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Mírian Romitti

Metabolismo dos Hormônios Tireoidianos no Carcinoma Papilar de Tireoide: Implicações na Tumorigênese e Crescimento Neoplásico

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Orientadora: Prof[a]. Dr[a]. Ana Luiza Maia

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- **Artigo de revisão:** Signaling Pathways in Follicular Cell-Derived Thyroid Carcinomas (review); publicado no International Journal of Oncology. 2013 Jan; 42(1):19-28.

- **Artigo original:** Type 3 deiodinase upregulation in papillary thyroid carcinoma is mediated by crosstalk between MAPK and Sonic Hedgehog pathways and is associated with cell proliferation

Além dos artigos já citados, ao longo do período do doutorado foram desenvolvidos os seguintes manuscritos relacionados ao tema oncogênese tireoidiana:

- Role of VEGF-A and Its Receptors in Sporadic and MEN2-Associated Pheochromocytoma. Ferreira C, Siqueira DR, **Romitti M**, Ceolin L, Brasil B, Meurer L, Capp C, Maia AL. International Journal of Molecular Sciences, v. 15, p. 5323-5336, 2014.

- Role of RET genetic variants in MEN 2-associated pheochromocytoma. Siqueira DR, Ceolin L, Ferreira CV, **Romitti M,** Maia SC, Zanini Maciel LM, Maia AL. European Journal of Endocrinology, v. 1, p. 1-10, 2014.

- Molecular Basis of Medullary Thyroid Carcinoma: The Role of RET Polymorphisms. Ceolin L, Siqueira DR, **Romitti M,** Ferreira CV, Maia AL. International Journal of Molecular Sciences, v. 13, p. 221-239, 2012.

- Additive effect of RET polymorphisms on sporadic medullary thyroid carcinoma susceptibility and tumor aggressiveness. Ceolin L, Siqueira DR, Ferreira CV, **Romitti M,** Maia SC, Leiria L, Crispim D, Ashton-Prolla P, Maia AL. European Journal of Endocrinology 2012; 166 (5):847-54.

- Is there a role for inherited TRβ mutation in human carcinogenesis?. Weinert LS,; Ceolin L, **Romitti M,** Camargo EG, Maia AL. Arquivos Brasileiros de Endocrinologia e Metabologia, v. 56, p. 67-70, 2012.

- The rare intracellular RET mutation S891A in an apparently sporadic medullary thyroid carcinoma: case report and review of the literature. Blom CB, Ceolin L, **Romitti M,** Siqueira DR, Maia AL. Arquivos Brasileiros de Endocrinologia e Metabologia, v. 56, p. 586-591, 2012.

LISTA DE ABREVIATURAS E SIGLAS

AKAP9 - A-kinase anchor protein 9

AKT - v-akt murine thymoma viral oncogene homolog

ARA70 - Synonymus with nuclear receptor coactivator 4

ATC - Anaplastic Thyroid Carcinoma

AU - Arbitrary unit

BCC - Basal cell carcinoma

BRAF - Serine/threonine-protein kinase B-Raf

cAMP - Adenosina monofosfato cíclico

CAT - Carcinoma Anaplásico de Tireoide

CDK - Cyclin-dependent kinase

CFT – Carcinoma Folicular de Tireoide

c-Kit - v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog

c-Myc - v-myc myelocytomatosis viral oncogene homolog

CMT – Carcinoma Medular de Tireoide

CPT – Carcinoma Papilar de Tireoide

CoA – Coactivator

CREB - cAMP response element-binding

CTBP2 - C-terminal binding protein

CTNNB1 - Catenin (cadherin-associated protein), beta 1

D1; *DIO1* - Deiodinase type 1; Desiodase tipo 1

D10S170 – Synonymus with H4 gene

D2; *DIO2* - Deiodinase type 2; Desiodase tipo 2

D3; *DIO3* - Deiodinase type 3; Desiodase tipo 3

DLK1 - Delta-like 1 homolog (Drosophila)

DMEM - Dulbecco's Modified Eagle Medium

DNA - Acido desoxiribonuicleico

dNTP - Deoxynucleotide triphosphates

DTC; CDT - Differentiated Thyroid Carcinoma; Carcinoma Diferenciado de Tireoide

E2F - E2F transcription factor

EGFR - Epidermal growth factor receptor

ELE1 - Synonymus with nuclear receptor coactivator 4

ERK - Extracellular-signal-regulated kinase

FBS - Fetal bovine serum

FOXO3 - Forkhead box O3

FTC - Follicular Thyroid Carcinoma

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase

GLI1 - GLI family zinc finger 1

GSK3-S - Glycogen synthase kinase 3 phosphorylation

HCl - Hydrochloric acid

HRAS - Harvey rat sarcoma viral oncogene homolog

INCA - Instituto Nacional de Câncer

K1 - Linhagem cellular humana de carcinoma papilar de tireoide $BRAF^{V600E}$ positiva

KCl - Potassium chloride

KRAS - Kirsten rat sarcoma viral oncogene homolog

LEF - Lymphoid enhancer factor

MAPK - Mitogen-activated protein kinase

MgCl2 - Magnesium chloride

miRNA; miR - MicroRNA

MMP - Matrix metalloproteinases

mRNA; RNAm - Messenger ribonucleic acid; RNA mensageiro

MST1 - Macrophage stimulating 1

MTC – Medullary Thyroid Carcinoma

NaOH - Sodium hydroxide

NCOA4 - Nuclear receptor coactivator 4

NF-κB - Nuclear factor-κB

NGF - Nerve growth factor

NRAS - Neuroblastoma RAS viral (v-ras) oncogene homolog

nTRE – Negative thyroid hormone response elements

NTRK1 - Neurotrophic tyrosine kinase receptor, type 1

P21 - Cyclin-dependent kinase inhibitor 1A

P27 - Cyclin-dependent kinase inhibitor 1B

P38 - Mitogen-activated protein kinase 14 (MAPK14)

PAX-8 - Paired box gene 8

PCCL3 - Linhagem celular de célula folicular tireoidiana de rato

PCR - Polymerase chain reaction; Reação em cadeia da polimerase

PDGFR - Platelet-derived growth factor receptors

PDK1 - Pyruvate dehydrogenase kinase isozyme 1

PI3K - Phosphatidylinositol 3-kinase

PIK3CA - Catalytic subunit p110α of PI3K

PIP3 - Phosphatidylinositol 3,4,5 phosphate

PPARγ - Peroxisome proliferator-activated receptor γ

PTC - Papillary Thyroid Carcinoma

PTEN - Phosphatase and tensin homolog

RAF - v-raf-1 murine leukemia viral oncogene homolog

RB - Retinoblastoma

RET - RE arrangement during transfection

RET/PTC - RET tyrosine kinase domain rearrangement with different partners

RFG - Synonymus with nuclear receptor coactivator 4

RXR - Receptor retinóide X

SB203580 - p38 inhibitor

SHH - Sonic Hedgehog

siRNA - Small interfering RNA

STAT - Signal transducer and activator of transcription proteins

T3 - Triiodothyronine; Triiodotironina

T4 - Thyroxine; Tiroxina

TCA - Trichloroacetic acid

TCF - T-cell factor

TGFβ - Transforming growth factor β

TH; HT - Thyroid hormone; Hormônio tireoidiano

TP53; p53 - Tumor protein p53

TPC-1 - Linhagem cellular humana de carcinoma papilar de tireoide RET/PTC1 positiva

TRE - Thyroid hormone response elements

TRα - Thyroid receptor α

TRβ - Thyroid receptor β

U0126 - MEK inhibitor

US - United States

UTR - Untranslated region

VEGFR - Vascular endothelial growth factor receptors

Wnt - Wingless in Drosophila

WRO - Linhagem cellular humana de carcinoma folicular de tireoide

αvβ3 – Integrin receptor

SUMÁRIO

RESUMO

O câncer de tireoide constitui o tipo de câncer endócrino mais comum, representando aproximadamente 1-1,5% de todas as doenças malignas humanas. O carcinoma papilar de tireoide (CPT) compreende o subtipo mais comum (~80% dos casos) e é caracterizado por um curso indolente e do prognóstico favorável. No entanto, cerca de 20-30% dos pacientes podem apresentar um curso clínico mais agressivo, com elevadas taxas de recidiva. Mutações pontuais nos genes *BRAF* e *RAS*, bem como rearranjos RET/PTC e NTRK1 são identificados em mais de 70% dos casos e levam a ativação aberrante da via MAPK. Estudos sugerem que a presença a mutação $BRAF^{V600E}$ estaria associada com o comportamento tumoral mais agressivo, no entanto seu papel como marcador prognóstico ainda não está bem definido.

Os hormônios tireoidianos (HT) influenciam uma grande variedade de eventos biológicos. A ativação do hormônio tiroxina (T4) no hormônio biologicamente ativo triiodotironina (T3), é catalisada pelas iodotironinas desiodases tipo 1 (D1, *DIO1*) e tipo 2 (D2, *DIO2*). Em contraste, a iodotironina desiodase tipo 3 (D3, *DIO3*) é responsável pela inativação dos hormônios T4 e T3. A ação orquestrada das desiodases é essencial na manutenção de níveis adequados dos HT. Estudos sugerem que alterações nos níveis dos HT estariam implicadas na transformação neoplásica, proliferação e sobrevida celular. Alterações na expressão das desiodases são frequentemente observadas em tumores humanos, sugerindo um possível papel como marcadores ou mesmo como moduladores da proliferação de células tumorais. Diminuição dos níveis da D2 e aumento da D3 foram demonstrados em diversas neoplasias, sugerindo que o hipotireoidismo local causado pela diminuição da ativação do HT e/ou aumento da inativação hormonal, poderia favorecer o crescimento tumoral. Recentemente demonstramos aumento da expressão da D3 no CPT e correlação positiva entre os níveis de expressão da enzima com o tamanho do tumor e doença avançada ao diagnóstico. A presença da mutação $BRAF^{V600E}$ foi associada aos níveis mais elevados da atividade enzimática. De modo interessante, a D3 não foi detectada em tumores medulares ou anaplásicos, sugerindo que mecanismos moleculares celular-específico possam influenciar na desregulação da expressão desta enzima.

No presente estudo observamos que aterações genéticas na via de sinalização MAPK, como a mutação $BRAF^{V600E}$ e o rearranjo RET/PTC, modulam a expressão da D3 no CPT. Além disso, a ativação da via Sonic Hedgehog também parece regular os níveis da D3 possivelmente através da cooperação com a via MAPK. De forma interessante, observamos

que o silenciamento da expressão da D3 foi capaz de reduzir significativamente a proliferação celular das células malignas tireoidianas. Estes dados em conjunto sugerem que a D3 pode exercer um papel importante na proliferação celular, possivelmente devido ao hipotireoidismo intracelular gerado, o que poderia contribuir para o crescimento e agressividade tumoral.

ABSTRACT

The thyroid cancer is the most common type of endocrine cancer, representing approximately 1-1.5% of all human malignancies. Papillary thyroid carcinoma (PTC) comprising the most common subtype (~ 80% of cases) and is characterized by an indolent course and favorable prognosis. However, about 20-30% of patients may have a more aggressive clinical course, with high recurrence rates. Point mutations in the *BRAF* or *RAS* genes or rearrangements RET/PTC or NTRK1 are identified in over 70% of cases and lead to aberrant activation of the MAPK pathway. Studies suggest that the presence of the $BRAF^{V600E}$ mutation would be associated with more aggressive tumor behavior; however its role as a prognostic marker is not well defined.

Thyroid hormones (TH) influence a variety of biological events. The activation of the hormone thyroxine (T4) to the biologically active hormone triiodothyronine (T3), is catalyzed by the iodothyronine deiodinases type 1 (D1, *DIO1*) and type 2 (D2, *DIO2*). In contrast, the iodothyronine deiodinase type 3 (D3, *DIO3*) is responsible for the inactivation of hormones T4 and T3. The orchestrated action of deiodinases is essential in maintaining adequate levels of circulating TH. Studies suggest that changes in the HT levels might be involved in neoplastic transformation, cell proliferation and survival. Expression changes in deiodinases are frequently observed in human tumors, suggesting a possible role as a marker or as modulator of tumor cell proliferation. Reduction in D2 and increase of D3 levels have been demonstrated in several tumors, suggesting that the local hypothyroidism caused by reduction of activation and/or increase in TH inactivation could contribute to tumor growth. Recently, we have demonstrated increased expression of *DIO3* in the PTC and a positive correlation between the enzyme levels and tumor size and advanced disease at diagnosis. Moreover, the presence of $BRAF^{V600E}$ mutation was associated with higher levels of enzyme activity. Interestingly, D3 was not detected in anaplastic and medullary thyroid tumors, suggesting that cell-specific molecular mechanisms may influence the expression of this enzyme.

In the present study, we have demonstrated that *DIO3* expression is modulated by specific MAPK genetic alterations, as $BRAF^{V600E}$ mutation and RET/PTC rearrangement, in PTC. Moreover, SHH activation might be also involved in *DIO3* upregulation in PTC, probably by cooperation with MAPK pathway. Finally, the reduction in cell proliferation after *DIO3* silencing support the hypothesis that the intracellular decreases in thyroid hormone

levels might be associated with induction of tumor growth and interfere in tumor aggressiveness.

INTRODUÇÃO

Câncer de tireoide

O câncer de tireoide constitui o tipo de câncer endócrino mais comum, representando aproximadamente 1-1,5% de todas as doenças malignas, com uma taxa de incidência anual de 12,9 casos para cada 100.000 habitantes (CURADO *et al.*, 2007). Estudos epidemiológicos indicam que a incidência de câncer de tireoide vem aumentando nos últimos anos, sendo considerado o de maior índice de crescimento nos Estados Unidos (6,6%) (PELLEGRITI *et al.*, 2013). No Brasil, a estimativa para o câncer da tireoide em mulheres para o ano de 2014 é de 9.200 casos novos, sendo o 4º tumor maligno mais freqüente em mulheres e o 13º em homens (INCA; http://www.inca.gov.br/estimativa/2014/estimativa-24042014.pdf).

O carcinoma diferenciado de tireoide (CDT), originário da célula folicular tireoidiana, representa mais de 90% dos tumores da glândula e compreende o carcinoma papilar de tireoide (CPT, ~80% dos casos) e o carcinoma folicular de tireoide (CFT; ~10% dos casos). O carcinoma anaplásico ou indiferenciado de tireoide representa cerca de 1% dos tumores tireoideanos, podendo se originar de novo ou ser resultado da progressão e/ou desdiferenciação dos CDT. O carcinoma medular de tireoide (CMT) é originado das células C ou parafoliculares tireoidianas e representam de 3-4% dos tumores malignos tireoidianos. O CPT é caracterizado por um curso indolente e por um prognóstico favorável. No entanto, cerca de 20-30% dos pacientes apresentam um curso clínico mais agressivo, com elevadas taxas de recidiva/persistência da doença (DELELLIS *et al.*, 2004). Atualmente, um grande desafio na prática clínica é identificar o risco específico de cada paciente a fim de permitir uma abordagem terapêutica mais individualizada e minimizar a morbidade relacionada ao tratamento. Ativação aberrante da via de sinalização mitogen-activated protein kinase (MAPK) devido a mutações ou rearranjos de genes é o evento genético mais comum no CPT.

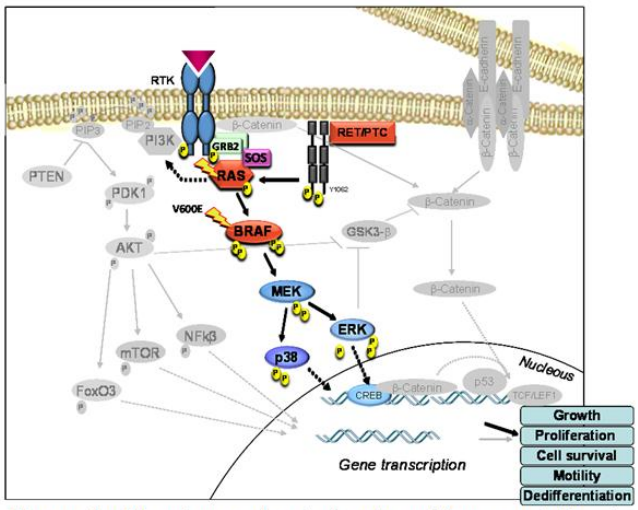

Figura 1: Mecanismos de sinalização celular associados à patogênese do CPT. Mutações nos genes BRAF ou RAS bem como rearranjos RET/PTC levam a uma ativação constitutiva da via MAPK. (Obtido de Romitti, *et al*, 2013)

Mutações pontuais no gene *BRAF* ou *RAS*, e rearranjos RET/PTC ou NTRK1 são mutualmente exclusivos e identificados em cerca de 70% dos casos (ROMITTI *et al.*, 2013). A figura 1 resume os mecanismos de sinalização envolvidos na patogênese do CPT. Estudos indicam que a presença de mutações no gene *BRAF* (~ 40% dos casos) estaria associada com um comportamento tumoral mais agressivo podendo ser considerado um marcador prognóstico, no entanto este papel ainda não está bem determinado (XING, 2005; LEE *et al.*, 2011; ZOGHLAMI *et al.*, 2014).

Metabolismo dos hormônios tireoidianos

Os hormônios tireoidianos (HT) são reguladores de uma grande variedade de eventos biológicos, dentre os quais desenvolvimento embrionário, crescimento, diferenciação e metabolismo em praticamente todos os tecidos. Embora a glândula tireóide secrete em sua maior parte tiroxina (T4), as ações dos hormônios tireoidianos são mediadas pelo hormônio biologicamente ativo, triiodotironina (T3) (YEN, 2001).

Os efeitos celulares dos HT são classificados como genômicos (nuclear) ou não-genômicos (citoplasma ou membrana através de receptores do tipo integrinas) (Figura 2). O mecanismo genômico é promovido principalmente pela ação T3 e requer o envolvimento dos receptores nuclear dos hormônios tireoidianos. Os genes *THRα* e *THRβ* codificam as isoformas dos receptores TRα1 e TRβ1-β3 (KIM *et al.*, 2012). A ligação do T3 aos receptores nucleares leva a ativação da transcrição, geralmente através da ligação com o receptor retinóide X (RXR), elementos de resposta aos hormônios tireoidianos (TREs) localizados nas regiões reguladoras dos genes alvo. A transcrição gênica é então regulada pelo balaço entre corepressores (CR) e coactivatores (CoA). Os elementos de resposta aos hormônios tireoidianos negativos (nTRE) podem mediar a repressão transcricional, no entanto neste caso o papel de coativadores e co-

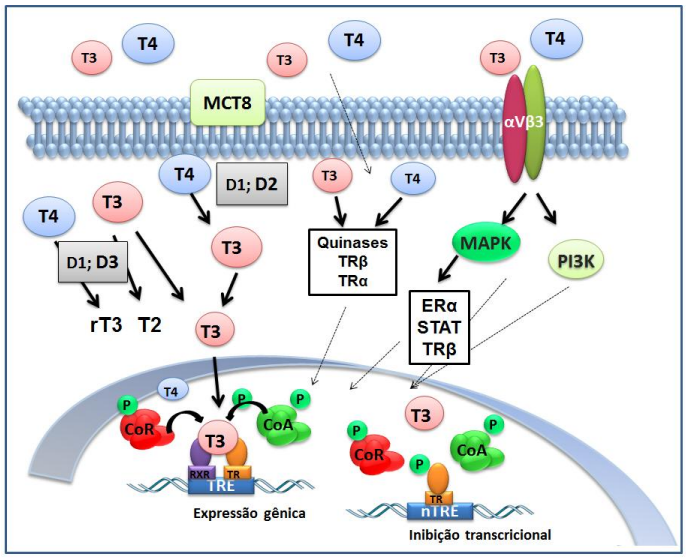

Figura 2: Mecanismos genômicos e não-genômicos de ação dos hormônios tireoidianos.

repressores não é bem definida (YEN, 2001). A natureza da resposta transcricional é determinada pelo tipo de célula, promotor e estado hormonal (HULBERT, 2000; ARANDA & PASCUAL, 2001). Em relação aos efeitos não-genômicos, estes são iniciados pela ligação do HT aos receptores do tipo integrina αvβ3, o que leva à ativação de diferentes vias de sinalização intracelulares, incluindo a MAPK, phosphatidylinositol 3-kinase (PI3K) e signal transducer and activator of transcription proteins (STAT), resultando em eventos celulares distintos, como a proliferação celular e angiogênese (DAVIS *et al.*, 2006; DAVIS *et al.*, 2008; CHENG *et al.*, 2010) (Figura 2).

A principal via de regulação dos níveis dos HT ocorre via ação das iodotironinas desiodases através da ativação e inativação hormonal. As desiodases tipos 1, 2 e 3 (D1, D2 e D3) constituem uma família de oxiredutases que contêm o raro aminoácido selenocisteína em seu sítio ativo, um resíduo essencial para uma atividade catalítica eficiente (CALLEBAUT *et al.*, 2003). A via da desiodação é um passo crítico na ativação e inativação do hormônio da tireoide, permitindo rápidas modificações no status tireoidiano intracelular de uma forma tecido-específica, sem afetar as concentrações circulantes dos mesmos. Assim, é possível controlar a concentração e a atividade intracelular de T3 independentemente dos níveis de T3 sérico.

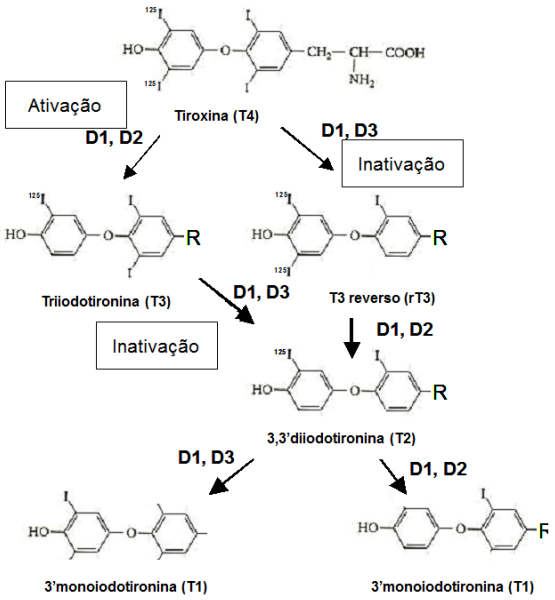

Figura 3: Metabolismo dos hormônios tireoidianos.

A principal via de produção da forma bioativa nos tecidos periféricos ocorre via desiodação do anel externo do T4, catalisada pelas iodotironinas desiodases tipo 1 (D1, *DIO1*) e tipo 2 (D2, *DIO2*). Em contraste, a iodotironina desiodase tipo 3 (D3, *DIO3*) é responsável por catalisar a inativação do T4 e T3 através da desiodação de anel interno dessas moléculas (Figura 1). Em humanos, os níveis mais altos de atividade da D1 são encontrados na tireoide, fígado e rim. A D2 é mais expressa na hipófise, cérebro, tireoide, pele, músculos esquelético e cardíaco (MAIA *et al.*, 2005; MEYER *et al.*, 2007). A D3 é altamente expressa no feto, placenta, útero, cérebro e pele. A expressão da D3 possui um papel essencial no desenvolvimento fetal, pois previne a exposição do embrião ao excesso de T3 o que está

associado com malformações, alterações no crescimento, retardo mental ou até mesmo morte (GALTON, 2005; MEYER, WAGNER e MAIA, 2007).

Expressão das iodotironinas desiodases em neoplasias

O hormônio tireoidiano, dentre outras ações estimula a diferenciação e proliferação celular. Diversos estudos indicam que as alterações nos níveis dos hormônios tireoidianos poderiam contribuir para a transformação neoplásica bem como na progressão tumoral (CHENG, 2005; KRESS *et al.*, 2009). A primeira associação entre o HT e câncer foi relatada em 1896, quando Beatson utilizou extrato tireoidiano como um potencial tratamento para câncer de mama (BEATSON, 1986). Nas últimas décadas, estudos sugerem que o hipotireoidismo pode ser um possível fator de risco para diversas neoplasias, como o câncer de fígado (REDDI *et al.*, 2007; HASSAN *et al.*, 2009) e neoplasias da tireóide (BOELAERT *et al.*, 2006; POLYZOS *et al.*, 2008; FIORE *et al.*, 2010). Em contraste, os baixos níveis dos HT parecem ser clinicamente favoráveis em glioblastomas de alto grau (HERCBERGS *et al.*, 2003). No entanto, no câncer de mama a conexão entre hipotireoidismo e patogênese tumoral ainda é uma questão controversa (CRISTOFANILLI *et al.*, 2005; ANGELOUSI *et al.*, 2012; HARDEFELDT *et al.*, 2012).

Recentemente, estudos *in vitro* e *in vivo* demonstram que mudanças nos níveis dos HT devido a desregulação na expressão das desiodases podem estar envolvidas na proliferação, diferenciação, sobrevivência e invasão celular em uma grande variedade tumores (LIN *et al.*, 2008; PERRA *et al.*, 2009; PINTO *et al.*, 2011). Alterações na expressão das desiodases já foram demonstradas em tumores benignos e malignos. Embora o papel do desiodases em neoplasias não seja totalmente compreendido, estudos avaliando o perfil de expressão da *DIO1* e *DIO2* relataram níveis diminuídos ou inalterados do RNA mensageiro (RNAm) na maioria das neoplasias tireoidianas, com exceção da atividade aumentada da D2 nos CFT e CMT (KIM *et al.*, 2003; ARNALDI *et al.*, 2005; MEYER *et al.*, 2008). Redução da expressão da D1 também foi descrita em amostras de adenocarcinoma renal, e estudos celulares indicam que essa alteração seria mediada através da indução dos microRNAs, miR-224 e miR-383, através de ligação direta na região 3'UTR do gene *DIO1*. De modo interessante, observou-se uma correlação inversa entre as alterações específicas na expressão de miR-224 no tumor com a expressão da D1 e com a concentração intracelular T3 (BOGUSLAWSKA *et al.*, 2011). de maneira semelhante, estudos em amostras de hepatocarcinoma identificaram um conjunto de

miRNAs envolvidos na regulação da região genômica DLK1-DIO3. Os autores mostram que a superexpressão do complexo DLK1-DIO3/miRNA foi associada a uma maior taxa de metástases e menor sobrevida global em pacientes com carcinoma hepatocelular (LUK *et al.*, 2011).

Alterações no equilíbrio entre ativação (D2) e inativação (D3) do HT parece ser fundamental na modulação do balanço entre proliferação e diferenciação celular (DENTICE *et al.*, 2007; DENTICE *et al.*, 2012). Níveis aumentados da D3, associados com redução da expressão D2, foram observados em amostras de carcinoma basocelular, bem como em modelos animais. Além disso, o crescimento de células tumorais implantadas em animais foi reduzido drasticamente após a inibição da D3, o que sugere que o hipotireoidismo intratecidual, resultante do aumento na inativação hormonal, pode ter um importante papel no processo de crescimento tumoral (DENTICE *et al.*, 2007). Do mesmo modo, a expressão oposta entre D3 e D2 também ocorre em células tumorais do cólon, e parece ser regulado via sinalização Wnt/β-catenina. Estudos experimentais com inibição desta via demonstraram redução nos níveis da D3 e indução da D2 e evidenciaram que a presença do T3 ocasionou uma redução significativa na proliferação, enquanto estimulou a diferenciação celular (DENTICE *et al.*, 2012). Recentemente realizamos estudos avaliando o papel da D3 nas neoplasias tireoidianas. Observamos aumento significativo da expressão da *DIO3* em amostras de CPT e, mais interessante, que o aumento da atividade foi associado com o tamanho tumoral e com doença avançada ao diagnóstico. Além disso, a mutação $BRAF^{V600E}$ esteve diretamente associada com os maiores níveis de RNAm e atividade da enzima. Curiosamente, não encontramos expressão da D3 em tumores medulares ou anaplásicos (ROMITTI *et al.*, 2012).

Com base nestes conhecimentos, o objetivo deste estudo foi avaliar o papel da D3 no processo neoplásico, particularmente os mecanismos de sinalização envolvidos na indução da D3 observada no CPT.

Parte I

Signaling Pathways in Follicular Cell-Derived Thyroid Carcinomas (review)

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Signaling pathways in follicular cell-derived thyroid carcinomas (Review)

MÍRIAN ROMITTI, LUCIELI CEOLIN, DÉBORA RODRIGUES SIQUEIRA, CARLA VAZ FERREIRA, SIMONE MAGAGNIN WAJNER and ANA LUIZA MAIA

Thyroid Section, Endocrine Division, Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

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Abstract. Thyroid carcinoma is the most common malignant endocrine neoplasia. Differentiated thyroid carcinomas (DTCs) represent more than 90% of all thyroid carcinomas and comprise the papillary and follicular thyroid carcinoma subtypes. Anaplastic thyroid carcinomas correspond to less than 1% of all thyroid tumors and can arise *de novo* or by dedifferentiation of a differentiated tumor. The etiology of DTCs is not fully understood. Several genetic events have been implicated in thyroid tumorigenesis. Point mutations in the *BRAF* or *RAS* genes or rearranged in transformation (RET)/papillary thyroid carcinoma (PTC) gene rearrangements are observed in approximately 70% of papillary cancer cases. Follicular carcinomas commonly harbor *RAS* mutations and paired box gene 8 (PAX8)-peroxisome proliferator-activated receptor γ (PPARγ) rearrangements. Anaplastic carcinomas may have a wide set of genetic alterations, that include gene effectors in the mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and/or β-catenin signaling pathways. These distinct genetic alterations constitutively activate the MAPK, PI3K and β-catenin signaling pathways, which have been implicated in thyroid cancer development and progression. In this context, the evaluation of specific genes, as well as the knowledge of their effects on thyroid carcinogenesis may provide important information on disease presentation, prognosis and therapy, through the development of specific tyrosine kinase targets. In this review, we aimed to present an updated and comprehensive review of the recent advances in the understanding of the genetic basis of follicular cell-derived thyroid carcinomas, as well as the molecular mechanisms involved in tumor development and progression.

Correspondence to: Professor Ana Luiza Maia, Thyroid Section, Endocrine Division, Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos 2350, 90035-003 Porto Alegre, Brazil
E-mail: almaia@ufrgs.br

Key words: follicular-derived thyroid carcinoma, genetic alterations, signaling pathways

Contents

1. Introduction

Thyroid carcinoma is the most common type of malignant endocrine neoplasia, accounting for approximately 1% of all new malignant diseases with an annual incidence of 5.9 and 17.3 per 100,000 in men and women, respectively (US 2005-2009) (1,2). Follicular cell-derived thyroid neoplasias include differentiated thyroid carcinoma (DTC), which represents more than 90% of all thyroid malignancies and comprise the papillary and follicular thyroid carcinomas (FTCs). The anaplastic thyroid carcinoma (ATC) corresponds to 1% of all thyroid tumors and can arise *de novo* or by the dedifferentiation of a papillary or follicular tumor (3). Medullary thyroid carcinoma (MTC) is a malignancy arising from the parafollicular C-cells and accounts for approximately 3-8% of all thyroid carcinomas (4).

The etiology of DTC is not yet fully understood. External radiation is the only exogenous factor which has been clearly identified as causing thyroid carcinoma, almost exclusively the papillary form. Iodine excess has been associated with the increase in the incidence of papillary thyroid carcinoma (PTC) (5,6). A number of genetic events have been described in thyroid carcinoma pathogenesis. Papillary carcinomas commonly present genetic alterations that lead to the activation of the mitogen-activated protein kinase (MAPK) pathway (7-9). In follicular carcinomas, the induction of both the MAPK and phosphatidylinositol 3-kinase (PI3K) cascades is frequently observed (10). On the contrary, anaplastic carcinomas harbor a wide set of additive genetic alterations, occurring mainly in the gene effectors of the MAPK, PI3K and β-catenin signaling pathways (11-13). These distinct signaling pathways have been implicated in follicular cell-derived thyroid cancer development and progression (14-16).

In this review, we aimed to present a comprehensive account of the recent advances in the understanding of the signaling pathways in follicular cell-derived thyroid carcinomas, as well as the molecular mechanisms involved in tumor development and progression.

2. Papillary thyroid carcinoma

PTC represents ~80% of all malignant thyroid tumors. The overall incidence of PTC is 7.7 per 100,000 and is increasing, in part due to the increase in the detection of small tumors (16). PTC is often diagnosed at approximately the 5th decade of life and is known to be a slow-growing tumor (17,18). Patients usually present with a palpable nodule and the absence of any other clinical findings is common (3). The majority of patients have a favorable outcome; however, ~10% of the cases have tumor recurrence and metastatic disease (18,19).

Aberrant activation of the MAPK pathway due to mutations or gene rearrangements is the most common genetic event in PTC (7-9). Point mutations in *BRAF* or *RAS* genes and (RET)/PTC or NTRK1 rearrangements are mutually exclusive and identified in more than 70% of PTCs (7-9). The Fig. 1A summarizes the major signaling pathways involved in PTC.

BRAF oncogene. Mutations in the *BRAF* gene are the most common genetic alteration in PTC, occurring in ~45% of cases (6). BRAF is a serine-threonine kinase protein, member of the RAF (v-raf-1 murine leukemia viral oncogene homolog) family, which comprises the serine/threonine-specific kinase effectors of the MAPK cascade (7,20,21). Briefly, the MAPK cascade effects initiate upon RAS activation, which recruits BRAF to the plasma membrane initiating its activation. Once activated, BRAF phosphorylates MEK, which in turn provides the signal to activate the tyrosine, ERK, in the cytosol and nucleus, leading to cell proliferation, migration and survival (22,23) (Fig. 1A). Approximately 95% of all *BRAF* mutations involve a T>A transversion at gene position 1799, resulting in valine to glutamate amino acid substitution at position 600 of the protein (V600E). Other described alterations in the BRAF gene include the A>G transversion at gene position 1801 (K601E), fusion with the A-kinase anchor protein 9 (*AKAP9*) gene and small in-frame insertions or deletions around codon 600 (24-26).

The presence of BRAF mutations in micro-PTC (~40%) and benign tumors (9,27,28) suggests a role of this alteration in the early stages of PTC development. $BRAF^{V600E}$ is an oncogenic protein with markedly elevated kinase activity that overactivates the MAPK pathway (34,35). Studies using $BRAF^{V600E}$-transgenic mice have shown the development of PTC with similar properties to those observed in human *BRAF*-positive PTCs (29), whereas mice with the constitutive or doxycycline-inducible *BRAF*-mutated gene develop infiltrative PTC with a high rate of extrathyroidal structures, vascular invasion and a poorly differentiated aspect (30,31). The induction of $BRAF^{V600E}$ mutation has been shown to abolish the expression of several thyroid-specific genes, radioiodine uptake and cause pronounced hypothyroidism, which may be partially explained by the down-regulation of the thyroid hormone activating type 1 and 2 deiodinases and induction of the thyroid hormone inactivating type 3 deiodinase, as recently described (31,33).

BRAF mutations are typically identified in classical and tall cell variant of PTC and are associated with a more aggressive tumor behavior (9,34,35). The high growth rates observed in $BRAF^{V600E}$ tumors may be explained partially by the MAPK-induced hyperphosphorylation with consequent inhibition of the retinoblastoma (RB) protein, dependent transcription factors (E2F) and p27 of cyclin-dependent kinase (CDK) activity (36). Moreover, the *BRAF* oncogene induces the expression of matrix metalloproteinases (MMPs), a large group of enzymes that regulate cell-matrix composition and are important factors of tumor invasiveness (37-39). Previous studies have suggested that MMP proteins are modulated according to the intensity of MAPK pathway activation and/or signal transducer and activator of transcription (STAT) expression, which may explain the mechanism of induction of these proteins in BRAF-mutated PTCs and the increased propensity of these tumors to invade surrounding tissues (37,40). The BRAF-mutated protein also induces nuclear factor-κB (NF-κB). Thyroid cells (WRO) harboring this oncogene display increased levels of activity in the NF-κB pathway, which results in the upregulation of anti-apoptotic factors and the induction of cell invasion (40).

Recently, a novel inhibitory mechanism that may operate in $BRAF^{V600E}$-induced PTC was shown. The presence of $BRAF^{V600E}$ mutation abolished the macrophage stimulating 1/forkhead box O3 (MST1/FOXO3) pathway transactivation in a thyroid cell line (FRO), resulting in the suppression of p21 and p27 CDK inhibitors and interrupting the apoptotic process. Accordingly, the development of $BRAF^{V600E}$ transgenic mice with the MST1 knockout leads to abundant foci of poorly differentiated thyroid carcinoma and large areas without follicular architecture or colloid formation, suggesting that the activity of the MST1/FOXO3 pathway determines the phenotype of $BRAF^{V600E}$ tumors (41).

RET/PTC rearrangements. The *RET* proto-oncogene, located on chromosome 10q11.2, encodes a tyrosine kinase receptor. The RET protein is usually expressed in cells derived from the neural crest and gain-of-function mutations are associated with MTC (42). In PTC, genomic rearrangements juxtapose the RET tyrosine kinase domain to unrelated genes, thereby creating dominantly transforming oncogenes, denominated RET/PTC. The RET/PTC rearrangements are the 2nd most common genetic alteration described in PTC and observed in ~13-43% of cases, mostly in pediatric cancers or in individuals exposed to ionizing radiation from nuclear accidents (12,43-45). At least 12 types of RET/PTC rearrangements have been reported, all originating from the RET fusion to different partners (44,46). RET/PTC1 comprises up to 60% of the rearrangements and is derived from an intrachromosomal rearrangement (10q), leading to the fusion of the RET tyrosine kinase domain to the *H4* gene (*D10S170*). The RET/PTC1 encodes a 585-amino acid protein with unknown function (47). RET/PTC3 accounts for 20-30% of the rearrangements and is formed by the RET gene fusion with the nuclear receptor coactivator 4 (*NCOA4*) gene (also known as *ELE1, RFG* or *ARA70*) (44,47).

Papillary tumors harboring the RET/PTC1 rearrangement commonly exhibit the classical papillary histology, whereas RET/PTC3 tumors normally present the solid variant (48). RET/PTC tumors tend to be small, with a favorable outcome and usually do not progress to a more aggressive behavior and/

or undifferentiated thyroid carcinoma (9,49,50). This alteration has also been associated with a younger age at diagnosis and a higher rate of lymph node metastasis (9,49). The high prevalence of RET/PTC in occult (42%) or microscopic PTC (77%) as well as in follicular adenoma (45%), may indicate a putative role of this rearrangement during the early stages of PTC development (51,52). Accordingly, studies performed using transgenic mice carrying RET/PTC1 and/or RET/PTC3 have shown that the PTC tumors which develop in these animals are similar to those occurring in humans (53,54).

The RET/PTC-derived mechanisms of tumor induction initiate with the fusion of protein partners, resulting in the ligand-independent autophosphorylation of the RET protein. The RET intracellular domain contains at least 12 autophosphorylation sites, and 11 of them are preserved in the RET/PTC protein (55). The Y1062 and Y1015 RET residues are constitutively phosphorylated and are required for cell transformation (56). These residues are essential binding sites for several proteins, which in turn, lead to the activation of the MAPK and PI3K/AKT signaling pathways and play an essential role in RET/PTC signaling with downstream cellular effects on migration and proliferation (57-59).

Another dysfunctional signaling pathway identified in 65-90% of RET/PTC-positive tumors is β-catenin, which is involved in gene transcription and cell adhesion regulation (60,61). The β-catenin pathway can be directly activated by several mechanisms: via RET tyrosine residue, cAMP response element-binding (CREB), glycogen synthase kinase 3 phosphorylation (GSK3-S) or via effectors of the MAPK and PI3K pathways (61,62). The increase in the free β-catenin protein pool promotes proliferation and invasion, possibly due to the interaction with transcriptional factors, such as the T-cell factor/lymphoid enhancer factor (TCF/LEF), c-Myc (v-myc myelocytomatosis viral oncogene homolog), or cyclin D1 (60,61,63).

RAS oncogene. *RAS* genes (H-*RAS*, K-*RAS*, and N-*RAS*) encode highly related G-proteins which play a central role in intracellular signal transduction by the activation of the MAPK and other signaling pathways, such as PI3K/AKT (see below) (15). *RAS* gene mutations are found in 10-43% of PTCs, particularly in the follicular variant (64-66). The RAS point mutations generally occur in codons 12, 13, or 61 of H-RAS, K-RAS, or N-RAS proteins. RAS-mutated PTC tends to be encapsulated and exhibits a low rate of lymph node metastasis (9,65). However, previous studies have reported that this mutation may also be associated with a more aggressive phenotype and a higher incidence of distant metastasis (66,67). The molecular mechanism proposed for *RAS*-derived tumorigenesis is the constitutive activation of distinct pathways involved in proliferation, differentiation and cell survival processes (66).

NTRK1 rearrangements. The neurotrophic tyrosine kinase receptor, type 1 (*NTRK1*) gene, located on chromosome 1, encodes the high-affinity nerve growth factor (NGF) receptor and is activated through the MAPK pathway (68). NTRK1 rearrangements are usually found in <10% of PTCs and result from the *NTRK1* gene fusion with different partners (69,70,71). Experimental evidence suggests that the *NTRK1* oncogene represents an early event in the process of thyroid carcinogenesis. Transgenic mice carrying *NTRK1* oncogene develop thyroid hyperplasia and PTC (72). Additionally, crossing NTRK1 mice with p27kip1-deficient mice has been shown to increase the penetrance of thyroid cancer and shorten the tumor latency period (73). NTRK1 rearrangements are associated with a younger age at diagnosis and a less favorable outcome (69,70).

3. Follicular thyroid carcinoma

The FTC represents 10-15% of thyroid cancers. These tumors are generally unifocal and present less lymph node involvement (<5%) than PTCs. By contrast, distant metastases, mainly to the lungs and bones, are more frequent at disease presentation (~20%) (4). Although former studies have indicated that FTCs, particularly the invasive form, have a poorer prognosis than PTCs (74,75), a recent study that evaluated more than 1,000 patients did not find differences in tumor-specific survival between PTC and FTC, after controlling for age, primary tumor size, extrathyroidal invasion or distant metastasis at diagnosis (76).

The most common genetic events observed in follicular carcinomas are point mutations in *RAS* genes and the rearrangements between the thyroid-specific transcription factor gene and the peroxisome proliferator-activated receptor gene [paired box gene 8 (PAX8)-peroxisome proliferator-activated receptor γ (PPARγ) rearrangements] (80%). Similarly to what is described in PTC, their oncogenic effects occur through the activation of the MAPK cascade; however, the induction of the PI3K pathway is an important event in follicular pathogenesis (15). Fig. 1B summarizes the major signaling pathways involved in FTC.

RAS oncogene. Activating mutations in the *RAS* gene are observed in 18-52% of follicular carcinomas and are associated with tumor dedifferentiation and a less favorable prognosis (77,78). A number of studies have suggested that *RAS* mutations are an early event in follicular thyroid tumorigenesis, since they are identified in up to 50% of benign follicular tumors (77,79,80,82,83). Studies using transgenic mice carrying the mutated N-*RAS* (Gln61Lys) oncogene demonstrated that these rodents developed follicular adenomas (11%), invasive follicular carcinomas (~40%) and, in certain cases, tumors with a mixed papillary/follicular morphology. Moreover, 25% of these carcinomas displayed large, poorly differentiated areas, with vascular invasion and with lung, bone or liver metastasis (81).

The RAS-mutated protein mediates its effects on cellular proliferation in part by activation of a cascade of kinases: RAF (A-RAF B-RAF and C-RAF), dual-specificity mitogen-activated protein kinases (MEK1/2), extracellular signal-regulated kinases (ERK1/2) and p38 mitogen-activated protein kinase. RAS also activates the PI3K pathway, via a direct interaction with the catalytic subunit of the protein. The PI3K activation leads to the accumulation of the 2nd messenger, phosphatidylinositol 3,4,5-trisphosphate (PIP3), resulting in pyruvate dehydrogenase kinase isozyme 1 (PDK1) and v-akt murine thymoma viral oncogene homolog (AKT) activation (85,86) (Fig. 1B). Previous studies using mice harboring a phosphatase and tensin homolog (*PTEN*) gene deletion and a $KRAS^{G12D}$ mutation, have shown that the separate activation of MAPK or PI3K pathways, is unable to transform thyroid follicular cells;

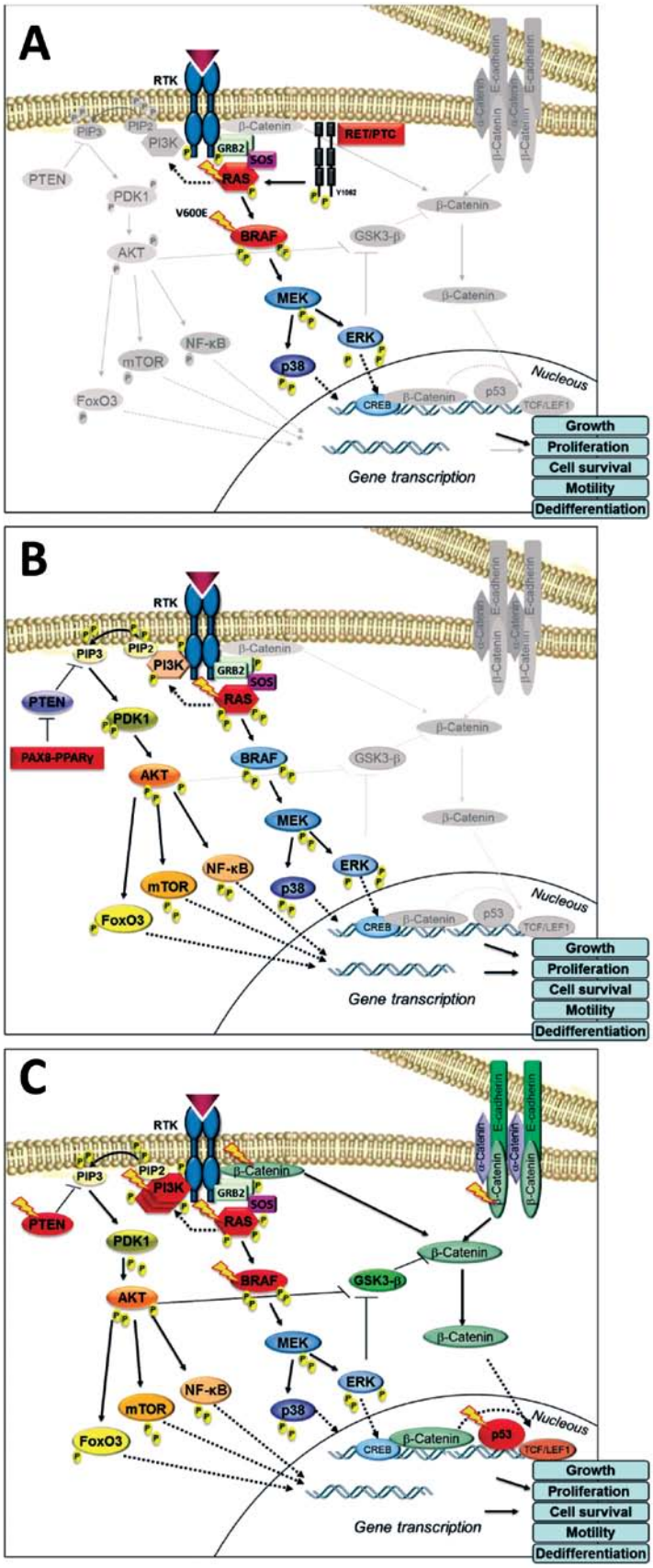

Figure 1 Schematic presentation of the signaling pathways involved in follicular-derived thyroid carcinoma. (A) In papillary thyroid carcinoma, BRAFV600E or *RAS* point mutations, or RET/PTC rearrangement result in a constitutively phosphorylated protein which leads to a potent activation of downstream effectors of the MAPK pathway. (B) In follicular thyroid carcinoma, RAS-mutated protein can mediate its cellular effects either by the activation of the MAPK cascade or the PI3K pathway, while PAX8-PPARγ rearrangement leads to the abrogation of the PTEN inhibitory effect and the PI3K signaling activation. (C) In anaplastic thyroid carcinoma, the MAPK cascade is induced by *RAS* or *BRAF* mutations, while copy gain or mutations of the PI3K and *PTEN* mutations are associated with the constitutive activation of PI3K/AKT pathway. Additionally, β-catenin mutations activate the β-catenin/E-cadherin pathway, whereas *TP53* gene alterations lead to aberrant cell cycle regulation.

however, their simultaneous activation is highly oncogenic, leading to locally invasive follicular carcinomas and distant metastasis (84).

PAX8-PPARγ rearrangements. The thyroid-specific transcription factor (*PAX8*) gene is a critical regulator of thyroid differentiation and growth (87). *PPARγ* is a ligand-dependent nuclear transcription factor highly expressed in adipose tissue, where it plays a critical role in adipocyte differentiation and fat metabolism regulation (88). The PAX8-PPARγ rearrangement arises through a chromosomal translocation, fusing the 5' portion of the *PAX8* gene with the entire coding sequence of the *PPARγ* gene (chromosomes 3p25 and 2q13). It is detected in ~35% of FTCs (10,89,90).

The PAX8-PPARγ rearrangement leads to strong induction of the PPARγ protein and the consequent abrogation of the normal PPARγ function (95,96). Under normal conditions, PPARγ inhibits cell proliferation and induces apoptosis via downstream pathways. The loss of these functions results in uncontrolled cell growth (14). *PPARγ* overexpression abolishes the PTEN-inhibitory effect on immunoactive AKT, which in turn induces the PI3K signaling pathway (58,97). The PAX8-PPARγ rearrangement also activates the MAPK, transforming growth factor β (TGFβ) and Wnt/β-catenin (wingless in *Drosophila*) signaling pathways. The increased expression of the C-terminal binding protein (*CTBP2)* gene has been observed in the PAX8-PPARγ-positive-tumors (95). CTBPs are co-repressor proteins associated with several transcriptional factors involved in Wnt, TGFβ and MAPK signaling activation, thus explaining their major role in follicular tumor development (98).

Patients with FTC harboring the PAX8-PPARγ rearrangement are usually diagnosed at a young age, have a small tumor size and the majority of tumors are overtly invasive at presentation (10,89). These findings, however, were not reproduced in other studies and the impact of PAX8-PPARγ on the biology and behavior of FTCs remains controversial (10,92).

Follicular adenomas have been shown to have lower frequency rates of PAX8-PPARγ rearrangements, suggesting that this chromosomal translocation may be involved in the early phases of the neoplastic process of FTC, possibly even in premalignant lesions (90,91,93). Transfection studies of PAX8-PPARγ in thyroid follicular epithelial cells have demonstrated accelerated growth rates and a lower number of cells in the G0/G1 resting state (14,94).

4. Anaplastic thyroid carcinoma

ATC, also known as undifferentiated thyroid carcinoma, is the most aggressive form of thyroid neoplasia. It can originate *de novo* or represent an advanced stage of follicular cell-derived thyroid tumors (4,99). Anaplastic tumors represent <1% of all thyroid tumors and their annual incidence is ~1-2 cases per 1,000,000 with a higher overall incidence in endemic goiter areas (100,101). The ATC typical presentation is advanced disease at diagnosis. Patients with anaplastic carcinoma usually have widespread local invasion and distant metastases, most frequent in the lung, pleura, bone and brain (100). This tumor has poor or no response to conventional therapeutic modalities. The median survival time after diagnosis is <1 year (102,103). A younger age (<60 years), smaller tumor size (<7 cm) and restricted disease have been associated with a lower mortality rate on multivariate analysis (104).

ATCs have been described as carrying multiple distinct genetic alterations with a high prevalence of mutations in MAPK effectors (13,21). Mutations in the *TP53* gene, β-catenin and PI3K cascade also play a critical role in ATC development, promoting the dedifferentiation of a previously well differentiated thyroid tumor (11,105,106). Fig. 1C summarizes the signaling pathways involved in ATC.

Mutations in gene effectors of the MAPK pathway. MAPK activating genetic alterations have been described to be involved in the development/progression of ATCs. ATC tumors present a significant prevalence of *RAS* (6-55%) and *BRAF* mutations (24-50%) (13,14,107). By contrast, RET/PTC, NTRK and PPARγ-PAX8 rearrangements are rarely observed in these undifferentiated tumors, supporting the hypothesis that DTCs associated with these rearrangements do not usually progress to anaplastic form (108,109).

$BRAF^{V600E}$ mutation is typically found in ATC tumors which contain areas of well-differentiated PTC, but also in poorly differentiated and anaplastic tumor areas. These observations suggest that although this mutation may occur early in tumorigenesis, it is not sufficient to initiate the dedifferentiation process. However, it is conceivable that *BRAF* mutations may predispose to additional genetic alterations which in turn activate more aggressive pathways and lead to dedifferentiation (15,110,111). Of note, $BRAF^{V600E}$ mutation has also been observed in lymph-node metastasis of ATCs (111). Of note, patients with ATCs harboring *BRAF* mutations have a higher mortality rate than those patients presenting with *RAS* or with no identified mutation, indicating a negative prognosis of these genetic alterations during all stages of thyroid cancer progression (13).

RAS mutations are found in a high prevalence in ATCs (6-55%) (13,14,77). A previous study suggested that the RAS effect may be due to the promotion of chromosomal instability, since the expression of constitutively activated RAS destabilizes the genome of PCCL3 thyroid cells, predisposing to large scale genomic abnormalities (112).

Genetic alterations in genes involved in the activation of the PI3K pathway

PIK3CA mutations and copy number gains. The *PIK3CA* gene encodes a catalytic subunit of PI3K and has been described to be mutated in 12-23% of ATC cases, normally restricted to the undifferentiated thyroid components. Previous studies have shown a preferential expression of *PIK3CA* mutations during the later stages of thyroid cancer, suggesting that this event may be more important in ATCs (12-23%) than in DTCs (PTCs, ~2% and FTCs, <10%) (11,106).

PIK3CA copy number gains are the 2nd most frequent event in ATC occurring in ~38-61% of tumors (14,106). Of note, this occurs almost exclusively in the undifferentiated component of the tumor. The copy number gain induces the activation of the PI3K cascade through the enhanced activity of AKT, leading to thyroid cancer progression. Of note, the *PIK3CA* mutations and copy number gain may co-exist with other somatic mutations in ATC, reinforcing the activation of the distinct signaling pathway in these tumors (11).

Table I. Clinical trials and follicular cell-derived thyroid tumors response.

Trade name	Compound	Target	Tumor type	No. of patients	Partial response[a] [% (n)]	Stable disease[b] [% (n)]	Refs.
Sorafenib	BAY 43-9006	BRAF ($BRAF^{V600E}$)	PTC	41	15 (6)	56 (23)	(127)
		VEGFR1-3, PDGFR,	DTC	31	25 (8)	-	(128)
		RET, RET/PTC	DTC	30	23 (7)	34 (10)	(129)
Axitinib	AG-013736	VEGFR1-3, PDGFR,	PTC	30	26 (8)	40 (12)	(131)
		c-Kit	FTC	15	40 (6)	46 (7)	
			ATC	2	50 (1)	-	
Pazopanib	W786034	VEGFR1/2, PDGFR	DTC	39	49 (18)	-	(132)
Motesanib	AMG706	VEGFR1-3, RET, c-kit	DTC	93	14 (13)	67 (62)	(133)
Gefitinib	ZD1839	EGFR	DTC	25	0	12 (3)	(134)
Selumetinib	AZD6244	MEK1/2	PTC (IR)	32	3 (1)	54 (21)	(135)
PLX4032	RG7204	$BRAF^{V600E}$	PTC	3	33 (1)	66 (2)	(130)

DTC, differentiated thyroid carcinoma; PTC, papillary thyroid carcinoma (IR, iodine-131 refractory); FTC, follicular thyroid carcinoma; ATC, anaplastic thyroid carcinoma. [a]Partial response: a decrease of at least 30% in the sum of the largest diameter of target lesions, relative to the corresponding sum at baseline. [b]Stable disease: the absence of shrinkage sufficient for a partial response and the absence of enlargement sufficient for progressive disease, relative to the corresponding sum at baseline.

PTEN gene alterations. *PTEN* is a tumor suppressor gene that antagonizes signaling through the PI3K pathway. Its action occurs by removing a phosphate group from the inositol ring of PIP3, which reduces the downstream activity of the AKT kinase, thereby inducing cell cycle arrest, apoptosis, or both (113). Several genetic alterations in the *PTEN* suppressor gene have been described in ATCs: 12% present a mutated form (106,108), 28% gene silencing (114) and 69% the hypermethylated *PTEN* gene (115). These alterations lead to PTEN inactivation by different mechanisms, with a prominent role in the pathogenesis of follicular epithelium-derived thyroid carcinomas, particularly in the most aggressive or undifferentiated forms (114,115). Moreover, PI3K activation produced by downregulated PTEN has been shown to correlate with regions of tumor invasion and metastasis (58,116). Of note, studies using transgenic mice with a deletion of *PTEN* or *RAS* mutations have shown that the presence of both genetic events is required to trigger this aggressive form of thyroid cancer (84).

TP53 mutations. The *TP53* gene encodes a nuclear protein that can induce cell cycle arrest, senescence and apoptosis in response to various stimuli. Alterations in the p53 pathway may contribute to carcinogenesis, disease progression and resistance to therapy (117). In thyroid tumors, *TP53* mutations are commonly observed in anaplastic carcinomas (~70%) and are rarely described in well-differentiated thyroid carcinomas (0-9%) (12,105,118). This suggests that *TP53* mutations are a late event in tumor progression and that this gene may play a critical role in the transformation of DTC into the anaplastic form (105). The frequent association of p53 inactivation with PI3K activation may contribute to genomic instability, leading cancer cells to become resistant to apoptosis and to escape from any growth restriction. This contributes to a rapidly enlarging neck mass as well as to chemotherapy and radiotherapy resistance commonly observed in these tumors (11).

β-catenin genetic alterations. Genetic alterations in the β-catenin (*CTNNB1*) gene are observed in ~65% of thyroid anaplastic tumors. Gain-of-function mutations can promote β-catenin nuclear translocation which consequently triggers the transcription process (119,120). The expression of E-cadherin, a component of the β-catenin pathway, normally expressed in thyroid tissue, is usually absent in undifferentiated thyroid carcinomas (121). These changes appear to play a pathogenic role in thyroid tumor invasion and regional lymph node metastasis, due to a decrease in intercellular adhesion and enhancement of cell motility (122). The lack of E-cadherin expression is associated with an adverse prognosis for patients with thyroid carcinoma (123).

5. Clinical Implications: Potential therapeutic targets

DTCs demonstrate indolent behavior in the majority of patients and can be effectively treated by surgery followed by radioactive iodine and/or thyroid hormone suppressive therapy (124,125). In patients with metastatic disease, radioactive iodine therapy can be effective in some cases, whereas suppressive thyroid hormone therapy can help to delay the pace of the disease (125,126). Nevertheless, for those patients with metastatic DTC that progresses despite radioiodine and thyroid hormone therapy, no effective treatments are currently available.

Over the last decades, cancer research has been predominantly focused on the genetic alterations and the advances in the understanding of the molecular events involved in differentiated thyroid carcinogenesis have allowed for the development of new therapies designed for patients with metastatic disease refractory to radioactive iodine treatment. Specific tyrosine multikinase inhibitors to target key molecules such as BRAF, RET/PTC rearrangements, vascular endothelial growth factor receptors (VEGFRs) and platelet-derived growth factor recep-

tors (PDGFR) have been evaluated as potential alternatives to DTC treatment. Table I summarizes the results obtained to date in several clinical trials. Phase II studies using BAY 43-9006 (sorafenib) have shown partial response (15-25%) and stable disease (34-56%) in progressive DTC patients and the median progression-free survival was significantly longer in patients harboring *BRAF* mutations (127-129). A recent study using PLX4032, an inhibitor of mutant BRAF, in metastatic melanoma patients evaluated the effect of this drug in 3 PTC patients. The response lasted 8 months in 1 patient (progression-free lasted for 12 months) and stable disease lasted 11 and 13 months in each of the other 2 patients (130). Although these compounds have demonstrated the most impressive clinical responses to date in the treatment of advanced thyroid cancer, the low rate of partial response, the rare report of complete responses and the emergence of eventual progression, point out to the need to develop either more effective single agents or to identify rational combinations of therapeutic targets.

6. Conclusion

Thyroid carcinogenesis consists of a complex process with a large number of molecular alterations among several thyroid neoplasias. The set of genetic alterations observed in follicular-cell derived thyroid carcinomas activates specific pathways, such as the MAPK, PI3K and β-catenin signaling pathways, which have been shown to play an important role in thyroid cancer initiation and progression. The screening for follicular cell-derived specific mutations in association with traditional diagnosis methods has improved the diagnostic accuracy, impacting the prognosis of these tumors. Moreover, the advances in the knowledge of the effects of thyroid oncogenes and related mechanisms of action have allowed for the development of multikinase inhibitor targets, promoting new perspectives on therapy to aggressive thyroid tumors.

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Parte II

MAPK signaling pathway activation modulates the thyroid hormone-inactivating type 3 deiodinase expression in human papillary thyroid carcinoma

MAPK signaling pathway activation modulates the thyroid hormone-inactivating type 3 deiodinase expression in human papillary thyroid carcinoma

Mírian Romitti[1], Simone Magagnin Wajner[1], Lucieli Ceolin[1], Carla Vaz Ferreira[1], Rafaela Vanin Pinto Ribeiro[1], Helena Cecin Rohenkohl[1], Shana de Souto Weber[1] Patrícia Lopes, Cesar Seigi Fuziwara[2], Edna Teruko Kimura[2], and Ana Luiza Maia[1]

[1]Thyroid Section, Endocrine Division, Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil.
[2]Department of Cell and Developmental Biology, Institute of Biomedical Sciences, Universidade de São Paulo, São Paulo, SP, Brasil.

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Correspondence: Dr. Ana Luiza Maia
Serviço de Endocrinologia
Hospital de Clínicas de Porto Alegre
Rua Ramiro Barcelos, 2350
CEP 90035-003 – Porto Alegre, RS, Brasil.
Phone/Fax: 55- 51- 33310207
E-mail: almaia@ufrgs

Abstract

Type 3 deiodinase (*DIO3*, D3) is reactivated in human neoplasias. Increased levels of D3 in papillary thyroid carcinoma (PTC) were associated with tumor size and metastatic disease. **Objective:** To investigate the signaling pathways involved in *DIO3* upregulation in PTC**. Material and Methods:** PTC cell lines (K1 and TPC-1 cells) were used to evaluate DIO3 regulation. *DIO3* mRNA levels were measured by real-time PCR, and D3 activity was measured by ion-exchange column chromatography. Protein expression was determined by Western blot analysis. *DIO3* gene silencing was performed with siRNA transfection. **Results:** *DIO3* mRNA levels and activity were readily detected in K1 ($BRAF^{V660E}$) and, at lower levels, in TPC-1 (RET/PTC1) cells (~5-fold, P<0.001; 14.9 *vs.* 8.1 fmol/mg.prot.24hs, P=0.02; respectively). Similarly, the levels of *DIO3* mRNA were higher in the PTC samples harboring the $BRAF^{V600E}$ mutation compared with those with the RET/PTC1 rearrangement or no mutation (8 *vs*. 5.8 *vs.* 5.4-fold; P<0.001; respectively). Specific inhibition of MEK (U0126; 10-20 μM) or p38 (SB203580; 10-20μM) was associated with decreases in *DIO3* expression in both cell lines. Additionally, the blockage of SHH activation by cyclopamine (10 μM) resulted in markedly reduced *DIO3* levels in K1 and TPC-1 cells. Interestingly, siRNA-mediated *DIO3* gene silencing decreased cyclin-D1 expression, while it increased the proportion of cells in the G1 phase of the cell cycle, thereby downregulating cell proliferation. **Conclusions:** Sustained activation of the MAPK and Sonic Hedgehog pathways modulates the levels of *DIO3* expression in PTC. Importantly, *DIO3* silencing was associated with decreases in cell proliferation, which further suggests a role of the molecule in tumor growth and aggressiveness.

Introduction

Thyroid hormone influences a wide variety of biological processes, including the balance between cell proliferation and differentiation. Thyroid hormone homeostasis is critically regulated by the synchronized activity of the iodothyronine deiodinases. Type 1 (D1; *DIO1*) and type 2 deiodinases (D2; *DIO2*) catalyze the conversion of the pro-hormone T4 (thyroxine) into the biologically active form T3 (triiodothyronine) via outer-ring deiodination. In contrast, type 3 iodothyronine deiodinase (D3; *DIO3*) catalyzes the inactivation of T4 and T3 via inner ring deiodination (1). Extensive data indicate an association between the thyroidal status and tumor pathogenesis. However, the role of deiodinases in thyroid cancer and other human neoplasias has not yet been established. Several studies have reported changes in the expression of deiodinases in benign and malignant tumors (2-4).

DIO3 reactivation in neoplastic tissues occurs at the transcriptional level, and it might to be driven by disruption in the activation of several signaling pathways (5-8). Interestingly, D3, a known fetal protein, was demonstrated to be reactivated in human neoplasias and associated with tumor behavior. Induced levels of D3 were demonstrated in proliferating keratinocytes as well as in mouse and human malignant basal cell carcinoma (BCC). The authors also demonstrated that the *DIO3* induction caused by sonic hedgehog (SHH)/GLI activation, led to reduction of intracellular active thyroid hormone levels, thus resulting in increased cyclin D1 and keratinocyte proliferation. Accordingly, D3 knockdown promoted a significant reduction in the growth of BCC xenografts in nude mice (5). Moreover, higher *DIO3* expression was demonstrated in human intestinal adenomas and carcinomas as compared with healthy intestinal tissue. D3 seems to be a direct transcriptional target of the β-catenin/TCF complex once that experimental attenuation of β-catenin reduced D3 levels and induced type 2 deiodinase. Additionally, under D3 inhibition, excess of T3 reduced cell proliferation and promoted differentiation in cultured cells and in xenograft mouse models (6).

Papillary thyroid cancer (PTC) is the most common malignant thyroid tumor, occurring in 85-90% of cases of malignant thyroid tumor (9, 10). Aberrant activation of mitogen-activated protein kinase (MAPK) signaling pathway is a hallmark in PTC and is generally caused by point mutations and/or gene rearrangements. The $BRAF^{V600E}$ point mutation is the most common genetic event, observed in ~50% of PTC cases, while RET/PTC rearrangement occurs in ~20% and *RAS* mutations in 10-15% of cases (11-14). We have recently demonstrated that there is an upregulation of *DIO3* in PTC samples. Interestingly, the

presence of $BRAF^{V600E}$ mutation was associated with the highest levels of *DIO3* mRNA and activity. Remarkable, increased D3 levels were associated with a larger tumor size and the presence of local and/or distant metastasis at diagnosis. Conversely, decreased levels *DIO2* were observed. Augmented D3 expression was also shown in follicular thyroid carcinoma but not in medullary or anaplastic thyroid carcinoma samples (8).

In the present study, we sought to determine the signaling pathways involved in *DIO3* upregulation in the PTC as well as to elucidate whether *DIO3* induction could interfere with cell proliferation.

Material and Methods

Cell Culture

Studies evaluating *DIO3* gene regulation were performed in two human PTC-derived cell lines, which endogenously express the *DIO3* gene; these were the K1 cell line, which carries the $BRAF^{V600E}$ mutation, and TPC-1 cells harboring the RET/PTC1 rearrangement. K1 cells were grown in DMEM: Ham's F12:MCDB 105 (2:1:1; Invitrogen) plus 2 mM glutamine and 10% fetal bovine serum (FBS). TPC-1 cells were grown in DMEM containing 5-10% fetal bovine serum. Additionally, we used a medullary thyroid carcinoma cell line, TT cells, to determine the effect of SHH on *DIO3* reactivation. TT cells were grown in RPMI (Invitrogen, Carlsbad, CA, USA) medium supplemented with 10% FBS. All cells were maintained at 37ºC in a humidified atmosphere of 5% CO2 and 95% air, and the culture medium was changed three times a week.

Human PTC samples

To the present study we selected PTC patients from the sample used in our previous study (Romitti, 2012). Neoplastic and surrounding normal human thyroid tissues were collected from fourteen unselect patients diagnosed with PTC at the Endocrine or Head and Neck Surgery Divisions at Hospital de Clínicas de Porto Alegre, Brazil. The attending physicians independently performed the surgery. Tumors were histologically classified according to WHO recommendations (15). The study was approved by the Ethical Committee of the Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil.

BRAF^{V600E} mutation and RET/PTC rearrangement analysis

Fourteen PTC samples and surrounding thyroid tissues were available for analysis. The BRAF^{V600E} analysis was performed by direct sequencing according previously described (Romitti, 2014).

For RET/PTC1 detection, total RNA was extracted from PTC samples using the Trizol Reagent and cDNA was generated using the Super Script III First-Strand Synthesis System (Invitrogen). Detection of RET/PTC rearrangement was performed by RT-PCR. Here, we used the forward primer for H4 gene and the reverse primer for the TK domain of RET (forward 5′-AGCGCCAGCGAGAGCGACACG-3′, reverse 5′-TACCCTGCTCTGCCTTTCAGATGG-3′; Nested: forward 5′-GTCGGGGGGCATTGTCATCT-3′, reverse 5′-AGTTCTTCCGAGGGAATTCC-3′). PCR conditions were performed according to a previously described protocol (16). Afterwards, ten microliters of the PCR product were analyzed by electrophoresis in a 1.5% agarose gel. TPC-1 cells were used as a positive control. Positive samples were subjected to direct sequencing to confirm the presence of RET/PTC rearrangement.

Real-time PCR

Total RNA was extracted from K1 and TPC-1 cells using the RNeasy minikit (Qiagen) while to PTC samples and surrounding thyroid tissues were used Trizol Reagent and 1 μg of RNA was reverse transcribed into cDNA using using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies), following the manufacturer's protocol for the oligo (dT) method. RT-qPCR experiments were performed in a 7500 Fast Real-Time PCR System Thermal Cycler with 7500 FAST System Sequence Detection 1.4 Software (Life Technologies - Applied Biosystems). Experiments were performed by real-time monitoring of the increase in fluorescence of SYBR Green dye. The oligonucleotides used were as follows: *DIO3*, 5′-TCCAGAGCCAGCACATCCT-3′ and 5′-ACGTCGCGCTGGTACTTAGTG-3′; *GAPDH*, 5´-ACCCACTCCTCCACCTTTG-3´ and 5´-CTCTTGTGCTCTTGCTGGG-3´; cyclophilin A (reference gene), 5′-GTCAACCCCACCGTGTTCTTC- 3′ and 5′-ACTTGCCACCAGTGCCATTATG-3′. Each sample was assayed in triplicate and a negative control was included in each experiment. Standard curves representing 5-point serial dilution of cDNA were analyzed and used as calibrators of the relative quantification of product generated in the exponential phase of the amplification curve. The r^2 was greater than 0.99, while the amplification efficiency was higher than 98%. Quantification of DIO3 and GAPDH cDNA were performed by relative

quantification using the comparative ΔΔCT method and expressed relative to the reference gene (cyclophilin A). Changes in gene expression were expressed as relative fold difference (n-fold change) or as arbitrary units (AU).

Inhibition of MAPK and SHH signaling

To evaluate the effect of MAPK signaling activation on *DIO3* induction in K1 and TPC-1 cell lines, we performed studies using specific inhibitors to the signaling effectors MEK (U0126: 10-20 μM; Sigma-Aldrich), p38 (SB203580: 10-20 μM; Sigma-Aldrich) and BRAF-mutated (PLX4032: 3 μM; Selleck Chemicals). Additionally, to assess the role of the Sonic Hedgehog pathway on *DIO3* regulation, we used the specific inhibitor of the Smoothened (a SHH signaling effector), cyclopamine (10 μM, Sigma-Aldrich). The recombinant Shh (1 μg/ml) was used to induce SHH activation in TT cells. Controls were incubated with medium + vehicle (1% DMSO). Cells were incubated during 24 hours and then were harvested and processed for total RNA or total protein extraction. All analyses were performed in triplicate in at least two independent experiments.

D3 activity assay

D3 activity was determined in PTC cells by ion-exchange column chromatography (17). After concluding the experiments, 300 μl of medium was collected, and the reaction was stopped with 200 μl of horse serum and 100 μl 50% TCA, which was followed by centrifugation at 12,000 *g* for 2 minutes to precipitate the nonmetabolized [125I]T3. The supernatant was used to determine the [125I]T2 and [125I]T1 levels. The Sephadex LH-20 column was equilibrated with 0.1 M HCl, and an equal volume of 0.1 M HCl was added to 500 μl samples and then mixed. Stepwise elution was performed by successive application of 2x 1 ml of 0.1 M HCl (for 125I– release), 6x 1 ml of 0.1 M NaOH-ethanol (8:1 v/v [125I] for T1 release), and 4x 1 ml of 50% ethanol in 0.1 M NaOH (1:1 v/v [125I] for T2 release). The 1-ml fractions were collected and counted for radioactivity. The D3 activity was calculated by multiplying the fractional conversion by the T3 concentration in the media and expressed as T3 inactivation (fmol/mg protein per 24 hours).

Western Blot Analysis

Cultured cells were lysed and prepared for Western blot analysis as previously described (18). Afterwards, 30-50 μg of each sample was fractionated by 8-12% SDS-PAGE and blotted onto an Immobilon PVDF membrane (Millipore, Billerica, MA, USA).

Nonspecific binding sites were blocked by incubation with 5% nonfat dry milk in Tris-buffered saline-0.1% Tween-20. The following primary antibodies were used: *anti-DIO3 (1:400; Novus Biologicals), anti-ERK1/2 (1:400; Santa Cruz Technologies), anti-phospho-ERK1/2 (1:400; Santa Cruz Technologies); anti-P38 (1:500; Cell Signaling); anti-phospho-P38 (1:200; Santa Cruz Technologies); anti-cyclin D1 (1:400; Santa Cruz Technologies); anti-Gli1 (1:400; Cell Signaling); anti-α-tubulin B7 (1:500; Santa Cruz Technologies); and anti-β-actin (1:10,000; Sigma).* The antigen-antibody complexes were visualized using horseradish peroxidase–conjugated secondary antibody and an enhanced chemiluminescence system (GE Healthcare). Expression was quantified using image densitometry with Image J Analysis Software.

Small interfering RNA transfection

Small interfering RNA (siRNA) studies were performed to evaluate the specific effects of *DIO3* inhibition on cell proliferation. The shorter-duplexes siRNAs were as follows: Silencer® Select *GAPDH* siRNA (#4390849, Ambion Inc, Life Technologies), used as positive control for inhibition experiments; Silencer® Select Negative Control (#4390843, Ambion Inc, Life Technologies) and Silencer® Pre-designed *DIO3* siRNA (#7631324, Ambion Inc, Life Technologies). Transfection studies were performed using Lipofectamine RNAiMAX reagent according to the manufacturers' instructions (Invitrogen by Life Technologies). A total of 15.10^4 cells/well (K1 and TPC-1) were plated in six-well plates and transfected with 40 pmol of GAPDH siRNA, 100 pmol of silencer negative and 100 pmol of *DIO3* siRNA. All analyses were performed in triplicate and in at least two independent experiments.

Cell proliferation assays

Absolute cell number count and flow cytometry were performed to evaluate cell proliferation. Initially, 15.10^4 cells/well (K1 and TPC-1) were plated in six-well plates, transfected with 100 pmol of *DIO3* siRNA and incubated for 48 hours. After 48 hs of treatment, the cells were trypsinized, and the absolute number of cells was counted using the Neubauer chamber. To evaluate the effect of *DIO3* expression on the cell cycle status, K1 cells were incubated with *DIO3* siRNA. After 48 hs, the cells were washed with PBS and then resuspended in 50 μg/mL propidium iodide and 0.1% Triton X-100 in sodium citrate solution. Cells were incubated on ice for at least 15 min. Marked cells were analyzed using a flow

cytometer Attune® Acousting Focusing Cytometer. The data generated were analyzed using the FlowJo software. All experiments were performed in triplicate.

Statistical analysis

DIO3 mRNA was expressed as arbitrary unit or *fold*, while D3 activity as the median ± SD. The number of cells in each cell cycle stage is shown as the frequency. To compare the D3 levels among the groups, we used t-Test or one-way ANOVA while Chi-Square test was performed to compare the differences in the proportion of cells in the different stages of the cell cycle. The Statistical Package for the Social Sciences 18.0 and Prism 5.0 software were used for all analyses, and $P<0.05$ was considered statistically significant.

Results

MAPK activation induces DIO3 levels in PTC cell lines

To estimate the role of MAPK activation in *DIO3* regulation, experimental studies were performed in two distinct human PTC cell lines, K1 cells carrying the $BRAF^{V600E}$ mutation and TPC-1 cells harboring the RET/PTC1 rearrangement. We observed that the levels of *DIO3* mRNA and activity were readily detected in both cell lines and were significantly higher in K1 cells compared to TPC-1 (~5-fold, $p<0.001$; 14.9 *vs.*8.1 fmol/mg.prot.24 hs, $p=0.02$; respectively; Figures 1A-B).

Next, we evaluated the oncogenic effects of $BRAF^{V600E}$ mutation on *DIO3* reactivation. The treatment of K1 cells with the specific *BRAF*-mutated inhibitor, PLX4032 (3 µM), caused reduction in ERK phosphorylation and *DIO3* levels (~2.5-fold; $P<0.001$, Figures 2A-B). The incubation of K1 cells with MEK inhibitor (10-20 µM) for 24hs, resulted in a substantial reduction in ERK phosphorylation (Figure 3A) and in a significant dose-dependent decrease of *DIO3* expression (5-10-fold $P<0.001$; Figures 3B). Likewise, we performed experiments using the p38 inhibitor (10-20 µM) and, as expected, p38 phosphorylation was substantially inhibited (Figure 3C), while slight reduction in *DIO3* transcripts was identified (~2-fold; $P<0.001$; Figure 3D). Similar results were obtained in TPC-1 cells under inhibition of MEK and p38 (1.5-6 and 2-3-fold; $P<0.001$; respectively, Figures 3E-G).

Next, we investigated the effect of MAPK genetic alterations on the *DIO3* levels in PTC samples and surrounding tissue collected from 14 patients. The mean age was 42.8 ±

14.5 years, and 78.6% were women. The median size tumor was 2.3 cm (0.8–10); 10 patients (71.4%) had lymph node metastasis, while 6 (42.9%) had distant metastasis at diagnosis. Seven (50%) out of the 14 PTC samples were positive for the $BRAF^{V600E}$ mutation, two (10%) carried RET/PTC1 rearrangement and 5 (40%) did not have any of these genetic alterations. *DIO3* mRNA was significantly increased in PTC samples compared with the surrounding thyroid normal tissue ($p<0.001$). Samples harboring $BRAF^{V600E}$ mutation had higher levels of *DIO3* expression compared with samples with RET/PTC1 rearrangement or those without any mutation (8 *vs.* 5.8 *vs.* 5.4AU, respectively; $P<0.001$; Figure 4).

Cooperation between MAPK and Sonic Hedgehog pathways drives the DIO3 upregulation in PTC

Previous studies have demonstrated in BCC samples that the activation of the SHH pathway would be driving the *DIO3* overexpression (5). To verify the requirement of GLI1, a downstream effector of SHH signaling, activation in D3 regulation, we blocked the SHH signaling using incubation with a chemical inhibitor, cyclopamine (10 μM). After 24 hs of treatment, we observed a reduction in the GLI1 protein (Figures 5A and C), which was followed by a marked decrease in the *DIO3* levels in K1 as well as in TPC-1 cells (12 and 2.5-fold; $P<0.001$; Figures 5B and D; respectively).

We also investigated whether the MAPK and SHH cascades could work in cooperation, promoting D3 induction in PTC. The levels of the SHH downstream effector, GLI1, were evaluated after the MEK and p38 proteins were inhibited. Interestingly, we observed a reduction in the GLI1 levels in K1 and TPC-1 cells after MAPK blockage, suggesting there is crosstalk between the signaling pathways (Figure 5E-H).

Next, we investigated whether the *DIO3* induction depends of SHH reactivation. TT cells, a MTC cell line known for presenting with low endogenous *DIO3* levels, were treated for 24 h with recombinant SHH (1 μg/ml). Interestingly, the SHH induction significantly increased the *DIO3* mRNA expression in MTC cells (2.5-fold; $P<0.0001$; Figure 5I) while reduced the *DIO2* levels in similar intensity (2.6-fold; $P<0.001$, Figure 5J).

***DIO3* attenuation is associated with reduction in cell proliferation of PTC cells**

To demonstrate the specific proliferative cell effect of *DIO3* upregulation, we silenced the *DIO3* gene in both cell lines, using *DIO3* specific siRNA (100 pmol). *GAPDH* siRNA (40

pmol) was used as a positive control. K1 and TPC-1 cells were transfected with the siRNAs and maintained for 48hs. The efficiency of silencing was established by reducing levels the positive control *GAPDH* (inhibition of 95% in K1 and ~90% in TPC-1 cells, $p<0.001$, data not shown). *DIO3* gene knockdown resulted in a ~90% blockage of *DIO3* transcripts and D3 protein in both PTC cell lines ($p<0,001$; Figures 6A and C). Interestingly, the *DIO3* inhibition was associated with significant reduction in the absolute cell number compared with control (~30%; $p<0.01$; Figures 6B and D). Further experiments evaluating the DIO3 effect on cell proliferation were performed in K1 cells and showed that the reduction in the *DIO3* levels was also associated with diminished levels of cyclin D1 protein (Figure 6E).

The evaluation of the cell cycle showed that the proportion of cells in G1 phase of cell the cycle was significantly augmented when *DIO3* was silenced, while the percentage of cells in S and G2 phases of cell cycle was reduced in the same proportion (~30%; $P<0.005$; Figure 6F).

Discussion

In the present study, we have demonstrated that genetic alterations in the MAPK pathway effectors, such as $BRAF^{V600E}$ mutation and RET/PTC1 rearrangement, increase D3 levels in papillary thyroid tumors. The SHH pathway also seems to be involved in *DIO3* upregulation once the signaling inhibition significantly reduces the *DIO3* expression. Interestingly, siRNA-mediated *DIO3* gene silencing decreased cyclin-D1 levels while increasing the proportion of cells in the G1 phase of the cell cycle, downregulating the proliferation of malignant thyroid cells.

The profile of *DIO3* gene expression shows that higher D3 activity is present in the developing organs while in mature tissues, it is predominantly expressed in the brain and skin (19, 20). A previously unrecognized role of D3 has been documented in both health and disease (17, 21, 22). Moreover, a potential role of D3 in tumorigenesis has been postulated because *DIO3* upregulation has been observed in several benign and malignant tumors (2, 3, 5-8, 23). *DIO3* regulation occurs at the transcriptional level, and it is likely that it is driven by activation of the MAPK pathway (24-26). Papillary thyroid carcinomas are known for carrying genetic alterations that lead to distinct an aberrant and constitutive activation of MAPK pathway (14). $BRAF^{V600E}$ is an oncogenic protein with markedly elevated kinase activity that over-activates the MAPK pathway, especially ERK signaling transduction (27, 28). Accordingly, we observed that $BRAF^{V600E}$ mutation was associated with the highest

levels of *DIO3* expression, which was mainly mediated by ERK phosphorylation. In RET/PTC rearrangements manifestation, the *DIO3* levels seems to be mainly regulated according the p38 phosphorylation status. Compared to BRAF-oncogene, RET/PTC rearrangement stimulates *DIO3* at a lower intensity, which is in part explained due a less potent MAPK induction after activation of other pathways such as the PI3K/AKT cascade (29, 30).

SHH signaling is critically important for embryogenesis and other cellular processes, such as proliferation and differentiation (31). Disruption in SHH signaling results in various human diseases and seems to contribute to neoplastic process promotion. The SHH reactivation occurs in up to 25% of human tumors and is associated with D3 induction (5, 7). Previously, Dentice *et al* demonstrated that the SHH pathway directly modulates *DIO3* upregulation in human and mouse BCC (5). Similarly, we observed that the *DIO3* levels were markedly inhibited by the SHH inhibitor, cyclopamine, indicating a direct effect of Shh/GLI1 signaling on gene regulation also in PTC cells. This effect of SHH on *DIO3* modulation was reinforced after that the induction of SHH activation in MTC cells resulted in a significant increase in the *DIO3* levels. Interestingly, our results suggest that there is cooperation between the MAPK and SHH pathways, once the MAPK blockage, by MEK and P38 inhibition, resulted in reduction in the total GLI1 levels. These data indicate that *DIO3* regulation in PTC might be driven by MAPK/SHH cooperation. This cross-regulation has been previously demonstrated in other pathological conditions (32, 33). In pancreatic cancer cells, the oncogenic effects of the oncogene KRAS were demonstrated to be mediated by SHH/GLI1 activation, and suppression of GLI activity led to selective attenuation of the oncogenic transformation activity in mutant KRAS-expressing cells (32). This data set reinforces the hypothesis that the D3 induction caused by MAPK and SHH activation leads to TH inactivation and can contribute to promoting a hypothyroid state at a cellular level.

Studies have suggested that alterations in the TH status might interfere with tumor pathogenesis. Clinical hypothyroidism seems to be a risk factor for several neoplasias, such as liver cancer, thyroid malignancies, high grade glioblastomas and human breast cancer (34-37). Alterations in the balance between TH inactivating (D3) and activating (D2) deiodinases and the consequent intracellular hypothyroidism seem to be critical for modulating the balance between cell proliferation and differentiation. Experimental studies in BCC and in colon tumor cells have shown that the increase in the D3 levels is associated with the induction of cell proliferation; *Dio3* knockdown caused a 5-fold reduction in the growth of xenograft tumor, while the T3 addition promoted differentiation (5, 6). Additionally, studies

in renal cancer have revealed that the loss of *DIO1* expression, mediated by miR-224 induction, resulted in diminishing intra-tumoral T3 concentration while increasing cell proliferation and apoptosis (38). Here, we also demonstrated that the siRNA-mediated *DIO3* gene attenuation caused a reduction in the total cell number as well as in the cyclin D1 levels. Additionally, *DIO3* seems to contribute to cell cycle progression, since its repression caused a partial stop in cell cycle progression in G1 phase. This set of results supports the hypothesis that the local hypothyroidism caused by *DIO3* overexpression could play an important role in tumor growth (5-7).

In conclusion, we have demonstrated that *DIO3* expression is modulated by specific MAPK genetic alterations in PTC. The $BRAF^{V600E}$ mutation seems to be a more potent inducer of *DIO3* than the RET/PTC rearrangement, and this is most likely because of a more potent induction of ERK phosphorylation. Moreover, SHH activation might also be involved in *DIO3* upregulation in PTC, which is most likely due to cooperation with the MAPK pathway. Finally, the reduction in the cell proliferation of D3-depleted PTC cells supports the hypothesis that the intracellular decreases in the thyroid hormone levels might be associated with the induction of tumor growth and might interfere with tumor aggressiveness.

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Declarations of conflicts of interest

The authors declare that there are no conflicts of interest that could be prejudice the impartiality of the reported research.

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Figure 1

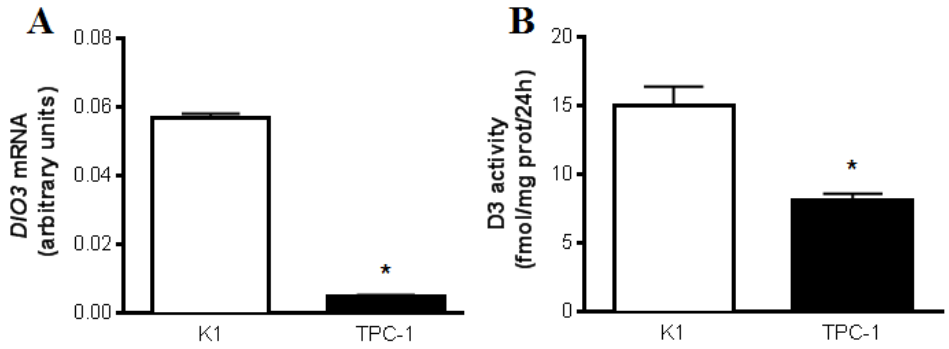

Figure 1 The *DIO3* mRNA levels (A) and activity (B) were readily detected in both PTC cell lines, K1 and TPC-1, and were significantly higher in K1 cells compared to TPC-1 (~5-fold, $p<0.001$; 14.9 *vs.*8.1 fmol/mg.prot.24 hs, $p=0.02$; respectively).

Figure 2

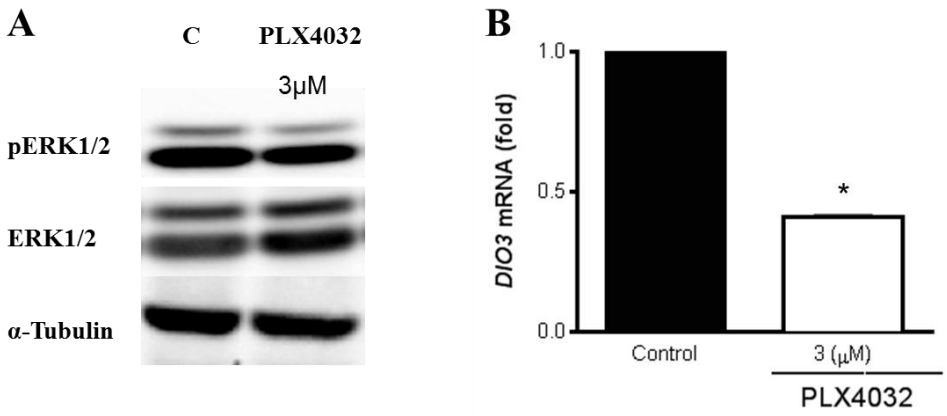

Figure 2 The treatment with the BRAF-mutated specific inhibitor for 24h, PLX4032 (3μM), led to a reduction in the ERK phosphorylation (~30%; A) and was associated with reduction in *DIO3* mRNA (~2.5-fold, $p<0.001$; B).

Figure 3

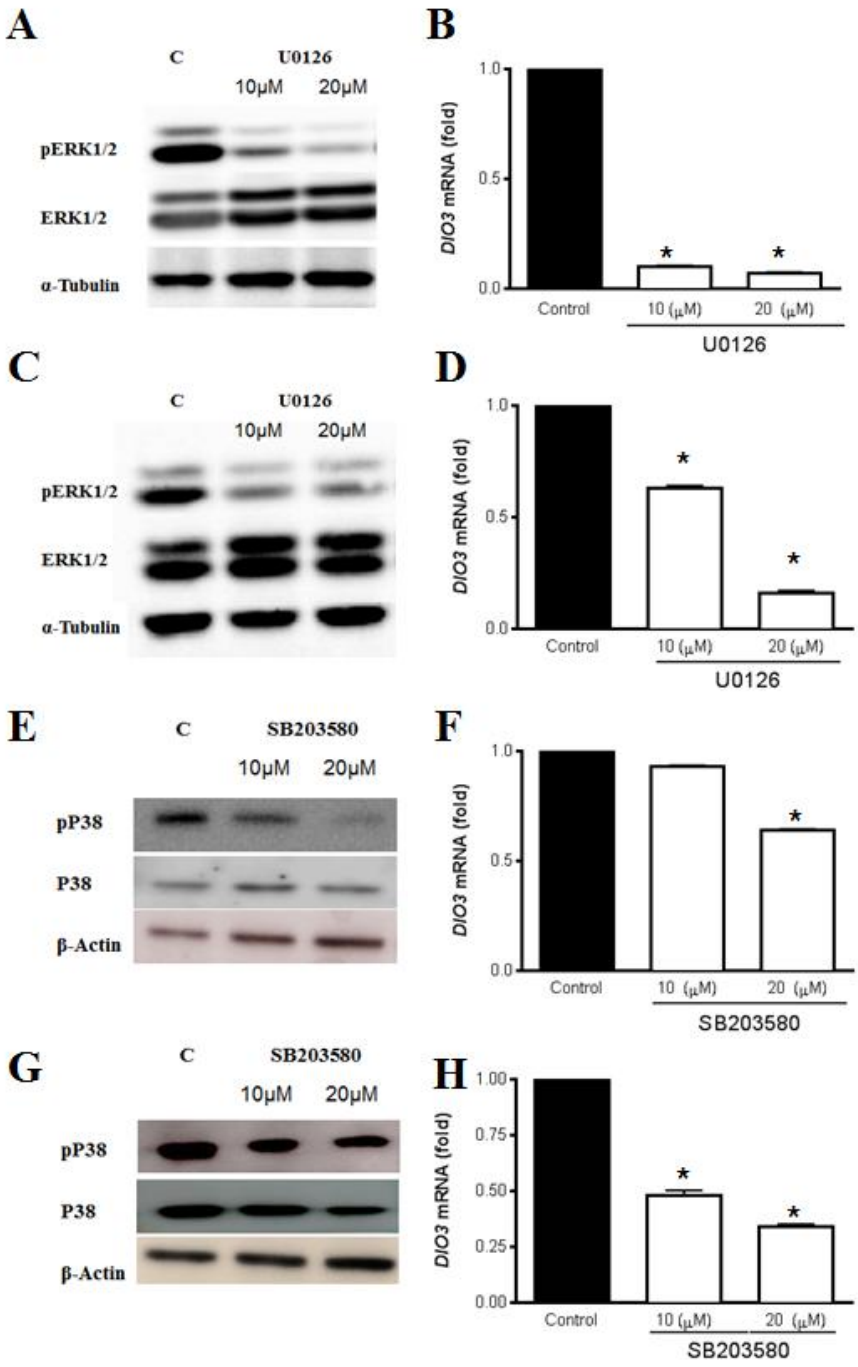

Figure 3 The MEK1 inhibition in K1 and TPC-1 cells, with U0126 incubation (10-20μM), caused a significant block in the phosphorylation of the ERK pathway (A and C) as well as a dose-dependent reduction in the *DIO3* levels (5-10 and 1.5-6-fold; P<0.001; respectively; B and D). The inhibition of p38 protein, by SB203580 (10-20μM), inhibited p38 phosphorylation (E and G) while also reduced the *DIO3* transcripts (~2 and 2-3-fold; P<0.01; F and H).

Figure 4

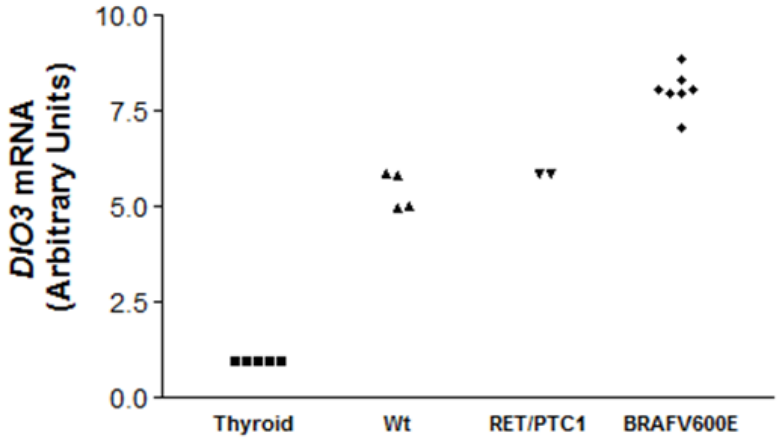

Figure 4 *DIO3* mRNA was significantly increased in PTC samples compared with the surrounding thyroid normal tissue ($p<0.001$). Samples harboring $BRAF^{V600E}$ mutation had higher levels of *DIO3* expression compared with samples presenting RET/PTC1 rearrangement or those without any mutation (8 *vs.* 5.8 *vs.* 5.4AU, respectively; $P<0.001$; Figure 4).

Figure 5

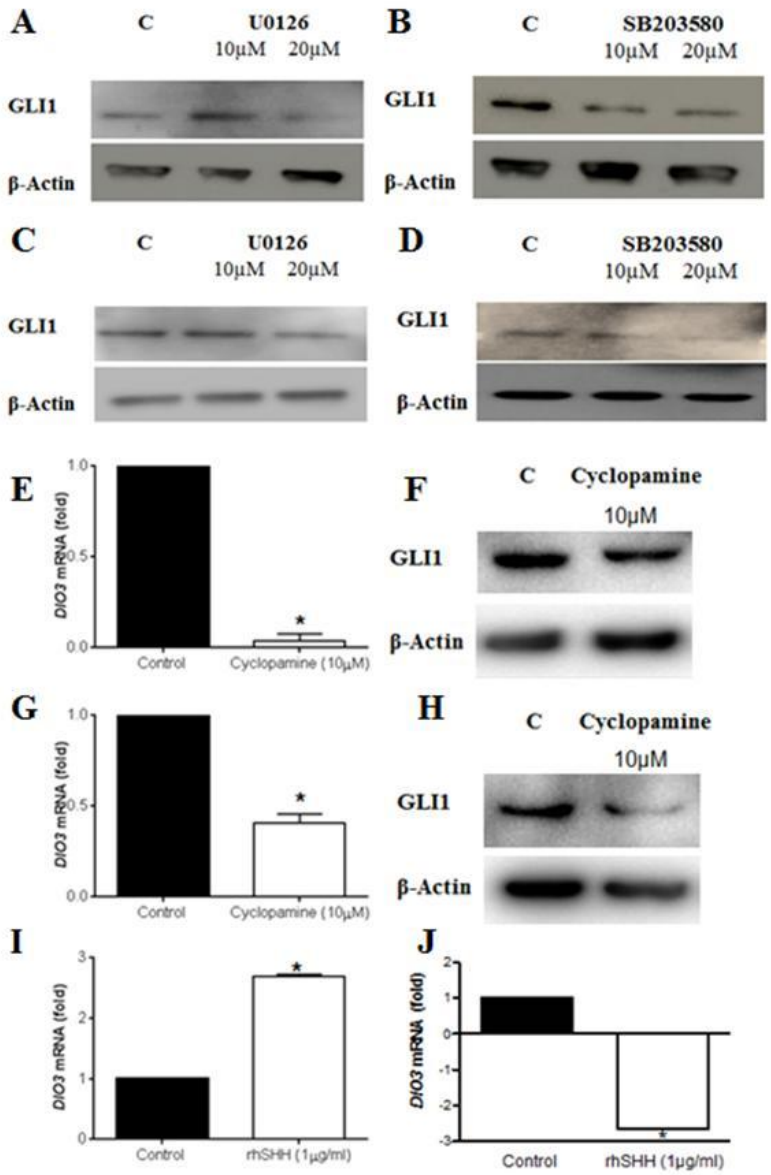

Figure 5 Inhibition of MAPK proteins, MEK and p38, reduced the levels of GLI1 protein in K1 and TPC-1 cells (A-D). Incubation of K1 and TPC-1 cells with a chemical inhibitor, cyclopamine (10 μM) diminished GLI1 protein (E and G) and considerably decreased the D3 levels in both cells (~12 and 2.5-fold; P<0.001; F and H; respectively). Next, TT cells (MTC cell line) was treated for 24h with 1 μg/ml of recombinant SHH which resulted in induction of *DIO3* mRNA (2.5-fold; P<0.0001; I) while reduced the *DIO2* levels in similar intensity (2.6-fold; P<0.001; J).

Figure 6

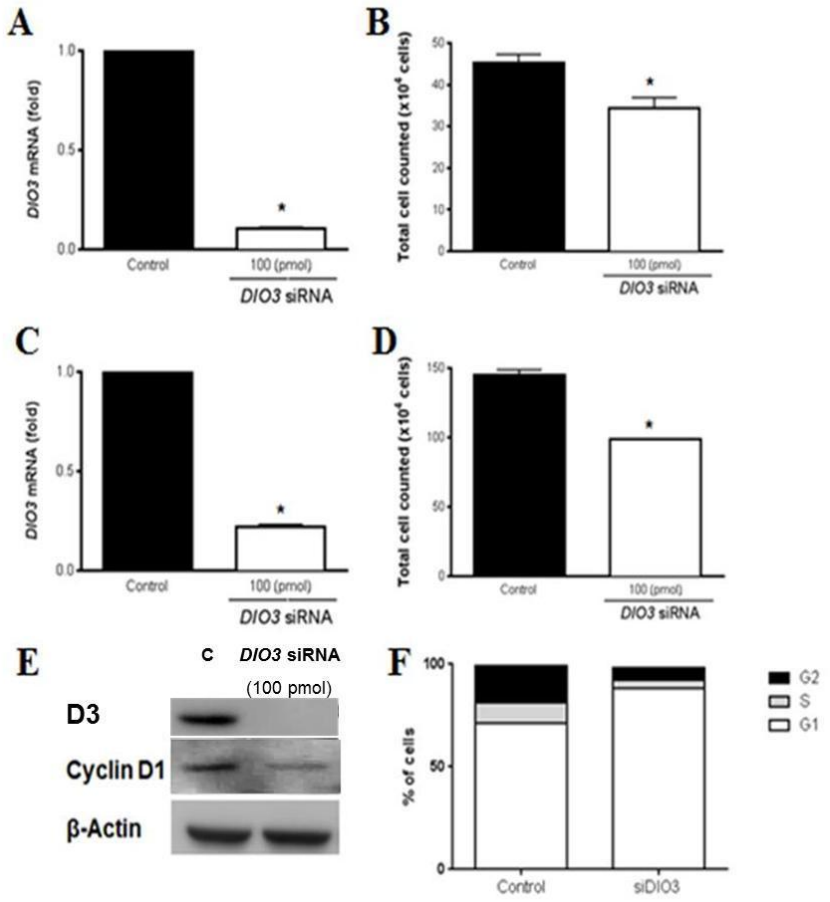

Figure 6 *DIO3* gene knockdown resulted in a 90% blockage of *DIO3* transcripts in K1 and TPC-1 cells (A and C). Moreover, the reduction in *DIO3* levels was also associated with a significant reduction in the absolute cell number compared with K1 and TPC-1 controls (~30%; B and D). Studies in K1 cells demonstrated that the *DIO3* silencing was associated with a reduction in cyclin D1 protein (E) while increased the proportion of cells in G1 phase of cell cycle (F).

CONCLUSÃO

No presente estudo demonstramos que alterações genéticas específicas na via de sinalização MAPK, modulam a expressão da D3 no CPT. A mutação $BRAF^{V600E}$ apresenta o efeito mais potente na indução da D3, quando comparado aos rearranjos RET/PTC ou ausência de alteração conhecida. A diferença na intensidade da indução, ocorre provavelmente devido aos diferentes níveis de indução da fosforilação da proteína ERK. Adicionalmente, a via de sinalização Sonic Hedgehog também desempenha papel determinante na regulação dos níveis da D3 no CPT, uma vez que a inibição da proteína GLI1, um dos principais efetores na sinalização desta via, foi capaz de reduzir substancialmente os níveis da D3. Além disso, demonstramos que a cooperação entre a via MAPK e SHH pode ser determinante na regulação da D3 no CPT. De forma interessante, observamos que o silenciamento do gene *DIO3* foi capaz de reduzir significativamente a proliferação celular das células malignas tireoidianas, através da redução dos níveis do regulador do ciclo celular, ciclina D1 e devido a uma parada de ciclo celular na fase G1.

Estes dados em conjunto sugerem que a D3 pode exercer um papel importante na proliferação celular, possivelmente devido ao hipotireoidismo intracelular gerado, o que poderia contribuir para o crescimento e agressividade tumoral. Além disso, esses resultados ampliam os conhecimentos sobre os eventos fisiopatológicos envolvidos na tumorigênese em humanos, com possibilidade de avanço em novas estratégias terapêuticas.

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Anexo A

Fluxogram de avaliação das amostras de CPT para análise das alterações genéticas, $BRAF^{V600E}$ e rearranjo RET/PTC1.