MARIA ISABEL CUNHA VIEIRA CORDIOLI

ESTUDO DO CARCINOMA DIFERENCIADO DA TIREOIDE PEDIÁTRICO: ASPECTOS CLÍNICOS, PERFIL MUTACIONAL E EXPRESSÃO DE GENES ESPECÍFICOS DA TIREOIDE

Tese apresentada à Universidade Federal de São Paulo – Escola Paulista de Medicina, para obtenção do título de Doutor em Ciências

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São Paulo
2016

Cordioli, Maria Isabel Cunha Vieira

Estudo do carcinoma diferenciado da tireoide pediátrico: aspectos clínicos, perfil mutacional e expressão de genes específicos da tireoide / Maria Isabel Cunha Vieira Cordioli. – São Paulo, 2016.

vii, 179f.

Tese (Doutorado) – Universidade Federal de São Paulo. Escola Paulista de Medicina. Programa de Pós-Graduação em Endocrinologia clínica.

Título em inglês: Study of pediatric differentiated thyroid carcinoma: clinical aspects, mutational profile and thyroid-specific genes expression.

1. Câncer de tireoide. / 2. Crianças e adolescentes. / 3. Mutações genéticas./ 4. Expressão gênica.

UNIVERSIDADE FEDERAL DE SÃO PAULO

ESCOLA PAULISTA DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM ENDOCRINOLOGIA CLÍNICA

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*Ao **Júlio**, meu grande companheiro e incentivador e ao **Pedro,** meu filho, que mudou o sentido da minha vida*

Agradecimentos

Agradeço, aos meus pais, Regina Melim Cunha e Dalmo Vieira Filho, meus exemplos de dedicação profissional, por todos os ensinamentos e princípios repassados, por me incentivarem na busca dos meus sonhos e acreditar que tudo é possível.

À minha irmã, Maria Luisa Spricigo Vieira, pelo apoio e incentivo e ao meu irmão, Manoel Francisco Cunha Vieira, pelos ensinamentos que a nossa convivência diária me traz. À minha amiga-irmã, Anamaria Teixeira dos Santos, minha companheira desde os primeiros anos de vida, pelo apoio e incentivo de sempre, dinda tão especial do meu filho e quem me deu um afilhado tão amado e especial, Bernardo.

Ao meu padrasto, Antônio Azevedo, e aos meus sogros, Celito e Cristina Cordioli, por todo o apoio e toda a ajuda nos cuidados do meu filho, essencial para que esse trabalho pudesse ser concluído.

Agradeço aos amigos do laboratório de Genética Molecular dos Tumores da Tireoide, em especial à Aline Araújo e à Gisele Oler, pelo apoio e incentivo na rotina de trabalho no laboratório e à Laís Moraes, por toda a ajuda e disponibilidade, fundamentais para que esse trabalho pudesse ser concluído. Agradeço também à professora Gianna Cavalheira e à Luiza Sisdelli pela parceria nos estudos de tumores pediátricos, a funcionária Angela Maria Faria, sempre tão prestativa, e à Maria José Carregosa, pela ajuda sempre que precisei.

Às minhas amigas Flávia Guimarães Merçon, Giovana Paranhos Campos Abraão e Letícia Iervolino, presentes que a vida em São Paulo me deu, pela acolhida, pelo incentivo de sempre e pelos encontros descontraídos que tornaram mais leves a pesada rotina de trabalho.

Às minhas amigas Renata Vianna e Maria Alice Bicalho, pelo incentivo, pelo apoio e por compartilharem comigo as angústias, desafios e alegrias em conciliar a vida profissional e as tarefas de mãe.

Às amigas Lúcia Amorim Moutinho, Gabriela Studkinski, Giovana Gallo, Thaís Meirelles, Luciana Decker, Carla Beirão, Bárbara Sturmer, Patrícia Pereira, Cristina Pires e Juliana de Souza, por compreenderem a minha ausência nos últimos tempos.

Aos meus amigos de trabalho da Policlínica e do CEAC, pelo incentivo e por compartilharmos os desafios do trabalho diário no SUS, em especial à Elaine Perugini, Monique Kowalski, Fabiana Scarton, Selma Loch e Filipe Perini.

Às médicas patologistas Maria Teresa de Seixas e Rosana Delcelo, pelo auxílio na seleção do material a ser analisado nesse trabalho. Ao médico patologista, Giancarlo Colombelli, pelo auxílio com a análise de imunoistoquímica, ainda em fase de elaboração.

Aos médicos preceptores do ambulatório de tireoide da UNIFESP, Jairo Tabacow Hidal, Luiza Kimiko Matsumura e João Roberto Maciel Martins pela convivência e ensinamentos nas discussões de casos de doenças tireoidianas.

Ao professor Hans Graf, quem me permitiu o primeiro contato com a Endocrinologia durante estágio realizado no SEMPR, pelos primeiros ensinamentos em doenças da tireoide e por ter me inspirado a seguir essa área de pesquisa na minha vida profissional.

Aos médicos Osmar Monte e Carlos Longui, por todos os ensinamentos e por terem tornado possível essa parceria de trabalho entre a Unifesp e a Santa Casa de São Paulo.

Ao meu coorientador, professor Adriano Namo Cury, exemplo de seriedade e ética como médico, por todos os ensinamentos em Endocrinologia e pelo incentivo como pesquisadora.

À minha orientadora, professora Janete Maria Cerutti, exemplo de dedicação e competência na vida acadêmica, por acreditar em mim, pelo incentivo, pela disponibilidade sempre que precisei e por todo o auxílio na elaboração desse trabalho. Que sorte a minha ter tido você como orientadora!

Ao meu filho, Pedro Vieira Cordioli, quem me ensinou o amor incondicional, quem veio ao mundo para mudar minha rotina, agora intercalada com muitas brincadeiras e momentos de descontração. Tua alegria e teu carinho me propiciam momentos inesquecíveis, tua existência me tornou uma pessoa melhor e as tuas boas sonecas diurnas e o teu sono noturno me permitiram concluir esse trabalho.

Ao meu marido, Júlio Cordioli, pelo incentivo de sempre, por compreender bem a minha ausência nos dias da semana em que eu estava em São Paulo e por dividir tão bem comigo as tarefas de cuidar e educar nosso filho.

À FAPESP, pelo apoio financeiro, fundamental para a compra de material necessário para essa pesquisa (processos 2014/06570-6 3 2023/03867-5).

Sumário

RESUMO

Nas últimas décadas, inúmeros estudos reportaram um aumento na incidência do câncer diferenciado de tireoide (CDT). Esse incremento também foi identificado na faixa etária pediátrica, principalmente entre adolescentes. Em comparação à população adulta, o CDT pediátrico apresenta diferenças clinico-patológicas significativas, com estadio mais avançado ao diagnóstico e com maiores taxas de recorrência local e à distância. Além disso, alguns autores sugerem que haja uma heterogeneidade clínica nesse grupo de pacientes mais jovens, através de estudos que evidenciaram uma maior ocorrência de extensão extra-tireoidiana e metástase para linfonodos e à distância em crianças (<10 anos) em comparação com adolescentes (10-18 anos).

As diferenças clínicas observadas no CDT entre crianças, adolescentes e adultos podem, em parte, serem decorrentes de graus distintos de diferenciação tumoral e, consequentemente, expressão diferencial dos genes responsáveis pela captação e metabolismo do iodo na tireoide. Além disso, diferenças no espectro de eventos genéticos pode ser também uma das razões para as diferenças clinico-patológicas evidenciadas de acordo com a idade.

Neste estudo, analisamos os níveis de expressão de genes relacionados à captação e ao metabolismo do iodo (*NIS, TG, TPO, PDS* e *TSH-R*) e o perfil molecular em uma série de CDT pediátrico predominantemente esporádico.

O primeiro artigo, ***Are we really at the dawn of understanding sporadic pediatric thyroid carcinoma?***, sumariza as principais características clinico-

patológicas e os principais eventos genéticos já descritos no CDT pediátrico e descreve as diferenças identificadas entre os grupos pediátrico e adulto e, no grupo pediátrico, entre os tumores esporádicos e induzidos por irradiação.

O segundo artigo, ***Thyroid-specific genes expression uncovered age-related differences in pediatric thyroid carcinomas,*** descreve os resultados da análise da expressão de genes associados à captação e ao metabolismo do iodo em uma casuística de CDT pediátrico predominantemente esporádico, com ênfase nas diferenças encontradas entre crianças e adolescentes, além da comparação com resultados de uma casuística adulta previamente estudada pelo nosso grupo. Pela primeira vez é descrito haver diferença na expressão dos genes *NIS*, *PDS* e *TSH-R* no grupo pediátrico, com uma menor expressão observada em crianças em comparação aos pacientes adolescentes.

O terceiro artigo, ***AGK-BRAF gene fusion is a recurrent event in sporadic pediatric thyroid carcinoma***, descreve, pela primeira vez, a ocorrência do rearranjo *AGK-BRAF* em pacientes com CPT pediátrico esporádico.

O quarto artigo, ***Fusion oncogenes are the main genetic events found in sporadic papillary thyroid carcinomas from children***, descreve a prevalência dos principais eventos genéticos já identificados no CPT pediátrico (BRAFV600E, NRAS Q61, *RET/PTC 1,2* e *3*, *ETV6-NTRK3* e *AGK-BRAF*) numa casuística de CPT pediátrico predominantemente esporádico e com um número de crianças investigadas superior aos demais estudos já publicados. Apesar de os eventos genéticos apresentarem prevalência semelhante entre crianças e adolescentes, o espectro de mutações identificado foi diferente. Em adolescentes observou-se uma maior prevalência da mutação BRAF V600E e de tumores com mais de um evento

genético identificado. Com relação ao perfil mutacional, nossos resultados corroboram os achados prévios de que o *RET/PTC* é o principal evento genético envolvido na patogênese do CPT pediátrico. A aparente ausência da mutação BRAF V600E no CDT em crianças e a menor prevalência no CDT em adolescentes, em comparação ao grupo adulto, sugere que a prevalência dessa mutação aumente com a idade e não desempenhe um papel importante no CDT pediátrico.

O melhor conhecimento acerca da patogênese do CDT pediátrico pode contribuir para o melhor manejo de nódulos tireoidianos e seguimento dos pacientes com CDT identificado. A melhor elucidação dos eventos genéticos envolvidos e do perfil de expressão gênica desses tumores pediátricos possibilitaria a aplicabilidade de testes moleculares na avaliação do risco de malignidade de nódulos tireoidianos e o desenvolvimento de terapias direcionados para os casos de tumores com maior grau de agressividade nessa faixa etária.

INTRODUÇÃO

Estudos epidemiológicos realizados nas diversas regiões do mundo tem demonstrado um aumento progressivo nas taxas de incidência do câncer diferenciado de tireoide (CDT) (1, 2). Assim como os dados reportados em outros países, estimativas brasileiras apontam para uma taxa ainda maior em mulheres em comparação aos homens, 5,7 casos e 1,08 casos/ 100 mil habitantes, respectivamente (3). Quanto a faixa etária pediátrica, um aumento na incidência do CDT tem sido identificado principalmente no sexo feminino e entre adolescentes (10-18 anos), em comparação a crianças (<10 anos) (4-6).

Na faixa etária pediátrica, assim como na população adulta, o carcinoma papilífero da tireoide (CPT) representa o tipo histológico mais comum de CDT e corresponde a cerca de 90% dos tumores (4, 7). O carcinoma folicular da tireoide (CFT), outro tipo histológico de CDT, ocorre mais raramente na população jovem e representa cerca de 5-10% dos casos de câncer de tireoide na população pediátrica (4, 7). Os carcinomas medular esporádico e anaplásico são raramente identificados em crianças e adolescentes (4).

O CDT pediátrico se apresenta comumente como um nódulo tireoidiano único (8-10). Apesar de raro na população pediátrica, os nódulos de tireoide identificados nessa faixa etária apresentam um maior risco de malignidade em comparação a nódulos diagnosticados em pacientes adultos (26 vs 5%) (11-13). A ocorrência de adenopatia cervical palpável ao diagnóstico também é um achado comum no CDT pediátrico (9, 10).

Em comparação à população adulta, o CDT pediátrico apresenta diferenças clinico-patológicas significativas. Nessa faixa etária mais jovem, a doença é comumente identificada em estágios mais avançados, com maior probabilidade de invasão local e metástase a distância (14-16), além de maiores taxas de doença persistente ou recorrente (15, 16). Uma outra diferença relatada é o tamanho tumoral, o qual tende a ser maior em crianças e adolescentes em comparação ao observado em pacientes adultos (14, 15). A ocorrência de microcarcinomas (tumores ≤ 10mm), um achado comum no CDT adulto, é pouco frequente no grupo pediátrico (17).

Apesar das significativas diferenças clínico-patológicas observadas entre o CDT adulto e o pediátrico, as recomendações sobre o manejo desses pacientes eram usualmente semelhantes (18). Apenas recentemente, foi criada uma força tarefa para a elaboração de um consenso destinado especificamente ao manejo de nódulos e de CDT pediátrico, publicado em 2015 (19).

A elevada ocorrência de tumor multifocal e com acometimento bilateral representa outra característica do CDT em crianças e adolescentes. Na faixa etária pediátrica, cerca de 30% dos pacientes apresentam tumor bilateral (9, 10) e a ocorrência de tumor multifocal é reportada em 30-88% dos casos (8-10, 20). Essa elevada taxa de acometimento bilateral e multifocal é um dos argumentos utilizados para se recomendar a realização de tireoidectomia total (TT) para a maioria dos pacientes pediátricos (19). Além disso, há evidências de que a realização de procedimentos cirúrgicos menos extensos do que a TT ou *near* TT está relacionada a maiores taxas de recorrência tumoral em pacientes pediátricos (21, 22).

A prevalência das diferentes variantes do CPT também difere de acordo com a faixa etária. Em pacientes pediátricos, especialmente em pacientes com idade inferior a 10 anos, o padrão clássico de apresentação do CPT, comumente observado em pacientes adultos, é menos prevalente. Nesses pacientes mais jovens, as variantes folicular, sólida e esclerosante difusa são mais comumente observadas (23-26).

Apesar do CDT pediátrico ser usualmente considerado na literatura como um grupo único de pacientes, alguns autores sugerem que haja uma heterogeneidade clínica nesses pacientes mais jovens. Estudos evidenciaram uma maior ocorrência de extensão extra-tireoidiana (20, 27) e metástase para linfonodos e à distância em crianças em comparação com adolescentes (20). Outros autores também relataram que a média de idade de pacientes pediátricos com metástase local ou à distância ao diagnóstico era significativamente inferior a idade de pacientes pediátricos com CDT não metastático (7, 8, 10). Além disso, crianças tendem a apresentar maiores taxas de recorrência em comparação ao grupo adolescente (27). Outros estudos que não subdividiram o grupo pediátrico em crianças e adolescentes, mas investigaram a influência da idade no seguimento desses pacientes, evidenciaram que a idade média ao diagnóstico de pacientes com doença recorrente era significativamente inferior ao grupo de pacientes com sobrevida livre de doença. (8, 9).

Na faixa etária pediátrica, a ocorrência de metástase pulmonar iodo-captante é um achado frequente (28, 29), o que pode explicar a melhor resposta à terapia com radioiodo, RAI (do inglês, *radioactive Iodine*), nesses pacientes, em comparação aos adultos (14, 29). De fato, alguns estudos demonstraram uma completa remissão da doença na maioria dos casos de CDT pediátrico com metástase pulmonar após tratamento com RAI, especialmente em adolescentes com

metástases pulmonares iodo-captantes (26, 28, 30). A maior prevalência de metástases à distância iodo-captantes e a melhor resposta à terapia com RAI sugere um maior grau de diferenciação do CDT pediátrico em relação ao adulto, ou seja, a existência de células tumorais mais semelhantes às células foliculares normais com relação à morfologia e função.

As diferenças clínicas observadas no CDT entre crianças, adolescentes e adultos podem, em parte, serem decorrentes de graus distintos de diferenciação tumoral e, consequentemente, expressão diferencial dos genes responsáveis pela captação e pelo metabolismo do iodo.

O transporte do íon iodeto para o interior das células foliculares tireoidianas representa a primeira etapa do processo de biossíntese dos hormônios tireoidianos. Esse transporte é realizado através de um mecanismo ativo mediado pelo transportador específico existente na membrana basolateral dessas células, o co-transportador sódio-iodeto, NIS (do inglês, Na^+/I^- *symporter*) (31). A seguir, o iodeto é transportado através da membrana apical da célula folicular pela proteína pendrina (PDS) e organificado (incorporado aos resíduos de tirosina da molécula de tireoglobulina (TG)) pela enzima tireoperoxidase (TPO) (**Figura 1**). Todas essas etapas descritas dependem da ação do hormônio tireotrófico (TSH) (31).

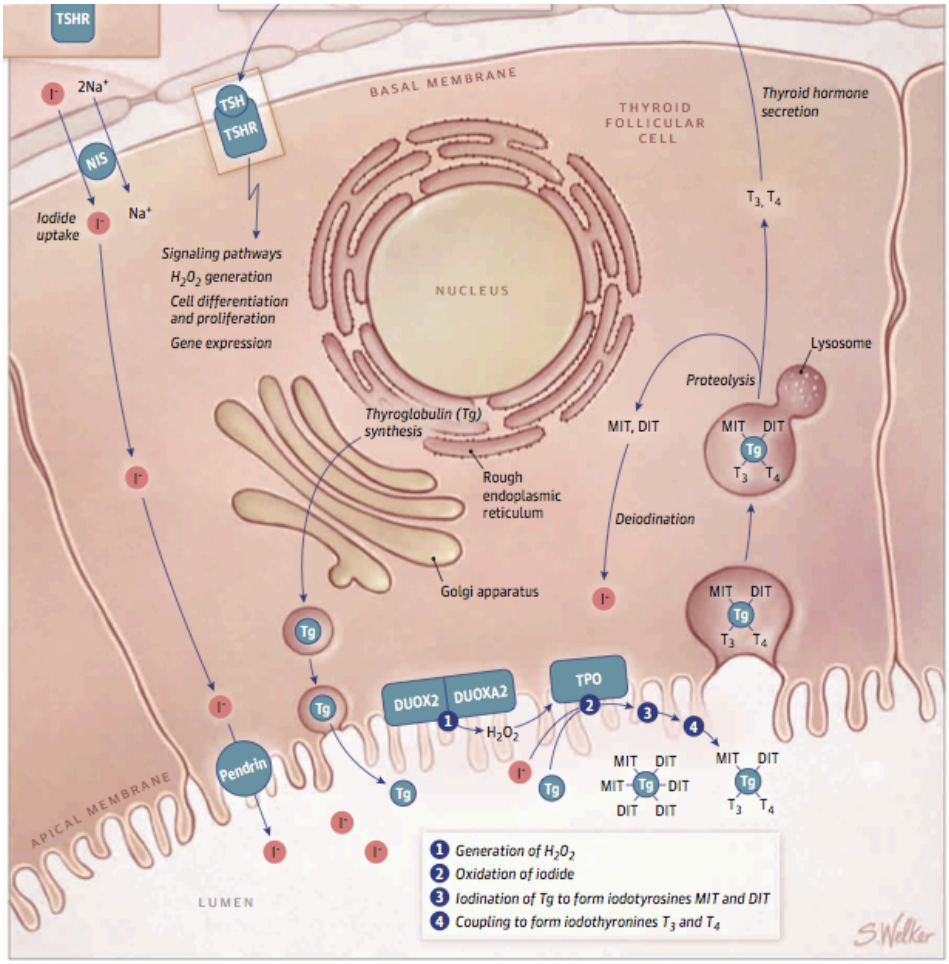

Figura 1. A primeira etapa da síntese dos hormônios tireoidianos é a captação do íon iodeto pelas células foliculares via proteína co-transportadora de sódio/iodeto (NIS), que é regulada pelo hormônio estimulador da tireoide (TSH) e pelo conteúdo intraglandular de iodo. O íon iodeto é transportado através da membrana apical da célula tireoidiana pela proteína pendrina (PDS), oxidado pela enzima tireoide peroxidades (TPO) e incorporado aos resíduos de tireoglobulina (TG) para formar a monoiodotirosina (MIT) e a diiodotirosina (DIT) que se acoplam para formar T3 (1 MIT + 1 DIT) e T4 (2 DITs). O T3 e T4 acoplados à TG são armazenados no lúmen folicular e liberados das células foliculares após endocitose e proteólise da TG (Fonte: Hanley, 2016) (32).

A hipótese de maior grau de diferenciação do CDT pediátrico em relação ao adulto implicaria numa maior expressão de *NIS* e de outros genes específicos da tireoide na população mais jovem. De fato, estudos prévios sugeriram que em tumores pediátricos a maior expressão de *NIS* está associada a uma menor taxa de recorrência (33, 34). Além disso, a atividade requerida de ^{131}I para se obter a

remissão da doença parece estar diretamente relacionada à expressão de NIS, sendo maior em pacientes com expressão de NIS indetectável (34, 35).

No entanto, estudos prévios que investigaram o papel da terapia com RAI no tratamento do CDT pediátrico, assim como os poucos estudos que investigaram a expressão de genes específicos da tireoide nesse grupo de pacientes, incluíram majoritariamente pacientes adolescentes e/ou com idade limite de até 21 anos (ao invés de 18 anos) e um número pouco significativo de pacientes crianças ou pré-púberes (14, 26, 28, 33, 34).

Em razão da heterogeneidade clínica existente no grupo pediátrico, faz-se necessária a realização de estudos comparativos da expressão dos genes associados a captação e metabolismo do iodo (**Figura 1**) nesse grupo de pacientes de acordo com a idade e/ou estadio puberal.

Alterações Genéticas nos Tumores Pediátricos da Tiroide

Nas últimas décadas, houve um grande avanço na compreensão dos mecanismos genéticos associados a patogênese do CDT em adultos. A grande maioria das alterações genéticas associadas a patogênese dos CDT da tiroide levam a ativação constitutiva da via de sinalização MAPK (do inglês, *Mitogen Activated Protein Kinases*) e via PI3K. Entre essas, as mutações nos genes *RAS* (*NRAS, KRAS* e *HRAS*) e *BRAF*, além das fusões do tipo *RET/PTC, BRAF* e NTRK tem sido associadas a etiologia do CPT. Mutações no gene *RAS* e fusões envolvendo PPARG, tem sido associadas a gênese do CFT.

A grande maioria dos estudos em tumores pediátricos tem avaliado a prevalência das mutações identificadas no CPT em adultos. Nesses estudos, tem-se observado uma diferença no espectro de mutações do CPT de acordo com a idade (36), mais especificamente, entre os grupos pediátrico e adulto (37, 38).

Ainda que estudos prévios tenham demonstrado haver diferenças no perfil mutacional entre o CPT pediátrico e adulto, uma parcela significativa desses estudos avaliou pacientes pediátricos com histórico de radiação (39-41). Evidências indicam que a prevalência dos eventos genéticos sabidamente associados à patogênese do CPT pediátrico seja diferente entre os grupos esporádico e pós radiação (42). Utilizando Sequenciamento de nova geração (NGS), um estudo recente identificou duas novas fusões (*ETV6-NTRK3* e *AGK-BRAF*) em CPT pediátricos expostos à radiação (42) (**Figura 2)**. Os autores também confirmaram que a mutação V600E no gene *BRAF,* com alta prevalência nos adultos, é pouco prevalente nos tumores pediátricos expostos à radiação ou esporádicos. Por outro lado, fusões RET/PTC são mais prevalentes nos tumores pediátricos, principalmente nos casos de exposição à radiação.

Outros estudos também avaliaram o perfil mutacional do CDT pediátrico esporádico. Conduto, o número de crianças incluídas na maioria desses estudos é pouco significativo (38, 42-44). Portanto, o conhecimento quanto ao papel dos principais eventos genéticos descritas no CDT adulto no CDT pediátrico esporádico, especialmente em crianças ou pacientes pré-púberes, ainda é escasso.

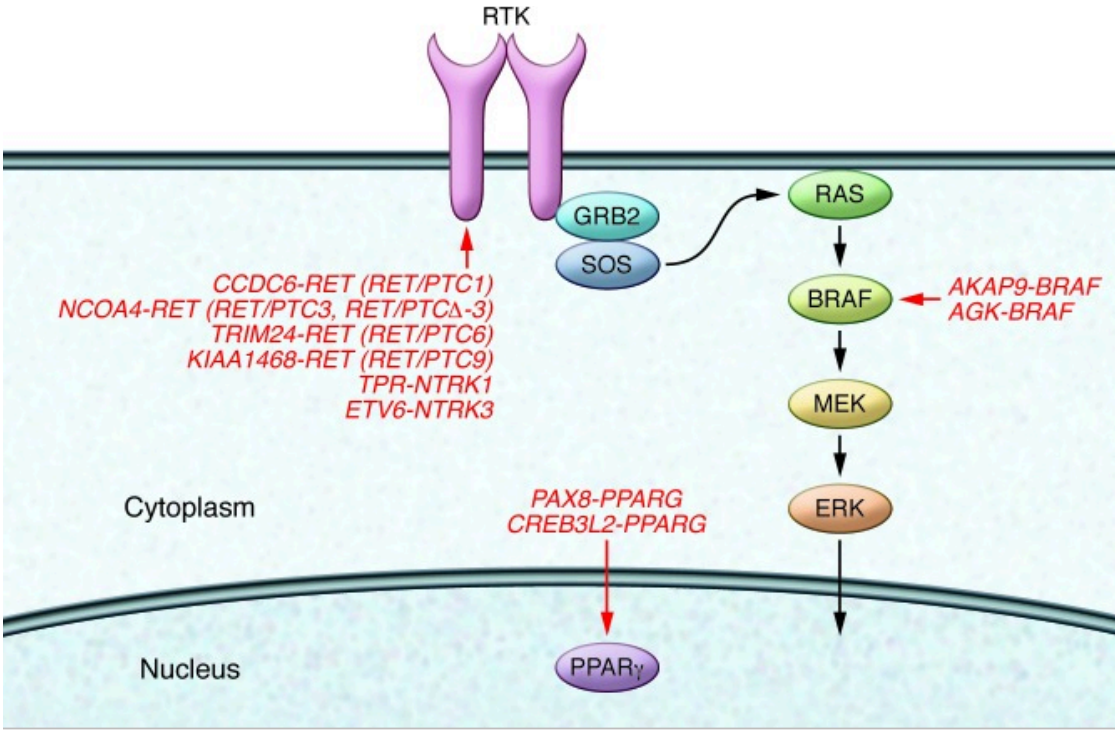

Figura 2. Resumo dos eventos genéticos identificados no CPT. Componentes da via MAPK incluem fusões dos receptores com atividade tirosino quinase *RET (RET/PTC)*, *NTRK1* e *NTRK3* ou *BRAF* quinase, além de mutações nos genes *RAS* e *BRAF*. Uma outra via identificada é representada pelos rearranjos envolvendo o receptor *PPARG*. (Fonte: Santoro, 2013) (45).

Apesar de o CDT pediátrico comumente se apresentar em estágios mais avançados ao diagnóstico e com maiores taxas de recorrência em relação ao CDT adulto, o prognóstico é excelente, com taxas de mortalidade mínimas ou mesmo zero no grupo pediátrico (8, 9, 27, 46). A análise comparativa do perfil mutacional e da expressão de genes específicos da tireoide entre tumores pediátricos e adultos poderia explicar as diferenças observadas nas características clinico-patológicas, na evolução e no prognóstico desses pacientes.

Além disso, faz-se necessária a inclusão de um número maior de crianças ou pacientes pré-púberes nos estudos de CDT, para que seja possível uma melhor elucidação quanto à existência de uma heterogeneidade clínica no grupo pediátrico e os motivos que poderiam explicar essa possível diferença.

O primeiro artigo, ***Are we really at the dawn of understanding sporadic pediatric thyroid carcinoma?***, apresenta uma extensa revisão da literatura sobre o CDT pediátrico e descreve dados epidemiológicos, características clínico-patológicas, fatores de risco e, especialmente, sumariza as informações sobre os eventos genéticos já descritos no CDT pediátrico esporádico e pós irradiação**.**

O segundo artigo, ***Thyroid-specific genes expression uncovered age-related differences in pediatric thyroid carcinomas***, descreve os resultados da análise da expressão de genes associados a captação e ao metabolismo do iodo em uma casuística de CDT pediátrico predominantemente esporádico, com ênfase nas diferenças encontradas entre crianças e adolescentes, além da comparação com resultados de uma casuística adulta previamente estudada pelo nosso grupo (47, 48).

O terceiro artigo, ***AGK-BRAF gene fusion is a recurrent event in sporadic pediatric thyroid carcinoma***, descreve, pela primeira vez, a ocorrência do rearranjo *AGK-BRAF* em pacientes com CPT pediátrico esporádico. Esse rearranjo foi recentemente descrito em um paciente adolescente com CPT e histórico de radiação na infância (42).

O quarto artigo, ***Fusion oncogenes are the main genetic events found in sporadic papillary thyroid carcinomas from children***, descreve a prevalência dos principais eventos genéticos já identificados no CPT pediátrico (BRAFV600E, NRAS Q61, *RET/PTC 1,2* e *3*, *ETV6-NTRK3* e *AGK-BRAF*) numa casuística de CPT pediátrico predominantemente esporádico e com um número de crianças investigadas superior aos demais estudos já publicados. Esse artigo foi submetido para a revista *Thyroid.* As considerações realizadas pelos revisores já nos foi

enviada, os questionamentos foram respondidos e o trabalho revisado foi submetido para análise dos revisores.

OBJETIVOS

Gerais:

- Analisar as características clínico-patológicas, caracterizar o perfil molecular e avaliar a expressão de genes relacionados à captação e ao metabolismo do iodo (*NIS*, *TG*, *TPO*, *PDS* e *TSH-R*) em uma casuística de CDTs pediátricos predominantemente esporádicos.

Específicos:

- Analisar a expressão dos genes *NIS*, *TG*, *TPO*, *PDS* e *TSH-R* em amostras de CDT pediátrico predominantemente esporádicos;
- Comparar o nível de expressão dos genes *NIS*, *TG*, *TPO*, *PDS* e *TSH-R* avaliado nas amostras de CDT pediátrico com a expressão observada em amostras de CPT em adultos;
- Investigar a prevalência das mutações BRAFV600E e NRAS Q61 e das fusões *RET/PTC 1,2* e *3*, *ETV6-NTRK3* e *AGK-BRAF* em uma série de CPT pediátricos, predominantemente esporádicos;
- Correlacionar os dados das análises de expressão gênica e caracterização molecular com as características clínico-patológicas na casuística investigada.

ARTIGO 1

Are we really at the dawn of understanding sporadic pediatric thyroid carcinoma?

Artigo publicado na revista *Endocrine Relate Cancer* em agosto de 2015

DOI: 10.1530/ERC-15-0381

Are we really at the dawn of understanding sporadic pediatric thyroid carcinoma?

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Short title: pediatric differentiated thyroid carcinoma

Keywords: sporadic pediatric papillary thyroid carcinomas, radiation-exposed papillary thyroid carcinomas, RET/PTC, BRAF, RAS and ETV6-NTRK3

Abstract

Data from the National Cancer Institute and from the literature have disclosed an increasing incidence of thyroid cancer in children, adolescents and adults. Although children and adolescents with thyroid cancer tend to present with more advance disease than adults, their overall survival rate is excellent; however, there is no clear explanation for the differences observed in the clinicopathological outcomes in these age groups. There has been an ongoing debate regarding whether the clinicopathological differences may be due the existence of distinct genetic alterations. Efforts have been made to identify these acquired genetic abnormalities that will determine the tumor's biological behavior and that will ultimately allow molecular prognostication. However, most of the studies have been performed in radiation-exposed pediatric thyroid carcinoma. Therefore, our understanding of the role of these driver mutations in sporadic pediatric DTC development is far from complete, and additionally, there is a strong need for studies in both children and adolescents. The aim of this review is present an extensive literature review with emphasis on the molecular differences between pediatric sporadic and radiation-exposed differentiated thyroid carcinomas and adult population.

Introduction

The incidence of thyroid cancer has increased worldwide over the last decades. Essentially, one of the largest annual increases from 2006-2010 was for cancer of thyroid (Davies and Welch 2006; Lise*, et al.* 2012; Siegel*, et al.* 2014b). Differentiated thyroid cancer (DTC) is most frequently diagnosed among adults aged 45-54, with a mean age at diagnosis of 50 {SEER Stat Fact Sheets:thyroid cancer, available at http://seer.cancer.gov/statfacts/html/thyro.html accessed on July, 2015} and with a female predominance. Currently, it is the fifth most common cancer in women in USA (Siegel *et al.* 2014b) and, in Italy, it is the second most frequent cancer in women below age of 45 (Pellegriti*, et al.* 2013). In São Paulo, Brazil, not only the thyroid cancer incidence rates were consistently higher than in USA but also the female predominance was higher than that reported in SEER (Veiga*, et al.* 2013).

Although rare in young population, DTC rates are also increasing significantly in children and adolescents. Among DTC, papillary thyroid cancer (PTC) accounts for nearly 90% and follicular thyroid carcinoma (FTC) accounts for about 5-10% of all thyroid cancer that occurs in pediatric population (Demidchik*, et al.* 2007; Hogan*, et al.* 2009).. It has been suggested that FTC is very rare and occurs in a slightly older age group (Jarzab*, et al.* 2005). Medullary thyroid carcinoma, poorly differentiated thyroid carcinoma and undifferentiated thyroid carcinoma are rare in young patients (Hogan *et al.* 2009) and are not the focus of this review.

Regardless the ethnicity, an increased trend in incidence rates of pediatric thyroid carcinomas was found in most regions of US in both genders (Vergamini*, et al.* 2014). When stratified by age, the annual incidence rate of cancer in children and

adolescents is 0.43 (5–9 years), 3.50 (10-14 years) and 15.16 per million (15-19 years) (Vergamini *et al.* 2014). Others have also demonstrated that the incidence rates increased with age (Hogan *et al.* 2009; Siegel*, et al.* 2014a). In fact, among 15-19 year old adolescents, thyroid is the eight most common cancer diagnosed and the second most common cancer among girls (Ward*, et al.* 2014; Wu*, et al.* 2003). Similar to that observed in adults, there is a female predominance (Hogan *et al.* 2009; Landau*, et al.* 2000; Lazar*, et al.* 2009).

The reasons for increasing incidences rates of pediatric thyroid cancer are currently unknown. Previous studies suggested that the increasing incidence of thyroid cancer among adults was predominantly due to better access to medical care and increased diagnostic scrutiny (Davies and Welch 2006). It is possible that advances in ultrasound imaging technologies have improved diagnosis and, consequently, over time, may have contributed to detect small and asymptomatic pediatric thyroid cancers.

Although increased diagnostic scrutiny may account for some of the rise, the increased incidence across all tumor sizes in young patients argue a favor of a true increase (Vergamini *et al.* 2014). Besides, it has been suggested that some of this rise may be due to environment factors and lifestyle changes. Finally, the increase in the incidence of PTC with no similar increase in the incidence of other histological types of thyroid cancer is an argument in favor that environment factors may contribute to the increase (Mazzaferri 1993).

There are significant molecular, pathological and clinical differences in DTC among children, compared to adult population. To indorse best practice standards for the diagnosis and management of thyroid cancer in pediatric population, a task force

appointed by the American Thyroid Association (ATA) recently provided the first recommendations specifically addressing the management of thyroid nodules and DTC in children and adolescents (Francis*, et al.* 2015). The authors suggested applying these recommendations to patients up to 18 years old, when the majority of pediatric patients have completed growth and development.

The aim of this review is to present an extensive literature review with emphasis on the molecular differences between the pediatric and adult population. Although several studies of pediatric DTC included individuals up to 21years of age, we mainly focused on studies that involved individual ≤18 years of age.

Clinical Presentation

The thyroid cancer in children usually presents as a solitary nodule (Grigsby*, et al.* 2002; Welch Dinauer*, et al.* 1998). The occurrence of palpable cervical adenopathy at diagnosis is also a common finding in pediatric DTC (Grigsby *et al.* 2002). Previous studies reported significant differences in the clinical presentation and outcomes of DTC in pediatric patients compared to adults (Jarzab and Handkiewicz-Junak 2007).

Although thyroid nodules are uncommon in the pediatric population, there is a greater risk of malignancy in nodules diagnosed in children and adolescents than in adults (26% *versus* 5%) (Gharib and Papini 2007; Niedziela 2006; Romei and Elisei 2012). Moreover, pediatric cases are more likely to present a more advanced stage of the disease at diagnosis, often a more aggressive local disease and higher rates of distant metastases (Alzahrani*, et al.* 2015; Chow*, et al.* 2004; Jarzab *et al.* 2005; Kumagai*, et al.* 2004; Zimmerman*, et al.* 1988). Neck lymph node metastasis at diagnosis was reported in nearly 90% of pediatric cases, while they were detected in

35% of adults (Zimmerman *et al.* 1988). Other series reported lymph node involvement at diagnosis in 40-90% of pediatric cases (Dinauer*, et al.* 2008; Landau *et al.* 2000; Lazar *et al.* 2009; Newman*, et al.* 1998; O'Gorman*, et al.* 2010), compared to 20–50% of adults (Ahn*, et al.* 2015; Zaydfudim*, et al.* 2008) (**Table 1**).

Distant metastasis was found in virtually 7-30% of pediatric patients compared to 2-9% of adults (Chow *et al.* 2004; Dinauer *et al.* 2008; Handkiewicz-Junak*, et al.* 2007; Hogan *et al.* 2009; La Quaglia*, et al.* 2000; Newman *et al.* 1998; O'Gorman *et al.* 2010; Zimmerman *et al.* 1988). Mostly pediatric patients present distant metastasis in the lungs**,** but few cases have been also reported in the brain, soft tissue or bone (Jarzab and Handkiewicz-Junak 2007; Newman *et al.* 1998) (**Table 1**).

Nevertheless, a marked heterogeneity within the pediatric group has been reported. Pediatric cases tend to be more symptomatic in the prepubertal group (Jarzab *et al*. 2005)**.** Children present with more aggressive local disease and are more likely to have lymph node metastases at diagnosis. In fact, it was demonstrated that prepubertal children had a greater degree of extrathyroid extension and lymph node involvement than adolescents (Alessandri*, et al.* 2000; Lazar *et al.* 2009). Additionally, they are more prone to develop distant metastases (Dinauer *et al.* 2008; Jarzab *et al.* 2005; Lazar *et al.* 2009; O'Gorman *et al.* 2010; Rivkees*, et al.* 2011) and they also experience recurrence more frequently and earlier than adolescents (Alessandri *et al.* 2000). The biological hypothesis for greater differentiation and responsiveness to treatment is discussed below.

The mean tumor size tends to be larger in pediatric patients. Comparison between 58 pediatric patients (< 17 years old) and 981 adults consecutive PTC patients treated at the Mayo Clinic revealed that the mean tumor size was greater in

pediatric cases (3.1 cm; +/- 1.7) than in adults (2.1 cm; +/- 1.7). The authors also showed that tumors larger than 4 cm were more prevalent in pediatric cases (36%) than in adults (15%) (Zimmerman *et al.* 1988). Furthermore, papillary microcarcinomas (≤ 1 cm) are rarely reported in pediatric cases (3% of cases), whereas microcarcinomas comprise about 30% of all thyroid carcinomas diagnosed in adults (Chow *et al.* 2004). It is likely that in populations undergoing extensive screening, small pediatric PTC will be detected. Excluding the screening programs conducted in Belarus area after Chernobyl accident in 1986 (Ashizawa*, et al.* 1997) and the screening of children from different Japanese prefectures after the Fukushima Daiichi Nuclear Power Plant accident Plan in 2011 (Hayashida*, et al.* 2013; Yasumura*, et al.* 2012), studies reporting the prevalence of small thyroid nodules in pediatric population are scarce. Ultrasound examination in children from Fukushima, Aomori, Yamanashi and Nagasaki prefectures revealed that between 35 and 51% of children who underwent thyroid ultrasound examination showed thyroid cysts and nearly 1% showed thyroid nodules ≤ 0.5 cm (Hayashida *et al.* 2013; Yamashita and Suzuki 2013).

Another difference between pediatric and adult DTC is the higher rates of bilateral and multifocal disease in childhood. Pediatric patients present bilateral disease in about 30% of cases (Grigsby *et al.* 2002; Lazar *et al.* 2009) and multifocal disease in 30-80% of cases (Gorman*, et al.* 2010; Grigsby *et al.* 2002; Welch Dinauer *et al.* 1998). This higher rate of bilateral and multifocal disease is one of the arguments used to recommend for a more comprehensive thyroid surgery in pediatric patients (Francis *et al.* 2015).

PTC variants, such as follicular variant of PTC (FVPTC) and diffuse sclerosing PTC (DSPTC) are more frequently found in pediatric patients than in adults (Neiva*, et*

al. 2012). Although there is no consensus on the prognosis of different histological type, it was recently demonstrated that DSPTC is frequently associated with bilateral disease, extrathyroidal extension, lymph node involvement, lung metastasis and lower rates of recurrence-free survival than of non-DSPTC (Koo*, et al.* 2009).

Treatment and Prognosis

Because pediatric DTC is an uncommon malignancy, randomized trials have not been applied to test best-care options in this group of patients (Rivkees *et al.* 2011). Therefore, the optimal initial and long-term treatment and follow-up remain controversial.

Despite a more advanced disease at presentation and a higher risk of recurrence, the prognosis of childhood DTC is generally fairly good. The reported mortality rate is low or even zero in some series (Alessandri *et al.* 2000; Henke*, et al.* 2014; Newman *et al.* 1998). For this reason, The ATA guideline for children with thyroid nodules and DTC developed recommendations based on the available scientific evidence and expert opinion (Francis *et al.* 2015). The authors suggested reconsidering the former recommendation that all children with DTC should be similarly treated with a more extensive surgery and routine RAI therapy (Rivkees *et al.* 2011). A more comprehensive surgical approach raises the risk of important clinical complications, mainly transient or permanent hypoparathyroidism and recurrent laryngeal nerve damage. The RAI therapy is associated with an increased in the risk of second primary malignancy, especially salivary cancer (Marti*, et al.* 2015).

The ideal surgical approach for the majority of patients is total thyroidectomy (TT) (Francis *et al.* 2015). However, in patients with a small unilateral tumor and without extrathyroidal extension, a near-TT can be considered to lower the risk of injury to either the recurrent laryngeal nerve or parathyroid glands (Francis *et al.* 2015; Rivkees *et al.* 2011). Previous studies that assessed the outcomes of a less comprehensive surgical approach in pediatric patients have shown a higher risk of relapse rates with lobectomy vs TT (Handkiewicz-Junak *et al.* 2007; Hay*, et al.*). Despite the high rate of cervical metastasis in pediatric DTC, routine central lymph node dissection is no longer recommended. The central neck dissection should be performed when there is evidence of central and/or lateral neck metastasis or gross extrathyroidal invasion (Francis *et al.* 2015)

Regarding RAI indications, the currently recommendation in pediatric DTC is for treatment of nodal or other locoregional disease that is not amenable to surgery as well as distant metastases that are iodine-avid. Moreover, the RAI therapy can also be consider in children with T3 tumors or extensive regional nodal involvement (Francis *et al.* 2015). Similar to adults, there is no evidence of benefit of RAI remnant ablation in pediatric patients with intra-thyroidal disease and no lymph node disease (Lamartina*, et al.*)

Risk Factors

The link between ionizing radiation during childhood and thyroid cancer has been known since 1950. The first sharp rise in the incidence of thyroid cancer was reported in epidemiological studies after external radiation to treat common childhood conditions such as acne, *tinea capitis*, and enlarged tonsils or thymus gland. A pool

analysis of seven studies demonstrated a high risk of thyroid cancer in subjects irradiated at a young age, even for radiation doses as low as 0.10 Gy. Although the risk of developing thyroid cancer is still present after more than 40 years after exposure, it is higher between 15 and 30 years. The risk decreased significantly with increasing age at exposure, with very little risk after age 20 (Ron*, et al.* 1995).

The second peak of thyroid cancer was observed in 1996, 10 years after the Chernobyl nuclear power station accident, when over 10^{18} Bq of radioactivity was released into the atmosphere, mainly ^{131}I and ^{137}Cs. The highest levels of contamination occurred in Belarus, Ukraine, and western Russia. Children and adults have been exposed to a relatively high dose of ^{131}I. Predominantly, through ingestion of contaminated food and drink, their thyroid has accumulated a high dose of ^{131}I. As childhood thyroid is very radiosensitive, one would expect a high prevalence of thyroid disease in those subjects exposed to radiation at a young age. In fact, the incidence rate of childhood thyroid carcinoma in the heavily contaminated region of Belarus reached 40 per million, while an annual incidence of 1 per million was reported in this area before the accident. The highest risk group was those patients aged 0–4 years at the time of exposure. After 1996, the incidence decline progressively and, after 2001, only sporadic cases (not exposed to radiation) were reported in pediatric patients (<15 years old) (Demidchik *et al.* 2007; Tuttle*, et al.* 2011; Williams 2008).

The radiation-associated risk of thyroid cancer to the exposed children and residents after the Fukushima Daiichi Nuclear Power Plant accident on March 2011 is still unclear. The RAI measured after the accident was one-tenth or less that measured after the Chernobyl accident, and the radiation exposure dose measured in children from neighboring regions after the accident was at a near negligible level.

The Fukushima Prefecture started the Fukushima Health Management Survey Project aimed at long-term health care administration and early medical diagnosis/treatment for prefectural residents. As the first round of screening, a thyroid ultrasound examination was conducted from October 2011 to March 2014 in nearly 300.000 individuals aged <18 years. From a total of 108 (0.8%) children with suspicious nodules, 84 had thyroid carcinoma, most (96%) were PTC (Yamashita and Takamura 2015). Although a not significant increase in the prevalence of thyroid cancer has been reported after the Fukushima Daiichi Nuclear Power Plant accident (Iwaku*, et al.* 2014), a sharp increase in the incidence of thyroid cancer was observed 4-5 years after the Chernobyl accident, and, therefore, it was preceded by a latency phase. Only a long-term follow-up will clarify whether a third peak of thyroid cancer might occur after the Fukushima Daiichi Nuclear Power Plant accident, .

These findings recognized the extreme sensitivity of children's thyroid to radiation, compared to adults. Many epidemiologic studies have explored whether the exposure to radiation during medical diagnostic and therapeutic procedures represent a risk factor for pediatric thyroid cancer. It has been demonstrated that the thyroid exposure to X-rays due to dental radiographic procedures (Memon*, et al.* 2010) or primary beam during computed tomography scan of the neck during childhood is associated with a low but not negligible risk of cancer (Mazonakis*, et al.* 2007; Pellegriti *et al.* 2013). Regarding therapeutic procedures, it is well known that survivors of pediatric cancer may suffer from late sequelae of treatment, including secondary malignant neoplasia in the irradiated region. Secondary thyroid carcinoma after radiotherapy to the neck has been reported in many publications. Interestingly, the risk of a subsequent thyroid cancer after a first tumor in childhood rose with increasing radiation dose (greatest risk 20–29 Gy) but doses higher than 30 Gy is

consistent with a cell-killing effect (Sigurdson*, et al.* 2005). As an example, the cumulative incidence for patients with up to 30 years of follow-up after the diagnosis of Hodgkin's lymphoma (HL) was 4,4% for thyroid carcinoma and the mean interval after HL diagnosis was 13.2 years (range, 4.0-29.2 years). The most frequent thyroid carcinoma identified in these patients is PTC (Dorffel*, et al.* 2000; Levy*, et al.* 2012; Marti*, et al.* 2012).

This pediatric thyroid cancer peak incidence and a "latency phase" reinforce that a long-term follow-up of patients should be undertaken for survivors of both the Fukushima Daiichi Nuclear Power Plant accident and any cancer during childhood involving radiotherapy to the thorax or head and neck region.

Hints from cancer biology

Recently, The Cancer Genome Atlas (TCGA) Research Network, using next-generation DNA and RNA-sequencing, copy-number variation, miRNA, methylomic, transcriptomic and proteomic profiles, combined with clinic-pathological data, characterizes the landscape of nearly 500 PTCs of adults. The study confirmed that PTC is associated with mutations in genes that code for proteins involved in the MAPK pathway such as *RET, BRAF* and *RAS*. The TCGA also identified new cancer-causing gene mutations that occur in PTC (*EIF1AX, CHEK2, PM1D*), as well as new fusion transcripts and somatic copy number alteration (recurrent 22q deletion and 1p amplification) that reduced the so called "dark matter'' of the PTC. The large collection of genetic alterations, combined with a comprehensive transcriptomic and proteomic analysis, revealed fundamental biological differences between PTCs. This increased knowledge helped stratify PTC into subgroups, which ultimately will refine

preoperative diagnosis of thyroid nodules and prognosis and treatment of adult PTC (The Cancer Genome Atlas Research Network, 2014).

Several studies have suggested that the spectrum of mutations may differ between tumors of pediatric patients and tumors of adults (Bongarzone*, et al.* 1996). Moreover, few studies have indicated that radiation-exposed and sporadic pediatric thyroid carcinomas are different biological types of cancer with the same histology (Nikiforov*, et al.* 1997).

To obtain a whole picture of the genomic landscape of the radiation-exposed pediatric thyroid carcinomas, a research team performed RNA-sequencing in five patients with thyroid carcinoma from the regions of Ukraine and who were younger than 10 years at the time of the Chernobyl nuclear accident. They selected patients who were negative for known BRAF mutations and known fusion transcripts (*RET/PTC, TPR-NTRK1, PAX8-PPARG* and *AKA9-BRAF*). Moreover, the research group performed low-pass whole-genome sequencing of five radiation-exposed and five patients with sporadic pediatric thyroid carcinoma who were from the same geographical regions (Ricarte-Filho*, et al.* 2013). The authors identified new kinase fusion oncogenes in radiation-exposed thyroid carcinomas. First, this study ratifies that the MAPK pathway plays a critical role in pediatric PTC development (Ricarte-Filho *et al.* 2013). Second, the prevalence of fusion oncogenes in radiation-induced tumors (84%) was much higher than the prevalence in sporadic cases (33%). This finding supports the concept that ionizing radiation induces chromosomal rearrangement but contests the notion that the prevalence of fusion oncogenes is similar in both sporadic and radiation-induced pediatric PTC. Last, it reinforces the idea that spectrum of mutations in pediatric tumors differ from adults.

The hints from molecular biology suggest that the clinical and pathological differences observed between pediatric and adults might be fundamentally due to their biological differences. Therefore, the therapy that may be recommended for an adult may not be appropriate for a child, which validates the development of unique pediatric guidelines (Francis *et al.* 2015).

The major known somatic events associated with radiation-exposed and sporadic pediatric thyroid carcinomas reported in the literature are summarized below (**Figure 1, Supplementary Tables 1 and 2**).

RET/PTC fusions transcripts

The *RET* (rearranged during transfection) gene, located in the chromosome 10q11.2, encodes for a cell membrane receptor tyrosine kinase (TK). *RET* rearrangement was initially described in an irradiated PTC (Fusco*, et al.* 1987). Through chromosome rearrangement, *RET* was fused to the NH_2 terminus of a heterologous gene denominated CCD6 (formerly named H4). *RET* gene is not expressed in normal follicular thyroid cells. However, the fusion product expresses intrinsic and constitutive TK activity. This not only was the first example of oncogene activation in solid tumors but also was the first *RET* rearrangement described in PTC and, hence, named *RET/PTC1* (Fusco and Santoro 2007).

In the subsequent years, other RET/PTC isoforms were identified in sporadic and radiation-exposed PTC. Currently, nearly 20 types of *RET/PTC* rearrangements were identified (The Cancer Genome Atlas Research Network, 2014; Fusco and Santoro 2007; Ricarte-Filho *et al.* 2013; Romei*, et al.* 2008). In all isoforms the TK

domain of RET is conserved and fused to other genes. Although *RET/PTC* rearrangement was described in benign lesions, in most series it was specifically found in PTC. An elegant work that was performed by the Nikiforov group shows that this thyroid specificity is likely due to nuclear architecture of thyroid cells, i.e. spatial proximity between partners and *RET* may influence their participation in the *RET/PTC* rearrangements in the human thyroid cell (Nikiforova*, et al.* 2000).

While in most series *RET/PTC* fusion is the second most common genetic event in PTC of adults (Romei and Elisei 2012), it is the main genetic event found in both sporadic and radiation-induced pediatric PTC (**Figures 1 and 2**).

In this systematic review of literature, we estimate the overall prevalence of *RET/PTC* in pediatric sporadic and radiation-exposed PTC. The overall prevalence of *RET/PTC* differs between sporadic and radiation-exposed pediatric PTC carcinomas (41% versus 58%, respectively) **(Figure 1)** (Student t-Test; *P*=0.034). The reported prevalence of *RET/PTC* in sporadic PTC varies from 22% (France/Italy) to 65% (USA), while its prevalence in radiation-exposed varies from 33% (Belarus) to 76% (Belarus) (**Figure 2, Supplementary Tables 1 and 2**). The highest incidence was found in post-Chernobyl pediatric PTC patients. As radiation exposure induces DNA double-strand breaks and *RET* gene and their partners are juxtaposed in the nuclei of thyroid cells**,** it facilitates chromosome recombination**.** Few studies reported PTC with concomitant *RET/PTC* in sporadic (Fenton*, et al.* 2000; Penko*, et al.* 2005) and radiation-induced PTC (Elisei*, et al.* 2001). The reported prevalence of concomitant *RET/PTC* rearrangements in sporadic cases was 2% and radiation-exposed was 1% **(Figure 1).**

Even if part of these differences can be attributed to geographic variability, the major differences in the prevalence of *RET/PTC* have been reported in radiation-exposed cases from Belarus area (Elisei *et al.* 2001; Klugbauer*, et al.* 1995; Kumagai *et al.* 2004; Nikiforov *et al.* 1997; Nikiforova*, et al.* 2004; Pisarchik*, et al.* 1998; Rabes*, et al.* 2000; Ricarte-Filho *et al.* 2013; Thomas*, et al.* 1999) (**Figure 2**). It has been suggested that other factors, probably influenced by ethnic or genetic background, may act independently from or in cooperation with radiations to trigger *RET* rearrangement (Elisei *et al.* 2001). It has also been suggested that tumor heterogeneity and the use of different detection method may contribute to the variability in the reported prevalence of RET/PTC (Zhu*, et al.* 2006) (Nikiforov and Nikiforova 2011).

Others have reinforced that tumor latency changes the prevalence and the type of *RET/PTC* rearrangement. Higher prevalence of *RET/PTC*3 rearrangements was found in faster developing tumors and in the most heavily contaminated areas (Rabes *et al.* 2000). Others have also found that *RET/PTC3* is preferentially found in radiation-associated pediatric PTC with a short latency period, whereas *RET/PTC1* mainly found in later-occurring PTC (Smida*, et al.* 1999).

Regarding the prevalence of different RET/PTC isoforms, *RET/PTC1* and *RET/PTC3* are by far the most prevalent isoforms identified in tumors from two groups (**Figure 1, Supplementary Tables 1 and 2**). *RET/PTC1* was found at comparable prevalence is sporadic (20%) and radiation-induced pediatric PTC (18%), while the prevalence of *RET/PTC3* was higher in radiation-exposed (33%) than in sporadic (10%) pediatric PTC (Fisher exact test; P=0.01). Although very few studies have examined the prevalence of *RET/PTC2*, this isoform was more prevalent in the sporadic group (Fenton, 2000; Nikiforov, 1997).

In the radiation-induced group, *RET/PTC3* fusion oncogene was associated with more aggressive variants such as solid variant and DSPTC, whereas *RET/PTC1* was mainly found in classical and FVPTC (Elisei *et al.* 2001; Rabes *et al.* 2000).

Even though it was described in a radiation-exposed PTC 28 years ago, it is still not clear whether *RET/PTC* rearrangement correlated with age or a more aggressive phenotype and histological subtype in sporadic pediatric PTC.

BRAF V600E mutation

The BRAF V600E, the T1799A nucleotide transversion which leads to a substitution of valine to glutamic acid, is the most common and specific genetic alteration found in PTC of adults (The Cancer Genome Atlas Research Network, 2014; Frasca*, et al.* 2008; Kimura*, et al.* 2003; Oler and Cerutti 2009; Xing 2005).

This review of the literature and appraisal of the overall prevalence of BRAF V600E in pediatric population shows that the prevalence of BRAF V600E is lower in radiation-exposed tumors (3%) than in sporadic cases (13%), although the observed differences did not reach statistical significance. In the sporadic group, the prevalence ranges from 0 to 37%, while in radiation-exposed group the prevalence ranges from 0-8% (**Figure 2**).

Though patient age was not specified in all series, none of the patients with *BRAF* mutation were younger than 10 years (Givens*, et al.* 2014; Lima*, et al.* 2004; Ricarte-Filho *et al.* 2013; Sassolas*, et al.* 2012). The lack of the BRAF V600E mutation in children and a lower prevalence of mutation in adolescents suggests that

the prevalence of BRAF V600E increases with age and that BRAF V600E may not play a major role in pediatric tumors.

Recently, a group reported a high prevalence (63%) of BRAF V600E mutation in pediatric PTC (Henke *et al.* 2014). The median age of patients enrolled in this study was 18.6 years and the number of patients younger than 10 years old and their *BRAF* mutation status were not mentioned. As the methodology used to detected BRAF V600E was PCR-RFLP, instead of PCR-sequencing, this study was not included in overall analysis.

All together, the prevalence of BRAF V600E is significantly lower than *RET/PTC* in both sporadic and radiation-exposed pediatric groups (P=0.0055).

RAS point mutations

Activating mutation in codons 12, 13, or 61 of *RAS* genes (*NRAS*, *KRAS* and *HRAS*) have also been described in PTC. According to the catalogue of somatic mutations in cancer (http://sanger.ac.uk/cosmic), *NRAS* is the most frequently mutated *RAS* isoform in PTC. The highest rates of mutation were found in exon 2 of NRAS (13%). The Q61K mutation results in substitution from a glutamine (Q) to a lysine (K), at position 61. Recently, *NRAS* was also reported as the second most common mutation found in PTC by the TCGA study (The Cancer Genome Atlas Research Network, 2014).

A strong association has been found between the presence of *RAS* mutations and histology in PTC of adults, with *RAS* mutations characterizing FVPTC (The Cancer Genome Atlas Research Network, 2014; Adeniran*, et al.* 2006; Park*, et al.*

2013; Rivera*, et al.* 2010; Zhu*, et al.* 2003). High prevalence of mutations in the *RAS* gene has been described in FTC (18-57%) and follicular thyroid adenoma (FTA) (24-53%) (Fukahori*, et al.* 2012). This mutation is also found in poorly differentiated and anaplastic carcinomas (18-31%) (Pita*, et al.* 2014).

Relatively few studies have evaluated the occurrence of *RAS* point mutation in pediatric DTC and the incidence rates ranges from 0 to 7% in sporadic tumors (Kumagai *et al.* 2004; Ricarte-Filho *et al.* 2013; Sassolas *et al.* 2012) and 0% in radiation-exposed tumors (Kumagai *et al.* 2004; Ricarte-Filho *et al.* 2013; Suchy*, et al.* 1998). In these studies only mutations at codon Q61 of *NRAS* were described. Although, Suchy *et al.* 1998 found mutations at codons 14 and 15 of *HRAS*, these were silent mutation or did not interfere with GTPase activity or protein binding capacity, respectively. Thus, different from adults, *RAS* mutations exert a minor role in the pathogenesis of pediatric PTC.

ETV6-NTRK3 fusions transcripts

The *ETV6-NTRK3* is a new fusion oncogene recently described in 7% of pediatric radiation-exposed PTC (Ricarte-Filho *et al.* 2013). The *ETV6-NTRK3* fusion results from an interchromosomal translocation, which juxtaposes exons 1-4 of *ETV6* to exons 12-18 of *NTRK3*. The chimeric transcript is able to activate MAPK and PI3K signaling pathways and promote cell growth of NIH-3T3 cells as well as colony formation in soft agar (Ricarte-Filho *et al.* 2013). Further validation analysis showed that 7% of sporadic pediatric PTC from the Ukraine area had *ETV6-NTRK3* fusion (Ricarte-Filho *et al.* 2013). The authors found that pathological characteristic of both radiation-exposed tumors and sporadic cases appeared to correlate with the nature

of underlying drive mutation; i.e., *ETV6-NTRK3* was mainly found in FVPTC. Finally, all tumors with *ETV6-NTRK3* fusion were from patients older than 13 years of age at surgery.

ETV6-NTRK3 was later detected in 14.5% post-Chernobyl PTCs (age range from 14 to 32 years) and in 2% of sporadic (age range from 15 to 97 years) (Leeman-Neill*, et al.* 2014). *ETV6-NTRK3* was the second most common rearrangement, after *RET/PTC*, in radiation-induced PTCs. One of the tumors with *ETV6-NTRK3* was from a patient who was aged 1 year at the time of the Chernobyl accident and another tumor was from a patient who was aged 10 years at the time of exposure. All radiation-induced PTCs in which *ETV6-NTRK3* fusion was identified had some component of a solid growth pattern and most were classified as FVPTC (Leeman-Neill *et al.* 2014). Importantly, the authors demonstrated that the presence of *ETV6-NTRK3* rearrangement, as well as *RET/PTC* and *PAX8-PPARγ* positive tumors, was significantly more common in tumors associated with higher dose exposure to ^{131}I than tumors that had point mutations (*NRAS*, *HRAS* and *BRAF*).

The prevalence of *ETV6-NTRK3* in pediatric sporadic PTC, its prognosis significance and whether in pediatric cases it is associated with older age (>10-18 years old) remains uncertain.

Other fusions transcripts

Others less prevalent fusion transcripts has been described in pediatric radiation-exposed PTC. The overall prevalence of these other fusion transcripts was 6% in a pediatric radiation-exposed PTC range from 3 to 19% (Ciampi*, et al.* 2005; Ricarte-Filho *et al.* 2013; Sassolas *et al.* 2012) and 0% in sporadic (Ricarte-Filho *et al.* 2013).

The *PAX8-PPARG* and *CREB3L2-PPARG* fusions were previously identified in follicular thyroid cancer (Kroll*, et al.* 2000; Lui*, et al.* 2008). *PAX8-PPARG* rearrangement is predominantly identified in FTC and less often in FVPTC (Placzkowski*, et al.* 2008). In adults, the *PAX8-PPARG* rearrangement occurs in up to 45-55% of FTC (Castro*, et al.* 2006; Sahin*, et al.* 2005), whereas the occurrence in follicular variant of PTC ranges from 0 to 35% (Castro *et al.* 2006; Zhu *et al.* 2003). In pediatric patients, the occurrence of *PAX8-PPARG* rearrangement was assessed only in one cohort of sporadic and radiation-exposed PTC patients. The authors did not find *PAX8-PPAG* in the sporadic group, whereas its prevalence was nearly 4% in the radiation-exposed group (Ricarte-Filho *et al.* 2013).

BRAF fusions have also been described in post-Chernobyl thyroid cancer, suggesting that this is a new mechanism of BRAF activation in human cancers (Ciampi *et al.* 2005; Ricarte-Filho *et al.* 2013). As far as we known, *AGK-BRAF* fusion was described in a tumor from one radiation-exposed PTC case who was 13 years old at surgery (Ricarte-Filho *et al.* 2013), while *AKAP9-BRAF* was identified in three tumors from radiation-exposed patients. Fuctional analyses revealed that both fusion oncogenes are able to activate MAPK pathway. None of the pediatric sporadic PTC evaluated presented the *AGK-BRAF* fusion transcript (Ricarte-Filho *et al.* 2013).

Is the expression of iodine uptake and metabolism proteins higher in pediatric DTC than in adults?

It is well know that iodine uptake is a result of an active transport mechanism mediated by the sodium iodide symporter (*NIS*) protein, which is found in the

basolateral membrane of thyroid follicular cells. It has served as an effective means for therapeutic doses of radioiodine to target and destroy cancer cells in which endogenous NIS is functionally expressed (Dadachova and Carrasco 2004). However, NIS-mediated radioiodine accumulation is often reduced in thyroid cancers due to decreased *NIS* expression/function (Liu*, et al.* 2012; Xing 2013).

An important difference between pediatric and adult DTC is the high prevalence of functional metastases and the greater differentiation and radioiodine responsiveness in pediatric DTC. Accordingly, it has been suggested that the expression of *NIS,* as well as other proteins involved in iodine uptake and metabolism in pediatric patients, is higher than their expression in adults (Espadinha*, et al.* 2009; Faggiano*, et al.* 2004; Patel*, et al.* 2002). Nonetheless, in some series, there is a higher prevalence of extrathyroidal extension, regional lymph node involvement and distant metastases in younger children than in adolescents (Alessandri *et al.* 2000; Dinauer *et al.* 2008; Francis *et al.* 2015; Jarzab *et al.* 2005; Lazar *et al.* 2009; O'Gorman *et al.* 2010; Rivkees *et al.* 2011; Vaisman*, et al.* 2011). Therefore, one could postulate that the expression of *NIS* in children is lower than its expression in adolescents and, therefore, treatment of pediatric DTC should be stratified into more than one group.

In fact, the hypothesis that DTC from pediatric patients usually has a higher expression of iodine-metabolizing genes than DTC from adults and older patients has little support in the available literature, especially for young children (< 10 years old). Either younger children were commonly underrepresented and/or patients over the age of 18 years at diagnosis were also included into the pediatric group. Moreover, only two studies specifically addressed the expression of iodine-metabolizing genes in pediatric patients (Espadinha *et al.* 2009; Patel *et al.* 2002). The former study

assessed the expression of *NIS* in the malignant tumor and compared to benign lesions as a substitute of normal thyroid. The authors did not find a significant difference between benign and malignant thyroid lesions (Patel *et al.* 2002). Because the overall recurrence risk was increased for those tumors that had undetectable *NIS* expression, the authors suggested that *NIS* expression is a favorable prognostic indicator for DTC in children and adolescents (Patel *et al.* 2002). Additionally, the authors studied patients up to 21 years of age and only 2 cases under the age of 10. No comparison was made between children and adolescents. The subsequent study suggested that the expression of *PDS*, *TPO* and *TSHR* mRNA is higher in the pediatric group compared to adult (22-59 years) and elderly patients (> 60 years). Nevertheless, among the 15 pediatric patients, only three cases were under 10 years of age, and there was no specific information regarding the expression of iodine-metabolizing genes in these patients (Espadinha *et al.* 2009).

Finally, it has been suggested that overactivation of the MAPK pathway, mainly through BRAF V600E mutation, leads to tumor dedifferentiation and, hence, reduced expression of proteins involved in iodine uptake and metabolism in PTC of adults (The cancer Genome Atlas Research Network 2014; Romei *et al.* 2008; Zhang*, et al.* 2014). However it is becoming clear that the BRAF V600E-mutated group consist of distinct subgroups with variable degrees of thyroid differentiation (The cancer Genome Atlas Research Network, 2014), which suggests that additional genetic events may be associated with dedifferentiation status of the thyroid.

Of note the prevalence of BRAF V600E mutation in pediatric PTC is much lower than the prevalence observed in adults (**Figures 1 and 2**). Whether other genetic alteration that activates the MAPK pathway may modulate the expression of *NIS* in pediatric groups is still uncertain.

Therefore, the data are unclear as to whether younger age indicates a greater risk for extensive disease or recurrence, and the hypothesis of a greater expression of genes such *NIS, TPO* and other proteins associated with iodine metabolism in pediatric patients would be associated with greater radioiodine responsiveness and overactivation of the MAPK pathways needs further evaluation.

Future plans

After the identification of new driver genes that are altered in radiation-exposed pediatric PTC cases lacking known genetic events (*RET/PTC*, *RAS*, *BRAF* mutations, *AKAP9-BRAF*, *TPR-NTRK1* and *PAX8-PPARγ*), significantly reduced the so-called dark matter. Nearly 84% had fusion, most oncoproteins activate MAPK pathways, suggesting that pediatric PTC are also MAPK-driver cancer. Conversely, the prevalence of drive fusion oncogenes in sporadic pediatric PTC was much lower. Nearly 30% of cases are negative for the fusion events and/or point mutations found in radiation-induced pediatric cohort (Ricarte-Filho *et al.* 2013). As the risk factor to the development of sporadic pediatric thyroid carcinoma is not known and the landscape of sporadic pediatric cancer likely differs significantly from the landscape of the radiation-exposed pediatric cases, it is expected that sporadic cases might have higher prevalence of point mutations than radiation-induced pediatric thyroid carcinomas. Further in-deep genome analysis of sporadic pediatric thyroid carcinoma is necessary to address and clarify this issue. Furthermore, such analysis may also help define whether pediatric tumors from children and adolescents represent different molecular subgroups.

The use of molecular diagnostic testing in thyroid nodules became a reality and aims to improve the accurate diagnosis in cytologically indeterminate thyroid nodules and, consequently, to avoid unnecessary surgical procedure. Although the evaluation and treatment of thyroid nodules in children should be the same as in adults (Francis *et al.* 2015), the molecular tests that are available for indeterminate thyroid nodules have not been validated in the pediatric patients. Although two studies have suggested a molecular test might improve the diagnosis of an indeterminate cytology in pediatric patients (Buryk*, et al.* 2013; Monaco*, et al.* 2012)}, it is still uncertain its usefulness. Although positive results may be associated with malignancy, the insufficient data associated with the fact that the "dark matter" of sporadic pediatric thyroid carcinomas has not yet been well characterized suggest that is too early to rely on negative genetic test to exclude malignancy. The in-deep genome analysis of the sporadic pediatric cases that had no known driver mutations will help define a panel of mutations/fusions that may be better applied to the diagnosis of pediatric thyroid nodules.

In conclusion, most of the efforts to determine the landscape of pediatric cases have been focused in radiation-exposed pediatric thyroid cancer, while most routine cases of thyroid nodules/cancer are indeed sporadic cases. As PTC is the most prevalent histological type of pediatric thyroid carcinoma, further efforts should be undertaken to define the genomic landscape of pediatric sporadic PTC.

Regarding treatment, although children with DTC have high rates of regional lymph node involvement and distant metastasis, the overall survival is good. Therefore, the extent of surgery and proper dose of ^{131}I should be better defined based on the risk of recurrence. Whether molecular classification will help to better classify pediatric thyroid carcinomas into subgroups and, therefore, refine diagnosis,

prognosis and treatment it is still a “dark matter”.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by research grants 2012/02902-9 and 2013/03867-5 from The São Paulo State Research Foundation (FAPESP) and 470441/2013-5 from The Brazilian Research Council (CNPq). LSM is a FAPESP scholar. JMC is a CNPq investigator.

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Table 1- Clinical features of pediatric thyroid carcinomas

Author	Cases (*n*)	RI exposure (%)	Mean age/ (range years)	Gender (female) (%)	Multi-focality (%)	Bilateral (%)	ET (%)	Cervical meta (%)	Distant meta (%)	Recur-rence (%)	Survival (%)
Zimmerman *et al.* (1988)	58	No	<17	69	NA	NA	24	90	7	30	76
Newman *et al.* (1998)	329	13	15.2 (0.4–20.8)	76	NA	NA	32	74	25	32	99
Welch Dinauer *et al.* (1998)	137	5	19 (3–21)	76	30.7	NA	NA	39	6	20	99
Fenton *et al.* (2000)	33	No	18 (6–21)	71	48	NA	NA	NA	NA	15	100
Alessandri *et al.* (2000)	38	21	12.6 (4.5–16.8)	74	ND	NA	42	60	5	45	100
Landau *et al.* (2000)	30	No	<16	77	23	NA	NA	57	10	40	70
Grigsby *et al.* (2002)	56	No	15.8 (4–20)	77	57	30	36	73	13	34	98
Kumagai *et al.* (2004)	29	No	11.3 (<15)	77	NA	NA	23	68	23	ND	NA
Lazar *et al.* (2009)	27	7	12.8 (6.1–17)	78	88.9	NA	52	67	41	18	100
Hogan *et al.* (2009)	1753	NA	15.9 (1–19)	81	NA	NA	NA	46	8	NA	NA
O'Gorman *et al.* (2010)	54	9	13 (F); 13.4 (M)	67	75.9	28	NA	46	15	NA	NA
Ito *et al.* (2012)	110	No	17 (7–19)	89	NA	NA	8	41	7	24	98
Sassolas *et al.* (2012)	28	NA	8–19	NA	NA	NA	32	50	7	NA	NA
Givens *et al.* (2014)	19	No	13.6 (2.8–18)	NA	NA	NA	42	68	26	11	NA
Henke *et al.* (2014)	27	No	18.6 (5.8–21.2)	79	NA	22	37	63	4	37	100
Alzahrani *et al.* (2015)	97	No	17 (8–20)	81	43	NA	53	78	16	34	100

NA, not available

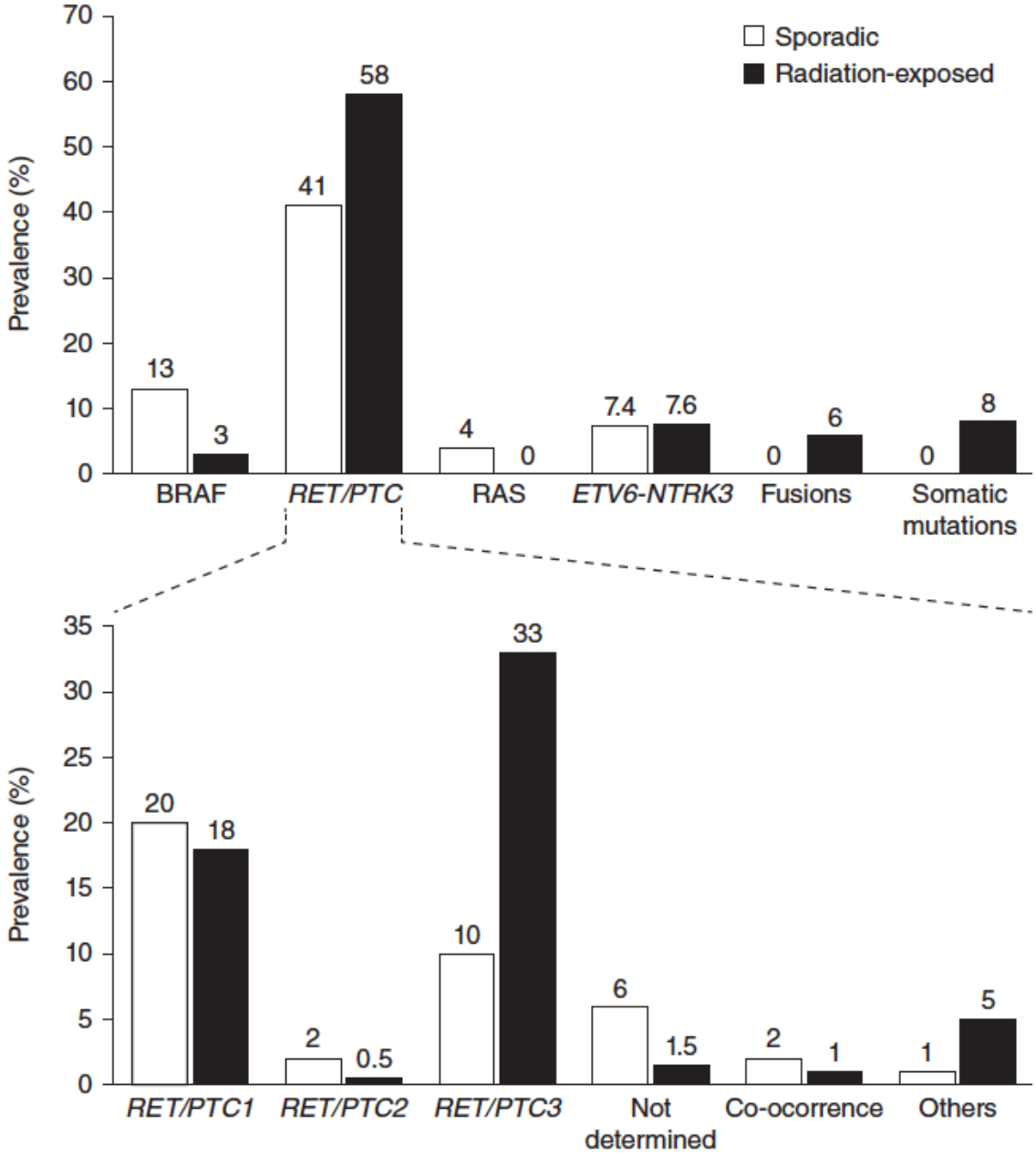

Figure 1. Overall prevalence of point mutations and rearrangements identified in sporadic pediatric and radiation-exposed pediatric papillary thyroid carcinomas. Other fusion group includes *PAX8-PPARG, AKAP9-BRAF, AGK-BRAF, NTRK1, CREB3L2-PPARG.* The other somatic mutations group includes BRAF V600_K601E and TSHR S425I. The prevalence of *RET/PTC* isoforms is show in details in the bottom panel. The prevalence and categories of mutations are detailed in supplementary Table 1 and 2.

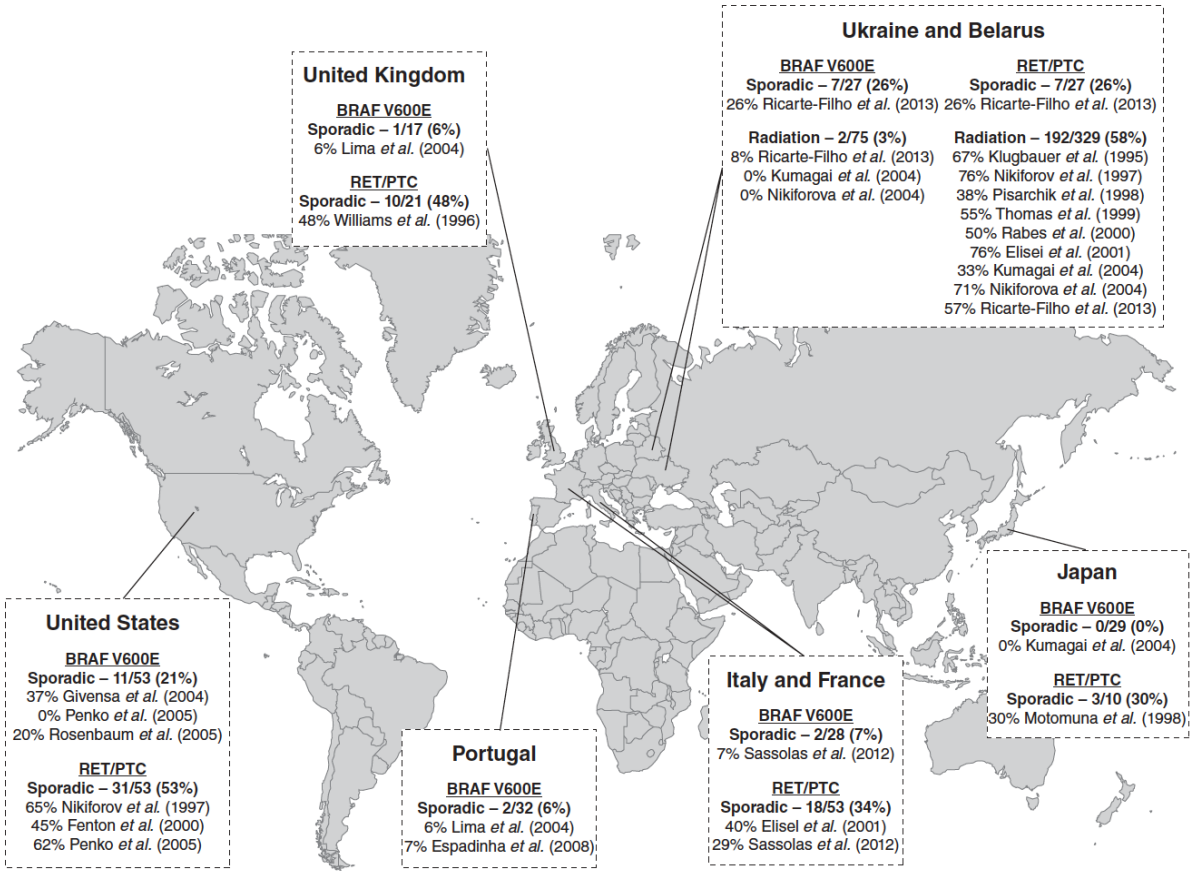

Figure 2. The worldwide prevalence of RET/PTC and BRAF V600E in sporadic and radiation-exposed PTC is shown. Studies from the same country were groups and the prevalence calculated.

Supplementary Table 1- Genetic events described in pediatric sporadic papillary thyroid carcinomas.

Mutations	Reference	Mean Age (Range ys)	Cases (*n*)	Prevalence (%)
RET/PTC			**169**	**69 (41%)**
	Williams *et al*. 1996	13 (7-14)	21	10 (48%)
	Nikiforov *et al*. 1997	13.7 (5-18)	17	11 (65%)
	Motomura *et al*. 1998	12.8 (9-14)	10	3 (30%)
	Fenton *et al*. 2000	18 (6-21)	33	15 (45%)
	Elisei *et al*. 2001	16 (8-18)	25	10 (40%)
	Penko *et al*. 2005	17.7 (0-21)	8	5 (62.5%)
	Sassolas *et al*. 2012	NA (8-19)	28	8 (29%)
	Ricarte-Filho *et al*. 2013	15.6 (5-18)	27	7 (26%)
BRAF V600E			**169**	**22 (13%)**
	Lima *et al*. 2004	NA (<18)	17	1 (6%)
	Penko *et al*. 2005	16.7 (10-21)	14	0
	Rosenbaum *et al*. 2005	15 (0-17)	20	4 (20%)
	Espadinha *et al*. 2008	14.2 (5-21)	15	1 (7%)
	Sassolas *et al*. 2012	NA (8-19)	28	2 (7%)
	Ricarte-Filho *et al*. 2013	15.6 (5-18)	27	7 (26%)
	Givens *et al*. 2014	13.6 (2.8-18)	19	7 (37%)
	Kumagai *et al*. 2004	11.3 (<15)	29	0
RAS			**84**	**3 (4%)**
	Sassolas *et al*. 2012	NA (8-19)	28	1 (3.6)%
	Ricarte-Filho *et al*. 2013	15.6 (5-18)	27	2 (7.4%)
	Kumagai *et al*. 2004	11.3 (< 15)	29	0

ETV6-NTRK3			**27**	**2 (7.4%)**
	Ricarte-Filho *et al*. 2013	15.6 (5-18)	27	2 (7.4%)
Other Fusions			**55**	**0**
PAX8-PPARG	Ricarte-Filho *et al*. 2013	15.6 (5-18)	27	0
AKAP9-BRAF	Ricarte-Filho *et al*. 2013	15.6 (5-18)	27	0
AGK-BRAF	Ricarte-Filho *et al*. 2013	15.6 (5-18)	27	0
NTRK1	Sassolas *et al*. 2012	NA (8-19)	28	0
	Ricarte-Filho *et al*. 2013	15.6 (5-18)	27	0
CREB3L2-PPARG	Ricarte-Filho *et al*. 2013	15.6 (5-18)	27	0
Other Somatic Mutations			**27**	**0**
BRAF V600_K601E	Ricarte-Filho *et al*. 2013	15.6 (5-18)	27	0
TSHR S425I	Ricarte-Filho *et al*. 2013	15.6 (5-18)	27	0

NA, not available

Supplementary Table 2 – Genetic events described in pediatric radiation-exposed papillary thyroid carcinomas.

Mutations	Reference	Mean Age (Range ys)	Cases (*n*)	Prevalence (%)
RET/PTC			**329**	**192 (58%)**
	Klugbauer *et al.* 1995	-	12	8 (67%)
	Nikiforov *et al.* 1997	11.2 (5-18)	38	29 (76%)
	Pisarchik *et al.* 1998	13.2 (11-17)	13	5 (38%)
	Thomas *et al.* 1999	NA (6-18)	67	37 (55%)
	Rabes *et al.* 2000	NA (≤14)	99	50 (50%)
	Elisei *et al.* 2001	12 (10-17)	25	19 (76%)
	Nikiforova *et al.* 2004	NA (6-20)	34	24 (71%)
	Ricarte-Filho *et al.* 2013	18 (13-22)	26	15 (57%)
	Kumagai *et al.* 2004	14.3 (≤15)	15	5 (33%)
BRAF V600E			**75**	**2 (3%)**
	Nikiforova et al. 2004	NA (6-20)	34	0
	Ricarte-Filho *et al.* 2013	18 (13-22)	26	2 (8%)
	Kumagai *et al.* 2004	14.3 (≤15)	15	0
RAS			**75**	**0**
	Suchy *et al.* 1998 *	NA	34	0
	Ricarte-Filho *et al.* 2013	18 (13-22)	26	0
	Kumagai *et al.* 2004	14.3 (≤15)	15	0
ETV6-NTRK3			**26**	**2 (7.6%)**
	Ricarte-Filho *et al.* 2013	18 (13-22)	26	2 (7.6%)

Other Fusions			**217**	**12 (6%)**
PAX8-PPARG	Ricarte-Filho *et al.* 2013	18 (13-22)	26	1 (4%)
AKAP9-BRAF	Ciamp *et al.* 2005	NA (7.8-21)	92	3 (3%)
	Ricarte-Filho *et al.* 2013	18 (13-22)	26	1 (4%)
AGK-BRAF	Ricarte-Filho *et al.* 2013	18 (13-22)	26	1 (4%)
NTRK1	Ricarte-Filho *et al.* 2013	18 (13-22)	26	1 (4%)
	Rabes *et al.* 2000	NA (≤14)	99	4 (4%)
CREB3L2-PPARG	Ricarte-Filho *et al.* 2013	18 (13-22)	26	1 (4%)
Other Somatic Mutations			**26**	**2 (8%)**
BRAF V600_K601E	Ricarte-Filho *et al.* 2013	18 (13-22)	26	1 (4%)
TSHR S425I	Ricarte-Filho *et al.* 2013	18 (13-22)	26	1 (4%)

NA, not available

*Mutations at codons 14 (silente mutation) and 15 of *HRAS* (did not interfere with GTPase activity or protein binding capacity).

ARTIGO 2

Thyroid-specific genes expression uncovered age-related differences in pediatric thyroid carcinomas

Artigo publicado na revista *International Journal of Endocrinology* em fevereiro de 2016

DOI: 10.1155/2016/1956740

Thyroid-Specific Genes Expression Uncovered Age-Related Differences in Pediatric Thyroid Carcinomas

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Abstract

Despite a more advanced stage of disease at presentation, a better response to radiodine (RAI) therapy and a reduced overall mortality have been reported in pediatric differentiated thyroid carcinoma (DTC) in comparison to adult DTC.. Few studies suggested that the better response to RAI therapy in pediatric patients might be associated with an increased expression of *NIS*. However, a marked heterogeneity within the pediatric group has been recognized. Children (<10 years old) usually present a more aggressive disease than adolescents (≥10-18 years old). By analyzing the expression of thyroid-specific genes in 38 sporadic pediatric tumors, we show that the expression of *NIS*, *PDS* and *TSHR* was lower in children than adolescents (P<0.05). A linear regression confirmed the association between *NIS* expression and age. Most significantly, *NIS* was expressed at similar levels in DTC from children and adults, whereas *PDS* and *TSHR* expression was even lower in DTC from children, compared to adolescents and adults. Our data suggest that biological behaviors of DTC in adolescents might differ from those in children and adults. Therefore, the premise that the expression of thyroid-specific genes is higher in tumors from pediatric patients than in adults is not entirely true and might be too oversimplified.

Key words: sporadic pediatric thyroid carcinoma, NIS, PDS and TSHR

Introduction

Thyroid cancer is the fastest increasing cancer worldwide [1]. Although the highest incidence rates are observed in the fifth decade and it is rare in the younger population, the incidence of thyroid cancer is also increasing in children (<10 years old) and adolescents (≥10-18 years old) [2, 3]. Thyroid cancer is the 2nd most prevalent cancer in females aged 15 to 19 years [4]. Similar to adults (> 18 years old), differentiated thyroid carcinomas (DTC) are the most common malignancy, with nearly 75-90% being papillary thyroid carcinoma (PTC) and the remainder follicular thyroid carcinoma (FTC) [2]

Previous studies reported significant differences in the clinical presentation and outcomes of DTC in pediatric patients (≤ 18 years old) compared to adults [5, 6]. Despite a more advanced stage of disease at presentation and higher rates of recurrences than adults, the overall mortality is lower [7-9]. Unlike adults, pediatric patients have a higher prevalence of pulmonary metastases, which almost always are functional [10-12]. This may explain why pediatric patients with DTC have better responsiveness to radiodine (RAI) therapy than the adults [10, 11]. In fact, some studies have shown that most pediatric DTC patients had a complete remission after RAI therapy, mainly those adolescents with iodine-avid pulmonary metastases [12-14].

The better response to RAI therapy in pediatric patients might infer greater degree of differentiation and higher expression of proteins involved in iodine uptake and metabolism. The transport of iodide into thyroid follicular cells is a result of an

active transport mechanism mediated by the sodium iodide symporter (NIS) protein, an integral plasma membrane glycoprotein located at the basolateral membrane of thyroid follicular cells [15]. Following active transport across the membrane, iodide is translocated across the apical membrane by pendrin (PDS) and organified by thyroid peroxidase (TPO). These actions are reliant on the thyroid-stimulating hormone (TSH), which interacts with the TSH receptor (TSHR) at the basolateral membrane of follicular cells [15].

Therefore, there is a strong need to investigate whether the expression of proteins involved in iodine-uptake and metabolism differs between pediatric and adult populations. In fact, it has been suggested that *NIS* expression was associated with lower risk of recurrence of pediatric thyroid carcinomas [16, 17]. Additionally, the dose of 131I require to achieve remission was directly related to the levels of *NIS* expression, being higher in those patients with undetectable *NIS* expression [17, 18]. The expression of *PDS* was also found diminished in pediatric patients [5, 6].

Nevertheless*,* a marked heterogeneity within the pediatric group has been reported. Some earlier studies suggested that children present with more aggressive local disease and are more likely to have lymph node metastases at diagnosis and, probably, more prone to develop subsequent distant metastases than adolescents [2, 7, 8, 11, 19, 20]. It has also been reported that children experience recurrence more frequently and earlier than adolescents [21, 22].

Therefore, there is a strong need for studies in both children and adolescents. Although efforts have been made, the role of RAI therapy in pediatric patients has been mainly assessed in adolescents. Most studies used a very small number of children or prepubertal patients [10, 23], which makes it difficult to

stratify pediatric patients into age groups or pubertal status and to test the hypothesis that the expression of genes associated with iodine uptake and metabolism might be higher in adolescents than in children. As most studies are retrospective or included a small number of children, it still remains unclear whether age influences the behavior of DTC within pediatric population [24].

To bridge some of the existing gap, this study investigated the expression of *NIS*, *PDS*, *TPO*, *TSHR* and thyroglobulin (*TG*) in a cohort of pediatric patients and correlated with clinicopathological features. To further explore a possible association of the expression of thyroid-specific genes with age, the expression analysis was performed in pediatric patients stratified into two age groups (<10 and ≥10-18 years). As our analysis suggests an impact of age on gene expression, the genes whose expression pattern differed between children and adolescents were further compared with their expression in DTC from adults.

Materials and Methods

Thyroid Samples

The series consists of 47 formalin-fixed paraffin-embedded (FFPE) sections from 38 primary tumors from pediatric patients who underwent thyroid surgery at Hospital São Paulo (Universidade Federal de São Paulo) and Hospital da Santa Casa de São Paulo between the years 1993 and 2012. The pediatric cohort included 35 PTCs, 3 FTCs and 9-matched normal thyroid tissues. All samples were reviewed by two pathologists (RD and MTSA). The clinical and pathological features are summarized in **Table 1**.

As recommended by the ATA guidelines for children with thyroid nodules and DTC, all of the pediatric patients were **≤** 18 years of age at the time of diagnosis [24]. The pediatric cases were further separated into two age groups: children (<10 years old) and adolescents (≥10-18 years old). Because information about pubertal development was not available for all of the patients, the age of 10 was used as the cut-off point. This cut-off point was recommended by the World Health Organization (WHO) and used to determine the effect of age on time to recurrence and mortality rates in pediatric DTC [21, 22].

The adult cohort included 115 PTC and 7 adjacent normal thyroid tissues obtained from patients who underwent thyroid surgery from 2000 through 2007 at Hospital São Paulo (Universidade Federal de São Paulo) and Hospital das Clínicas de São Paulo (Universidade de São Paulo). The clinical and pathological features are summarized in **Table 2**.

The control groups (normal thyroid tissues) included only those samples from the contralateral nodule of patients with unilateral DTC (i.e, no evidence of either benign or malignant thyroid disease in the contralateral nodule). The study was conducted under the approval of the Review Boards and Research Ethical Committees of the affiliated institutions.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from 10-μm-thick FFPE sections using the Recover All Total Nucleic Acid isolation kit (Ambion Inc., Austin, TX). Total RNA (500 ng) was treated with DNAse and reverse-transcribed into cDNA with oligo-dT_{12-18} (50 μM) and random hexamers (50 ng) using a Superscript III transcriptase kit (Invitrogen Corp., Carlsbad, CA).

Expression of Thyroid-Specific Genes in Thyroid Samples by Quantitative RT-PCR

The thyroid samples were screened for the expression of target genes (*NIS, TG, TPO, PDS* and *TSHR*) and the reference gene (*RPS8*) by quantitative RT-PCR (qRT-PCR), as previously described [25, 26]. Briefly, an aliquot of cDNA was used in a 12-μL PCR reaction containing SYBR Green PCR Master Mix (PE Applied Biosystems) and 3 pmol of each specific primer. The PCR reaction was subjected to 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The qPCR reactions were performed in triplicate, and the Ct was obtained and averaged (SD < 0.85). The relative expression (RE) was calculated according to the comparative ΔΔCt

method. Normal thyroid samples were used as the control group. The choice to use the *RPS8* as the reference gene is based on previous analyses from our group that identified this gene as the best suitable reference gene for thyroid tissues [27]. The PCR primers are summarized in **Supplementary Table 1.**

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA) and R 3.1.3. (R software). Mann-Whitney U test or Student's t-test was used to compare the continuous variables, and Fisher's exact test was used for dichotomous variables. The associations were tested using discriminant analysis or linear regression when applicable. The results with $P < 0.05$ were considered to be statistically significant.

Results

Clinical and Pathological Features of Pediatric Patients

The mean age at diagnosis was 11.84 years (range, 4 to 18 years). The mean age of the pediatric control group was 11.7 years (range from 7 to 17 years) and, therefore, similar to that observed in pediatric thyroid cancer group. The female to male ratio was 29:9, with female predominance mainly in the adolescent group. The pathological findings included multifocality in 17 (45%), extrathyroidal extension in 16 (42%), lymph node metastases at diagnosis in 28 (74%) and lung metastasis in 10 (26%) patients. Two of the patients had a family history for PTC, and four cases had a history of previous radiation exposure during childhood to treat another cancer (**Table 1**). When compared to adults, higher rate of cervical metastases was identified in pediatric patients compared to adults (74% vs. 42%; *P*=0.0007), as well as pulmonary metastases (26% vs. 3%; *P*<0.0002) (**Table 2**).

To better understand clinical, pathological and expression differences between children and adolescents, the patients were classified into two age groups. Thirteen cases (34%) were <10 years of age, and 25 cases (66%) were ≥10-18 years of age. An increased prevalence of extrathyroidal extension was observed among the children compared to the adolescents (69% versus 28%; *P*=0.0448). Children also had a higher tendency to develop lung metastasis than adolescents (46% versus 16%; *P*=0.0620) (**Table 1**).

Expression of Thyroid-Specific Genes and Correlation with Clinical and Pathological Features and Molecular Status

The expression of *TG*, *NIS, PDS,* and *TPO* was significantly lower in pediatric thyroid carcinomas compared to the normal thyroid tissue (**Figure 1**; $P<0.05$). Importantly, the expression of *NIS, PDS* and *TSHR* was consistently lower in children than in adolescents (**Figure 2**; $P<0.05$). Considering the clinical and pathological features associated with poor prognosis, *NIS* and *TSHR* mRNA expression was significantly lower in those tumors with extrathyroidal extension. Besides, *NIS* mRNA expression was notably lower in those tumors from patients with lung metastases (**Figure 3)**. Multiple linear regression analysis confirmed the association between age and *NIS* expression, regardless of the other clinicopathological features.

As *NIS*, *PDS* and *TSHR* were differentially expressed between children and adolescent groups, we further compared those changes to the changes we observed in adult population [25, 26]. We observed that children and adults have similar *NIS* mRNA expression. Remarkably, *NIS* expression was significantly higher in tumors from adolescents than tumors from adults ($P<0.05$) (**Figure 4**). The range of *TSHR* and *PDS* expression in children was considerably lower as compared to adults ($P<0.05$) (**Figure 4**).

Seeing that adult population is characterized by a wide age range (20–70 years) and that older patients have more aggressive disease, we separated younger adults (< 45 year old) from older adults and evaluated the expression of *NIS, PDS* and *TSHR* in these groups. However, the expression of these genes did not differ between these two age groups (data not presented).

Discussion

Previous studies reported significant differences in the clinical presentation and outcomes of DTC among pediatric patients when compared to adults. Pediatric patients have larger tumor size, have more extensive local disease, are more likely to present with lymph node and distant metastases and have a higher frequency of functional metastases. The discrepancy between more aggressive disease at diagnosis and higher recurrence rates but a more favorable progression-free survival is quite remarkable [11]. It is still not clear whether these differences observed between pediatric and adult DTC lie in the existence of distinct gene expression and/or mutational profile. It has been suggested that the rare progression to less-differentiated tumor and the better response to RAI therapy are due to higher expression of key genes involved in thyroid function, including *NIS* [11].

Another intriguing point is the heterogeneity reported within pediatric group. As most studies included a very small number of children, it is still unclear whether younger age is associated with an increase risk for extensive disease. Studies, in which the number of children is roughly 25% of the pediatric cohort, showed that young age is correlated with a higher risk for extensive disease or recurrence [8, 19, 20]

In this work, we identified a significant decrease in *TG, NIS, PDS,* and *TPO* expression in pediatric DTC compared to paired-normal thyroid tissues. Moreover, the age-related analysis revealed that the expression of *NIS*, *PDS* and *TSHR* was significantly lower in children than in adolescents. Remarkably, children had a

higher rate of extrathyroidal extension and a trend towards a higher prevalence of distant metastases than adolescents.

Although one study previously investigated the expression of *NIS* in children and adolescents, no significant difference in *NIS* expression was found between benign and malignant thyroid tumors [17]. Though no difference was found between benign and malignant tumors, because the overall recurrence risk was increased for tumors that had undetectable NIS expression, the authors suggested that NI*S* expression is a favorable prognostic indicator for DTC in children and adolescents [17]. Although these results appear to be in contrast with ours, in the former study, the expression of NIS in the malignant tumor was compared to benign lesions as a substitute of normal thyroid. In the present study the expression of NIS in malignant tumors was compared to its expression in normal thyroid. That is essential, as, previous studies have demonstrated that *NIS* expression was lower in benign thyroid lesion compared to normal thyroid tissue [28, 29]. Additionally, the authors studied patients up to 21 years of age and only 2 cases under the age of 10. Hence, no comparison was made between children and adolescents. Remarkably, *NIS* expression was not detected in these patients under the age of 10 [17].

Another study suggested that the expression of *PDS*, *TPO* and *TSHR* mRNA is higher in the pediatric group (5-21 years) compared to adults (22-59 years). Nevertheless, among the 15 pediatric patients, only 3 cases were under 10 years of age, and there was no specific information regarding the expression of iodine-metabolizing genes in these patients [16].

As in our study the expression of *NIS*, *PDS* and *TSHR* was significantly different between children and adolescents, the premise that the expression of iodine-metabolizing genes is higher in all DTC from pediatric patients than in DTC from adults might not be entirely true and seems too oversimplified. The differences observed among children and adolescents in the present study, at molecular level, may explain the striking differences reported within the pediatric group in terms of the clinical and pathological features.

To help to clarify the issue whether the level of expression of iodide-metabolizing genes in children and adolescents differs from that of adults, we next compared the expression of *NIS*, *PDS* and *TSHR*, which were found differentially expressed between children and adolescents, with their expression in adult population. We found that the expression of *NIS* is comparable in tumors from children and adults. Most significantly, the expression of *NIS* was higher in tumor from adolescents compared with children and adults. *PDS* and *TSHR* expression were even lower in children than in adolescents and adults. The higher expression of *NIS, PDS* and *TSHR* in adolescents suggested a greater degree of differentiation of thyroid carcinomas in this age group. The opposite is also true, that is,. lower expression of this thyroid-specific genes may indicate a lower grade of tumor differentiation and, therefore, a more aggressive thyroid tumor behavior in children.

There are some limitations to our study. First, it is limited by the inherent biases of a retrospective analysis and therefore the lack of a proper follow-up of the patients. Second, because previously published guidelines about DTC management were mainly addressed for adult DTC, the management of pediatric

DTC varies according to different medical services. The patients enrolled in this study were followed in two different hospitals with different criteria regarding the surgical procedures, indications for RAI ablation and dosing regimens and follow-up protocol. The first guideline specifically addressing the management of thyroid nodules and DTC in children and adolescents was only recently released [24]. For this reason, we decided not to analyze the relationship between gene expression and response to RAI treatment.

In conclusion, to the best of our knowledge, this is the first study to report a differential thyroid-specific gene expression profile within the pediatric group classified according to age. Our data suggests that the biological behavior of tumors in adolescents is different compared to tumors in patients under the age of 10 and adults. The identification of age-related differences may allow a subclassification of pediatric tumors into genetic and clinical subtypes and will certainly add to our ability to predict clinical outcomes and to develop future treatment strategies tailored to the differences. Whether genetic events might explain the phenotypic differences, warrants further investigation.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by research grants 2012/02902-9 and 2013/03867-5 from The São Paulo State Research Foundation (FAPESP) and 470441/2013-5 from The Brazilian Research Council (CNPq). LSM is a FAPESP scholar. JMC is a CNPq investigator.

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Table 1. Summary of the clinicopathological features of pediatric thyroid carcinoma

	Total	**Patients <10 yr old**	**Patients ≥ 10-18 yr old**	***P* value**
	***n*=38**	***n*=13**	***n*=25**	
Mean age ± SD	11.84 (± 4.4)	6.76 (± 1.92)	14.48 (± 2.63)	<0.0001
Gender				
Female	29	8 (62%)	21 (84%)	0.2262
Male	9	5 (38%)	3 (16%)	
Tumor size (cm) mean ± SD	2.65 (± 1.48)	2.28 (± 1.49)	2.83 (± 1.48)	0.2383
Risk Factors				
Family History	2	1 (8%)	1 (4%)	1.00
Exposure to radiation	4	0	4 (16%)	0.2779
Extrathyroidal extension	16	9 (69%)	7 (28%)	**0.0448**
Multifocal disease	17	4 (31%)	13 (52%)	0.3068
LN metastases	28	11 (85%)	17 (68%)	0.4413
Distant metastases	10	6 (46%)	4 (16%)	0.0620

Table 2. Summary of the clinicopathological features of pediatric and adult thyroid carcinoma

	Pediatric	**Adult**	***P* value**
	***n*=38**	***n*=115**	
Mean age ± SD	11.84 (4-18ys)	45.29 (20-76)	
Gender			
Female	29 (76%)	96 (83%)	0.3387
Male	9 (24%)	19 (17%)	
Extrathyroidal extension	16 (42%)	39 (34%)	0.5596
Multifocal disease	17 (45%)	56 (49%)	0.7082
LN metastases	28 (74%)	48 (42%)	**0.0007**
Distant metastases	10 (26%)	4 (3%)	**0.0002**

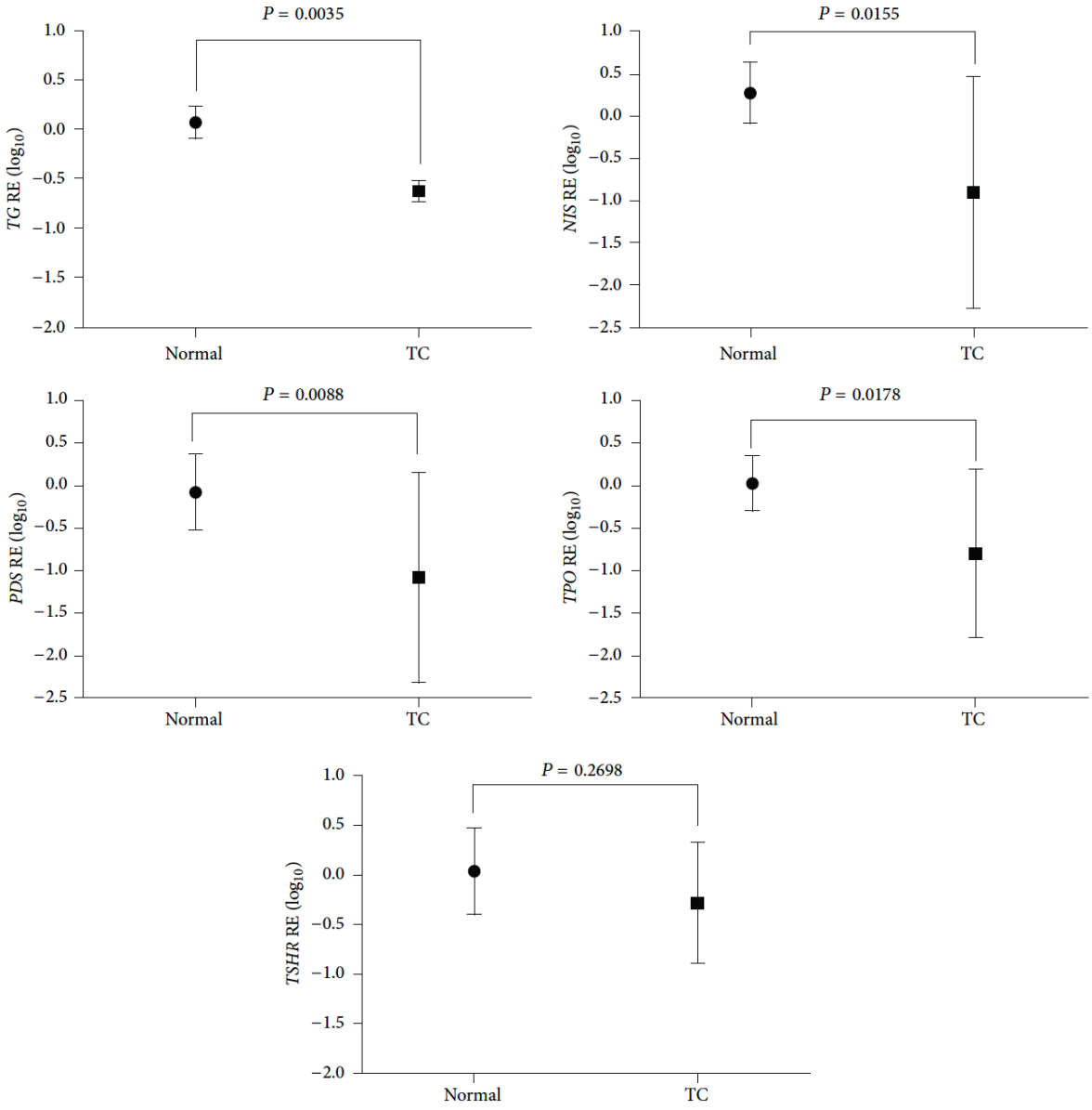

Figure 1. Relative expression (RE) of thyroid-specific genes in differentiated thyroid carcinomas (DTC) (*n*=38) and normal thyroid tissues (*n*=9) from pediatric patients. The graphics shows the mean value (±SD) of log-transformed data. *P* <.05 were considered statistically significant.

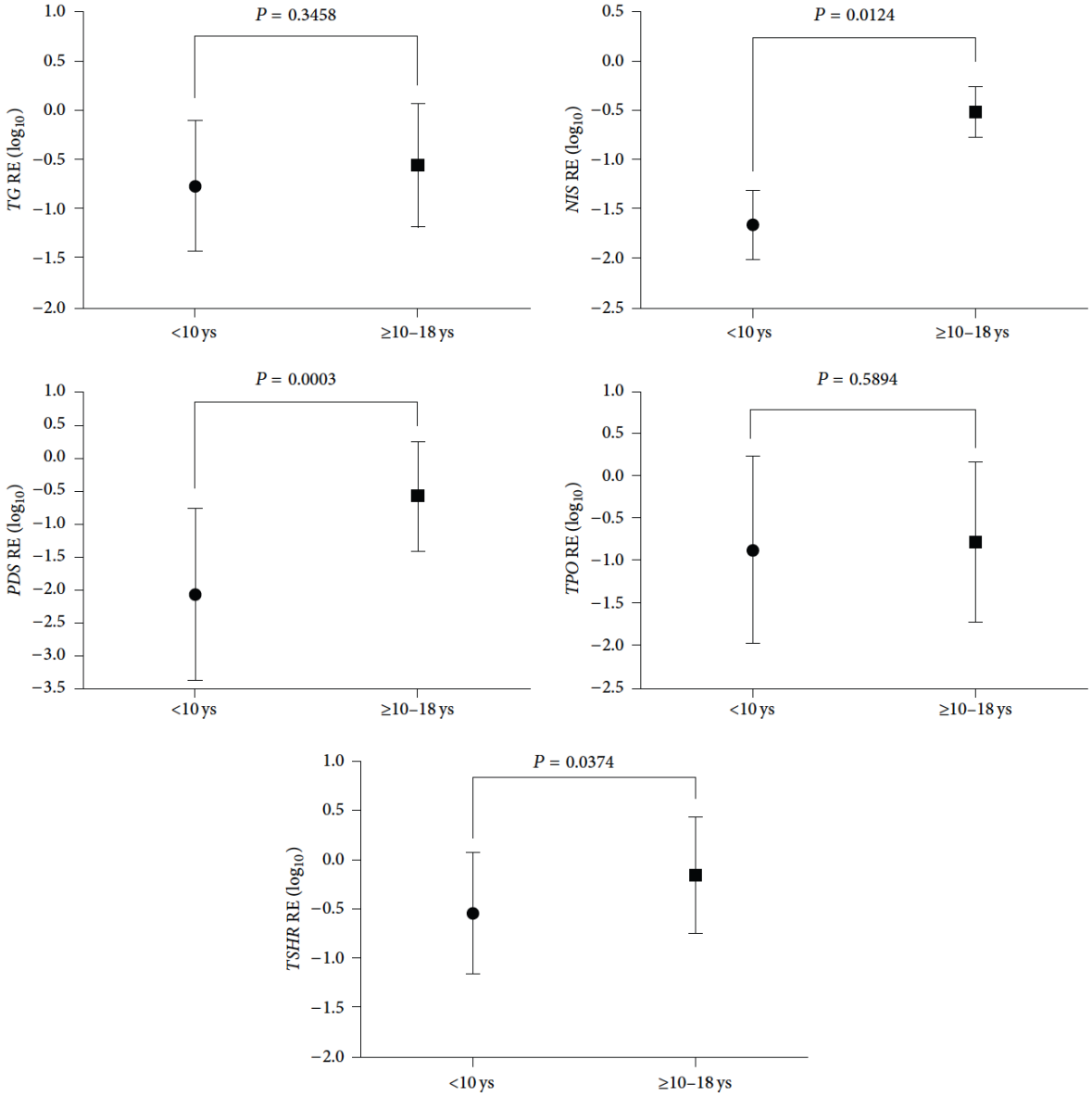

Figure 2. Relative expression (RE) of thyroid-specific genes in pediatric thyroid carcinomas classified according age: children (*n*=13; <10 years old) or adolescents (*n*=25; ≥ 10 years old). The graphics shows the mean (±SD). Data was log-transformed before analysis. *P* <.05 were considered statistically significant

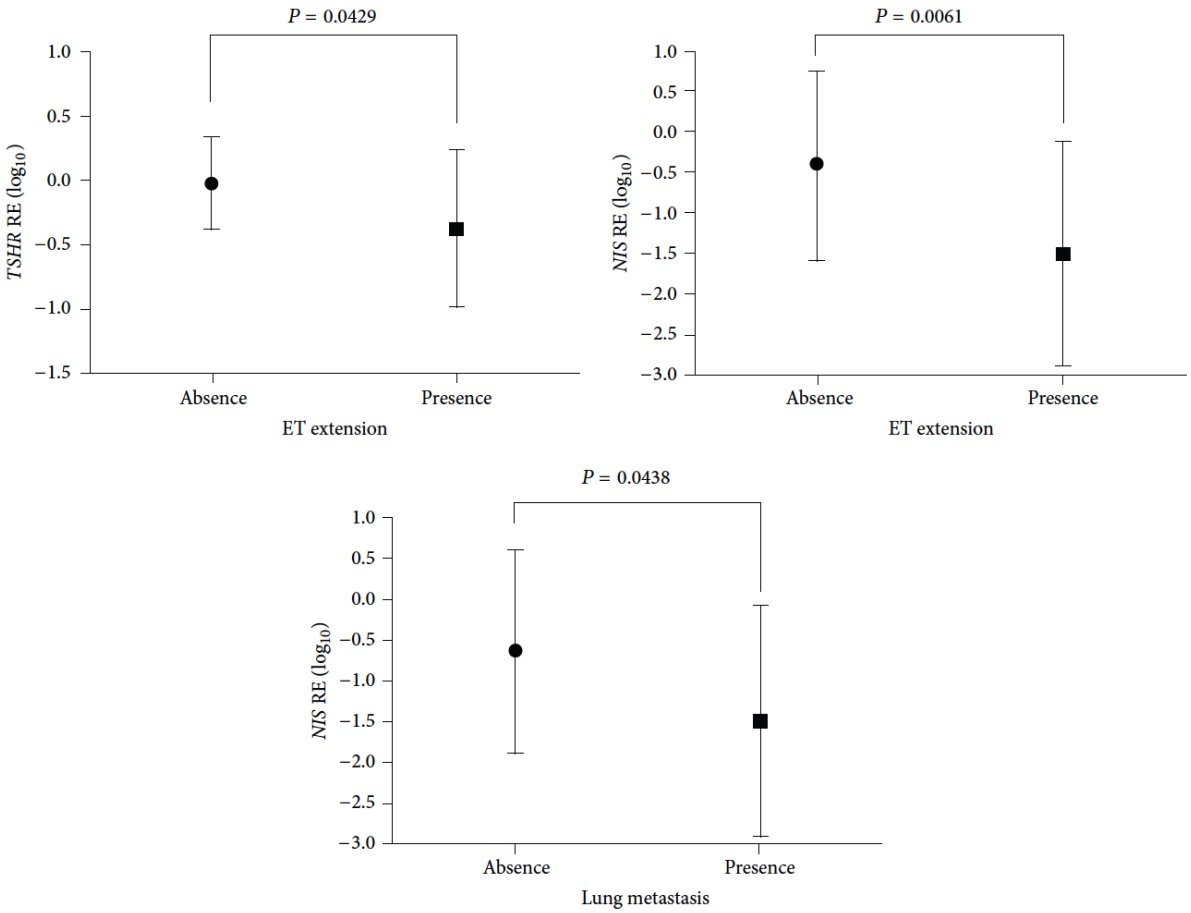

Figure 3. Relative expression (RE) of *TSHR* and *NIS* in pediatric thyroid carcinomas classified according to the presence (*n*=22) or absence (*n*=16) of extrathyroidal (ET) extension; and RE expression of *NIS* in patients classified according to the presence (*n*=10) or absence (*n*=28) of distant metastasis. The graphics shows the mean value (±SD) of log-transformed data. *P* <.05 were considered statistically significant.

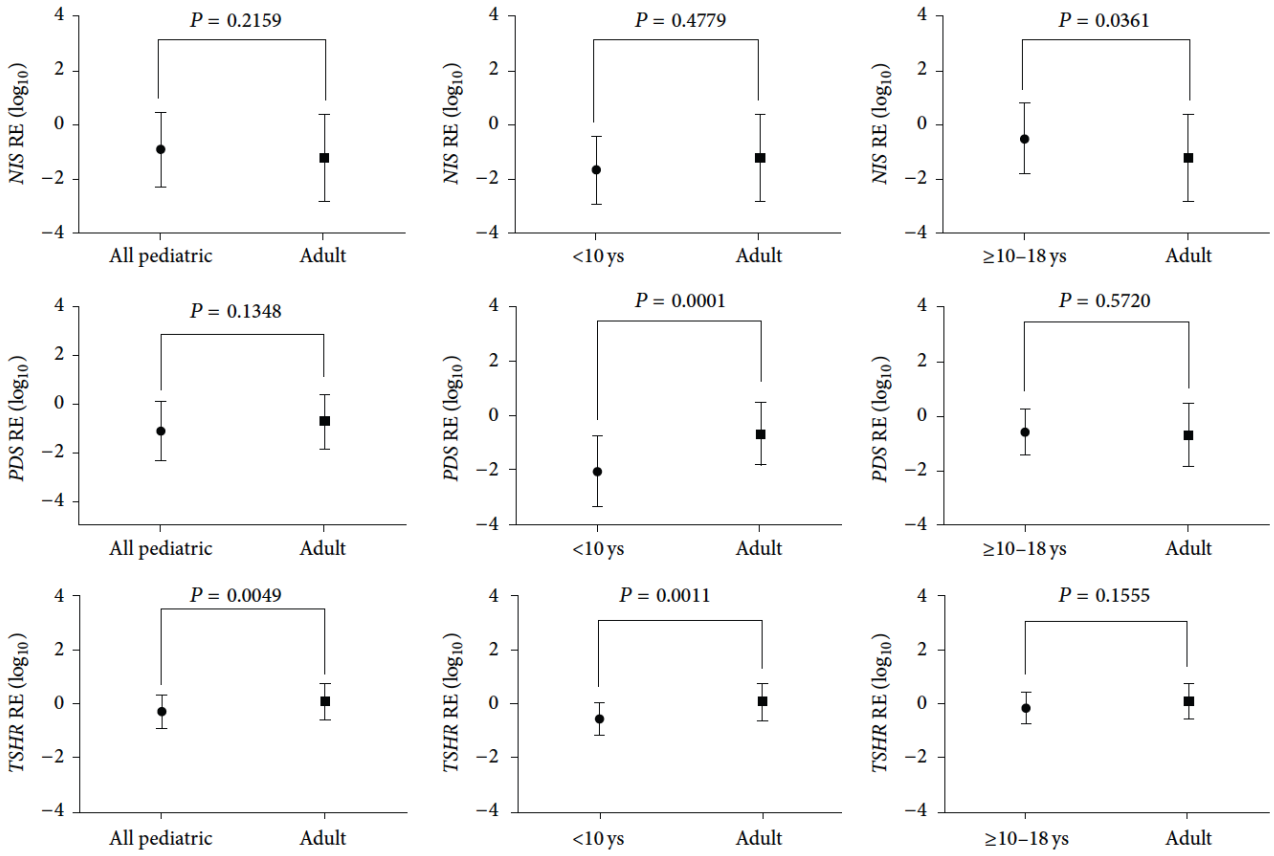

Figure 4. Relative expression (RE) of *NIS*, *PDS* and *TSHR* in DTC patients stratified by age: all pediatric (≤ 18 years old; *n*=*38*), children (<10 years old; *n*=13), adolescents (≥10-18 years old; *n*=25) and adults (≥ 19 years old; *n*= 115). The graphics shows the mean (±SD) expression level of log-transformed data. *P* <.05 were considered statistically significant.

Supplementary Table 1. Primer sequences and expected PCR product size

Genes		Primer Sequence (5' - 3')	Expected Product size (bp)
TSHR[a]	Sense	ACATGACGTCAATCCCTGTG	105
	Antisense	TGAAAGCATATCCTTGGACTG	
NIS[a]	Sense	CAGAACCACTCCCGGATCAA	81
	Antisense	ACCCACCACAAAAGTCCAGAA	
TPO	Sense	TTGTACAACGGGTTCCCACT	103
	Antisense	GGAGGTCAGAATAGCGGTCA	
PDS	Sense	TCAAGAGGGTCAAGGTTCCA	102
	Antisense	TCAAGTTCTTCTTCCGTCAGC	
TG	Sense	TTCAGTGAGCTGCTCCCCAATC	165
	Antisense	ATCTTCTCTTAGCCCAGATCCAGCC	
S8[a]	Sense	AACAAGAAATACCGTGCCC	125
	Antisense	GTACGAACCAGCTCGTTATTA	

a [27]

ARTIGO 3

AGK-BRAF gene fusion is a recurrent event in sporadic pediatric thyroid carcinoma

Artigo publicado na revista *Cancer Medicine* em julho de 2016

DOI: 10.1002/cam4.698

AGK-BRAF gene fusion is a recurrent event in sporadic pediatric thyroid carcinoma

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Short running title: *AGK-BRAF* in sporadic pediatric thyroid carcinoma

Keywords (3-6): *AGK-BRAF*, pediatric thyroid cancer, papillary thyroid carcinoma, BRAF V600E

Abstract

Thyroid cancer is the fastest increasing cancer worldwide in all age groups. Papillary thyroid carcinoma (PTC) is the most common type of thyroid cancer in both adults and children. PTC genomic landscape has been extensively studied in adults, but information regarding sporadic pediatric patients is lacking. Although BRAF V600E mutation is highly prevalent in adults, this mutation is uncommon in pediatric cases. As adult and pediatric PTC is mitogen-activated protein kinase-driven cancer, this altered pathway might be activated by different genetic events. The aim of this study was to investigate the occurrence of AGK-BRAF fusion gene, recently described in radiation-exposed pediatric PTC, in a cohort of predominantly sporadic pediatric PTC. The series consists of 30 pediatric PTC younger than 18 years of age at the time of diagnosis and 15-matched lymph node metastases (LNM). Primary tumors and matched LNM were screened for the presence of the AGK-BRAF fusion transcript by RT-PCR. To confirm the identity of the amplified products, randomly selected samples positive for the presence of the fusion transcripts were sequenced. Moreover, *BRAF* dual color, break-apart probes confirmed *BRAF* rearrangement. Overall, the *AGK-BRAF* fusion gene was detected in 10% (3/30) of primary tumors. For one of these cases paired LNM was also available, which also show the presence of AGK-BRAF fusion gene. This study described, for the first time, the presence of *AGK-BRAF* in sporadic pediatric PTC. Understanding the molecular events underlying pediatric PTC may improve preoperative diagnosis, allow molecular prognostication and define a therapeutic approach toward sporadic PTC patients.

Introduction

An increasing incidence of thyroid cancer has been reported in most populations worldwide [1, 2]. Thyroid cancer is the fifth most common cancer in women in the United States, accounting for approximately 5% of all cancer [3]. Recently, a rise in thyroid cancer incidence rate has also been reported in pediatric patients, mainly among adolescents [3, 4]. In fact, thyroid cancer is the second most prevalent cancer in females with 15-19 years of age [5]. Similar to adults, the great majority of pediatric follicular cell-derived thyroid carcinomas are papillary thyroid carcinomas (PTC), with nearly 75-90% of cases [6].

The clinical presentation and outcomes of thyroid carcinoma differ between pediatric and adult population. Although pediatric patients are more likely to present a more advanced stage of disease at diagnosis and higher risk of recurrent and persistent disease than adults, they usually have an excellent overall survival [7, 8]. Furthermore, a great heterogeneity within the pediatric group has been reported. Some studies have suggested worse outcome for children compared to adolescents [9, 10].

It is still not clear whether the clinicopathological differences observed between pediatric and adult population may be due to the existence of distinct genetic alterations. In fact, the frequency and spectrum of mutations in adult PTC is markedly different than that in pediatric PTC [11, 12]. Some studies have also reported a different spectrum of mutations within pediatric group. Actually, the prevalence of the BRAF V600E mutation, the most common genetic event found in adult PTC [13], is significantly lower in sporadic and radiation-exposed pediatric PTC [14, 15], [18]. On the

other hand, a high prevalence of genetic rearrangements has been described in both sporadic [11, 16] and radiation-exposed pediatric thyroid carcinomas [17, 18].

Interestingly, *BRAF* rearrangements, in which the BRAF kinase domain is fused to a variety of 5' partners, have been reported in several solid tumors types [19] as well as in PTC[20]. The fusion gene (*AKAP9-BRAF*) was found in radiation-exposed PTCs and results from an inframe fusion of the exons 1-8 of the *AKAP9* gene (A-kinase anchor protein 9) to exons 9-18 of BRAF gene[20].

As *BRAF* fusion represent an alternative mechanism of BRAF activation, one could hypothesize that *BRAF* could be activated in pediatric PTCs through rearrangement. In fact, recently, *AGK-BRAF* rearrangement was described in one case of radiation-exposed pediatric PTC, but was not identified in pediatric cases from patients with unknown radiation exposure[18]. This rearrangement was caused by an inversion of the long arm of chromosome 7, which juxtaposes the exons 1 and 2 of the AGK (acylglycerol kinase) to exons 8-18 of *BRAF*[18]. The expression of this fusion oncogene promotes a constitutive activation of MEK and ERK phosphorylation, thus activating the mitogen-activated protein kinase (MAPK) cascade[18]. *AGK-BRAF* was later described in one PTC from adult patient with apparently no history of radiation exposure[21].

To elucidate alternative mechanisms of aberrant *BRAF* activation, this study investigated the prevalence of *AGK-BRAF* fusion oncogene in a cohort of sporadic pediatric PTC.

Material and Methods

Thyroid Samples

The series consists of 45 formalin-fixed paraffin-embedded (FFPE) sections from 30 primary PTC and 15 matched lymph node metastases (LNM) from patients who underwent thyroid surgery from 1993 through 2012 at Hospital São Paulo (Universidade Federal de São Paulo) and Hospital da Santa Casa de São Paulo. All samples were reviewed by two pathologists (RD and MTSA). As recommended by the ATA guidelines, all pediatric patients included in this study were ≤ 18 years of age at the time of diagnosis[22]. The study was conducted under the approval of the Review Boards and Research Ethical Committees of the affiliated institutions.

RNA isolation and cDNA synthesis

Total RNA was isolated from 10-µm-thick FFPE sections using the Recover All Total Nucleic Acid isolation kit (Ambion Inc., Austin, TX). Total RNA (500 ng) was treated with DNAse and reverse-transcribed into cDNA with both 50 µM oligo$(dT)_{12-18}$ and 50 ng random hexamers using a Superscript III transcriptase kit (Invitrogen Corp., Carlsbad, CA).

Transient Transfection of AGK-BRAF fusion oncogene in thyroid cells

FTC 238 thyroid carcinoma cells, established from a lung metastases of a human follicular thyroid carcinoma, purchase from the European Collection of Cell Cultures (ECACC, Health Protection Agency, Salisbury, UK) were cultured in

Dulbecco's modified essential medium (DMEM):Ham's F12 (1:1) medium supplemented with 5% fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA). FTC 238 cells were transiently transfected with 10 μg of pLVX-*AGK-BRAF* plasmid by electroporation using a Gene Pulser II (Bio-Rad Laboratories Inc., Hercules, CA, USA). The oncogene-transfected cells were harvested, and the total RNA was isolated using TRIzol Reagents (Invitrogen Corp.) and reverse-transcribed into cDNA, as above-mentioned. The cDNA generated from cells expressing the fusion transcripts was used as a positive control. The pLVX-*AGK-BRAF* plasmid was kindly donated by Dr. James Fagin (Memorial Sloan-Kettering Cancer Center).

Detection of AGK-BRAF fusion transcript

All of the samples were screened for the presence of *AGK-BRAF* fusion transcript by RT-PCR, as previously described[18]. Briefly, cDNA (2 μL) was subjected to PCR amplification using 1.0 unit Platinum Taq DNA Polymerase (Invitrogen Corp.) and 2 pmol of each primer, as described. The samples were incubated at 95°C for 10 minutes and then subjected to 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and polymerization at 72°C for 30 seconds, with a 5-min final extension at 72°C. The efficiency of cDNA synthesis was tested using RPS8 as internal control, as previously described[23]. A positive and negative control was included in each PCR run. The PCR products were resolved on a 2% agarose gel and visualized on a Bio-Rad Gel Doc EZ system (Bio-Rad). To confirm the identity of the amplified products, positive samples were sequenced using the BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA). The

primers used to detect the *AGK-BRAF* fusion transcript, located in exon 2 of *AGK* and exon 8 of *BRAF*, were previously described and validated[18].

Dual-color break-apart fluorescence in situ hybridization (FISH)

A commercially available, dual-color, break-apart assay, was used to test possible breakage of *BRAF* gene resulting from structural rearrangements. The two differentially labeled probes, flanking the *BRAF* gene, were cohybridized in two *AGK-BRAF*-positive PTCs. Targeted tumor areas were circled, following review of the corresponding H&E slide by a pathologist (RD), prior to the FISH assay. A 3-µm thick unstained tissue sections were deparaffinized, rehydrated, and incubated in the pretreatment solution and washed according to manufacturer's protocol (DAKO, Glostrup, Denmark). Slides were then washed then incubated with 5 µL solution containing the labeled FISH *BRAF* probes and IQFISH Fast Hybridization buffer (SureFISH break-apart probles; Agilent Technologies, Palo Alto, CA) denatured at 80° C for 10 minutes and hybridized overnight at 37° C. Posthybridization wash was performed in stringent wash buffer at 65ºC and two nonstringent washes at room temperature. The slides were then mounted with 10 µL of Mounting Buffer with 4',6-diamidino-2-phenylindole (DAPI) as a counterstaining. The FISH results were evaluated with fluorescent microscope Zeiss (Zeiss, Oberkochen, Germany) using ISIS Karyotype Image System (Metasystems, Altlussheim, Germany). At least 100 non-overlapping and intact nuclei were evaluated.

Results

Clinical and pathological features

We systematically investigated the prevalence of *AGK-BRAF* mutation in all primary tumors and matched LNM. Age ranged from 4 to 18 years old (mean=11.36 years). Twenty-one patients (70%) were females and 9 (30%) were males. The study included 12 classical PTC (CPTC), 12 follicular variant of PTC (FVPTC), 4 diffuse sclerosing variant of PTC (DSVPTC) and 2 other variants of PTC. The clinical and pathological features evaluated are summarized in **Table 1**.

Recurrent *AGK-BRAF* rearrangement in sporadic pediatric PTCs

In order to optimize RT-PCR reaction, we primarily used cDNA obtained from FTC cells transiently transfected with plasmid containing the AGK-BRAF fusion gene. Different primer concentrations and PCR conditions were assayed. AGK-BRAF was found in two primary tumors. Moreover, in one patient *AGK-BRAF* rearrangement was identified in the LNM, while the fusion gene was not observed in the paired primary tumor. As this patient presented a multifocal PTC (case 21), additional foci were selected for further investigation. One out of five foci presented the *AGK-BRAF* fusion oncogene. Overall, the *AGK-BRAF* fusion gene was found in nearly 10% (3/30) of primary tumors and in about 6% (1/15) of LNMs (**Table 1**; **Fig. 1**). The presence of *AGK-BRAF* fusion oncogene was confirmed by sequencing analysis (**Fig. 1**).

Additionally, two cases that were positive for *AGK-BRAF* by RT-PCR were selected to test possible breakage of the *BRAF* gene and, therefore, to confirm the *BRAF* rearrangement. In addition to the fused yellow or red-green signal, the selected fields showed the presence of red and green split signals (**Fig. 1).** The split-apart BRAF signal was identified in 30% and 36% of cells. Nearly 70% of cells exhibited only two fused yellow or red-green signals, confirming tumor heterogeneity.

AGK-BRAF **fusion oncogene and clinical-pathological features of sporadic pediatric PTC**

Among three patients positive for *AGK-BRAF*, the mean age at diagnosis was 11.66 years (range 7-15 years). Two tumors with *AGK-BRAF* fusion were of classical histology and one of follicular variant. Extrathyroidal extension was observed in all patients with *AGK-BRAF* rearrangement. The prevalence of patients with distant metastasis at diagnosis and multifocality was higher in the *AGK-BRAF*-positive groups than in *AGK-BRAF*-negative groups (**Table 2**).

Discussion

The Cancer Genome Atlas (TCGA) Research Network, using different platforms combined with clinicopathological data, characterized the genomic landscape of nearly 500 PTCs of adults. The study confirmed that PTC is a MAPK-driven cancer, identified new cancer-causing gene mutations, as well as new fusion transcripts and somatic copy number alteration. These findings reduced the so called "dark matter'' of the PTC. Importantly, the large collection of genetic alterations, combined with a comprehensive transcriptomic and proteomic analysis, exposed fundamental biological variances between PTCs. This increased knowledge helped to stratify PTC into subgroups, which eventually will improve preoperative diagnosis of thyroid nodules, prognosis and treatment of adult PTC[21].

Despite intensive efforts, much less is known about the genetic alterations that are, in fact, "driver genes" in pediatric PTC. Although pediatric PTCs are also a MAPK-driven cancer, the spectrum of mutations differs between adults and pediatric tumors. Furthermore, radiation-exposed and sporadic pediatric PTCs likely have different genetic landscapes. In fact, Nikiforov *et al.*[17] provided the first evidence that they have different molecular signature. The authors reported that the prevalence of *RET/PTC* rearrangements is markedly different between sporadic and radiation-exposed pediatric PTC. Not only the overall prevalence of *RET/PTC* diverges between sporadic and radiation-exposed PTC but also the prevalence of *RET/PTC3* isoform was higher than *RET/PTC1* in radiation-exposed cases[12, 17].

Recently, another group described that the proportion of samples harboring fusion oncogenes in radiation-exposed pediatric PTC is markedly higher (85%) from

that seen in non radiation-exposed group (33%), while point mutations have been mainly found in nonradiation-exposed patients than in radiation-exposed PTC patients [18].

Most of the efforts to determine the landscape of pediatric cases have been concentrated in radiation-exposed pediatric thyroid cancer, while in the routine most cases of thyroid cancer are actually sporadic cases.

The molecular differences between adult and pediatric PTC and the fact that fewer genetic events were described in pediatric PTC may impact on the utility of molecular testing for diagnosis of thyroid nodules in children. In fact, the ATA Guidelines for Children with thyroid nodules and differentiated thyroid cancer suggested that, although in adults molecular testing aids in the management of thyroid nodules with indeterminate cytopathology, insufficient data exist in children to rely on negative genetic studies. Therefore, the test cannot be recommended in routine clinical on pediatric patients practice until further studies are conducted[22].

This study identified the presence of *AGK-BRAF* fusion gene in sporadic pediatric PTC. Although BRAF V600E mutation is uncommon in both radiation-exposed and sporadic pediatric PTC[12,15,14], our findings reveal that BRAF fusion might be an alternative mechanism of MAPK pathway activation. It has been previously demonstrated that expression of *AGK-BRAF* in NIH3T3 and COS-7 cells promotes constitutive activation of MAPK signaling pathway and induces NIH3T3 cell growth and colony formation [18].

The frequency of *BRAF* fusion in this cohort of sporadic cases, one of the largest of literature, was validated using different approaches. FISH analysis allowed us to detected clonal rearrangements and to ratify tumor heterogeneity. Finally, dual-

color, break-apart *BRAF* probe will help us to detect the presence of any fusion within the BRAF gene in sporadic pediatric cancer.

It has been suggested that biological differences may explain the clinical and pathological features differences between pediatric and adult patients. It still remains unclear whether *AGK-BRAF* correlates with clinicopathological parameter in PTC such as age, presence of metastases, histological subtypes and advanced clinical stages. In this study, *AGK-BRAF* fusion appears to be related to a more aggressive biological behavior, as extrathyroidal extension was seen in all patients with *AGK-BRAF* rearrangement. Additionally, multifocality, lymph node, and distant metastasis at diagnosis were observed in two patients out of three patients with *AGK-BRAF* rearrangement. Unfortunately, no clinical information is available for the previously described *AGK-BRAF*-positive radiation-exposed PTC[18]. Nevertheless, further analysis, ideally multicenter studies, is needed to confirm this hypothesis and to better elucidate the biological behavior of sporadic pediatric PTC with *AGK-BRAF* fusion gene.

In summary, our findings provide additional insight to our current understanding of tumor biology of sporadic pediatric PTC. Further efforts should be undertaken to define the genomic landscape of sporadic pediatric PTC. The knowledge of the molecular events underlying this group of patients would be extremely useful to improve the accurate diagnosis of thyroid nodules and prevent unnecessary thyroid surgeries, allow molecular prognostication and define a therapeutic approach toward sporadic PTC patients.

Funding Support:

The study was supported by research grants from The São Paulo State Research Foundation (FAPESP), grant numbers 2012/02902-9 and 2013/03867-5. LM is a FAPESP scholar. JMC is a Brazilian Research Council (CNPq) investigator.

Conflict of Interest Statement:

The authors have reported no conflicts of interest.

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Table 1. Summary of the clinic-pathological features and occurrence of AGK-BRAF fusion oncogene in pediatric thyroid carcinoma

Case	PTC Variant	Age (yr)	Gender	Tumor Size (cm)	Multifocality	Lymph node Metastasis	Distant Metastasis	Extrathyroidal extension	TNM	Radiation Exposure	*AGK-BRAF*
1	Classical	7	M	1.4	No	Yes	No	No	T1N1M0	No	No
2*	Follicular	18	F	4.5	No	Yes	No	NA	T3N1M0	No	No
3	Classical	4	M	1.7	No	Yes	Yes	Yes	T4N1M1	No	No
4	Classical	13	F	3.2	No	No	No	No	T2N0M0	No	No
5*	Diffuse Sclerosing	17	F	1.5	Yes	Yes	No	No	T1N1M0	No	No
6	Classical	4	M	0.7	No	Yes	No	Yes	T3N1M0	No	No
7*	Classical	18	F	3.5	Yes	Yes	No	No	T2N1M0	No	No
8	Follicular	17	F	2.5	No	No	No	No	T2N0M0	No	No
9*	Classical	7	F	6	Yes	Yes	Yes	Yes	T4N1M1	No	No

10*	Follicular	12	M	3	Yes	Yes	Yes	Yes	T4N1M1	No	No
11	Classical	5	F	3	No	Yes	Yes	Yes	T4N1M1	No	No
12	Diffuse Sclerosing	13	F	2.5	Yes	Yes	No	No	T2N1M0	No	No
13*	Diffuse Sclerosing	9	M	1.7	Yes	Yes	Yes	Yes	T4N1M1	No	No
14*	Classical	18	F	3.5	No	Yes	No	No	T2N1M0	No	No
15	Follicular	12	F	1.8	No	No	No	No	T1N0M0	No	No
16	Classical	12	F	NA	Yes	Yes	Yes	Yes	T4N1M1	No	No
17	Follicular	6	F	NA	NA	Yes	No	NA	TxN1M0	No	No
18*	Follicular	13	M	2	Yes	Yes	No	Yes	T4N1M0	No	No
19	Encapsulated	10	F	2	No	No	No	No	T1N0M0	No	No
20*	Classical	15	M	4.5	Yes	Yes	Yes	Yes	T4N1M1	No	Yes
21* ♈	Follicular	13	F	5	Yes	Yes	Yes	Yes	T4N1M1	No	Yes

22*	Classical	16	F	2	Yes	Yes	No	No	T1N1M0	No	No
23*	Follicular	8	F	2.5	No	Yes	No	No	T2N1M0	No	No
24*	Solid	8	M	0.9	Yes	Yes	Yes	Yes	T4N1M1	No	No
25*	Diffuse Sclerosing	9	F	2.1	No	Yes	No	Yes	T4N1M0	No	No
26	Classical	7	F	1.6	No	No	No	Yes	T3N0M0	No	Yes
27*	Follicular	6	F	3.5	Yes	Yes	Yes	Yes	T4N1M1	No	No
28	Follicular	14	F	1.7	No	No	No	No	T1N0M0	No	No
29	Follicular	18	F	1	No	Yes	No	No	T1N1M0	No	No
30	Follicular	12	M	2.2	Yes	Yes	No	No	T2N1M0	No	No

*PTC samples with matched and Lymph node metastasis

ϒ PTC samples with matched Lymph node metastasis which was positive for AGK-BRAF

Table 2. Pediatric PTC characteristics according to the prevalence of *AGK-BRAF*

	Total No.	***AGK-BRAF* Negative No (%)**	***AGK-BRAF* Positive No (%)**
	(*n*=30)	(*n*=27)	(*n*=3)
Age ± SD (mean/ys)	11.36	11.33	11.66
Tumor Size ± SD (mean/ cm)	2.55	2.41	3.7
Gender			
Female	21	19 (70)	2 (66)
Male	9	8 (30)	1 (34)
Extrathyroidal extension	14	11(40)	3 (100)
Multifocal disease	14	12 (44)	2 (66)
LN metastases	24	22 (81)	2 (66)
Distant metastases	10	8 (29)	2 (66)

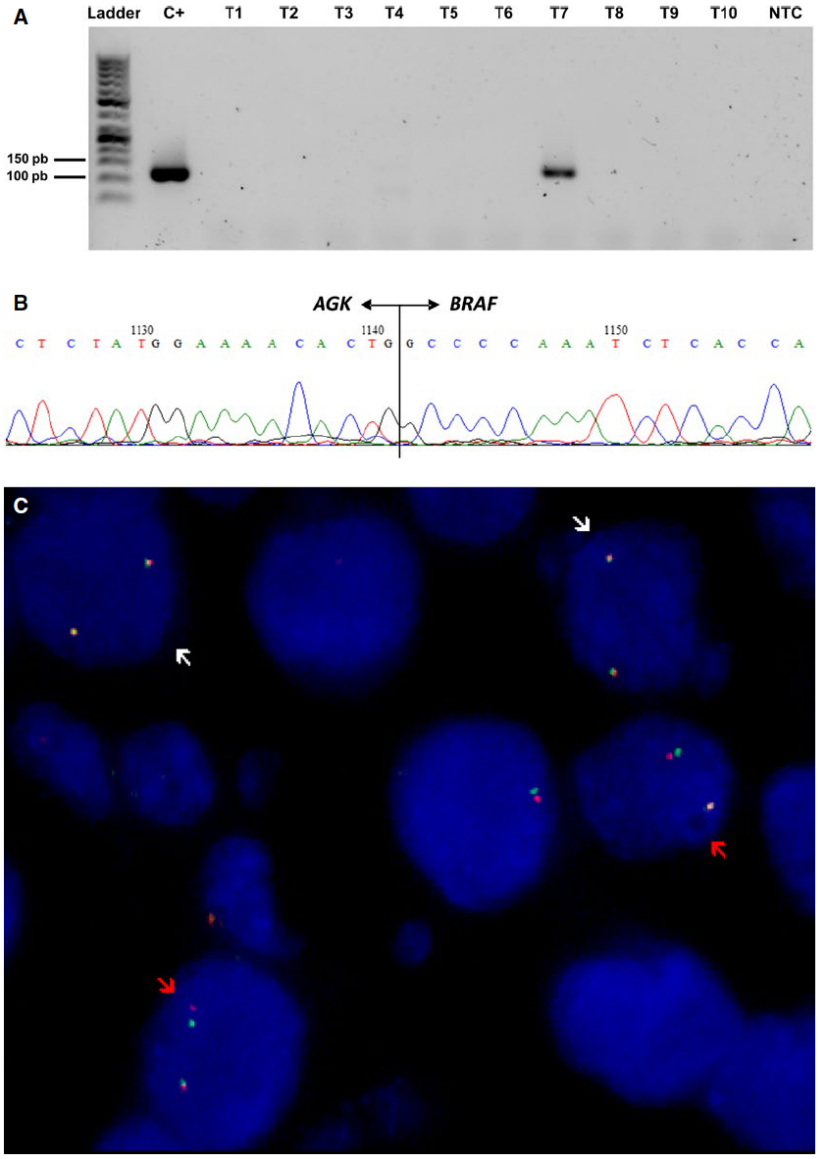

Figure 1. Screen for the presence of *AGK-BRAF* fusion oncogene in sporadic pediatric PTC. Representative results of RT-PCR analysis performed in sporadic pediatric PTC (T1-T10). Positive (C+) and negative controls (NTC) were included in each run. Positive cases showed the proper size range (113bp), as showed in C+ and case 20 (T7) (**A**). Sanger sequencing confirmed the presence of *AGK-BRAF* fusion oncogene (**B**). Dual-color, break-apart FISH confirmed the BRAF rearrangement (C). Nuclei exhibiting rearrangement showed the presence of one red and green split signals (red arrow), in addition to the fused yellow or red-green signal (white arrow).

ARTIGO 4

Fusion oncogenes are the main genetic events found in sporadic papillary thyroid carcinomas from children

Artigo aceito para publicação na revista *Thyroid* em outubro de 2016

Fusion oncogenes are the main genetic events found in sporadic papillary thyroid carcinomas from children

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Running title: Mutational status in pediatric thyroid carcinomas.

Keywords: pediatric thyroid carcinomas, *BRAF, RET/PTC, ETV6-NTRK3, AGK-BRAF*

Abstract

Background: Previous studies reported significant differences in the clinical presentation and outcomes of papillary thyroid carcinoma (PTC) in pediatric patients compared to adults. Previous studies have suggested that the clinicopathological differences observed between pediatric and adult PTC may be due the existence of distinct genetic alterations. However, the knowledge of genetic events in pediatric PTC is based primarily on studies in radiation-exposed PTC or in few studies that enrolled predominantly adolescent patients. The aim of this study was to characterize the known oncogenic alterations of the MAPK pathway found in adult and radiation-exposed PTCs in a cohort of predominantly sporadic pediatric PTC patients.

Methods: Thirty-five pediatric PTCs were screened for the most prevalent fusions (*RET/PTC1, RET/PTC2, RET/PTC3, ETV6-NTRK3* and *AGK-BRAF*) and point mutations (BRAF V600E and NRAS Q61) described in sporadic pediatric PTC. The mutational status was correlated with clinicopathological data.

Results: Mutations were found in 20 out of 35 (57%) PTC cases. Fusion oncogenes were the main genetic alterations found. *RET/PTC1-3* rearrangements were found in 13 (37%), *ETV6-NTRK3* in 3 (9%), *AGK-BRAF* in 4 (11%) and BRAF V600E in 3 (9%). No mutation was found in NRAS Q61. BRAF V600E was associated with older age and larger tumor size (P <0.05) and *RET/PTC3* was associated with a larger tumor size and multifocality (P <0.05).

Conclusions: The genetic signature in this cohort was remarkably different than that observed in adults. Although observed at a lower prevalence, the spectrum of mutations was quite similar to that described in radiation-exposed pediatric PTC. As mutations were unidentifiable in over 40% of the PTC cases, more comprehensive

studies conducted in these patients will help to decipher the genetic landscape of sporadic pediatric PTC.

Introduction

The incidence of thyroid cancer has been rising sharply over the past few decades worldwide (1, 2) and it is the fastest-increasing cancer in both men and women (3). Although rare in the younger population, the incidence is also increasing among children and adolescents (4, 5). Thyroid cancer is the 2nd most prevalent cancer in females 15-19 years of age (6). Differentiated thyroid carcinomas (DTC) are the most common malignancy in all age groups, with nearly 80-90% being papillary thyroid carcinomas (PTC) and the remainder follicular thyroid carcinomas (FTC) (4, 7).

Previous studies reported significant differences in the clinical presentation and outcomes of PTC in pediatric patients compared to adults. Although pediatric PTC patients usually present at a more advanced stage of disease at diagnosis and have a higher risk of recurrence, the overall mortality is lower (8, 9). Nevertheless, a marked heterogeneity within the pediatric group has been reported. Children (<10 years) present with more aggressive local disease and are more likely to have lymph node metastases at diagnosis and are probably more prone to develop subsequent metastases than adolescents (≥10-18 years) (4, 10-13). They also experience recurrence more frequently and earlier than adolescents (14).

There has been an ongoing debate about whether the clinicopathological differences observed between pediatric and adult PTC may be due to the existence of distinct genetic alterations (15). Efforts have been made to identify these acquired genetic abnormalities in tumors from pediatric patients that will determine the tumors' biological behavior, and that will ultimately allow molecular prognostication. However,

most studies were performed in pediatric PTC after radiation exposure such as the Chernobyl disaster.

The prevalence of the *BRAF* V600E mutation, the most common genetic event found in adult PTC (16, 17), is significantly lower in sporadic and radiation-exposed pediatric PTC (18-20). *RET/PTC* rearrangement, the second most common event in adult PTC, is the major genetic alteration found in sporadic (21-24) and radiation-exposed pediatric PTC (20, 24-26). Moreover, *RAS* mutations are a rare genetic event in pediatric PTC. The incidence rates range from 0% to 7% in sporadic cases (15, 20, 27, 28), while no mutation was reported in radiation-exposed tumors (15, 20, 29). In the few pediatric patients in whom *RAS* point mutations were detected, only the NRAS Q61R was described

Recently, the *ETV6-NTRK3* fusion oncogene was identified as the second most common rearrangement found in radiation-exposed pediatric PTC (20, 30). A similar prevalence was described in pediatric PTC with no known radiation exposure from the same geographic area (20). Remarkably, the authors also reported *AGK-BRAF* rearrangements in 4% (1/26) of radiation-exposed pediatric PTC patient, but this genetic alterations was not found in sporadic cases (20). However, our group reported *AGK-BRAF* fusions in 3/30 (10%) of sporadic pediatric PTC patients (31), suggesting the possibility of genetic and environmental differences between ethnicities and geographic regions**.**

Given that the prevalence and specificity of each genetic event varies geographically in pediatric populations, additional studies, performed across different populations, are required to gain insights into disease biology and to develop strategies for molecular diagnosis and prognosis, as well as treatments for pediatric

thyroid cancer.

In the present study, we screened a cohort of 35 Brazilian patients with sporadic pediatric PTC for the most common genetic alterations found in adult and pediatric PTC diagnosed in other countries and correlated these findings with clinicopathological features.

Materials and Methods

Patients and samples

The series consists of 35 formalin-fixed paraffin embedded (FFPE) sections from primary tumors from patients who underwent thyroid surgery at Hospital São Paulo (Universidade Federal de São Paulo) and Hospital da Santa Casa de São Paulo. The samples were reviewed by two pathologists (RD and MTSA). The study included 16 classical PTC (CPTC), 13 follicular variant of PTC (FVPTC), 4 diffuse sclerosing variant of PTC (DSVPTC), and 2 other variants of PTC.

All of the patients were **≤** 18 years of age at the time of diagnosis, as recommended by the American Thyroid Association (ATA) guidelines for children with thyroid nodules and DTC (32). The pediatric cases were further separated into two age groups: children (<10 years old) and adolescents (≥10-18 years old). Because information about pubertal development was not available for all of the patients, the age of 10 was used as the cut-off. This cut-off point was recommended by the World Health Organization (WHO) and used to determine the effect of age on time to recurrence and mortality rates in pediatric DTC (14, 33, 34).

The clinical and pathological features evaluated are summarized in **Supplementary Table 1**. The study was conducted under the approval of the Review Boards and Research Ethical Committees of the affiliated institutions.

DNA isolation and BRAF V600E and NRAS Q61R Genotyping

DNA was isolated from 10 µm-thick formalin-fixed paraffin-embedded (FFPE) sections using the NucleoSpin Tissue kit (Macherey-Nagel, Duren, Germany). The DNA was quantified using a NanoDrop 2000c spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Somatic mutations in *BRAF* (BRAF V600E) and NRAS (Q61) were screened by PCR. Exon 15 of the *BRAF* gene was amplified by PCR as previously reported (35). Exon 3 of the *NRAS* was amplified as follows: 94°C for 5 minutes, followed by 40 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds. The PCR products were resolved by electrophoresis, purified and submitted to cycle sequencing using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The samples were sequenced at least twice and in both directions. PCR primers are summarized in **Supplementary Table 2**.

RNA isolation and cDNA synthesis

Total RNA was isolated from 10 µm-thick FFPE sections using the Recover All Total Nucleic Acid isolation kit (Ambion Inc., Austin, TX). Total RNA (500 ng) was treated with DNAse and reverse-transcribed into cDNA with oligo-dT_{12-18} (50 µM) and random hexamers (50 ng) using a Superscript III reverse transcriptase kit (Invitrogen Corp., Carlsbad, CA), following manufacturer's instructions.

Expression of fusion oncogenes in thyroid cell lines

PCCL3 cells (normal follicular thyroid cells derived from Fischer rats) were cultured in Ham's F12 medium (Life Technologies, Carlsbad, CA) supplemented with 5% FBS, TSH (1 mU/mL), hydrocortisone (10 ng/mL), transferrin (5 µg/mL), and

insulin (10 μg/mL) (Sigma-Aldrich, Saint Louis, MO). Transient transfections were performed using 10 μg of plasmids encoding RET/PTC1, RET/PTC2, RET/PTC3, ETV6-NTRK3 and AGK-BRAF. The oncogene-transfected cells were harvested, and the total RNA isolated using TRIzol Reagents (Invitrogen Corp) and reverse transcribed into cDNA using oligo$(dT)_{12-18}$ and Superscript III reverse transcriptase, as previously described (36). The cDNA generated from cells expressing the fusion transcripts was used as positive control.

RET/PTC2, RET/PTC3, ETV6-NTRK3 and *AGK-BRAF* plasmids were kindly donated by Dr. James Fagin (Memorial Sloan-Kettering Cancer Center), and the *RET/PTC1* construct was donated by Massimo Santoro (Università di Napoli Federico II).

Detection of *ETV6-NTRK3* fusion transcript

All of the samples were screened for the presence of the *ETV6-NTRK3* fusion transcript by RT-PCR, as previously described (20). Briefly, cDNA (2 μL) was subjected to PCR amplification using 2 pmol of each specific primer and Platinum Taq DNA Polymerase (Invitrogen Corp). The PCR products were resolved on a 2.5% agarose gel and visualized on a Bio-Rad Gel Doc EZ system (Bio-Rad). The presence of the fusion transcripts was confirmed by direct sequencing. The primers used were previously described and validated (20) (**Supplementary Table 2**).

Detection of *AGK-BRAF* fusion transcript

The *AGK-BRAF* fusion was previously investigated in 30 cases of PTC (31). In this study a total of 35 patients were screened for the presence of the *AGK-BRAF* fusion transcript by RT-PCR, as previously described (31). The presence of *AGK-BRAF* was confirmed by FISH analysis.

Detection of *RET/PTC* rearrangements by quantitative RT-PCR

The thyroid samples were screened for *RET/PTC1*, *RET/PTC2* and *RET/PTC3* rearrangements according to standard quantitative RT-PCR. Briefly, cDNA (2 μL) was subjected to PCR amplification using 1X SYBR Green PCR Master Mix (PE Applied Biosystems) and predesigned primers that span the known breakpoints specific for each of the *RET/PTC* transcripts (23) or an internal control (*RPS8*). After an incubation at 50°C for 10 minutes and 95°C for 10 minutes, the PCR reaction was subjected to 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The qPCR reaction was performed in triplicate, and the threshold cycle (Ct) was obtained using Applied Biosystem software. Post-amplification fluorescent melting curve analysis was performed. The PCR products were analyzed by electrophoresis on a 2% agarose gel and visualized on a Bio-Rad Gel Doc EZ system (Bio-Rad, Hercules, CA, USA). The samples were considered positive when at least two replicates had amplification curves that crossed the threshold before cycle 38, the melt curve aligned with the positive control, and the gel analysis revealed a band of the expected product size. To confirm the identity of the amplified products, randomly selected samples positive for *RET/PTC* isoforms were sequenced using the BigDye Terminator Cycle Sequencing Kit (PE Applied

Biosystems). The primers used to detect the *RET/PTC* fusion transcripts were previously described and validated (23) (**Supplementary Table 2**).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA) software. Shapiro-Wilk test was used to verify the normality of distribution. Mann-Whitney U test or the Student's t-test was used to compare continuous variables and Fisher's exact test was used for dichotomous variables. Results with $P< 0.05$ were considered to be statistically significant and a trend with borderline significance was considered when P was ≥ 0.05 and <0.10.

Results

Clinical and pathological features

The mean age at diagnosis was 11.8 years (range, 4 to 18 years). The female to male ratio was 26:9, with female predominance mainly in the adolescent group. The pathological findings included multifocality in 17 (49%), extrathyroidal (ETE) extension in 17 (49%), lymph node metastasis (LNM) in 28 (80%), and lung metastasis in 11 (31%) patients. There was no death from PTC in patients enrolled in this study. Two of the patients had a family history for PTC, and three cases had a history of previous radiation exposure during childhood to treat another cancer. Demographic data were previously reported (37) and are summarized in **Supplementary Table 1**.

BRAF V600E and NRAS Q61 mutation in sporadic pediatric PTC

We systematically investigated the prevalence of the *BRAF* V600E mutation in 35 pediatric PTC. The *BRAF* mutation was found in 3 (9%) patients (**Figure 1)**. The age of the patients ranged from 16 to 18 years old (mean 17.33). None of the patients had a mutation in exon 3 of *NRAS.*

ETV6-NTRK3 in sporadic pediatric thyroid carcinomas

ETV6-NTRK3 was identified in 3 (9%) patients with PTC (**Figure 1**). Although different isoforms of

ETV6-NTRK3 rearrangements were previously identified in PTC, in this study, the fusion transcript that juxtaposes exons 1-4 of *ETV6* to exons 12-18 of *NTRK3* was observed in all *ETV6-NTRK3*-positive samples. Sequencing analysis confirmed the identity of the *ETV6-NTRK3* rearrangements identified on gel analysis. None of the cases positive for *ETV6-NTRK3* had a radiation exposure history.

AGK-BRAF was identified in both sporadic and radiation-exposed pediatric PTC

The *AGK-BRAF* fusion was detected in 4 (11%) patients, one case had a radiation exposure history for the treatment of Hodgkin's lymphoma. The remaining cases were sporadic pediatric PTC patients (31).

RET/PTC rearrangements in sporadic pediatric PTC

Overall, *RET/PTC* was identified in 13 (37%) patients. *RET/PTC1* alone was identified in 4 (11%), *RET/PTC2* in 1 (3%) and *RET/PTC3* in 4 (11%). The co-occurrence of *RET/PTC1* and *RET/PTC3* was identified in 1 (3%), *RET/PTC1* and *ETV6-NTRK3* was detected in 1 (3%), and *RET/PTC3* and *AGK-BRAF* was found in 2 (6%) patients (**Figure 1)**. Only one case with a *RET/PTC3* and *AGK-BRAF* rearrangement had a radiation exposure history. Sequencing analysis confirmed the identity of the *RET/PTC* rearrangements identified on gel analysis.

Correlation of mutational status with clinicopathological features

Of the 35 PTC screened using a candidate gene approach, 20 (57%) were positive for BRAF V600E, *RET/PTC1, RET/PTC2, RET/PTC3*, *ETV6-NTRK3* or *AGK-BRAF* (**Figure 1***)*.

When the tumors harboring fusion oncogenes and BRAF point mutations were grouped and compared with tumors negative for genetic alterations, the mutation-positive cases were of larger size than the mutation-negative cases ($P<0.05$). When each genetic event was independently analyzed, tumors harboring *RET/PTC*3 or *BRAF* V600E were of a larger size than the mutation-negative cases ($P<0.05$).

When each mutation was considered as an independent variable, *RET/PTC3* was correlated with multifocality ($P=0.0325$) and a tendency to ETE ($P=.0867$), and *BRAF* V600E was significantly associated with older age of the patients ($P=0.0221$).

The presence of genetic event was not significantly correlated with cervical or distant metastases. Eighteen out of twenty (90%) tumors positive for mutations had LNM at diagnosis, whereas 10/15 (67%) tumors without mutations had LNM at diagnosis ($P=0.1122$). Distant metastases were identified in 7/20 (35%) tumors with an identified genetic alteration, and in 4/15 (27%) tumors negative for mutations. Moreover, when each mutation was considered individually, none of the genetic alterations were correlated with the presence of cervical or distant metastases.

When tumors were classified according histological subtypes, a trend to a higher prevalence of rearrangement in FVPTC (70%) than in CVPTC (31%) (9/13 vs. 5/16; $P=0.0618$) was identified. Of note, a trend to an increased incidence of *RET/PTC* fusion transcripts was identified in FVPTC (46%) compared to CVPTC (19%) (6/13 vs. 2/16; $P=0.0648$)**.** Therefore, there was an apparent correlation with

the nature of the causal mutation. Consistent with previously reported findings (38), *RET/PTC1* was a common event found in DSVPTC.

Interestingly, the prevalence of genetic alterations did not differ significantly between the two age groups: 6/12 (50%) and 14/23 (61%) of alterations occurred in the <10 and ≥10-18 age groups, respectively (**Figure 1**). *RET/PTC*, the most prevalent genetic alteration, was equally prevalent in both age groups, which may explain the lack of correlation between genetic mutation and clinicopathological findings in children and adolescents. Of note, *BRAF* V600E mutation was found only in the ≥10-18 age group.

Discussion

In this cohort, the lack of the *BRAF* V600E mutation in children and a lower prevalence (9%) of this mutation in pediatric PTC compared to adults PTC is in agreement with previous reports (18-20, 39). Our findings suggest that the prevalence of *BRAF* V600E is not only lower in pediatric sporadic PTC than in adults, but that its prevalence also increases with age. Although similar findings were previously reported (27, 39), a higher prevalence of *BRAF* V600E was recently described in sporadic pediatric PTC (28, 40, 41). It is worth mentioning that these last reports enrolled predominantly adolescent patients. Moreover, the study with the highest prevalence of *BRAF* V600E considered pediatric patients up to 21 years of age (41).

*RET/*PTC fusion transcripts were previously identified as the central genetic event in both radiation-exposed and sporadic pediatric PTC (24). Indeed, a *RET/PTC* rearrangement was the most prevalent mutation found in this patient cohort. Although the prevalence (37%) was lower than the one reported in radiation-exposed PTC (24, 38, 42), our data are consistent with those observed in sporadic pediatric PTC from different geographical areas (27, 43). *RET/PTC1* and *RET/PTC3* were the most prevalent isoforms identified in our cohort. Although *RET/PTC3* was mainly found in the patients who were exposed to ^{131}I from the Chernobyl accident (24, 42), only 1 patient with the *RET/PTC3* rearrangement had a previous radiation history in the cohort presented here. To the best of our knowledge, this is the first study to report the prevalence of *RET/PTC* fusions in pediatric patients from Brazil.

Because both the prevalence and distribution of isoforms diverge widely among different countries, the difference observed in the distributions of the *RET/PTC* isoforms in our series could be, at least in part, due to the exposure to different environmental factors and genetic background. Another potential explanation is that a large proportion of the tumors were follicular variants of PTC in our series. Previous studies reported such an association between *RET/PTC3* and solid and follicular variant PTC (24, 42).

ETV6-NTRK3 was reported as the second most common rearrangement found in radiation-exposed PTC diagnosed among individuals who were < 18 years of age at the time of the Chernobyl accident (20, 30). In this cohort, the prevalence of *ETV6-NTRK3* (9%) was consistent with that reported in adult (5%) and sporadic pediatric (7%) PTC (20, 30).

While the mutations described above occur in a mutually exclusive pattern in most tumors, we identified one tumor with concomitant presence of a *RET/PTC1* and *RET/PTC3* rearrangement*,* another tumor with *RET/PTC1* and *ETV6-NTRK3,* and two tumors with *RET/PTC3* and *AGK-BRAF* fusions. Although *ETV6-NTRK3* was initially identified in radiation-exposed pediatric PTC lacking known common driver mutations in PTC (20), further validation analyses showed the co-occurrence of *ETV6-NTRK3* with *RET/PTC1* or with *BRAF* V600E (30).

Although the studied mutations were equally prevalent in children and adolescents, the spectrum of mutations notably diverges between these age groups. The occurrence of *BRAF* V600E mutation and concomitant rearrangements were found only in adolescents. These findings suggest that there are differences in tumor biology according to age.

Although the *BRAF* V600E mutation was previously reported to be correlated with a more aggressive tumor phenotype in adults (17, 44, 45), we did not find such a correlation in sporadic pediatric PTC. Other groups have previously reported an absent association between biological behavior of pediatric PTC and the presence of the *BRAF* V600E mutation (15, 41). These findings reflect the fact that in this age group, *BRAF* V600E is uncommon and the aggressiveness of PTC is likely associated with other genetic events. Whether additional changes in tumor microenvironment, metabolism, microRNA pattern or other genetic events that activate the MAPK pathway such as the *ETV6-NTRK3* or *AGK-BRAF* fusion oncogenes (20) are associated with a more aggressive phenotype, is still unclear.

Although *ETV6-NTRK3* reportedly activates both MAPK and PI3K signaling pathways and *AGK-BRAF* activates MAPK (20), the ability of these fusions proteins to activate these pathways and the biological effects, compared to *BRAF* V600E and *RET/PTC*, are still uncertain.

Remarkably the *RET/PTC3* isoform was associated with pathological features related with tumor aggressiveness, such as larger tumor size and multifocality, and there was a trend towards a higher prevalence of ETE. *RET/PTC3* was previously associated with solid-follicular growth pattern in radiation-exposed pediatric PTC and more aggressive disease, whereas *RET/PTC1* was mainly associated with no invasive disease (23, 24, 42, 43).

There was no statistically significant difference between the presence of a genetic mutation and other features related to a worse prognosis, such as local or distant metastasis, in this study. Nevertheless, further studies, ideally involving a multicenter approach, are needed to confirm the absent of such an association.

There are some limitations to our study. First, our study is limited by the inherent biases of a retrospective analysis and, therefore, the lack of a proper follow-up with all of the patients. Second, it is limited by a small sample size. However, most of the previous studies usually enrolled a similar or even smaller number of sporadic pediatric PTC patients (15, 19, 20, 27, 28, 43). The much lower incidence of sporadic pediatric PTC, specially in patients under 10 years old, compared to adult PTC, makes it difficult to collect a larger number of patients.

The investigation of the most prevalent mutations in adult PTC, including the recently identified *ETV6*-NTRK3 and *AGK-BRAF* fusion transcripts, in a cohort of pediatric sporadic PTC, contributes to better understanding the tumor pathogenesis in young population. We observed that the prevalence of mutations in this cohort was remarkably different compared to adult PTC, even in the same geographical area (17, 46). Nearly 9% of the patients had activating mutations, while 49% had rearrangements. Although the prevalence of fusion oncogenes was lower than in radiation exposed PTC (20), they were the most prevalent genetic event found in this cohort. Importantly, about 43% of the pediatric patients were negative for any of the studied mutations, suggesting that the most prevalent mutations found in adults and radiation-exposed PTC are not the major events in sporadic pediatric PTC. Because the tissue samples used in this study were particularly enriched in tumors cells, we believe that the false-negative rate is very low.

In summary, the clinical features of PTC are markedly different according to age, and this fact is probably associated with the differences in molecular profiles. The high number of patients in whom no genetic event was identified suggests that other genetic/epigenetic factors may be associated with the pathogenesis and biological behavior of sporadic pediatric PTC. More comprehensive studies of the

PTC negative for mutations are needed in order to decipher their genetic signature and to reduce the so-called “dark matter” in the genome of sporadic pediatric PTC.

Acknowledgments

The study was supported by research grants 2013/03867-5 and 2015/60330-8 from The São Paulo State Research Foundation (FAPESP) and grant 470441/2013-5 from The Brazilian Research Council (CNPq). LM and AUB are recipients of fellowships from FAPESP. JMC is a recipient of a scholarship of Research Productivity from CNPq.

Author Disclosure Statement

The authors have no conflicts of interest to declare.

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Supplementary table 1. Summary of the demographic data of pediatric PTC

	All patients No.	**Patients <10 yr old** No. (%)	**Patients ≥ 10-18 yr old** No. (%)	***P* value**
	***n*=35**	***n*=12**	***n*=23**	
Age (± SD) (mean)	11.80 (±4.48)	6.58 (± 1.88)	14.52 (± 2.59)	**<0.0001**
Gender				
Female	26	7 (58)	19 (83)	0.2204
Male	9	5 (42)	4 (17)	
Mean tumor size (cm) ± SD	2.63 (± 1.44)	2.28 (± 1.49)	2.81 (± 1.41)	0.2179
Risk Factors				
Family History	2	1 (8)	1 (4)	1.00
Exposure to radiation	3	0	3 (13)	0.5361
Histological subtypes				
Classical PTC	16	6 (50)	10 (43)	0.7362
Follicular variant of PTC	13	3 (25)	10 (43)	0.4630
Others [a]	6	3 (25)	3 (13)	0.3912
Extrathyroidal extension	17	9 (75)	8 (35)	**0.0238**
Multifocal disease	17	4 (33)	13 (57)	0.2890
LN metastases	28	11 (92)	17 (73)	0.3800
Distant metastases	11	6 (50)	5 (22)	0.1297

[a] Others variants of papillary thyroid carcinoma (PTC) included diffuse sclerosing, solid and ›sulated variant

Supplementary Table 2. Primer sequences and expected PCR product size

Genes		Primer Sequence (5' - 3')	Expected Product size (bp)
RET/PTC1[a]	Sense	CAAAGCCAGCGTTACCATCG	81
	Antisense	CCTTCTCCTAGAGTTTTTCC	
RET/PTC2[a]	Sense	GAAATTGTGGGGCATCGACC	108
	Antisense	CCTTCTCCTAGAGTTTTTCC	
RET/PTC3[a]	Sense	CAAGCTCCTTACATACC	134
	Antisense	CCTTCTCCTAGAGTTTTTCC	
ETV6-NTRK3[b]	Sense	ACACACACAGCCGGAGGTCATAC	90
	Antisense	AGTGGGCTGGCTGAGTCCTCC	
AGK-BRAF[b]	Sense	CTGCTGACCTGGGGAGGCCATT	113
	Antisense	TCATCTGCTGGTCGGAAGGGCTG	
BRAF[c]	Sense	TCATAATGCTTGCTCTGATAGGA	195
	Antisense	CCTCAATTCTTACCATCCACAAAA	
RAS	Sense	TAGCATTGCATTCCCTGTGG	193
	Antisense	CGCCTGTCCTCATGTATTGG	

[a] (23), [b] (20), [c] (35)

Supplementary Table 3. Clinicopathological features and genetic alterations in pediatric papillary thyroid cancer

Case	Age (yr)	Gender	Variant	Cervical Meta	Distant Meta	Tumor Size (cm)	ET Extension	TNM	BRAF	RET/PTC1	RET/PTC2	RET/PTC3	ETV6-NTRK3	AGK-BRAF
1	9	F	DSVPTC	Yes	No	2.1	Yes	T4N1M0	No	Yes	No	No	No	No
2	8	M	SVPTC	Yes	Yes	0.9	Yes	T4N1M1	No	Yes	No	No	No	No
3	6	F	FVPTC	Yes	Yes	3.5	Yes	T4N1M1	No	No	No	Yes	No	No
4	7	F	CVPTC	Yes	Yes	6	Yes	T4N1M1	No	No	No	Yes	No	No
5	8	F	FVPTC	Yes	No	2.5	No	T2N1M0	No	No	No	No	Yes	No
6	7	F	CVPTC	No	No	1.6	Yes	T3N0M0	No	No	No	No	No	Yes
13	12	M	FVPTC	Yes	No	2.2	No	T2N1M0	No	Yes	No	Yes	No	No
14	13	F	DSVPTC	Yes	No	2.5	No	T2N1M0	No	Yes	No	No	Yes	No
15	18	F	CVPTC	Yes	No	3.5	No	T2N1M0	No	Yes	No	No	No	No
16	18	F	FVPTC	Yes	No	1	No	T1N1M0	No	Yes	No	No	No	No
17	14	F	FVPTC	No	No	1.7	No	T1N0M0	No	No	Yes	No	No	No
18	12	M	FVPTC	Yes	Yes	3	Yes	T4N1M1	No	No	No	Yes	No	No
19	13	M	FVPTC	Yes	No	2	Yes	T4N1M0	No	No	No	Yes	No	No
20	13	F	FVPTC	Yes	Yes	5	Yes	T4N1M1	No	No	No	Yes	No	Yes
21	15	F	CVPTC	Yes	No	NA	Yes	T3N1M0	No	No	No	Yes	No	Yes
22	15	F	FVPTC	Yes	No	4.5	Yes	T3N1M0	No	No	No	No	Yes	No
23	15	M	CVPTC	Yes	Yes	4.5	Yes	T4N1M1	No	No	No	No	No	Yes
24	18	F	CVPTC	Yes	No	3.5	No	T2N1M0	Yes	No	No	No	No	No
25	18	F	FVPTC	Yes	No	4.5	No	T3N1M0	Yes	No	No	No	No	No
26	16	F	CVPTC	Yes	No	6	No	T3N1M0	Yes	No	No	No	No	No

PTC, papillary thyroid carcinoma; DSVPTC, diffuse sclerosing PTC; SVPTC, solid PTC; FVPTC,follicular variant PTC; CVPTC, classic PTC; NA, not available; ET Extension, Extrathyroidal Extension.

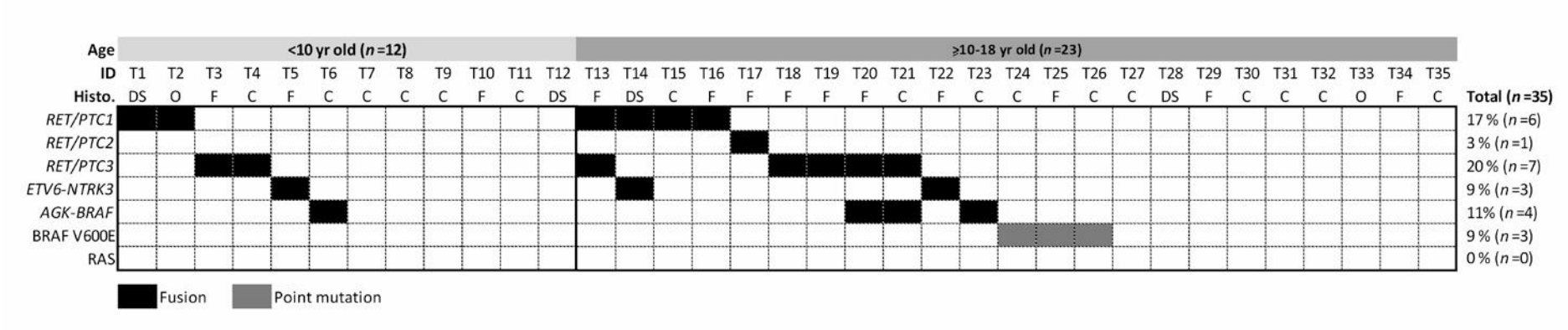

Figure 1. Prevalence of *RET/PTC1, RET/PTC2, RET/PTC3*, *BRAF V600E, ETV6-NTRK3, AGK-BRAF* and *NRAS* in all of the pediatric PTC (*n*=35). The prevalence of genetic alterations in pediatric PTC was classified according to age: children (<10 years old) or adolescents (≥ 10-18 years old) and histological variants. DS, diffuse sclerosing variant of PTC; F, Follicular variant of PTC; C, classical PTC; O, other variants of PTC (solid and encapsulated variant of PTC).

DISCUSSÃO

Inúmeros estudos evidenciaram a existência de diferenças nas características clínico-patológicas do CDT entre os grupos pediátrico e adulto. Os pacientes mais jovens comumente apresentam a doença em estágios mais avançados ao diagnóstico e com maiores taxas de doença recorrente ou persistente (15, 49). Contudo, o prognóstico do CDT pediátrico é, na maioria das vezes, excelente (8, 9, 49). Além disso, alguns estudos ressaltam uma heterogeneidade clínica no grupo pediátrico, com uma maior tendência a metástase local e à distância em crianças em comparação aos adolescentes (8-10, 20, 27). As características clínico-patológicas do CDT pediátrico descritas em estudos de diferentes regiões do mundo estão detalhadas e sumarizadas no **artigo 1**.

Uma outra importante diferença mencionada na literatura entre os grupos pediátrico e adulto é a elevada prevalência de metástase pulmonar iodo-captantes (28, 30) e a melhor resposta ao tratamento com RAI no grupo mais jovem (14, 50), o que poderia explicar a maior sobrevida em relação aos pacientes adultos. Essa melhor resposta à terapia com RAI no grupo pediátrico sugere um maior grau de diferenciação tumoral, o que resultaria numa maior expressão do gene *NIS*, além de outros genes associados à captação e ao metabolismo do iodo. No entanto, os estudos prévios que investigaram o papel da terapia com RAI e a expressão dos genes associados a captação e metabolismo do iodo no CDT pediátrico avaliaram, majoritariamente, pacientes adolescentes e um número pouco significativo, ou mesmo ausente, de crianças ou pacientes pré-púberes (14, 26, 28, 33, 34). Em razão da diferença observada em alguns estudos na apresentação clínica do CDT

entre crianças e adolescentes, a afirmação de que o grupo pediátrico como um todo apresenta um maior grau de diferenciação tumoral e uma maior expressão de genes específicos da tireoide em comparação com adultos, apresenta pouca evidência na literatura.

O **artigo 2** descreve o resultado da análise da expressão dos genes *NIS, TG, TPO, PDS e TSH-R* em uma casuística de CDT pediátrico. Além disso, compara o nível de expressão destes genes nos casos agrupados de acordo com a idade (<10 anos vs. ≥10-18 anos). Reportamos, pela primeira vez na literatura, diferença na expressão dos genes *NIS*, *PDS* e *TSH-R* entre esses grupos, com uma menor expressão observada nos casos <10 anos em comparação aos pacientes ≥ 10-18 anos. Esse resultado corrobora a hipótese de haver uma heterogeneidade no grupo pediátrico e sugere que exista uma distinção no grau de diferenciação tumoral entre crianças e adolescentes.

Além disso, no **artigo 2** é descrito também a comparação da expressão desses genes (*NIS*, *PDS* e *TSH-R*) nos subgrupos <10 anos vs. ≥10-18 anos, com dados obtidos da expressão destes genes em uma casuística adulta previamente investigada pelo nosso grupo (47, 48). Essa análise evidenciou, pela primeira vez na literatura, uma maior expressão de *NIS* no grupo de CDT de pacientes adolescentes (≥10-18 anos), em comparação aos casos de crianças (<10 anos) e adultos, que mostraram taxas reduzidas e semelhantes de expressão de *NIS*. Por outro lado, observou-se que no CDT em crianças a expressão de *PDS* e *TSH-R* é inferior ao encontrado no CDT em adolescentes e adultos. Esses resultados sugerem um menor grau de diferenciação tumoral em crianças, o que pode explicar, em parte, o maior grau de invasão tumoral observada por alguns autores no CDT nessa faixa

etária. Além disso, essa diferença identificada na expressão de genes associados à captação e ao metabolismo do iodo pode justificar, em parte, as diferentes respostas ao tratamento observadas de acordo com a faixa etária, com maiores taxas de recorrência e metástase durante o seguimento observadas em crianças (10, 27, 51).

Estudos prévios sugerem haver diferenças no perfil mutacional do CDT pediátrico em relação ao grupo adulto. No entanto, assim como o observado nos demais estudos que investigaram o CDT pediátrico, informações acerca do perfil molecular do CDT em crianças ou pacientes pré-púberes são escassas na literatura, pelo número pouco significativo ou mesmo ausente de pacientes dessa faixa etária incluídos nos estudos (38, 42). Além disso, uma parcela significativa dos estudos moleculares realizados em pacientes com CDT pediátrico avaliou pacientes com histórico de irradiação (39-41). Portanto, o conhecimento dos mecanismos moleculares envolvidos na patogênese do CDT pediátrico esporádico, especialmente em crianças, ainda é escasso.

O **artigo 1** sumariza os principais eventos genéticos já descritos no CPT pediátrico e descreve as diferenças de prevalência identificadas entre os grupos pediátrico e adulto e, no grupo pediátrico, entre os tumores esporádicos e induzidos por irradiação.

A diferença observada no perfil mutacional do CPT pediátrico entre os pacientes com e sem histórico de exposição à radiação corrobora os resultados de alguns estudos prévios que sugerem que os mecanismos moleculares envolvidos na patogênese do CDT nesses dois grupos sejam diferentes (52). A maior prevalência de genes de fusão no CPT pediátrico induzido por radiação reforça a hipótese de que a radiação ionizante promove quebras na molécula de DNA e a formação de

rearranjos cromossômicos, principal mecanismo envolvido na patogênese do CPT nesse grupo de pacientes. Por outro lado, a menor prevalência de genes de fusão nos casos de CPT pediátricos esporádicos e a maior proporção de pacientes em que nenhum evento genético foi identificado, sugere que possa haver outros mecanismos moleculares envolvidos na patogênese do CPT pediátrico esporádico.

O rearranjo genético *RET/PTC,* descrito em inúmeros estudos como o segundo principal evento genético do CPT adulto (53), representa a principal alteração genética identificada no CPT pediátrico, segundo análise dos estudos descritos no **artigo 1** (54). Nesse grupo de pacientes pediátricos, a prevalência de *RET/PTC* é maior nos CPT expostos a radiação em comparação aos casos de CPT esporádicos (58 % *vs* 41%, respectivamente). Com relação a prevalência das isoformas de *RET/PTC*, o *RET/PTC1* e o *RET/PTC3* são os mais comumente identificados, com taxas semelhantes de ocorrência de *RET/PTC1* em tumores pediátricos esporádicos (20%) e induzidos por radiação (18%), mas com maior prevalência da isoforma *RET/PTC3* nos casos com histórico de radiação (33%), em comparação aos casos considerados esporádicos (10%) (54).

A mutação BRAF V600E, principal evento genético descrito no CDT adulto (55), apresenta menor prevalência no grupo pediátrico (3% nos tumores com histórico de exposição à radiação e 13% nos casos esporádicos), sem descrição na literatura de identificação desse evento em crianças (54). A aparente ausência dessa mutação no CDT em crianças e a menor prevalência no CDT em adolescentes, em comparação ao grupo adulto, sugere que a prevalência da mutação BRAF V600E aumente com a idade e não desempenhe um papel importante no CDT pediátrico. Por fim, fusões envolvendo o gene *BRAF,* com consequente ativação da via MAPK, podem representar um evento alternativo nos CPT (54).

Mutações nos genes *RAS*, identificadas principalmente na variante folicular do CPT, no carcinoma folicular e nos carcinomas pouco diferenciados e anaplásicos da tireoide em pacientes adultos (56-58), apresentam uma menor prevalência no grupo pediátrico (54). Estudos realizados em CDT pediátrico esporádico descreveram taxas de prevalência de mutações no gene *NRAS* entre 0-7%, sem identificação de mutações desse gene em tumores pediátricos induzidos por radiação. Em todos os casos pediátricos, apenas mutações no códon 61 do gene *NRAS* foram identificadas (54).

O rearranjo *ETV6-NTRK3* foi recentemente descrito em 7% dos pacientes pediátricos com histórico de radiação e em 7% de paciente provenientes da mesma região, mas sem exposição à radiação ionizante (42). Contudo, o impacto no prognóstico dos pacientes nos quais esse rearranjo é identificado, permanece incerto.

Rearranjos do gene *BRAF* foram também descritos em pacientes expostos à radiação ionizante. O gene de fusão *AKAP9-BRAF* foi inicialmente descrito em 3 casos de tumores pediátricos induzidos por radiação (59). Recentemente, a fusão *AGK-BRAF* foi descrita em um caso de tumor pediátrico induzido por radiação (42).

O **artigo 3** descreve, pela primeira vez, a ocorrência do rearranjo *AGK-BRAF* em pacientes com CPT pediátrico esporádico. Além disso, dentre os 3 pacientes em que esse rearranjo foi identificado, todos apresentavam extensão tumoral extra-tireoidiana e 2 apresentavam metástase pulmonar ao diagnóstico. A identificação do rearranjo *AGK-BRAF* contribui para o melhor conhecimento da patogênese do CPT pediátrico esporádico. No entanto, estudos futuros são necessários para a melhor elucidação das características do CPT relacionado a ocorrência desse gene de

fusão e para confirmar ou não essa aparente relação entre a ocorrência desse rearranjo e tumores com maior grau de invasividade.

O **artigo 4** objetivou descrever a prevalência dos principais eventos genéticos já identificados no CPT e a correlação desses eventos com características clinico-patológicas numa casuística de CPT pediátrico predominantemente esporádico. O número de crianças ou pacientes pré-púberes investigadas nesse estudo é superior aos demais estudos realizados em casos de CDT pediátrico (38, 42).

Apesar de o percentual de pacientes com eventos genéticos identificados ter sido semelhante entre crianças e adolescentes, o espectro de mutações identificado foi diferente. Em adolescentes, uma maior prevalência da mutações pontuais e de tumores com mais de um evento genético identificado foi observada.

Com relação à ocorrência do rearranjo *RET/PTC*, nossos resultados corroboram os achados prévios de que esse gene de fusão é o principal evento genético envolvido na patogênese do CPT pediátrico. A prevalência identificada no nosso estudo (37%) é inferior a descrita em CPT pediátricos com histórico de exposição à radiação (39, 52, 60), mas semelhante a encontrada em outras casuísticas de CPT pediátrico esporádico (38, 60).

A mutação BRAF V600E foi encontrada em apenas 3 pacientes investigados, um deles com 16 anos e dois deles com idade de 18 anos ao diagnóstico. A baixa prevalência dessa alteração genética identificada nessa casuística de CPT pediátrico (9%) é semelhante as taxas encontradas em estudos prévios (37, 38, 41, 61). Esse resultado corrobora a hipótese de que a prevalência da mutação BRAF V600E no CPT pediátrico é inferior a verificada no CPT adulto e tende a aumentar com a idade (38).

A prevalência do rearranjo *ETV6-NTRK3* verificada no nosso estudo (9%) é semelhante ao descrito inicialmente no CDT pediátrico esporádico e induzido por radiação (42). Estudos futuros, idealmente multicêntricos, para que se obtenha uma maior casuística, são necessários para a melhor compreensão dos mecanismos envolvidos na patogênese do CDT relacionado a esse rearranjo.

A ausência da mutação NRAS Q61 nessa casuística de CDT pediátrico predominante esporádico é compatível com estudos prévios, que identificaram uma baixa prevalência dessa mutação nessa população (54).

Desde a publicação do **artigo 1**, em 2015, outros estudos que investigaram os aspectos moleculares do CDT pediátrico predominantemente esporádico, principalmente em pacientes adolescentes, foram publicados. A prevalência de mutações na região promotora do gene *TERT* (Telomerase reverse transcriptase), descritas recentemente em tumores de tireoide e relacionadas com maior agressividade tumoral (62), parecem não desempenhar um papel significativo na patogênese do CDT pediátrico, com taxas de prevalência variando entre 0-1,8% (63-65). Uma elevada prevalência de genes de fusão do NTRK (25%), em especial o rearranjo *ETV6-NTRK3*, foi identificada em CPT pediátricos esporádicos na região nordeste dos Estados Unidos (EUA) (66). Esses resultados requerem uma confirmação com estudos que incluam um número maior de pacientes, além da investigação de possíveis fatores ambientais. A prevalência de mutações nos genes *RAS* também foi investigada nesses estudos mais recentes, com taxas reportadas entre 0-5,3% e, assim como em estudos prévios, apenas a mutação NRAS Q61 foi identificada (65-67). A mutação BRAF V600E foi avaliada em todos esses estudos recentes, com taxas de prevalência que variaram entre 20-48% (63-67). Nos dois estudos que reportaram maiores taxas de prevalência (40-48%), a pesquisa da

mutação BRAF V600E foi realizada através de sequenciamento de nova geração e a casuística era composta predominantemente por pacientes pediátricos com idade superior a 10 anos (53/54 casos) (65, 66).

Diferentes técnicas moleculares (análise de expressão gênica e pesquisa de mutações) estão sendo utilizadas na prática para auxiliar a avaliação de risco de malignidade de nódulos tireoidianos. O objetivo desses métodos complementares é auxiliar no manejo de nódulos tireoidianos cuja análise citológica evidencia um risco intermediário de malignidade (classificação Bethesda classes III e IV). No entanto, em razão da falta de validação nos pacientes pediátricos, em especial crianças ou pré-púberes, os testes moleculares atualmente disponíveis no mercado são recomendados apenas para pacientes com idade superior a 18 anos.

Raramente, o CDT pediátrico metastático pode evoluir de forma progressiva e sintomática, sem opções de terapêutica cirúrgica e sem resposta a terapia com RAI (21). Para esses pacientes, atualmente, não há opções de tratamento efetivo (19). O uso da terapia alvo molecular, apesar dos importantes efeitos colaterais associados, representa uma opção terapêutica no manejo do CDT adulto metastático progressivo (68). O conhecimento sobre esse tipo de terapia em pacientes pediátricos ainda é escasso e restrito a poucos relatos de casos de uso do sorafenib nesse grupo de pacientes (69, 70). O melhor conhecimento acerca da patogênese do CDT pediátrico pode contribuir para o desenvolvimento de terapias alvo-dirigidas para essa faixa etária mais jovem.

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IRMANDADE DA SANTA CASA DE MISERICÓRDIA DE S PAULO
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Tel.: (11) 2176-7689 E-mail: eticamedbernadete@santacasasp.org.br

São Paulo, **26** de **outubro** de **2011**.

Projeto nº319/11
Informe este número para identificar seu projeto no CEP

Ilmo.(a).Sr. (a).
Dr. Adriano Namo Cury
Departamento de Pediatria

O Comitê de Ética e Pesquisa da ISCMSP, em **reunião ordinária**, dia **26/10/11** e no cumprimento de suas atribuições, após revisão do seu projeto de pesquisa: **"Carcinoma diferenciado da tireóide na infância: investigação da expressão do marcador CD68 e da mutação V600E do gene BRAF"** emitiu parecer enquadrando-o na seguinte categoria:

[X] **Aprovado (Inclusive TCLE);**

[ ] **Com pendências** há modificações ou informações relevantes a serem atendida em 60 dias, (enviar as alterações em duas cópias);

[ ] **Retirado**, (por não ser reapresentado no prazo determinado);

[ ] **Não aprovado:** e;

[ ] **Aprovado** (inclusive **Termo de Consentimento Livre e Esclarecido), e encaminhado para apreciação da Comissão Nacional de Ética em Pesquisa – MS - CONEP, a qual deverá emitir parecer no prazo de 60 dias.** Informamos, outrossim, que, segundo os termos da Resolução 196/96 do Ministério da Saúde a pesquisa só poderá ser iniciada após o recebimento do parecer de aprovação da CONEP.

Profª Drª Maria Helena Vianello Matthiensen
Vice-Presidente

Prof. Dr. Nelson Keiske Ono
Presidente do Comitê de Ética em Pesquisa – ISCMSP

Conforme a Resolução 196/96, o relatório parcial deve ser apresentado de forma detalhada ao CEP, inicialmente em 26/04/2012 e a cada seis meses. Conforme ofício circular 0226/CONEP/CNS, datado de 29-10-2010, a suspensão de estudo e relatório final deverão ser apresentados conforme modelo elaborado pela CONEP. Impressos disponíveis em nosso site: www.santacasasp.org.br

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Universidade Federal de São Paulo
Escola Paulista de Medicina

Comitê de Ética em Pesquisa
Hospital São Paulo

São Paulo, 3 de outubro de 2012

CEP Nº **0746/11**
CONEP Nº:

Ilmo(a) Sr(a)

Pesquisador(a): PALOMA DA SILVA BESSON

Disciplina/Departamento: Genética

Título do estudo: Carcinoma da tiroide na infância: investigação da expressão dos genes C1ORF24, ITM1 E PVALB e de alterações nos genes BRAF E RET

Prezado(a) Pesquisador(a),

O Comitê de Ética em Pesquisa da Universidade Federal de São Paulo/Hospital São Paulo ANALISOU E APROVOU o(a) Solicitação de alteração do pesquisador principal, de: PALOMA DA SILVA BESSON, para: MARIA ISABEL CUNHA VIEIRA CORDIOLI; inclusão de nova metodologia; e alteração do título, de "Carcinoma da tiroide na infância: investigação da expressão dos genes C1ORF24, ITM1 E PVALB e de alterações nos genes BRAF E RET", para: " investigação da expressão do marcador CD163 e dos genes C1ORF24, ITM1 e PVALB, NIS, TG, TSH-R e TPO e da mutação V600E do gene BRAF no carcinoma da tiroide na infância" do projeto de pesquisa acima referenciado.

Atenciosamente,

Prof. Dr. José Osmar Medina Pestana
Coordenador do Comitê de Ética em Pesquisa da
Universidade Federal de São Paulo/Hospital São Paulo

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