fosfato

PIP3: Fosfatidilinositol-3,4,5-fosfato

PRAS40: *Proline-rich Akt substrate of 40 kDa*

Rheb: Proteína homóloga a Ras enriquecida no cérebro

RHEB: Ras GTPase enriquecida no cérebro

SD: *Segmental Duplication*

SEGA: *Subependymal giant cell Astrocytomas*

SNC: Sistema Nervoso Central

SNP: *Single Nucleotide Polymorphism*

SS: Sequenciamento direto de Sanger

SSCP: *Single-strand conformation polymorphism*

SUS: Sistema Único de Saúde

TPM: Transcritos por milhão

VUS: *Variant of Uncertain Significance* (Variante de Significado Incerto)

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Capítulo I

Introdução Geral, Justificativa e Objetivos

1 **INTRODUÇÃO GERAL**

1.1 Genodermatoses

As genodermatoses são um grande grupo que engloba cerca de 560 doenças hereditárias e heterogêneas com manifestações cutâneas e frequente acometimento de outros órgãos. Embora as genodermatoses sejam raras, com uma taxa de prevalência de menos de 1 em 50.000 a 200.000 pessoas, elas geralmente são doenças crônicas, graves e podem ser fatais (Gerstenblith et al. 2010; Aşkın et al. 2020).

As características elementares mais comuns das doenças genéticas da pele incluem problemas de erosão na camada mais externa da epiderme, erros no processo de cornificação, de pigmentação, de vascularidade e/ou a presença bolhas cutâneas. Porém, apenas uma pequena parcela das genodermatoses que acometem a pele apresenta unicamente sintomas relacionados a problemas de pele em si (Silverberg and Sidbury 2020). A grande maioria apresenta também envolvimento sistêmico e os sistemas mais frequentemente envolvidos são os sistemas neurológico, ocular, musculoesquelético e ósseo (Feramisco et al. 2009). A predisposição aumentada a diversas neoplasias na infância e na vida adulta também é observada (Frommherz et al. 2021).

Algumas genodermatoses são altamente incapacitantes, representando um enorme impacto na qualidade de vida dos doentes e também de seus familiares, devido a exclusão social, dificuldade na inserção profissional, vulnerabilidade a nível psicológico e cultural, bem como redução da expectativa de vida (Silverman, 2008). São doenças congênitas que frequentemente cursam com sintomas a o nascimento ou no início da vida, mas em alguns casos podem ser reconhecidas somente anos após.

As genodermatoses são classificadas em distúrbios de queratinizarão (ictiose, hiperqueratose palmo-plantar, epidermólise bolhosa), desordens

relacionadas com a produção de colágeno (síndrome de Ehlers-Danlos, pseudoxantoma elástico) e síndromes de pré-disposição a câncer (neurofibromatoses e esclerose tuberosa) (Silverberg 2020). As manifestações cutâneas das genodermatoses estão descritas no Quadro 1 (Aşkın et al., 2020).

Quadro 1. Manifestações cutâneas de genodermatoses (adaptado de Aşkın et al., 2020)

Ictiose

Xerose

Membrana de colódio

Fenótipo arlequim

Hiperpigmentação

Hipopigmentação

Despigmentação

Poiquiloderma

Desordens vasculares

Ectasia

Esteira telangiectática

Marcas vasculares congênitas

Malformações vasculares

Defeitos do tecido conjuntivo

Hamartomas

Aplasia cutis

Epidermólise bolhosa

Transtornos de fotossensibilidade / metabolismo da porfirina

Porfirias

Distúrbios metabólicos heme

Envelhecimento avançado

Transtornos mediados por metabolismo

Ocronose

Acrodermatite enteropática

1.1.1 Bases genéticas e moleculares das genodermatoses

Análises usando o banco de dados online do catálogo OMIM (*Online Mendelian Inheritance in Man*) da Universidade Johns Hopkins, publicadas no estudo de Feramisco e colaboradores (2009), identificaram 560 doenças de pele genéticas distintas. Dessas 560, apenas 60 são consideradas ‘‘genodermatoses puras’’, exibindo apenas fenótipos de pele, sem manifestações sistêmicas. Uma explicação para isso é que são poucos genes que regulam as características da pele de uma forma restrita, sem codificar produtos que atuem em diferentes rotas metabólicas. Cerca de 80% das genodermatoses identificadas no catálogo OMIM apresentam um único gene identificado como a causa do fenótipo, 9% têm dois diferentes genes associados, e cerca de 4% têm três ou mais genes relacionados à causa da doença. A maioria dos genes (81,6%) se relaciona apenas com uma doença; 11,6%, 4% e 2,8% estão associados a duas, três e quatro ou mais doenças, respectivamente (Feramisco et al. 2009).

O modo de herança também é variado entre as genodermatoses, sendo as mutações autossômicas recessivas e dominantes as mais frequentes. A assinatura genética das genodermatoses encontradas no OMIM pode ser vista na Figura 1, adaptada de Feramisco e colaboradores (2009). Diferentes mutações nos 501 genes codificantes de proteínas únicas podem causar distúrbios com fenótipos de pele distintos. O gene encontrado mutado com mais frequência foi o gene alfa-1 (*COL7A1*) que codifica o colágeno do tipo VII, ocorrendo em 9 genodermatoses distintas, mas relacionadas (Feramisco et al. 2009). Outras alterações moleculares comumente encontradas nas genodermatoses incluem mutações em genes estruturais da epiderme que codificam proteínas como a filagrina, uma proteína estrutural fundamental no desenvolvimento e manutenção da barreira cutânea ou diferentes tipos de queratinas, que subsequentemente causam ictioses, alterações na epiderme que causam extremo ressecamento e descamação; mutações em genes que codificam componentes integrais dos complexos de adesão supramolecular das células (por exemplo, os desmossomos), relacionadas à doenças de pele com a presença bolhas; ou ainda, mutações em genes envolvidos nas vias de reparo de DNA, como por

exemplo, na doença rara xeroderma pigmentoso (XP) e em genes supressores de tumor (Lehmann et al. 2017).

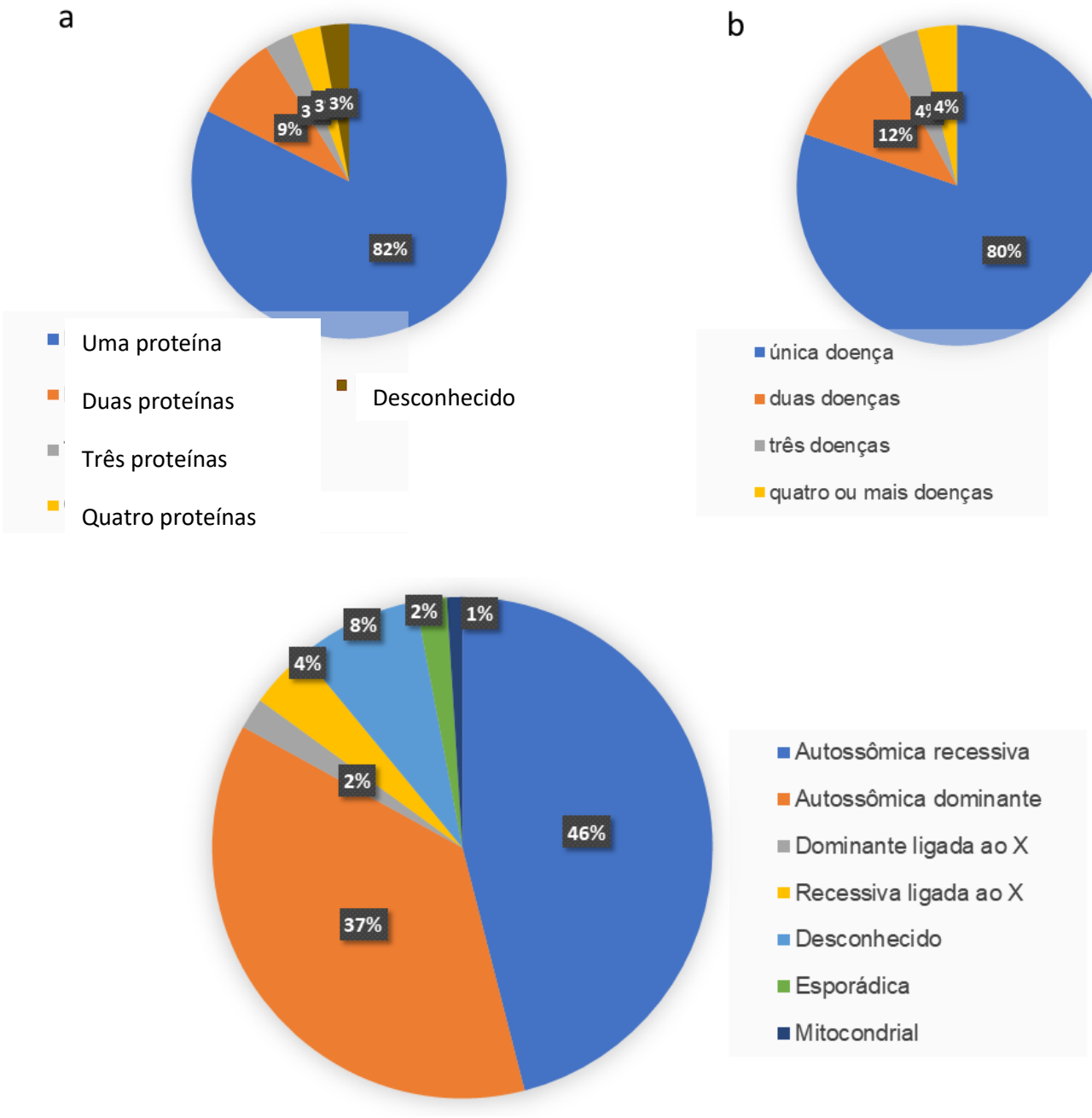

Figura 1. Análise genotípica de genodermatoses registradas no catálogo OMIM (a) Número de proteínas mutadas associadas à ocorrência das genodermatoses (b) Número de doenças causadas pelos diferentes genes associados às genodermatoses (c) Formas de herança descritas nas genodermatoses. Adaptado de Feramisco (2009).

1.1.2 Identificando uma genodermatose

Devido à variabilidade de sintomas, diagnosticar uma genodermatose apenas com investigação clínica pode ser um desafio. Para um diagnóstico clínico completo, o exame dermatológico deve avaliar a pele, cabelos, unhas, dentes e membranas mucosas do paciente, sendo complementado com análise através de uma lâmpada de Wood para detecção de manchas hipocrômicas invisíveis à inspeção ocular. As manifestações extra cutâneas podem ser identificadas através de exames laboratoriais ou de imagem. A avaliação da história médica do paciente e testes laboratoriais de histopatologia, microscopia eletrônica, imunofluorescência, bioquímicos e moleculares podem ajudar a confirmar o diagnóstico (Tantcheva-Poór et al. 2016).

O diagnóstico molecular é importante no diagnóstico diferencial das genodermatoses e é um importante complemento mesmo naquelas doenças com diagnóstico clínico consagrado para fins de aconselhamento genético. Os testes moleculares tornam-se fundamentais para detectar alterações genéticas associados com a causa da maioria das doenças (Tantcheva-Poór et al. 2016) e devem incluir a investigação das regiões codificadoras e flanqueadoras, com análise de sequência e de rearranjos gênicos. Os testes de escolha atualmente envolvem painéis multigênicos e sequenciamento de nova geração (NGS, *Next Generation Sequencing*), ficando o sequenciamento bidirecional de Sanger reservado para análise preditiva de variantes específicas pré-identificadas em uma família. A análise de rearranjos gênicos pode ser realizada pelo NGS ou por técnicas específicas para detecção de variações no número de cópias (do inglês *Copy Number Variation* - CNV) como o *Multiplex Ligation-dependent Probe Amplification* (MLPA) (Aşkın et al. 2020). Além disso, eventualmente, o sequenciamento do exoma ou genoma completo podem ser utilizados para uma

análise mais ampla nos casos com manifestações clínicas atípicas ou naqueles em que não se identificam variantes causais nos genes canônicos (Schaffer 2016).

Diversas genodermatoses exibem, além dos achados cutâneos e extra cutâneos, predisposição aumentada para o desenvolvimento de múltiplos tumores. A suspeita e o diagnóstico precoce de uma genodermatoses são fundamentais para que as medidas corretas de manejo do paciente e posteriormente de seus familiares em risco possam ser implementadas, aumentando as chances de prevenção de diversas neoplasias (Aşkın et al. 2020). Em um estudo de revisão recente, Ladd e colaboradores descrevem 34 genodermatoses que cursam com maior predisposição a tumores, incluindo as RASopatias, ataxia telangiectasia, xeroderma pigmentoso, neurofibromatose tipo 1 e esclerose tuberosa (Ladd et al. 2020).

Dentre as genodermatoses associadas a malignidade, duas das mais frequentes são associadas a genes supressores de tumor e são o foco deste estudo: a neurofibromatose tipo 1 (NF1 OMIM #162200), que afeta 1 em 3.000 indivíduos (Uhlmann and Plotkin 2012) e a esclerose tuberosa (ET OMIM #191100), que ocorre na frequência de 1 em 6.000 nascidos vivos, de ambos os sexos (Henske et al. 2016).

1.2 Neurofibromatose tipo 1

As neurofibromatoses são reconhecidas como um grupo heterogêneo de desordens herdáveis, caracterizadas por alterações pigmentares cutâneas, tumores da bainha neural na pele e nos nervos espinhais, predisposição a neoplasias e outras alterações em múltiplos órgãos/tecidos. De fato, as neurofibromatoses são doenças multisistêmicas e se dividem em dois principais tipos, neurofibromatoses tipo 1 e tipo 2 (Uhlmann and Plotkin 2012). A neurofibromatose tipo 1 (NF1) é o tipo mais comum, também conhecida como doença de Von Recklinghausen, é caracterizada por neurofibromas cutâneos benignos da bainha do nervo periférico (células de Schwann) e por neurofibromas

plexiformes, que acometem cerca de 50% dos pacientes (Rosser and Packer 2002). Outros achados comuns são as anormalidades pigmentares cutâneas, incluindo manchas café-com-leite e efélides axilares e inguinais, presentes na maioria das crianças (Ferner 2007). Os pacientes também podem apresentar nódulos de Lisch na íris, xantogranulomas, observados ainda na primeira infância e possivelmente relacionados a fenótipo de leucemia mieloide crônica em crianças e hiperpigmentação difusa. Outra característica clínica importante da NF1 é a dificuldade de aprendizagem por déficit cognitivo que acomete pelo menos 50% dos portadores de NF1 (Eoli et al. 2019).

1.2.1 Sintomatologia e Diagnóstico da Neurofibromatose tipo 1

As manchas café-com-leite podem ser observadas na grande maioria dos pacientes com NF1 ainda em idade jovem e algumas podem ser congênitas, enquanto os neurofibromas cutâneos são de aparecimento mais tardio, entre a adolescência e a vida adulta (Friedman and Birch 1997; Rasmussen and Friedman 2000). Neurofibromas dérmicos, espinhais e plexiformes podem causar sintomas importantes, dependendo da sua localização, compressão mecânica, neural e/ou vascular (Rosser and Packer 2002).

Alterações esqueléticas frequentemente observadas são escoliose, pseudoartrose/displasia tibial e displasia da asa do esfenoide, macrocefalia e em alguns casos baixa estatura (Ferner 2007; Ladd et al. 2020). Anormalidades vasculares estão associadas a mortalidade significativa nos pacientes e incluem hipertensão grave, geralmente secundária à estenose da artéria renal; doença cerebrovascular, possivelmente levando a hemorragia cerebral; e hipertensão arterial pulmonar (Stewart et al. 2007).

Neurofibromas dérmicos e plexiformes associados às alterações pigmentares da NF1 podem levar a deformidades com impacto significativo na autoestima (Teles et al. 2012). Os neurofibromas plexiformes podem evoluir para tumores malignos da bainha de nervo periférico (MPNST), com capacidade de

metastatizar extensivamente. O risco de desenvolvimento de MPNST ao longo da vida para pacientes com NF1 é cerca de 10% (Karajannis and Ferner 2015).

Em geral, os pacientes com NF1 tem um risco 2,7 vezes maior de desenvolver câncer, com um risco cumulativo de 20% em pacientes com mais de 50 anos de idade (Friedman and Birch 1997). O risco cumulativo vital de câncer nestes pacientes varia de 4% e 52% (Walker et al. 2006; Gerstenblith et al. 2010). Entre os tumores que acometem os pacientes com NF1 estão os MPNSTs, gliomas (especialmente no nervo óptico) e outros tumores do Sistema Nervoso Central (SNC). Os gliomas da via óptica são astrocitomas pilocíticos de baixo grau capazes de envolver o nervo óptico, quiasma, trato e/ou hipotálamo, que podem ocasionar perda visual e/ou desregulação endócrina (Savar and Cestari 2008). Nos adultos, tumores de mama (mulheres jovens, antes dos 50 anos), tumores renais (feocromocitoma) e outros tumores sólidos não são incomuns, assim como tumores endócrinos pancreáticos, tumores glômicos, rabdomiossarcomas e somatostatinomas duodenais (Ratner and Miller 2015).

Os critérios para o diagnóstico clínico de NF1 foram estabelecidos inicialmente em 1987 pela *National Institutes of Health* (NIH) *Consensus Conference* (EUA), sendo constantemente revisados (última revisão em 2019). Segundo estes critérios, o diagnóstico clínico definitivo de NF1 pode ser realizado em uma criança de quatro anos de idade, se dois ou mais dos critérios descritos estiverem presentes, já que aproximadamente 85% dos pacientes apresentam manifestações clínicas típicas da doença até esta idade. As crianças que herdam NF1 de um dos pais geralmente são diagnosticadas no primeiro ano de vida, principalmente pelas múltiplas manchas café-com-leite, que se desenvolvem na infância precoce em mais de 95% desses casos (Nunley et al. 2009). Os critérios estabelecidos pelo consenso do NIH são presença de: 1) seis ou mais manchas “café-com-leite”, com mais de 0,5 cm no maior diâmetro em indivíduos pré-puberais, ou mais de 1,5 cm no maior diâmetro em indíviduos pós-puberais; 2) dois ou mais neurofibromas de qualquer tipo ou um neurofibroma plexiforme; 3) efélides nas regiões axilares ou inguinais; 4) glioma óptico; 5) dois ou mais

hamartomas na íris; 6) uma lesão óssea típica, como displasia esfenoide ou pseudartrose tibial; 7) um familiar de primeiro grau (pai, irmão ou filho) com NF1 definido conforme critérios acima. O número de complicações possíveis e o padrão da doença variam bastante entre os indivíduos, mesmo dentro de uma família, atestando que nesta doença há expressividade variável (Friedman 1998) [Atualizado em 06 Jan 2019]).

1.2.2 O gene *NF1* e a proteína neurofibromina

A neurofibromatose tipo 1 é causada por um número aproximadamente igual de variantes germinativas espontâneas e autossômicas dominantes herdadas, no gene supressor tumoral *NF1*. Trata-se de um grande gene localizado no cromossomo 17q11.2, com 58 éxons e 350kb, que codifica a proteína hidrofílica neurofibromina de 2818 aminoácidos e 250 kiloDaltons (kDa) (Ars et al. 2003). A inativação do gene *NF1* ocasiona a ativação constitutiva de sistemas envolvidos no aumento da proliferação celular e, consequentemente, na chance de desenvolvimento de tumores (Garcia-Linares et al. 2011).

Semelhante ao surgimento dos carcinomas basocelulares na síndrome de Gorlin, acredita-se que os neurofibromas da NF1 surjam após um "segundo evento" mutacional no gene *NF1*, levando à perda de heterozigosidade (Colman et al. 1995). Diversos mecanismos de perda do segundo alelo do gene *NF1* são possíveis eventos de inativação somática, como variantes adquiridas de perda de função, metilação do promotor do gene, alteração no número de cópias, silenciamento epigenético ou mediado por micro-RNAs (Garcia-Linares et al. 2010).

A proteína neurofibromina (NF1) é principalmente expressa em células da crista neural, incluindo neurônios, células de Schwann, melanócitos, oligodendrócitos e leucócitos (Moles et al. 2012). Trata-se de uma grande proteína citoplasmática com muitos domínios, que ainda não tem sua função completamente elucidada, mas que atua no controle da via Ras (rat sarcoma),

uma das vias de proliferação celular e ativação de outros genes supressores tumorais (Barbacid 1987; Sherekar et al. 2020). Até o momento, as informações estruturais detalhadas sobre neurofibromina são limitadas a estruturas cristalizadas de alta resolução dos domínios GAP (Scheffzek et al. 1998), do domínio Sec-PH e o complexo ternário entre KRAS4b (Welti et al. 2011), e do domínio EVH1 de interação com SPRED1 (outra proteína relacionada a genodermatoses) e o GRD de NF1. Não existe informação detalhada sobre a estrutura ou função dos restantes 80% da proteína, mas atualmente foi demonstrado que sua estrutura depende de dímeros de alta afinidade que podem se formar e ser estabilizados dentro da célula (Sherekar et al. 2020).

A neurofibromina possui importante região de 360 aminoácidos (compreendendo os éxons 21-27) que mostra homologia com o domínio catalítico da GTPase ativadora de proteína (GAP) de mamíferos (Xu et al. 1990). A ligação do nucleotídeo guanina à proteína Ras media a transdução de sinais que regulam o crescimento celular. A ligação de GTP (guanina tri-fosfatada) à Ras ativa a sinalização, enquanto a hidrólise para GDP (guanina di-fosfatada) cessa o sinal, inibindo o crescimento celular (Barbacid et al. 1987). O domínio relacionado à GAP da neurofibromina também hidrolisa GTP (guanina tri-fosfatada), bem como possui propriedades consistentes com regulação negativa dos múltiplos efetores ativados por Ras, como PI3K (*Phosphatidylinositide 3-kinase*) e MAPK (*mitogen-activated kinase*), que estão envolvidos na proliferação celular, síntese de DNA e apoptose. Resumidamente, a neurofibromina regula a atividade da Ras GTP, convertendo-a em sua forma inativa e suprimindo o crescimento celular (Gerstenblith et al. 2010). Uma outra importante proteína ativada por Ras e envolvida nos processos de proliferação celular, síntese de proteínas e autofagia é a proteína alvo de rapamicina (mTOR) (Figura 2).

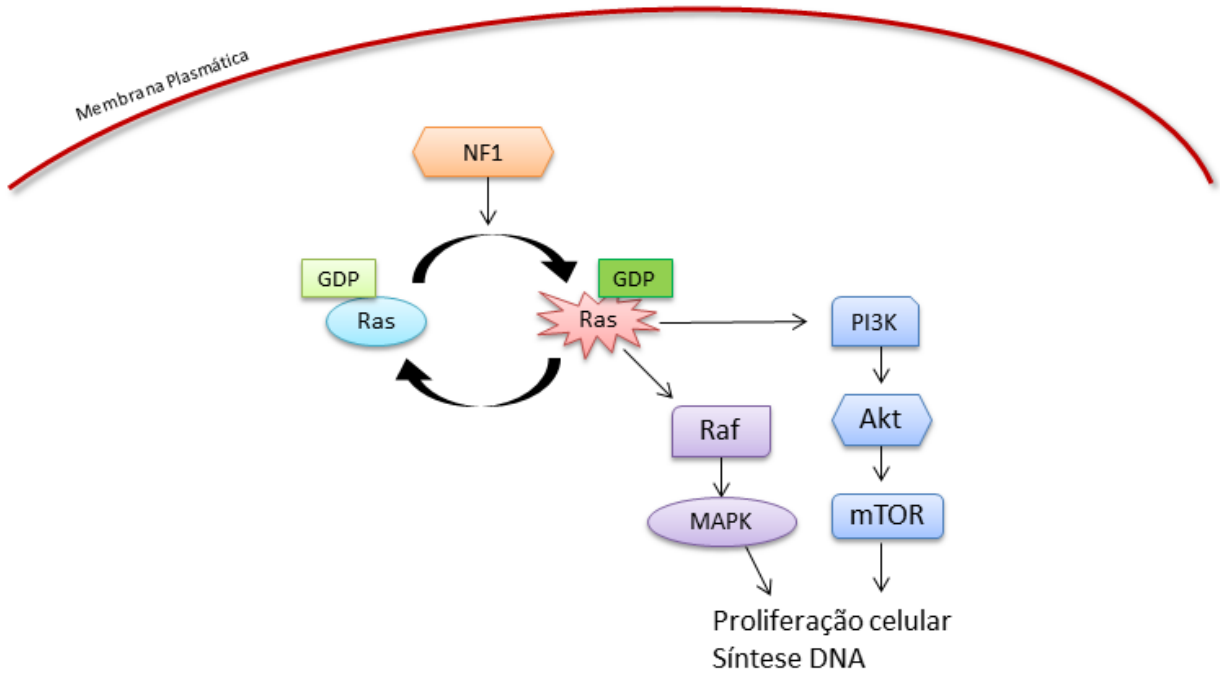

Figura 2: Envolvimento da proteína neurofibromina (NF1) nas vias de proliferação celular. Adaptado de Le and Parada 2007.

Estudos recentes tem se dedicado a determinar o mecanismo exato de funcionamento da neurofibromina. Devido a variabilidade de fenótipos encontrados na doença NF1, é possível que outros domínios desta proteína, além do GAP, tenham algum um papel importante na patogênese. Por ser uma proteína grande e de múltiplos domínios, a neurofibromina tem interação com várias outras proteínas intracelulares: proteínas da família SPRED, tubulina, cinesina-1, proteína quinase A, proteína quinase C, caveolina e proteína precursora de amilóide (revisado por Sherekar et al. 2020). Curiosamente, SPRED1 inibe a proteína Raf, e variantes no gene *SPRED1* são responsáveis por uma síndrome fenotipicamente muito semelhante a NF1 que consiste em múltiplas máculas café com leite, efélides axilares e macrocefalia chamada Síndrome de Legius (Brems et al. 2007).

1.2.3 Variantes no gene *NF1*

A NF1 é um distúrbio autossômico dominante com penetrância completa, e cerca de 50% dos casos apresentam variantes germinativas patogênicas/potencialmente patogênicas (P/LP) *de novo*. Nos casos familiais se observa que o fenótipo da doença é muito variável e a origem dessa variabilidade ainda não está completamente elucidada. Há relatos de variabilidade até mesmo em gêmeos monozigóticos, sugerindo inclusive um papel de alterações epigenéticas nessa expressividade variável (Vogt et al. 2011). Sendo assim, conclui-se que eventuais correlações genótipo-fenótipo não podem ser explicadas apenas pelo alelo mutante em questão, e que outras variantes constitutivas, variantes somáticas e alterações epigenéticas podem estar resultando na grande variabilidade de expressão (Dunning-Davies and Parker 2016).

Mais de 3200 variantes germinativas P/LP diferentes do gene NF1 foram identificadas até hoje (*The Human Gene Mutation Database* - HGMD, 2021), envolvendo deleções, duplicações, variantes em sítios de processamento, de perda de sentido, de sentido trocado, translocações e alterações da região 3' não-traduzida do gene. Não existe um único *hot spot* mutacional em *NF1* e sabe-se que variantes somáticas nesse gene são importantes na aquisição de resistência a drogas, como por exemplo aos inibidores de BRAF e de EGFR, ao tamoxifeno e ao ácido retinóico em melanoma, câncer de pulmão e mama e neuroblastoma, respectivamente (Friedman 1998) [Atualizado em 06 Jan 2019]). Da mesma forma, são observadas altas taxas de mutação somática em *NF1* no melanoma cutâneo, câncer de pulmão, carcinoma de ovário e glioblastoma, que geralmente não resultam no fenótipo de NF1, que é causada por variantes germinativas no gene. A identificação de uma alta frequência de mutações somáticas no gene *NF1* encontrada em tumores esporádicos indica que a neurofibromina provavelmente desempenha um papel crítico no desenvolvimento destes tumores, muito além do papel já evidente na NF1 (Philpott et al. 2017).

A maioria das variantes P/LP germinativas no gene *NF1* são únicas, ou seja, encontradas em uma ou poucas famílias e mais de 80% resultam em uma proteína truncada, geralmente pela alteração do processamento de RNA mensageiro (Rasmussen and Friedman 2000; Ars et al. 2003). Com o desenvolvimento de tecnologias cada vez mais precisas e robustas, diversas novas variantes vêm sendo identificadas em diferentes populações nos últimos anos. Um percentual significativo dessas variantes é classificado como *de novo* (Rosset et al. 2018; Chen et al. 2019; Yao et al. 2019; Koczkowska et al. 2020).

1.2.4 Rearranjos genômicos e grandes deleções em *NF1*

A maioria das variantes já descritas em *NF1* (~85% a 90%) são substituições, inserções ou deleções de uma única base. Outras mutações encontradas no gene são deleções ou duplicações simples ou multi exon (~2%) e grandes rearranjos em diferentes regiões no braço longo (q) do cromossomo 17 chamados de microdeleções, abrangendo *NF1* e um número variável de genes adjacentes (~5% a 10%) (Kluwe et al. 2004; Valero et al. 2011). A análise de rearranjos pela técnica de MLPA constitui a primeira etapa de investigação nos protocolos para identificação de NF1, permitindo identificar múltiplas deleções e duplicações de éxons únicos ou múltiplos (Ishida and Gupta, 2021). De uma forma geral a análise molecular na NF1 tem sido considerada complexa pelo grande tamanho do gene *NF1*, presença de pseudogenes e grande variedade de possíveis anormalidades. As estratégias de diagnóstico recomendam que a pesquisa de grandes deleções seja feita como investigação inicial pela prevalência destas alterações, seguida de estratégias para detecção de mutações de ponto em pacientes sem rearranjos detectados (Kluwe et al. 2004).

As microdeleções são alterações importantes, observadas em cerca de 5% a 15% dos pacientes com *NF1* (Clementi et al. 1996) em que além do gene *NF1*, vários outros genes estão co-deletados. As microdeleções mais frequentes (tipos 1, 2 e 3) variam em tamanho, sendo a maior delas (tipo 1) com tamanho aproximado de 1.4 Mb, envolvendo a região inteira do gene NF1 e outros 13

genes, 5 pseudogenes e 2 genes de microRNA no seu entorno (Dorschner et al. 2000; López Correa et al. 2000) (Figura 2). A maioria dos genes co-deletados nesses grandes rearranjos não possuem função totalmente conhecida (De Raedt et al. 2003). Essas microdeleções são causadas por recombinação homóloga não alélica entre sequências parálogas localizadas a montante (NF1-REPa) e a jusante (NF1-REPc) do gene *NF1* (Dorchner et al. 2000; Jenne et al. 2003). Essas sequências, também presentes na região NF1-REPb, são compostas por cópias do pseudogene *LRRC37BP*, pequenos fragmentos do pseudogene *SMURF2* e por sequências parálogas derivadas do gene *LPHN1*, localizado no cromossomo 19 (Jenne et al. 2003).

A microdeleção tipo 2 é menor (1.2 Mb) e tem seus pontos de quebra localizados no gene *SUZ12* e em seu pseudogene *SUZ12P* (Petek et al. 2003; Kehrer-Sawatzki et al. 2004). Por último, a microdeleção tipo 3, de 1.0 Mb de comprimento, apresenta os pontos de quebra dentro de repetições de pequenas cópias, denominadas NF1-REPb e NF1-REPc (Bengesser et al. 2010) (Figura 3).

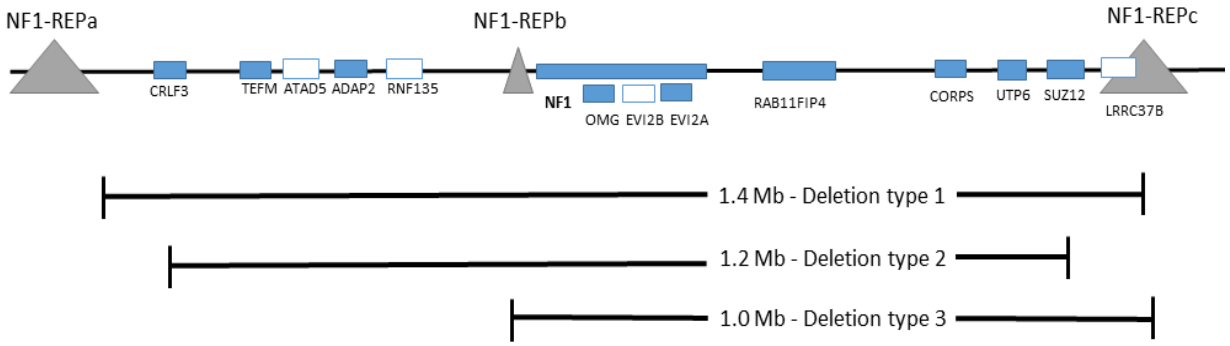

Figura 3: Representação de microdeleções (tipos 1, 2 e 3) em pacientes com NF1. Triângulos cinza representam as regiões de ponto de interrupção NF1REPa, NF1REPb e NF1REPc. Os retângulos azuis indicam genes funcionais. Adaptado de (Kehrer-Sawatzki and Cooper 2012).

1.2.5 Correlação genótipo-fenótipo e análises evolutivas em microdeleções

Embora existam múltiplas alterações genéticas diferentes envolvidas na causa das NF1, ainda não há muitos estudos que investigam grandes rearranjos e os associam com o fenótipo. Três correlações genótipo-fenótipo claras já foram observadas: (a) a deleção de todo o gene *NF1* associada ao aparecimento precoce e numeroso de neurofibromas cutâneos, anormalidades cognitivas mais frequentes e mais graves do que na NF1 causada por variantes pontuais, mãos e pés grandes e dismorfia facial (Mautner et al. 2010); (b) a deleção de 3pb na extremidade do éxon 17, que confere menor risco de desenvolvimento de neurofibromas cutâneos ou plexiformes (Upadhyaya et al. 2007) e (c) indivíduos com todo o *locus NF1* duplicado não tem o fenótipo característico de NF1, mas sim, deficiência mental e convulsões (Grisart et al. 2008). Em relação as microdeleções, um artigo publicado recentemente apresenta dois casos de duas meninas de 2 e 4 anos de idade não-relacionadas. A primeira apresentou uma deleção na posição 17q11.2, com de cerca de 1 Mb (pontos de interrupção nas posições 29.124.299 e 30.151.654), que envolveu os genes *NF1*, *CRLF3, ATAD5, TEFM, ADAP2, RNF135, OMG, EVI2B, EVI2A* e *RAB11FIP4* como sintomas manchas café com leite e efélides axilares, nódulos de Lisch, estrabismo, palato arqueado alto, má oclusão, cifoescoliose grave, pé calcâneo valgo bilateral, hipotonia generalizada leve, hiperatividade e déficits de fala e habilidades relacionadas. A segunda menina apresentou uma deleção heterozigótica de NF1, com as posições 29.124.299 e 30.326.958 como seus pontos de quebra, e que incluiu além dos genes deletados na primeira paciente, além dos genes *COPRS*, *UTP6* e *SUZ12* parcialmente. Seus sintomas incluíam atraso no crescimento e desenvolvimento, estenose pulmonar supravalvar, manchas café com leite, efélides axilares, características dismórficas craniofaciais, pescoço curto com pterígio, anormalidades nos membros e focos de displasia neural. A análise nos pais demonstrou uma origem *de novo* das deleções em ambos os casos (Serra et al. 2019).

Determinar as funções dos genes frequentemente deletados nas microdeleções pode facilitar o entendimento dos diferentes fenótipos entre os pacientes que apresentam esse tipo de grande rearranjo. A maioria dos genes está relacionada com o desenvolvimento e com o controle do ciclo celular, porém os genes *RNF135*, *SUZ12*, *ADAP2*, *UTP6* e *OMG* merecem um foco maior quando se trata de relação genótipo-fenótipo. A haploinsuficiência do gene *RNF135* parece estar relacionada com alta estatura, macrocefalia, bem como com dismorfias faciais em pacientes com microdeleção, com a primeira e a última característica tornando-se menos aparentes na vida adulta, reforçando a importância da investigação durante a infância (Spiegel et al. 2005; Douglas et al. 2007).

A melhor compreensão dos efeitos das microdeleções que envolvem o gene *NF1* e de outros genes co-deletados pode contribuir para o entendimento dos mecanismos de doença e do desenvolvimento dos fenótipos associados. Historicamente, a maneira mais utilizada para investigar biomoléculas era centrada na caracterização de moléculas individuais para entender seu papel no desenvolvimento de um fenótipo. Entretanto, essa estratégia não é efetiva para elucidar a evolução de uma doença complexa (Murray et al. 2007). Neste contexto, análises moleculares *in silico* têm sido cada vez mais utilizadas como método de rastreamento de potenciais alvos terapêuticos, ainda mais quando levamos em consideração a crescente disponibilidade de dados genômicos. A filogenia molecular e a análise comparativa da estrutura gênica são ferramentas interessantes para melhorar a compreensão das relações evolutivas e funcionais de genes estudados (Wang et al. 2013). Um exemplo disso é a inferência de que a conservação de uma sequência proteica está frequentemente relacionada a um papel funcional crítico dessa macromolécula na célula (Siepel et al. 2005).

1.3 Esclerose tuberosa

Semelhante a NF1, a esclerose tuberosa (ET) também é uma doença neurocutânea que afeta vários sistemas. Relatada pela primeira vez por Bourneville em 1880, a esclerose tuberosa foi nomeada após a caracterização de lesões corticais cerebrais que se assemelham a tubérculos e a calcificação periventricular em pacientes com a doença (Gómez 1995; Henske et al. 2016).

A esclerose tuberosa (ET) é uma desordem autossômica dominante e multissistêmica (ORPHA:805) que afeta crianças e adultos, e apresenta manifestações neurológicas que incluem epilepsia, deficiência intelectual e autismo (Kingswood et al. 2017). É causada por variantes germinativas P/LP de perda de função em um dos genes supressores de tumor relacionados: *TSC1* (OMIM #605284) ou *TSC2* (OMIM #191092) (Henske et al. 2016). Trata-se de uma doença com penetrância parcial e expressividade variável que tem o potencial de afetar praticamente qualquer órgão do corpo, com manifestações clínicas identificadas em diferentes fases do desenvolvimento (Northrup et al. 2013). É caracterizada também pela ocorrência de tumores benignos (hamartomas) em diversos órgãos, sendo a pele, cérebro, rins, pulmões e coração os sítios mais comuns. Esses hamartomas podem causar disfunções nos órgãos em quem se desenvolvem, do ponto de vista funcional ou estrutural, levando, por exemplo, a complicações secundárias a compressão de estruturas nobres adjacentes ou perda de parênquima funcional em alguns órgãos (Northrup et al. 2013). A prevalência de ET na população é de aproximadamente 1 a cada 20.000 indivíduos, sem predileção sexual ou racial (Ebrahimi-Fakhari et al. 2017a).

1.3.1 Aspectos clínicos da esclerose tuberosa

As manifestações clínicas da ET vão desde o surgimento dos hamartomas até sintomas secundários como acometimento neurológico que incluem convulsões, transtornos do desenvolvimento relacionados a espectro autista e

prejuízos cognitivos, além do comprometimento de pele e mucosas que estão envolvidos em cerca de 70% dos casos (Henske et al. 2016). Alguns sintomas podem estar presentes desde o nascimento e a maioria dos sintomas é observada até os quatro anos de idade, como é o caso, por exemplo, das máculas hipomelanóticas ou hipopigmentadas (MH) congênitas que geralmente são os primeiros sinais visíveis, muitas vezes precedendo a epilepsia. Além das MH, outras características causam menor comprometimento, como angiofibromas faciais e fibromas subungueais (Ebrahimi-Fakhari et al. 2017a). No início da terceira década de vida, MH, nódulos subependimais e angiomiolipomas renais são vistos em mais de 80% dos pacientes, enquanto a epilepsia e angiofibromas faciais são observados em mais de 70% dos pacientes (Ladd et al. 2020).

Os critérios de diagnóstico para ET endossados pela organização *Tuberous Sclerosis Alliance* incluem categorias principais e secundárias. O diagnóstico é feito com a observação de duas características principais (excluindo linfangioleiomiomatose e angiomiolipoma renal isoladamente) ou uma característica principal e duas secundárias (Roach et al. 1998; Gerstenblith et al. 2010). A Tabela 1 resume todos os critérios de diagnóstico clínico e genético revisados e atualizados para ET (com base nas referências Northrup et al. 2013; Ebrahimi-Fakhari et al. 2017b).

Tabela 1. Critérios para diagnóstico clínico e genético da esclerose tuberosa

Critérios diagnóstico genético		
A identificação de qualquer variante patogênica nos genes *TSC1* ou *TSC2* no DNA de um tecido normal é um critério independente e suficiente para o diagnóstico, independente dos achados clínicos. Variantes patogênicas são definidas como: (1) Variantes que inativam a função das proteínas TSC1 ou TSC2; (2) Variantes que impedem a síntese de proteínas: grandes deleções genômicas; (3) Variantes silenciosas cujos efeitos na função da proteína podem ser determinados por ensaio funcional;		
Critérios diagnóstico clínico		
Características dermatológicas e odontológicas		
Características principais	Frequência	Início dos sintomas
≥3 Máculas hipomelanocíticas, com pelo menos 5 mm de diâmetro	observadas em cerca de 90% dos pacientes	nascimento ou infância
≥3 angiofibromas ou placas fibrosas cefálicas	angiofibromas ocorrem em cerca de 75% dos pacientes, placas fibrosas em cerca de 25%	entre 2 a 5 anos de idade
≥2 fibromas ungueais	em adultos chega a 80%	adolescência e vida adulta
Nevo de tecido conjuntivo (*Shagreen patch*)	observadas em cerca de 50% dos pacientes	primeira década de vida
Características secundárias	Frequência	Início dos sintomas
Lesões *confetti* na pele, de 1 a 3 mm de diâmetro	frequências variam de 3% a 58% dos pacientes	variado
≥3 cavidades no esmalte dental	Mlynarczyk G et al. Reportou em todos os 50 adultos analisados	adolescência e vida adulta
≥2 fibromas intraorais	ocorre em cerca de 20% a 50% dos pacientes	variado
Características oftalmológicas		
Características principais	Frequência	Início dos sintomas
Hamartomas múltiplo de retina	são encontrados em cerca de 30% a 50% dos pacientes, não é comum encontrar múltiplas lesões no mesmo paciente	infância
Características secundárias	Frequência	Início dos sintomas
Máculas acrômicas na retina	cerca de 39% dos pacientes	variado
Características do Sistema Nevoso Central		
Características principais	Frequência	Início dos sintomas
Displasia cortical	tubérculos corticais ocorrem em cerca de 90% dos pacientes	desenvolvimento fetal
Nódulos subependimais (SEM) e astrocitoma de células gigantes (SEGA)	SEM é observado em 80% dos pacientes; SEGA em 5% a 15% dos pacientes	infância e adolescência
Características pulmonares		
Características principais	Frequência	Início dos sintomas
Linfangioleiomiomatose (LAM)	observado em 30% a 40% das pacientes mulheres; alcançando cerca de 80% de mulheres afetadas após os 40 anos de idade	adolescência e vida adulta
Características renais		
Características principais	Frequência	Início dos sintomas
≥2 angiomiolipomas	observado em cerca de 80% dos pacientes	infância e vida adulta
Características secundárias	Frequência	Início dos sintomas
Cistos múltilplos renais	muito variada	adolescência e vida adulta
Características endócrinas		
Características principais	Frequência	Início dos sintomas
Angiomiolipoma adrenal	presente em 1/4 dos pacientes	variado

Alguns dos principais sintomas da ET abrangem o SNC, resultando em epilepsia, déficit cognitivo, transtorno do espectro autista e déficit de atenção e hiperatividade, causando prejuízos que se manifestam desde a primeira infância (Grabole et al. 2016). A epilepsia é o sintoma mais comum, acometendo até 90% dos pacientes e costuma representar o primeiro sinal da doença, visto que mais de dois terços dos pacientes apresentam epilepsia antes do segundo ano de vida (Kingswood et al. 2017). Mais tarde, diferentes tipos de crises convulsivas podem surgir, com episódios focais ou generalizados. A deficiência intelectutal que pode acometer cerca de 60 a 70% dos pacientes é frequentemente ligado a convulsões com grande variabilidade na gravidade, e em alguns pacientes ainda são presentes outras anomalias de comportamento, como esquizofrenia (Northrup et al. 2019a). A gravidade dos sintomas no SNC se relaciona com a presença de alterações do desenvolvimento do SNC, localização, número e tamanho de hamartomas cerebrais – os chamados “tubérculos corticais” e presença de outros tumores como astrocitomas. As três principais lesões intracranianas associadas à ET são os tubérculos corticais, nódulos subependimais e astrocitomas subependimais de células gigantes (do inglês *subependymal giant cell astrocytomas* - SEGA) (Altermatt et al. 1991). Tipicamente, os SEGAs são tumores glioneuronais de crescimento lento. As apresentações clínicas mais comuns dos pacientes com SEGAs incluem convulsões, deficiência intelectual, déficit cognitivo, distúrbios visuais, dores de cabeça e vômitos. SEGAs são encontrados em 5-20% dos pacientes com ET e constituem mais de 90% dos tumores intracranianos associados a doença, sendo responsáveis por 25% da mortalidade atribuída a ET (Jansen et al. 2006; Jansen et al. 2019; de Vries et al. 2020).

Além do comprometimento do SNC, no coração, rabdomiomas intracardíacos podem comprometer funções ventriculares e resultar em obstrução do fluxo sanguíneo. Esses tumores são observados em indivíduos afetados por ET durante a vida fetal, no entanto, geralmente regridem ao longo dos primeiros meses ou anos de vida. Cerca de 80% dos pacientes com rabdomiomas

cardíacos ao nascimento são posteriormente diagnosticados com ET (Northrup et al. 2013; Henske et al. 2016).

As manifestações renais são uma importante fonte de morbidade e mortalidade em ET, tendo sido apontados como a segunda causa de morte prematura na ET (Feliciano 2020). Em adultos com ET, a doença renal é a causa da morte mais comum. Mais de 70% dos pacientes de ET desenvolvem angiomiolipomas, tumores benignos compostos de tecido vascular, muscular liso e adiposo que são geralmente encontrados nos rins, sendo múltiplos e bilaterais e podendo alcançar grandes dimensões (Henske et al. 2016). A frequência e o tamanho dos angiomiolipomas (observado em 80% dos pacientes) aumenta com a idade. À medida que se expandem, pode ocorrer aneurisma vascular resultando em hemorragia maciça ou insuficiência renal devido ao efeito mecânico da massa tumoral (Ladd et al. 2020).

A principal manifestação pulmonar de ET é a linfangioleiomiomatose (LAM), associada com expansão intersticial do pulmão com células musculares lisas benignas que conseguem se infiltrar em todas as estruturas pulmonares (Carsillo et al. 2000; Sato et al. 2004). Essa é uma das manifestações que podem ocorrer mais tardiamente, com pacientes apresentando sintomas na terceira a quarta década de vida. Alterações parenquimatosas pulmonares císticas compatíveis com LAM são observadas em 30 a 40% das mulheres com ET. As mesmas alterações são observadas em cerca de 10 a 12% dos homens com ET, porém alterações sintomáticas são bastante raras (Carsillo et al. 2000). A manifestação oral mais comum da esclerose tuberosa inclui alargamento gengival, que geralmente se associa com pequenos nódulos fibrosos. Os fibromas orais na esclerose tuberosa podem aparecer na mucosa bucal e no dorso da língua. Além disso, defeitos de esmalte dentário podem ser observados em pacientes com esclerose tuberosa (Gosnell et al. 2021).

Como mencionado anteriormente, há significativa variabilidade na expressão do fenótipo, mesmo entre os membros acometidos em uma mesma família. Por fim, em diferentes séries de caso, a proporção de casos familiais e

esporádicos (casos isolados) de ET tem sido descrita como sendo de 40% e 60%, respectivamente (Henske et al. 2016).

1.3.2 Os genes *TSC1* e *TSC2* e as proteínas hamartina e tuberina

O gene *TSC1* (NM_000368.4, NG_012386.1), localizado no cromossomo 9q34, abrange cerca de 53kb de DNA genômico, com 23 éxons que codificam a proteína hamartina, uma proteína hidrofílica de 1164 aminoácidos e 130 kDa. O éxon 23 é o maior éxon de *TSC1* (5.407 pb), contribuindo para o grande comprimento da região 3'UTR não-traduzida dos transcritos. O maior íntron é o primeiro (9.447 pb). A média de tamanho dos éxons é de 374 pb, enquanto dos íntrons é de 798 pb. A proteína hamartina interage e estabiliza a proteína ativadora de GTPase, tuberina, codificada pelo gene *TSC2* (van Slegtenhorst et al. 1998).

O gene *TSC2* (NM_000548.3, NG_005895.1), localizado no cromossomo 16p13.3, compreende cerca de 40kb de DNA genômico e possui 41 éxons que codificam a proteína tuberina, com 1807 aminoácidos e 180 kDa, a qual possivelmente atua como uma chaperona para a hamartina. O maior éxon de *TSC2* tem o tamanho de 488 pb (éxon 34) e o maior íntron tem 4.820 pb (íntron 16). A média de tamanho entre os éxons é de 133 pb, enquanto a média de tamanho dos íntrons é de 798 pb (Dabora et al. 2001; Nellist et al. 2015).

A proteína hidrofílica hamartina (TSC1, NP_000359.1) possui uma região de 266 resíduos de aminoácidos próximo das porções C-terminal e N-terminal que é necessária para a interação com a tuberina (TSC2), com a qual forma um homodímero. Na ausência de TSC2, o gene *TSC1* produz hamartina insolúvel, apresentando distribuição citoplasmática pontual, visualizada por análises de imunofluorescência. Se ambas as proteínas estão sendo co-expressas, TSC1 passa a ser solúvel e é possível visualizar uma distribuição citoplasmática mais homogênea das duas proteínas (van Slegtenhorst et al. 1998).

A proteina tuberina (TSC2, NP_000539.2) contém um domínio N-terminal relativamente hidrofílico (Maheshwar et al. 1996) e uma região conservada de 183 aminoácidos próxima à porção C-terminal, que possui homologia com as proteínas da superfamília Ras GTPases rap1GAP e mSpa1 (Maheshwar et al. 1997). O domínio C-terminal é codificado pelos éxons de 31 a 38 e atua como um GAP para a via Ras GTPase enriquecida no cérebro (RHEB), catalizando a hidrólise de GTP para GDP dependente de RHEB. A porção N-terminal da tuberina é necessária para a interação com a hamartina, permitindo que as duas proteínas formem um complexo estável e funcional (van Slegtenhorst et al. 1998).

O complexo harmatina-tuberina, também chamado de complexo TSC1-TSC2 ou complexo TSC, regula negativamente a sinalização do complexo 1 de alvo da rapamicina em mamíferos, chamado de complexo mTORC1 (Northrup et al. 2018). O complexo TSC interage fisicamente no aparelho de Golgi e regula negativamente o crescimento e proliferação celular (van Slegtenhorst et al. 1998) além de modular a sinalização dependente de PI3K através de mTOR (Tee et al. 2002).

O desenvolvimento da maioria dos hamartomas na ET, assim como dos tumores associados a NF1, segue a teoria de dois eventos proposta por Knudson em 1970 para explicar a oncogênese em retinoblastoma. O primeiro evento corresponde à mutação germinativa, 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 do mesmo gene (Garcia-Linares et al. 2011; Polchi et al. 2018) (Figura 4). Muitos relatos têm demonstrado que a inativação pós-traducional do complexo TSC surge através da ativação da proteína Akt (Zhang et al. 2015), um efetor de PI3K, que é conhecido por ativar a tradução de mTOR. Outro mecanismo de tumorigênese em ET é a inativação do complexo hamartina-tuberina depois da fosforilação por várias quinases, como a quinase regulada por sinal extracelular (ERK) (Jozwiak et al. 2008).

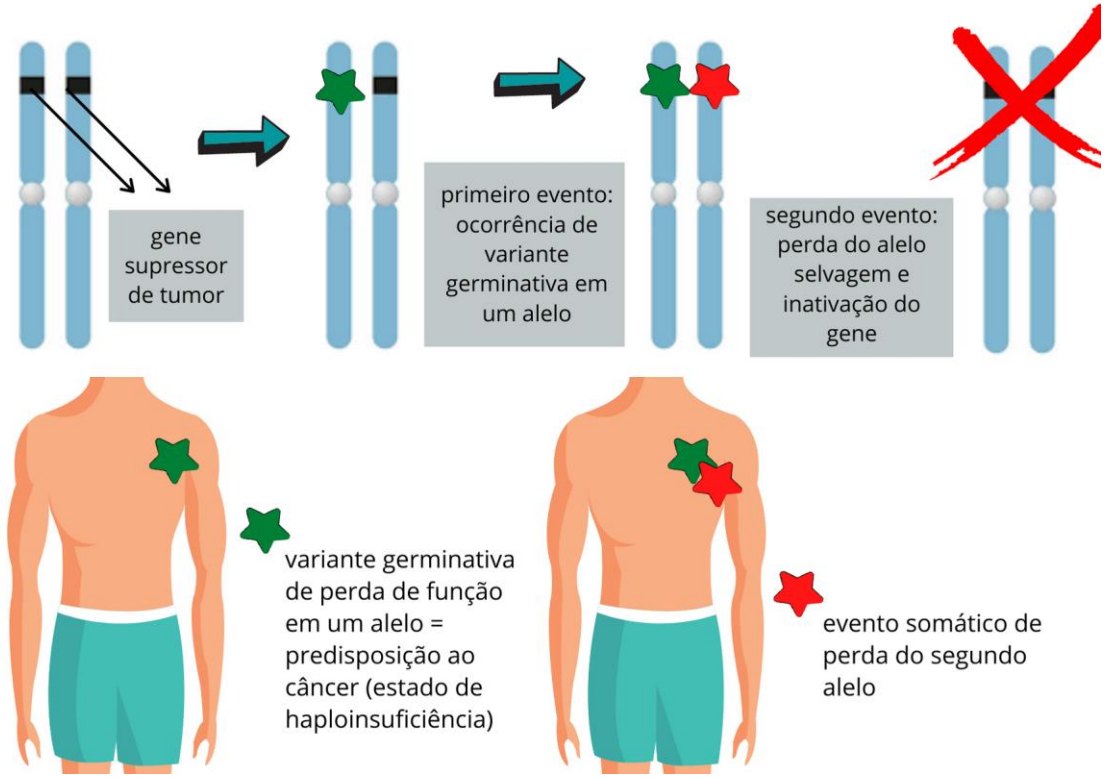

Figura 4: Hipótese de aquisição de segundo evento mutacional para o surgimento dos hamartomas e malignidades em esclerose tuberosa. Figura original.

1.3.3 Variantes nos genes *TSC1* e *TSC2*

Similar a NF1, a ET exibe penetrância completa com ampla variabilidade fenotípica, mesmo dentro de famílias que compartilham a mesma variante patogênica (Schwartz et al. 2007; Napolioni and Curatolo 2008). Em estudos com grandes famílias e várias gerações de pacientes acometidos com esclerose tuberosa, as variantes patogênicas nos genes *TSC1* e *TSC2* ocorrem na proporção de 1: 1. No entanto, as mutações em *TSC2* são responsáveis pela maioria dos casos isolados (não familiais), aparentemente devido a uma taxa aumentada de eventos *de novo.* Da mesma forma, há relatos de maior gravidade de sintomas em portadores(as) de variantes patogênicas em *TSC2* e isso talvez se deva a ocorrência mais precoce do segundo evento mutacional que já foi descrita para esse gene (Dabora et al. 2001; Sancak et al. 2005). Assim, indivíduos com variantes em *TSC2* apresentam, em comparação com aqueles que tem variantes em *TSC1*, déficit cognitivo mais acentuado, maior número de

tubérculos corticais, hamartomas retinais e angiofibromas faciais (Dabora et al. 2001; Lendvay and Marshall 2003).

Dezenas de mutações em *TSC1* e *TSC2* estão descritas na literatura, a maioria delas mutações pontuais no DNA, seguidas das grandes deleções e duplicações de éxons inteiros. Deleções genômicas nos dois genes correspondem a aproximadamente 7% de todas as variantes P/LP de esclerose tuberosa e 17% das alterações descritas são de sentido-trocado (HGMD, 2021). Variantes P/LP germinativas nas regiões codificadoras e intrônicas adjacentes de *TSC1* e *TSC2* são identificadas em cerca de 80% a 95% dos pacientes com diagnóstico clínico definido e variantes P/LP *de novo* são responsáveis por cerca de 65% a 75% dos casos de esclerose tuberosa. Essas variantes levam a uma ativação da via de sinalização PI3AKT, que resulta em estímulo à proliferação celular (Ladd et al. 2020).

As variantes P/LP no gene *TSC1* são geralmente pequenas deleções e inserções, além de mutações sem sentido. Já as variantes P/LP no gene *TSC2* compreendem, além dessas já citadas, grandes deleções e rearranjos. As variantes em *TSC2* são responsáveis pela maioria dos casos, embora mutações em ambos os genes possam produzir fenótipos alterados (Gerstenblith et al. 2010). As grandes deleções de *TSC2* também podem resultar em deleções do gene adjacente *PKD1,* responsável pela doença renal policística, o que resulta em indivíduos com ET e múltiplos cistos renais identificados desde a infância (Brook-Carter et al. 1994).

Algumas variantes de sentido trocado em *TSC2* já foram associadas a um fenótipo mais leve da ET, embora não seja possível estabelecer uma previsão de gravidade pelo tipo e localização da mutação (Jansen et al. 2006; Napolioni and Curatolo 2008; Northrup et al. 2019a).

1.3.4 Impacto de variantes de perda de função em *TSC1* e *TSC2* nas vias de sinalização intracelular envolvidas na Esclerose Tuberosa

O complexo mTORC1 é um importante regulador de crescimento, proliferação celular, autofagia e tradução de RNA mensageiro, sensível a nutrientes e formado por diferentes subunidades. A principal delas, a subunidade catalítica mTOR, é uma proteína quinase altamente conservada em vertebrados (Hay and Sonenberg 2004) que se encontra associada a outras proteínas, como a proteína regulatória Raptor, o alvo da subunidade do complexo alvo da rapamicina LST8 (MLST8) e as subunidades mais recentemente identificadas: o substrato AKT rico em prolina de 40kDa (PRAS40) e a proteína de interação mTOR contendo domínio DEP (DEPTOR). Todas essas estruturas associadas formam o complexo mTORC1 (Wullschleger et al. 2006).

Quando ativado, através de recursos energéticos adequados, disponibilidade de oxigênio e fatores de crescimento, este complexo é capaz de estimular a tradução de proteínas nas células, bem como a biogênese ribossomal e a importação de nutrientes. Em contrapartida, com a disponibilidade do composto rapamicina (principal inibidor do complexo) e componentes estressores, os mecanismos de autofagia e transcrição responsiva a fatores de estresse são inibidos (Hahn-Windgassen et al. 2005) (Figura 5). A via de mTOR integra vários sinais para regulação de crescimento celular, e os três principais efetores da via são os fatores de crescimento, os nutrientes disponíveis e a energia.

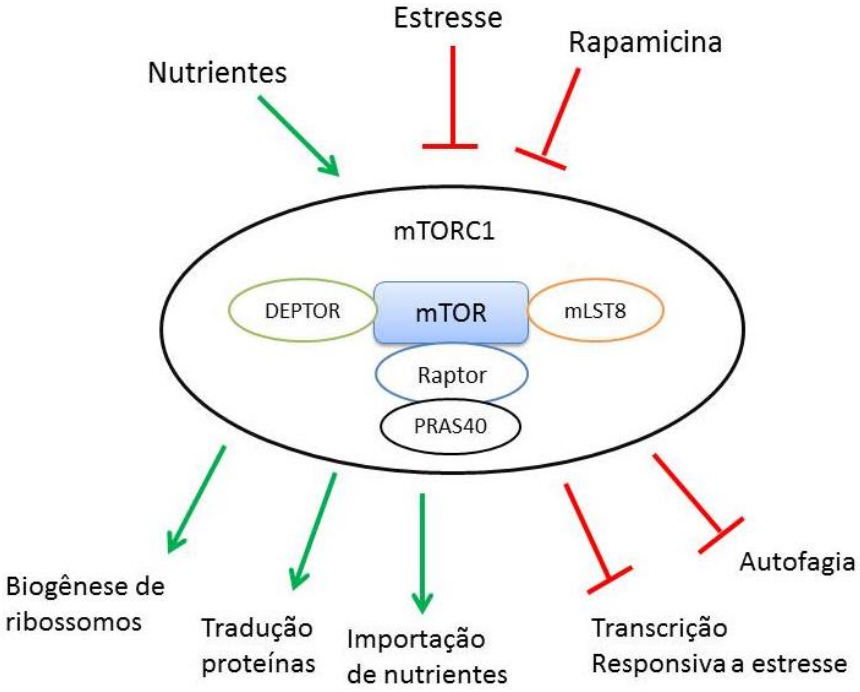

Figura 5: Esquema representativo do complexo mTORC1, suas subunidades e os principais estímulos e respostas da via na qual está inserido, em mamíferos. Estímulos que regulam positivamente a ativação do complexo são demonstrados com setas verdes. Estímulos que regulam negativamente são apresentados como barras vermelhas. Figura adaptada de (Hay and Sonenberg 2004; Wullschleger et al. 2006).

Nutrientes, especialmente aminoácidos, também regulam a sinalização de mTORC1. A pouca disponibilidade de aminoácidos, principalmente a ausência de leucina, resulta na rápida defosforilação dos efetores da via de mTOR: proteína ribossomal S6 quinase beta-1 (S6K1) e fator eucariótico de iniciação de tradução ligador de proteína 4E (4E-BP1), enquanto a adição de aminoácidos restaura a fosforilação dos mesmos alvos de maneira dependente de mTORC1 (Hay and Sonenberg 2004). É proposto que a regulação de mTORC1 por aminoácidos ocorra através da inibição do complexo TSC (Gao et al. 2002) ou, de maneira alternativa, independente de mTORC1 pela estimulação de Rheb, que permite a sinalização de mTORC1 na ausência de aminoácidos (Harputlugil et al. 2014).

Além disso, a ligação de Rheb e mTOR é regulada pela presença de aminoácidos, enquanto a carga de GTP em Rheb é independente de aminoácidos

(Longa et al. 2001) (Figura 6). Em relação aos altos níveis energéticos necessários para a síntese de proteínas, crescimento e proliferação das células que ocorrem com a ativação de mTORC1, o complexo é sensível ao status energético da célula através da proteína quinase ativada por AMP (AMPK), que é ativada em resposta a baixa energia na célula (alto nível de AMP por ATP). AMPK ativada é capaz de fosforilar diretamente a tuberina (TSC2) e assim aumentar a sua atividade GAP, levando à inibição de mTORC1 (Huang et al. 2009). A regulação do suprimento de energia celular também pode ser um mecanismo para ativar o mTORC1. De acordo com o modelo proposto por Hahn-Windgassen e colaboradores, a AKT ativa mTORC1 não apenas pela fosforilação direta de tuberina, mas também pela manutenção de altos níveis de ATP, através da manutenção da absorção de nutrientes, que causam a diminuição da taxa AMP/ATP e consequentemente essa proporção diminuída seria a responsável por inibir a fosforilação e ativação de tuberina (Hahn-Windgassen et al. 2005) (Figura 6).

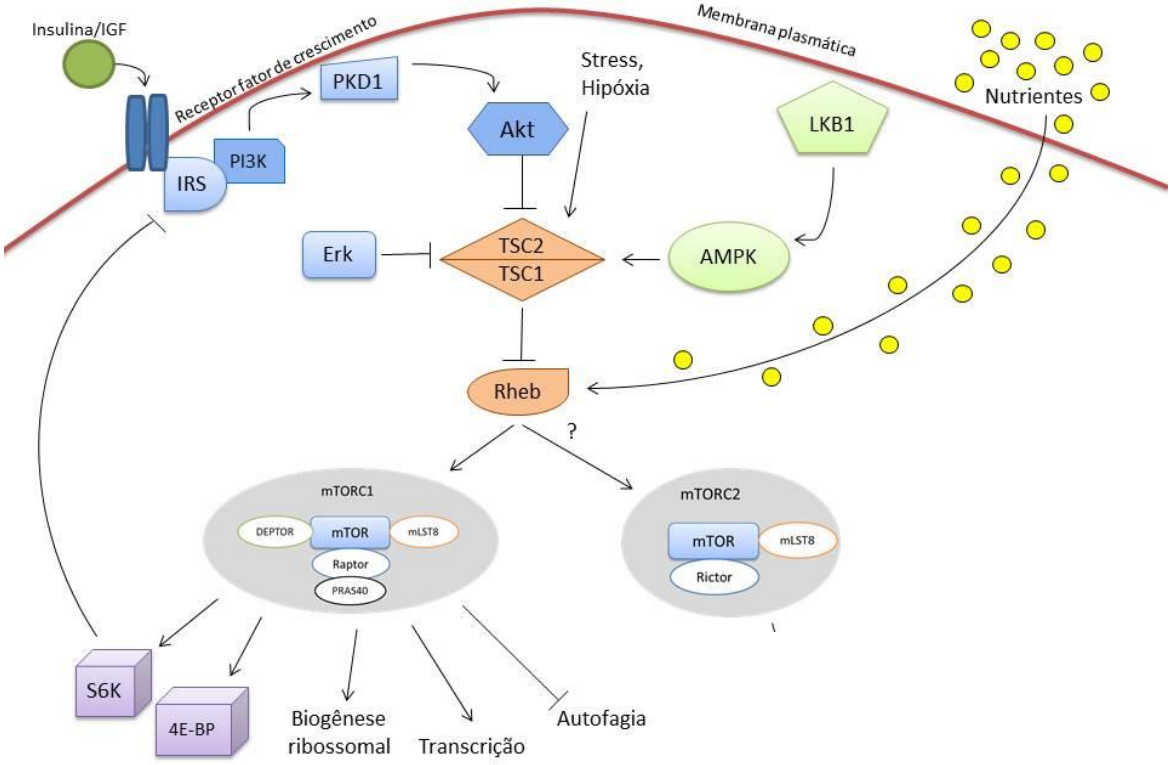

Figura 6: Sinalização da via de mTOR, seus principais estímulos (insulina, fatores de crescimento, nutrientes, stress e hipóxia), reguladores (Akt, complexo TSC, Rheb, AMPK, mTORC1 e mTORC2) e respostas (alvos SK6 e 4E-BP, biogênese ribossomal, transcrição e autofagia). A via de mTORC1 responde a fatores de crescimento através da PI3K. Trata-se de uma família de enzimas envolvidas em funções celulares, tais como crescimento, diferenciação, proliferação, motilidade, sobrevivência e tráfego intracelular. A ligação de insulina ou de fatores de crescimento semelhantes à insulina (IGFs) a seus receptores leva ao recrutamento e à fosforilação do substrato do receptor de insulina (IRS), e ao subsequente recrutamento de PI3K. PI3K se liga a IRS e converte fosfatidilinositol-4,5-fosfato (PIP2), presente na membrana celular para fosfatidilinositol-3,4,5-fosfato (PIP3). O acúmulo de PIP3 é antagonizado pela fosfatase lipídica PTEN. PIP3 co-recruta PDK1 e Akt para a membrana, resultando na fosforilação e ativação de Akt por PKD1. O complexo mTORC1 é ligado à via PI3K através das proteínas hamartin e tuberina do complexo TSC, que regula negativamente a sinalização mTOR. Em resposta a presença de insulina, a tuberina é fosforilada e funcionalmente inativada pela Akt, permitindo que mTORC1 realize suas funções. Por funcionar como uma proteína ativadora de GTPase (GAP) para a pequena GTPase Rheb (proteína homóloga a Ras enriquecida no cérebro), a tuberina é capaz de regular mTORC1. Rheb liga-se

diretamente ao domínio quinase de mTOR, ativando a via de maneira dependente de GTP. Adaptada de (Wullschleger et al. 2006).

1.3.5 O mecanismo de autofagia e a sua relação com a ET

A autofagia é um mecanismo natural, bem regulado e destrutivo que elimina componentes disfuncionais ou não necessários nas células (Yu et al. 2010). É um processo que permite a degradação de componentes celulares "velhos" e danificados e a reciclagem de componentes úteis, com a captura de frações do citoplasma e organelas pelas próprias células e o consumo dessas nos seus lisossomos (Yu et al. 2010; Galluzzi et al. 2015). O resultado dos produtos consumidos é utilizado no metabolismo celular, para gerar energia e construir novas proteínas e membranas. Em condições de restrição de nutrientes, a autofagia garante uma fonte interna de nutrientes para a geração de energia e consequentemente a sobrevivência das células e é um poderoso promotor da homeostase metabólica tanto em nível celular quanto em nível de organismo completo (Rabinowitz and White 2010).

Existem três formas de autofagia: a macroautofagia, a microautofagia e a autofagia mediada por chaperonas, além da mitofagia, um tipo de autofagia direcionada para mitocôndrias. As vias de autofagia são mediadas por genes relacionados, chamados de genes ATG (*autophagy-related genes*) e suas enzimas associadas (Jung and Lee 2010).

A macroautofagia é a via principal, usada primariamente para erradicar organelas celulares com dano ou proteínas que não estão sendo usadas no momento. Em linhas gerais, envolve a formação de dupla membrana conhecida como autofagossomo, ao redor do componente marcado para degradação. O autofagossomo se desloca através do citoplasma da célula até o lisossomo, e as duas organelas se fundem. Dentro do lisossomo, o conteúdo do autofagossomo é degradado através das hidrolases lisossomais ácidas (Jung and Lee 2010) (Figura 7).

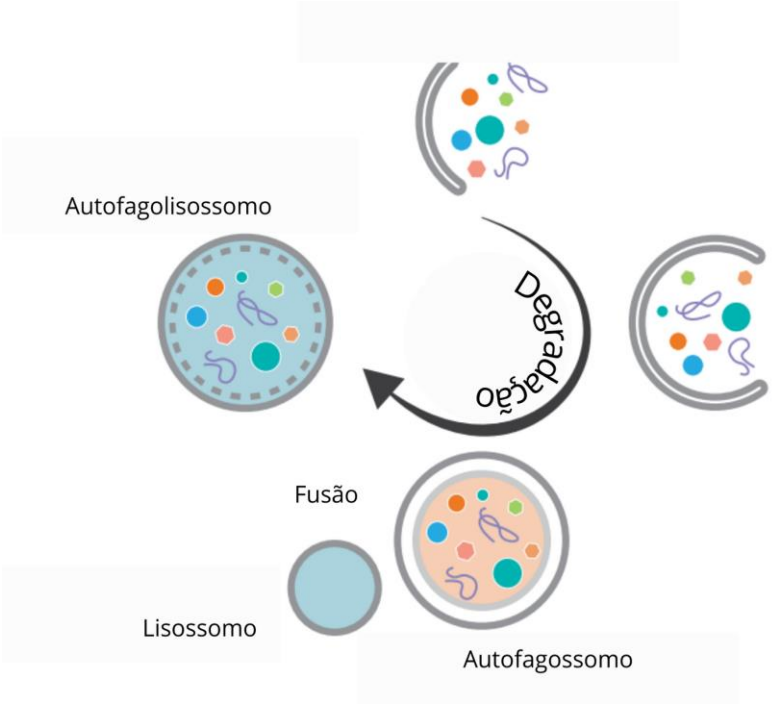

Figura 7: Autofagia depende da formação de autofagossomos que se fusionam com os lisossomos, formando os autofagolisossomos. Após a indução da autofagia, uma estrutura inicial de membrana dupla começa a se formar ao redor do material a ser degradado. Essa estrutura cresce e engloba o conteúdo citosólico, formando um autofagossomo maduro. A fusão entre autofagossomo e lisossomo produz um autofagolisossomo, onde ocorre a degradação ativa do material citosólico necessário para a célula. Adaptado de (Novus Biologicals 2017).

Em leveduras e mamíferos, a formação do complexo Atg1/ULK1 é essencial para a formação dos autofagossomos, além do complexo mTORC1 que desempenha um papel crítico, acoplando a sensibilidade aos níveis de nutrientes aos processos anabólicos e catabólicos. Quando os nutrientes estão disponíveis, mTORC1 é ativado e fosforila ULK1 que regula negativamente a autofagia, enquanto a biogênese ribossomal e a síntese proteica são estimulados (Rabinowitz and White 2010; Amaravadi et al. 2019). De modo inverso, a limitação de nutrientes desacopla a ligação mTORC1/ULK1, libertado ULK1 para se ligar a Atg1, aumentando a nucleação e o alongamento dos autofagossomos. Esse processo inativa mTORC1, inibindo o crescimento celular e estimulando a autofagia (Novus Biologicals 2017). Outro importante regulador da atividade de mTORC1, e consequentemente da autofagia é o complexo TSC, conforme

demonstrado na Figura 6. A etapa de expansão das membranas de autofagossomos envolve a indução da proteína citosólica Atg8, também chamada de LC3. Após formadas, as membranas encapsulam o conteúdo a ser degradado por proteases, lipases, nucleases e glicosidases. As permeases lisossomais liberam os produtos da degradação no citosol, tais como aminoácidos, lipídeos, nucleosídeos e carboidratos, onde estarão disponíveis novamente para as vias sintéticas e metabólicas (Rabinowitz and White 2010; Klionsky et al. 2021).

Defeitos na via de autofagia podem contribuir para a tumorigênese por permitirem o acúmulo de proteínas e compostos defeituosos nas mitocôndrias das células, que podem levar a instabilidade genética. Em contraste com as células nos tecidos normais, os ambientes tumorais são privados de nutrientes, fatores de crescimento e oxigênio, como um resultado da vascularização anormal desses locais (Yu et al. 2010; Yu et al. 2011). Nesse sentido, o efeito da autofagia nos tumores é paradoxal. Embora possa prevenir o início de alguns tumores, ela também pode dar suporte ao seu crescimento, garantindo aporte nutricional nas regiões tumorais com hipóxia, distantes dos vasos sanguíneos e assim sustentando o crescimento tumoral nessas regiões (Rabinowitz and White 2010). Portanto, o papel metabólico da autofagia pode contribuir para garantir disponibilidade consistente de nutrientes internos, permitindo a sobrevivência das células neoplásicas em períodos de escassa nutrição externa. Corroborando essa observação é fato que muitas das vias que regulam a autofagia se encontram desreguladas em câncer e alguns compostos terapêuticos buscam ativar essas vias. Alguns fazem isso diretamente, por inibição de mTORC1, enquanto outros inibem suporte nutricional ou vias de sinalização em fluxo ascendente (Yu et al. 2010).

1.3.6 Tratamentos para esclerose tuberosa: inibidores de mTOR

As variantes germinativas P/LP encontradas nos pacientes com ET tornam as proteínas que compõem o complexo TSC incapazes de bloquear a sinalização de mTOR, tornando-a constitutivamente ativa e descontrolada. Esse desequilíbrio pode levar ao estabelecimento dos tumores observados na síndrome, em especial

hamartomas, comumente localizados em regiões de difícil acesso e possibilidade remota de dissecção (Galluzzi et al. 2015).

Recentemente, como uma alternativa para as cirurgias, o tratamento com inibidores seletivos de mTORC1 tem sido proposto (Li et al. 2019). Embora esses inibidores sejam efetivos, possuem um grande custo financeiro. Além disso, pacientes com ET tratados com o inibidor mais comum, a rapamicina, por longos períodos de tempo, desenvolvem uma série de efeitos colaterais indesejados que em alguns casos até os fazem abandonar o tratamento (Canpolat et al. 2018). Os principais efeitos colaterais ocorrem devido à falta de especificidade da rapamicina, que acaba por bloquear além da sinalização de mTOR hiperativa nos tumores, a via mTOR de outras populações de células que desempenham funções fundamentais no organismo, como por exemplo a estabilização dos níveis glicêmicos (Boutouja et al. 2019).

Diversos estudos clínicos já demonstram a eficácia dos fármacos inibidores da via de mTOR rapamicina e seus análogos, especialmente para o tratamento de tumores em pacientes com ET (Verhoef et al. 1999; Peron et al. 2016). Sabendo que mTORC1 é um inibidor chave da autofagia através da fosforilação direta de ULK1, a disfunção que ocorre no complexo TSC de pacientes com esclerose tuberosa pode promover uma oportunidade única de investigar as implicações da desregulação da autofagia em doença humana, com apenas “três degraus” de separação entre as proteínas TSC e a regulação da via (Yu et al. 2011) (Figura 8).

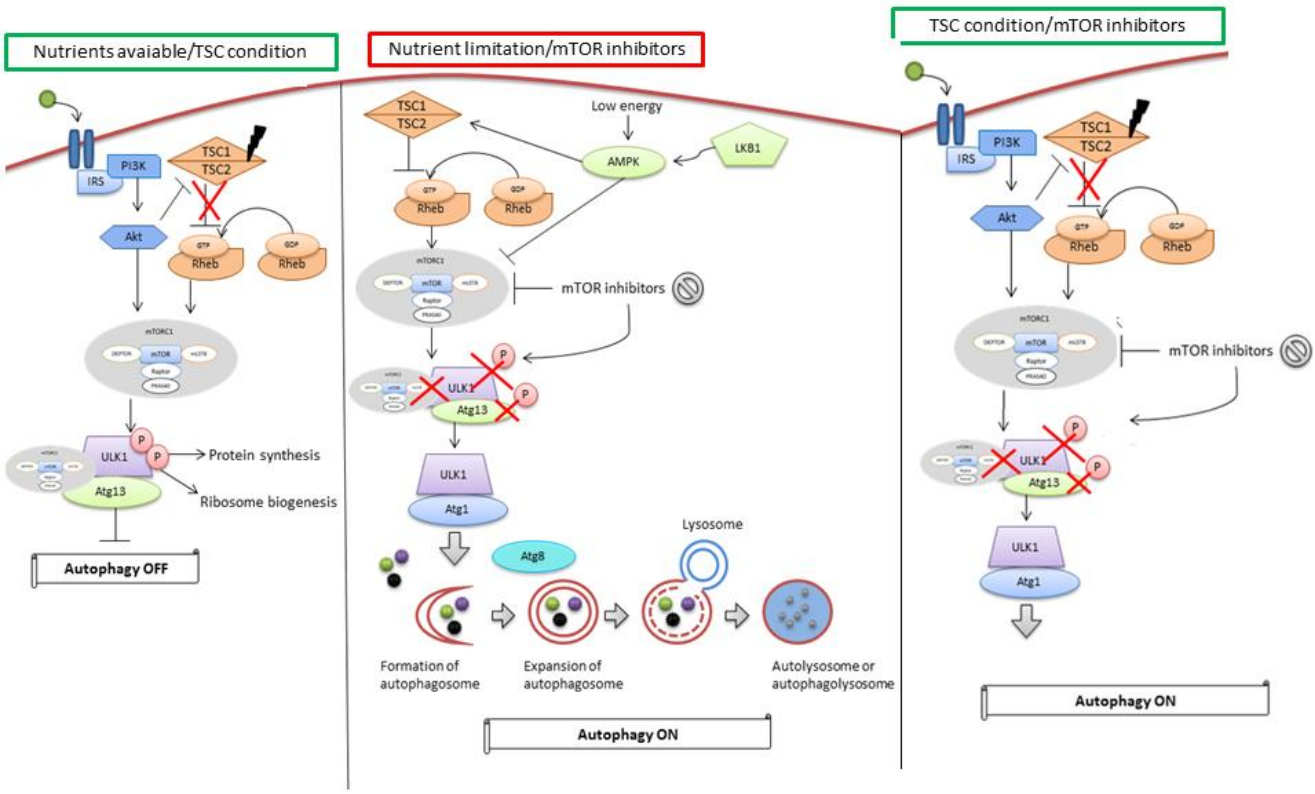

Figura 8: Sinalização de autofagia com nutrientes disponíveis ou na doença ET e com limitação de nutrientes ou presença de inibidores de mTOR. Neste primeiro cenário, o complexo TSC é incapaz de evitar a conversão de Rheb-GDP em Rheb-GTP causando a ativação de mTORC1. O mTORC1 ativado causa a inativação do complexo ULK1 através da fosforilação de ULK1 e ATG13, suprimindo assim a autofagia e permitindo a síntese de proteínas e biogênese do ribossomo. Por outro lado, no segundo cenário, sob limitações de nutrientes ou com a presença de inibidores de mTOR, mTORC1 é inativado, o que permite que o complexo ULK1 seja ativado junto com proteínas Atg que desencadeiam a formação de vesícula de autofagossomos em torno do conteúdo celular que precisa ser degradado. O autofagossomo se funde com o lisossoma formando autolisossomo que degrada o conteúdo interno da célula da vesícula. Paralelamente à indução da autofagia, a inativação do mTORC1 inibe o crescimento celular. Figura original.

Através de ensaios genéticos e farmacológicos de inibição da autofagia, foi possível verificar que células *TSC2*-deficientes são altamente dependentes do processo de autofagia para sobreviver. Os inibidores de mTOR induzem a

autofagia, que pode promover vantagens adaptativas para as células tumorais presentes nos harmatomas. Eles podem levar as células a um tipo de "dormência", onde a proliferação é bloqueada devido a inibição da tradução de proteínas mediada por mTORC1 mas a sobrevivência ao longo do tempo é possível devido a ativação da autofagia mediada pela mesma molécula (Yu et al. 2011).

Nesse sentido, investigar a condição da via de autofagia na ET e o seu estímulo e resposta a outros compostos que atuem nessa via tem sido proposto. A metformina é um agente anti-hiperglicêmico utilizado para o tratamento de diabetes mellitus não dependente de insulina. O exato mecanismo de ação da metformina não é bem elucidado, mas sabe-se que ela pode exercer um efeito inibitório na via de sinalização mTOR (Amin et al. 2019).

A inibição de mTORC1 por metformina pode ocorrer diretamente via AMPK através da fosforilação e inibição de Raptor, que é a proteína adaptativa e reguladora no complexo mTORC1 e pela ativação dos genes *TSC1* e *TSC2* (Howell et al. 2017) (Figura 6). Evidências em modelo animal demonstram que a metformina atua na mTOR, porém os possíveis benefícios terapêuticos para pacientes com ET ainda são controversos. Kalender e colaboradores (2010) demonstraram que em fibroblastos embrionários de camundongos (MEFs) normais e sem o gene *TSC2* (*TsC2* $^{-/-}$) tratados com metformina houve inibição de mTORC1 (Kalender et al. 2010).

Em modelo tumoral de camundongos com mutação heterozigota em *TSC2* (*TsC2* $^{+/)}$ não foi verificada redução no tamanho tumoral quando comparado ao uso da rapamicina, sugerindo benefícios terapêuticos limitados do uso da metformina em harmatomas (Auricchio et al. 2012). Por outro lado, em tumores renais de modelo murino *TsC2* $^{+/-}$ a metformina foi capaz de reduzir a sinalização mTOR apenas nos tecidos normais e não nos tumores dos animais (Dowling et al. 2016). Em células hepáticas desse mesmo modelo a metformina inibiu a sinalização de mTOR através de um mecanismo dose-dependente envolvendo a via AMPK e o complexo TSC (Howell et al. 2017). Alguns estudos apontam para o

potencial da metformina em controlar a inibição de mTOR e de atuar na prevenção, evitando a recorrência dos tumores associados a ET. As vantagens dessa potencial intervenção, além de ter potencialmente poucos efeitos colaterais, é o custo que é bem inferior ao de outros inibidores de mTOR (Amin et al. 2019).

1.4 Fluxograma geral

Na Figura 9 está representado graficamente um fluxograma geral com a temática desta tese de doutorado e os capítulos que a compõem a tese.

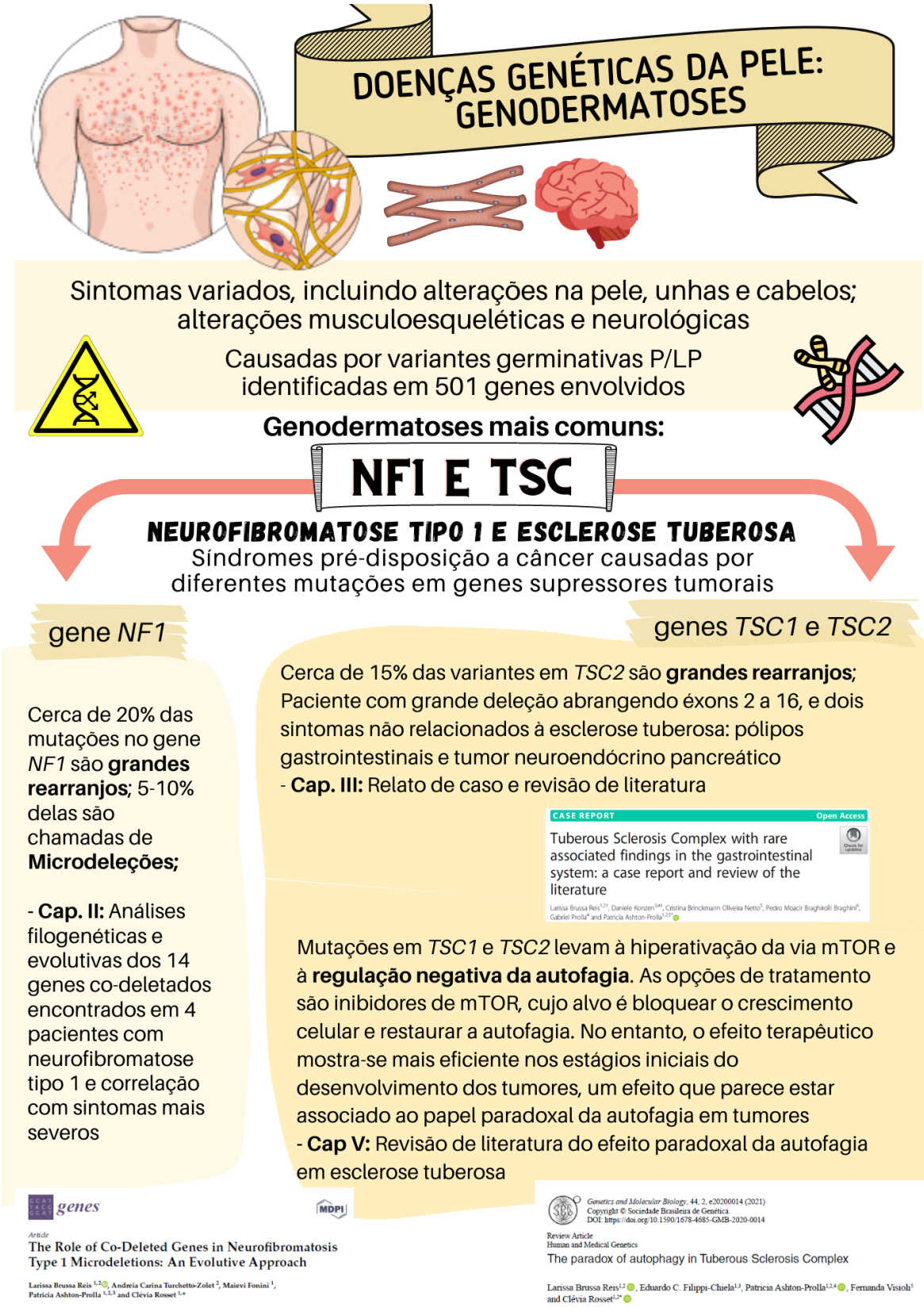

Figura 9: Esquema geral dos assuntos abordados na Tese. Figura original.

2. JUSTIFICATIVA

O ambulatório de Oncogenética do Hospital de Clínicas de Porto Alegre iniciou as atividades em 2006. Em apenas dois anos, o número de atendimentos de pacientes com diversas síndromes de predisposição ao câncer já havia aumentado consideravelmente. Em 2019, mais de 300 famílias com genodermatoses foram atendidas no ambulatório. Esse número vem aumentando a cada ano, o que incentivou o grupo de Oncogenética a iniciar as pesquisas nesta área, visando melhorar o atendimento e acompanhamento desses pacientes.

As genodermatoses podem ter importante repercussão na qualidade de vida dos pacientes e de suas famílias, especialmente nos casos que tem fenótipos mais graves. A exclusão social, a deficiência os múltiplos sintomas que podem estar associados a comorbidades secundárias e em alguns casos redução da expectativa de vida são alguns dos fatores que tornam os pacientes mais gravemente acometidos especialmente vulneráveis. Além disso, apenas tratamentos sintomáticos estão disponíveis para a maioria dos pacientes e o atendimento clínico pode ser complexo, envolvendo além da genética médica diversas especialidades médicas, como dermatologia, neurologia, pediatria, nefrologia e urologia, oftalmologia, oncologia, cirurgia, cardiologia, radiologia além das áreas de apoio psicologia e serviço social. Nesse sentido, existem ainda lacunas importantes no nosso entendimento acerca de questões relacionadas com o mecanismo de doença, predição de risco de morbidades associadas e manejo/tratamento farmacológico das genodermatoses.

A NF1, com manifestações e gravidade que variam em cada paciente, podem tornar o diagnóstico e o acompanhamento clínico dos pacientes afetados um desafio para as várias especialidades envolvidas. Nossa compreensão é particularmente incompleta sobre correlações genótipo-fenótipo e capacidade de predição de evolução clínica nos pacientes com alterações complexas, como os rearranjos gênicos. Estas alterações podem ocorrer em até 5% a 10% dos casos e como poucas informações estão disponíveis sobre a sua repercussão, em

especial sobre o impacto de genes co-deletados nas microdeleções, estudos de caracterização *in silico* podem trazer informações adicional sobre a importância funcional destes genes, facilitando a escolha de genes alvo para estudos posteriores e a identificação de correlações genótipo-fenótipo. Assim, mais informações acerca do papel dos genes co-deletados podem contribuir para predição das morbidades associadas, talvez ajudando a identificar grupos de pacientes de maior risco que se beneficiariam com programas de prevenção e acompanhamento clínico, por exemplo, programas de detecção precoce de neoplasias.

Em relação à ET, embora uma parcela importante se estabeleça claramente a partir de variantes P/LP germinativas em genes claramente associados, uma parcela significativa de pacientes apresenta variantes de significado incerto (do inglês *Variant Uncertanly Significance* – VUS) ou até mesmo nenhuma variante detectável em *TSC1/TSC2*. O mesmo ocorre em pacientes com o quadro clínico de NF1. Assim, torna-se necessário avaliar outros potenciais mecanismos de doença como alterações em sítios de processamento de RNA. Em termos de tratamento, as terapias utilizadas hoje para a doença são os inibidores da via de mTOR. Por fim, embora promissores, os tratamentos farmacológicos de pacientes com ET com rapamicina e seus análogos são de alto custo, podem apresentar efeitos colaterais importantes e podem não ser efetivos no controle e na erradicação dos tumores dos pacientes com ET. Ademais, no SUS a rapamicina é o único medicamento da classe hoje disponível. Tratamentos com compostos alternativos como metformina e soro de baixa glicose que são conhecidos como indutores de autofagia podem ser testados quanto à sua eficácia no controle das manifestações neoplásicas em pacientes com esclerose tuberosa.

A presente tese de Doutorado pretende contribuir para aprimorar o entendimento acerca de lacunas existentes em subgrupos clínicos específicos de NF1 e ET, além da patogênese de ET e da relação das alterações moleculares subjacentes com manifestações clínicas e potenciais estratégias terapêuticas.

3 OBJETIVOS

Caracterizar a patogênese da NF1 e ET utilizando abordagens evolutivas e moleculares.

3.2 Objetivos Específicos

3.2.1 Realizar análises filogenéticas *in silico* que auxiliem na compreensão do impacto de grandes rearranjos patogênicos envolvendo 14 genes co-deletados em pacientes com NF1 e microdeleções, bem como avaliar correlações genótipo-fenótipo específicas para o fenótipo mais grave desses pacientes;

3.2.2 Descrever um paciente com alteração no gene *TSC2* e polipose gastrointestinal e tumor neuroendócrino pancreático, alterações ainda não descritas em pacientes com esclerose tuberosa;

3.2.3 Avaliar possíveis alterações em sítios de processamento de RNA nos genes *TSC1* e *TSC2,* em pacientes com ET portadores de VUS ou sem variante identificada na sequência de DNA;

3.2.4 Revisar a participação dos genes *TSC1* e *TSC2* como reguladores da macro autofagia, o papel das rotas alternativas de autofagia, como privação de nutrientes e fatores de crescimento, e de compostos moduladores de autofagia em ET, além do papel paradoxal da autofagia na formação de tumores da síndrome;

3.2.6 Avaliar os níveis de macro autofagia e de fluxo autofágico em linhagens de fibroblastos de pacientes com ET, que apresentam mutação germinativa em heterozigose, comparando com linhagens controle, com e sem tratamento com potenciais agentes terapêuticos.

Capítulo II

Artigo publicado:

“*The Role of Co-Deleted Genes in Neurofibromatosis Type 1 Microdeletions: An Evolutive Approach”*

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Article

The Role of Co-Deleted Genes in Neurofibromatosis Type 1 Microdeletions: An Evolutive Approach

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Abstract: Neurofibromatosis type 1 (NF1) is a cancer predisposition syndrome that results from dominant loss-of-function mutations mainly in the *NF1* gene. Large rearrangements are present in 5–10% of affected patients, generally encompass *NF1* neighboring genes, and are correlated with a more severe NF1 phenotype. Evident genotype–phenotype correlations and the importance of the co-deleted genes are difficult to establish. In our study we employed an evolutionary approach to provide further insights into the understanding of the fundamental function of genes that are co-deleted in subjects with *NF1* microdeletions. Our goal was to access the ortholog and paralog relationship of these genes in primates and verify if purifying or positive selection are acting on these genes. Fourteen genes were analyzed in twelve mammalian species. Of these, four and ten genes showed positive selection and purifying selection, respectively. The protein, RNF135, showed three sites under positive selection at the RING finger domain, which may have been selected to increase efficiency in ubiquitination routes in primates. The phylogenetic analysis suggests distinct evolutionary constraint between the analyzed genes. With these analyses, we hope to help clarify the correlation of the co-deletion of these genes and the more severe phenotype of NF1.

Keywords: genotype–phenotype correlations; microdeletions; neurofibromatosis type 1; phylogenetic analysis

1. Introduction

Neurofibromatosis type 1 (NF1) (OMIM # 162200) is an autosomal dominant tumor predisposition syndrome affecting both sexes in all ethnic groups, with an estimated incidence of one per 3000 [1]. NF1 results from dominant loss-of-function (haploinsufficiency) mutations mainly in the *NF1* gene, but this is not the only mutational event that explains the phenotype. NF1 clinical diagnosis is based on criteria approved by the National Institutes of Health Consensus Development Conference [2]. The NF1 phenotype is extremely variable and one possible explanation for this variation is a large number of different mutations in different regions of the *NF1* gene [3]. Nearly 20% of *NF1* mutations are single or multiexon deletions or duplications; 5–10% of these *NF1* deletions are known as microdeletions, surrounding *NF1* and its bordering genes. Four types of microdeletions (type 1, 2, 3, and atypical) have been identified and are different in breakpoint location and size [4]. Patients with microdeletions have been described to have a higher risk of malignant peripheral nerve sheath

tumors, lower average intelligence, connective tissue dysplasia, skeletal malformations, dysmorphic facial features, cardiovascular malformations, and a higher burden of cutaneous neurofibromas and previous onset of benign neurofibromas [5–9]. Some authors suggest that increased malignancy may be elucidated by variations in the expression of tumor suppressor genes placed in co-deleted regions [10,11], as some of the genes in the deletion interval may have tumor suppressive functions. The analysis of conserved domains in genes in this cluster indicates that many of these genes are presumably involved in the regulation of cell growth and morphology [12]. NF1 clinical symptoms are variable even when comparing individuals with *NF1* microdeletions, probably due to the variability characteristic of all patients with NF1, regardless of the type of *NF1* gene mutation involved, but also due to changes in deletion size and breakpoint location [13]. Nonetheless, some variability in expression of the clinical symptoms has been detected even within the group of patients that are hemizygous for the same number of genes [14], which could be explained by the expression level of non-deleted genes, as well as by environmental factors. All these findings make the study of genotype–phenotype correlations and the determination of the importance of the co-deleted genes difficult. For this reason, a new approach to investigate the importance of these genes has been proposed by this study, using evolutionary and phylogenetic analyses.

Evolutionary analyses take into account the natural selection, which includes purifying or negative selection and positive selection or neutrality. Most of the genes are affected by purifying selection that decreases the frequency of mutations that disadvantage carriers in a given environment [15,16]. Interspecies neutrality tests use data concerning the divergence between closely related species to detect relatively ancient selective events. Interspecies tests include the non-synonymous and synonymous (dN/dS) test that detects selection acting on protein-coding loci by comparing the ratio of non-synonymous (dN) to synonymous (dS) substitutions [17]. Phylogenetic analyses are important because they enrich our understanding of how genes, genomes, and species evolve. The evolutionary history of living species is usually inferred through the phylogenetic analysis of molecular and morphological information using assorted mathematical models.

In the present study we employed an evolutionary approach to provide further insights into the understanding of the fundamental function of genes that are co-deleted in patients with NF1 microdeletions. Our main goal was to access the ortholog and paralog relationship of these genes in primates and verify if purifying or positive selection are acting on these genes. With these analyses, we hope to help to clarify the correlation of the co-deletion of these genes and the more severe phenotype of NF1.

2. Methodology

2.1. Background

In a previous study conducted by our group ninety-three unrelated NF1 probands who met the NIH diagnostic criteria were recruited at the Oncogenetics clinics of the Hospital de Clínicas de Porto Alegre, located in the state of Rio Grande do Sul, southern Brazil. The study was approved by the Institutional Ethics Committee Board of Hospital de Clínicas de Porto Alegre, under registration number 13-0260, Clinical evaluation and molecular characterization were enrolled in these patients. We found large gene rearrangements in four patients (6%) by multiplex ligation-dependent probe amplification (MLPA) analysis followed by confirmation using chromosomal microarray [18]. One patient presented the classical 1.4 Mb type 1 microdeletion, spanning from *SUZ12P1* to *LRRC37B*, including 22 genes (16 functional). Two patients showed a similar deletion, but their breakpoints differed slightly (one within *CRLF3* intron 1 and *SUZ12* intron 5, including 19 genes and spanning 1.15 Mb and the other within *CRLF3* intron 4 and the *SUZ12* gene, including 18 genes and spanning 1.13 Mb). Finally, one patient showed an atypical microdeletion, with breakpoints between *SUZ12P1* and *LRRC37BP*, spanning 890 kb and 15 genes. The fourteen genes deleted in at least one of the four patients were

selected for the phylogenetic and selection analyses in this study: *CRLF3, ATAD5, TEFM, ADAP2, RNF135, NF1, OMG, EVI2B, EVI2A, RAB11FIP4, COPRS, UTP6, SUZ12*, and *LRRC37B*.

2.2. Genes and Organisms Selected for Analysis

The following fourteen genes that are frequently co-deleted in NF1 patients with microdeletions were included in the analyses: cytokine receptor like factor 3 (*CRLF3*), ATPase family AAA domain containing 5 (*ATAD5*), transcription elongation factor, mitochondrial (*TEFM*), ArfGAP with dual PH domains 2 (*ADAP2*), ring finger protein 135 (*RNF135*), neurofibromin 1 (*NF1*), oligodendrocyte myelin glycoprotein (*OMG*), ecotropic viral integration site 2B (*EVI2B*), ecotropic viral integration site 2A (*EVI2A*), RAB11 family interacting protein 4 (*RAB11FIP4*), coordinator of PRMT5 and differentiation stimulator (*COPRS*), UTP6 small subunit processome component (*UTP6*), SUZ12 polycomb repressive complex 2 subunit (*SUZ12*), and leucine rich repeat containing 37B (*LRRC37B*). As the focus of evolutionary analysis was to perform evolutionary analysis of the deleted genes in primates, our ingroup included eight primate species (*Homo sapiens, Pan troglodytes, Pongo abelii, Gorilla gorilla gorilla, Nomascus leucogenys, Macaca mulatta, Callithrix Jacchus*, and *Tarsius syrichta*). The *Canis lupus familiaris, Bos taurus, Mus musculus*, and *Rattus norvegicus* were used as outgroup. All the included species have their complete genome sequence available in online databases.

2.3. Sequence Alignment and Phylogenetic Analyses

In order to obtain gene sequences in the organisms described above, BlastX was performed for each gene from each organism in the Ensembl Databases (EMBL-EBI and Wellcome Trust Sanger Institute) and the National Center for Biotechnology Information (NCBI) using human sequences encoding the 14 genes as queries. The human genes are located in the frequently deleted region of chromosome 17 and were obtained from the Metazome database (Joint Genome Institute and Center for Integrative Genomics) and confirmed in NCBI. Full-length coding sequencing (CDS) and protein sequences were aligned using MUSCLE [19] implemented in MEGA 7.0 [20] with default parameters. The multiple alignments were manually inspected and edited when necessary in the final analysis. The phylogenetic relationships were reconstructed following nucleotide and protein sequence alignments using a Bayesian method carried out in BEAST v1.8.4 [21]. JModelTest 2.1 [22] and ProTest 2.4 [23] were used to select the best model of nucleotides and protein evolution. The birth-death process was selected as a tree prior to Bayesian analysis, and it was run for 10,000,000 generations with Markov chain Monte Carlo (MCMC) algorithms for both amino acid and nucleotide sequences. Tracer v1.6 [24] was used to verify the convergence of the Markov chains and the adequate effective sample sizes (>200) and the normal distribution curve. Trees off the curve were burned by tree annotator with a posterior probability limit of 0.005. Trees were visualized and edited using FigTree v1.4.3 [25].

2.4. Selection Analysis

Using the alignments of each gene and the respective phylogenetic trees as inputs, the rates of nonsynonymous to synonymous substitutions (dN/dS or ω), homogeneity, and positive selection could be determined using maximum-likelihood models in the program CodeML in PAML v4.9 [26]. First, we used the models allowing ω to vary among sites (site models). The most effective models were: the basic model (M0), which estimates uniform ω ratio among all sites; the site models including M1 (nearly neutral), M2 (selection), M3 (discrete), M7 (beta distribution, $\omega > 1$ disallowed) and M8 (beta distribution, $\omega > 1$ allowed). The likelihood-ratio test (LRT) test is obtained by calculating twice the log-likelihood difference between the alternative and null models ($2\Delta L$). The LRT was performed between the following pairs of the models: M0 vs. M3; M1 vs. M2, and M7 vs. M8. These LRT statistics approximately follow a chi-square distribution, and the number of degrees of freedom is equal to the number of additional parameters in the more complex model [27]. A significantly higher likelihood of the alternative model compared to the null model suggests positive selection. CodeML was also used to estimate different values of ω among the sites and branches according to the branch site model

comparing the alternative model (model = 2, Nsites = 2, fix_omega = 0, and omega = 0) with its null model (model = 2, Nsites = 2, fix_omega = 1, and omega = 1) [28]. The model assumes that the branches in the phylogeny are divided in the foreground (the one of interest for which positive selection is expected) and background (those not expected to exhibit positive selection). We set CodeML to estimate branch lengths by using random starting points (fix_blenght = −1). All models were run using the F3x4 option for expected codon frequencies based on third codon positions. Finally, the naive empirical Bayes (NEB) and the Bayes empirical bayes (BEB) approaches were used to calculate the posterior probability (PP) of each site belonging to the site class of positive selection within each alternative model.

3. Results

3.1. Phylogenetic Relationships of the Co-Deleted Genes

The clinical features of all previously analyzed patients are shown in Table S1 and described in full in Rosset et al., 2018 [18]. The deletions found in the patients are shown in Figure S1. One patient showed a typical type one deletion and the other three patients showed a typical *NF1* microdeletions.

In order to investigate the presence of the missing genes in the microdeletion region of NF1 patients in other organisms, the fourteen human genes *CRLF3, ATAD5, TEFM, ADAP2, RNF135, NF1, OMG, EVI2B, EVI2A, RAB11FIP4, COPRS, UTP6, SUZ12*, and *LRRC37B* were used as query in BLAST searches against the genomes of seven primates and another four mammalian species. The results showed that three genes were found deleted in primate species, such as *ATAD5* in *Pongo abelii, RNF135* in *Nomascus leucogenys*, and *LRRC37B* in *Callithrix Jacchus*. Figure 1 shows the deleted genes in humans and in the comparative species.

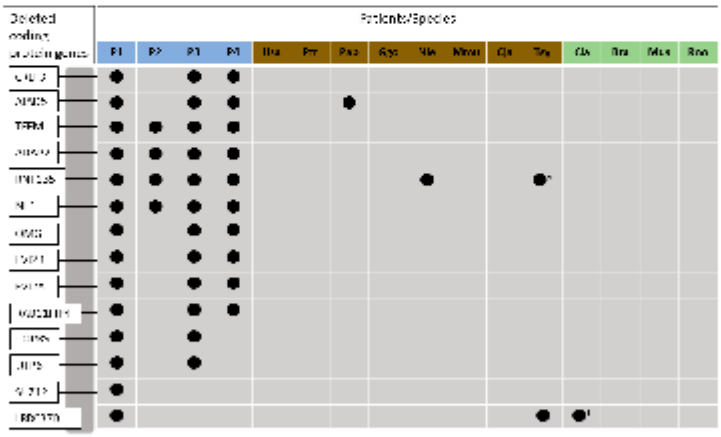

Figure 1. Deleted genes analyzed in this study and in comparative species used for the phylogenetic analysis. Blue squares represent patients with *NF1* microdeletions: patient 1, patient 2, patient 3, and patient 4, respectively. Brown squares represent ingroup species: *Homo sapiens, Pan troglodytes, Pongo abelii, Gorilla gorila gorila, Nomascus leucogenys, Macaca mulatta, Callithri Jacchus e Tarsius syrichata*, respectively. Green squares represent outgroup species: *Canis lupus familiaris, Bos taurus, Mus musculus e Rattus norvegicus*, respectively. Deleted genes in each species are represented with dark circles. Asterisk indicates that the *Tarsius syrichata RNF135* gene, and *Canis lupus LRRC37B* are absent because their sequences are mostly incomplete.

The search for deleted gene sequences in the databases NCBI and Esembl reveals that all genes are located in the same chromosomal unity in humans. The other species show some different locations,

such as *Nomascus leucogenys* that has only *CRLF3* and *ADAP2* on chromosome 14 and other genes on chromosome 19, and *Mus musculus* that has all genes on chromosome 10 with the exception of *COPRS* that is located on chromosome 16. The chromosomal locations of all genes in the analyzed species are shown in Table S2. The best models of nucleotides and protein alignments obtained by JModelTest 2.1 and ProTest 2.4 are shown in Table S3. Phylogenetic analysis was performed for each gene of the ingroup and outgroup species. In general, trees with nucleotide and amino acid sequences showed similar topologies. However, the order of organisms was not always the same and the posterior probabilities were higher in the most phylogenies reconstructed based on nucleotide sequences (Figures S2–S13). Only *RNF135* and *LRRC37B* genes showed better values in the trees imputed with their amino acid sequences (Figures S14 and S15). Additionally, these two genes showed considerable variation among their sequences in the species studied, (red cells from Table S3). The results concerning *LRRC37B* trees are difficult to interpret since this gene has several pseudogenes in different species and it is not possible to infer if all these sequences are functional. *LRRC37B*, *UTP6*, and *EVI2B* trees grouped *Homo sapiens* and *Pan troglodytes*, and *Mus musculus* and *Rattus norvegicus*, however, they did not group *Canis lupus familiaris* and *Bos Taurus*. *SUZ12* trees did not group *Homo sapiens* and *Pan troglodytes*, instead the tree grouped *Homo sapiens* and *Gorilla gorilla gorilla*. *COPRS* trees grouped *Homo sapiens* and *Pan troglodytes* and did not group *Canis lupus familiaris* and *Bos taurus* and *Mus musculus* and *Rattus norvegicus*. Nucleotide and amino acid trees generated with *ATAD5*, *ADAP2*, *NF1*, *OMG*, *EVI2A*, and *TEFM* sequences showed all clades in correct positions. Only *ATAD5* gene was deleted in *Pongo abeli*.

CRLF3 is probably a negative regulator of cell cycle [12] and was conserved in all analyzed species. However, the trees do not show *Homo sapiens* and *Pan troglodytes* with a common ancestor. Nucleotide sequence tree grouped *Homo sapiens* and *Gorilla gorilla gorilla*, whereas amino acid tree grouped *Homo sapiens* and *Pongo abelii*. Nucleotide tree showed better posterior values but did not group *Bos taurus* and *Canis lupus familiaris*. Finally, *RNF135* nucleotide and amino acid trees did not group *Homo sapiens* and *Pan troglodytes*; both grouped *Bos taurus* and *Canis lupus familiaris* and *Mus musculus* and *Rattus norvegicus*. This gene was not found in *Nomascus leucogenys* and was incomplete in *Tarsius syrichta*. Moreover, the *RNF135* gene could act as a transcription factor, due to the presence of zinc finger domains and the lower conservation found outside the sequence corresponding to protein domains, a common feature in transcription factors.

3.2. Selection Analysis of the Co-Deleted Genes

All genes presented a statistically significant value for M3 × M0 analysis, indicating ω heterogeneity among all sites (Table 1). *RNF135*, *UTP6*, *LRRC37B*, and *EVI2B* showed statistically significant values for the comparison between the M8 × M7 models ($p < 0.005$, $p < 0.025$, $p < 0.001$, and $p < 0.025$, respectively), indicating positive selection. In addition, *LRRC37B* was the only gene that also presented a statistically significant difference in the comparison of M2 × M1 models (Table 1). The naive empirical Bayes (NEB) and Bayes empirical bayes (BEB) analyses demonstrated which sites are under selection (Tables S4–S7), however, presented statistically significant values only for sites in the *RNF135* gene. Twenty-seven amino acid sites showed positive selection, with statistical significance for the NEB model (21 sites with l-value >0.005 and 6 sites with *p*-value >0.001) (Table S4). Figure 2 shows the positively selected sites and positions, including sites located in the RING finger domain of *RNF135*.

Table 1. CodeML analysis of the entire dataset using the site models.

Gene Family	Model/Likelihood	Comparison	Parameters/Significance
CRLF3	M0/-3789,882728 M1/-3774,195335 M2/-3777,450354 M3/-3774,195335 M7/-3774,904179 M8/-3774,50421	M3 vs. M0 M2 vs. M1 M8 vs. M7	$2\Delta L = 31.37$ (df = 4)/$p << 0.001$ * $2\Delta L = -6.51$(df = 2)/$p < 0.99$ $2\Delta L = 0.79$(df = 2)/$p < 0.5$
ATAD5	M0/-22006,87329 M1/-21865,9053 M2/-21865,9053 M3/-21856,31708 M7/-21859,57933 M8/-21857,4487	M3 vs. M0 M2 vs. M1 M8 vs. M7	$2\Delta L = 301.11$ (df = 4)/$p < 0.001$ * $2\Delta L = 0$ (df = 2)/$p < 0.99$ $2\Delta L = 4.26$ (df = 2)/$p < 0.5$
ADAP2	M0/-4320,93576 M1/-4283,476747 M2/-4283,476747 M3/-4278,962315 M7/-4279,346509 M8/-4279,258106	M3 vs. M0 M2 vs. M1 M8 vs. M7	$2\Delta L = 83.94$ (df = 4)/$p < 0.001$ * $2\Delta L = 0$ (df = 2)/$p < 0.99$ $2\Delta L = 0.176$ (df = 2)/$p < 0.99$
RNF135	M0/-5802,616454 M1/-5745,76494 M2/-5744,363479 M3/-5744,351541 M7/-5750,260024 M8/-5744,430178	M3 vs. M0 M2 vs. M1 M8 vs. M7	$2\Delta L = 116.5$ (df = 4)/$p < 0.001$ * $2\Delta L = 2.80$ (df = 2)/$p < 0.1$ $2\Delta L = 11.65$ (df = 2)/$p < 0.005$ *
NF1	M0/-20275,12645 M1/-20240,46408 M2/-20240,46408 M3/-20238,58367 M7/-20239,50865 M8/-20238,71939	M3 vs. M0 M2 vs. M1 M8 vs. M7 M3 vs. M0	$2\Delta L = 73.08$ (df = 4)/$p < 0.001$ * $2\Delta L = -4 \times 10^{-6}$ (df = 2)/$p < 0.99$ $2\Delta L = 1.57$ (df = 2)/$p < 0.99$
UTP6	M0/-6507,150936 M1/-6433,008949 M2/-6433,008949 M3/-6429,850049 M7/-6434,194703 M8/-6429,931052	M3 vs. M0 M2 vs. M1 M8 vs. M7	$2\Delta L = 154.60$ (df = 4)/$p < 0.001$ * $2\Delta L = 0$ (df = 2)/$p < 0.99$ $2\Delta L = 8.52$ (df = 2)/$p < 0.025$ *
SUZ12	M0/-4810,097946 M1/-4803,610839 M2/-4803,610839 M3/-4802,77363 M7/-4802,776934 M8/-4802,777078	M3 vs. M0 M2 vs. M1 M8 vs. M7	$2\Delta L = 14.64$ (df = 4)/$p < 0.005$ * $2\Delta L = 0$ (df = 2)/$p < 0.99$ $2\Delta L = -0.000288$ (df = 2)/$p < 0.99$
OMG	M0/-3421,827547 M1/-3412,43271 1 M2/-3393,13422 M3/-3412,246309 M7/-3412,265355 M8/-3412,256186	M3 vs. M0 M2 vs. M1 M8 vs. M7	$2\Delta L = 20.99$ (df = 4)/$p < 0.001$ * $2\Delta L = 0$ (df = 2)/$p < 0.99$ $2\Delta L = 0.018$ (df = 2)/$p < 0.99$
LRRC37B	M0/-16071,4708 M1/-16018,91532 M2/-16013,97878 M3/-16011,07676 M7/-16018,02362 M8/-16011,36949	M3 vs. M0 M2 vs. M1 M8 vs. M7	$2\Delta L = 120.7$ (df = 4)/$p < 0.001$ * $2\Delta L = 9.87$ (df = 2)/$p < 0.005$ * $2\Delta L = 13.30$ (df = 2)/$p < 0.001$ *

Table 1. *Cont.*

Gene Family	Model/Likelihood	Comparison	Parameters/Significance
EVI2A	M0/-2792,694611 M1/-2771,253237 M2/-2770,590286 M3/-2770,464944 M7/-2772,654596 M8/-2770,510367	M3 vs. M0 M2 vs. M1 M8 vs. M7	2ΔL = 44.45 (df = 4)/p < 0.001 * 2ΔL = 1.32 (df = 2)/p < 0.99 2ΔL = 4.28 (df = 2)/p < 0.1
EVI2B	M0/-5998,097719 M1/-5964,792227 M2/-5963,862998 M3/-5962,167576 M7/-5965,886294 M8/-5962,759212	M3 vs. M0 M2 vs. M1 M8 vs. M7	2ΔL = 71.86 (df = 4)/p < 0.001 * 2ΔL = 1.85 (df = 2)/p < 0.1 2ΔL = 6.25 (df = 2)/p < 0.025 *
RAB11FIP4	M0/-8519,576811 M1/-8500,408316 M2/-8500,408316 M3/-8412,192643 M7/-8412,081211 M8/-8410,442254	M3 vs. M0 M2 vs. M1 M8 vs. M7	2ΔL = 214.76 (df = 4)/p < 0.001 * 2ΔL = 0 (df = 2)/p < 0.99 2ΔL = 3.27 (df = 2)/p < 0.1
TEFM	M0/-4523,914053 M1/-4479,041925 M2/-4477,690271 M3/-4476,141837 M7/-4478,690601 M8/-4476,734719	M3 vs. M0 M2 vs. M1 M8 vs. M7	2ΔL = 95.54 (df = 4)/p < 0.001 * 2ΔL = 2.70 (df = 2)/p < 0.1 2ΔL = 3.91 (df = 2)/p < 0.1
CORPS	M0/-2021,525059 M1/-2001,718453 M2/-2001,718453 M3/-2001,71364 M7/-2001,856893 M8/-2001,724449	M3 vs. M0 M2 vs. M1 M8 vs. M7	2ΔL = 39.62 (df = 4)/p < 0.001 * 2ΔL = 0 (df = 2/p < 0.99 2ΔL = 0.26 (df = 2)/p < 0.5

* Statistical significant result obtained by Fisher chi-square.

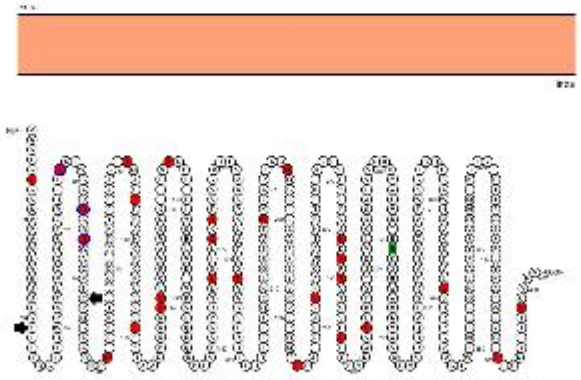

Figure 2. Sequence of human protein RNF135 and their positive selected sites. Representation of the structure of the cytoplasmic protein RNF135 and its 432 amino acids. The orange rectangle represents the cell membrane. The small circles represent the amino acids of the protein, with the names indicated by the letters contained within the circles. The beginning and end of the sequence corresponding to the RING finger domain are indicated by the first and second black arrows respectively. The circles painted in red represent the 27 amino acids that are under positive selection. The green square represents the *N*-glycosilated motif.

The *CRLF3*, *ATAD5*, *ADAP2*, *NF1*, *SUZ12*, *OMG*, *EVI2A*, *RAB11FIP4*, *TEFM*, and *CORPS* genes did not present statistically significant values for the comparisons of M2 × M1 and M8 × M7 models, indicating that they may be under purifying selection (Table 1).

4. Discussion

Tumor occurrence in NF1 follows the two hit mechanism proposed by Knudson in 1971 for retinoblastoma. The first hit corresponds to the germ line mutation (haploinsufficiency) that is enough to cause the initial NF1 symptoms. The second hit is somatic and stimulates tumorigenesis in specific tissues involved in NF1 disease [29]. Only a few genotype–phenotype correlations have been established to date in NF1 [30–32]. One of the most evident is associated with *NF1* microdeletions, independent of the second hit acquisition. The architecture of the genomic regions flanking the *NF1* gene in 17q11.2 in humans is characterized by low copy repeats (LCRs). LCRs predisposes to large deletions mediated by mutational mechanisms occurring in the germline of an unaffected parent or during mitotic postzygotic cell divisions [33]. Initially, the breakpoints of large rearrangements were characterized by microdeletion junction-specific PCR assay of 54 NF1 patients. The recombinant event occurred in 46% of cases in a recombination hotspot region of the flanking NF1REPs, also causing three intervals of recombinant events [34]. Later, four types of microdeletions (type 1, 2, 3, and atypical) were identified; the most frequent type of microdeletion is type 1 *NF1* deletion which encompass 1.4 Mb and include 14 protein-coding genes as well as four microRNA genes (Figure S1) [35–37]. The non-allelic homologous recombination (NAHR) events causing type 1 *NF1* deletions are mediated by the low copy repeats, NF1-REPa, and NF1-REPc [36]. Type 2 deletions encompass only 1.2 Mb and are associated with hemizygosity for 13 protein-coding genes. They are also mediated by NAHR but their breakpoints are located within *SUZ12* and its highly homologous pseudogene *SUZ12P1* which flank NF1-REPc and NF1-REPa, respectively (Figure S1) [37]. Type 3 *NF1* deletions are very rare; these 1.0 Mb deletions occur in only 1–4% of all patients with gross *NF1* deletions and are mediated by NAHR between NF1-REPb and NF1-REPc leading to hemizygosity for a total of nine protein-coding genes (Figure S1). Atypical large *NF1* deletions do not exhibit recurrent breakpoints and are variable considering the number of genes located within the deleted region [38].

In our previously studied cohort [18], one of the NF1 probands showed the well described *NF1* type 1 microdeletion. Although the other three patients showed *NF1* deletion breakpoints similar to type 2 and type 3 microdeletions, they were considered atypical (Table S2 and Figure S1). 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* codifies a protein that contains a RING finger domain at the N terminus and a B30.2/SPRY domain at the C terminus. RING domains are specialized zinc-finger motifs that can have ubiquitin and sumo ligase activity [39]. *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 [39]. The dysmorphic facial character as seen in patients with *NF1* microdeletions is generally missing in patients with intragenic *NF1* mutations. One of the patients from our previous study (Table S1) had an *NF1* deletion including NF1-REPa to NF1-REPb and *RNF135* and had several dysmorphic features as well as tall stature. However, since only the study conducted by Douglas et al. (2007) [39] analyzed patients with intragenic *RNF135* mutations but lacking *NF1* microdeletions, more studies are necessary to assess the role of *RNF135* co-deletion in NF1. In our study, a different approach was applied to try to elucidate the importance of this gene in *NF1* microdeletions. The phylogenetic analysis revealed the evolutionary relationships of each gene among the primate species analyzed. These results showed that some genes are not in agreement with the species tree, suggesting distinct evolutionary constraint between them, which could be expected for genes that present divergence of function during species evolution and are under distinct selection pressure. The distinct statistical values for clades on the phylogenies considering nucleotide and protein sequences could be explained by the differences in nucleotide and

protein variation. These differences could be explained by the high synonymous substitution found for some genes. The phylogenetic trees were used to estimate the dN/dS value for molecular evolutionary rate analysis and revealed a distinct selection pattern for each gene. Most genes are under purifying or negative selection, but some present some positively selected sites.

Our results indicate that there is heterogeneity of ω between the sites of all analyzed genes in the different species. This indicates that, in the same position, the amino acids vary among the different groups investigated. Four genes present evidence of being under positive selection (*RNF135*, *UTP6*, *LRRC37B*, and *EVI2B*), which possibly increased their adaptive values. Of these, the only gene that presented statistically significant differences for its sites in the NEB model was *RNF135* (Table S4). The protein RNF135 showed twenty-seven sites under positive selection, and three of them are located in the RING finger domain of the protein. The second positively selected residue is a glutamic acid, with a negative net charge in humans and other primates (*Macaca mulatta*, *Callithrix Jacchus*, *Pongo abelii*, and *Pan troglodytes*). In organisms that are phylogenetically more distant to humans (e.g., *Mus musculus*), this site has a positively charged amino acid. The third residue is an aspartic acid, a negative amino acid in the human protein. In the primates *Pan troglodytes*, *Pongo abelii*, and *Macaca mulatta*, the neutral amino acid glicine was selected; in *Mus musculus* the amino acid in this position is an arginine. Finally, the fourth selected residue is an arginine in humans and other main primates; in *Mus musculus*, a valine, a polar hidrofobic amino acid, is in this position. These differences in amino acids and their charges could have been selected in humans and other primates to allow better tridimensional conformation of the RNF135 protein and, consequently, a higher efficiency in its ubiquitination function. Most of the ring finger proteins contain two zinc atoms grouped with cistein or cistein-histidin rich clusters. The consensus sequence of this domain is C – X2 – C – X9–39 – C – X1–3 – C – X2 – C – X4–48 – C – X2 – C [40]. The *RNF135* gene has this consensus region, represented in Figure 2, which encompass the second, third, and fourth sites under positive selection (shown in red in Figure 2). Genes under this type of selection have important functions and these data support that *RNF135* haploinsufficiency contributes to human disease. Other genes showed positive selection in our study (*UTP6*, *LRRC37B*, and *EVI2B*), but without a statistically significant difference in the NEB model (Tables S5–S7).

The other ten genes showed evidence of being under purifying selection, which is related to the importance of maintaining their function during the evolution of organisms. They are fairly conserved genes that are present in all organisms investigated, with the exception of the *ATAD5* gene in *Pongo abelii*. These genes in the co-deleted region may also contribute to the phenotype in microdeleted patients. *SUZ12* (also known as *JJAZ1*) is critical in embryonic development [41]. There are evidences that biallelic *SUZ12* loss promotes malignant peripheral nerve sheath tumor (MPNST) progression in NF1 [42]. The SUZ12 protein is a component of the Polycomb repressive complex 2 (PRC2) and is involved in the epigenetic silencing of many different genes by establishing di- and tri-methylation of histone H3 lysine 27 [43]. Loss of histone H3 lysine 27 trimethylation was observed in 50–70% of MPNSTs. By contrast, in benign neurofibromas, the H3 lysine 27 trimethylation is retained, serving as a diagnostic marker for malignant transformation [44]. Another gene, *OMG*, which encodes the oligodendrocyte myelin glycoprotein is an important inhibitor of neurite overgrowth [45]. Haploinsufficiency of the *OMG* gene has been proposed to be associated with learning disability. Cardiovascular malformations observed in patients with *NF1* microdeletions could be related to hemizygosity of the *ADAP2* gene. This conclusion is drawn from the observation that *ADAP2* is highly expressed during early stages of heart development in both mice and humans [46]. Haploinsufficiency of *UTP6* seems to reduce cellular apoptosis, increasing the risk of tumor development [47]. *ATAD5* (ATPase family AAA domain-containing protein 5) is involved in the stabilization of stalled DNA replication forks by regulating proliferating cell nuclear antigen (PCNA) ubiquitination during DNA damage bypass, thereby promoting the exchange of a low-fidelity translesion polymerase back to a high-fidelity replication polymerase [48]. Embryonic fibroblasts from more than 90% of haploinsufficient Atad5+/m mice developed tumors such as sarcomas, carcinomas, and adenocarcinomas that exhibited high levels of genomic instability [49].

5. Conclusions

In our study, we found that four of the frequently co-deleted genes in NF1 patients with microdeletions present evidence of being under positive selection, including specific amino acid sites of RNF135 protein in its main functional domain (RING finger), reinforcing its importance and contribution to human disease. Moreover, the other ten co-deleted genes showed evidence of being under purifying selection, which is related to the importance of maintaining their function during the evolution of organisms. Our in silico analysis could help clarify the function and contribution of the co-deleted genes to disease and, in the future, co-deleted genes could possibly be included in the *NF1* molecular diagnostic workup.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4425/10/11/839/s1. Figure S1: Representation of existing microdeletions in patients with neurofibromatosis (type 1, 2 and 3 deletions) and deletions found in the previously analyzed patients, Figure S2: Phylogenetic tree of *CRLF3* gene reconstructed based on nucleotides sequences, Figure S3: Phylogenetic tree of *ATAD5* gene reconstructed based on nucleotides sequences, Figure S4: Phylogenetic tree of *TEFM* gene reconstructed based on nucleotides sequences, Figure S5: Phylogenetic tree of *ADAP2* gene reconstructed based on nucleotides sequences, Figure S6: Phylogenetic tree of *NF1* gene reconstructed based on nucleotides sequences, Figure S7: Phylogenetic tree of *OMG* gene reconstructed based on nucleotides sequences, Figure S8: Phylogenetic tree of *EVI2B* gene reconstructed based on nucleotides sequences, Figure S9: Phylogenetic tree of *EVI2A* gene reconstructed based on nucleotides sequences, Figure S10: Phylogenetic tree of *RAB11FIP4* gene reconstructed based on nucleotides sequences, Figure S11: Phylogenetic tree of *COPRS* gene reconstructed based on nucleotides sequences, Figure S12: Phylogenetic tree of *UTP6* gene reconstructed based on nucleotides sequences, Figure S13: Phylogenetic tree of *SUZ12* gene reconstructed based on nucleotides sequences, Figure S14: Phylogenetic tree of *RNF135* gene reconstructed based on amino acid sequences, Figure S15: Phylogenetic tree of *LRRC37B* gene reconstructed based on amino acid sequences; Table S1: Clinical characterization of the NF1 patients previously analyzed, Table S2: Information about the sequences obtained in the Ensembl and NCBI databases for all species included in this study, Table S3: The best model of nucleotides and protein alignment, Table S4: Positively selected sites in *RNF135* and amino acid differences between species, Table S5: Positively selected sites in *UTP6* and amino acid differences between species, Table S6: Positively selected sites in *LRRC37B* and amino acid differences between species.

Author Contributions: L.B.R. participated in the literature review, in silico analysis, manuscript design, conceptualization, and writing; A.C.T.-Z. participated in the in silico analysis and manuscript writing and revising for intellectual content; M.F. participated in the in silico analysis and methodology design; P.A.-P. participated in the manuscript writing and revising for intellectual content; C.R. participated on the literature review, manuscript design, conceptualization, writing and revising.

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Capítulo III

Relato de caso publicado:

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CASE REPORT Open Access

Tuberous Sclerosis Complex with rare associated findings in the gastrointestinal system: a case report and review of the literature

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Abstract

Background: Tuberous Sclerosis Complex (TSC) is a complex and heterogeneous genetic disease that has well-established clinical diagnostic criteria. These criteria do not include gastrointestinal tumors.

Case presentation: We report a 45-year-old patient with a clinical and molecular diagnosis of TSC and a family history of cancer, presenting two rare associated findings: gastrointestinal polyposis and pancreatic neuroendocrine tumor. This patient was subjected to a genetic test with 80 cancer predisposing genes. The genetic panel revealed the presence of a large pathogenic deletion in the *TSC2* gene, covering exons 2 to 16 and including the initiation codon. No changes were identified in the colorectal cancer and colorectal polyposis genes.

Discussion and conclusions: We describe a case of TSC that presented tumors of the gastro intestinal tract that are commonly unrelated to the disease. The patient described here emphasizes the importance of considering polyposis of the gastrointestinal tract and low grade neuroendocrine tumor as part of the TSC syndromic phenotype.

Keywords: Tuberous sclerosis complex, Adenomatous colonic, Rectal polyposis, Pancreatic neuroendocrine tumor, Case report

Background

Tuberous Sclerosis Complex (TSC) is a genetic disorder with multiorgan involvement, a broad phenotype with inter and intra-familiar variability and well-established clinical diagnostic criteria (Table 1) [1–4]. The incidence of TSC is approximately 1 in 6000–10,000 live births, and in Europe its prevalence has been estimated to be 8.8/100,000 [5]. Germline pathogenic variants in *TSC1* and *TSC2* are identified in 75–90% of patients with the clinical diagnosis and at least 60% of TSC patients do not have a family history of the disease and are considered sporadic [6].

In this report, we describe a patient with the clinical and molecular diagnosis of TSC presenting with two rare associated findings: gastrointestinal polyposis and a pancreatic neuroendocrine tumor. A review of the literature on the subject is provided.

Case presentation

The patient, a 45-year-old male, was referred for genetic assessment due to clinical findings suggestive of

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Table 1 Criteria for the clinical diagnosis of TSC [1]

Criteria	Description	Observed in the proband
Major	Facial angiofibroma	✓
	Ungueal/peri-ungueal fibroma	
	Hypomelanotic macules	
	Subependymal nodules	✓
	Cortical tubers	✓
	Subependymal giant cell astrocitoma (SEGA)	
	Multiple nodular retinal hamartomas	
	Cardiac rhabdomyoma	
	Renal angiomyolipoma	✓
	Lymphangiomyomatosis	
Minor	Multiple dental enamel macules	
	Rectal polyps	✓
	Osseous cysts	
	Abnormal migration tracts of the White matter	
	Gengival fibromas	
	Non-renal hamartomas	
	Multiple renal cysts	
	"Confetti" skin lesions	

Definitive TSC: Two major criteria or one major and two minor criteria; Probable TSC: One major and one minor criterion; Possible TSC: One major and two minor criteria

Tuberous Sclerosis Complex (TSC) and polyposis of the gastrointestinal tract. Past medical history included symptoms such as significant seizures since infancy, mild cognitive impairment and adult-onset psychiatric symptoms. These symptoms prompted investigation with a brain magnetic resonance imaging (MRI), which showed subependymal nodules and cortical tubers, two major diagnostic criteria of TSC. Physical examination revealed facial angiofibroma but no additional cutaneous abnormalities were observed. Ophthalmologic, cardiac e pulmonary evaluations did not reveal presence of retinal hamartomas, cardiac rhabdomyomas or pulmonary lymphangioleiomyomatosis. Abdominal computed tomography (CT) scans showed an expansive lesion with heterogeneous enhancement, located in the lower pole of the right kidney, measuring 5.5 cm × 4.0 cm which was later confirmed as a renal angiomyolipoma, another classical sign of TSC. Multiple nodular lesions with arterial enhancement were identified in the liver, the largest one measuring 7.0 × 5.0 cm with features suggestive of secondary implants of unknown origin. In addition, abdominal imaging also showed an expansive lesion in the pancreatic body, with heterogenous enhancement, involving the splenic artery and measuring approximately 6.0 × 4.0 cm. In addition, the patient also had a long history of diarrhea and underwent colonoscopy and upper gastrointestinal endoscopy, revealing presence of more than 50 gastric, colonic and rectal polypoid formations (2 mm to 5 mm).

Family history of cancer was significant for presence of 2 relatives with central nervous system tumors (father and brother diagnosed at ages 62 and 57 years, respectively). Eight additional cancer unaffected siblings were reported. There was also no report of any other family member with clinical features of Tuberous Sclerosis Complex or other genetic conditions. Considering the clinical features of TSC and polyposis of the digestive tract, germline genetic testing was proposed with a next generation sequencing panel validated for large rearrangement screening including 80 cancer predisposition genes in a commercial laboratory. Genes in the panel included: *ALK, APC, ATM, AXIN2, BAP1, BARD1, BLM, BMPR1A, BRCA1, BRCA2, BRIP1, CASR, CDC73, CDH1, CDK4, CDKN1B, CDKN1C, CDKN2A (p14ARF), CDKN2A (p16INK4a), CEBPA, CHEK2, DICER1, DIS3L2, EPCAM, FH, FLCN, GATA2, GPC3, GREM1, HRAS, KIT, MAX, MEN1, MET, MLH1, MSH2, MSH6, MUTYH, NBN, NF1, NF2, PALB2, PDGFRA, PHOX2B, PMS2, POLD1, POLE, POT1, PRKAR1A, PTCH1, PTEN, RAD50, RAD51C, RAD51D, RB1, RECQL4, RET, RUNX1, SDHAF2, SDHB, SDHC, SDHD, SMAD4, SMARCA4, SMARCB1, SMARCE1, STK11, SUFU, TERC, TERT, TMEM127, TP53, TSC1, TSC2, VHL, WRN, WT1* genes. The patient died due to complications of the disease a few months after genetic evaluation. Informed consent to publish this case report was obtained *post-mortem* from his spouse.

Regarding pathology of the tumors, the haematoxylin and eosin stain (HE) performed in lesion of the right kidney revealed round cell renal tumor with typical morphology (Fig. 1a). The liver lesions were biopsied, showing a histologic pattern suggestive of a low-grade neuroendocrine tumor (NET) (Fig. 1c and e). Biopsies of the pancreatic lesion diagnosed a low-grade neuroendocrine pancreatic tumor (PanNET). Based on the major phenotypic criteria identified in the patient, the clinical diagnosis of TSC with a rare manifestation (PanNET) was established. Partial polypectomies were performed resecting three polyps from the gastric body, two polyps from the right colon and four polyps from the rectum. Histologic examinations of the gastric and colonic/rectal polyps revealed fundic gland polyps and tubular adenomas with low-grade dysplasia, respectively (Fig. 2). Immunohistochemistry (IHC) was performed in the biopsy of the right kidney lesion and demonstrated positive expression of melanoma antigen (Melan A) (Fig. 1b), melanosomal glycoprotein gp100 antigen (HMB45) and smooth muscle actin antigen. The lesions in the liver were confirmed by IHC, showing positivity for multiple citokeratins antigens (40, 48, 50 e 50,6 kDa), chromogranin A antigen (CGA) (Fig. 1d), and synaptophysin (Sinapto) (Fig. 1f).

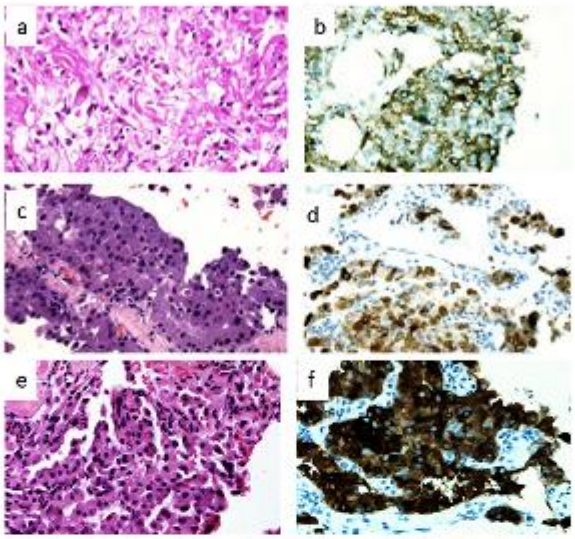

Fig. 1 Histologic and immunohistochemistry analyses of the renal and hepatic lesions identified in the proband. **a** and **b**: Kidney biopsy: round cell neoplasia of the kidney (renal angiomyolipoma). **a**) HE: haematoxylin and eosin stain, **b**) Melan A: melanoma antigen. **c**, **d**, **e** and **f**: Liver biopsy (low grade endocrine neoplasia). **c**) HE: haematoxylin and eosin stain, **d**) CGA: chromogranin A antigen, **e**) HE: haematoxylin and eosin stain, **f**) Sinapto: synaptophysin (HE and IHC, 200x)

Germline genetic testing revealed presence of a large pathogenic deletion in *TSC2* gene encompassing exons 2 to 16 and including the initiation codon. No alterations in colorectal cancer/colorectal polyposis genes (*APC, AXIN2, BMPR1A, CDH1, CHEK2, EPCAM, GREM1, MLH1, MSH2, MSH3, MSH6, MUTYH, NTHL1, PMS2, POLD1, POLE, POLE, PTEN, SMAD4, STK11, TP53*) were identified.

Discussion and conclusions

TSC is an autosomal dominant disease associated with cancer predisposition and multisystemic involvement mainly due to hyperactivation of the mTOR pathway, secondary to loss of function mutations in *TSC1* and *TSC2* [7]. Approximately 15% of the pathogenic variants identified in *TSC2* and 8% of those identified in *TSC1* are large gene rearrangements (LGR) [8], and therefore, genotyping using a methodology that allows LGR detection is important in a diagnostic workup. Although criteria for clinical diagnosis of TSC are well established, expressivity is highly variable, even within families with multiple carriers of the same pathogenic variant and simplex cases with de novo mutations are not

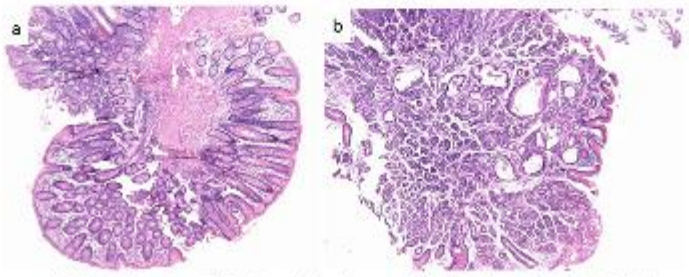

Fig. 2 Tubular adenoma and Fundic gland polyp (HE). Panel a: tubular adenomas. Panel b: fundic gland polyps (HE, 200x)

uncommon reaching up to 86% in some cohorts [9]. The recent, increased access to multigene panel testing to investigate suspected hereditary cancer has resulted in molecular diagnosis of individuals without the classic clinical criteria or apparently "sporadic" tumors or isolated clinical features of the disease.

In this report, we describe a patient fulfilling criteria for the clinical diagnosis of TSC, such as cortical tubers, facial angiofibroma and renal angiomyolipoma (Table 1 and Fig. 1a and b) carrying a previously described large *TSC2* rearrangement with two uncommon clinical manifestations of the disease: gastrointestinal adenomatous polyposis and a metastatic pancreatic neuroendocrine tumor. The occurrence of numerous colonic and rectal polyps, characterized in this patient as tubular adenomas, is a symptom associated with gastrointestinal polyposis and colorectal cancer syndromes, such as Familial adenomatous polyposis (FAP), a rare autosomal, dominant hereditary disease [10]. FAP is caused by a germline mutation in the *APC* gene [11]. Besides FAP, other syndromes could be associated, including mismatch repair deficiency (biallelic *MLH1*, *MSH2*, *MSH6*, *PMS2* gene mutations), polymerase proofreading-associated polyposis (*POLD1*, *POLE* genes), juvenile polyposis (*SMAD4*, *BMPR1A* genes) and *MUTYH*-associated polyposis [12]. Sequence changes and exonic deletions/duplications were evaluated in all of these associated genes and negative results exclude these syndromes in this patient.

The heterozygous *TSC2* exon 2–16 deletion identified is also known as deletion of exons 1–15 in the literature. Truncating variants including gross deletions in *TSC2* are known to be pathogenic. The 5′ end of the deletion remained undetermined as it was beyond the assayed region and the 3′ boundary was probably within intron 16 of the *TSC2* gene. This deletion is expected to result in complete removal of the TSC1 binding domain (T1BD) the N-terminus of the TSC2 protein in one of the alleles. This domain is critical for TSC1-TSC2 interaction (formation of TSC complex) and abnormal or absent TSC complex results in TSC2 ubiquitination and degradation. This in turn eliminates inhibition of the conversion of Rheb-GTP which accumulates and directly activates the mTORC1 pathway [13, 14].

Three previous reports describe TSC patients carrying the same germline *TSC2* exon 2–16 (a.k.a. exon 1–15) deletion [15–17]. However, according to the information available, none of them presented the uncommon clinical features reported here (neuroendocrine tumors or gastrointestinal polyps) (Tables 2 and 3); although it is possible that due to their ages, these phenotypes would not yet be identifiable. Interestingly, Mortaji et al., 2018 described an adult TSC patient who presented both, a pancreatic NET and gastrointestinal (GI) polyps. But different from the case presented here, they were hamartomatous/inflammatory polyps [40]. Although rectal polyps are included as a minor clinical diagnostic criterion for TSC, there is no mention to polyps in other portions of the GI tract and the vast majority of polyps described in TSC patients are hamartomatous [19, 43]. Gastric fundic polyps (FGPs) are considered hamartomas and tuberin protein (codified by *TSC2* gene) seems to play an important role in pathogenesis of sporadic FGPs by deregulation of cell proliferation. The altered cellular localization of tuberin interrupts its interaction with hamartin protein (codified by *TSC1*) preventing the formation of TSC complex that regulates mTORC1 pathway, responsible for cell proliferation and protein synthesis signaling pathways. In addition, altered cellular localization of tuberin may preclude its negative regulation of gene transcription mediated by tuberin-associated proteins glucocorticoid receptor (GCR) [44]. We identified only one case report of an adolescent TSC patient with tubular adenomatous polyps of the GI tract. The report by Digoy et al. (2000), and the case reported here, presented with a high number of GI tract polyps (unlikely somatic in origin) and a negative comprehensive evaluation of known polyposis genes, reinforce that GI polyposis with different histologies is likely part of the TSC phenotype and

Table 2 Previous reports of GI tract polyposis in TSC patients

Reference	Age	TSC features	GI tract alterations	Mutant gene[a]
[18]	17 yo female	Mental retardation, brain astrocytoma, facial angiofibroma, hypomelanotic macules, renal angiomyolipoma	Rectal adenocarcinoma and multiple (> 50) tubular adenomas	NA
[19]	42 yo female	Seizures, renal and liver angiofibromas, multiple subependymal calcifications of the brain, lymphangioleiomyomatosis of the lungs, cerebromalacia	Multiple gastric (fundic) hamartomas	NA
[20]	51 yo female	Epilepsy, mild cognitive impairment, ungueal fibromas.	More than 50 sessile polyps of small size scattered through the left colon and rectum	*TSC1* c.1257delC (p.Arg420Glyfs*20)

[a]NA Not assessed

Table 3 Previous reports of neuroendocrine tumors in individuals with a clinical or clinical and molecular diagnosis of TSC

Reference	Summary	Mutant gene[a]
	Pituitary NET	
[21]	Case report: 12 yo male with a GH-oma and acromegalic gigantismo.	NA
[22]	Case report: 25 yo female with hyperprolactinaemia, amenorrhoea and galactorrhoea after delivery of 3rd child.	NA
[23]	Case report: 32 yo male with an ACTH-oma and Cushingoid features.	NA
[24]	Case report: 13.5 yo male with an ACTH-oma, short stature, abnormal distribution of fat tissue and rounded face, plethora and acne.	NA
	Parathyroid NET	
[25]	Case report: 20 yo female with parathyroid hyperplasia, and on autopsy multiple endocrine adenomatosis affecting, in addition to the parathyroid, the pituitary (a non-functioning pituitary adenoma), adrenals and pancreas (islet cell tumour).	NA
[26]	Case report: 14 yo female with a parathyroid adenoma, anorexia, occasional nausea and vomiting, polydipsia, polyuria, constipation and generalized osteoporosis	NA
[27]	Case report: 15 yo male with a parathyroid adenoma and acute pancreatitis	NA
	Rectal NET	
[28]	Case report: 18 yo female with Proteus syndrome and TSC, subcortical tubers, developmental delay, seizure disorder, bilateral renal angiomyolipomas, ventricular rhabdomyomas, choledochal cyst, epidermal inclusion cysts, skin tags, synchronous well-differentiated L-cell rectal neuroendocrine tumor and leiomyomatosis-like lymphangioleiomyomatosis of the rectum.	*TSC2*
	Pancreatic NET	
[29]	Case report: 24 yo female with insulinoma and symptomatic hypoglycaemia and novel onset of seizures	NA
[30]	Case report: 23 yo male with insulinoma and recurrent seizures presented after 15 years of being seizure free	NA
[31]	Case report: 34 yo male with a pancreatic gastrinoma, presenting with reflux esophagitis and massive weight loss	NA
[32]	Case report: 28 yo male with insulinoma and behavioral changes characterised by episodes of agitation and, at other times, lethargy	NA
[33]	Case report: 18 yo female with insulinoma with symptomatic hypoglycaemia.	NA
[34]	Case report: 12 yo male with a malignant islet cell tumour	*TSC2* (nonsense)
[35]	Case report: 43 yo male with insulinoma and episodes of Episodes of sweating and dizziness.	NA
[36]	Case report: 6 yo male with a malignant islet cell tumour of pancreas	*TSC2* (nonsense)
[37]	Case report: 39 yo male with a pancreatic islet cell tumor and lichenified hyperpigmented plagues (paraneoplastic process)	*TSC2* (1 bp ins)
[38]	Case report: 31 yo male with TSC, multiple congenital subependymal nodules, bilateral cortical tubers, seizures and a malignant (metastatic) pancreatic neuroendocrine tumor.	NA
[39]	Description of 5 patients with TSC (clinical diagnosis) and pancreatic tumors, 2 of them confirmed pancreatic neuroendocrine tumors, localized in the pancreatic tail (5 yo male with a 26 mm lesion and 12 yo male with a 10 mm lesion).	NA
[40]	Case report: 35 yo female with TSC, adenoma sebaceum, shagreen patch and hypopigmented macules, bilateral renal angiomyolipomas and Hurthle cell adenoma. Multiple benign hamartomatous and inflammatory-type polyps in the cecum, sigmoid colon, and rectum. Pancreatic well-differentiated neuroendocrine tumor.	*TSC1* (2 bp del)
	Pheochromocytoma	
[41]	Case report: 29 yo female with a pleomorphic adrenal pheochromocytoma, recurrent fever and abdominal pain. Abdominal recurrence involving the spinal cord	NA
	Carcinoid tumor	
[42]	Case report: 34 yo female with renal cysts and a bronchial carcinoid presenting by hemoptysis 2 years after diagnosis of "sporadic" lymphangiomyomatosis (LAM). On post-mortem examination LAM was observed in the lungs, mediastinal lymph nodes, kidneys and uterus. LOH for the *TSC1* mutation observed in several tissues but not in the carcinoid tumor.	*TSC1* (nonsense)

[a]NA Not assessed

should be considered in the differential diagnosis [18]. Of note, glandular fundic polyps and tubular adenomatous polyps could be two different expressions of the same germline variation.

Finally, pancreatic neuroendocrine tumors (PanNET) are most commonly sporadic but have been reported previously in association with TSC and in other inherited cancer syndromes such as von Hippel-Lindau

disease, Neurofibromatosis type 1 and Multiple endocrine neoplasia type 1 [45]. Most TSC patients diagnosed with NETs have pancreatic NETs, but NETs in other organs must be considered as part of the TSC phenotype. Recent studies have shown that most TSC patients with Pancreatic NETs have a germline pathogenic variant in *TSC2* gene, as observed in our case. The multiple reports of NET in TSC patients and recent evidence for a pivotal role of TSC1 and TSC2 proteins in NET development and tumor's response to mTORC1 modulating interventions, point to a direct relationship between loss of function variants in *TSC1* and *TSC2* and NET suggesting that TSC clinical criteria should be modified to include NETs [46–48]. To our knowledge, there are no previous reports of tubular adenomatous polyposis in multiple segments of the GI tract in carriers of *TSC2* germline pathogenic variants (Table 3). In a previous report describing molecular features of TSC patients, none of the probands reported GI tract polyposis [49].

In conclusion, there is currently no recommendation for GI polyp or PanNET screening, probably given the rarity of these findings, in TSC patients. Gastric and colorectal polyps and PanNETs are also not considered as phenotypic criteria for the clinical diagnosis of the syndrome. The patient described here, with confirmed molecular diagnosis of TSC underscores the importance of considering GI tract polyposis and NETs as part of the syndromic phenotype.

Abbreviations

TSC: Tuberous Sclerosis Complex; MRI: Magnetic resonance Imaging; CT: Computed tomography; HE: Hematoxylin and eosin; NET: Neuroendocrine tumor; PanNET: Neuroendocrine pancreatic tumor; IHC: Immunohistochemistry; Melan A: Melanoma antigen; HBM45: Melanosomal glycoprotein gp100 antigen; CGA: Chromogranin A antigen; Sinapto: Synaptophysin; LGR: Large gene rearrangements; FAP: Familial adenomatous polyposis; TBD: TSC1 binding domain; GI: Gastrointestinal; FGPs: Gastric fundic polyps; GCR: Glucocorticoid receptor

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Authors' contributions

L.B.R, D.K. and P.A-P collected data and wrote the manuscript. P.A-P, G.P., P.B and C.B.O.N treated the patient and interpreted the data. P.B. ordered diagnostic laparoscopy and colonoscopy and was the primary clinical oncologist of the case. G.P. was consulted for a second opinion and referred the patient for genetic evaluation. P.A-P was involved with conception of the report and acted as a supervisor. All the authors read and approved the final manuscript.

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Not applicable.

Consent for publication

The spouse of the patient described here, has provided written informed consent (for the) submission of this case report since the patient was already deceased. Consent to publish included the Images In Figure(s) 1a, 1b, 1c, 1d, 1e, 1f, 2a and 2b.

Competing interests

The authors declare that they have no conflict of interest or financial disclosure.

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Publisher's Note

Capítulo IV

Resultados parciais: artigo em preparação

Caracterização de possível *exon skipping* em pacientes com esclerose tuberosa com variantes de significado incerto ou sem mutação de DNA nos genes *TSC1* e *TSC2*

Caracterização de *exon skipping* em pacientes com esclerose tuberosa com variantes de significado incerto ou sem variantes patogênicas identificadas nos genes *TSC1* e *TSC2*

Introdução e justificativa

Esclerose Tuberosa (ET) é uma genodermatose causada por variantes patogênicas nos genes supressores de tumor *TSC1* (OMIM #605284, localização cromossômica 9q34.13 ou TSC2 (OMIM #191092, localização cromossômica 16p13.3) (Lendvay and Marshall 2003; Northrup et al. 2019b). Exibe um modo de herança autossômica dominante com uma incidência que varia de 1/6.000 a 1/10.000 nascimentos. Trata-se de uma desordem caracterizada pelo surgimento de harmatomas (tumores benignos de crescimento lento) multisistêmicos, que afetam comumente o cérebro, coração, rins, pulmões, retina e pele (Napolioni and Curatolo 2008; Henske et al. 2016). Outras características comuns incluem desordens envolvendo Sistema Nervoso Central e se manifestam através de sintomas como epilepsia, comprometimento intelectual e transtorno do espectro autista (Henske et al. 2016; Kingswood et al. 2017).

Northrup e colaboradores (2013) apresentaram uma classificação atualizada dos sintomas para o diagnóstico clínico da doença, incluindo características principais e secundárias. Devido a variabilidade dos sintomas, o mesmo grupo implementou o critério de diagnóstico genético, com a detecção de variante patogênica nos genes *TSC1* ou *TSC2* como suficiente para o diagnóstico molecular de ET (Northrup et al. 2013), seguindo os critérios *do American College of Medical Genetics and Genomics* (ACMG) para variantes patogênicas reportadas no DNA.

Cerca de 90% dos pacientes com critérios clínicos claros para ET apresentam alguma variante genética patogênica nos genes *TSC1* ou *TSC2* (Peron et al., 2018). Existem atualmente mais de 2000 variantes patogênicas em *TSC1/TSC2* descritas no *Leiden Open Variation Database* (LOVD www.lovd.nl/TSC1 e www.lovd.nl/TSC2) (Fokkema et al. 2011). Dessas variantes,

21–26% estão localizados em *TSC1* e 69–79% em *TSC2*. No entanto, em aproximadamente 5% a 25% dos casos com diagnóstico clínico bem definido, não é identificada nenhuma variante patogênica de DNA em *TSC1* ou *TSC2* através das técnicas moleculares convencionais, tais como sequenciamento direto de Sanger (SS), amplificação de sonda dependente de ligação multiplex (MLPA) e sequenciamento de nova geração (NGS) (Camposano et al. 2009; Nellist et al. 2015; Tyburczy et al. 2015).

Mosaicismo somático e variantes intrônicas funcionais nos genes envolvidos foram reportados recentemente em pacientes sem mutação identificada nas regiões codificantes (Nellist et al. 2015; Tyburczy et al. 2015). Um grande estudo com 325 indivíduos com diagnóstico definitivo de complexo de esclerose tuberosa, reunindo casos familiais e isolados identificou 4% dos pacientes com variantes de significado incerto e 29% sem mutação identificada (Au et al. 2007a). Já um estudo em uma amostra de 66 pacientes mexicanos com ET demonstrou variantes intrônicas em *TSC1* ou *TSC2* em 7% e 10% dos casos, respectivamente, e três pacientes sem mutação identificada nas regiões codificantes (Reyna-Fabián et al. 2020). Por fim, Nellist e colaboradores (2015), identificaram variantes de significado clínico incerto em 4 de 7 pacientes com ET. As variantes identificadas incluíram alterações em mosaico, alterações localizadas profundamente nas sequências intrônicas e alterações que afetam as regiões promotoras que não teriam sido identificadas usando análises baseadas apenas em regiões codificantes.

Os genes *TSC1* e *TSC2*, como a maioria dos genes eucarióticos contém sequências não codificantes em proteínas (íntrons) e regiões codificantes (éxons) que devem ser removidos e unidos, respectivamente, no processo de *Splicing* (processamento) de RNA mensageiro precursor (chamado pré-mRNA), que resulta no RNA mensageiro maduro, que posteriormente será traduzido nas proteínas correspondentes (Au et al. 2007b). A interrupção deste processo por variantes de sequência consenso nas junções éxon/íntron pode levar ao processamento aberrante de éxons (Carsillo et al. 2000; Kaufmann et al. 2002).

Splicing é o processo de maturação de um pré-RNA mensageiro (RNA precursor). Nesse processo, as regiões não codificantes (íntrons) são retiradas do pré-mRNA, que passa a conter somente as regiões codificantes(éxons). O *splicing* pode ocorrer durante e/ou após a transcrição do pré-mRNA, tornando-o RNA mensageiro maduro para ser traduzido. Esse processo está diretamente relacionado a diversidade proteica dos organismos. Por ser um processo complexo e com regulação fina, uma variante em um sítio de reconhecimento da junção éxon/íntron ou em um elemento regulador pode causar um erro no processo, gerando um produto aberrante, que pode, muitas vezes, inativar um gene, com graves consequências. Estima-se atualmente que erros no processo de *splicing* causem cerca de 10% das doenças genéticas (Chen and Manley 2009; Matera and Wang 2014).

Objetivo do estudo: Investigar o RNA mensageiro em indivíduos com critérios clínicos de ET e sem variantes patogênicas identificadas nos genes *TSC1* ou *TSC2* identificados por Rosset et al. 2017b.

Metodologia

Recrutamento

Foram selecionados sete pacientes com diagnóstico clínico de ET, três deles com diagnóstico molecular de VUS e quatro sem variante identificada por NGS e MPLA nos genes *TSC1* e *TSC2*. As características dos pacientes recrutados estão resumidas na Tabela 1.

Aspectos éticos

Este projeto foi aprovado pelo CEP HCPA sob o número 2015-0049. Foi aplicado o Termo de Consentimento Livre e Esclarecido a todos os pacientes convidados a participar do estudo.

Preparo das amostras

Extração de RNA total, obtido a partir da fração leucocitária de amostra de sangue dos pacientes, utilizando o kit *PureLink RNA mini kit* (Thermo Fisher

Scientific, USA), seguido de etapa de conversão para cDNA utilizando *GoScript™ Reverse Transcriptase cDNA Synthesis* (Promega) através da transcrição reversa.

Avaliação do cDNA

Para verificar se há alteração a nível de RNA, especialmente alterações afetando os sítios de processamento foram desenhados *primers* específicos para os éxons correspondentes, incluindo junções éxon-íntron. Os amplicons foram submetidos à amplificação por PCR e visualizados por eletroforese em gel de agarose, tendo seus tamanhos comparados a um marcador de peso molecular. Amostras sem alteração no éxon correspondente também foram incluídas na mesma eletroforese como controles. Nos casos com potenciais alterações de *splicing*, para confirmação, análise ortogonal por sequenciamento de Sanger será realizada.

Resultados parciais

Até o momento foram recrutados 7 pacientes e suas características clínicas e moleculares estão apresentadas na Tabela 1. Já foi obtido o consentimento para o estudo, a coleta de sangue, a extração de RNA, a quantificação e a conversão para cDNA de todas as amostras, que no momento se encontram congeladas a -80°C.

Adicionalmente, foram desenhados e obtidos pares de primers contemplando todos os éxons codificantes dos genes *TSC1* e *TSC2* em blocos, conforme a Tabelas 2. Encontram-se padronizadas as reações de amplificação de dezoito pares de *primers* (realizado utilizando cDNA comercial).

Tabela 1. Características clínicas e moleculares de pacientes ET com variantes de significado incerto ou sem variante patogênica identificada na região codificadora de *TSC1* e *TSC2.*

Caso familial ou isolado	Gênero	Sintomas	Genótipo	Gene	Posição	Classificação	MLPA *TSC1/TSC2*
1 Isolado	M	tubérculos corticais e máculas hipomelanóticas	WT/c.664-10 T>C	*TSC1*	íntron 7	VUS	Negativo
2 Isolado	M	máculas hipomelanocíticas, tubérculos corticais, angiomiolipoma renal, rabdomioma cardíaco múltiplo, fibroma gengival, convulsões	WT/c.2011G>T	*TSC2*	éxon 19	VUS	Deleção dos éxons 41, 42 em *TSC2* e do éxon 1 em *PKD1*
3 Familial	F	angiofibromas, angiomiolipoma renal, fibromas ungueais, cisto hepático, máculas hipomelanocíticas	WT/c.975+8G>A	*TSC2*	íntron 10	VUS	Negativo
4 Isolado	M	máculas hipomelanocíticas, angiomiolipoma renal	Não detectada por NGS e MLPA				Negativo
5 Desconhecido	M	tubérculos corticais, rabdomioma cardíaco único, spams, máculas hipomelanocíticas	Não detectada por NGS e MLPA				Negativo
6 Desconhecido	F	angiofibromas, angiomiolipoma renal, convulsões, limfangioleiomiomatose, catarata bilateral nódulos calcificados do parênquima cerebral bilateral, labirintite	Não detectada por NGS e MLPA; Variante adicional no gene *GATA*				Negativo
7 Desconhecido	M	máculas hipomelanocíticas, angiomiolipoma renal, convulsões	Não detectada por NGS e MLPA				Negativo

Tabela 2. Sequências de *primers* do gene *TSC1* e *TSC2*.

Gene *TSC1*

Região	Sequência de *Primers*	Tamanho de Fragmento (bp)
Bloco 1: éxons 1-3	F: GGGGAGGTGCTGTACG R: CCACGGTCAGAATTGAGG	341
Bloco 2: éxons 4-6	F: AGAGAACCTCAATTCTGACC R: ACGTGGCCTGGTTTCT	426
Bloco 3: éxons 7-10	F: GAAGAAACCAGGCCACG R: GCTCCAAAGAGTAGCTTGTG	547
Bloco 4: éxons 11-14	F: TGAACCACCACAAGCTACTC R: CTAGATATTGCAGCTTCTTCTTTAT	436
Bloco 5: éxons 15-16	F: AAAGATAAAGAAGAAGCTGCAAT R: AGCCTCCAAAGTGGGTC	622
Bloco 6: éxons 17-19	F: GACCCACTTTGGAGGCT R: TTGAGAGCTTTTGGGAAACC	482
Bloco 7: éxons 20-22	F: GTTTCCCAAAAGCTCTCAAAC R: CCGTCATTACAACAGTCAAGC	506

Gene *TSC2*

Região	Sequência de Primers	Tamanho do Fragmento (bp)
Bloco 1: éxons 1-3	F: GCCACGGTCAGAATTGAG R: AGAACCTCAATTCTGACCGT	344
Bloco 2: éxons 4-5	F: AGAACCTCAATTCTGACCGT R: GTCCATCTTGAGACATTTTAGT	278
Bloco 3: éxons 6-10	F: GAAGAGCTGGCTGACTTTG R: CATGGCCTGGTAAAATGATG	510
Bloco 4: éxons 11-15	F: CCATCATTTTACCAGGCCA R: ATCACCTTCTCGATGATGTC	644
Bloco 5: éxons 16-18	F: GACATCATCGAGAAGGTGAT R: CTCTCTCTGGCTCCATGT	376
Bloco 6: éxons 19-22	F: TACATGGAGCCAGAGAGAG R: GCCAGAGTGGACAGGAA	611
Bloco 7: éxons 23-27	F: TGGAGTTCCTGTCCACTC R: CAGGAGACCTCTTCGGG	609
Bloco 8: éxons 28-30	F: CCGAAGAGGTCTCCTGTG R: TTGATGTCCGAGGAGAAAGG	548
Bloco 9: éxons 31-33	F: GAGGCCCACAGGGAAC R: CTGGAGACTGAGGACGAC	423
Bloco 10: éxon 34	F: GGTCGTCCTCAGTCTCC R: GCAGGAACACGAAACTGG	504
Bloco 11: éxons 35 -39	F: AACCCCAGTTTCGTGTTC R: CTCCATGTCTTTCCTGCAC	591
Bloco 12: éxons 40 -41	F: CAGGAAAGACATGGAGGG R: GGCTTCCTCGCAGATCC	214

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Review Article
Human and Medical Genetics

The paradox of autophagy in Tuberous Sclerosis Complex

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Abstract

Tuberous sclerosis complex (TSC) is an autosomal dominant genetic disorder caused by germline mutations in *TSC1* or *TSC2* genes, which leads to the hyperactivation of the mTORC1 pathway, an important negative regulator of autophagy. This leads to the development of hamartomas in multiple organs. The variability in symptoms presents a challenge for the development of completely effective treatments for TSC. One option is the treatment with mTORC1 inhibitors, which are targeted to block cell growth and restore autophagy. However, the therapeutic effect of rapamycin seems to be more efficient in the early stages of hamartoma development, an effect that seems to be associated with the paradoxical role of autophagy in tumor establishment. Under normal conditions, autophagy is directly inhibited by mTORC1. In situations of bioenergetics stress, mTORC1 releases the Ulk1 complex and initiates the autophagy process. In this way, autophagy promotes the survival of established tumors by supplying metabolic precursors during nutrient deprivation; paradoxically, excessive autophagy has been associated with cell death in some situations. In spite of its paradoxical role, autophagy is an alternative therapeutic strategy that could be explored in TSC. This review compiles the findings related to autophagy and the new therapeutic strategies targeting this pathway in TSC.

Keywords: Autophagy, mTOR signaling, Tuberous Sclerosis Complex.

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Tuberous Sclerosis Complex: Epidemiology and manifestations

Tuberous sclerosis complex (TSC - ORPHA: 805) has an extremely variable disease profile that has the potential to affect any organ of the body and is characterized mainly by the development of hamartomas in the skin, brain, kidneys, lungs, and heart (Northrup and Krueger, 2013). Hamartomas are defined as benign proliferation of mature tissues that grow aberrantly and often with disorganized architecture, which may occur in any body site (Tjarks *et al.*, 2019). The incidence of TSC is approximately 1 in 6,000 live births, with prevalence in the population of 1 in 20,000 individuals, with no sexual or racial predilection (Sahin *et al.*, 2016). The clinical manifestations of TSC vary widely. The involvement of the skin and mucous membranes is marked, with alterations identified in approximately 70% of the cases (Henske *et al.*, 2016). The onset of symptoms may happen soon after birth and to four years of age. Congenital hypopigmented macules (HM) in the skin, the first visible symptom, usually precedes epilepsy. In addition to HM, there are other similar skin features, such as facial angiofibromas and subungual fibromas (Sahin *et al.*, 2016). Regarding the central nervous system (CNS), epilepsy is the most common symptom, affecting up to 90% of TSC patients, of whom two-thirds show symptoms before the second year of life. Cognitive deficit affects approximately 60 to 70% of patients and is often linked to seizures (Northrup and Krueger, 2013). The severity of seizures and other symptoms of the CNS depend on the presence, location, number and size of the brain hamartomas - called tubers. The three major intracranial lesions associated with TSC are cortical tubers, subependymal nodules and subependymal giant cell astrocytomas (SEGAs) (Shepherd *et al.*, 1991), which are found in 5 to 20% of patients. SEGAs constitute more than 90% of the intracranial tumors associated with the disease and are responsible for 25% of the mortality attributed to TSC (Nabbout *et al.*, 1999; Adriaensen *et al.*, 2009). Regarding other sites that may be affected in TSC, cardiac rhabdomyomas (benign tumors of the heart) are observed in up to 50% of patients and it can be detected in the fetus as early as 22 weeks gestation. Tumors may be single or multiple, may reach 3–25 mm, and are usually located in the cardiac ventricles

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along the septum. There is a strong association of germline *TSC1* or *TSC2* mutations and cardiac rhabdomyomas, with a mutation being identified in up to 93% of affected patients, in both patients with single and multiple tumors. Tumors may compromise ventricular functions, resulting in obstruction of blood flow and very rarely lead to arrhythmia, valvular defects, or cardiac failure. In most cases, however, rhabdomyomas are not hemodynamically relevant and do not increase in size, in fact, they tend to involute and disappear after the age of 3 (Tworetzky *et al.*, 2003, Altmann *et al.* 2019; Uysal and Sahin, 2020). Renal abnormalities are another important morbidity factor in TSC and are considered the second leading cause of premature death. More than 70% of TSC patients develop multiple and bilateral angiomyolipomas, which usually occur in the kidneys and reach large dimensions (Henske *et al.*, 2016). Although benign in nature these tumors can rupture and bleed and ultimately cause renal failure. Algorithms for the management of AML have been developed and treatment intervention is recommended for TSC-associated AML >3cm in diameter. Therefore, they should be followed closely for timely intervention. Renal cysts are also commonly identified, with and without angiomyolipomas and can result in hypertension or kidney failure (Hatano and Egawa, 2020; Uysal and Sahin, 2020). The main pulmonary manifestation of TSC is lymphangioleiomyomatosis (LAM), which is associated with the infiltration of smooth muscle cells in all lung structures (Adriaensen *et al.*, 2011). This manifestation may occur later, with patients presenting symptoms in the third to fourth decade of life. LAM-compatible cystic lung parenchymal abnormalities are observed in 30 to 40% of women and in 10 to 12% of men with TSC, but symptomatic changes are quite rare in men (Adriaensen *et al.*, 2011; Cudzilo *et al.*, 2013). Some reports consider that LAM is almost exclusively observed in adult women with TSC, suggesting that it is an estrogen-dependent phenotype, which has been actually demonstrated in animal studies. (Uysal and Sahin, 2020; Xu *et al.*, 2020). In a seminal clinical trial led by McCormack *et al.* (2011), which included 89 patients with LAM, Sirolimus stabilized lung function, reduced serum VEGF-D levels, and was associated with a reduction in symptoms and improvement in quality of life. This and other studies led to the FDA approval of Rapamycin (Sirolimus) for lymphangioleiomyomatosis treatment in 2015.

The criteria for clinical and genetic diagnoses of TSC are shown in Table S1 (based on Refs. Northrup and Krueger, 2013; Sahin *et al.*, 2016). At least 60% of TSC patients have no family history of the disease (Henske *et al.*, 2016).

Genetics and metabolic Pathways involved in Tuberous Sclerosis Complex

TSC is an autosomal dominant disease caused by mutations that inactivate one of the two tumor-suppressor genes *TSC1* (OMIM 605284) or *TSC2* (OMIM 191092). The *TSC1* gene, located on chromosome 9q34, spans approximately 53 kb of genomic DNA, with 23 exons coding for the protein hamartin, a hydrophilic protein of 1164 amino acids and 130 kDa that interacts and stabilizes the GTPase activating protein tuberin, which is encoded by the *TSC2* gene. The *TSC2* gene, located on chromosome 16p13.3, comprises approximately 40 kb of genomic DNA and has 41 exons that generate a protein of 1807 amino acids and 200 kDa, possibly acting as a chaperone for hamartin. These two proteins together, along with TBC1D7 (Tre2-Bub2-Cdc16-1 domain family member 7) (Dibble and Manning, 2013), form the hamartin-tuberin complex, also called the TSC1/TSC2 complex or TSC complex. This complex acts as a GTPase activating protein (GAP) to inhibit the Ras-related small GTPase protein RHEB (Ras homologue enriched in brain) (Menon *et al.*, 2014), which, in turn, regulates activation of the rapamycin target complex 1 in mammals (mTORC1) (Northrup *et al.*, 2020). TSC complex is also required for proper activation of a second complex, called mTORC2 (Huang *et al.*, 2008).

mTOR complexes

The mTORC1 complex is an important regulator of cell growth, proliferation and translation of mRNAs and is sensitive to growth factors, nutrients and the energy status of the cells. This complex is formed by different subunits. The major catalytic subunit is mTOR, a highly conserved protein kinase that regulates cell cycle progression in vertebrates (Hay and Sonenberg, 2004). mTOR is associated with other proteins, such as the regulator-associated protein of mammalian target of rapamycin called Raptor, mLST8 (mammalian lethal with sec-13 protein 8, also known as GβL), and the recently identified subunits PRAS40 (proline-rich Akt substrate of 40 kDa) and DEPTOR (DEP domain-containing mTOR-interacting protein). mTORC2 is structurally and functionally distinct from mTORC1. Although mTORC2 is conserved, as mTORC1, it has a distinct catalytic subunit, called Rictor. mTORC2 controls the actin cytoskeleton and it is rapamycin insensitive, whereas mTORC1 is rapamycin sensitive (Wullschleger *et al.*, 2006).

Response to growth factor, nutrients and energy status

mTORC1 responds to growth factors via the phosphatidylinositol-4,5-bisphosphate 3-kinase pathway (PI3K). In response to the presence of insulin, tuberin is phosphorylated and functionally inactivated by Protein kinase B (Akt). The phosphorylation impairs the ability of the TSC complex to exert its GTPase activity that converts Ras homolog enriched in brain (RHEB) GTP-binding to RHEB-GDP. The accumulation of RHEB-GTP potentially activates mTORC1, which phosphorylates and inhibits the 4E-BP1 substrate and activates the substrates ribosomal protein S6 kinase beta-1 (S6K1) and beta-2 (S6K2) (Wullschleger *et al.*, 2006), which promotes protein translation. In a negative feedback loop, mTORC1 and S6K1 directly phosphorylate insulin receptor 1 (IRS1) and block the signal transduction from insulin or insulin-like growth factor 1 (IGF-1) to PI3K (Huang and Manning, 2008) (Figure 1). Nutrients, especially amino acids, also regulate mTORC1 signaling. Nutrients inhibit the TSC complex by phosphorylation of S6K1 and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) (Gao *et al.*, 2002). Alternatively, the nutrients can regulate the TSC complex independently of mTORC1 by inducing RHEB stimulation, which causes the rapid dephosphorylation of the same targets (Saucedo *et al.*, 2003) (Figure 1). In

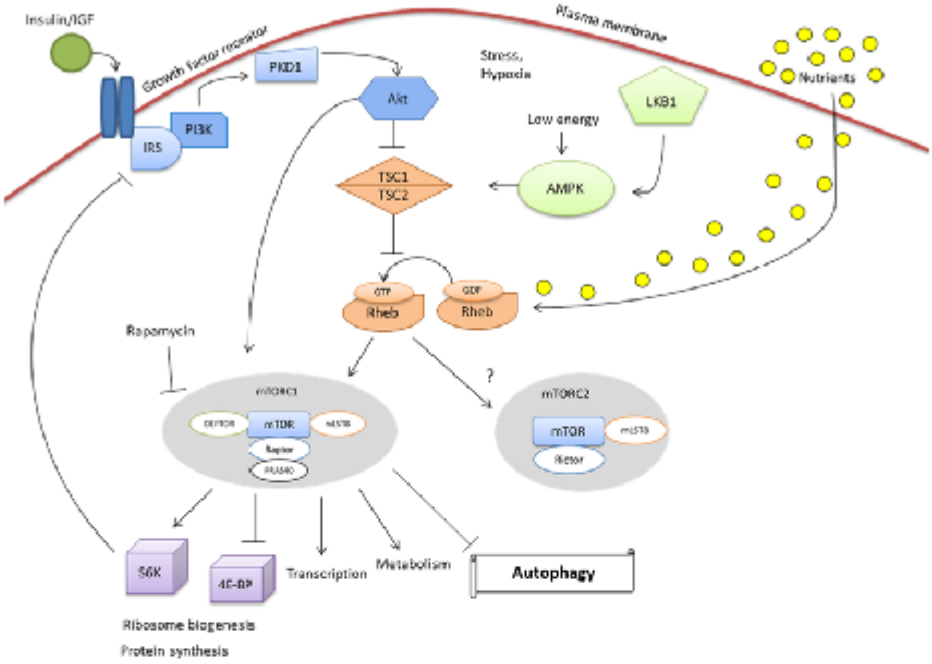

Figure 1 – Signaling of TSC and mTOR complexes. TSC complex negatively regulates mTOR signaling. In response to the presence of insulin, tuberin is phosphorylated and functionally inactivated by Akt, allowing mTORC1 to perform its functions. Nutrients regulate mTORC1 signaling by the inhibition of the TSC complex. mTORC1 complex is sensitive to the energetic status of the cell through AMPK, which is activated in response to low energy in the cell level of AMP/ATP. Activated AMPK leads to inhibition of mTORC1. It is not completely clear which factors act upstream of mTORC2. Growth factors and amino acids regulate actin polymerization, suggesting that they may also regulate this complex through TSC and Rheb. Once activated, mTORC2 increases the cascade of mTOR signaling.

relation to the high energy levels required for its activation, the mTORC1 complex is sensitive to the energy status of the cell as mediated through AMP-activated protein kinase (AMPK), which is activated in response to low energy at the cellular level of AMP/ATP. Activated AMPK is able to phosphorylate tuberin directly and thus increases its GTPase activity, leading to inhibition of mTORC1 (Inoki *et al.*, 2003). It has been suggested that the tumor suppressor LKB1 is linked to the TSC-mTORC1 signaling pathway. Upon energy deprivation and in conjunction with AMP, LKB1 activates AMPK, which in turn phosphorylates and activates TSC2, resulting in the inhibition of mTORC1 (Wullschleger *et al.*, 2006). The factors that act upstream of mTORC2 are not well known. Growth factors and amino acids regulate actin polymerization, suggesting that they may also regulate the mTORC2 complex through TSC and RHEB (Jacinto *et al.*, 2004). Once activated, mTORC2 phosphorylates the downstream target Akt to increase the mTOR signaling cascade (Xie and Proud, 2014) (Figure 1).

mTORC1 is a major negative regulator of autophagosome formation (Hung *et al.*, 2012). In yeast and mammals, the activation of the Atg1/ULK1 complex, formed by ULK1-ATG13-FIP200, is essential for the canonical formation of autophagosomes (Hosokawa *et al.*, 2009). The activity of this complex is negatively regulated by mTORC1. When nutrients are available, mTORC1 is activated and the ULK1 complex is inactivated through the phosphorylation of ULK1 and ATG13, thus suppressing autophagy; on the other hand, in this context, ribosomal biogenesis and protein synthesis are stimulated. Conversely, under nutrient limitations, mTORC1 is inactivated, which enables the ULK1 complex to be activated, which triggers the formation of autophagosomes. In parallel to autophagy induction, the inactivation of mTORC inhibits cell growth (Balgi *et al.*, 2009).

Therapies targeting mTOR complexes in TSC

Some therapies directed to the inhibition of mTOR complexes in the TSC context have been proposed. First-generation mTOR inhibitors consist of rapamycin (MacKeigan and Krueger, 2015). Rapamycin is an immunosuppressor that forms an inhibitory complex with the immunophilin FKBP12, which then binds and inhibits the ability of mTORC1 to phosphorylate downstream substrates such as S6Ks and 4EBPs. Since 2001 it has been approved for a large number of therapeutic uses in the United States and demonstrates beneficial results in reducing the volume of SEGAs (Franz *et*

al., 2006) and renal angiomyolipomas (Cabrera-López et al., 2012), reducing the number of facial angiofibromas (Koenig et al., 2018), and controlling epileptic seizures (Zou et al., 2014). Rapamycin analogs (also called rapalogs) are being increasingly used not only for hamartomatous and oncological manifestations of TSC but also as adjunct therapies for epilepsy and intellectual disability among others desabilities (Franz and Capal, 2017). More recently, second-generation mTOR inhibitors have been developed. In contrast to rapamycin and its analogs, these molecules do not target FKBP12 but inhibit both mTORC1 and mTORC2 directly by blocking their catalytic sites through competition with ATP. These new agents, called Everolimus and Sirolimus are potent inhibitors of cell proliferation and may have therapeutic benefits in TSC. Some studies indicate that inhibition of mTORC1 and mTORC2 together could be more appropriate than the use of rapalogs alone, since the dual approach could prevent activation of mTORC2 that may result from the inhibition of mTORC1 alone (Julien et al., 2010). Knowing that mTORC2 phosphorylates Akt to activate it and thus promotes cell survival through many downstream signaling targets, the loss of mTORC2-mediated Akt activation in cells without a functional TSC complex may effectively suppress apoptosis-inducing stimuli. For these reasons, many investigators prefer to consider both the aberrant activation of mTORC1 and the inactivation of mTORC2 when developing therapeutic strategies for TSC (Huang et al., 2008). Important clinical trials developed in recent years that have changed systemic treatments in TSC patients include EXIST-1 trial, completed in 2016 that demonstrated the efficacy and safety of Everolimus in SEGAs (Kingswood et al., 2014; Franz et al., 2016) and EXIST-2 trial that demonstrated the benefit of mTOR inhibitors for renal angiomyolipomas (AMLs) and resulted in Everolimus approval by the FDA for asymptomatic and growing renal AMLs larger than 3 cm (Bissler et al. 2013). Some years later, Bissler et al. (2017) treated 112 patients with Everolimus in an extension phase of the EXIST-2 study for an average of 46.9 months and observed that 58% achieved some AML response, the majority being reduction in renal lesion volumes with no AML-related bleeding or nephrectomies being reported. The most common adverse events suspected to be treatment-related were stomatitis, hypercholesterolemia, acne, aphthous stomatitis and nasopharyngitis but less than 10% of patients withdrew treatment due to an adverse event. In addition Bissler et al. (2018) reported that in a series of 33 patients with TSC treated with Everolimus for SEGAs, renal angiomyolipoma response was achieved by 75.8% of patients with sustained reductions in tumor volume over nearly 4 years of treatment, reaching ≥50% in most (≥80%) patients. Beneficial effects of Everolimus on autism and attention-deficit/hyperactivity disorder symptoms have been reported also (Kilincaslan et al., 2017). Regarding tests involving Sirolimus, MILES trial included 89 patients with LAM and reported stabilized lung function, reduced serum VEGF-D levels that were associated with a reduction in symptoms and improvement in the quality of life for TSC patients with LAM (McCormack et al. 2011). These results led to the FDA approval of Sirolimus in TSC-associated LAM in 2015. It is important to consider that both mTORC1 and mTORC2 inhibitors cause a variety of side effects that can lead to life-threatening outcomes, including sepsis and death. In addition, another point that requires further research relates to the long-lasting effects of these treatments, particularly in the context of the high incidence of lifetime clinical features and the adult- onset symptoms of TSC patients (Trelinska et al., 2015). For most TSC-related hamartomas, lifelong treatment will likely be mandatory, since several lesions may regrow and complications of these tumors may recur when medications are discontinued. One of the challenges in TSC treatment is that different clinical manifestations of the disease may require different therapeutic interventions. A key point is timing of mTOR inhibition for each symptom and what other pharmacologic and nonpharmacologic interventions could be used in combination. The main mTOR inhibitors tested in patients with TSC-related manifestations are summarized in Table S2.

Basics of autophagy

Autophagy is a physiological and well-regulated cellular mechanism that degrades dysfunctional or unnecessary factors, enabling the recycling of cellular components and the maintenance of energy and structural homeostasis. It occurs through the capture of cytoplasmic fractions and organelles by autophagosome, which are digested in lysosomes. The final products of lysosomal digestion are returned to the cytosol. They are then used in cellular metabolism to generate energy and to build new proteins, organelles and membrane components. When nutrients are restricted, autophagy is increased to ensure an internal source of nutrients for energy supply and, consequently, for cell survival. Therefore, it is a powerful mechanism to promote metabolic homeostasis at both the cellular and organism levels (Rabinowitz and White, 2010).

Autophagy pathways are mediated by autophagy-related proteins called ATGs and their associated enzymes. There are three commonly described forms of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). Macroautophagy is the major pathway and primarily eradicates damaged or malformed organelles or proteins. In general, it involves the detachment of a portion of the endoplasmic reticulum (ER) called the phagophore, which elongates to form a double membrane organelle known as an autophagosome, which captures the cellular components to be degraded. The expansion of the autophagosomal membranes involves the incorporation of cytosolic microtubule-associated protein 1 – light chain 3 (MAP1LC3 - hereafter called LC3 only) in the membrane of the growing autophagosome. LC3, the mammalian homolog of the yeast ATG8 gene, is diffused throughout the cytoplasm (LC3 forms LC3-I). LC3-I assumes the LC3-II form when it is added to phosphatidylethanolamine, which is incorporated into the autophagosome. The autophagosome moves along microtubules and fuses to a lysosome to form an autolysosome or autophagolysosome, where the cellular content is degraded with acidic lysosomal hydrolases (Jung et al., 2010). Lysosomal permeases return the products of digestion into the cytosol, such as amino acids,

lipids, nucleosides and carbohydrates, where they will be available for structural and metabolic pathways (Rabinowitz and White, 2010).

Autophagy and its relationship with mTOR and TSC

Rapamycin treatment or induced starvation in human cells and mouse embryonic cells (MEFs) results in the dephosphorylation of ULK1, restoring autophagy (Hung *et al.*, 2012). Furthermore, glucose starvation reduces ATP levels and activates AMPK, which is potentiated by LKB1, a protein kinase that phosphorylates AMPK. Activated AMPK inhibits mTORC1 and, as a consequence, positively regulates autophagy, thus making this pathway critical for monitoring cellular energy status and stress conditions. Moreover, autophagy can be induced by suppressing growth factor signaling pathways. The growth factor signaling in the IGF-1-PI3K-Akt pathway regulates mTORC1 to negatively regulate autophagy (Jung *et al.*, 2010).

Chaperone-mediated autophagy (CMA) selectively degrades cytosolic proteins in lysosomes and contributes to the maintenance of proteostasis and cellular adaptation to stress. CMA substrates are delivered by a cytosolic chaperone to the surface of the lysosome, where they are unfolded and internalized through a membrane translocation complex. In murine models, lysosomal mTORC2 and Akt regulate the activity of CMA (Arias *et al.*, 2015). Recently, the cochaperone BAG3 has been shown to coordinate protein synthesis and autophagy through the spatial regulation of mTORC1. BAG3 acts on the recruitment of the TSC complexes that inhibit the positive regulation of mTORC1 in the synthesis of cytoskeleton-associated actin fibers. In addition, when protein synthesis is necessary, BAG3 mediates the sequestration of the TSC complex, alleviating the inhibition of the mTORC1 that remains in the cytoplasm. In human muscle, an association of TSC1 with the exercise-induced cytoskeleton was described, indicating the coincidental activation of mTORC1 in the cytoplasm (Kathage *et al.*, 2017).

Given that mTORC1 is a key inhibitor of autophagy through direct phosphorylation of ULK1, studying the states of TSC deficiency may provide an opportunity to investigate the implications of autophagy dysregulation in human pathophysiology (Kim *et al.*, 2011). Through genetic and pharmacological inhibition of autophagy, it was possible to verify that TSC2-deficient tumor cells derived from LAM could be dependent on autophagy to survive. The induction of autophagy by mTOR inhibitors may enhance hamartoma cell adaptations to stress through a type of dormancy, in which proliferation is blocked due to inhibited mTORC1-mediated protein translation, leading to survival over time due to the activation of autophagy (Yu *et al.*, 2010). This mechanism is important when considering the role of autophagy in cancer. Defects in autophagy may contribute to cell transformation and the initiation of tumorigenesis by causing the accumulation of defective proteins and organelles, thereby increasing oxidative stress and genetic instability. Parkhito *et al.* showed that lowering autophagy blocked TSC tumorigenesis across genetic down-regulation of p62/sequestosome 1 (SQSTM1), the autophagy substrate that accumulates in TSC tumors as a consequence of low autophagy levels. This substrate strongly inhibited the growth of TSC2-null xenografted tumors, demonstrating that autophagy is a critical component of TSC tumorigenesis and suggesting that mTORC1 inhibitors may have autophagy-dependent prosurvival effects in TSC, and revealing two distinct therapeutic targets for TSC: autophagy and the autophagy target p62/SQSTM1. (Parkhitko *et al.*, 2011). More recently, p62 has also been associated with the maintenance of intracellular pools of glutamine, glutamate and glutathione, necessary to limit mitochondrial dysfunction in tumor cells with hyper activated mTORC1, being suggested as a possible therapeutic target in these tumors (Lam *et al.*, 2017).

In summary, there is a consensus that autophagy acts as a chemopreventive mechanism in normal cells to prevent their transformation. In contrast to cells in normal tissues, the tumor and surrounding environment are often chronically deprived of nutrients, growth factors, and oxygen as a result of abnormal vascularization. In this context, autophagy may support tumor growth and the adaptation of tumor cells to metabolic stress as a mechanism to provide nutritional support (Rabinowitz and White, 2010). Thus, depending on the disease status, autophagy can either be beneficial or detrimental. For instance, the activation of the ULK1 pathway by compounds that act in the AMPK pathway can counteract inhibition of mTORC1 in *Tsc2*-knockdown neurons in mice suggesting that autophagy inducers can have therapeutic potential to treat TSC-associated neuronal pathologies (Di Nardo *et al.*, 2014).

Autophagy in TSC and other conditions with mTOR deficiency

To better understand the role of autophagy in TSC, we searched the PubMed database on May 16, 2019, to retrieve original articles describing the role of autophagy in TSC that were published in English and available in the literature between 2008 and 2019. The following keywords were used: "autophagy" and "tuberous sclerosis complex", which resulted in the initial retrieval of 183 articles. We excluded the reviews, studies before 2008 and studies that did not relate to the scope of interest. Twenty-six articles were identified as studies related to the subject of this review and the studies are summarized in Table 1.

Autophagy seemed to play a role in various disease processes observed in TSC. For instance, insufficient autophagy in human melanocytes has been identified as responsible for hypopigmentation in specific sites in the skin, contributing to perhexiline the development of HM, which is one of the main clinical manifestations of TSC (Yang *et al.*, 2018). Furthermore, some neuronal characteristics of TSC have been described as associated with autophagy. The level of autophagic proteins was different in dysmorphic neurons than it was in balloon cells (the term used for the abnormal cells observed in cortical tubers) or normal neurons, reflecting different degrees of activation of mTOR pathways in TSC cells (Miyahara *et al.*, 2013). Overactived mTOR, together with impaired autophagy, may produce an excess of synaptic protein in neurons of patients with autism spectrum disorders (ASD, a common morbidity in TSC patients). This finding suggests that these two mechanisms may underlie the synaptic pathology of ASD (Tang *et al.*, 2014). Injured autophagy

Table 1 – Original articles describing the role of autophagy in TSC conditions

TSC Sample	Goal of the manuscript	Autophagy findings	Reference
[A]MEF *TsC2* knock-out and knock-in	Coumpounds perhexiline, niclosamide, amiodarone and rottlerin reversibly inhibit mTORC1 and stimulate autophagy	The chemicals activate autophagy in cells growing in nutrient rich conditions	Balgi *et al*., 2009
[A]MEF *TsC2* knock-out and knock-in, 621-101 and [B]ELT3 *TsC2*-deficient cells, and renal lesions in mice heterozygous to *TsC2*	Autophagy is a critical component of TSC tumorigenesis, and the authors suggesting that rapamycin may have autophagy-dependent prosurvival effects	The combination of mTORC1 and autophagy inhibition was more effective than these isolated treatments, both for suppression of spontaneous tumor cells growth and of xenografts.	Parkhitko *et al*., 2011
Cortical tubers removed from 7 patients and 5 controls of cortical tissue taken from non-TSC patients with epilepsy	Evaluation of induction of autophagy via mTOR that occurred in TSC-associated cortical tuber samples	Suppression of autophagy in cortical tubers presumably via the mTOR pathway	Miyahara *et al*., 2013
Brain tissues of conditional *TsC1* and phosphatase and tensin homolog knock-out mice	Autophagy was suppressed in samples investigated, which display seizures and aberrant mTOR activation; the conditional deletion of *Atg7* in mouse neurons is sufficient to promote of spontaneous seizures	The impaired autophagy contributes to epileptogenesis	McMahon *et al*., 2012
Primary cells from tuber samples of patients with TSC and frozen cells from a case of [C]FCD	Defects in autophagy in [C]FCD and in TSC share the altered mTOR pathway; this could be, in part, reversed *in vitro* by rapamycin	Abnormal activation of mTOR may contribute directly to a defect in autophagy in [C]FCD and TSC	Yasin *et al*., 2013
[D]FAO cells, MCF-7 cells expressing constitutively active AKT (myr-AKT) and peroxisome-deficient human Zellweger cells	TSC has a role in the response to [E]ROS in peroxisome and recognizes peroxisome as a signaling organelle involved in the regulation of mTORC1	TSC localized in peroxisomes functioned as a Rheb GTPase activator protein to suppress mTORC1 and induce autophagy	Zhang *et al*., 2013
Rat hippocampal neuronal cultures, [A]MEFs, [A]HEK293T cells, human TSC neurons collected from patients with intractable epilepsy	*Tsc2*-deficient neurons have increased autolysosome accumulation and autophagic flux despite mTORC1-dependent inhibition of ULK1; investigation of previously uncharacterized cellular mechanism that contributes to altered neuronal homeostasis in TSC disease	Loss of *Tsc2* gene in rat neurons results in autophagic activity via AMPK-dependent activation of ULK1; in *Tsc2*-knockdown neurons the AMPK activation is the dominant regulator of autophagy	Di Nardo *et al*., 2014
Transgenic mice with deletion of the *TsC1* gene by *CreLoxP*, breast tumor cells, isolated from mammary tumors created with the injection of these cells into nude mice	Creation of system that allows deletion of *TsC1* in tumor cells in an inducible manner; demonstration directly that deletion of *TsC1* and consequent activation of mTORC1 promoted tumor growth and metastasis, besides increased glucose starvation-induced autophagy and Akt activation	Glucose starvation-induced autophagy was increased significantly in *Tsc1*-null tumor cells, which could promote tumor cell survival and contribute to the increased tumor growth in vivo	Chen *et al*., 2014
MEFs *TsC2* knock-out and knock-in, *Tsc2*-null cystadenoma cell line, cells derived from mouse renal tumor	*TsC2*-null cells have distinctive autophagy-dependent [F]PPP alterations, enhanced glucose uptake and utilization, decreased mitochondrial oxygen consumption, and increased mitochondrial [E]ROS production; *TsC2*-deficient cells can be therapeutically targeted focusing on their metabolic vulnerabilities	[F]PPP is a key autophagy-dependent compensatory metabolic mechanism; [F]PPP inhibition with [G]6-AN in combination with autophagy inhibition suppressed proliferation and prompted the activation of [H]NF-κB and [I]CASP1 pathways in *TsC2*-deficient only	Parkhitko *et al*., 2014
Cell line stably expressing *TSC2* and [J]LAM *TSC2* knock-out cells were obtained from patient sample, wild and knockout for *TsC2* [A]MEFs	Rapamycin and resveratrol combination treatment blocked rapamycin-induced upregulation of autophagy and restored inhibition of Akt; this combination selectively promoted apoptosis of *TSC2*-deficient cells	Resveratrol caused inhibition of autophagy and targeting for apoptosis in *TSC2*-null cells	Alayev *et al*., 2015b

Table 1 – Cont.

TSC Sample	Goal of the manuscript	Autophagy findings	Reference
Dendritic spine and frozen samples from [L]ASD patients and controls, postmortem tissue of the temporal lobe from patients with [L]ASD, *Tsc2* heterozygous mice; *Tsc1* conditional knockout mouse line	mTOR-regulated autophagy is required for developmental spine pruning; and activation of neuronal autophagy corrects synaptic pathology and social behavior deficits in [L]ASD models with hyperactivated mTOR	The activation of neuronal autophagy corrects synaptic pathology and social behavior deficits in [L]ASD models with hyperactivated mTOR	Tang *et al.*, 2014
Transgenic knock-in and knock-out mice for the *Tsc1* and *Yap* genes by CreLoxP, cell line HEK293, MEFs knock-in and knock-out for *Tsc2*.	Regulation of [H]YAP by mTOR and the autophagy pathway like a novel mechanism of growth control; this molecular mechanism was required for the tumorigenesis of TSC-related kidney lesions and in [C]PEComas and the [H]YAP may serve as a potential therapeutic target for TSC and other diseases with dysregulated mTOR activity	The data favor a model in which the control of [H]YAP lysosomal degradation by mTOR matches [H]YAP activity with nutrient availability in growth permissive conditions	Liang *et al.*, 2014
[A]MEFs *Tsc1* and *Tsc2* knock-out and knock-in, [B]ELT3 cells, Angiomyolipoma-derived tuberin-deficient cells; HeLa cells, GFP-LC3-expressing WI38 fibroblasts.	Hamartin interacts with [F]PLK1; [F]PLK1 protein levels are increased in hamartin and tuberin-deficient cells and [I]LAM patient-derived specimens and that this increase is rapamycin-sensitive	PLK1 inhibition attenuated autophagy, and repressed the expression and protein levels of key autophagy genes and proteins and the protein levels of Bcl2 family members, suggesting that PLK1 regulates both autophagic and apoptotic responses	Valianou *et al.*, 2015
CB17-SCID mice, [B]ELT3 cells. For xenograft tumor establishment, 2.53106 cells were inoculated bilaterally into the posterior back region of mice	Combination of rapamycin and resveratrol is effective in reducing of tumors in TSC2-deficient in vivo; support of the model whereby the synergistic interaction of rapamycin and resveratrol results in a reduction of xenograft tumors	Rapamycin and resveratrol combination therapy not only arrested tumor growth but also by eliminating lesions, possibly through induction of apoptosis, besides also induced to suppression of autophagy induction	Alayev *et al.*, 2015b
[A]MEF *Tsc2* knock-out	The results indicate that an AMPK/p27 axis is promoting a survival mechanism that could explain in part the relapse of TSC tumors treated with rapamycin	Rapamycin induced increase of autophagic levels after 24h of serum deprivation; inhibition of AMPK with compound C inhibited basal levels of autophagy	Campos *et al.*, 2016
Cardiac-specific *Tsc2*-knock-out mice	Effects of hyperactivation of mTORC1 on cardiac function and structure; analysis of hearts revealed misalignment, aggregation and a decrease in the size and an increase in the number of mitochondria, but the mitochondrial function was maintained.	Autophagic flux was inhibited, while the phosphorylation level of [R]S6 or [R]4E-BP1 was increased; autophagy plays an important role in the maintenance of cardiac function and mitochondrial quantity and size in the heart	Taneike *et al.*, 2016
MIN6 cell line; [A]MEF knock-out *Tsc1* and *Tsc2*, and [M]HEK293T cells.	Role of lysine acetylation in *TSC2*, in the regulation of mTORC1, autophagy and cell proliferation; effects of treatments with nicotinamide and resveratrol on mTORC1 signaling and autophagy modulation are *TSC2*-dependent	Nicotinamide increased TSC2 acetylation, and lead to mTORC1 activation and cell proliferation. In contrast, resveratrol avoided *TSC2* acetylation, inhibiting mTORC1 signaling and promoting autophagy	Garcia-Aguilar *et al.*, 2016
A7r5 rat skeletal muscle biopsies from musculus vastus lateralis collected 72 h after the last training activity, and post-exercise biopsies collected 45 min after acute resistance exercise	Cochaperone BAG3 stimulates translation through spatial regulation of mTORC1, inhibiting and recruiting the TSC complex to the cytoskeleton, where autophagy is initiated; mTORC1 inhibition in the remaining cytoplasm is relieved and translation efficiency increased	BAG3 insufficiency results in a severe imbalance of protein synthesis and protein degradation, and in autophagic levels	Kathage *et al.*, 2017
[M]HEK293 cells	*TSC2* acted as a negative regulator of autophagy after olaquindox treatment, and also played a pro-apoptotic function	Olaquindox induced autophagy by reducing *TSC2* expression in [M]HEK293 cells	Li *et al.*, 2017

Table 1 – Cont.

TSC Sample	Goal of the manuscript	Autophagy findings	Reference
HeLa cells, primary human fibroblasts, human diploid fibroblasts, [A]MEF knock-out *Tsc2* and wild-type	During the acquisition of senescence occurs the constitutive activation of mTORC1, which is resistant to both serum and amino acid starvation; persistent mTORC1 simultaneously prevents senescent cells from realizing their full autophagic potential, which would otherwise lead to cell death	Constitutive mTOR activity in senescent cells was supported by high levels of autophagy, and increased autophagy contributes to mTORC1 deregulation and to the survival of senescent cells during starvation	Carroll *et al*., 2017
The generation of a *TSC*-cell model by isolating [L]NSPCs from the brain of six-week-old *Tsc1* mice.	Migration deficit observed in *Tsc1*-deficient [L]NSPCs depends on the state of TFEB activation; treatments that promote [V]TEFB nuclear translocation restore *Tsc1*-deficient [L]NSPCs migration independently of mTORC1	[V]TFEB overexpression has been shown to reactivate autophagy and restore radial migration in new-born neurons where those processes were impaired	Magini A *et al*., 2017
[A]MEFs *Tsc2* knock-out and knock-in and controls	General autophagy induction after uncoupling of oxidative phosphorylation by [X]CCCP agent, and your importance in PINK1/parkin regulation which allows the directioning of uncoupled mitochondria to autophagahy degradation	Stimulation by [X]CCCP resulted in increased of [Y]LC3B-II protein in controls cells when compared to *Tsc2*-deficient cells; *Tsc2*-deficient cells showed less autophagy	Bartoloma A *et al*., 2017
Paraffin-embedded sections from skin lesions of [W]HM from TSC patients and samples from corresponding sites of healthy donors; normal [H]HEMn-MP from moderately pigmented donors	Insufficient autophagy leads to reduced pigmentation in *TSC2*-silenced melanocytes; dysregulated autophagy contributes to hypopigmentation in patients with TSC in response to mTOR hyperactivation; enhancing both mTOR-dependent and -independent autophagy stands to improve depigmentation in TSC-model melanocytes	The results suggest that insufficient autophagy is a likely contributor to epidermal pigmentation abnormalities resulting in the hypomelanotic macules that are hallmarks of TSC.	Yang *et al*., 2018

[A]MEF: mouse embryonic fibroblasts; [B]ELT3: Eker rat uterine leiomyoma–derived cells; [C]FCD: focal cortical dysplasia; [D]FAO: rat liver; [E]ROS: reactive oxygen species; [F]PPP: pentose phosphate pathway; [G]6-AN: 6-aminonicotinamide; [H]NF-κB: nuclear factor kappa B; [I]CASP1: Caspase-1; [J]LAM: Limfangioleiomyomatosos; [L]ASD: autism spectrum disease; [M]HEK293T: human embryonic kidney 293; [N]YAP: hippo–Yes-associated protein 1 pathway; [O]PEComas: tumours showing perivascular epithelioid cell differentiation; [P]PLK1: polo-like kinase 1; [Q]Bcl-2: B-cell lymphoma 2; [R]4E-BP1: eukaryotic initiation factor 4E -binding protein 1; [S]S6: ribosomal protein; [T]MIN6: mouse insulinoma 6; [L]NSPCs: neural stem/progenitor cells; [V]TFEB: transcription factor EB; [X]CCCP: carbonyl cyanide m-chlorophenyl hydrazone; [Y]LC3: microtubule-associated protein 1A/1B-light chain 3; [W]HM: hypopigmented macules; [H]HEMn-MP: human neonatal epidermal melanocytes.

may also contribute to epileptogenesis. The suppression of autophagy in the brain tissues of conditional *TSC1*-knockout mice resulted in aberrant mTOR activation and seizures. The conditional deletion of *Atg7* in mouse neurons was sufficient to promote the development of spontaneous seizures (McMahon *et al.*, 2012).

The decrease of autophagy in some *in vitro* and *in vivo* studies was explained by the inactivation of *TSC1* or *TSC2* due to loss-of-function mutations, making the formation of the regulatory complex of mTORC1 infeasible. Due to this impaired regulation, the mTOR signaling pathway remained continuously active, leading to the uncontrolled proliferation of mutated cells and, as a consequence, to tumor formation. Interestingly, restoration of autophagy did not always affect tumors. In some cases, autophagy-related mTOR overactivation led to endoplasmatic reticulum (ER) stress and unfolded protein response (UPR) activation and to the formation of intracellular protein aggregates. These aggregated proteins contributed to cellular toxicity in different TSC cell culture models (Babcock *et al.*, 2013; Di Nardo *et al.*, 2009). Additionally, autophagy induction promoted tumor cell survival by enabling continuous cell growth in the tumoral microenvironment, which is generally characterized by starvation.

A few studies have also assessed the effect of dietary, non-pharmacological approaches associated with autophagy induction in TSC-associated tumors. The pro-autophagic metabolic interventions studied include glucose deprivation, ketogenic diet (KD) and carbohydrate restrictions. Harputlugil *et al.* (2014) showed that TSC1 was fundamental to the hepatoprotective effect played by protein restriction in ischemia-reperfusion injury. In agreement with this, TSC2-deficient MEFs were hypersensitive to amino acids starvation and hypoxia, in an ATG7-dependent manner. Indeed, the knockdown of *Atg7* in *Tsc2*$^{+/+}$ cells sensitized them to amino acid deprivation (Ng *et al.*, 2011). Considering the link between TSC and neoplasias, in a study with 5 TSC patients, ketogenic diet did not produce beneficial effects to patients with TSC-related tumors, with no signs of growth suppression or tumor regression (Chu-Shore and Thiele, 2010). Considering the management of epilepsy in children with TSC, cognition and behavior were improved after ketogenic diet initiation, in addition to reducing seizure frequency (Park *et al.*, 2017). However, it is important to mention that autophagy was not measured in these models using ketogenic diet.

The increase in the rate of glycolysis in parallel with the reduction of oxidative phosphorylation is a typical hallmark of tumorigenesis (DeBerardinis *et al.*, 2008). The overactivation of PI3k/Akt/mTOR pathway promotes glycolysis and glucose-dependence, leading *TSC1/TSC2* mutant tumor cells entering apoptosis after glucose withdraw (Maher *et al.*, 2004). Indeed, *TSC2* suppresses apoptosis in contexts of energy deprivation (Inoki *et al.*, 2003). Using a TSC tumor xenograft model Jiang *et al.* showed that animals receiving 2-deoxyglucose (2-DG, a modified form of glucose that cannot be used for glycolysis) showed reduced proliferation of tumor cells and the smallest tumors comparing to animals receiving Western-style diet or unrestricted carbohydrate-free. In addition, tumors from animals exposed to carb-free diets were larger and showed areas of necrosis and inflammation. Alternative energy substrates such as ketone bodies and monounsaturated oleic acid supported the growth of *Tsc2*$^{-/-}$ cells *in vitro*. This result suggests that glycolytic inhibition and glucose deprivation may be considered in TSC therapy (Jiang X *et al.*, 2011). However, it is important to keep in mind that pro-autophagic interventions used in these studies and other strategies to induce autophagy may modulate not only this mechanism, but also others including basal metabolism. Thus, attributing the effect of these interventions exclusively and directly to autophagy can lead to a misunderstanding if appropriated methodologies are not used. In fact, many studies with an 'autophagy-centric' bias do not consider other mechanisms. This aspect has to be lead into consideration in studies that aim to assess the effect of pro-autophagy strategies to treat TSC tumors.

TSC, UPR and autophagy

High basal levels of ER stress were detected in different TSC-deficient cells (MEF cells, oligodendrocyte lineage and ELT3 leiomyoma rat smooth muscle cell line) (Ozcan *et al.*, 2008; Babcock *et al.*, 2013; Jiang *et al.*, 2016). ER stress in mutant TSC cells is caused by increased cell proliferation which results in higher protein synthesis that overloads the ER capacity of protein folding. Moreover, autophagy impairment caused by mTOR overactivation in TSC-deficient cells also contributes to ER stress, since misfolded protein aggregates instead of being degraded by autophagy accumulates within ER lumen. In these cells, ER stress was detected due to activation of targets related to the UPR. The UPR is a cellular response triggered to relieve ER stress and reestablish protein homeostasis (Hetz and Papa, 2018). Therefore, UPR induces chaperones expression to improve ER capacity of protein folding, and the ERAD system (endoplasmic reticulum-associated degradation) to eliminate unfolded or misfolded proteins by proteasome or to eliminate protein aggregates by autophagy (Hwang and Qi, 2018).

Higher levels of UPR targets as PERK, XBP1, CHOP and GRP78 were detected in *Tsc1*$^{-/-}$ MEFs when compared to wild-type cells, as well as increased UPR parameters in *TSC2*-mutant tumors (Ozcan *et al.*, 2008; Qin *et al.*, 2010; Tenkerian *et al.*, 2015, Johnson *et al.*, 2015). It was also observed that mTOR overactivaction of TSC-deficient cells results in c-MYC activation, which in turn, is able to induce UPR and ATF4 activation, by direct binding to its promoter, this may be a regulatory pathway (Babcock *et al.*, 2013). TSC-deficient cells while presenting overactivation of mTORC1 show lower levels of mTORC2, as a compensatory negative feedback mechanism. It was shown that disruption of mTORC2 also contributed to the activation of the PERK – eIF2a arm of UPR, independently of mTORC1 (Tenkerian *et al.*, 2015).

Increased basal ER stress rendered TSC-deficient cells highly sensitive to cell death induced by pharmacological ER stressors as thapsigargin, tunicamycin and bortezomib (Ozcan *et al.*, 2008, Johnson *et al.*, 2015; Tenkerian *et al.*, 2015). Increased apoptosis in TSC mutant versus wild-type cells suggests that these drugs can be a promising therapy option for disease control, since it would selectively kill tumor cells, while non-tumor and non-stressed cells would be able to cope

to transient stress. Adjunct treatment with rapamycin was able to reduce UPR activation and rescued TSC mutant MEF cells from apoptosis induced by thapsigargin and tunicamycin (Ozcan *et al.*, 2008), as well rescued leiomyoma cells from apoptosis induced by bortezomib treatment (Babcock *et al.* 2013). These results confirm that overactivation of mTORC1 in TSC mutant cells is at least partly responsible for ER stress and UPR activation.

Since autophagy is triggered upon ER stress to eliminate excess proteins aggregates, and to mediate cell death under intense and prolongated stress, it is important to verify its relationship with ER stress on TSC-deficient cells. However, most of the studies that induced or checked ER stress in TSC deficient cells have not described autophagy levels. In TSC normal cells, drug-induced ER stress triggers autophagy-mediated cell death in MEF cells and ER stress-induced autophagy was attributed to the downregulation of mTOR pathway. On the other hand, when compared to wild-type cells, TSC -mutant cells with constitutive activation of mTOR were more resistance to ER stress-induced autophagy (Qin *et al.*, 2010; Kang, *et al.*, 2011).

Nelfinavir is an ER stressor drug which was tested on *Tsc2*$^{-/-}$ MEF cells and it was able to reduce mTOR signaling while increased autophagy levels, observed by decreased SQSTM1 protein and increased LC3 lipidation to the lower resolving LC3-II isoform. Chloroquine, an autophagy inhibitor, also enhanced nelfinavir-induced cell death in these cells. A combination of nelfinavir and chloroquine potentiated ER stress and affected autophagy resulting in *Tsc2*$^{-/-}$ cells death, while cells with normal expression of mTOR were tolerant. The combination of an ER-stressor drug and an autophagy inhibitor also seems to be a promising therapeutic option for TSC (Johnson *et al.*, 2015).

Autophagy therapies in TSC- and mTOR-deficient contexts

Many of the pathways that regulate autophagy are dysregulated in cancer development, and some therapeutic compounds have been designed to restore or inactivate these pathways (Table 1). Some of these compounds directly inhibit mTORC1, while others inhibit mTORC1 indirectly by reducing the nutritional support for cells or inhibiting the upstream targets in the mTOR signaling pathways (Yu *et al.*, 2011). Some of these therapies leverage the combined therapeutic effect of the mTORC1 and mTORC2 complexes. However, therapies that focus on both complexes can induce considerable toxicity and drug resistance mechanisms. Recently, several studies have focused on the discovery of new compounds that act in these pathways, aiming to control exacerbated cell proliferation and autophagy. Among the alternative compounds that regulate autophagy by TSC2 are olaquindox, which induces autophagy and promotes apoptosis in HEK293 cells (Li *et al.*, 2017). Perhexiline, niclosamide, amiodarone (approved drugs) and rottlerin (pharmacological reagent) inhibit mTORC1 signaling and stimulate autophagy (Balgi *et al.*, 2009).

In most cases, rapamycin treatment leads to the restoration of autophagy in tumor cells. This restoration can stop tumor growth in some cases. However, tumors may regrow after prolonged treatment with rapamycin due to the inhibition of the AMPK pathway, as demonstrated in MEFs from the Tsc2-null model (Campos *et al.*, 2016). The impairment of autophagic flux and the accumulation of autophagosomes observed in the Tsc2-KD neurons of mice were also dependent on the AMPK pathway. After treatment with rapamycin, LC3-II accumulation and increased AMPK-ULK1 activation revealed that this accumulation of autolysosomes was insensitive to rapamycin, indicating a mTORC1-independent mechanism regulating autophagy (Di Nardo *et al.*, 2014). Indeed, AMPK can directly activate the ULK1 complex in an mTOR-independent manner (Kim *et al.*, 2011). Conversely, there is evidence suggesting that persistent mTORC1 signaling in the TSC context reduces the capacity of senescent cells to undergo autophagy, which leads to cell death. This seemingly contradictory role for autophagy as a prosurvival and cell death mechanism of senescent cells is a phenomenon that may also contribute to the tumorigenesis and neurodegeneration in TSC conditions (Carroll *et al.*, 2017).

Metformin and resveratrol have been given greater attention in recent years as possible means to stimulate autophagy in TSC. Metformin is an antihyperglycemic agent used for the treatment of noninsulin-dependent diabetes mellitus. The exact mechanism of action of metformin is not well elucidated, but a possible inhibitory effect on the mTOR signaling pathway has been recognized (Amin *et al.*, 2018). Inhibition of mTORC1 by metformin can occur (a) through the phosphorylation and inhibition of Raptor and (b) through the activation of the *TSC1* and *TSC2* genes (Howell *et al.*, 2017) (Figure 2). Normal and non-TSC2 (*Tsc2*$^{+/+}$) embryonic mouse cells treated with metformin showed an upregulation of mTORC1 (Kalender *et al.*, 2010). In the tumor model of mice with heterozygous mutations in *TSC2* (*Tsc2*$^{+/-}$) there was no reduction in tumor size after metformin treatment compared with those treated with rapamycin, suggesting limited therapeutic benefits of metformin in treating hamartomas (Auricchio *et al.*, 2012). In murine kidney *Tsc2*$^{+/-}$ tumors, metformin was able to reduce mTOR signaling only in normal tissues but not in tumor cells (Dowling *et al.*, 2016). In liver cells of the same model, metformin inhibited mTOR signaling through a mechanism involving the AMPK pathway and the TSC complex (Howell *et al.*, 2017). None of these studies assessed autophagy after treatment. Some studies have raised the prospect of metformin use to suppress the initiation and recurrence of TSC-associated tumors, which would induce fewer side effects and have a lower cost than other mTOR inhibitors (Amin *et al.*, 2019).

Resveratrol is another compound investigated for its effect on mTOR inhibition and on the stimulation of autophagy and apoptosis. Resveratrol (3,5,4'-trihydroxystilbene) is a polyphenolic phytoalexin derived from stilbene. The compound is present in high concentrations in red wine, the imbibing of which has been associated with a lower incidence of heart disease. Other benefits of resveratrol include its anti-inflammatory, antioxidant (Wishart *et al.*, 2018) and neuroprotective effects (Quincozes-Santos *et al.*, 2013). It has been shown to regulate cell proliferation, apoptosis, and angiogenesis and to ameliorate DNA damage (Alayev *et al.*, 2015), and regulate mitochondrial activity, which is important for the treatment of obesity and diabetes (Agarwal and Baur,

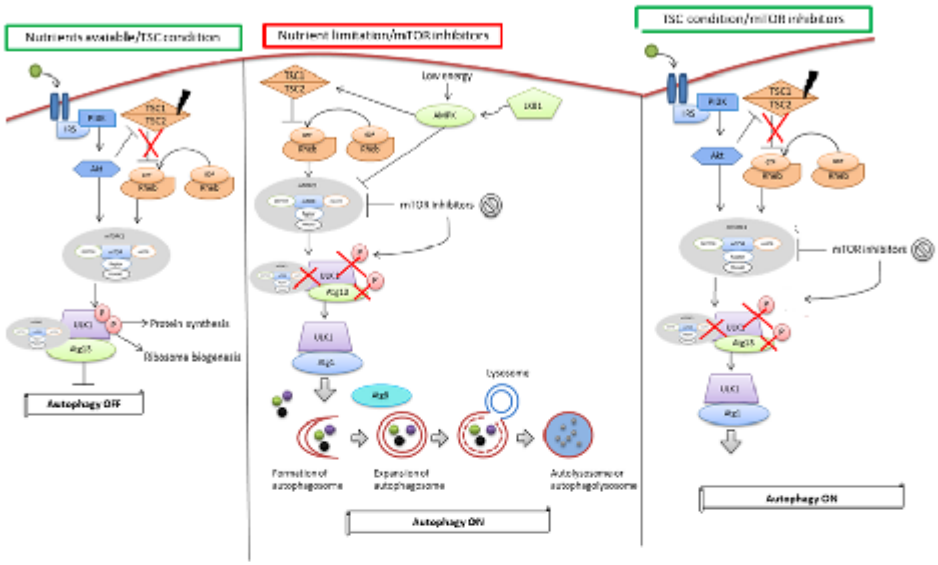

Figure 2 – Autophagy signaling with nutrients available or TSC condition and with nutrient limitation or presence of mTOR inhibitors. In this first scenario, the TSC complex is unable to prevent to conversion of Rheb-GDP to Rheb-GTP causing the activation of mTORC1. mTORC1 activated causes inactivation of ULK1 complex through the phosphorylation of ULK1 and ATG13, thus suppressing autophagy and allowed the protein synthesis and ribosome biogenesis. Conversely, in the second scenario, under nutrient limitations or with the presence of mTOR inhibitors, mTORC1 is inactivated, which enables the ULK1 complex to be activated together with Atg proteins which triggers the formation of doble membrane vesicule called autophagosomes around the cell content that need to be degraded. Autophagosome merges with lysosome forming autolysome or autophagolysosome that degrades this vesicule internal cell content. In parallel to autophagy induction, the inactivation of mTORC inhibits cell growth.

2011). Similar to metformin, resveratrol suppresses the activity of mTORC1 through the AMPK pathway (Wang *et al.*, 2018), and induction of autophagy by resveratrol has been shown to occur directly via the mTOR-ULK1 pathway (Park *et al.*, 2016). In addition, resveratrol inhibits the activation of many proteins upstream of the mTORC1/S6K1 signaling pathway, including PI3K and can modulate autophagy by directly inhibiting S6K1 activation (Armour *et al.*, 2009) (see Figure 1). Additionally, resveratrol seems to also regulate the activation of mTORC2 (Gurusamy *et al.*, 2010). Recent work has shown that resveratrol is capable of inducing apoptosis and autophagy in breast cancer tumor cells resistant to cisplatin treatment, non-small cell lung cancer cells and renal cell carcinoma cells (Park *et al.*, 2016; Chang *et al.*, 2017; Liu *et al.*, 2018; Wang *et al.*, 2018). Because of the ability of resveratrol to modulate the autophagy and apoptosis pathways, its efficacy was investigated in the context of TSC. It has been reported that resveratrol prevented the positive regulation of autophagy induced by rapamycin, inhibiting the cleavage of LC3 and the formation of autophagosomes in *TSC2*$^{-/-}$ cells. In this context, resveratrol was able to prevent p62 degradation in a *TSC2*-dependent manner (Alayev *et al.*, 2014). A combination of rapamycin and resveratrol tested in an animal model was able to specifically inhibit the PI3K/Akt/mTORC1 signaling pathway, activating apoptosis and reducing cell survival in a *TSC2*$^{+/-}$ xenograft tumor model of LAM but not in cells expressing *TSC2* (Alayev *et al.*, 2015).

New treatment alternatives focused on autophagy modulation

A few treatment initiatives have already been reported for tumors with mTORC1 aberrant activation. These alternatives primarily target metabolic compensatory mechanisms triggered by autophagy inhibition but not solely through mTORC1 inhibition. The first example was published in the report by Parkhitko *et al.* (2014), who used chloroquine, a molecule that blocks lysosome–autophagosome fusion and lysosomal function, to suppress macroautophagy and chaperone-mediated autophagy. In this study, the authors demonstrate that TSC2-null cells have distinctive autophagy-dependent pentose phosphate pathway (PPP) alterations. With this in mind, they directly targeted mTORC1-independent autophagy by antimetabolite 6-aminonicotinamide. The authors showed a 40% reduction in cell proliferation after 96 h of treatment. In *Tsc*$^{+/-}$ mice with spontaneously formed cystoadenomas, the authors showed a 50% reduction in macrolesions and microlesions after 4 months of treatment (Parkhitko *et al.*, 2014). The second example was related to Polo-like kinase 1 (PLK1) inhibitors. These compounds were also shown to be potential therapeutic agents for the treatment of tumors

with dysregulated mTORC1 signaling. The pharmacological inhibition of PLK1 by the small molecule BI-2536 significantly decreased the viability and clonogenic survival of TSC1- and TSC2-deficient cells in vitro. This inhibition of PLK1 was associated with increased apoptosis, suggesting that this kinase can regulate both autophagy and apoptosis (Valianou *et al.*, 2015).

Conclusions and perspectives

Tuberous Sclerosis Complex, although relatively rare, is the second most common disorder in the genodermatoses category of disorders. The disease can manifest in the early stages of life and often results in social exclusion and vulnerability at the medical, psychological and cultural levels. Severe epilepsy disorder may occur in very young patients. In addition, there is increased morbidity and a reduction in life expectancy, especially when hamartomas related to the syndrome emerge, especially as intracardiac and brain tumors. To date, mTOR inhibitors have been widely used to attenuate the clinical manifestations of the disease. However, several challenges still exist in the management of TSC using rapalogs, including their cost, side effects, definition of the ideal timing of treatment initiation for different disease symptoms and a better understanding of partial efficacy of these drugs in certain applications. In this scenario, alternative or combined treatments must be sought, and modulators of autophagy are promising candidates. The mTOR inhibitor-induced autophagy in tumor cells may have a dual effect by either interrupting or enhancing tumor growth. Currently, it is a consensus that autophagy is fundamental to control cell homeostasis and tissue functioning since its loss has been associated with the development of several pathologies. Autophagy deficiency has been observed in the early steps of tumor initiation, for instance. In the other hand, the adaptability to stress provided by autophagy can contribute to metabolic adaptation and tumor growth in advanced stages of carcinogenesis (Amaravadi *et al.*, 2019). *TCS2*-deficient fibroblasts, for instance, are more dependent on exogenous nutrients, and the knockout of *ATG5* reduced even more cell adaptability to nutrient deprivation (Filippakis *et al.*, 2018). In animal models, the above-mentioned dynamic of autophagy in cancer is reproducible, but the evidence from human samples suggests that this process is more complex (Galluzzi *et al.*, 2015). Actually, it seems that the dominant effect played by autophagy (i.e. whether chemoprevention or tumor supporting) may depend on the tumor type, genetic and epigenetic background, the event involved in the carcinogenesis, among other variables. The fact is that the paradoxical role of autophagy in promoting or suppressing TSC tumorigenesis and modulating other clinical behaviors is inherent to the functioning of the mechanism, which controls multiple steps and, in the other hand, is controlled by several others (Amaravadi *et al.*, 2019). The large number of mechanisms controlling and being controlled by TSC complex and mTOR adds even more variables to the models (Filippakis *et al.*, 2018). These concepts must be kept in mind constantly in the discussion of pharmacological or non-pharmacological strategies that induce autophagy, either through the inhibition of mTOR or through other mechanisms. In several studies, however, a direct role has been attributed to autophagy even when the mechanism is not properly or exclusively assessed. This occurs, for instance, in studies showing that Rapamycin is effective in controlling tumor growth and/or reducing tumor volume in animal models (Kenerson *et al.*, 2005; Lee *et al.*, 2009) and in TSC patients (Cabrera-López *et al.*, 2012; Mao *et al.*, 2017; Li *et al.*, 2019). Indeed, these evidences suggest that the chemopreventive role played by autophagy could be dominant. But other studies challenge the establishment of a unique conclusion, as exemplified by the observation that TSC individuals, despite not showing the altered incidence of renal tumors in relation to controls, developed renal cancer too much earlier in life (Peron *et al.*, 2016).

Therefore, two aspects need to be raised at this point. The first one concerns to the multitude of mechanisms controlled by TSC complex and mTOR in the interface between health and disease, from autophagy to protein synthesis, the activity of immune cells, cell metabolism, cell growth, and death (Boutouja *et al.* 2019). Attributing the effect of TSC mutations or Rapamycin exclusively to autophagy can be a misinterpretation. The second point is that tumors and other pathologies are not coordinated by a unique cell type or mechanism. Tumor microenvironment, for instance, is formed by dozens of cell types, including normal, immune and mesenchymal cells. The autophagy of all these players might be modulated when this mechanism is activated, and a growing body of recent evidence has shed some light on the crosstalk between tumor cells autophagy and the tumor microenvironment (Janji *et al.*, 2018). Notwithstanding, the dominant role played by autophagy modulation, in this context, may depend not only on the genetic status of *TSC1/TSC2* but also the metabolism, the immune status, endocrine functions and other signaling molecules. With this in mind, it is important to maintain the criticism when interpreting results from mTOR inhibitors or metabolism modifications (Medvetz *et al.*, 2015). In this sense, testing compounds that modulate autophagy in a more specific manner or animal models with the co-occurrence of *TSC1/TSC2* and *ATG* mutations could provide more accurate evidence regarding the role of autophagy in TSC (Filippakis *et al.*, 2018). And, in addition to Rapamycin, new classes of mTOR inhibitors and compounds that modulate other abovementioned mechanisms could be combined to reach optimal clinical responses.

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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.

Author Contributions

LBR participated on literature review, manuscript design, conceptualization and writing; ECFC participated on manuscript writing and revising for intellectual content, PAP participated on manuscript writing and revising for

intellectual content, FV participated on literature review, manuscript writing and revising for intellectual content and CR participated on literature review, manuscript design, conceptualization, writing and revising for intellectual content.

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Supplementary Material

The following online material is available for this article:

Table S1 – Clinical and Genetic criteria for the diagnosis of Tuberous Sclerosis Complex.

Table S2 – The main mTOR inhibitors tested in patients with TSC-related manifestations.

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Capítulo VI

Resultados parciais:

Fluxo autofágico em fibroblastos de pacientes com esclerose tuberosa e variante germinativa patogênica em *TSC2* após tratamentos com compostos moduladores da autofagia

Introdução

A doença esclerose tuberosa é a segunda genodermatose mais incidente no Brasil e no mundo, acometendo cerca de 1 em cada 6.000 nascidos-vivos de ambos os sexos (Northrup et al. 2018). Trata-se de uma condição genética autossômica dominante causada por mutações nos genes supressores tumorais *TSC1* ou *TSC2,* que acomete múltiplos sistemas, que vão desde o surgimento de tumores (hamartomas) no sistema nervoso central, pulmões e rins, até incapacidades cognitivas, deficiência intelectual e autismo e que não tem cura e possui poucas opções de tratamento, utilizados apenas para a tentativa de controle dos sintomas Northrup et al. 2013).

As terapias utilizadas hoje para a doença são os inibidores da via de mTOR (Cabrera-López et al., 2012). Embora promissor, o tratamento com rapamicina e seus análogos possui alto custo, pode apresentar efeitos colaterais e não ser efetivo no controle e na erradicação dos tumores dos pacientes (Bissler et al. 2013). Os análogos da rapamicina ainda não estão amplamente consolidados nem liberados para uso dos pacientes atendidos pelo SUS, com uma série de questões em aberto relacionadas principalmente a variabilidade da resposta terapêutica. De maneira geral, esses inibidores atuam na supressão da via de mTOR, que se encontra hiperativada, e na indução do processo de autofagia nas células dos pacientes. A autofagia regula os níveis de nutrientes na célula, sendo capaz de estacionar ou estimular o desenvolvimento tumoral (Rabinowitz e White 2010). O uso de bafilomicina A1, que atua como um inibidor da fusão entre o autofagossomo e o lisossomo, permite avaliar o fluxo autofágico das células. Combinado com a rapamicina, permite verificar a indução de autofagia pela droga, além da detecção da presença de etapas finais da autofagia por vias alternativas (Klionsky et al. 2021).

O composto metformina (antidiabético de uso oral) é capaz de diminuir o influxo de nutrientes na célula, e, consequentemente, estimular a autofagia (Liu et al. 2018; Amin et al. 2019). O soro com baixa glicose (HBSS), por possuir menor quantidade de nutrientes, pode simular um efeito de dieta hipocalórica nas células, e, consequentemente estimular a autofagia. Devido a esse potencial,

metformina e uma simulação de dieta hipocalórica podem ser testados como tratamentos alternativos para a esclerose tuberosa, com menor custo e menos efeitos colaterais em comparação com os inibidores de mTOR. Por esse motivo, verificar a resposta da indução da autofagia em fibroblastos de pacientes com esclerose tuberosa (que possuem variante patogênica nos genes supressores tumorais *TSC1* ou *TSC2*) pode ser uma boa alternativa para a avaliação desses compostos no sentido de prevenção de surgimento e recorrência de tumores na doença. Além disso, pode diminuir os custos com ressecções cirúrgicas e exames de avaliação de tumores, melhorando a qualidade de vida dos pacientes.

Objetivos do estudo: Explorar o fluxo autofágico e a indução da autofagia, além dos níveis de mitofagia, apoptose e necrose em células de paciente ET e variante patogênica em heterozigose no gene *TSC2* e um indivíduo controle sem ET, com e sem o tratamento padrão de controle da doença (rapamicina) e comparar com o uso de compostos alternativos conhecidos por estimular a autofagia, como metformina e soro com baixa glicose.

Metodologia

Amostra

Para as investigações foram utilizadas uma linhagem de fibroblastos de indivíduo com ET e variante patogênica germinativa em *TSC2* (c.4375C>T p. Arg1459Ter) e uma linhagem de individuo sem ET, que se encontram estabilizadas em cultivo.

Aspectos éticos

Este projeto foi aprovado no CEP HCPA sob o número 2019-0153.

Tratamentos moduladores da autofagia

Os fibroblastos do caso e do controle foram tratados com: rapamicina a 200nM; ou com rapamicina a 200nM mais bafilomicina a 100nM; ou com bafilomicina a 100nM; ou com metformina a 20mM; ou com 1uL de HBSS; ou apenas com veículo DMSO.

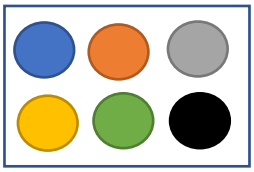
Investigação fluxo autofágico por imunocitoquímica e anticorpo LC3

Foi realizada a investigação de fluxo autofágico nas células com a técnica de imunocitoquímica utilizando o anticorpo marcador da proteína autofágica LC3 para avaliar importantes aspectos da autofagia, tais como a quantidade, o tamanho e a localização subcelular dos autofagossomos, moléculas fundamentais para o início do processo e dos autofagolisossomos, marcadores da fase final da autofagia.

Preparo das placas de cultivo celular

Foram preparadas placas com lamínulas tratada previamente com poli-l-lisina. As lamínulas foram esterilizadas por 12 horas em álcool 70%, secaram por 2 horas na luz ultravioleta, foram alocadas no fundo de placas de cultivo de células com 6 poços. Foi adicionado 1000uL e cima de cada lamínula por uma hora. Depois disso, garrafas de cultivo contendo as células do paciente e do controle foram tripsinizadas, e foram plaqueadas $1x10^4$ células por poço, em cima da lamínula. Após oito dias e 75% de confluência nos poços, as células receberam os tratamentos conforme o esquema abaixo:

1 placa por paciente; 1 poço por condição:



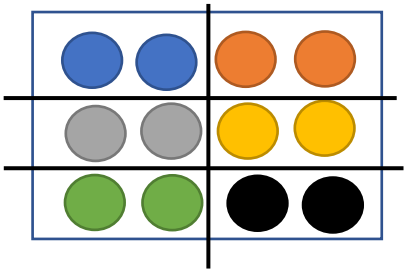
- Controle DMSO
- Rapamicina
- Rapamicina + Bafilomicina
- Bafilomicina
- Metformina
- HBSS

Após os tempos de cada tratamento as células foram fixadas em álcool absoluto gelado. As lamínulas foram preparadas para a imunocitoquímica com 1uL de anticorpo em 400uL diluente para cada 4 poços, e foram visualizadas e

fotografadas em microscópio de fluorescência para análises posteriores da presença, número e tamanho dos autofagossomos e autofagolisossomos após cada tratamento.

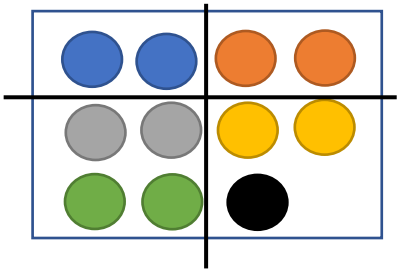
Citometria

Análises de citometria foram utilizadas para caracterizar autofagia total, mitofagia, apoptose e necrose nas células do paciente e controle. As células do paciente com ET e do controle foram cultivadas em placas de 12 poços, na quantidade de $3.5x10^4$ células por poço. Após atingir a confluência de 75% a 80% as células foram tratadas conforme o esquema abaixo:

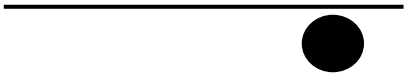


Desfecho de Autofagia, marcação com Laranja de acridina, Mitotracker (200nM, 20min a 37°C)

- Controle DMSO
- Rapamicina 6h
- Rapamicina 6h + Bafilomicina 2h
- Bafilomicina 2h
- Metformina 12h
- HBSS 12h



Desfecho de viabilidade e morte celular por Apoptose, marcação com Iodeto de propídeo e Anexina V + FITC (1,5 ug/mL, 15min, temperatura ambiente)



Controle DMSO

Rapamicina 24h

Rapamicina 24h + Bafilomicina 2h

Bafilomicina 2h

Metformina 24h

HBSS 24h

Resultados parciais

Até o momento foi possível realizar todos os experimentos com as duas linhagens celulares: fibroblastos de paciente com ET e a variante patogênica do tipo SNV (c.4375C>T p. Arg1459Ter) no gene *TSC2* e fibroblastos de indivíduo sem ET (controle). Os resultados estão sendo avaliados e o manuscrito está em preparação.

Capítulo VII

Discussão Geral, Conclusões, Perspectivas, Referências Bibliográficas

DISCUSSÃO GERAL

As genodermatoses NF1 e ET podem ser consideradas síndromes de predisposição hereditária ao câncer pois são as síndromes com maior risco associado para desenvolvimento de certos tumores, como os MPTNMs. Nessas síndromes, a caracterização molecular mais completa possível dos acometidos tem importância crucial para o manejo e prognóstico do paciente, assim como para o aconselhamento genético de suas famílias (Gerstenblith et al. 2010; Ladd et al. 2020).

Como os sinais e sintomas mais precoces das genodermatoses são manifestações cutâneas típicas, a suspeita destes diagnósticos geralmente é levantada por um médico dermatologista. O diagnóstico precoce de pacientes com NF1 e ET é de fundamental importância para garantir o correto acompanhamento e para reduzir o risco e/ou aumentar as chances de diagnóstico precoce de tumores, impactando assim o prognóstico (Gerstenblith et al. 2010). Embora o diagnóstico geralmente possa ser feito pelo exame clínico e alguns exames adicionais (em especial de imagem), o diagnóstico molecular pode ser de grande auxílio na confirmação da suspeita clínica em especial nos casos com sinais e sintomas menos óbvios (Frommherz et al. 2021).

Uma vez feito o diagnóstico completo de uma genodermatose, a conduta é centrada fundamentalmente no acompanhamento multidisciplinar seguindo diretrizes clínicas específicas para a faixa etária do(a) paciente. No entanto, não é infrequente que pacientes com manifestações típicas sejam diagnosticados apenas na vida adulta, quando já apresentam complicações mais acentuadas das doenças (Frommherz et al. 2021). No Brasil, a indisponibilidade do diagnóstico molecular no SUS, limitações de acesso ao aconselhamento genético e também a alguns tratamentos, são fatores que podem levar ao diagnóstico e manejo sub-ótimo das genodermatoses.

NF1 é causada, na maioria dos pacientes, por variantes germinativas patogênicas na região codificadora do gene *NF1*. No entanto, uma parcela importante de pacientes não tem nenhuma alteração molecular identificada com análises convencionais do gene. Além disso, além da heterogeneidade molecular

no caso de pacientes com variantes identificadas em *NF1*, a expressividade variável da síndrome pode estar relacionada a outros genes os quais podem atuar como modificadores de fenótipo (Sabbagh et al. 2009; Vogt et al. 2011; Pasmant et al. 2012; Kowalski et al. 2020).

O cromossomo 17, onde o gene *NF1* está localizado, tem o segundo maior conteúdo gênico entre todos os cromossomos. Ele contém vários genes, incluindo *PMP22*, *PAFAH1B1*, *YWHAE*, *RAI1*, que estão relacionados a doenças genéticas em humanos (Zody et al. 2006). Flanqueados por duplicação segmental (SD), alternativamente denominados repetições com baixo número de cópias (LCRs), esses genes são frequentemente afetados por duplicações ou deleções recorrentes, implicadas em uma série de distúrbios genômicos em humanos, incluindo neurofibromatose tipo 1 (Lupski and Stankiewicz 2005). Cerca de 4,7%-11% dos pacientes com NF1, apresentam grandes deleções abrangendo o gene *NF1* e outros genes adjacentes, na região cromossômica 17q11.2. Essas grandes deleções são denominadas 'microdeleções de NF1' (Cnossen et al. 1997; Rasmussen and Friedman 2000; Kluwe et al. 2004).

Desde a primeira identificação de pacientes com microdeleções NF1, se observou que a maioria desses pacientes costuma apresentar fenótipo mais grave e características atípicas. Entre estas destacam-se dismorfias faciais, deficiências severas de aprendizagem, grande número de neurofibromas, alta estatura, mãos e pés grandes com tecido mole redundante, hiperflexibilidade das articulações e anomalias cardíacas congênitas (Venturin et al. 2004; Spiegel et al. 2005; Zhang et al. 2015; Kehrer-Sawatzki et al. 2020; Scala et al. 2021). Outro ponto importante é que os indivíduos com microdeleções NF1 têm um risco aumentado de desenvolvimento de MPNST com diagnósticos em idade mais jovem (Evans et al. 2002; De Raedt et al. 2003; Mautner et al. 2010; Evans et al. 2012). No capítulo II desta tese apresentamos uma descrição de quatro pacientes com microdeleções envolvendo NF1 e que também apresentam fenótipo mais grave, incluindo presença de MPNST em idade jovem (paciente com 26 anos), macrocefalia, escoliose e características dismórficas menos frequentes como fissuras palpebrais oblíquas, orelhas proeminentes, estatura alta e

hipogonadismo. Apenas um desses pacientes apresentou microdeleção típica (tipo 1), enquanto os demais rearranjos apresentaram tamanhos atípicos. Foi realizado uma abordagem de conservação evolutiva dos genes co-deletados envolvidos nos diferentes tamanhos de microdeleções encontrados nos pacientes, com o objetivo de acessar a relação ortóloga e paráloga desses genes em primatas e outras espécies de mamíferos. Além disso, foi possível verificar o tipo de seleção agindo sobre esses genes e seus aminoácidos, buscando contribuir para o estabelecimento da importância desses genes para o fenótipo agravado.

A detecção e caracterização precisa das microdeleções NF1 é de grande importância clínica, principalmente por causa da sobreposição fenotípica que pode existir entre NF1 e outras Rasopatias (por exemplo, síndrome de Legius). O entendimento do espectro das diferentes deleções, e do papel de outros genes co-deletados no fenótipo é de importância para o entendimento da história natural e para aconselhamento genético dos pacientes (Kluwe et al. 2004; Wimmer et al. 2006; Etzler et al. 2008). É provável que a variabilidade clínica de NF1 resulte de uma combinação de fatores genéticos, ambientais e estocásticos. Tal complexidade e a diversidade de variantes moleculares que ocorrem nesta doença tornam, difícil o estabelecimento de correlações genótipo-fenótipo.

Os grandes primatas possuem um grau elevado de *duplicons*, sendo os *duplicons* centrais os mais abundantes e frequentemente relacionados com o surgimento de novos genes, apresentando diferenças drásticas de expressão gênica e de variação estrutural (Jin et al., 2004; Johnson et al., 2001). A presença de um *duplicon* central ao longo do braço q do cromossomo 17 acabou levando ao surgimento da família gênica *LRRC37* durante a evolução dos primatas, por exemplo (Jin et al., 2004). No artigo, são descritos quatorze genes co-deletados que foram analisados em oito espécies de primatas. As análises filogenéticas apresentadas demonstram que alguns desses genes não estão em concordância em todas as árvores filogenéticas das espécies analisadas, sugerindo distinta restrição evolutiva entre eles (Reis et al. 2019). Essa constituição pode ser esperada para genes que apresentam divergência de função durante a evolução das espécies e estão sob distintas pressões de seleção e reforçando que a

presença desses genes em humanos é fundamental para a constituição de um organismo saudável.

Dos quatorze genes analisados, dez genes apresentaram evidências de estarem sob seleção purificadora (*CRLF3, ATAD5, TEFM, ADAP2, NF1, OMG, EVI2A, RAB11FIP4, COPRS* e *SUZ12)* e quatro genes apresentaram evidências de estarem sob seleção positiva (*RNF135*, *UTP6*, *LRRC37B* e *EVI2B*), o que possivelmente aumentou seus valores adaptativos.

De Raedt e colaboradores estimaram que o risco ao longo da vida de MPNST em pacientes com microdeleções NF1 é quase o dobro do observado na população geral de NF1. O risco aumentado de malignidade pode ser explicado por variações na expressão de um ou vários genes (genes supressores de tumor em vez de oncogenes) localizados na região da microdeleção de 1,4 Mb (tipo 1) (De Raedt et al. 2003).

A perda funcional de *SUZ12*, associada à perda funcional de *NF1*, foi considerada um dos fatores para o desenvolvimento de tumores malignos da bainha do nervo periférico (Zhang et al. 2014). Ao mesmo tempo, essa perda funcional acaba ativando um interruptor epigenético que sensibiliza tumores ao tratamento de inibidores de bromodomínios, uma estratégia terapêutica promissora (De Raedt et al. 2014). Devido à elevada expressão de *SUZ12* e *ADAP2* nos tecidos cardíacos, esses genes são fortes candidatos a estarem relacionados com má formação cardiovascular (Venturin et al. 2004). Quanto aos demais genes, o gene *OMG* apresenta envolvimento com dificuldade de aprendizagem em pacientes com NF1 (Terzi et al. 2011), além de regular o crescimento celular (Vourc'h et al. 2003). Já a haploinsuficiência de *UTP6* parece conferir às células de pacientes com a microdeleção uma menor suscetibilidade a apoptose, aumentando o risco de desenvolvimento de tumores (Piddubnyak et al. 2007).

Análises de expressão gênica de amostras de 23 neurofibromas dérmicos, 13 neurofibromas plexiformes e 13 MPNSTs de pacientes com NF1 demonstraram cinco genes regulados positivamente: *OMG* e *SUZ12* em

neurofibromas plexiformes e *ATAD5, EVI2A* e *COPRS* em MPNSTs. Esses resultados são consistentes com um modelo no qual os genes superexpressos agem como oncogenes. No entanto, é possível também que na região das microdeleções NF1 contenha um regulador negativo desconhecido que seria inativado pela deleção e essa inativação regularia positivamente oncogenes vizinhos (Pasmant et al. 2011). Em nosso estudo, esses genes demonstraram evidências de estarem sob seleção purificadora, o que está relacionado à importância de manter sua função durante a evolução dos organismos.

Os resultados encontrados em nosso estudo apoiam a hipótese de que a haploinsuficiência de *RNF135* contribui para a ocorrência de doenças em humanos. Além disso, a proteína RNF135 apresentou vinte e sete sítios sob seleção positiva, sendo três deles localizados no domínio de dedo RING da proteína, que podem ter sido selecionados para aumentar a eficiência nas rotas de ubiquitinação em primatas (Reis et al. 2019). Outros trabalhos também demonstram correlações genótipo-fenótipo envolvendo a haploinsuficiência de *RNF135* resultando em crescimento excessivo (Ferrari et al. 2017), dificuldade de aprendizagem e características dimórficas (Stewart et al. 2007; Serra et al. 2019). O gene *RNF135*, especificamente, apareceu hipoexpresso (regulado negativamente) nas amostras MPNST investigadas por Pasmant e colaboradores (2011), apontando para o seu envolvimento na predisposição a tumores observada em pacientes com microdeleção NF1 (Pasmant et al. 2011). Sabendo que a inativação do gene RNF135 pode ocorrer por diversas alterações e não só pela sua deleção, os achados do nosso estudo pretendem contribuir para a inclusão de investigação molecular no gene *RNF135*, além do gene *NF1* para pacientes com suspeita de NF1.

Assim como NF1, a esclerose tuberosa é condição genética autossômica dominante ainda sem cura e com poucas opções de tratamento (Kingswood et al. 2017; Rosset et al. 2017a). Variantes nos genes supressores tumorais *TSC1* e *TSC2* são as responsáveis por essa genodermatose (van Slegtenhorst et al. 1998). O gene *TSC1* (NG_012386.1) possui 23 éxons, sendo que os éxons 1, 2 e

os primeiros 80 pares de bases do éxon 3 compreendem a região 5’ não traduzida do gene. Seu RNA mensageiro canônico (NM_000368.4) possui 8626 nucleotídeos no total e é expresso em quase todos os tecidos. Já o gene *TSC2* (NG_005895.1) possui 42 éxons, sendo que o éxon 1 e os primeiros 29 pares de bases do éxon 2 fazem parte da região 5’ não traduzida do gene, e a região 3’ não traduzida compreende os últimos 102 pares de bases do éxon 42. O RNA mensageiro canônico desse gene (NM_000548.3) consiste em 5675 nucleotídeos, e similar ao *TSC1*, é expresso na maioria dos tecidos, com as maiores quantidades de expressão no cerebelo (Gutmann et al. 2000)

O banco de dados ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) registra 2593 alterações no gene *TSC1*, sendo 2067 nucleotídeos de única base (SNPs), 282 deleções, 153 duplicações, 173 inserções e 25 do tipo indel (inserção seguida de deleção). Para o gene *TSC2* o mesmo banco de dados registra 6030 alterações, divididas em: 4964 alterações tipo SNP, 612 deleções, 385 inserções, 321 duplicações e 66 alterações do tipo indel (Landrum et al. 2018; ClinVar, 2021). Embora a grande maioria das alterações descritas nesses sejam SNPs, existe uma parcela significativa de rearranjos, que incluem deleções, inserções, duplicações e alterações indel.

O estudo de Longa e colaboradores (2001) analisou as diferenças de rearranjos entre os genes *TSC1* e *TSC2* de pacientes com ET e identificou 19 deleções parciais ou completas, três envolvendo *TSC1* e dezesseis envolvendo *TSC2*. Resumidamente, três características principais parecem distinguir as deleções entre os genes: (1) tamanho de exclusão: todas as deleções de *TSC1* detectadas estavam dentro da unidade de transcrição, enquanto 12 das 16 deleções de *TSC2* tinham pelo menos um ponto de interrupção externo; (2) a localização dentro do gene: todas as deleções de *TSC1* estavam confinadas à extremidade 3’ não traduzida do gene, resultando na mesma mutação de mudança de quadro de leitura após o aminoácido K875, enquanto os pontos de interrupção internos de *TSC2* mostraram-se espalhados ao longo do gene; (3) preferência por sequências recombinatórias: seis de oito pontos de interrupção de

TSC2 internos mapeiam dentro de repetições Alu, enquanto nenhuma das três deleções de *TSC1* parecem ser mediadas por repetições Alu (Longa et al. 2001).

No capítulo III desta tese é apresentado o relato de caso de um paciente jovem com fenótipos que não são comumente relacionados à ET: pólipos gastrointestinais e tumor neuroendócrino pancreático. O paciente apresentava uma grande deleção no gene *TSC2*, englobando éxons 2 a 16, e incluindo códon de iniciação (Reis et al. 2020). Os rearranjos gênicos correspondem a aproximadamente 15% das variantes patogênicas encontradas no gene *TSC2* (Padma Priya and Dalal 2012) e a alteração observada no paciente relatado resultou na completa remoção do domínio de ligação TSC1 (T1BD) na porção N-terminal da proteína TSC2. Esse domínio é fundamental para a interação de TSC1-TSC2 e formação do complexo TSC, que quando ausente resulta na ubiquitinação e degradação da proteína TSC2 e hiperativação da via de mTORC1 (Hodges et al. 2001; Huang and Manning 2008).

Estudos recentes envolvendo caracterização de alterações genéticas em ET demonstram a ocorrência de deleções envolvendo mais de um éxon de *TSC2*. Em uma amostra de 21 pacientes chineses, foram encontradas duas deleções de códon único e uma grande deleção envolvendo os éxons 10 a 16 (He et al. 2020). Em uma amostra ainda maior de chineses, foram encontradas grandes deleções apenas no gene *TSC2*, em 5 de 19 pacientes sem mutação identificada por investigação na sequência de DNA, que corresponderam a 7,9% de todas as mutações encontradas no estudo. Destas, uma envolveu os éxons 1 ao 37, uma foi identificada como deleção do éxon 16 e três foram deleções completas de *TSC2* combinadas com a deleção do último éxon do gene adjacente *PKD1*, causador da doença renal policística autossômica dominante tipo 1 (PKD1) (Lin et al. 2019). A deleção envolvendo os genes *TSC2* e *PKD1* é associada a síndrome de deleção contígua TSC2-PKD1 que pode envolver diferentes éxons dos dois genes e ocasiona lesões policísticas renais progressivas de início precoce (Brook-Carter et al. 1994; Boehm et al. 2007; Consugar et al. 2008). Mais recentemente, deleções nesses dois genes foi associada ao surgimento de rabdomioma cardíaco (Osumi et al. 2020) e espasmos infantis (Matsubara et al. 2021).

Além de contribuir para a caracterização de apresentações fenotípicas mais raras da síndrome, este relato de caso ressalta a importância da investigação molecular de rearranjos. Os estudos apresentados nessa discussão demonstram a variabilidade das alterações moleculares na ET, indicam a importância do protocolo diagnóstico molecular clínico que deve incluir a cobertura total do gene e análise deste, tanto por sequenciamento de nova geração quanto por um método que permita detecção de grande rearranjos (Tyburczy et al. 2015).

Variantes patogênicas nos genes *TSC1* e *TSC2* são identificadas em 75% a 90% dos pacientes com diagnóstico clínico de esclerose tuberosa, porém uma parcela significativa de pacientes permanece sem identificação da causa molecular (Dabora et al. 2001; Padma Priya and Dalal 2012; Nellist et al. 2015; Kingswood et al. 2017; Rosset et al. 2017a). Em uma coorte de 490 casos, Sancak et al. identificaram 29 variantes (6%) que não puderam ser classificadas como patogênicas ou neutras a partir dos dados clínicos e genéticos disponíveis, sendo classificadas como VUS (Sancak et al. 2005). Outra questão importante é que apesar do notável progresso na pesquisa de esclerose tuberosa na última década, é que o teste molecular convencional, mesmo seguido de rastreio de grandes rearranjos, não é suficiente para identificar variantes patogênicas nos genes *TSC1* e *TSC2* em 10% a 25% dos casos (NMI = *non-mutated individuals*) (Camposano et al. 2009). Nesse contexto, o desenvolvimento de metodologias que possam avaliar o impacto funcional de VUS, bem como explorar outras regiões (p.ex. reguladoras ou intrônicas) do gene em pessoas não mutadas (NMI) com fenótipo de ET, torna-se bastante relevante (Nellist et al. 2015). No capítulo IV desta tese, são apresentados os resultados parciais de uma abordagem de caracterização de variantes de significado incerto (VUS) encontradas em três pacientes, localizadas no íntron 7 de *TSC1*, íntron 10 e éxon 19 de *TSC2* e possíveis alterações em quatro pacientes sem identificação de variante a nível de DNA. A detecção de uma VUS ou a ausência de qualquer variante em um(a) paciente com fenótipo clínico característico de ET é um desafio para a prática

clínica e aconselhamento genético e com essa abordagem pretende-se colaborar com essa questão.

Em uma investigação de processamento aberrante de éxons nos transcritos dos genes *NF1*, *NF2* e *TSC2* em amostras de tumores, realizada por Kaufmann e colaboradores (2002), foram encontradas alterações *in-frame* em cinco dos sete éxons investigados do gene *NF1*, em dois dos três éxons do gene da neurofibromatose tipo 2 (*NF2*) e em um de três éxons do gene *TSC2* em todos os tecidos tumorais humanos testados, bem como em linhagens de células tumorais. Esses achados demonstram que *o* processamento aberrante é um processo muito comum em tumores e pode ser atribuível à rara ocorrência de estruturas alternativas no local do sítio doador do processamento, que não são reconhecidas pelo spliceossomo. Em células *HeLa*, o processamento aberrante ocorreu mais frequentemente em temperaturas elevadas e baixo pH, condições frequentemente encontradas em tecidos tumorais (Kaufmann et al. 2002).

Os capítulos IV e V desta tese tem por objetivo investigar a hiperativação do complexo mTORC1 em decorrência de alterações em seu principal complexo regulador, TSC. Como já abordado anteriormente, variantes inativadoras nos genes *TSC1* e *TSC2* levam à ativação sustentada da via homólogo Ras enriquecido no cérebro (RHEB) da mTORC1, uma proteína quinase que regula o tamanho celular em muitos organismos. mTORC1, por sua vez, inibe processos catabólicos, incluindo autofagia, e ativa processos anabólicos, incluindo tradução de mRNA (Feliciano 2020). Assim, mTORC1 é o principal regulador negativo da formação canônica dos autofagossomos, através da ativação do complexo Atg1/ULK1 (Hosokawa et al. 2009). A hiperativação de mTORC1 é uma característica fundamental na patogênese da ET e de outras doenças genéticas do neurodesenvolvimento, coletivamente denominadas MTORopatias (Ebrahimi-Fakhari et al. 2017b) e em ET está associada principalmente a ocorrência de defeitos de desenvolvimento e predisposição a tumores, especialmente do sistema nervoso central (Crino 2011; Costa-Mattioli and Monteggia 2013). Alterações no processo de autofagia também já foram descritas nas manchas hipomelanociticas, sinais comuns em ET (Yang et al. 2018).

Embora mTORC1 regule negativamente a autofagia, o impacto da autofagia na sinalização de mTORC1, em particular *in vivo*, ainda não é completamente elucidado. Recentemente foi demonstrado por Wang e colaboradores (2019) que células progenitoras neurais de camundongos com deleção do gene *Tsc1* e do gene essencial da autofagia *Fip200,* sob condições de estresse energético, requerem autofagia para manter a hiperativação de mTORC1 apesar da deficiência do complexo TSC. A hiperativação de mTORC1 nessas células aumentou a glicólise aeróbica e o armazenamento de lipídios via lipofagia que servem como uma fonte de energia alternativa, para atender ao aumento da demanda de energia. A inibição da lipofagia ou de sua via catabólica a jusante reverteu os fenótipos defeituosos e reduziu a tumorigênese em modelos de camundongo, descrevendo um novo paradigma de regulação de mTORC1 por lipofagia seletiva e catabolismo lipídico (Wang et al. 2019). Na investigação de dezenove espécimes de tubérculos corticais de ET ressecados cirurgicamente não foi encontrada diminuição significativa do nível de autofagia em neurônios dimórficos e células gigantes, o que pode estar relacionado ao mecanismo compensatório da via de sinalização da AMPK frente a baixos níveis energéticos (Ge et al. 2019). Esses resultados revelam uma função cooperativa da autofagia seletiva no acoplamento da disponibilidade de energia com a patogênese da ET, além do papel paradoxal da autofagia na doença, abordado detalhadamente no capítulo V desta tese.

As intervenções terapêuticas farmacológicas disponíveis hoje para tratar aspectos específicos da esclerose tuberosa são os inibidores da via de mTOR (Schwartz et al. 2007; Franz et al. 2016; Canpolat et al. 2018). Embora promissor, o tratamento com rapamicina e seus análogos para indicações específicas (certos tumores) na ET possuem alto custo, podem apresentar efeitos colaterais significativos e não são totalmente efetivos no controle e na erradicação dos tumores (Franz et al. 2016; Canpolat et al. 2018). Outros inibidores, chamados de análogos da rapamicina têm sido estudados (Schwartz et al. 2007) mas também apresentam alto custo, ainda são em sua maioria indisponíveis no SUS e carecem de maiores informações relacionadas principalmente a variabilidade da resposta terapêutica para que possam ser usadas mais amplamente na prática clínica.

De maneira geral, os inibidores da via mTOR atuam na supressão da via e na indução da autofagia (Lee et al. 2009; Li et al. 2019). O processo de autofagia, por sua vez, regula os níveis de nutrientes na célula, sendo capaz de tanto de reprimir quanto estimular o desenvolvimento tumoral (Galluzzi et al. 2015; Medvetz et al. 2015; Reis et al. 2021) e pode responder a diferentes compostos reguladores do metabolismo celular como metformina e soro de baixa glicose. Esses compostos têm sido usados como indutores de autofagia em alguns estudos (Kossoff et al. 2005; Kalender et al. 2010; Jiang et al. 2011; Auricchio et al. 2012; Howell et al. 2017; Amin et al. 2019). Devido a esse potencial, nossa proposta foi de testá-los como tratamentos alternativos para o controle de potenciais tumores na ET, considerando que a indução da autofagia pode estabilizar os níveis exacerbados da via de mTOR (Ebrahimi-Fakhari et al. 2017b; Wang et al. 2019; Feliciano 2020). O capítulo VI apresenta uma abordagem exploratória de uso desses compostos em comparação com a rapamicina, a fim de verificar uma possível indução e restauro da autofagia em uma linhagem primária de fibroblastos de paciente com variante patogênica em heterozigose de *TSC2* e uma linhagem controle. Nossa hipótese é de que esses compostos possam restaurar a via de autofagia nas células não-tumorais, na tentativa de impedir a progressão tumoral das células. Futuramente, havendo comprovação do racional teórico aqui estabelecido, ensaios clínicos seriam o próximo passo para avaliar a efetividade destas intervenções na prática clínica.

CONCLUSÕES

Esta tese de doutorado teve como principal objetivo utilizar abordagens evolutivas e moleculares para contribuir na caracterização da neurofibromatose 1 e esclerose tuberosa, bem como ampliar o entendimento da patogênese da esclerose tuberosa investigando processos celulares relacionados a autofagia. Nessa proposta, uma parte significativa das atividades previstas envolviam atividades experimentais em bancada e recrutamento ativo de pacientes, as quais foram severamente afetadas, por restrições de recursos financeiros e pelas restrições impostas pela pandemia do novo coronavírus a partir de março de 2020. Em relação aos objetivos específicos originalmente propostos, estão descritas a seguir as principais conclusões obtidas nessa tese.

Com as análises realizadas envolvendo NF1, através das análises filogenéticas *in silico* foi possível contribuir com a compreensão do impacto de grandes rearranjos envolvendo quatorze genes co-deletados em pacientes com microdeleções. A análise filogenética sugere restrição evolutiva distinta entre os genes analisados. Foi demonstrado que quatro dos genes frequentemente co-deletados estão sob seleção positiva, incluindo aminoácidos específicos da proteína RNF135 em seu domínio funcional principal, reforçando sua importância e contribuição para as doenças humanas. Além disso, os outros dez genes co-deletados mostraram-se sob seleção purificadora, que está relacionada à importância de manter sua função durante a evolução dos organismos. Através dos resultados deste estudo esperamos ajudar a esclarecer a correlação da co-deleção desses genes e o fenótipo mais grave do NF1 e lançar luz sobre a importância da inclusão do gene *RNF135* na avaliação diagnóstica molecular. Embora os resultados da tese ainda sejam exploratórios, ele se junta a outras evidências que demonstram o possível efeito deste gene como um modificador de fenótipo.

Na doença ET, apresentamos descrição de relato de caso que também apresenta um grande rearranjo, dessa vez envolvendo a deleção de 15 éxons incluindo o códon de iniciação do gene *TSC2* e a presença de tumores

gastrointestinais e tumor neuroendócrino pancreático, duas associações raras, além da ausência de alterações genéticas em genes associados a câncer colorretal e polipose colorretal. A descrição deste paciente juntamente com uma revisão da literatura atual apresentados neste trabalho, lançam luz sobre a importância de considerar polipose do trato gastrointestinal e tumores neuroendócrinos. Tratando-se de investigação de mecanismo da ET, apresentamos uma revisão bibliográfica sobre a participação dos genes *TSC1* e *TSC2* como reguladores da autofagia, bem como o papel das rotas alternativas de autofagia, como privação de nutrientes e fatores de crescimento, e de compostos indutores de autofagia em ET. Além disso, descrevemos o papel paradoxal da autofagia na formação de tumores da síndrome, que pode atuar na sobrevivência de tumores estabelecidos, fornecendo precursores metabólicos durante a privação de nutrientes; e paradoxalmente, a autofagia excessiva é associada à morte celular em algumas situações. A melhor compreensão da genética molecular dessas doenças, bem como definição de correlações genótipo-fenótipo poderá influenciar o manejo destes pacientes e familiares, incluindo aconselhamento genético e manejo terapêutico.

PERSPECTIVAS

Considerando os resultados gerados pela presente tese, destacando a caracterização molecular dos pacientes com NF1 e ET, as seguintes perspectivas emergem para o melhor entendimento dessas doenças pelo grupo:

- Estudo similar ao apresentado no capítulo IV, com a caracterização dos transcritos de RNA de pacientes com NF1 e variantes sinônimas ou ausência de variante patogênica nas sequencias codificadoras de DNA, previamente analisados pelo grupo (Rosset et al. 2018);

- Ampliação das análises *in vitro* para incluir um número maior de linhagens celulares de pacientes com ET e diferentes variantes patogênicas e também avaliar o efeito dos compostos em tumores comumente identificados na ET;

- Avaliação de outros compostos conhecidos por modular a via da autofagia, tais como resveratrol e tioxantona e comparação com a rapamicina em fibroblastos de pacientes com ET e diferentes mutações germinativas nos genes *TSC1* e *TSC2*, a fim de encontrar outro composto com potencial modulador de autofagia;

- Utilização de sistema de edição gênica CRISPR-Cas9 para indução mutacional no segundo alelo (originalmente sem mutação) de fibroblastos de pacientes ET com mutação germinativa, com o objetivo de mimetizar a perda de heterozigosidade e avaliar possíveis marcadores tumorais nas células editadas.

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8. PRODUÇÃO CIENTÍFICA ADICIONAL DURANTE O DOUTORADO

Artigo científico publicado no período, relacionado à Tese de Doutorado:

"Systems Biology Approaches Reveal Potential Phentoype-Modifier Genes in Neurofibromatosis Type 1"

Cancers, 2020, 12, 2416, doi: 10.3390/cancers12092416

Artigo científico publicado no período não relacionado à Tese de Doutorado:

"Skin pigmentation polymorphisms associated with increased risk of melanoma in a case-control sample from southern Brazil"

BMC Cancer, 2020, 20, 1069, doi: 10.1186/s12885-020-07485-x

Comunicação breve publicada no período, não relacionada à Tese de Doutorado:

"MIR605 rs2043556 is associated with the occurrence of multiple primary tumors in TP53 p. (Arg337His) mutation carriers"

Cancer Genetics, 2020, 240, doi: 10.1016/j.cancergen.2019.11.005

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Systems Biology Approaches Reveal Potential Phenotype-Modifier Genes in Neurofibromatosis Type 1

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Abstract: Neurofibromatosis type (NF1) is a syndrome characterized by varied symptoms, ranging from mild to more aggressive phenotypes. The variation is not explained only by genetic and epigenetic changes in the *NF1* gene and the concept of phenotype-modifier genes in extensively discussed in an attempt to explain this variability. Many datasets and tools are already available to explore the relationship between genetic variation and disease, including systems biology and expression data. To suggest potential NF1 modifier genes, we selected proteins related to NF1 phenotype and *NF1* gene ontologies. Protein–protein interaction (PPI) networks were assembled, and network statistics were obtained by using forward and reverse genetics strategies. We also evaluated the heterogeneous networks comprising the phenotype ontologies selected, gene expression data, and the PPI network. Finally, the hypothesized phenotype-modifier genes were verified by a random-walk mathematical model. The network statistics analyses combined with the forward and reverse genetics strategies, and the assembly of heterogeneous networks, resulted in ten potential phenotype-modifier genes: *AKT1, BRAF, EGFR, LIMK1, PAK1, PTEN, RAF1, SDC2, SMARCA4,* and *VCP*. Mathematical models using the random-walk approach suggested *SDC2* and *VCP* as the main candidate genes for phenotype-modifiers.

Keywords: neurofibromatosis type 1; phenotype-modifier genes; systems biology

1. Introduction

Neurofibromatosis type 1 (NF1) is a disease with a worldwide birth incidence of 1 in 2500 and a prevalence of at least 1 in 4000 [1]. The main clinical features are café-au-lait spots, axillary and inguinal freckling, cutaneous and subcutaneous neurofibromas, and Lisch nodules, occurring in almost every NF1 patient. Other less -ommon characteristics are scoliosis, macrocephaly, learning disabilities, plexiform neurofibromas, and multiple other benign and malignant tumors [2,3]. Inter-familial and intra-familial variability in NF1 is extensive: cutaneous neurofibromas may vary in number from dozens to thousands; about 30% to 50% of patients are affected by large plexiform neurofibromas; only about

10% of them develop malignant peripheral nerve sheath tumors (MPNSTs), an aggressive sarcoma and one of the most critical symptoms [2,4,5]. Other tumors outside the central nervous system occur in different frequencies between NF1 patients: low grade pilocytic astrocytomas, pheochromocytoma, gastrointestinal stromal tumor, thyroid tumors, ovary and lung tumors, breast cancer, juvenile myelomonocytic leukemia, myelodysplastic syndrome, osteosarcoma, and rhabdomyosarcoma [3–5].

NF1 is caused by dominant loss-of-function mutations in the tumor suppressor gene *NF1*, which encodes neurofibromin, an interactor of Ras GTPase proteins [6]. Although NF1 is a monogenic disorder of dominant character, only a few associations between a specific *NF1* variant and the disease phenotype have been reported to date. Four genotype–phenotype correlations are well described in the literature: NF1 patients harboring microdeletions have been reported to have an increased risk of malignant peripheral nerve sheath tumors, lower average intelligence, connective tissue dysplasia, skeletal malformations, and dysmorphic facial features [7–9]; the 3-bp in-frame deletion c.2970_2972delAA was previously associated with absence of neurofibromas [10]; the missense variant p.Arg1809Cys was associated with developmental delay and/or learning disabilities, pulmonic stenosis, and Noonan-like features, but no external plexiform neurofibromas [11]; and missense mutations affecting *NF1* codons 844–848 were associated with a more severe clinical presentation [12].

Apart from the aforementioned correlations, NF1patients with the same mutation may develop severe symptoms or a mild clinical expression [13–15]. Modifier genes, environmental factors, epigenetic factors, or a combination of them may be responsible for the remaining variability [16,17]. Modifier genes include any genes, protein-coding sequences, microRNA, and long noncoding RNA that influence one or various features of the NF1 phenotype. Primarily, modifier genes were found to be associated with phenotype variation in NF1 in large family studies, and posteriorly, NF1 animal models and knock-in and knockdown strategies have reinforced these assumptions [18]. Several strategies to discover and understand modifiers genes have been developed to help to explain the NF1 variability and were reviewed recently [19–25]. These strategies have identified important candidate modifier genes, and some hypotheses and associations have been established so far [16,20,22]. However, many NF1 characteristics and variability remain unexplained.

Systems biology is an integrative field that combines molecular biology experiments and computational analysis. Its aim is to understand the simplest interactions in the complexity of an organism by the evaluation of interaction networks [26,27]. By integrating genomics, proteomics, and phenotype information, it is possible to evaluate how each of these elements acts as disturber-mechanism in a specific network. This strategy consists of a very effective and economical approach to explore the disease, and might even be applied if there is little information obtained from differential gene expression studies. Hence, through systems biology tools it is possible to perform genomics research by introducing a forward or reverse strategy. The former is a strategy used by evaluating the candidate genes and how they could explain the phenotype, whilst the second strategy starts from the outcome (here NF1), and evaluates which the genes and mechanisms could be connected to it [28]. The use of a deep phenotype characterization is a good approach in conditions with heterogeneous phenotypes, when combined with next-generation sequencing data [29]. For a better comprehension of these molecular mechanisms, ontologies databases have been widely used for a correct assortment of the gene function and in the phenome characterization [30].

In this context, by using this approach, the present study searches for novel candidate NF1 modifier genes. Considering that the modifier genes could play a role in the NF1 signaling pathway or other related and unrelated pathways, in silico analyses were performed through systems biology tools involving the *NF1* gene, its protein–protein interaction network, and its related genes or phenotype ontologies. Network statistics suggested ten candidate genes and mathematical models highlighted the roles of two of them as NF1 phenotype modifiers.

2. Results

A scheme presenting the main steps of the present study is available in Figure 1. To better comprehend the parameters used in each analysis, please see the Methods section.

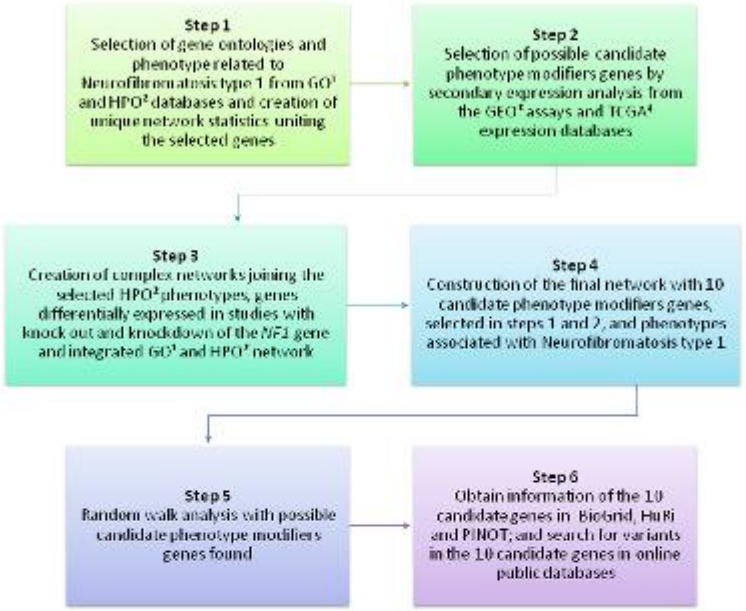

Figure 1. Main steps of the present study. The scheme shows the main steps in chronological order to identity potential NF1 phenotype-modifier genes. Gene Ontology (GO)[1]; Human Phenotype Ontology (HPO)[2]; Gene Expression Omnibus (GEO)[3]; The Cancer Genomic Atlas (TCGA)[4].

2.1. Gene and Phenotype Ontologies Analyses

Gene Ontology (GO) describes a biological domain considering three aspects: molecular function, cellular component, and biological process. The Human Phenotype Ontology (HPO) provides a standardized vocabulary of phenotypic abnormalities encountered in human diseases. NF1 GO biological processes and NF1 HPO were analyzed by two coworkers individually. The chosen ontologies are listed in Table S1.

HPO selection provided 1697 genes related to NF1 phenotype (OMIM 162200), whilst GO filter yielded 1449 genes included in the same ontologies previously selected for the *NF1* gene. When comparing both strategies, it was observed that HPO and GO analysis had 265 genes in common (Figure 2a). To assemble a network of the ontologies' selected genes, we used the STRING tool, observing protein–protein interactions (PPI) that were previously described by experimental assays. The separate networks generated for GO and HPO analyses are represented in Figure S1 and Figure S2, respectively. A combined network, comprising NF1 direct interactions (first neighbors) for both GO and HPO strategies is available in Figure 2b.

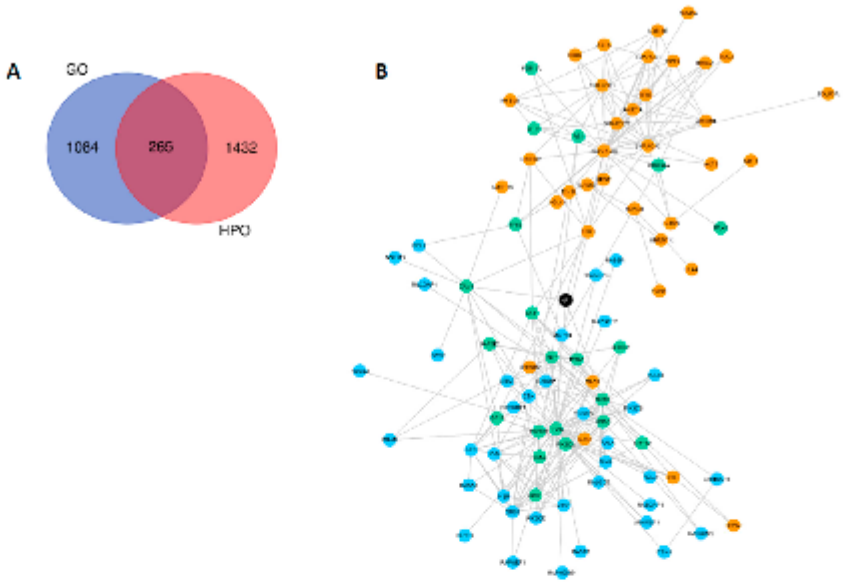

Figure 2. NF1 Gene Ontology (GO) and NF1 Human Phenotype Ontology (HPO) results. (**A**) Venn diagram showing in red genes related with neurofibromatosis type 1 exclusively found by the HPO project in the selected ontologies for NF1; genes exclusively found by the GO consortium using the ontologies selected for NF1 are shown in blue; and in purple genes shared by both GO and HPO analysis. (**B**) A combined network using the STRING tool using the 1697 genes selected exclusively in HPO (orange nodes); 1449 genes selected exclusively in GO (blue nodes); and 265 genes observed in both HPO and GO (green nodes). The network shows only direct protein–protein interactions with NF1 (first neighbors).

2.2. *Network Statistics*

To verify the nodes with relevant roles in the information flow from the network assembled in the previous section (Figure 2), systems biology network statistics were applied using Cytoscape v.3.7.2 software. Two main parameters were observed: (I) betweenness centrality, a measure based on the communication paths, meaning the nodes with high betweenness centrality could be important in the control of the information flow; and (II) the closeness centrality measure, which is based on the fastness of this information flow (from the central node to the others) [31]. The resulting network had 1561 nodes, making it difficult to visualize the main nodes. A simplified version, representing only the first neighbors, can be assessed in Figure 3.

According to this strategy, AKT1 presents the highest levels of betweenness and closeness centrality. However, despite NF1 being a highly connected protein in the network evaluated, it presented low levels of betweenness and closeness centrality, as can be observed by the node size (small) and color (light yellow, compared to the dark orange elements). Hence, we aimed to evaluate HPO and GO networks separately using the same approach to minimize the possibility of overlooking potential phenotype-modifier genes, as described in the following section.

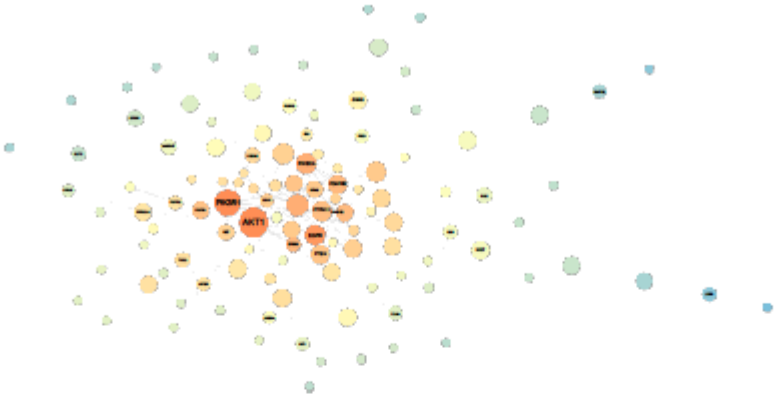

Figure 3. Betweenness centrality and closeness analysis of the STRING network previously generated in Figure 2b using GO and HPO. Large nodes have a more central role in communication among other nodes (hub/hub-like nodes), i.e., more connections. The darker orange the nodes are, the faster information flows towards the central node; i.e., they have the potential to impact the whole network even when having few connections (bottleneck nodes). Thus, nodes can be visualized in four categories: (1) large/dark-orange nodes = hub/bottleneck nodes; (2) large/blue nodes = hub/non-bottleneck nodes; (3) small/dark-orange nodes = non-hub/bottleneck nodes; and (4) small/blue nodes = non-hub/non-bottleneck hubs. NF1 is represented by the yellow node on the right side of AKT1.

2.3. *Forward and Reverse Genetics Strategies*

As mentioned before, GO and HPO databases are related, respectively, to the gene function and phenotype association. Hence, the observations of their independent networks, previously represented in Figures S1 and S2, were based on the forward and reverse genetics concepts.

When evaluating betweenness and closeness centrality by the forward genetics strategy (GO network), six genes were selected: *AKT1*, *RAF1*, *LIMK1*, *BRAF*, *EGFR*, and *PTEN*. In the other analysis, the reverse genetics approach (evaluating the HPO network) provided six genes as well: *PAK1*, *VCP*, *AKT1*, *SMARCA4*, *RAF1*, and *PTEN*. Together, the strategies provided nine candidate genes for neurofibromatosis phenotype modifiers. Besides, the network communities were evaluated, and *AKT1* was identified as the network main hub, whilst the *RAF1* gene had the highest score for authority. The authority score estimates the importance of the node itself, and the hub score measures this importance based on the other nodes which are linked to the main hub. Despite the network statistics having provided these candidates, we wanted to observe whether or not their expression was altered in the absence of *NF1*. Therefore, the next step was designed to conduct gene expression analyses to evaluate this hypothesis.

2.4. *Differential Gene Expression Networks*

To comprehend the differential gene expressions (DGEs) of the candidate phenotype-modifier genes, we performed secondary expression analysis on the data available in two public repositories: The Cancer Genome Atlas (TCGA) and the Gene Expression Omnibus (GEO).

Using the GEO database, *NF1* knockdown and knockout assays were selected: GSE14038 and GSE115406. The log fold-change (logFC) and false discovery rate (FDR) values for the ten potential modifier genes in each dataset are presented in Table S2.

In TCGA, we selected seven different types of tumors for which samples with nonsense mutations in *NF1* were available. We evaluated tumors that presented *NF1* nonsense mutations against tumors with wildtype *NF1*. The logFC and adjusted *p*-values, after the FDR correction, for the seven tumors evaluated and ten candidate genes are available in Table S3. Despite the somatic origins of these tumors,

we believe this information is valuable in order to check how NF1-loss could affect the global gene expression in a tissue/site-specific way, and check for signatures more related to a certain phenotype.

For both TCGA and GEO assays, few genes were evidenced to be significantly differentially expressed, demonstrating that the expression of all the candidates, and for the *NF1* gene, is strictly regulated. We did not identify a variable expression profile between the tumors evaluated, and knockout assays. Therefore, we performed other systems biology analysis with the PhenomeScape application v.1.0.4 from Cytoscape software, assembling a complex network (Figure 1, step 3). For that, we used as input (I) the ontologies selected from HPO database (Figure 1, step 1); (II) the network generated in STRING tool, as also mentioned in step 1 (Figure 1); and (III) expression data from studies selected from GEO database (Figure 1, step 2). The resulting upregulated genes (overexpressed) were presented in red and the downregulated (lower expression) in green. These networks are available in Figures S3–S7. *NF1* is downregulated (lower expression) in the knockdown and knockout studies, and upregulated (overexpressed) in the evaluation of malignant tumors when compared to benign neurofibromas. *NF1* is absent from the network when its expression is not significantly altered in the expression dataset.

Besides the genes previously selected in the forward and reverse genetics analysis, when evaluating the complex networks, we observed that the *SDC2* gene also had its expression altered when *NF1* was affected. Furthermore, *SDC2* is the first neighbor of *NF1*, which means both genes share a direct protein–protein interaction.

2.5. Systems Biology Approaches Reveal 10 NF1 Phenotype-Modifier Candidate Genes

Table 1 shows the final list of the 10 genes selected as potential phenotype-modifiers in this study and summarizes the strategies by which they were found. We then generated a complex network comprising all the candidate phenotype-modifier genes selected so far, and the NF1 phenotypes they are related to (Figure 4); the phenotypes were provided by the PhenomeScape tool, according to the data available in HPO database. Finally, we used a mathematical model to evaluate whether one of those genes could be a stronger candidate as a phenotype-modifier than the others, which is described in the following section.

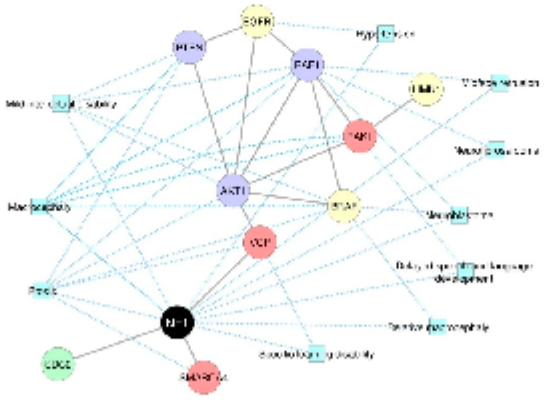

Figure 4. A complex network evaluation comprising the 10 candidate phenotype-modifier genes and the NF1 phenotypes they are related to. The ten candidate NF1 phenotype-modifier genes suggested by our analysis are represented with the NF1 phenotypes they are related. Yellow nodes: genes selected with the forward genetics strategy; red nodes: genes selected with the reverse genetics strategy; purple nodes: genes selected by forward and reverse genetics strategies; green node: gene selected by evaluating the differential gene expression in the complex network. Blue squares: phenotypes provided by the PhenomeScape tool, according to the associations presented in the HPO database.

Table 1. Potential phenotype-modifier genes selected in this study. Characterization of the 10 potential phenotype-modifier genes and the approaches used for their selection.

Gene (OMIM)	Aliases	Cytogenetic Location	Summary	PINOT [1]	STRING [2]	BioGrid	Human Interactome	HPO	GO	Phenome Scape	Direct Strategy	Forward Strategy
AKT1 (*164730)	*AKT, CWS6, PKB, PKB-ALPHA, PRKBA, RAC, RAC-ALPHA*	14q32.33	Serine/threonine kinase - development of the human nervous system; mediator of growth factor-induced neuronal survival; can inactivate components of apoptosis			X		X	X		X	X
BRAF (164757)	*NS7; B-raf; BRAF1; RAFB1; B-RAF1*	7q34	Serine/threonine kinase - role in regulating the MAP kinase/ERK signaling pathway						X		X	
EGFR (131550)	*ERBB, ERBB1, HER1, NISBD2, PIG61, mENA*	7p11.2	Cell surface protein - acts as a receptor for members of the epidermal growth factor family which induces cell proliferation	X		X			X		X	
LIMK1 (601329)	*LIMK; LIMK-1*	7q11.23	Serine/threonine kinase - regulates actin polymerization; it is ubiquitously expressed during development; associated with cytoskeletal structure						X		X	
IMK1 (602590)	*IDDMSSD; p68-IMK; IMKalpha; alpha-IMK IDS; DEC;*	11q13.5-q14.1	Serine/threonine kinase - cytoskeleton reorganization and nuclear signaling; regulates cell motility and morphology; essential for the RAS-induced malignant transformation					X				X
PTEN (601728)	*CWS1; GLM2; MHAM; TEP1; MMAC1; PTEN1; PTENbeta*	10q23.31	Phosphatidylinositol-3,4,5-triphosphate 3-phosphatase that functions as a tumor suppressor	X		X		X	X		X	X
RAF1 (164760)	*NS5; CRAF; Raf-1; c-Raf; CMD1NN*	3p25.2	MAPs kinase - involved in the cell division cycle, apoptosis, cell differentiation and cell migration			X		X	X		X	X
SDC2 (142460)	*HSPG; CD362; HSPG1; SYND2 BRG1; CSS4;*	8q22.1	Syndecan proteoglycan protein - mediates cell binding, cell signaling, and cytoskeletal organization	X	X	X	X			X		
SMARCA4 (603254)	*SNF2; SWI2; MRD16; RTPS2; BAF190; SNF2LA; SNF2L3; hSNF2b; BAF190A; SNF2-beta*	19p13.2	Part of the large ATP-dependent chromatin remodeling complex required for transcriptional activation of genes normally repressed by chromatin	X	X			X				X
VCP (601023)	*p97; TERA; CDC48*	9p13.3	Plays a role in protein degradation, intracellular membrane fusion, DNA repair and replication, regulation of the cell cycle, and activation of the NF-kappa B pathway	X	X	X	X	X				X

[1] PINOT = Protein Interaction Network Online Tool; [2] STRING = Search Tool for Recurring Instances of Neighboring Genes.

2.6. Random Walk Analysis

A random walk is a mathematical model known as a random process. It is based on the idea that a gene (node) is an imaginary particle that performs a succession of random steps (interactions) in a network [32]. Our aim was to evaluate whether these random steps could lead the gene to the phenotype, which was set as neurofibromatosis type 1 (OMIM 162200). For this goal, we performed the random walk analysis with the *RandomWalkRestartMH* package in R v.3.6.2.

According to this mathematical model, the *NF1* gene only had to take "one step" (one interaction) to reach the phenotype OMIM 162200. The genes *SDC2* and *VCP* had to take only two steps (two interactions) (Figure 5), whilst the other eight candidates needed more interactions to cause the syndrome (Figure S8). Genes *LIMK1* and *PAK1* also needed a higher number of potential interactions, and hence more steps, than the others.

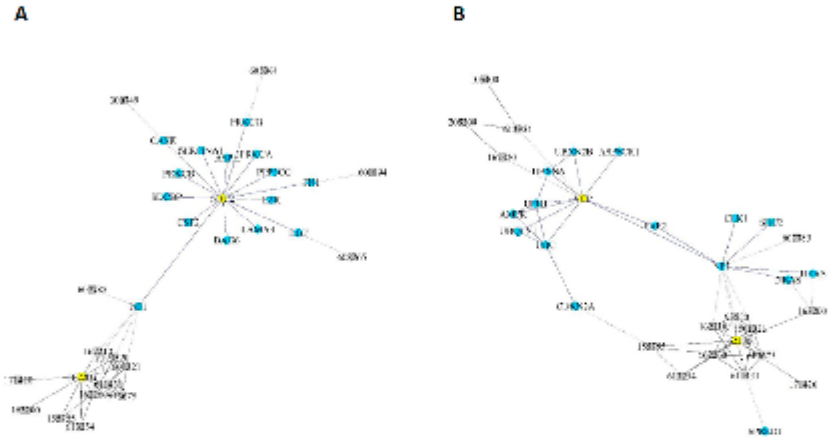

Figure 5. Random walk analysis. The minimum steps (interactions) between the selected genes (nodes) and the neurofibromatosis 1 phenotype were calculated. Two analyses are shown: (**A**) one for *SDC2*; and (**B**) one for *VCP*.

With this analysis, we confirmed all the candidates as potential phenotype-modifier genes. However, the results using this model pointed to *SDC2* and *VCP* as being more directly connected to the NF1 phenotype.

2.7. Literature Review and Genomic Databases Evaluation

To check for genetic variants already described in our 10 candidate genes, we looked at two databases: The Genome Aggregation Database (gnomAD v. 2.1.1), which spans 125,748 exomes and 15,708 genomes from unrelated individuals; and ClinVar, which aggregates information about genomic variation and its relationship to human health. For gnomAD, we found a total of 11,211 variants (Table 2). *SMARCA4* and *EGFR* have the highest numbers of variants (2575 and 2000, respectively); *SDC2* and *PTEN* the lower (335 and 456, respectively).

In ClinVar, germline variants were reported for all genes (N = 5216), with the exception of *SDC2*. Almost half (46.4%) of them were submitted as variants of uncertain significance (VUS). *EGFR* has the highest rate of VUS (67.3%), while all the 36 *LIMK1* variants are classified as benign/likely benign (B/LB). On the opposite way, *PTEN* and *BRAF* have the highest number of pathogenic/likely pathogenic (P/LP) variants, corresponding to 32.5% and 23.9% of all reported variants, respectively (Table 3).

Table 2. Variants reported in gnomAD for the 10 candidate genes. The total numbers and percentages of variants are presented according to their annotations.

GENE	All	Missense	Synonymous	Splice Site	Frameshift	Inframe Del/Ins	Intronic	Nonsense	Stop Lost	Start Lost	5′UTR	3′UTR
AKT1	932	166 (17.81%)	155 (16.63%)	80 (8.58%)	3 (0.32%)	4 (0.43%)	435 (46.67%)	1 (0.11%)	0	0	36 (3.86%)	52 (5.58%)
BRAF	1073	230 (21.44%)	170 (15.84%)	56 (5.22%)	1 (0.09%)	8 (0.75%)	561 (52.28%)	2 (0.19%)	1 (0.09%)	0	13 (1.21%)	31 (2.89%)
EGFR	2000	682 (34.10%)	387 (19.35%)	117 (5.85%)	15 (0.75%)	2 (0.10%)	867 (43.35%)	16 (0.80%)	1 (0.05%)	0	9 (0.45%)	104 (5.20%)
LIMK1	1133	322 (28.42%)	214 (18.89%)	58 (5.12%)	5 (0.44%)	1 (0.09%)	469 (41.39%)	3 (0.26%)	1 (0.09%)	0	44 (3.88%)	16 (1.41%)
PAK1	774	128 (16.54%)	121 (15.63%)	58 (7.49%)	4 (0.52%)	6 (0.78%)	401 (51.81%)	5 (0.65%)	1 (0.13%)	0	8 (1.03%)	42 (5.43%)
PTEN	456	83 (18.20%)	77 (16.89%)	17 (3.73%)	5 (1.10%)	0	223 (48.90%)	5 (1.10%)	0	0	28 (6.14%)	18 (3.95%)
RAF1	1005	264 (26.27%)	145 (14.43%)	65 (6.47%)	4 (0.40%)	2 (0.20%)	490 (48.76%)	7 (0.70%)	0	2 (0.20%)	8 (0.80%)	18 (1.79%)
SDC2	335	102 (30.45%)	52 (15.52%)	18 (5.37%)	5 (1.49%)	3 (0.90%)	114 (34.03%)	0	0	5 (1.49%)	22 (6.57%)	14 (4.18%)
SMARCA4	2575	473 (18.37%)	551 (21.40%)	164 (6.37%)	4 (0.16%)	22 (0.85%)	1318 (51.18%)	0	1 (0.04%)	0	9 (0.35%)	33 (1.28%)
VCP	928	135 (14.55%)	191 (20.58%)	72 (7.76%)	1 (0.11%)	1 (0.11%)	488 (52.59%)	0	0	0	20 (2.16%)	20 (2.16%)

Table 3. Germline variants submitted to ClinVar database for each candidate gene. The number of variants is presented according to its classification. Related syndromes and other relevant conditions for NF1 phenotype are also summarized.

GENE	Classification *					Related Syndromes ***	Other Relevant Reported Conditions
	All **	B/LB	P/LP	CI	VUS		
AKT1	182	94	8	4	76	Cowden, Proteus	E17K variant was associated with 22 conditions, including breast cancer
BRAF	334	125	80	12	117	Cardiofaciocutaneous, Dandy-Walker malformation, LEOPARD, PHACE, Noonan	Astrocytoma, glioma
EGFR	199	54	4	7	134	Cowden, not otherwise specified (NOS) hereditary cancer	Cerebral arteriovenous malformation, inflammatory skin and bowel disease
LIMK1	36	36	0	0	0	-	-
PAK1	6	2	3	-	1	-	intellectual developmental disorder with macrocephaly, seizures, and speech delay
PTEN	1567	367	510	22	668	Bannayan-Riley-Ruvalcaba, Cowden, Hereditary breast and ovarian cancer, NOS Hereditary cancer-predisposing, Proteus-like	Macrocephaly/autism, Phophatase and Tensin (PTEN) Homolog hamartoma tumor
RAF1	412	155	43	17	197	LEOPARD, Noonan	Chordoma, retinoblastoma, and
SDC2	-	-	-	-	-	-	leri pleonosteosis are reported in patients carrying the copy number gain of 8q22.1, which includes *SDC2*
SMARCA4	2310	980	95	81	1154	Coffin-Siris, NOS Hereditary cancer-predisposing, Rhabdoid tumor predisposition	Craniopharyngioma, intellectual deficiency, medulloblastoma, neurodevelopmental disorder, neuroblastoma
VCP	170	64	18	10	78	-	Amyotrophic lateral sclerosis, paget disease, Charcot-Marie-Thoth disease

* LB = likely benign; B = benign; LP = likely pathogenic; P = pathogenic; CI = conflicting interpretation; VUS = variant of uncertain significance. ** Unprovided interpretations and drug response variants were not considered. *** Syndromes/conditions reported for benign/likely benign variants, drug response variants, and not-interpreted variants were not considered.

Finally, we also explored TCGA and Genomics Evidence Neoplasia Information Exchange (GENIE) datasets to check for tumor samples harboring both genetic alterations in one of our candidate genes and *NF1*. Due to lack of samples or to the higher mutational and clinical heterogeneity, we managed to make reasonable assumptions only for *AKT1*, *VCP*, and *SDC2*. More details are presented in the discussion section.

3. Discussion

It is evident that genetic variants in *NF1* do not act alone to determinate disease phenotype. Many factors may contribute to disease variability, including environmental factors, the occurrence of epigenetic alterations, and somatic second hits in NF1-associated tumors. The accumulation of somatic NF1 mutations is much more difficult to evaluate since each tumor needs to be sequenced individually, but it may be responsible for some level of NF1 variability. Other symptoms, like delayed mental development, are less influenced by second hit mutations. Genetic modifiers in a single locus or the interaction between several genes may suppress or enhance disease severity, including genes involved in the pathways other than the NF1-Ras-mTOR pathway. There is evidence that genetic modifiers explain a major fraction of phenotypic variation in NF1 [16]. A few genes and their variants have already been described as phenotype modifiers in literature and were reviewed and summarized in Table 4, but they are still insufficient to explain all the variability found in NF1 patients.

Recently, a review pointed out the main methods with which to discover novel phenotype-modifier genes in Mendelian diseases and formulate hypotheses about other pathways than Ras-NF1 that could be phenotype modifiers [44]. The most used methods to select candidate modifier genes are whole-genome sequencing, genome-wide association studies, and experimental approaches using animal models. Other studies also select candidate modifier genes using differential gene expression analysis [20]. These strategies have proved their value in identifying a few phenotype-modifier genes to date; however, they have some disadvantages, such as the high costs involved, being time-consuming, the use and maintenance of animal models, and the confounding factors in studies with selected NF1 patients [18].

One of these limitations was observed in our expression analysis, for which a few candidate genes were actually differentially expressed. Furthermore, differential expression analysis using TCGA tumor samples (Table S2) generated distinct results when compared to GEO controlled knockdown/knockout experiments (Table S3). Expression may depend on the tumor heterogeneity, i.e., the number of cells that are actually not expressing functional NF1, and its location, since the expression profiles of *NF1* and related genes are tissue-dependent. Gene expression is by far the most common analysis among multi-omics studies. Despite that, in many studies it is not possible to obtain a clear scenario of the biological mechanisms disrupted in a disease by evaluating only the mRNA levels. Known disease genes are often not differentially expressed in affected individuals, once the mutations may only alter the protein function or post-translational mechanisms. As a consequence, much information contained in transcriptomics datasets are ignored, demanding an alternate strategy to evaluate these multi-omics assays [45]. On the other hand, the differential gene expression networks also allowed the selection of the *SDC2* gene as a new candidate, once its expression was altered when *NF1* was affected. This example highlighted the need to evaluate the multi-omics data in a more integrated and multidisciplinary perspective [46].

Table 4. List of genes and proteins previously described as NF1 phenotype modifiers in the literature.

Genes/Proteins	Consequence	Methodology Aspects	Reference
ADCY8	Genetic polymorphisms in *ADCY8* are correlated with glioma risk in NF1 in a sex-specific manner, elevating risk in females while reducing risk in males	- Genotyping of NF1 patients using Affymetrix whole-genome human SNP array - Primary astrocyte cultures from NF1-CKO mice and treatment with dideoxyadenosine to induce ADCY inhibition - cAMP regulator expression with qPCR and ELISA	Warrington et al. 2015 [33]
ANRIL allele T of SNP rs2151280	Higher number of plexiform neurofibromas; rs2151280 reduced *ANRIL* transcript levels	- High-resolution array comparative genomic hybridization (aCGH) of PNFs from NF1 patients	Pasmant et al. 2011 [22]
ATP6V0B SNP rs7161 *DPH2* SNP rs4660761 *MSH6* SNP rs1800934	*ATP6V0B* is associated with melanosome biology rs7161 and rs4660761 associated with café-au-lait macule (CALM) count; rs1800934 associated with development an NF1-like phenotype	- Lymphoblastoid cell lines with NF1-associated phenotypes - Gene expression (microarray and qPCR) - Sequencing of genes with increased expression in patients with high count CALM - Meta-analysis	Pemov et al. 2014 [19]
CRLF3, ADAP2, RNF135, UTP6, SUZ12, OMG, LRRC37B, EVI2A, EVI2B, RAB11FIP4, RAB11FIP3, TEFM, ATAD5, CORPS, NF1 large 17q11 deletions encompassing the entire NF1 locus and neighboring genes	Dysmorphic features, learning disabilities, cardiovascular malformations, childhood overgrowth, a higher tumor burden and earlier onset of benign neurofibromas, and probably, a higher incidence of malignant peripheral nerve sheath tumors (MPSTs)	-MLPA, breakpoint-spanning PCR and FISH in NF1 deletion patients	Mautner et al. 2010 [13]
CXCR4 and its ligand, CXCL12	Highly expressed in mouse models of NF1-deficient MPNSTs, but not in normal precursor cells; Suppression of CXCR4 activity decreases MPNST cell growth in culture and inhibits tumorigenesis in allografts and in spontaneous genetic mouse models of MPNST; Demonstrated conservation of these activated molecular pathways in human MPNSTs	- NF1-deficient skin-derived precursor models (SKPs) and gene expression microarray (normal SKPs; pretumorigenic SKPs with either Nf1 deletion or Nf1 and p53 deletion) - qPCR, westerblot and IHC of CXCR4 and CXCL12 - CXCR4 shRNA for knockdown in SKP MPNST cells - Tissue microarray from plexiform neurofibromas in NF1 patients harboring MPNSTs and MPNSTs samples from NF1 and sporadic patients - CXCR4 cDNAs sequencing	Mo et al. 2013 [34]
GDNF R93W germline variant and maternally inherited *NF1* mutation	Congenital megacolon development	- Investigation of a family carrying variants in *GDNF* and *NRTN* genes with cutaneous manifestations of NF1 and megacolon - Haploinsufficient animal models for *Nf1* and *Trp53* that developed MPNSTs	Bahuau et al. 2001 [35]

Table 4. *Cont.*

Genes/Proteins	Consequence	Methodology Aspects	Reference
miR-34a	Down-regulation of miR-34a founded in most MPNSTs compared to neurofibromas; The p53 inactivation and subsequent loss of expression of miR-34a may contribute to MPNST development	- Microarray of MPNSTs, neurofibromas, Schwannomas, and synovial sarcomas - MPNST cell lines to check for miR-34a and other p53-dependent miRNAs by qRT-PCR after overexpressing wild-type p53	Subramanian et al. 2010 [36]
miR-21	Important in MPNST tumorigenesis and progression through its target, PDCD4	- Global miRNA expression profiling of MPNSTs and neurofibromas - qPCR of differentially expressed miRNAs in MPNSTs, 11 NFs, and 5 normal nerves and MPNST cell lines - Knockdown of miR-21 in MPNST cells	Itani et al. 2012 [37]
miR-204	Down-regulation of miR-204 contributes to development and tumor progression of MPNSTs	- Global miRNA expression profiling of MPNSTs and benign NF1 neurofibroma tissues - qPCR of differentially expressed in tumor tissues and MPNST cell lines - Lentiviral system for miR-204 transfection in NF1 and non-NF1 MPNST cell lines - Non-NF1 MPNST cells Xenograft	Gong et al. 2012 [38]
MSH2, MSH6, MSH3, MLH1, PMS2	Phenotype overlapping between NF1 and Constitutional mismatch repair deficiency (CMMRD) Association with rare childhood malignancies	- Literature review about co-occurrence of symptoms and variants in genes associated with CMMRD and NF1	Wimmer, Rosenbaum and Messiaen 2017 [39]
SDHB	Cause gastrointestinal stromal tumor (GISTs)	- SDHB expression by immunohistochemically in NF1-associated GISTs	Wang, Lasota and Miettinen 2011 [40]
Serotonin receptor *5'UTR* 5-HT$_6$ - HTR6 protein	Disrupting HTR6-neurofibromin interaction prevents agonist-independent HTR6-operated cAMP signaling in the prefrontal cortex, an effect that might underlie neuronal abnormalities in NF1 patients; 5-HT$_6$ receptor may be considered as a potentially therapeutic target to correct some NF1-related cognitive deficits	- Nf1+/− heterozygote mice - HEK-293T and NG108-15 cell lines - Immunoprecipitation followed by Western blot analysis	Deraredj Nadim et al. 2016 [41]
SPRED1 nonsense, frameshift and missense mutations	Complete *SPRED1* inactivation is needed to generate CALMs	- GWAS in unaffected and affected individuals. - *SPRED1* cDNA sequencing - Melanocyte cell culture from normal skin and CALM of NF1 patient -Mouse embryonic fibroblasts	Brems et al. 2007 [42]
TERT mRNA and telomerase activity	Telomere dysfunction may play a role in driving genomic instability and clonal progression in NF1-associated MPNST	- High-resolution Single Telomere Length Analysis (STELA) of cutaneous and diffused plexiforme neurofibromas, and MPNSTs	Jones et al. 2017 [43]

Network analysis makes it possible to combine different multi-omics studies, a strategy that has been applied in several personalized medicine studies evaluating genetic syndromes with phenotypic variability [47–49]. Recently, many projects and consortiums were created, gathering a huge amount of public results with germline and somatic mutation databases, transcriptomics, proteomics, and metabolomics data that can now be evaluated with a systems biology tools [44]. The analysis proposed here can be seen as an optimization in the search for candidate genes acting as phenotype modifiers in NF1, which can later be confirmed by the more robust molecular and functional assays. A brief description of the potential phenotype modifiers found in this study and their variants is provided below to show mechanistic insights and facilitate experimental studies. They are also summarized in Tables 1–3, respectively.

The ontologies selection was an important step in our analysis, especially because *NF1* is important in several molecular mechanisms. If not filtered, our analysis could be later compromised by evidencing genes that are not so deeply associated to neurofibromatosis type 1, but which are more frequently studied in cancer (i.e., *TP53* and developmental genes). This strategy has also been applied in studies that do not identify (or do not have access to) differentially expressed genes [50,51]. For Human Phenotype Ontology (HPO), our workflow was based on a deep phenotyping strategy (computational analysis of detailed, individual clinical abnormalities) [29], according to the heterogeneity of neurofibromatosis type 1 visualized in our patients. We also aimed to avoid ontologies related to congenital anomalies outside the NF1 spectrum of phenotypes, especially the ones that could have led to embryo lethality or severe impairments (major malformations) that would have been diagnosed before NF1. Embryo development is a critical, stepwise controlled process that can be disrupted by genetic or environmental factors, such as maternal infections or exposures [52]. Since the data on *NF1* expression in the embryonic period are scarce, and we do not have information about the maternal genome or environment, we focused on the functional anomalies (more related to the fetal period) that are better characterized in neurofibromatosis type 1.

In our forward strategy, we found the epidermal growth factor receptor (EGFR) that acts upstream of NF1 in the Ras signaling pathway. EGFR belongs to a family of receptor tyrosine kinases that are anchored to the cytoplasmic membrane. EGFR is frequently over-activated in cancer and studies have shown that it is not expressed by normal Schwann cells but it is overexpressed in subpopulations of NF1 mutant Schwann cells [53]. The great involvement of this gene in different cancers and its differential expression patterns in NF1-enriched tissues may indicate that the occurrence of minor variants in this gene could act as phenotype modifiers in NF1. There are 2000 *EGFR* variants registered in the gnomAD database, 34.1% of them missense mutations. In ClinVar, most of the 199 catalogued germline variants are VUS (N = 134). Among the four P/LP variants, one (C326F) was related to Cowden syndrome. Variants in promoter and UTR regions might also have a potential as phenotype modifiers, since animal models have already shown that high levels of *EGFR* expression modify the initiation of neurofibromas, increasing their numbers [54].

AKT1, *BRAF*, *LIMK1*, *PTEN*, and *RAF1* genes were also suggested by the forward strategy. They encode proteins that act downstream of NF1 in the Ras signaling pathway. Phosphoinositide 3-kinase PI3K/AKT is one of the most frequently activated pathways in cancer. This activation may occur through mutation of multiple genes, including *PTEN*, *PIK3R1*, and *mTORC1* [55]. *AKT1* presented the highest levels of betweenness and closeness centrality in systems biology network analysis, demonstrating itself to be the most relevant gene in the information flux among our selected genes (Figure 3).

AKT1 germline mutations are mainly associated with Cowden syndrome, characterized by the appearance of hamartomas, and an increased risk of developing multiple cancers, especially breast cancer [56]. One particular pathogenic variant, E17K, is reported to be linked to 22 different conditions in ClinVar. This variant was also found in gnomAD in a European individual. To explore how this alteration could modify the phenotype when co-occurring with *NF1* mutations, we looked at mutational and clinical data deposited in the GENIE database (v7.0). When excluding *AKT1*-mutated

patients and considering only *NF1* mutations with more deleterious effects (nonsense, frameshift, and splicing variants), the most frequent cancer types are non-small cell lung cancer (12%), glioma (10%), and melanoma (9%). However, when grouping samples with both *AKT1*-E17K and *NF1* mutations, breast cancer becomes the most prevalent, corresponding to 73% of all tumors. The link between breast cancer susceptibility and *NF1* alterations was already established [18]. However, the mechanism that leads to this specific phenotype remains to be elucidated and *AKT1* emerges as a strong candidate.

PI3K-Akt pathway activity is negatively regulated by phosphatase and tensin homolog protein (PTEN) [57]. PTEN is a tumor suppressor and its inactivation has a role in plexiform neurofibroma tumorigenesis and progression to high-grade peripheral nerve sheath tumors in the context of NF1 loss in Schwann cells, which is a very variable symptom in NF1, and may also participate in the mechanism of tumorigenesis of other tumors related to NF1 [58,59]. There are not many variants catalogued for *PTEN* in gnomAD (N = 456), but 1546 were already reported in ClinVar, with 35% still being classified as VUS. The phenotypes related to *PTEN* variants are, as expected, similar to *AKT1*, including Cowden syndrome and hereditary breast and ovarian cancer syndrome. Considering the importance of both *AKT1* and *PTEN* in tumorigenesis, variants in the corresponding genes, not necessarily pathogenic, could act as a modifier of NF1 disease and need to be further investigated.

In contrast, BRAF studies in NF1 patients were already conducted. *BRAF* gene encodes a protein belonging to the RAF family of serine/threonine protein kinases. This protein plays a role in regulating the MAPK/ERK signaling pathway, which affects cell division, differentiation, and secretion. Germline mutations in *BRAF* were previously associated with cardiofaciocutaneous, Noonan, and Costello syndromes [60]. In ClinVar, 334 germline variants were already submitted, 80 being classified as P/LP and 117 of the remainder as VUS. A recent study analyzed a cohort of 100 patients clinically suspected of NF1 and identified 73 *NF1* mutations and two *BRAF* novel variants. The clinical features of NF1 patients with co-occurrence of *NF1-BRAF* mutations were severe, and *BRAF* variants may have a synergistic role in determining NF1 phenotype [61].

Another member of the same family, the *RAF1* gene, was suggested by the forward strategy. The *RAF1* gene encodes a serine/threonine kinase protein that functions downstream of RAS and activates MEK1 and MEK2. In GENIE, *RAF1* mutations are observed in various cancers. LEOPARD and Noonan syndromes were already associated in ClinVar with 6 and 28 pathogenic *RAF1* variants, respectively. The other 197 variants were still classified as VUS and included conditions such as other rasopathies, chordoma, and retinoblastoma.

Finally, by the forward strategy, *LIMK1* was suggested as a phenotype modifier. The N-terminal domain of LIM kinase 1 (*LIMK1*) regulates actin dynamics, affects cell adhesion and migration by phosphorylating cofilin, and negatively regulates the Rac1/Pak1/LIMK1/cofilin pathway [62]. *NF1* is an upstream regulator of *LIMK1* by acting on cofilin phosphorylation. When *NF1* is mutated, this pathway is affected, possibly influencing neuronal development and cognitive deficits associated with the disease [62,63]. We found 1133 *LIMK1* variants in gnomAD, but only 36 reported as germline in ClinVar, all of them classified as benign/likely benign. That may indicate that mutated *LIMK1* alone is not pathogenic, but it does not exclude a combined effect of *NF1* variants acting as a phenotype modifier.

By the reverse strategy, *AKT1*, *PTEN*, and *RAF1* were also suggested, reinforcing the possible roles of these genes in NF1 phenotypes. Additionally, *PAK1*, *SMARCA4*, and *VCP* were found. The kinase PAK1 is a Rac/CDC42-dependent serine/threonine kinase that acts by activating several kinases such as RAF, ERK, and LIMK1, and other related pathways by activating TGFα and VEGF. PAK1 is required for the malignant growth of RAS transformants in NF1 neurofibrosarcoma cell lines [64,65]. There are not many PAK1 variants registered in gnomAD (N = 774) and only six in ClinVar. However, two LP variants (Y131C and Y429C) reported in ClinVar were associated with an intellectual developmental disorder with macrocephaly, seizures, and speech delay, phenotypes that are reported in NF1 patients.

SMARCA4 is a central component of the switch/sucrose-non-fermentable (SWI/SNF) chromatin remodeling complex. Inactivating mutations and loss of expression in several components of this complex have been implicated in carcinogenesis [66–68]. Thus, variants in one of these genes might

influence the NF1 phenotype. *SMARCA4* has the highest number of variants in both gnomAD and ClinVar among our candidate genes: 2575 and 2310, respectively. Peripheral nerve sheath tumors were already reported in patients with the syndrome carrying *SMARCB1* mutations, which belongs to the same family of *SMARCA4* [14]. Loss of *SMARCB1* was also related to Schwannoma, another phenotype found in NF1 despite being more frequent in NF2 [69].

The valosin-containing protein (*VCP*) appeared as a strong candidate for being an NF1 phenotype modifier by our random-walk analysis. *VCP* gene is associated with the multisystem degenerative autosomal dominant disorder of inclusion body myopathy with Paget disease of bone and frontotemporal dementia (IBMPFD) and mutations were related to 1–2% of amyotrophic lateral sclerosis cases [70]. However, missense mutations in *VCP*, and low-effect and low-penetrant mutations in this gene, have controversial roles in causing disease. Neurofibromin interacts with VCP through its Leucine- Rich Domain (LRD)-domain [71]. Patients with NF1 who have mutations in the LRD coding region were described to be more prone to developing cognitive deficits than those with mutations elsewhere in the *NF1* gene [72]. In the same study, it was observed that point mutations in the LRD coding region in the *NF1* gene abolished the ability of NF1 to interact with VCP, while *VCP* mutants were shown to have reduced affinity for NF1. Interestingly, non-disease-associated polymorphisms in the LRD region of the *NF1* gene may increase the risk of an IBMPFD patient developing dementia. In the same way, polymorphisms in the *VCP* gene that code for domains that interact with NF1 might influence the NF1 phenotype. These data obtained from literature research reinforce the accuracy of our systems biology analysis and random-walk mathematical model, which pointed *VCP* as a strong NF1 phenotype modifier candidate.

It would not be a surprise if *VCP* variants were found co-occurring with *NF1* alterations, especially the ones in ATPAse domains 1 and 2 (D1 and D2), responsible for interacting with neurofibromin's LRD-domain. For example, in ClinVar only two variants are reported as pathogenic D1/D2, one in each domain. On the other hand, 34 remain as VUS, 23 in D1, and 11 in D2. In gnomAD, more than half of the (N = 491) cataloged *VCP* variants are located in D1 and D2 domains. Looking at TCGA, there are few samples (N = 28) with *NF1* mutations co-occurring with variants in *VCP* D1/D2 domains, most of them (47.2%) from uterine corpus endometrial carcinomas.

The last gene that was suggested by our analysis is *SDC2*, which was found by differential gene expression networks and pointed to as a strong candidate by our mathematical model. The heparan sulfate part of SDC2 interacts with extracellular matrix proteins and growth factors to act as an adhesion molecule and as a coreceptor [73]. Variants in this gene might be associated with autism spectrum disorder [74]. Interestingly, some studies showed a higher frequency of autism spectrum disorder in NF1 children, and this is a variable condition in NF1 that might be influenced by variants in other genes [75]. Despite *SDC2* emerging as a strong candidate in our study, only 335 variants were found in the gnomAD database and none in ClinVar, suggesting a highly conserved gene. However, the lack of *SDC2* in ClinVar may merely reflect its absence from gene panels used for diagnostic purposes. On the other hand, TCGA somatic samples carrying both *NF1* and *SDC2* mutations are scarce (N = 14/10,437), most of them (57.1%) also related to uterine corpus endometrial carcinomas. This finding does not exclude the gene as a phenotype modifier, but variants co-occurring with *NF1* alterations might be a rare event.

The literature search for variants and functions of the candidate modifier genes identified by our strategy shows that this is an economical and accurate way to filter and select genes that would be further validated by experimental assays. As a perspective, variants in the ten genes selected by our strategy will be searched in NF1 patients with different symptoms. Many strategies could be used to subsequently evaluate and validate the selected genes. One of them is to identify variants in these genes or other nearby genes and genotype those variants in NF1 patients with different symptoms and control populations, followed by statistical methods to identify correlations with the phenotype. Moreover, in-vitro and in-vivo studies are also useful for validating previously selected genes, focusing on CRISPR/Cas9 assays to induce partially and complete loss of the proteins. Our candidate genes

could be also included in commercial gene panels with a low impact on their coast, which would help to feed public databases such as ClinVar.

One obvious limitation of the present study is the lack of proper validation for the candidate phenotype-modifier genes using benchwork. Hence, the results obtained must be evaluated with caution. Experimental validation is necessary and strongly recommended before clinical extrapolation. However, our purpose was to provide a new look in the strategies for evaluation of neurofibromatosis using the huge amount of data already available in shared public-curated datasets. For example, the protein–protein interactions identified by us were previously validated by several in vitro assays and here combined in a network. Together with our network analysis, we also performed random walk, a robust mathematical model that has been applied in the analysis of biological multiplex heterogeneous networks [76,77]. We hope this complex and robust systems biology approach will help to better understand the neurofibromatosis type 1 and its phenotypic variation.

4. Materials and Methods

4.1. Selection of NF1 Ontologies

The complete list of NF1 gene ontologies (GO) and phenotype ontologies were obtained in the AmiGO and Human Phenotype Ontology (HPO) databases, respectively. In modifier studies, the selection of which phenotypes to study is a key step, and in NF1, several phenotypic features are time-dependent [19]. Then, we selected from both lists (GO and HPO) the ontologies related to the less frequent and variable characteristics and not necessarily time-dependent, presented by NF1 patients, as reported in the literature and in our clinical experience in the Oncogenetics clinics of Hospital de Clinicas de Porto Alegre [16,78,79]. For example, cutaneous neurofibromas are common and may occur in up to 99% of NF1 patients in a cohort; this is a variable characteristic, mainly in the number of neurofibromas, but it is less variable when considering their presence in NF1 patients. Thus, we focused on ontologies related to characteristics that occur in a smaller number of patients to try to explain the variability of less common but more aggressive NF1 symptoms, such as breast cancer, delayed mental development, plexiform neurofibromas, and facial dysmorphism. The processes and phenotypes selected for the analysis involved NF1 and NF1-related signaling pathways, such as the MAPK cascade and regulation of the Ras pathway, considering the upper ontology in the hierarchy of each database. Hence, some ontologies were not selected because there was an upper term in the hierarchy that encompassed these ontologies. It is worth mentioning that we followed a guide for the correct selection of ontologies to try to limit the bias introduced by the choice of terms and keywords [80]. The processes were evaluated by two independent researchers and selected for subsequent analysis when both researchers considered it relevant.

4.2. Systems Biology Analysis

Networks were generated using STRING database v.11, comprising protein–protein interactions (PPI) for *Homo sapiens*. Only experimental interactions were selected, with a minimum required interaction score set in 0.400 (default). The assembled networks were transferred to Cytoscape v.3.7.2 software, with which the network statistics was obtained. Big nodes represented proteins with high betweenness centrality scores and warm colors comprised proteins with high closeness centrality measures.

Comparison between networks was performed with DyNet application for Cytoscape v.3.7.2. Complex networks comprising HPO selected phenotypes, gene expression, and PPI networks were assembled using the PhenomeScape app, also in Cytoscape v.3.7.2, using the default settings.

4.3. Gene Expression Evaluation

RNA-seq and microarray secondary analysis were performed using studies selected in Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) databases. For the GEO studies,

we looked for *NF1* knockdown or knockout assays and selected only the ones performed in human tumor cells. The data extraction was performed manually, and the robust multiarray averaging (RMA) normalization was applied using *oligo* or *affy* R packages (R v.3.6.2). The differentially expressed genes were obtained using the *limma* package (R v.3.6.2).

Firstly, for TCGA, we selected seven somatic tumors with nonsense mutations in *NF1* (*NF1*-ns): bladder urothelial carcinoma (BLCA), brain lower grade glioma (LGG), breast invasive carcinoma (BRCA), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), colon adenocarcinoma (COAD), glioblastoma multiforme (GBM), and pheochromocytoma and paraganglioma (PCPG). Despite tumors having a somatic origin, this information can be useful to check how alterations in *NF1* could affect the global gene expression in a specific tissue/tumor (phenotype). We then compared these samples against wild type *NF1* tumors to check which genes were differentially expressed only in the *NF1*-ns group. The gene expression analysis for TCGA data was performed by extracting the data with *TCGAbiolinks* package and evaluating differential gene expression with *edgeR* package. All analyses were performed in R v.3.6.2.

4.4. Random Walk Analysis

The heterogeneous networks comprising both the genes and phenotypes selected were assembled in the *RandomWalkRestartMH* package, in R v.3.6.2, and the random walk analysis was performed with the same package.

4.5. Database Research

Other databases consulted to obtain data for the potential NF1 phenotype-modifier genes were: (1) BioGrid, for curated protein interactions; (2) PINOT tool, for literature data on curated protein interactions; (3) STRING database, for protein–protein interactions; and (4) The Human Reference Protein Interactome (HuRI), for the binary protein–protein interactions.

4.6. Variant Datasets

To explore variants in our candidate genes already reported in the general population or with clinical significance, we consulted The Genome Aggregation Database (gnomAD) v 2.1.1 and the ClinVar archive. For gnomAD, variants were classified according to its annotation. In ClinVar, only variants reported by at least one submitter as a germline were considered and classified according to their interpretations.

Finally, an additional analysis was performed consulting the 79,720 tumor samples made available by the AACR Project GENIE and the 10,967 samples from the TCGA PanCancer Atlas studies, using the cBioPortal for Cancer Genomics. Samples were filtered according to their mutational status: *NF1*-mutated patients including only nonsense, frameshift, and splicing; and patients with selected variants in our candidate genes, if available. Then, the clinical data were accessed and confronted with the mutational status to check which cancer types were predominant when *NF1* was exclusively altered and when *NF1* variant co-occurred with variants in our candidate genes.

5. Conclusions

We presented here a not yet explored systems biology strategy to investigate *NF1* phenotype modifiers. The public availability of multi-omics datasets makes possible the use of robust tools to generate complex networks including protein–protein interactions, differential expression data, and phenotypes, reinforced by mathematical models such as random-walk. Combining all these strategies, we found 10 candidate genes as potential NF1 phenotype modifiers. Resources and time may be scarce to carry out association studies and systems biology analyses makes possible to better explain the genetic heterogeneity of this complex syndrome. Our results must be interpreted cautiously in clinical application and may guide further in-vitro and in-vivo validation studies, saving time and

financial resources. The approach presented here may guide further in-vitro and in-vivo validation studies, saving time and financial resources.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6694/12/9/2416/s1. Figure S1: Network for GO analysis. Figure S2: Network for HPO analysis. Figure S3: A complex network generated using GEO MPNST dataset, protein–protein interactions, and HPO ontologies as input data. Figure S4: A complex network generated using GEO neurofibroma dataset, protein–protein interactions, and HPO ontologies as input data. Figure S5: A complex network generated using GEO *NF1*-shRNA, protein–protein interactions, and HPO ontologies as input data. Figure S6: A network generated using GEO CRISPR-induced *knockout* of *NF1*, protein–protein interactions, and HPO ontologies as input data. Figure S7: A network generated using GEO neuroblastoma cell line, protein–protein interactions, and HPO ontologies as input data. Figure S8: Random walk analysis calculating the minimum steps (interactions) that a candidate gene (node) takes to reach the neurofibromatosis 1 phenotype. Table S1: Selected NF1 ontologies, Table S2: *NF1* knockdown and knockout assays in GEO database, Table S3: Tumors with *NF1* nonsense mutations from TCGA database, Table S4: List of genes and proteins previously described as NF1 phenotype modifiers in the literature.

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Skin pigmentation polymorphisms associated with increased risk of melanoma in a case-control sample from southern Brazil

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Abstract

Background: Melanoma is the most aggressive type of skin cancer and is associated with environmental and genetic risk factors. It originates in melanocytes, the pigment-producing cells. Single nucleotide polymorphisms (SNPs) in pigmentation genes have been described in melanoma risk modulation, but knowledge in the field is still limited.

Methods: In a case-control approach (107 cases and 119 controls), we investigated the effect of four pigmentation gene SNPs (*TYR* rs1126809, *HERC2* rs1129038, *SLC24A5* rs1426654, and *SLC45A2* rs16891982) on melanoma risk in individuals from southern Brazil using a multivariate logistic regression model and multifactor dimensionality reduction (MDR) analysis.

Results: Two SNPs were associated with an increased risk of melanoma in a dominant model: rs1129038AA and rs1426654AA [OR = 2.094 (95% CI: 1.106–3.966), $P = 2.3\ 10^{-2}$ and OR = 7.126 (95% CI: 1.873–27.110), $P = 4.0\ 10^{-3}$, respectively]. SNP rs16891982CC was associated with a lower risk to melanoma development in a log-additive model when the allele C was inherited [OR = 0.081 (95% CI: 0.008–0.782), $P = 3\ 10^{-2}$]. In addition, MDR analysis showed that the combination of the rs1426654AA and rs16891982GG genotypes was associated with a higher risk for melanoma ($P = 3\ 10^{-3}$), with a redundant effect.

Conclusions: These results contribute to the current knowledge and indicate that epistatic interaction of these SNPs, with an additive or correlational effect, may be involved in modulating the risk of melanoma in individuals from a geographic region with a high incidence of the disease.

Keywords: Melanoma, SNPs, Pigmentation

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Background

Melanoma is the most aggressive skin tumor, and its incidence has been correlated with latitude of residence, occurring most frequently in fair-skinned individuals [1]. In fact, the risk of developing melanoma diverges markedly according to skin pigmentation and geographical area, mainly due to the causative effect of ultraviolet radiation [2, 3]. Currently, Australia and New Zealand have the highest incidence and mortality rates of melanoma in the world, with incidence reaching 33.6/100,000 and 33.3/100,000, respectively [4]. In those two countries, the risk of developing melanoma before age 75 years is 1/24 and 1/34 for males and females, respectively [5]. In Brazil, melanoma represents 4% of all skin cancers, and 6260 new diagnoses are estimated in 2018. The highest incidence rates per region are expected for southern Brazil, reaching 8.4/100.000 [6]. These high rates are attributed to geographical location– the southernmost state of Brazil, Rio Grande do Sul, is within the same latitude as Australia (30.0346° South) [7]. Social practices with intense and often unprotected sun exposure and a majority-European ancestry are associated with lighter pigmentation of the skin in these individuals [7, 8].

Approximately 10% of melanomas are caused by germline mutations in cancer predisposition genes [9]. These include genes predominantly associated with melanoma (such as *CDKN2A* and *CDK4*) but also genes related to multiple solid tumors including melanoma. Examples are, among others, the *BAP1* gene, the *PTEN* gene related to Cowden's syndrome and *XPD*, *XPC* and *XPA* genes related to Xeroderma pigmentosum. Most identifiable heritable mutations associated with hereditary melanoma have variable penetrance [10]. In addition to germline mutations, our understanding of the contribution of single nucleotide polymorphisms (SNP) proposed as risk modulators for melanoma is increasing [11]. Several common SNPs, usually of low penetrance, are commonly investigated in polygenic risk models. These models can assess the joint effect of independent SNPs in genes with lower and intermediate penetrance such as *MC1R*, *ARNT*, *CDK10*, identified by the GWAS, and can assist in the identification of individuals with a higher risk of susceptibility to melanoma [12]. Some of these SNPs are located in genes of the melanogenic pathway, and some have been described in association with melanoma in different populations across the world [13]. A complicating aspect of such studies is that, in multifactorial disorders such as melanoma, genetic and nongenetic factors, such as admixture, population substructure, and evolution patterns, can severely confound the results and result in false-positive associations [14]. Thus, differences in allele frequency between cases and controls could be associated with differences in ancestry rather than reflect an association of genes with disease [15].

The Consortium for Analysis of Diversity and Evolution in Latin America (CANDELA) is a multidisciplinary international study that involves researchers focused on studying the biological diversity of Latin Americans, analyzing samples from Mexico, Colombia, Peru, Chile, and Brazil for a wide range of issues relevant to anthropological, biological and medical research in these populations. In 2014, an analysis to evaluate a possible association between 18 SNPs in genes involved in the pigmentation pathway and Melanin Index (MI) was performed within the Brazilian cohort of Consortium for the Analysis of the Diversity and Evolution of Latin America (CANDELA) with participants born in Rio Grande do Sul (RS) and Bahia (BA), in the South and Northeast of Brazil, respectively. As a result of this analysis, four SNPs were associated with differences in MI in these populations: rs1126809 (p.Arg402Gln) on tyrosinase (*TYR*), rs1129038 (3'UTR) in the hect domain and rcc1-like domain (*HERC2*), rs1426654 (p.Thr111Ala) in solute support family 24, member 4 (*SLC24A5*) and rs16891982 (p.Phe374Leu) in the family of solute, member 2 (*SLC45A2*) carriers. Among these four SNPs, allele A of rs1426654 and allele G of rs16891982 were associated with less melanin content in the 352 participants of RS cohort ($P < .001$) [16].

In this study, we aimed to assess the association of these four SNPs with melanoma risk in southern Brazil, a region with important contribution of European ancestry and with the highest indices of melanoma in the country.

Methods

Samples and genotyping

This case-control study was conducted at a public University Hospital, Hospital de Clínicas de Porto Alegre (HCPA) in southern Brazil, was approved by the Institutional Review Board under number 07–139, and all participants provided informed consent. Overall, 255 unrelated individuals were recruited for the study between September 2007 and November 2008. All participants were born in the State Rio Grande do Sul, and of these, 120 had been diagnosed with melanoma. Among the melanoma patients, 19 had a family history of melanoma (melanoma in first-, second-, and/or third-degree relatives) and/or multiple primary melanomas and 101 had features of sporadic melanoma. All diagnoses were confirmed by pathology reports. The 135 individuals of the control group were recruited consecutively among patients who presented to the outpatient clinic of the same dermatology department for an initial consultation or regular follow-up for diseases other than skin cancer. None of the individuals included in the control group reported a family history of the disease, and within the patients and control groups, there were no familial relations. All patients willing to participate were clinically examined and demographic

variables, pigmentation traits (eye and hair color), skin type, tanning ability, quantitative/qualitative presence of nevi, and data from primary lesions of patients with cutaneous melanoma were documented. Genomic DNA was obtained from peripheral blood, and genotyping of SNPs rs1126809 (*TYR*), rs1129038 (*HERC2*), rs1426654 (*SLC24A5*) and rs16891982 (*SLC45A2*) based on the results of the Consortium for the Analysis of the Diversity and Evolution of Latin America (CANDELA, http://www.ucl.ac.uk/silva/candela) was performed using Human Custom *TaqMan*® SNP Genotyping Assays 40X (Applied Biosystems, USA; Assay IDs: AHBKFKH; C_48033–10; C_2908190_10; C_2842665_10, respectively). Genotyping were conducted using 20 ng of genomic DNA in a *StepOneTM Real-Time PCR System* (Applied Biosystems, USA). Allelic discrimination and analysis was performed using *the Real-Time PCR software v.2.2*. The study was conducted according to the Declaration of Helsinki Principles.

Ancestry analysis

Because the population structure due to admixture is a known confounding factor in association studies, the proportion of African, European, and Amerindian ancestry of all individuals recruited was evaluated using a previously published panel containing 61 biallelic short insertion/deletion polymorphisms (INDELs) [17].

Statistical analysis

Genotype and allele frequencies were obtained by simple counting. Differences between groups were compared using Pearson's chi square or Fisher's exact tests. All tests were two-tailed, and significance was set at 0.05. Wilcoxon test was performed to compare ancestry profiles between cases and controls, skin types and carriers and noncarriers of fixed alleles in European populations. The study was conducted considering two scenarios: with and without population structure control.

After obtaining the proportion of African, European, and Amerindian ancestry of the individuals recruited for this study we observed that some individuals had strikingly different ancestry profiles when compared to the majority of the sample, indicating populational substructure (Additional file 1). To control for this substructure, we performed 10,000 bootstrap simulations and calculated the average 95% confidence interval of these simulations to obtain the ancestry distribution profile. Using this confidence interval, we were able to identify samples exceeding the interval. This approach identified 29 individuals, which were removed to reduce sample substructure, which could skew the analysis. The remaining samples were used in all subsequent analyses, such as in the Hardy-Weinberg equilibrium test, logistic regression and MDR. To estimate the risk of melanoma associated with selected variants, we calculated odds ratios and their 95% confidence intervals using multivariate logistic regression analysis and controlled for the following confounders: age (discrete variable); sex; skin type according to the Fitzpatrick scale in 6 subtypes, hair color, number of nevi (more or less than 50 nevi); European and African ancestry. Eye color was not used as a confounding variable because it is a covariable of the color of skin and hair. We chose these variants for adjustment since they are established risk factors for melanoma [18]. All statistical analyses were performed using SPSS®, version 18 (IBM, USA) and R.

Multifactor dimensionality reduction

Higher-order gene-gene interactions among the SNPs associated in the logistic regression analysis were used in a nonparametric and genetic model-free multifactor dimensionality reduction (MDR) approach (version 3.0.4.). Bivariate MDR analysis was performed to verify the contribution of each SNP in the interaction and included *HERC2* rs1129038, *SLC24A5* rs1426654, and *SLC45A2* rs16891982. The model with the highest testing balance accuracy and with major cross-validation consistency was selected as the best model. Statistical significance was determined using a 1000-fold permutation test.

Results

Sample

Clinical features of the 254 individuals recruited are summarized in Table 1. Most individuals were female, older than 50 years at recruitment and fair-skinned (56.8% skin types I and II). The mean age was higher in the control group and the number of nevi was higher in the case group. A trihybrid ancestry profile with predominant European contribution was observed. Although admixture in the Brazilian population is expected and significant, in our sample, a homogeneity of European ancestry and a difference of European and African ancestry profiles between cases and controls were observed ($P = .004$ and $P = .008$, respectively). Additionally, mean European ancestry in individuals with skin types I and II was different than that observed in those with skin types III, IV and V (0.946, CI 95% [0.934–0.959] versus 0.902, CI 95% [0.875–0.928], $P = .002$). Moreover, carriers of almost all fixed alleles in European populations had a different ancestry profile when compared with noncarriers (the European ancestry profile A in rs1426654 was 0.928, CI 95% [0.915–0.942] and 0.746, CI 95% [0.021–1.470] in carriers and noncarriers, respectively, $P = .033$; the European ancestry profile G in rs16891982 was 0.936, CI 95% [0.924–0.947] and 0.740, CI 95% [0.561–0.918] in carriers and noncarriers, respectively, $P < 0.001$). Among melanoma patients, the average age at diagnosis was 53.72 years (SD15.5), and 23.5% had intraepithelial tumors, with the most common

Table 1 Characteristics of samples included in this study

	Global n = 255 (%)	Cases n = 120 (%)	Controls n = 135 (%)	P
Sex				
Female	157 (61.6)	73 (60.8)	84 (62.2)	0.461
Age[a]				
≥ 50 years old	180 (70.6)	78 (65)	102 (75.6)	
< 50 years old	75 (29.4)	42 (35)	33 (24.4)	0.012
Mean ages (SD)	57.01	56.31 (15.5)	58.28 (13.7)	
Skin type				
I	9 (3.5)	7 (5.8)	2 (1.5)	
II	136 (53.3)	66 (55)	70 (51.8)	
III	100 (39.2)	42 (35)	58 (43)	
IV	7 (2.7)	4 (3.3)	3 (2.2)	
V	3 (1.2)	1 (0.8)	2 (1.5)	0.281
Hair color				
Blond	52 (20.4)	30 (25)	22 (16.3)	
Red	11 (4.3)	9 (7.5)	2 (1.5)	
Light Brown	82 (32.1)	39 (32.5)	43 (31.8)	
Dark Brown	89 (35)	32 (26.7)	57 (42.2)	
Black	21 (8.2)	10 (8.3)	11 (8.1)	0.017
Eyes color				
Blue	70 (28.1)	39 (33.3)	31 (23.5)	
Green	47 (18.9)	27 (23)	20 (15.1)	
Brown	131 (52.6)	51 (43.6)	80 (60.6)	
Black	1 (0.4)	0	1 (0.7)	0.074
Number of nevi				
≥ 50	41 (16.5)	33 (28.2)	8 (6.1)	
< 50	207 (83.5)	84 (71.8)	123 (93.9)	0.000
Number of dysplasic nevi				
≥ 5	17 (6.8)	15 (12.8)	2 (1.5)	
< 4	231 (93.1)	102 (87.1)	129 (98.5)	0.000
Ancestry profile[b]				
European	0.971	0.945	0.911	0.004
African	0.010	0.022	0.039	0.008
Native-American	0.013	0.032	0.048	0.388

Abbreviation: *SD* standard deviation
[a]Samples age at recruitment
[b]Ancestrality profiles obtained for Ancestral Informative Markers (AIMs) indels panel. Date presented as median of percentile ancestry component

histological subtype being superficial spreading melanoma (Table 2).

Population substructure control

Using the 95% confidence interval calculated for each ancestry, we detected a population substructure (mean European ancestry was 0.971, CI 95% [0.583–0.991], mean African ancestry was 0.010, CI 95% [0.002–0.206], and mean Native-American ancestry was 0.013, CI 95% [0.003–0.280], see Additional file 1A. A total of 29 samples (13 cases and 16 controls) were outside the ancestry confidence interval and were excluded from the analysis of Hardy-Weinberg equilibrium and logistic regression (see Additional file 1B) in order to control for the population substructure (the entire list of excluded samples can be found in Additional Table 1).

Table 2 Clinical features of patients with melanoma and Histologic aspects of their tumors

Mean age at diagnosis (SD)	53.72 (15.5)
Breslow thickness/TNM Staging[a]	**n (%)**
in situ/ Tis	26 (22.6)
≤ 1.0 mm/ T1	41 (35.6)
1.01–2 mm/ T2	23 (20)
2–4 mm/ T3	14 (12.2)
> 4 mm/ T4	11 (9.6)
Histological subtype	
Acral lentiginous	2 (1.8)
Superficial spreading	78 (72.2)
Nodular melanoma	20 (18.5)
Lentigo maligna	8 (7.4)
Multiple primary melanoma	
Yes	8 (6.7)
No	112 (93.3)
Family History of melanoma	
Yes	19 (15.8)
No	101 (84.2)

Abbreviations: *SD* standard deviation, *TNM* classification of malignant tumours
[a]The Breslow thickness and TNM Staging according to National Comprehensive Cancer Network (NCCN) guidelines version 1.2011

Genotyping

Genotyping results are summarized in Table 3. Initially, we undertook a separate analysis of the SNP frequencies in individuals with and without a family history of melanoma in the case group and did not identify a significant difference between groups (data not shown). Therefore, we have opted to continue the additional analyses including all individuals in the case group (those with and without a family history of melanoma). Linkage disequilibrium was not observed, and only rs16891982 in *SLC45A2* did not follow Hardy-Weinberg equilibrium when considering the entire sample. After controlling for substructure, all SNPs were in Hardy-Weinberg equilibrium ($\alpha = 0.05$; rs1126809 *TYR* $\chi^2 = 0.089$, $P = 1$; rs1129038 *HERC2* $\chi^2 = 1.361$, $P = 1$; rs1426654 *SLC24A5* $\chi^2 = 0.796$, $P = 1$; rs16891982 *SLC45A2* $\chi^2 = 3.182$, $P = 0.498$). Further analysis showed a statistically significant difference in genotypic and allelic frequencies between cases and controls for rs1426654, rs16891982, and

Table 3 Allelic and genotypic frequencies of *TYR* rs1126809, *HERC2* rs1129038, *SLC24A5* rs1426654, *SLC45A2* rs16891982 variants

Gene	Chr.	Location	Position[a]	SNP	MAF	Genotypes	Cases n (%)	Controls n (%)	*P*
TYR	11	c.1205G > A	Exonic (p.Arg402Gln)	rs1126809	A = 0.22	GG	72 (46.7)	82 (53.2)	0.660[b]
						GA	45 (48.9)	47 (51.1)	
						AA	3 (33.3)	6 (66.6)	
HERC2	15	c.13272 + 874C > T	Intronic (3'UTR)	rs1129038	G = 0.45	AA	51 (42.5)	35 (25.9)	**0.016[b]**
						GA	45 (37.5)	62 (45.9)	
						GG	24 (20)	38 (28.1)	
SLC24A5	15	c.331A > G	Exonic (p.Ala111Thr)	rs1426654	G = 0.06	AA	117 (97.5)	111 (82.2)	**< 0.001[b]**
						GA	2 (1.7)	23 (17)	
						GG	1 (0.8)	1 (0.7)	
SLC45A2	5	c.1122C > G	Exonic (p.Phe374Leu)	rs16891982	C = 0.14	GG	103 (85.8)	93 (68.9)	**0.002[b]**
						CG	16 (13.3)	32 (23.7)	
						CC	1 (0.8)	10 (7.4)	

Abbreviations: Chr chromosome, SNP single nucleotide polymorphism, *MAF* minor allelic frequency
[a]Position in genome and protein change according to dbSNP
[b]Fisher chi-square for associating comparing the distribution of categorical variables in cases and controls subjects. *P* values are two-sided;
[c]Pearson chi-square for associating comparing the distribution of categorical variables in cases and controls subjects. *P* values are two-sided;

rs1129038. Comparisons of allelic frequencies between the main population databases and other population data of southern Brazilians are shown in Additional Table 2. Allelic frequency data reinforce similarities between South Brazilians and Europeans.

Genetic variants and skin pigmentation

Details from the comparative data on the associations between genotypic frequencies and skin pigmentation parameters in cases and controls are shown in Additional Table 2. With the exception of SNP *TYR* rs1126809, all the SNPs were associated with certain phenotypes. The SNP *HERC2* rs1129038 AA genotype was more frequent in individuals with light skin and eyes and blond hair in both cases and controls ($P < .001$ for all analysis). The SNP *SLC24A5* rs1426654 was also associated with lighter skin and eye color, but only in the control group ($P = .0017$). The SNP *SLC45A2* rs16891982 GG genotype was more frequent in individuals with fair skin and hair both in cases and controls ($P > .001$ and $P = .008$; $P < .001$ and $P = .004$, for cases and controls, respectively). The same genotype was associated with light eye color only among cases ($P < .001$).

Genetic variants as risk factors for melanoma

Clinical features of the melanoma patients are summarized in Table 2. Three of the four SNPs were associated with melanoma outcome. The *HERC2* rs1129038AA and *SLC45A2* rs16891982GG genotypes and the rs1426654A allele were more frequently observed in cases than controls ($P = .0016$, $P = .002$, and $P < 0.001$, respectively). In a regression logistic model, including the following risk factors: sex, age, hair color, skin type, number of nevi, and African ancestry, these associations remained strong, suggesting that they may be independent risk factors. Odds ratios (OR) for melanoma associated with genetic effect models that were obtained before and after genetic substructure control and are shown in Table 4. The dominant model for *HERC2* rs1129038 and *SLC24A5* rs1426654 was considered the best model, and in both, an increase in OR was observed after substructure control: [OR = 2.212 (95% CI: 1.106–4.426), $P = .025$] and [OR = 13.996 (95% CI: 1.711–113.995), $P = .014$], respectively. For *SLC45A2* rs16891982, the most suitable genetic model was log-additive, showing a slight reduction in the effect modification after substructure reduction when compared to the entire sample [OR = 0.068 (95% CI: 0.007–0.692), $P = .023$] and [OR = 0.081 (95% CI: 0.008–0.782), $P = .030$].

Additionally, we performed nonparametric Multifactor dimensionality reduction (MDR, v. 3.0.4.) to detect and characterize gene-gene interactions among *HERC2* (rs1129038), *SLC24A5* (rs1426654), and *SLC45A2* (rs16891982) in risk of developing melanoma (Moore et al., 2006). Significant two- ($P < .001$) and three-locus interactions ($P = .031$) were identified in our analysis (Table 5). According to these results, the best model for predicting melanoma development was the combination of the three factors (*HERC2* rs1129038, *SLC24A5* rs1426654, and *SLC45A2* rs16891982). More details about the criteria for selecting the best model can be found in [19]. The largest main effect with the higher information gain (IG) was observed for *SLC24A5* rs1426654 (5.63%), with 100% accuracy for this model. The contribution of the other two markers, *SLC45A2* rs16891982 (3.80%) and *HERC2* rs1129038 (2.34%),

Table 4 Odds ratio (OR) and Genetic Effects Models with and without Genetic Substructure evaluation

SNP	Effect	OR[a]	IC (95%)	P	AUC	IC (95%)
With Genetic Substructuring, *n* = 254						
rs1126809	Dominant	1.121	0.639–1.965	0.691	0.661	0.593–0.729
	Additive	1.921	0.412–8.961	0.406	0.660	0.592–0.729
	Recessive	1.999	0.444–9.003	0.367	0.660	0.592–0.728
rs1129038	**Dominant**	**2.121**	**1.105–4.070**	**0.024**	**0.714**	**0.651–0.777**
	Additive	0.510	0.220–1.183	0.117	0.714	0.651–0.777
	Recessive	1.137	0.569–2.270	0.716	0.707	0.643–0.771
rs1426654	**Dominant**	**7.164**	**1.868–27.472**	**0.004**	**0.729**	**0.667–0.791**
	Additive	1.538	0.091–25.909	0.765	0.728	0.666–0.790
	Recessive	0.509	0.029–8.937	0.644	0.697	0.632–0.763
rs16891982	Dominant	2.188	1.077–4.443	0.030	0.687	0.621–0.754
	Additive	**0.081**	**0.008–0.782**	**0.030**	**0.692**	**0.626–0.758**
	Recessive	9.278	0.939–91.701	0.057	0.665	0.598–0.733
Without Genetic Substructuring, *n* = 225						
rs1126809	Dominant	1.180	0.645–2.157	0.591	0.682	0.611–0.753
	Additive	3.077	0.502–18.849	0.188	0.688	0.618–0.758
	Recessive	2.951	0.494–17.643	0.235	0.689	0.619–0.759
rs1129038	**Dominant**	**2.212**	**1.106–4.426**	**0.025**	**0.705**	**0.635–0.774**
	Additive	0.390	0.157–0.967	0.042	0.698	0.629–0.767
	Recessive	1.501	0.715–3.151	0.283	0.695	0.625–0.765
rs1426654	**Dominant**	**13.966**	**1.711–113.995**	**0.014**	**0.725**	**0.659–0.790**
	Additive	0.000	0.000	0.998	0.713	0.659–0.788
	Recessive	0.000	0.000	1.000	0.693	0.623–0.762
rs16891982	Dominant	2.624	1.220–5.643	0.014	0.708	0.639–0.776
	Additive	**0.084**	**0.008–0.872**	**0.038**	**0.711**	**0.643–0.779**
	Recessive	9.261	0.902–95.118	0.061	0.688	0.618–0.757

Abbreviations: SNP single nucleotide polymorphism, *OR* odds ratio, *IC* confidence interval
[a]OR values was adjusted to the following risk factors: sex, Age, hair color, Skin type, Number of nevi, and African ancestry

indicated that they also have an important role in predicting melanoma risk. This interaction represents a high redundancy effect, which can be interpreted as an additive or correlation effect (Fig. 1). These finding are confirmed when analyzing the interaction graph with genotypic associations (Fig. 2). The combination of *SLC24A5* rs1426654AA and *SLC45A2* rs16891982GG was significantly associated with melanoma risk regardless of the genotype of *HERC2* rs1129038, confirming the lower weight of *HERC2* (2.34%) in the analysis of gene interaction. However, even isolated *HERC2* rs1129038 analysis shows that the AA genotype confers an increased risk for melanoma. Logistic regression models were created with the risk genotypes pointed out by MDR for rs1426654 and rs16891982 (AA and GG genotypes, respectively) and compared to a model without these risk alleles to identify the best model. The comparison, using ROC curve analysis, demonstrated that

Table 5 SNPs interaction by the multifactor dimensionality reduction (MDR) analysis

Models	Balanced Accuracy CV Training	Balanced Accuracy CV Testing	Cross-validation consistency	P[1]
rs1129038	0.5900	0.5190	5/10	0.564
rs1426654 and rs16891982	0.6216	0.6170	10/10	**< 0.001**
rs1129038, rs1426654 and rs16891982	0.6347	0.5863	10/10	**0.031**

[1]Evaluated using a 1000-fold permutation test to compare observed testing accuracies with those expected under the null hypothesis of null association

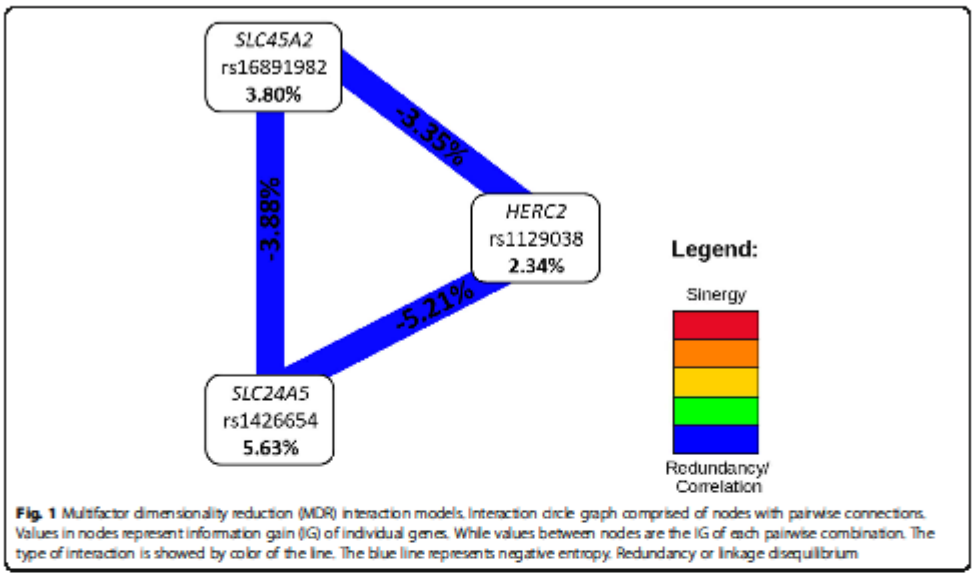

Fig. 1 Multifactor dimensionality reduction (MDR) interaction models. Interaction circle graph comprised of nodes with pairwise connections. Values in nodes represent information gain (IG) of individual genes. While values between nodes are the IG of each pairwise combination. The type of interaction is showed by color of the line. The blue line represents negative entropy. Redundancy or linkage disequilibrium

the model with the risk alleles is more appropriate than the model without these alleles (AUC 0.702, 95% CI 0.637–0.766 versus AUC 0.669, 95% CI 0.602–0.736).

Discussion

Based on a study developed by CANDELA, we investigated 4 variants previously associated with skin pigmentation in southern Brazil in melanoma patients and unaffected controls from the same geographic region [16]. A model of logistic regression including ancestry, melanoma risk factors and SNPs *HERC2* rs1129038 and *SLC24A5* rs1426654 in dominant models of inheritance showed significant associations with melanoma. The *SLC45A2* rs16891982CC SNP, in a log-additive model, was associated with a lower risk for developing the disease.

Pigmentation is a polygenic trait. and different variants have been associated with melanin levels in populations over the world [20]. One of the four SNPs investigated rs1129038, occurs in the untranslated region of *HERC2*, and three. *TYR* rs1126809, *SLC24A5* rs1426654, and *SLC45A2* rs16891982, are present in genes involved in the synthesis of melanosomes, the vesicles where melanin production and deposition occurs. In fact, rs1426654 and rs16891982 polymorphisms are determinants of pigmentation in Europeans [21], as well as in other populations [20, 22]. Our findings corroborate the association of some genotypes with lighter pigmentation and predominant European ancestry. The association of European ancestry and fair skin, eyes and hair was previously demonstrated in a sample of 1594 individuals from the same geographic region of the present study [23]. and the different ancestry profiles between darker and lighter individuals has also been previously reported [24]. Although our sample is not representative of the tri-hybrid pattern seen in most Brazilians [25], it reflects the massive colonization by Europeans in the specific region of the study [16, 23]. Just as there are regional differences in the proportions of ancestral populations in Brazil, we also expect heterogeneity in the frequency of genetic variants of specific genes, especially those related to skin, eye and hair pigmentation. Although research with admixed populations can be useful for allele detection involved in susceptibility to common diseases, the population substructure is a potential bias and should be controlled [26, 27]. In addition to a limitation in sampling (the sample was partially paired), the Hardy-Weinberg deviation found in our allelic frequencies for *SLC45A2* rs16891982 can also be explained by the occurrence of interethnic mix and population substructure. Previously identified in Europe and in other Brazilian populations [28].

The nonsynonymous SNP *TYR* rs1126809 (p.Arg402Gln) has been previously associated with light pigmentation of skin and is frequent in Caucasians. Its presence results in the reduction of activity of tyrosinase, a key enzyme in of the melanin production pathway, and some authors reported an increased risk of melanoma in carriers. Both in Europe and Australia [29]. However, we did not observe a consistent

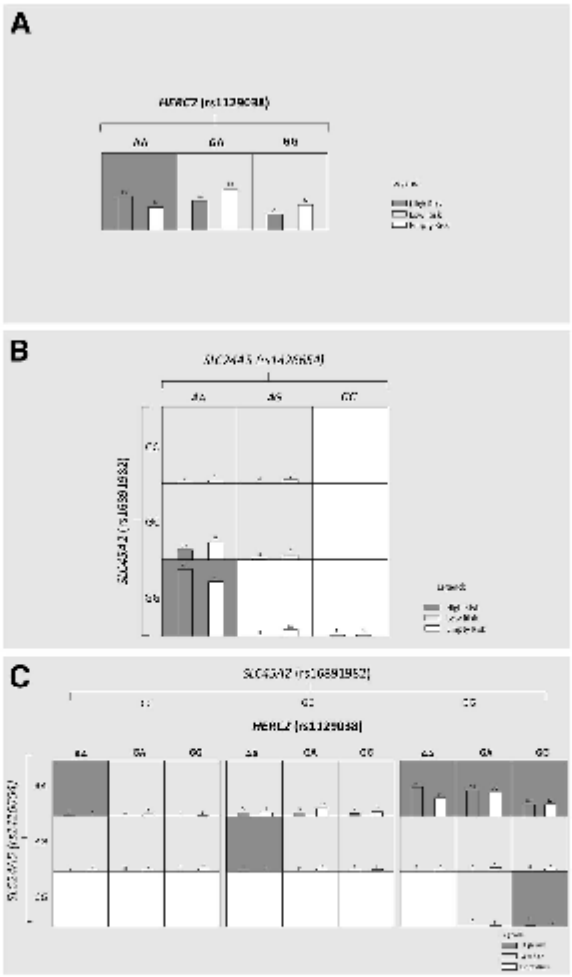

Fig. 2 The SNPs interaction to risk with melanoma development. A high-frequency genotype combination is displayed in Dark Square while low-frequency combinations are in lightly shaded. For each cell, The left bar indicates the absolute number of cases and the right bar the absolute number of controls. **a** The effect of *HERC2* rs1129038AA genotype. **b** The combination effect of *SLC24A5* rs1426654AA and *SLC45A2* rs16891982GG genotypes. **c** The combination effect of *SLC24A5* rs1426654AA, *SLC45A2* rs16891982GG, and *HERC2* rs1129038AA genotypes

association of this SNP with pigmentation nor with risk for melanoma in our series.

On other hand, *HERC2* rs1129038, which was previously associated with lighter eye pigmentation in European populations [30, 31], showed a significantly association with fair skin. Eyes and hair in our sample. Forensic associations have described this SNP as a good predictor of blue eyes in Europeans [30, 32] and Brazilians [33]. and our findings reinforce these predictions.

Finally, SNPs *SLC24A5* rs1426654 and *SLC45A2* rs16891982 were associated with fair skin, eyes, and hair and with melanoma. *SLC24A5* rs1426654 (p.Thr111Ala) was first described in zebrafish as responsible for the golden phenotype due to a delay in melanin production

during embryonic development [34]. In melanocyte cultures, homozygous GG leads to an increase in *SLC24A5* gene transcripts and a consequent increase in tyrosinase activity and melanin production [35]. The decrease of G allele frequency is gradual from Africa to Europe, indicating that a selection pressure in favor of the A allele acted on the determination of fair skin in places where the intensity of UV radiation is lower [36, 37]. Evidence of natural selection makes this SNP a frequent component of ancestral and forensic informative panels [38]. In our study, we confirmed the association of the AA genotype with fair skin and light eyes. and we identified allelic frequencies consistent with those observed in European populations [3] and in previous studies of Brazilians from other regions [39]. Likewise, SNP *SLC45A2* rs16891982 is also widely studied regarding its relationship with pigmentation in different populations. *SLC45A2* encodes the membrane-associated transporter protein carrier involved in melanin synthesis, and experimental studies in zebrafish. Mice and yeast have clearly demonstrated that the presence of the missense variant rs16891982 (p.Phe374Leu) results in decreased protein activity [40]. This SNP is also considered an ancestry informative marker (AIM), since it is able to differentiate European populations due to G allele frequency. Which is similar to the rs1426654 A allele [41]. These findings are aligned with the theory of vitamin D synthesis. Which proposes that light skin is a feature selected to compensate for the lower solar incidence in populations living far from the Equator [42] and with increased ability of the skin to respond to ultra violet (UV) radiation [43].

Alleles associated with lighter pigmentation were also associated with melanoma in our study, and this result remained significant after analysis with multivariate logistic regression adjusted for the risk factors ancestry, gender, age, eye, hair, and skin color, and number of Nevi. We considered this analysis essential to identify whether the variants studied could be considered independent risk factors for the occurrence of melanoma. Thus, SNPs *HERC2* rs1129038 and *SLC24A5* rs1426654 remained strongly associated with risk for the development of melanoma in a dominant model. The presence of homozygous genotypes of either SNP (AA for rs1426654 and AA for rs1129038) were associated with increased melanoma risk, while the *SLC45A2* rs16891982 C allele was associated with protection for melanoma as shown previously in a GWAS study in Greece composed of 284 patients and 284 controls (OR = 0.51. 95% CI 0.34–0.76; $P = 0.001$) [44]. On the other hand, an Australian sample with individuals of 100% Northern European ancestry (1.062 cases and 1.262 controls) showed the same allele associated with the risk to melanoma in logistic regression models including pigmentation features and ancestry, similar to the one presented here (OR = 2.04. 95% CI 1.27–3.40) [45] The results remained unchanged after population substructure analysis. Furthermore, the independent effects of each of these SNP were also accessed by MDR analysis, and the analysis considering the entire sample showed a redundancy interaction between the same SNPs that displayed significance through logistic regression ($P = .031$). The interaction illustrated in Fig. 1 shows that these three genes act redundantly to increase the risk of melanoma. The genotypic combinations *SLC24A5* rs1426654AA and *SLC45A2* rs16891982GG present a greater contribution in determining the risk for the disease, presenting a possible epistatic effect similar to that found between *SLC45A2* and *VDR* [46], *SLC45A2* and *OCA2*. and *MC1R* and *SLC24A5* [45].

The two most important limitations of our study are sampling process (individuals showing mostly Euro descendant ancestry in the entire sample) and relatively limited sample size. However, despite these limitations, our results are in line with previous studies and demonstrate that SNPs in genes related to pigmentation confer an independent increase in the risk for developing melanoma. In determining complex human traits in general, common genetic variants tend to have small effect sizes individually, but together. They may reveal important information and contribute to the assessment of individual risk for complex diseases such as cancer [47]. The development and evaluation of predictive models that combine environmental and genomic risk factors can help improve melanoma prevention and population screening by motivating risk reduction behaviors, especially in regions with high incidence rates. High UV radiation exposure and predominantly European ancestry [48].

Additional studies should be performed to verify whether the same scenario occurs in other regions of Brazil and Latin America. Although an association between *SLC24A5* rs1426654 and *SLC45A2* rs16891982 and melanoma has been previously described in Europeans, to our knowledge, this is the first study that confirms this association in a South American high-risk population.

Conclusions

In this case-control study conducted in Southern Brazil, SNPs *SLC24A5* rs1426654 and *SLC45A2* rs16891982 were associated with an increased risk for melanoma, which was found to be additive and independent of pigmentation profile. These results contribute to the current knowledge about melanoma risk factors in individuals from a geographic region with a high incidence of the disease.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12885-020-07485-x.

Additional file 1. Ancestry profile of samples (A) Individual European, African, and Native American ancestry inferred from 61 ancestry-informative markers in our all sample. Patients (green) and controls (orange) were compared with individuals from the putative parental populations used to infer admixture: Europeans, African, and Native Americans. (b) Individual European, African, and Native American ancestry inferred from 61 ancestry-informative markers in sample after substructure reduction. Patients (green) and controls (orange) were compared with individuals from the putative parental populations used to infer admixture: Europeans, African, and Native Americans. Admixture was estimated using STRUCTURE V.2.3.4 software.

Additional file 2: Additional Table 1. Samples excluded in order to reduce Population Substructure. **Additional Table 2.** Allelic and genotipic frequencies of SNPs *TYR* rs1126809, *HERC2* rs1129038, *SLC24A5* rs1426654, and *SLC45A2* rs16891982 in Southern Brazil samples and in main populational databases

Abbreviations

SNP: Single nucleotide polymorphisms; MDR: Multifactor dimensionality reduction; CANDELA: Consortium for Analysis of Diversity and Evolution in Latin America; MI: Melanin Index; RS: Rio Grande do Sul; BA: Bahia; HCPA: Hospital de Clínicas de Porto Alegre; CI: Interval confidence; OR: Odds ratio; IG: Information gain; AIM: Ancestry Informative marker; UV: Ultravioleta radiation

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Authors' contributions

LBR, GSM and PAP conceived the experiment(s); LBR, RMB and VCJ, conducted the experiment(s); LBR, FSLV, VCJ, SS and AMR-DS analyzed the results. All authors reviewed the manuscript. The author(s) read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

The study was conducted according to the Declaration of Helsinki Principles, and the protocol approved by the Institutional Review Board of Hospital de Clínicas de Porto Alegre (HCPA) under number 07–139, and all participants provided informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Short Communication

MIR605 rs2043556 is associated with the occurrence of multiple primary tumors in *TP53* p.(Arg337His) mutation carriers

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Multiple primary tumors

ABSTRACT

Li-Fraumeni and Li-Fraumeni-like (LFS/LFL) Syndrome are cancer predisposition syndromes caused by germline pathogenic variants in *TP53* and are associated with an increased risk of multiple early-onset cancers. In Southern and Southeastern Brazil, a germline founder variant with partial penetrance located in the oligomerization domain of TP53, c.1010G>A p.(Arg337His, commonly known as R337H), has been detected in 0.3% of the general population. Recently, the functional *MIR605* variant rs2043556 (*A>G*) has been identified as a novel LFS phenotype modifier in families with germline *TP53* DNA binding variants. In this study, our goal was to verify *MIR605* rs2043556 allele frequencies and further explore its possible effects on the phenotype of 238 Brazilian individuals carrying *TP53* p.(Arg337His). The *MIR605* rs2043556 G allele was detected in 136 (57.1%) individuals, including 25 homozygotes (10.5%), and although it had been previously associated with an earlier mean age of tumor onset, this effect was not observed in this study ($p = 0.8$). However, in p.(Arg337His) mutation carriers, the GG genotype was significantly associated with the occurrence of multiple primary tumors ($p = 0.005$). We provide further evidence of *MIR605* rs2043556 G allele's effect as a phenotype modulator in carriers of germline *TP53* pathogenic variants.

Introduction

Li-Fraumeni Syndrome (LFS) (OMIM # 151623) and its variant, Li-Fraumeni-like Syndrome (LFL), are autosomal dominant cancer predisposition syndromes characterized by a high risk for development of multiple tumors at a young age [1,2]. Germline pathogenic variants in the *TP53* gene are identified in approximately 70% and 40% of families that meet clinical criteria for LFS and LFL, respectively [3,4]. The protein product of *TP53*, p53, is a transcription factor whose ability to mediate tumor suppression has been extensively studied. p53 exerts its multiple antiproliferative functions through the transcriptional control of several target genes and through protein-protein interactions [5].

In southern and southeastern Brazil, the *TP53* founder variant c.1010G>A or p.(Arg337His, commonly known as R337H), located in the oligomerization domain (OD) of the gene, is detected in 0.3% of the general population. Although penetrance is reduced in relation to DNA binding domain (DBD) variants, its frequency is higher than what is observed for any other germline variant possibly present in the gene [6]. Currently, c.1010G>A is associated with a broad spectrum of tumors, similar to the one observed in

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families with LFS/LFL caused by DBD variants [7–9]. Recent studies have prioritized epidemiological aspects of this mutation, while the mechanism of cancer predisposition associated with this founder variant and its penetrance is still not completely understood [8,9]. Despite existing evidence linking the LFS/LFL phenotype with differences in mutant p53 activity in terms of cellular localization and functional effect, these genotype-phenotype correlations do not fully explain the global and intra-familial heterogeneity observed in carriers of this Brazilian founder mutation [10,11].

In this context, several recent studies have explored the role of coexisting or secondary genetic factors that might modify p53 function. Among these are microRNAs (miRNAs), a class of 18- to 25-nucleotide-long single-stranded non-coding RNAs involved in the post-transcriptional regulation of gene expression [12,13]. These small molecules play a role in several biological processes, including cell proliferation, differentiation, apoptosis, and development, acting as oncogenes or tumor suppressors [14–16]. In addition, it has been shown that several miRNAs contribute to a refined p53 expression control by interacting directly with target sites on the 3'UTR of the *TP53* mRNA and, thus, they could be considered clinically relevant oncogenes. A few examples of indirect p53 regulation through a miRNA network have been described, including the effect of miR-605, which directly modulates *MDM2* expression, the main p53 activity negative regulator [17,18]. MiR-605 also appears to be inducible by p53 in response to cell stress mechanisms [19]. Furthermore, the presence of a *MIR605* gene variant, rs2043556 (*A>G*), has been associated with an increased risk for developing different cancers [16]. More recently, this same single nucleotide polymorphism (SNP) was identified as a functional variant and a novel genetic modifier of the LFS phenotype, specifically associated with an earlier mean age of tumor onset in Canadian families with *TP53* pathogenic DBD variants [20]. Hence, the objective of this study was to determine the allelic and genotypic frequencies as well as the phenotypic effect of *MIR605* SNP rs2043556 (*A>G*) in Brazilian LFS/LFL individuals carrying the germline founder variant p.(Arg337His).

Materials and methods

Study subjects and ethical aspects

Patients selected for the study were recruited from three tertiary care hospitals in southern and southeastern Brazil. They all fulfilled the Chompret criteria for LFS/LFL and were carriers of a germline *TP53* pathogenic variant, either p.(Arg337His) (main study group) or a DBD variant (comparison group). A total of 238 p.(Arg337His) carriers were recruited, 65 from Hospital de Clínicas de Porto Alegre (HCPA, in the city of Porto Alegre, southern Brazil), 66 from A.C. Camargo Cancer Center (city of São Paulo, southeastern Brazil), and 107 from Hospital de Câncer de Barretos (city of Barretos, southeastern Brazil). Six LFS patients carrying germline DBD variants from HCPA were recruited and included in a comparison group. Genetic analyses were previously approved by research ethics committees of the involved collaborating centers (registered under the Certificate of Presentation for Ethical Appreciation – CAAE nº 52641616.0.0000.5327).

MIR605 *SNP genotyping*

TaqMan® allelic discrimination analyses of variant rs2043556 were performed according to Applied Biosystems® standard protocols (Applied Biosystems, Carlsbad, USA), using fluorescent allele-specific probes (reference number C_11737438_10). *TP53* p.(Arg337His) genotyping was performed following previously published protocols using custom TaqMan® assays [21]. Sanger sequencing confirmed all samples with a variant identified by TaqMan®. Sequencing of *TP53* exon 10 encompassing p.(Arg337His) was performed according to IARC standard protocols (primer sequences and PCR conditions available at http://p53.iarc.fr/download/tp53_directsequencing_iarc.pdf), while *MIR605* was analyzed according to the primers described by Id Said & Malkin [20].

Statistical analysis

Genotype and allele frequencies were estimated by simple counting. Differences between groups were compared using the Kruskal-Wallis' (median age at tumor onset), Pearson's chi-squared (multiple primary tumors and cancer personal history) or Fisher's exact tests (tumor type). All tests were two-tailed, significance was set at p less than 0.05 and statistical analyses were done using SPSS® version 18 (SPSS Inc., Chicago, USA).

Results

Clinical data on LFS/LFL patients included in the main study group are summarized in Supplementary Table 1. Among the 238 p.(Arg337His) carriers, the variant G-allele for *MIR605* rs2043556 was detected in 136 individuals (57.1%), including 25 homozygotes (GG genotype, 10.5%) (Table 1). Moreover, the G-allele had a high frequency in this series (0.34), similar to the frequency observed in population databases, especially those related to Latin cohorts (Supplementary Table 2). Presence of the *MIR605* rs204356 G allele was not associated with age of tumor onset ($p = 0.8$, Fig. 1, Supplementary Table 3) or tumor type ($p = 0.3$) (Table 2) or personal history of cancer ($p = 0.1$, Supplementary Table 4). Genotypic frequency distribution was also not related with the median age of breast and adrenocortical cancers (two common tumor types found in LFS/LFL families, Supplementary Table 5). However, the GG genotype was significantly associated with the occurrence of multiple primary tumors ($p = 0.005$) (Table 2). For instance, among these *TP53* p.(Arg337His) and rs204356[GG]-positive patients with tumor diagnosis in multiple primary sites, one female patient developed very early-onset breast and thyroid cancers (at 23 and 25 years, respectively; data not shown). Of note, 26 of 122 cancer-affected patients (10.3%) developed more than one primary tumor (Table 2). Among these 26 patients, 4 (15.4%) had synchronous tumors; for the remaining metachronous tumors, the average time between diagnoses was approximately 6 years (Supplementary Table 6).

In addition, *MIR605* rs2043556 was genotyped in a small group of LFS patients with DBD mutations (comparison group, $N = 6$). Clinical data from these patients is summarized in Supplementary Table 7. The variant G-allele was detected at a frequency of 0.33, very similar to the one observed in the p.(Arg337His) group. Interestingly, one patient in the comparison group heterozygous for the *TP53* p.(Arg273His) variant (identifier LFS-4) was GG homozygous for the *MIR605* SNP and was diagnosed with multiple tumors

Table 1
Genotypic and allelic frequencies of *MIR605* rs2043556 (*A>G*) in *TP53* p.(Arg337His) mutation carriers with the LFS/LFL phenotype.

Genotype frequencies	N (%) $n = 238$
AA	102 (42.9%)
AG	111 (46.6%)
GG	25 (10.5%)
AG+GG	136 (57.1%)
Allele frequency	
G*	0.34

* Risk/variant allele.

Genotype SNP rs2043556	Median age in years (IR)[a]	p
AA	35.5 (31.75)	
AG	31.5 (41.75)	0.808[b]
GG	40 (36)	

[a] IR, interquartile range.
[b] Kruskal-Wallis test.

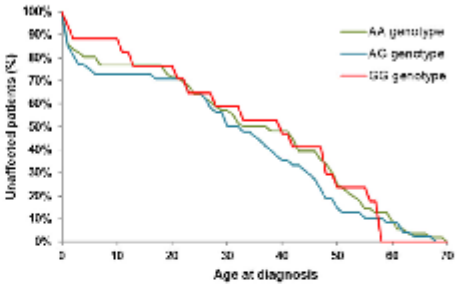

Fig. 1. Distribution of the median age of first cancer diagnosis according to *MIR605* rs2043556 genotype in *TP53* p.Arg337His carriers. The plot on the right shows the percentage of cancer-unaffected individuals harboring the founder variant for each *MIR605* genotype as a function of age of cancer onset.

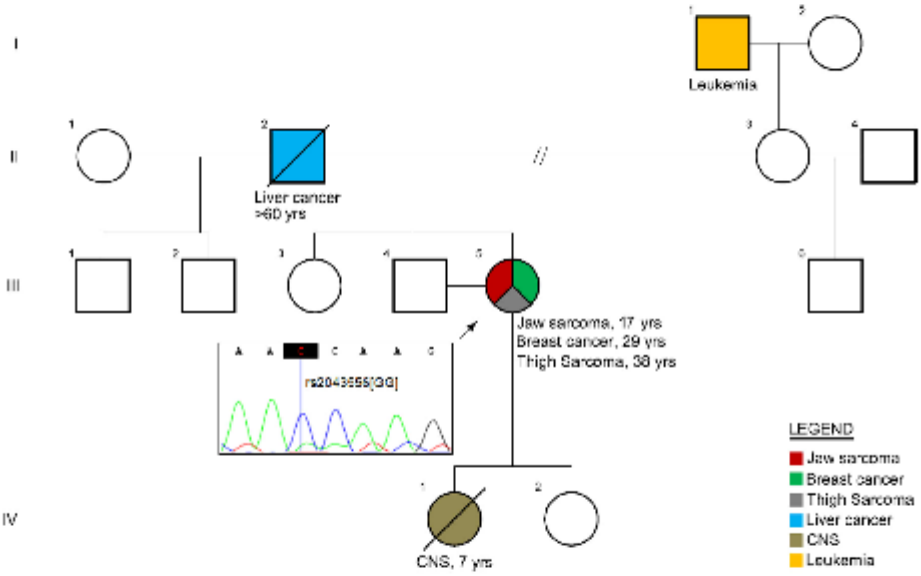

Fig. 2. Pedigree of an LFS proband (indicated by the black arrow; identifier LFS-4 in Supplementary Table 7) fulfilling classical clinical criteria and harboring the *TP53* DNA-binding domain germline variant p.(Arg273His), along with the *MIR605* variant G-allele in homozygosis (confirmed by Sanger sequencing as shown by the lower-left panel). Yrs, years; CNS, central nervous system tumor.

(sarcoma at age 17, breast cancer at age 29 and a new sarcoma at age 38 years). Fig. 2 shows the pedigree of this proband. To our knowledge, this is the first description of a patient with a DBD p53 pathogenic variant carrying the *MIR605* rs2043556 G-allele in homozygosis. Sanger sequencing confirmed the GG genotype in all cases identified by TaqMan® analyses (Supplementary Fig. 1).

Discussion

In a previous report, *MIR605* rs2043556 (*A*>*G*) was proposed to be a genetic modifier of the age of cancer onset in a Canadian cohort of LFS patients (classical LFS criteria) composed predominantly of *TP53* DBD mutation carriers [20]. Presence of the G-allele in heterozygosity was associated with a 10-year acceleration in the mean age of tumor onset ($p = 0.04$) in this LFS case series. Importantly, none of the participants in this previously mentioned study were carriers of the Brazilian founder mutation p.(Arg337His). We did not observe the same effect in our cohort, and although this could be due to the different genetic background between Canadian and Brazilian populations, the most important difference between both studies is the localization of the germline *TP53* mutations of their participants (DBD *vs.* OD). The modifying effect of *MIR605* rs2043556 G allele in heterozygosity on the age of cancer diagnosis previously observed in carriers of highly penetrant DBD mutations might be insufficient to cause a detectable phenotypic change in carriers of "milder", less penetrant *TP53* variants such as p.(Arg337His). In addition, it is worth emphasizing that a significant proportion of cancer diagnoses reported in p.(Arg337His) carriers occurs in adults, except for adrenocortical carcinomas and brain tumors, which occur more frequently in pediatric patients.

Table 2
Genotypic frequencies of *MIR605* rs2043556 in *TP53* pArg337His carriers according to tumor type and number of cancer diagnoses.

Genotype SNP rs2043556	Breast cancers, N (%)	Adrenocortical carcinomas, N (%)	CNS tumors[a], N (%)	Sarcomas and osteosarcomas[b], N (%)	Other tumor types[c], N (%)	p	Single tumor diagnosis, N (%)	Multiple primary tumors diagnosis, N (%)	p
AA	18 (40)	10 (38.5)	6 (66.7)	5 (33.3)	18 (64.3)		44 (45.8)	12 (46.1)	
AG	19 (42.2)	12 (46.1)	3 (33.3)	9 (60)	7 (25)	0.308[d]	44 (45.8)	6 (23.1)	**0.005**
GG	8 (17.8)	4 (15.4)	0 (0)	1 (6.7)	3 (10.7)		8 (8.3)	8 (308)	

[a] CNS, central nervous system.
[b] Soft tissue sarcomas and osteosarcomas.
[c] Twenty-eight patients developed other tumor types, including prostate, thyroid, lung, kidney, uterine cancers, colorectal, gastric cancers, pheochromocytoma, melanoma, leukemia, lymphoma, and multiple myeloma.
[d] Fisher exact test.
[e] Pearson's chi-squared test.

A remarkable finding in the present study is a significant association between the homozygous GG genotype and the development of more than one primary tumor, suggesting that the G-allele might have a dose dependent effect associated with increased cancer predisposition, or might exert an influence on predisposition to a second primary tumor. This association between *MIR605* rs2043566 and multiple cancer diagnoses seems to be limited to *TP53* p.(Arg337His) carriers, since in the previous study ($N = 55$) [20] and in our small cohort ($N = 6$), among LFS patients with *TP53* DBD mutations, the frequency of multiple primary tumors did not differ between individuals harboring the variant G-allele and those with the AA genotype.

Furthermore, Id Said and Malkin (2015) [20] showed in their functional experiments that, in *MIR605* rs2043556 AG heterozygotes, the miR-605 processing efficiency from its precursor to its mature form was compromised, leading to reduced mature miR-605 levels. Therefore, it is reasonable to hypothesize that GG homozygotes could have dramatically impaired miRNA processing, triggering severe deregulation effects on MDM2 and/or p53 function. This effect would be profoundly deleterious in the presence of a germline *TP53* DBD mutation, but less so in the presence of a "milder" *TP53* OD mutation. In addition to MDM2 and p53, other validated individual target regulated by miR-605 includes Sec24D (miRTarBase database, http://mirtarbase.mbc.nctu.edu.tw/php/search.php#mirna), a cellular trafficking protein lacking a well-established role in carcinogenesis [22], as well as MAPK signaling pathway was shown to be induced by miR-605 overexpression, leading to tumor suppressive effects in *TP53* mutant cell lines according to the preliminary evidence reported by Malkin's group [23]. In agreement with this hypothesis, our study identified, for the first time, a considerable amount of *MIR605* rs2043556[GG] homozygotes meeting LFS/LFL clinical criteria and harboring the *TP53* OD mutation p.(Arg337His), differing from the results of the study by Id Said and Malkin (2015) in which no GG homozygotes were identified [20]. When considering the G allele frequencies described in population databases worldwide, the fact that none of the individuals in the Canadian study had the G-variant allele in homozygosis is striking. This difference in GG genotype frequency (0/55 *vs.* 25/258) between Canadian and Brazilian LFS/LFL cohorts exhibited a statistically significant difference, reinforcing that these findings are not random events ($P = 0.006$).

In conclusion, our results support previous studies in showing that the *MIR605* rs2043556 G allele is a potential phenotype modifier not only in LFS/LFL patients with DBD mutations but also in those with OD mutations, as demonstrated here in carriers of the exon 10 founder *TP53* variant p.(Arg337His). In these patients, we identified an association between the presence of the *MIR605* rs2043556 GG genotype and occurrence of multiple primary tumors. Larger studies including patients with different *TP53* germline mutations preferentially accompanied by functional analysis of the *MIR605* rs2043556 risk allele are required to confirm this hypothesis. Taken together, these findings emphasize the importance of analyzing miRNA genes that directly or indirectly regulate p53 expression as potential phenotype modifiers and as promising therapeutic targets in LFS/LFL.

Internet resources

IARC *TP53* Database. April 5, 2019. <http://p53.iarc.fr/Download/TP53_DirectSequencing_IARC.pdf>. miRTarBase Database. September 20, 2019. <http://mirtarbase.mbc.nctu.edu.tw/php/search.php#mirna>.

Declaration of Conflict Interest

H. C. R. G. has a disclosure with AstraZeneca Brazil (financial support for lectures) and Hermes Pardini Institute (Consultancy). Other authors do not have any conflict of interest to declare.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cancergen.2019.11.005.

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TRABALHOS CIENTÍFICOS ADICIONAIS: DIVULGAÇÃO CIENTÍFICA

A divulgação científica, também conhecida como popularização da ciência, comunicação pública da ciência e jornalismo científico engloba as atividades que buscam fazer a difusão do conhecimento científico para públicos não especializados. A divulgação científica é fundamental para o desenvolvimento da ciência, uma vez que ela é responsável pela circulação de ideias e divulgação de resultados de pesquisas para a população em geral. Desta forma, através da divulgação científica é possível potencializar o debate científico e instigar novos talentos para atividades de ciências (Massarani et al., 2002; Massarani et al., 2019). A divulgação científica iniciou-se há mais de cinco mil anos. Mais recentemente, a popularização da ciência tem sido interpretada também como um instrumento para tornar disponíveis conhecimentos e tecnologias que ajudem a melhorar a vida das pessoas e que deem suporte a desenvolvimentos econômicos e sociais sustentáveis. Tais ações podem ter ainda um importante papel de apoio às atividades de ensino. Atualmente, a divulgação científica ocorre em praticamente todos os formatos e meios de comunicação: documentários de televisão, revistas de divulgação científica, artigos em periódicos, websites e blogs (Porto, 2009).

Em março, a Organização Mundial da Saúde (OMS) definiu o surto da doença Covid-19, causada pelo SARS-Cov2 (novo coronavírus), como uma pandemia. O primeiro caso da pandemia de Covid-19 foi identificado em Wuhan, na China, em 31 de dezembro de 2019. Desde então, os casos começaram a se espalhar rapidamente pelo mundo: primeiro pelo continente asiático, e depois por outros países. Em fevereiro, a transmissão da Covid-19 no Irã e na Itália chamaram a atenção pelo crescimento rápido de novos casos e mortes. Pouco tempo depois, foi confirmada a primeira morte por Covid-19 no Brasil, em São Paulo (Brasil Escola, 2019; Portal Fiocruz, 2020; Johns Hopkins CSSE, 2021).

Os sintomas de Covid-19 são altamente variáveis, variando de nenhum a doenças com risco de morte. O vírus se espalha através das pessoas,

principalmente pelo ar e pela proximidade entre infectados e não infectados, sendo as vias de transmissão mais comuns a boca, o nariz ou os olhos, através da tosse, espirro ou fala. Embora seja menos comum, o novo coronavírus também pode se espalhar através de superfícies contaminadas. As pessoas permanecem contagiosas por até duas semanas e podem espalhar o vírus mesmo se forem assintomáticas. As medidas preventivas recomendadas incluem distanciamento social, uso de máscaras faciais em público, ventilação e filtragem de ar, lavagem das mãos, cobertura da boca ao espirrar ou tossir, desinfecção de superfícies e monitoramento e autoisolamento para pessoas expostas ou sintomáticas. Várias vacinas foram desenvolvidas em tempo recorde e estão sendo distribuídas ao redor do mundo. Os tratamentos atuais se concentram nos sintomas enquanto drogas terapêuticas que inibem o vírus são desenvolvidas. Autoridades em todo o mundo responderam implementando restrições a viagens, *lockdowns*, controles de locais de trabalho e fechamentos de instalações. Muitos lugares também trabalharam para aumentar a capacidade de testar e rastrear os contatos dos infectados (*Centers for Disease Control and Prevention*, 2021; NHS, 2021).

A velocidade de espalhamento do novo coronavírus, bem como o crescente número de óbitos em decorrência da doença, foi acompanhada de uma crescente desinformação por parte da população brasileira. Nesse contexto, surgiu a necessidade de divulgação científica com ações voltadas principalmente ao enfrentamento da pandemia, ao combate à desinformação e às *fake news* e para fomentar as vacinas contra a Covid-19. Desde o início da pandemia, diversas iniciativas voluntárias encabeçadas por cientistas e pesquisadores buscam auxiliar na divulgação de informações científicas confiáveis na tentativa de frear o curso da pandemia, diminuir o número de mortes e conscientizar a população sobre a adesão às medidas de enfrentamento e o incentivo à vacinação. Desde maio de 2020 eu integro três dessas iniciativas, realizando trabalho voluntário de produção e revisão de textos de divulgação científica, conteúdo didático para redes sociais, como postagens, imagens, vídeos, podcasts e áudios de Whatsapp explicativos e atualização de mídias sociais como Instagram e Twitter.

1. Rede Análise COVID-19

A Rede Análise COVID-19 (https://redeaanalisecovid.wordpress.com/) surgiu no final de fevereiro de 2020 nasceu de uma iniciativa na rede social Twitter (https://twitter.com/analise_covid19) para a criação de um espaço construtivo e de discussão a partir da reunião de pesquisadores, profissionais e pessoas engajadas no tema, como uma necessidade de coletar, analisar e divulgar informações respaldadas na ciência sobre a pandemia da COVID-19 no mundo e no Brasil. Desde então, a Rede vem crescendo e ganhando espaços nas redes sociais (44 mil seguidores no Twitter e mais de 3500 mil seguidores no Instagram - https://www.instagram.com/analise.covid19/) levando informação científica, combatendo a desinformação e instigando a formação de novos grupos e colaborando com diversas redes voltadas para a comunicação e divulgação científica. Nosso principal objetivo e comprometimento é com a informação científica acessível.

Alguns textos de minha autoria que foram publicados pela Rede Análise Covid-19:

Temática	Título texto	Link de acesso
Divulgação de estudo científico	Entenda por que a reabertura de pré-escolas e creches antes da distribuição de uma vacina segura contra o novo coronavírus não parece uma boa ideia	https://redeaanalisecovid.wordpress.com/2020/08/05/entenda-porque-a-reabertura-de-pre-escolas-e-creches-antes-da-distribuicao-de-uma-vacina-segura-contra-o-novo-coronavirus-nao-parece-uma-boa-ideia/
	O risco de COVID-19 para grávidas: evidências de alterações moleculares associadas com pré-eclampsia na infecção por SARSCoV-2	https://redeaanalisecovid.wordpress.com/2020/11/10/o-risco-de-covid-19-para-gravidas-evidencias-de-alteracoes-moleculares-associadas-com-pre-eclampsia-na-infeccao-por-sarscov-2/
	Revisão sistemática e meta-análise: O que vários estudos têm a nos dizer sobre a carga viral e tempo de transmissão da doença?	https://redeaanalisecovid.wordpress.com/2020/12/01/revisao-sistematica-e-meta-analise-o-que-varios-estudos-tem-a-nos-dizer-sobre-a-carga-viral-e-tempo-de-transmissao-da-doenca/
	O que sabemos sobre os assintomáticos para a COVID-19	https://redeaanalisecovid.wordpress.com/2020/12/07/o-que-sabemos-sobre-os-assintomaticos-para-a-covid-19/

	Participantes de testes das vacinas para a COVID-19: a questão dos placebos	https://redeaanalisecovid.wordpress.com/2020/12/16/participantes-de-testes-das-vacinas-para-a-covid-19-a-questao-dos-placebos/
Combate às *fake news*	Tratamento precoce e Kit Covid	https://redeaanalisecovid.wordpress.com/2021/01/20/tratamento-precoce-e-kit-covid/
	Ivermectina: será mesmo a droga milagrosa?	https://redeaanalisecovid.wordpress.com/2020/12/22/ivermectina-sera-mesmo-a-droga-milagrosa/
	Checamos: Vacina RNA, Metais, Enzima Luciferase, 5G e Criptomoedas	https://redeaanalisecovid.wordpress.com/2020/12/11/checamos-vacina-rna-metais-enzima-luciferase-5g-e-criptomoedas/
	Evitar flúor, suplementar com magnésio e água alcalina, só isso basta?	https://redeaanalisecovid.wordpress.com/2020/11/24/evitar-fluor-suplementar-com-magnesio-e-agua-alcalina-so-isso-basta/
Vacinas	“Vacina feita às pressas”?	https://redeaanalisecovid.wordpress.com/2021/01/06/vacina-feita-as-pressas/
	“Vacina feita às pressas”? Parte 2	https://redeaanalisecovid.wordpress.com/2021/01/06/vacina-feita-as-pressas-parte-2/

2. União Pró Vacina

A União Pró-Vacina (https://sites.usp.br/iearp/uniao-pro-vacina/) é uma iniciativa organizada pelo Instituto de Estudos Avançados (IEA) Polo Ribeirão Preto da USP em parceria com o Centro de Terapia Celular (CTC), o Centro de Pesquisa em Doenças Inflamatórias (CRID) e a Ilha do Conhecimento, e hoje reúne pesquisadores, cientistas e divulgadores científicos de todo o Brasil. O objetivo é unir instituições acadêmicas e de pesquisa, poder público, institutos e órgãos da sociedade civil para combater a desinformação sobre vacinas, planejando e coordenando atividades conjuntas. Entre as ações realizadas estão: produção de material informativo; intervenções em escolas, espaços públicos e centros de saúde; eventos expositivos; combate às informações falsas e desenvolvimento de games.

3. Todos Pelas Vacinas

Todos pelas vacinas é uma rede nacional criada por 21 organizações ligadas à divulgação científica e ao combate à desinformação, unidas com um único objetivo: promover a conscientização sobre a importância da vacinação contra a Covid-19 e outras doenças preveníveis por vacinas. Fazem parte da iniciativa a Sociedade Brasileira de Imunologia, Sociedade Brasileira de Microbiologia, Associação Brasileira de Saúde Coletiva, Sociedade Brasileira para o Progresso da Ciência e Sociedade Brasileira de Virologia, além de contar com o apoio da Sociedade Brasileira de Medicina Tropical, Sindicato dos Professores pela Universidade Pública, Associação Nacional dos Procuradores da República, entre outros. O site https://www.todospelasvacinas.info reúne centenas de materiais de divulgação científica, incluindo artes, perguntas e respostas, material para educadores, podcasts, artigos, fios publicados no Twitter, jogos didáticos, material informativo para crianças e memes. Alguns materiais produzidos por mim para as redes União Pró Vacina e Todos Pelas Vacinas incluem vídeos e áudios de combate à desinformação sobre tratamento precoce e kit covid (https://drive.google.com/drive/folders/12T3zD89S3H1gCjGVmBPufDGHcT0_b4P

M), o uso de ivermectina para tratar Covid-19 (https://drive.google.com/drive/folders/12T3zD89S3H1gCjGVmBPufDGHcT0_b4PM), campanha de vacinação da gripe comum (https://drive.google.com/drive/folders/12T3zD89S3H1gCjGVmBPufDGHcT0_b4PM) e outros, disponíveis para download.

4. Movimento Biotecnologia Brasil

O Movimento Biotecnologia Brasil (https://www.movbiotecbrasil.com/) é uma instituição fundada em 2015 que visa mobilizar os cidadãos em prol da implementação planos, programas, projetos, ações e atividades de ensino para o efetivo desenvolvimento da Biotecnologia e da profissão de Biotecnologista no Brasil. A proposta é defender a interação do Governo Federal e das Universidades Federais com o setor empresarial, laboratórios públicos e privados e institutos de pesquisa, buscando criar oportunidades para os diversos setores que utilizam a biotecnologia no país. Fui selecionada como Coordenadora Estadual do Rio Grande do Sul em junho de 2020 e desde então venho participando da realização de ações, eventos e cursos educativos online, além de produção de conteúdo digital para as redes sociais do Movimento.

Atividades que desenvolvi pelo Movimento Biotecnologia Brasil:

- Palestrante no Congresso Biotecnologia Brasil, com a palestra online intitulada “O papel do Biotecnologista no enfrentamento do SARS-CoV2” - julho de 2020;
- Organização do Simpósio Biotecnologia Brasil - novembro de 2020;
- Criação de minicurso “Biologia Sintética na Produção de Vacinas” no Simpósio Biotecnologia Brasil - novembro de 2020
- Criação de minicurso “Biologia Sintética na Produção de Fármacos” no Simpósio Biotecnologia Brasil - novembro de 2020
- Organização da Jornada de Biotecnologia Brasil – fevereiro de 2021
- Palestrante na Jornada de Biotecnologia Brasil, com a palestra online intitulada “Como começar sua carreira de cientista dentro da faculdade” – fevereiro de 2021

Referências Bibliográficas

CDC (11 de fevereiro de 2020). Coronavirus Disease 2019 (COVID-19). Centers for Disease Control and Prevention (em inglês). Acesso em 12 de abril de 2021.

Coronavirus COVID-19 Global Cases by the Center for Systems Science and Engineering (CSSE) at Johns Hopkins University (JHU). ArcGIS. Johns Hopkins CSSE. Acesso em 12 de abril de 2021.

Massarani, L., de Abreu, W. V., & Norberto Rocha, J. (2019). Apoio a projetos de divulgação científica: análise de edital realizado pela Fundação Oswaldo Cruz. RECIIS, V.13(2): 391-410. http://dx.doi.org/10.29397/reciis.v13i2.1646

Massarani, L. et al. (2002). Ciência e Público: caminhos da divulgação científica no Brasil (PDF). Rio de Janeiro: Casa da Ciência – Centro Cultural de Ciência e Tecnologia da Universidade Federal do Rio de Janeiro. 232 páginas. ISBN 85-89229-01-7. Consultado em 22 de abril de 2021.

NHS Scotland Coronavirus (COVID-19): General advice». www.nhsinform.scot (em inglês). Consultado em 17 de fevereiro de 2021.

Porto, Cristiane de Magalhães (org.) (2009). Divulgação científica independente na internet como fomentadora de uma cultura científica no Brasil (PDF). Difusão e cultura científica. alguns recortes. Salvador: UFBA. p. 93-112. ISBN 978-85-232-0619-2. Consultado em 25 de abril de 2021.

5. Lista de participações na mídia – Divulgação Científica

Participação Seminário Conselho Estadual de Saúde do Rio Grande do Sul: https://www.facebook.com/ConselhoEstadualdeSaudeRs/videos/803659940536186

Participações em matérias de websites:

Participação matéria BBC Brasil https://www.bbc.com/portuguese/brasil-56824217

Participação matéria UOL Viva Bem: https://www.uol.com.br/vivabem/noticias/redacao/2021/04/01/antietico-uso-de-preso-como-cobaia-ja-foi-negado-por-d-pedro-ii-no-brasil.htm

Participação na Revista Seleções *Reader Digest* https://www.selecoes.com.br/saude-bem-estar/entenda-por-que-e-importante-se-vacinar-contra-a-covid-19/

Participação TRT World news https://www.pri.org/stories/2021-01-05/brazilians-flock-coast-during-height-tourist-season-while-coronavirus-cases-surge

Participação matéria G1 Bem Estar https://g1.globo.com/bemestar/coronavirus/noticia/2021/01/08/teste-de-covid-19-por-saliva-utiliza-tecnica-mais-simples-e-barata-que-o-rt-pcr-veja-perguntas-e-respostas.ghtml

Participação Revista Veja https://veja.abril.com.br/blog/diario-da-vacina/a-falacia-dos-tratamentos-precoces-contra-a-covid-19/

Participação Portal R7 de notícias https://noticias.r7.com/saude/se-pais-vacinar-1-milhao-por-dia-76-teriam-uma-dose-em-5-meses-20032021

04/03/2021 Participação IEA USP http://www.iea.usp.br/noticias/iniciativa-da-uniao-pro-vacina-resgata-participacao-das-mulheres-na-historia-da-vacinologia

Entrevista Portal Notícias Piauí: https://www.clubenoticias.com/noticia/12449/butanvac-saiba-mais-sobre-essa-tecnologia-e-como-ela-pode-ser-importante-no-combate-a-covid-19

Participações em lives e programas de televisão:

Entrevista CBN Madrugada https://cbn.globoradio.globo.com/media/audio/336008/sao-anuncios-que-trazem-esperanca-mas-devem-ser-re.htm

Entrevista Jornal da Record das 20h
https://www.youtube.com/watch?v=9QRha_pXHPY

The International Journalists' Network - Webinar #33: Rumos aos 3 mil mortos por dia: o que mais podemos fazer?
https://www.youtube.com/watch?v=ak2KGRYnPnY

Entrevista TVT Programa Central do Brasil https://www.youtube.com/watch?v=L9-PZa6Jhe0

Entrevista Programa Cruzando Conversas RDC TV https://lnkd.in/dtT6k9F

Participação Rádio Show Time: Oncogenética e Covid-19 - 12/03
https://soundcloud.com/radioshowtimeoficial/evening-news-12032021

Entrevista Rádio Oceano FM 91.1 FM

Participação Vacina Talks: UPVacina:
https://www.youtube.com/watch?v=aGt5cTAtyYY

Live IGTV Rede Análise Covid-19 - Vacinas: uma questão política, mas não partidária https://www.instagram.com/tv/CG3colRjjGQ/

Live IGTV Rede Análise Covid-19 - O quão perigosa pode ser a 2ª onda da Covid-19 https://www.instagram.com/tv/CHJejoaDNvQ/

Live IGTV Rede Análise Covid-19 - A Covid Longa: sintomas que persistem após a cura https://www.instagram.com/tv/CHbgAMTj9Fw/

Live IGTV Rede Análise Covid-19 - Covid-19: Desvendando as fake news
https://www.instagram.com/tv/CHth5pPDdUx/

Live Rede Análise Covid-19 YouTube - Dados da Pandemia: Uma verdade inconveniente https://www.youtube.com/watch?v=XckFqIte-XY&t=2s

Live Rede Análise Covid-19 YouTube - "Então é Natal!" E o que faremos para minimizar os riscos? https://www.youtube.com/watch?v=p3TGyp6Bw6c&t=17s

Live Movimento Biotecnologia Brasil - Biologia Sintética na produção de Fármacos
https://www.youtube.com/watch?v=3ibGJQ02g3k

Live Movimento Biotecnologia Brasil - Biologia Sintética na produção de Vacinas
https://www.youtube.com/watch?v=KZGHf0kmoys&t=1132s

Mesa Redonda Movimento Biotecnologia Brasil - Biologia sintética sob o olhar da bioética: riscos, promessas e responsabilidades
https://www.youtube.com/watch?v=4RfDW4Pec_k&t=2247s

Live Bem-estar Capital - Vacinação e Controle da Pandemia
https://www.youtube.com/watch?v=FICxC3KkoEI

Live Aspectos bioquímicos do novo coronavírus - Uninassau Pernambuco
https://www.instagram.com/p/CJuTU9THAtI/

Live IGTV Elas na Ciência - Elas na Live: Covid-19
https://www.instagram.com/tv/CKfFI_eHZF7/

Live IGTV Revista Com Tempo https://www.instagram.com/tv/CLAwVacpMNk/

Participação ZS WEB TV Ijuí - 15/01/2021
https://www.facebook.com/watch/live/?v=3944059018946440&ref=watch_permalink

Participação ZS WEB TV Ijuí
https://www.facebook.com/zalmirsoaresnoticias/videos/472189493972843

Participação Rádio Jornal Ibiá
https://www.facebook.com/watch/live/?v=818980658689425&ref=watch_permalink

Participação TV Thati Programa Ribeirão em Movimento
https://www.facebook.com/watch/?v=459142975468238

Participação Manawa Rádio Web: Programa Horizontes
https://www.facebook.com/568679746639413/videos/239428761193496

Threads no Twitter: https://threadreaderapp.com/user/laribrussa

Palestras em Eventos:

Simpósio Acadêmico de Biotecnologia da Uni Federal de Uberlândia - Lina Biotec - palestrante

Conbiotec: Congresso Biotecnologia Online - palestrante

Centro Acadêmico Bárbara McClintock, da graduação de Biotecnologia da USP - palestrante https://www.youtube.com/channel/UCRQKMpSN2ovX_3bKSt2g2Gg

ANEXOS

Prêmio de produtividade do Programa de Pós-graduação em Genética e Biologia Molecular da Universidade Federal do Rio Grande do Sul

1º Lugar categoria Doutorado – 2020

UFRGS
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

CERTIFICADO

PPGBM
Programa de Pós-Graduação
em Genética e Biologia Molecular
da UFRGS

Certificamos que **Larissa Brussa Reis** recebeu o 1° lugar no prêmio de Aluno com Maior Produtividade no ano de 2020 (Área Humana/Médica - Nível Doutorado), concedido pelo Programa de Pós-Graduação em Genética e Biologia Molecular (PPGBM) da Universidade Federal do Rio Grande do Sul (UFRGS).

Porto Alegre, 20 de janeiro de 2021.

Prof. Dr. Thales Renato Ochotorena de Freitas
Coordenador PPGBM-UFRGS

Elmo Jurandir Antunes Cardoso
Coord. Administrativo PPGBM-UFRGS