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EXPRESSÃO E REGULAÇÃO DA ENZIMA DESIODASE TIPO 3 EM NEOPLASIAS MAMÁRIAS

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2019

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.

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Orientadora: Profa. Dra. Ana Luiza Maia

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"*Todos os problemas da humanidade decorrem da incapacidade do homem de ficar quieto em uma sala sozinho*."

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- **Artigo de revisão:** Role of thyroid hormones in the neoplastic process: an overview; publicado no Endocrine-Related Cancer. 2017 Nov; 24(11):R367-R385. doi: 10.1530/ERC-17-0192. Impact Factor 5.331

- **Artigo de revisão com dados originais:** Current concepts and challenges to unravel the role of iodothyronine deiodinases in human neoplasias; publicado no Endocrine-Related Cancer. 2018 Dec 1;25(12):R625-R645. doi: 10.1530/ERC-18-0097. Impact Factor 5.331

- **Artigo original:** Decreased expression of the thyroid hormone-inactivating enzyme type 3 deiodinase is associated with lower survival rates in breast cancer

Dados preliminares do artigo original da presente tese foram apresentados e/ou aceitos para apresentação nos seguintes eventos científicos:

- XVII Encontro Brasileiro de Tireoide, 2016, Gramado/RS

  Expressão da desiodase tipo 3 no câncer de mama

- XVI Latin American Thyroid Congress, 2017, Rio de Janeiro/RJ.

  The type 3 deiodinase is highly expressed in breast cancer

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- XX Congresso Brasileiro de Oncologia Clínica, 2017, Rio de Janeiro/RJ

  A desiodase tipo 3 está hiperexpressa no câncer de mama

- Endo 2018, 2018, Chicago/EUA

  The Role of Type 3 Deiodinase Expression in Breast Cancer

- AACR (American Association of Cancer) Annual Meeting, 2019, Atlanta/EUA

  Loss of deiodinase type 3 expression distinguishes patients with poor prognosis in breast cancer

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Além dos artigos que fazem parte da presente tese, ao longo do período de doutoramento participei como autor/co-autor das seguintes publicações:

Cartas:

- PCSK9 Inhibitors and Cardiovascular Events. Goemann IM, Londero TM, Dora JM. New England Journal of Medicine (NEJM). 2015 Aug 20;373(8):773-4. doi: 10.1056/NEJMc1508222. Impact Factor: 79.258
- Cardiometabolic Effects of CASCADE Trial Explained by Mediterranean Diet. Moreira AM, Londero TM, Goemann IM, Schaan BD. Annals of Internal Medicine. 2016 Apr 19;164(8):573-4. doi: 10.7326/L16-0015_1. Impact Factor: 19.384

Capítulos de livro:

- Goemann IM, Kramer, CK, Schaan BD. Feocromocitoma. In: Barros, E; Albuquerque GC; Xavier, RM e organizadores. Laboratório na Prática Clínica - Consulta Rápida – 3 ed. Porto Alegre: Artmed, 2016. cap. 23, p. 188-193.
- Goemann IM, Gerchman, F. Dislipidemias. In: Silvveiro, SP, Satler, F e colaboradores. Rotinas em Endocrinologia – 1 ed. Porto Alegre: Artmed, 2015. cap 14, p. 74-90.

Anais de Congresso:

- Londero TM, Moreira AMS, Garcia, SP, Costenaro, F, Goemann IM, Cipriani GF, Viecceli C, Rodriges TC, Czepielewski MA. Is cushing's syndrome remission associated with diabetes regression? Analysis of retrospective cohort of 108 patients with cushing's disease. Diabetology & Metabolic Syndrome 2015, 7(Suppl 1):A106. doi:10.1186/1758-5996-7-S1-A106. Impact Factor 2.413
- Viecceli, C, Garcia SP, Londero TM, Moreira AMS, Goemann IM, Cipriani, GF, Zelmanovitz, T. The ketosis-prone diabetes diagnosis dilemma-a case report. Diabetology & Metabolic Syndrome 2015, 7(Suppl 1):A104. Impact Factor 2.413
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LISTA DE ABREVIATURAS E SIGLAS

3D	3 dimensões
5-FU	5-fluorouracil
αvβ3	Integrin receptor
ANOVA	Análise de variância
AJCC	American Joint Committee on Cancer
BCC	Basal cell carcinoma
cAMP	Adenylyl cyclase
ccRCC	Clear cell renal cell carcinoma
cDNA	Complementary DNA
CI	Confidence interval
CoA	Coactivator
CO	Colonic organoid
CoR	Correpressor
COX	Cyclooxygenase
CRC	Colorectal cancer
CPM	Counts per million
CSC	Cancer stem cells
DC	Dendritic cell
DCIS	Ductal carcinoma *in situ*
DEX	Dexamethasone
DGE	Differential Gene Expression
DIO1, D1*	Type 1 deiodinase, desiodase tipo 1
DIO2, D2*	Type 2 deiodinase, desiodase tipo 2
DIO3, D3*	Type 3 deiodinase, desiodase tipo 3
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECM	Extracellular matrix
ER	Estrogen receptor
FDR	False discovery rate
FFPE	Formalin-fixed paraffin-embedded
FTC	Follicular thyroid carcinoma
GC-1	A thyroid hormone receptor β-selective agonist
GEPIA	Gene Expression Profiling Interactive Analysis

GH	Growth hormone
GTEx	Genotype-Tissue Expression
HCC	Hepatocarcinoma
HE	Haematoxylin-eosin
hESC	Human embryonic stem cell
hPSCs	Human pluripotent stem cells
HR	Hazard ratio
IDC	Invasive ductal carcinoma
ILC	Invasive lobular carcinoma
IRD	Inner ring deiodination
iPSCs	Induced pluripotent stem cells
K_m	Michaelis constant
KO	Knock-out
MCL	Myeloid cell leukemia
mESC	Mouse embryonic stem cells
miRNA	Micro ribonucleic acid
MMP	Metalloproteinase
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cells
MTC	Medullary thyroid carcinoma
ORD	Outer ring deiodination
OS	Overall survivall
PAM50	The Prosigna Breast Cancer Prognostic Gene Signature Assay
PCR	Polimerase chain reaction
PR	Progesteron receptor
PRL	Prolactin
PTC	Papillary thyroid carcinoma
Prx	Peroxiredoxin
PTU	6-propyl-2-thiouracil
RNA	Ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
rT_3	Reverse triiodothyronine, 3,3′,5′-triiodothyronine
RV	Resveratrol
Sec	Selenocysteine residue

SECIS	Sec insertion sequence
siRNA	Small interfering RNA
shRNA	Short Hairpin RNA
Src	Tyrosine-protein kinase
T_2	3,3′-diiodothyronine, 3,3′-diiodotironina
T_3	Triiodothyronine, triiodotironina
T_4	Thyroxine, 3,3′,5,5′-tetraiodothyronine, tiroxina
Tcf	T-cell factor
TCGA	The Cancer Genome Atlas
TCL	T-cell lymphomas
Tet	Tetracycline
TH	Thyroid hormone
TPA	12-O-tetradecanoyl-phorbol-13-acetate
TR	Thyroid hormone receptor
TRE	Thyroid hormone response element
TSH	Thyroid stimulating hormone, thyrotropin, tireotropina
UTR	Untranslated region

*Durante o período de doutoramento houve uma tendência na literatura para a modificação do nome das enzimas desiodases 1,2 e 3, de D1/D2/D3 para DIO1/DIO2/DIO3 a fim de entrar em conformidade com as normas atuais do HUGO Gene Nomenclature Committee e UniProt (http://www.uniprot.org). Assim, no primeiro artigo o leitor encontrará a nomenclatura antiga das enzimas referidas, enquanto nos dois últimos a nomenclatura já encontra-se de acordo com as diretrizes atuais.

RESUMO

O câncer de mama é uma doença altamente heterogênea, sendo que a identificação de biomarcadores que predigam o comportamento biológico do tumor contribuem para definição do prognóstico e estratégica terapêutica. Os hormônios tireoidianos (HT) são reguladores essenciais de diversos processos celulares, e alterações no *status* do HTs interferem na progressão tumoral através de virtualmente todos os marcos do câncer ("*hallmarks of cancer*"). Estudos clínicos têm associado os níveis de HTs a risco de desenvolvimento de câncer de mama, enquanto estudos *in vitro* têm demonstrado que os HTs influenciam a proliferação, apoptose e migração de células tumorais mamárias. A enzima desiodase tipo 3 (DIO3) é a principal enzima na inativação dos hormônios tireoidianos, e alterações na expressão dessa enzima tem sido descritas em diversas neoplasias humanas.

Na primeira parte desta tese, o leitor encontrará um artigo de revisão sobre o papel dos hormônios tireoidianos no processo neoplásico e seus efeitos sobre cada *hallmark* do câncer. Na segunda parte, é apresentado um levantamento de dados originais e revisão sobre a expressão das desiodases - enzimas que ativam e inativam os hormônios tireoidianos – em diferentes neoplasias humanas, e seu potencial efeito sobre o processo tumoral. Na terceira parte, é apresentado o artigo original desta tese, com objetivos, metodologia, resultados e discussão dos mesmos.

O objetivo deste trabalho foi avaliar a expressão e valor prognóstico da DIO3 em câncer de mama em humanos. Para isso foram utilizadas duas coortes retrospectivas de pacientes com câncer de mama. A expressão da enzima DIO3 foi avaliada através de técnica de imunohistoquímica em tecido de mama de 53 pacientes e quantificada através de H-Score em uma coorte primária. Subsequentemente, os resultados foram validados em uma segunda coorte de 1094 pacientes com câncer de mama utilizando-se dados de *RNA sequencing* (RNA-Seq*)* da base de dados *The Cancer Genome Atlas* (TCGA). Em ambas as populações, os dados de expressão foram correlacionados com dados clínico-patológicos dos pacientes, a significância prognóstica da expressão da enzima foi avaliada através de regressão de Cox e a avaliação de sobrevida foi realizada por método de Kaplan-Meier. O padrão de metilação de DNA da região genômica do gene *DIO3* em mama foi analisado utilizando-se dados clínicos e de metilação de DNA de 890 pacientes provenientes da base de dados do TCGA. Adicionalmente, a regulação da enzima foi avaliada em linhagens celulares derivadas de câncer de mama (células MCF-7 e MDA-MB-231).

A expressão proteica de DIO3 foi encontrada em 35/39 (89.7%) das amostras de carcinoma ductal invasor, com H-Score médio de 104.9 ± 55, e em apenas uma amostra de três analisadas de carcinoma lobular invasor (H-Score=86). O mRNA do gene *DIO3* está expresso em tecido mamário normal e tumoral, com expressão de mRNA reduzida em tumores em relação a tecido normal (logFC =-1.54, P ajustado <0.00001). A intensidade de expressão de DIO3 não se correlacionou com características clínico-patológicas dos pacientes na coorte primária, como tamanho tumoral, presença de metástase linfonodal ou à distância, positividade para receptor de estrógeno (RE), receptor de progesterona (RP) ou receptor epidérmico humano 2 do fator de crescimento (HER2). Entretanto, na mesma coorte, em análise univariada utilizando-se mortalidade como desfecho primário, a negatividade para expressão da DIO3 se associou a maior risco de morte (HR 4.29 [IC 95%, 1.24-14.7] P=0.021), sendo que pacientes com ausência de expressão de DIO3 tiveram menor sobrevida em relação à pacientes que expressavam DIO3 (73.3 meses [IC 95%, 41 a 105] vs. 122 meses [IC 95%, 109 a 135]; log-rank P=0.012). Validamos estes achados na segunda coorte (N=1094), onde a baixa expressão do gene *DIO3* se correlacionou com maior tamanho tumoral (P=0.019) e negatividade para RE (P=0.022). Confirmando os achados da coorte primária, baixa expressão de *DIO3* se associou a menor sobrevida global (HR 1.6 [IC 95% 1.18-2.26]; P=0.003) em modelo univariável e se manteve como preditor independente de prognóstico em modelo multiváriavel ajustado para idade, tamanho tumoral, presença de metástase linfonodal e à distância, status de RE e RP (HR 1.55 [IC 95% 1.07-2.24]; P=0.02). A sobrevida global em 5 anos foi de 90.4% (IC 95%, 86.4%-94.5%) no grupo com alta expressão de *DIO3* e 77.4% (IC 95%, 71.3%-84.1%) no grupo com baixa expressão.

A análise de metilação de DNA revelou que a região do gene *DIO3* encontra-se hipermetilada em tecido tumoral relação ao tecido normal (p<0.0001), em especial os sítios CpGs localizados na região promotora do gene.

A análise da regulação de *DIO3* em linhagem celulares MCF-7 e MDA-MB-231 demonstrou indução do mRNA de *DIO3* quando ambas as linhagens celulares foram submetidas a tratamento com 10 nM de triiodotironina (T_3) por 24h. Além disso, ocorreu inibição dose-dependente do mRNA quando as células MCF-7 foram tratadas com dexametasona em doses de 10 e 100 nM, efeito que não se observou em células MDA-MB-231. A inibição da via *mitogen-activated protein kinase* (MAPK) com utilização do inibidor MEK-específico U0126 (10 uM) levou à redução de 50% na expressão de mRNA de *DIO3* (P=0.004) em células MCF-7.

Em conclusão, nossos resultados indicam que a enzima DIO3 encontra-se expressa em tecido mamário normal e em câncer de mama. De modo interessante, a diminuição ou perda expressão de *DIO3*/DIO3 foi fator independente para menor sobrevida em duas populações distintas. A redução da expressão da DIO3 em câncer de mama pode ser explicada ao menos em parte por hipermetilação da região promotora do gene neste tipo tumoral. Em linhagem celular MCF-7, a enzima mantém suas características de regulação pré-transcricional por T_3, dexametasona e modulação pela via da MAPK. Esses resultados apontam para a DIO3 como marcador prognóstico em câncer de mama, sendo a redução de sua expressão associada a pior sobrevida.

ABSTRACT

Breast cancer is a highly heterogeneous disease and the identification of biomarkers that predict tumor biological behavior is warranted in improving patient survival. Thyroid hormones (THs) are critical regulators of cellular processes, and TH status alterations are known to contribute to cancer progression through all the hallmarks of cancer. Clinical studies associate THs levels with breast cancer mortality, and THs have been shown to influence breast cancer proliferation, apoptosis, and migration in *in vitro* models. Type 3 deiodinase (DIO3) is the main enzyme responsible for TH inactivation and disturbed DIO3 expression has been demonstrated in several human neoplasias.

In the first part of this thesis, the reader will find a review article concerning the role of the thyroid hormones in the neoplastic process and their effect on each hallmark of cancer. In the second part, we present original data and a review on current evidence of deiodinases – enzymes that activate and inactivate thyroid hormones - expression in human neoplasias, as well as their potential role in the neoplastic process. In the third part, we present the main aim of this thesis, our methods, results, and their discussion.

We aimed to evaluate expression patterns and the prognostic significance of DIO3 in breast cancer in humans. The expression of DIO3 was evaluated through immunohistochemistry in a primary cohort of 53 samples of breast tissue and quantified by the H-Score method. Subsequently, these results were validated in a second cohort of 1094 patients using the RNA sequencing (RNA-Seq) data from The Cancer Genome Atlas (TCGA) database. We assessed DIO3 expression in both populations according to retrieved clinicopathological information. The prognostic value of DIO3 expression was evaluated through Cox regression analysis, and survival analysis was assessed by the Kaplan-Meier method. DNA methylation and clinical data for 890 samples from the TCGA study were obtained to evaluate levels of methylation of the *DIO3* gene region in breast cancer. We also evaluated *DIO3* regulation in breast cancer cell lines MCF-7 and MDA-MB-231.

DIO3 protein expression was present in both normal and tumoral breast glandular tissue. DIO3 expression in FFPE tissues of breast cancer was positive in 35/39 (89.7%) of Invasive Ductal Carcinoma (IDC), with a mean H-Score of 104.9 ± 55, and only in 1 of 3 samples of invasive lobular carcinoma (ILC) (H-Score=86). *DIO3* mRNA expression was found to be reduced in tumor samples when compared to healthy tissue, (logFC =-1.54, adjusted $P<0.0001$). DIO3 staining intensity did not correlate with clinicopathologic

characteristics in the primary cohort such as tumor size, the presence of lymph node or distant metastasis, estrogen or progesterone receptor positivity or HER2 positivity. However, the univariate analysis with overall survival (OS) as the primary outcome, loss of DIO3 expression was associated with increased mortality (HR 4.29 [95% CI, 1.24-14.7] P=0.021). Patients with negative DIO3 expression had worse OS than those positive DIO3 expression (73.3 months [95% CI, 41 to 105)] vs. 122 months [95% CI, 109 to 135]; log-rank P=0.012). We then validated this finding in the second cohort (N=1094). Interestingly, low *DIO3* expression was associated with greater tumor size (P=0.019) and estrogen receptor negativity (P=0.022), Confirming our results in the primary cohort, low *DIO3* expression was associated with worse overall survival in a univariate model (HR 1.6 [95% CI, 1.18-2.26]; P=0.003) and remained as an independent prognostic factor in a multivariate model adjusted for age, tumor size, lymph node and distant metastasis, ER and PR status (HR 1.55 [95% CI, 1.07-2.24]; P=0.02). The estimated rate of overall survival at five years in the Kaplan–Meier analysis was 90.4% (95% CI, 86.4% - 94.5%) in the high *DIO3* group and 77.4% (95% CI, 71.3% - 84.1%) in the low *DIO3* group. DNA methylation analysis revealed that *DIO3* gene promoter is hypermethylated in tumoral samples when compared to normal tissue (p <0.0001).

Additional experiments were performed to determine *DIO3* regulation in breast cancer cells. In MCF-7 and MDA-MB-231 cells, *DIO3* was subject to T_3 stimulation (10 nM). We observed a dose-dependent inhibition of *DIO3* when MCF-7 cells were treated with dexamethasone 10 and 100 nM, an effect that was not observed in MDA-MB-231 cells. Also in MCF-7 cells, mitogen-activated protein kinase (MAPK) pathway inhibition using specific MEK inhibitor U0126 (10 uM) resulted in 50% reduction of *DIO3* expression (P=0.004).

In conclusion, our results demonstrate that DIO3 is expressed in normal and tumoral breast tissue. We showed that low DIO3 expression was an independent factor associated with reduced overall survival in two different populations of breast cancer. Loss of DIO3 expression in breast cancer can be explained at least in part by hypermethylation of the promoter region of the gene. The enzyme maintains its regulation by T_3, dexamethasone and it is subject to MAPK modulation in MCF-7 cells. In summary, our results point to DIO3 as a new prognostic marker in breast cancer, as loss of its expression is associated with reduced overall survival.

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Parte I

Role of thyroid hormones in the neoplastic process: an overview

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TITLE: ROLE OF THYROID HORMONES IN THE NEOPLASTIC PROCESS: AN OVERVIEW

SHORT TITLE: THYROID HORMONES AND NEOPLASIAS

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ABSTRACT

Thyroid hormones (TH) are critical regulators of several physiological processes, which include development, differentiation, and growth in virtually all tissues. In past decades, several studies have shown that changes in TH levels caused by thyroid dysfunction, disruption of deiodinases and/or thyroid hormone receptor (TR) expression in tumor cells, influence cell proliferation, differentiation, survival, and invasion in a variety of neoplasms in a cell type-specific manner. The function of THs and TRs in neoplastic cell proliferation involves complex mechanisms that seem to be cell-specific, exerting effects via genomic and non-genomic pathways, repressing or stimulating transcription factors, influencing angiogenesis and promoting invasiveness. Taken together, these observations indicate an important role of TH status in the pathogenesis and/or development of human neoplasia. Here, we aim to present an updated and comprehensive picture of the accumulated knowledge and the current understanding of the potential role of TH status on the different hallmarks of the neoplastic process.

INTRODUCTION

The association between thyroid hormone (TH) status and cancer was reported as early as 1896, when Beatson used thyroid extract as a potential treatment for breast cancer [1]. Since then, an impressive expansion of knowledge has established THs as key regulators of several physiological processes, including the embryonic development, growth, and metabolism of virtually all tissues [2]. Additionally, recent data have demonstrated critical roles of THs in cell proliferation, differentiation, and survival [3; 4; 5; 6; 7; 8].

The human thyroid gland mainly secretes thyroxine (T4), but the active hormone, triiodothyronine (T3), mediates most of the hormonal actions. The main pathway for the production of the bioactive form in peripheral tissues occurs via outer ring deiodination of T4 through the action of iodothyronine deiodinase types 1 and 2 (*DIO1;* D1 and *DIO2;* D2). In contrast, type 3 iodothyronine deiodinase (*DIO3*; D3) is mainly responsible for TH inactivation via inner-ring deiodination of both T4 and T3 [9]. Intracellular T3 bioavailability is controlled in a tissue-specific manner, depending mainly on its activation by D2 and inactivation by D3. Notably, proper deiodinase function depends on the availability of a yet unidentified thiol cofactor that acts as a reducing agent during the catalysis [10]. Conditions that result in dysregulation of the intracellular redox state possibly interfere with endogenous cofactor(s) levels, thereby impairing deiodinase activity [11].

THs exert their effects through genomic (nuclear) and nongenomic (cytoplasmic or membrane TH receptor (TR)) pathways. The genomic mechanisms are mediated mostly by T3 through nuclear TRs. The TRα and TRβ genes encode the TH-binding TR isoforms TRα1 and TRβ1-β3 [12]. T3 binds to nuclear TRs that activate the transcription of target genes by binding to TH response elements (TREs) located in the regulatory regions. Gene transcription is regulated by an exchange of corepressor (CoR) and coactivator (CoA) complexes. Negative TREs (nTREs) can mediate ligand-dependent transcriptional repression. However, in this case, the roles of CoAs and CoRs are not well defined [2]. The nature of the transcriptional response is determined by cell type and hormone status [13; 14]. On the other hand, the nongenomic effects are initiated by TH binding to integrin αVβ3 receptor, which leads to the activation of different signaling pathways, including mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), signal transducers and activators of transcription (STAT) pathways. These cascades result in distinct cellular events, such as cell division, proliferation, and angiogenesis [15; 16; 17; 18; 19].

In past decades, several clinical studies have indicated that an altered TH status might be a risk factor for the development of tumors, such as liver, breast, colon, prostate and thyroid malignancies [20; 21; 22; 23; 24; 25; 26; 27]. However, other studies have described TH alterations as clinically favorable, such as hypothyroidism for high-grade glioblastomas [28]. Several *in vitro* and *in vivo* studies have demonstrated that THs influence a myriad of oncological events and control the balance between proliferation and differentiation, which is one of the most important hallmarks of TH action in cancer cells [3; 29; 30]. Changes in TH levels caused by thyroid dysfunction or the disruption of deiodinases and/or TR expression in tumor cells influence cell proliferation, differentiation, survival and invasion in a variety of neoplasms in a cell type-specific manner [31; 32; 33]. The function of THs and TRs in neoplastic cell proliferation involves complex mechanisms that seem to be cell type-specific, exerting effects via distinct pathways, repressing or stimulating transcription factors, influencing angiogenesis and promoting invasiveness [2; 29]. Here, we aim to present an updated picture of recent advances in the current understanding of the potential effects of TH status on the different hallmarks of the neoplastic process.

1. Overview of the neoplastic process

The hallmarks of the neoplastic process include sustained proliferation signaling, resistance to growth suppressors, evasion of programmed cell death, replicative immortality, sustained angiogenesis and promotion of invasion and metastasis [34]. In the past decade, two emerging characteristics have extended our understanding of this process: reprogramming energy metabolism and evasion from immune destruction, both contributing to a favorable tumor microenvironment [35; 36; 37].

The acquisition of multiple cancer hallmarks depends on a succession of alterations in the cellular genome [35]. Alterations affecting the DNA-maintenance machinery, such as defects in genes involved in the detection and repair of DNA damage, or tumor suppressor genes, have been associated with the progression of the neoplastic process [38; 39; 40; 41].

Solid tumors can also recruit new blood vessels through the secretion of angiogenic factors. Vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF; FGF2) and platelet-derived growth factor (PDGF) are examples of molecules that promote the proliferation and migration of vascular endothelial cells and can severely constrain angiogenesis and tumor growth [42; 43].

Programmed cell death is a natural mechanism that is as important for healthy tissue growth as controlled cell proliferation. In order to grow indefinitely, cancer cells must overlap apoptosis mechanisms, disabling the cellular apoptosis-inducing circuitry. The intracellular apoptotic machinery depends on a family of proteolytic enzymes called caspases, which participate in a process that can be initiated by either extracellular or intracellular death signals. Caspase activation is tightly regulated by members of the B-cell lymphoma 2 (BCL2) and inhibitors of apoptosis proteins families, proteins that can either be pro- or anti-apoptotic [44; 45].

Another distinct attribute of cancer cells that is functionally important for tumor development involves major reprogramming of the cellular energy metabolism to support continuous cell growth and proliferation, replacing the metabolic program that operates in most normal tissues [46]. Neoplastic cells typically generate more reactive oxygen species (ROS) than normal cells, a mechanism that can be partially explained by oncogenic signaling and downregulated mitochondrial function [47; 48]. ROS promote DNA damage and signaling mediation, and their presence may contribute to the transformation of cells [49].

More recently, disruption of the mechanisms involved in cellular autophagy has emerged as a new hallmark of cancer [50]. Controlled autophagy prevents intracellular components, such as proteins, lipids, and organelles, from accumulating, which can be harmful to cells [51].

As the effects of THs on these processes are variable and complex, we comprehensively organized our review according to the cancer hallmarks described above (Figure 1). The emerging effects of TH analogs on tumorigenesis and the disruption of signaling caused by TR mutations have been discussed elsewhere [43; 52; 53; 54; 55; 56] and are not included in this review.

2. The roles of THs on the cellular hallmarks of cancer

2.1. TH effects on sustained proliferative signaling pathways

A vital capacity acquired by cancer cells involves their ability to sustain chronic proliferation through different pathways [45; 57; 58; 59; 60]. THs influence cell growth, acting either as growth factors or as cell growth inhibitors through several proliferation pathways.

Davis and colleagues (1999) demonstrated for the first time the nongenomic actions of THs in the induction of the MAPK pathway in HeLa and CV-1 cells [61]. T4 promotes the phosphorylation of MAPK and the co-immunoprecipitation of nuclear tyrosine

phosphorylated MAPK with STAT-1a and STAT-3 [62]. This effect causes the MAPK-mediated serine phosphorylation of TRβ1, which dissociates the TRβ1 and the co-repressor silencing mediator for retinoid receptors or TRs, thus affecting the nuclear receptor via a mechanism independent of the binding of T3 to TRβ1 [63]. For this process to occur, a cell membrane T4 receptor is required. Later, the same group showed that a member of the plasma membrane heterodimeric integrin protein family, integrin αVβ3, binds T4 preferentially over T3 [17]. Presently, most of the nongenomic effects of THs are known to be mediated by activation of the integrin αVβ3 receptor, which sends several survival mechanism signals to the cell, including the stimulation of ERK- and AKT-dependent pathways [19].

MAPK pathway

The activation of MAPK (ERK1/2) by physiological levels of T4 influences tumor proliferation, as has been demonstrated in glioma [64], follicular thyroid carcinoma (FTC) and papillary thyroid carcinoma (PTC) [18], undifferentiated pheochromocytoma [65], and myeloma [66] (Figure 2). In human breast cancer cells, T4 induces proliferation nongenomically, requiring ERK1/ERK2 and phosphorylating the estrogen receptor alpha (ERα). This observation highlights the crosstalk between THs and estrogen signaling pathways in certain cancer cells, culminating in specific intranuclear events [67]. Another example of THs and estrogen crosstalk is the induction of proliferation in human lung cancer cells, which is initiated via the cell surface integrin αVβ3 [68].

T3 also activates MAPK nongenomically but only at supraphysiological levels [63; 69]. Studies in glioma cell lines have shown that T3 suppresses proliferation and induces redifferentiation in a mechanism independent of ERK 1/2 activation, suggesting a potential role of TRα1 [70]. In contrast, other studies have demonstrated that both T4 and T3 induce cell proliferation in glioblastoma and pheochromocytoma cells via ERK1/2 pathway activation [7; 65]. In ovarian tumor cells, physiological concentrations of T3 and T4 induce MAPK-dependent cell proliferation and support cell survival in a process that requires an intact TH-integrin interaction for ERK activation [71].

The interaction between THs and the RAS signaling pathway also deserves attention due to its important role in carcinogenesis. RAS proteins act as key membrane signaling mediators by transferring information from this cellular compartment to the nucleus. RAS activates several pathways to regulate cell growth, survival, differentiation, and angiogenesis; MAPK is a key downstream target of these pathways [72]. Activating mutations in *RAS* genes and the consequent aberrations in the expression of the RAS-MAPK complex are implicated in

several human cancers [73; 74]. Cyclin D1, which is critical for cell cycle progression, is one of the main elements mediating the proliferative effects of RAS oncogenes [75]. T3, acting through TRα1 and TRβ1, not only blocks the RAS-mediated proliferation of neuroblastoma cells via the regulation of cyclic AMP response elements but also represses their transcriptional activity, thus reducing the cyclin D1 levels and consequently the cell proliferation [76]. Studies performed using hepatocarcinoma (HCC) cells and breast cancer cells originally lacking TRs have shown that the reexpression of TRβ1 abolishes tumor growth and migration [77] while preventing tumor formation by RAS-transformed cells in nude mice, even under hypothyroid conditions [52]. In neuroblastoma (Neuro-2a) cells overexpressing TRβ1, T3 treatment blocks cell proliferation through an arrest of cells in G0/G1 and induces morphological and functional cell differentiation through acetylcholinesterase activity [78]. Taken together, these data indicate that a loss of the expression and/or function of TRs could result in a selective advantage for malignant transformation in RAS-dependent tumors.

PI3K/protein kinase B pathway

The PI3K/protein kinase B (AKT) pathway also plays a pivotal role in the regulation of cell growth and proliferation and its deregulation contributes to cellular transformation in a variety of neoplasms [79; 80]. Several nongenomic and genomic TH actions in tumors occur via the PI3K pathway. Incubation of endothelial cells with T3 increases the association of TRα1 with the p85α subunit of PI3K by non-transcriptional mechanisms, leading to the phosphorylation and activation of AKT [81]. Notably, in a mouse model of FTC, a TRβ mutant can activate the PI3K regulatory subunit p85α, affecting signaling in both the nuclear and extranuclear compartments [80]. Experimental data obtained using PTC and neuroblastoma cell lines show that T3 promotes the activation of ERK, AKT, and Src. T3 can also induce AKT phosphorylation nongenomically through TRβ1 [82; 83]. In insulinoma cell lines (rRINm5F and hCM) that express TR isoforms TRα1, TRα2, and TRβ1, T3 induces cell proliferation and is also able to promote survival due to a regulation of different cellular apoptotic proteins, specifically activating the PI3K pathway [84]. In non-tumoral β-cells, T3 action in the AKT pathway is also mediated by TRβ1, which contributes to the stimulation of proliferation and survival both in a rapid and long-term manner [85]. Interestingly, in contrast, T3 treatment enhances PI3K activity in glioblastoma cells but leads to nonproliferative downstream functions [7]. Taken together, these observations show the critical role of T3 nongenomic effects on the rapid PI3K-AKT/PKB-mTOR activation in normal and neoplastic cells [83; 85; 86; 87; 88].

Unlike T3, T4 is unable to activate PI3K nongenomically, supporting the concept that the integrin αVβ3 receptor contains two specific sites in the hormone-binding domain. One site binds T3 exclusively, activates PI3K via Src kinase. The second site binds both T4 and T3, which in turn, activates ERK1/2-dependent tumor cell proliferation (Figure 2) [7].

Recently, alternative mechanisms for T3- and T4-dependent AKT activation have been proposed. In human umbilical vein endothelial cells (HUVECs), neither T4- nor T3-induced AKT phosphorylation was attenuated by the addition of tetrac (which blocks T4 from binding to the integrin αVβ3 receptor) suggesting that integrin αVβ3 is not involved in the nongenomic actions of THs in these cells, and raising the question whether membrane-localized TRs are involved in such rapid actions of THs. Of interest, the blockade of D2 activity abolished AKT phosphorylation, indicating that the conversion of D2-catalysed T4 to T3 is required for TRα1/PI3K-mediated nongenomic actions of T4 in HUVECs [89].

Wnt/β-catenin pathway

The Wnt signaling pathway has a critical role in the embryonic development and regeneration of tissues. Mutations and/or deregulated expression of the Wnt pathway can induce cancer [90; 91]. β-Catenin, a central mediator in the Wnt pathway, interacts with E-cadherin to control cellular functions [92]. The relationship between T3 and the Wnt pathway was demonstrated by an elegant study performed by Miller and colleagues [93], which showed that T3-induced cell proliferation is associated with the immediate silencing of Wnt signaling in rat pituitary cells. Later studies in colon cancer cells demonstrated that T3/TRβ1 suppress the transcription of cyclin D1 by wild-type β-catenin [94]. Therefore, T3/TR signaling can negatively regulate the Wnt pathway by inhibiting transactivation by β-catenin/Tcf on the cyclin D1 promoter. The physical interaction of β-catenin and TRβ was also demonstrated in a mouse model of thyroid cancer. T3 binding to TRβ weakened the β-catenin/TRβ interaction, increasing the amount of β-catenin available to be degraded via the proteasomal pathway [95].

β-catenin also interacts with TRα1, but causes different effects when compared to β-catenin/TRβ interaction. TRα1 is primarily responsible for cell cycle regulation and proliferation in the normal intestinal epithelium [96]. In these cells, T3-activated-TRα1 receptor directly controls the transcription of the β-catenin in vitro, promoting cell proliferation [97]. TRα1 overexpression also enhances the intestinal tumorigenic process in a predisposed genetic background. In human CaCo2 cells, TRα1 interacts with the β-catenin/Tcf4 complex, leading to a reduced TRα1 functionality. In this model, TRα1 is recruited to interact with Wnt-responsive element regions in pre-cancerous and cancerous intestinal lesions and

stabilizes Wnt effectors on their target genes [98; 99]. Remarkably, the Wnt/β-catenin pathway modulates the colonic epithelium T3 concentration through the coordinated effects of D3 and D2 enzymes (Figure 2). D3 is a downstream target upregulated by Wnt/β-catenin, while unknown mechanisms downregulate D2. In colon cancer cells, D3 depletion causes intracellular T3 levels to rise, promoting differentiation and reducing proliferation [100]. These observations demonstrate the complexity of the interactions among THs, deiodinases, and the Wnt pathway in the balance of cell proliferation and differentiation. Notably, the effects of THs on colorectal cancer stem cells (CSCs) enhance the chemotherapy sensitivity and might be clinically important in the colon cancer therapy [101].

TH and Wnt/β-catenin interactions are also involved in the hepatocellular physiopathology by regulating the cell cycle during development and regeneration in the liver [102; 103; 104]. T3 enhances the activation of β-catenin in hepatocytes by increasing its phosphorylation through the activation of protein kinase A (PKA), indicating that T3-PKA-β-catenin crosstalk is essential for normal hepatocyte proliferation [105]. Wnt-β-catenin signaling is constitutively activated in HCC [106] but a contributing role of THs in liver tumor proliferation through this pathway remains to be demonstrated.

Sonic hedgehog (SHH) pathway

SHH signaling promotes cell differentiation and organ formation during embryogenesis [107]. SHH remains active in some organs through adulthood, and the deregulation of this pathway can result in uncontrolled cell proliferation [108]. Notably, SHH signaling is required not only for cancer initiation but also for growth and survival of several types of cancer [4; 108; 109; 110; 111].

Basal cell carcinoma (BCC), the most prevalent cancer in light-skinned individuals, is associated with increased levels of D3, the main TH-inactivating enzyme. SHH, through Gli family zinc finger 2 (Gli2), directly induces D3 expression, which in turn reduces intracellular T3 levels and increases cell proliferation, indicating that D3 overexpression is a major player in BCC progression. Indeed, D3 depletion (or T3 treatment) significantly reduces proliferation and cyclin D1 levels in malignant keratinocytes [4]. T3 treatment or D3 depletion also downregulates miR21, a key miRNA involved in oncogenesis. In an opposite manner, miR21 positively regulates *DIO3* expression in BCC through grainyhead-like transcription factor 3 (GRHL3) [112]. The crosstalk between the SHH and MAPK pathways for D3 upregulation has also been demonstrated in human PTC cell lines [113; 114]. Similarly, D3 depletion reduces cell proliferation and decreases cyclin D1 levels [114]. Taken together, these data support the link

between D3 overexpression and SHH/Gli2 pathway reactivation, suggesting that decreased intracellular levels of THs may be a critical factor for tumor growth, at least in some types of cancer.

Other less characterized TH effects in neoplastic process

TH effects on other signaling pathways have also been described. In T-cell lymphomas (TCL), T3 activates αvβ3 integrin signaling inducing cell proliferation and angiogenesis, in part, via the upregulation of VEGF.[6; 115]. Interestingly, a paradoxical effect was found in mouse models inoculated with TCLs, in which high circulating levels of THs favored T lymphoma growth, while hypothyroidism promoted tumor dissemination [116]. Moreover, *in vitro* short-term TCL exposure to THs led to proliferation, while a longer treatment increased tumor cell apoptosis [116; 117]. In embryonic carcinoma cells, T3 treatment decreased the growth rate via the rapid downregulation of E2F1, a key regulator of proliferation. This effect is dependent on the presence of active TRs [118].

Recently, an interaction was demonstrated between TRβ and nuclear corepressor 1 (NCoR), a coregulatory protein that mediates transcriptional repression via certain nuclear receptors. TRβ increases NCoR levels, thus suppressing the transcription of prometastatic genes whereas decreased NCoR leads to increased tumor growth, invasion, and metastasis, suggesting that NCoR is a critical mediator of the suppressive actions of TRβ in tumor growth and metastasis [119].

2.2. Evading growth suppressors

TH and TRs can act as tumor suppressors in specific types of tumors. These TH-mediated effects have been studied mostly in hepatic neoplastic and non-neoplastic cells, where T3 was shown to inhibit cell proliferation and to induce differentiation. T3 has a suppressive effect on the growth of specific liver tumors such as hepatoma, where the proliferative inhibitory effect of T3 is mediated by TGF-β upregulation [120]. T3/TR signaling mediates Dickkopf 4 (DKK4) expression that inhibits the proliferation and migration of hepatoma cells via blockade of the Wnt signaling pathway [121]. Similarly, THs inhibit cell proliferation by promoting p21 stability through endoglin upregulation [122]. Moreover, in TRα1-overexpressing hepatoma cells, T3/TR signaling promotes inhibition of liver cancer cell growth via downregulation of the *ubiquitin-like with PHD and ring finger domains 1* (*UHRF1*). [123].

Interestingly, the treatment of preneoplastic hepatocytes with T3 or GC-1 (a TRβ antagonist) leads to a loss of markers associated with neoplastic processes, such as glutathione S-transferase and gamma glutamyl transpeptidase. Meanwhile, T3 promotes the reacquisition of the activity of glucose 6-phosphatase and adenosine triphosphatase, two enzymes expressed in normal hepatocytes. Notably, the reduction in the number of preneoplastic lesions occurs despite an increase in cell proliferation, indicating that active TRs negatively influence the carcinogenic process through the redifferentiation of preneoplastic hepatocytes [124]. In a similar manner, T3 reduced the tumor development and metastasis rate in rats exposed to cycles of TH therapy. These data suggest that T3 could act as an anticarcinogenic molecule, most likely leading to hepatocyte redifferentiation [125].

Similarly, studies evaluating the effect of THs on glioma cell lines demonstrated T3-dependent cell redifferentiation at nearly physiological concentrations of the hormone. Remarkably, more aggressive tumors were more sensitive to the T3 inhibitory effects over cell proliferation, an effect that was mediated, at least in part, by TRα1 overexpression [70]. Consistently with these observations, it has been shown that several genes related to neuroblastoma cell differentiation are responsive to THs [126].

On the other hand, TR action on tumor proliferation and metastasis might occur independently of the presence of T3 [127]. Nevertheless, these effects have become increasingly difficult to study, in part due to the heterogeneous expression of TRs among different cancer types (and even within the same tumor type), the presence of TR mutations deregulating downstream pathways, and, as mentioned above, due to parallel nongenomic effects of T3/T4 on the cellular metabolism [56; 128]. Indeed, TRs, particularly the TRβ isoform, can act as tumor suppressors, with a functional loss of TRα1/β promoting tumor development and metastasis [76; 77; 127; 129].

2.3. Evading cell death and enabling replicative immortality

TH actions have also been demonstrated in the evasion of programmed cell death, an important feature of neoplastic transformation [18; 31; 130]. In brief, apoptosis can be divided into two major circuits: the extrinsic and intrinsic apoptotic programs. The extrinsic apoptosis pathway involves the interaction of ligands, such as tumor necrosis factor (TNF)-α and Fas ligand, with specific receptors on the cell surface. THs decrease TNF-α, Fas receptor, and Fas ligand expression and the activity of caspase-3, thus suppressing apoptosis in non-tumoral models [131]. An anti-apoptotic role of THs is also supported by the effect of T3 on apoptosis regulators. T3 decreases the cellular abundance of caspases and the pro-apoptotic Bcl-2-

associated X protein (BAX) and increases the expression of the anti-apoptotic X-linked inhibitor of apoptosis protein (XIAP)[6; 132]. When considering the intrinsic apoptosis pathway, there is evidence that T3 administration protects hypothyroid rat liver cells from apoptosis induced by oxidative stress in a non-tumoral model [133]. TH also regulates proteins involved in the intrinsic apoptosis pathway. For example, T3 induces the expression of myeloid cell leukemia 1 (MCL-1), a Bcl-2-related protein located in the outer mitochondrial membrane [134], while T4 downregulates expression of the BAX gene, the gene product of which is proapoptotic at mitochondria. These anti-apoptotic effects of THs are in accordance with the evidence that molecules inhibiting T4 action (tetrac/nanotetrac) have pro-apoptotic effects on tumor growth [135].

The nongenomic effects of T4 in the apoptotic pathway occur, at least in part, via induction of the MAPK pathway, initiated through the integrin αVβ3 receptor [18]. The T4-induced MAPK activation results in the serine phosphorylation of the oncogene suppressor p53, STAT1α, STAT-3 and TRβ1, leading to proliferative and anti-apoptotic effects [62; 63; 130; 136]. The T4 anti-apoptotic effect was demonstrated in human PTC and FTC cell lines incubated with resveratrol (RV), an apoptosis-inducer that also initiates signaling via the plasma membrane integrin αVβ3 [18; 31]. In glioma cells, RV increases the nuclear content of cyclooxygenase-2 (COX2) via MAPK induction, while the incubation of RV-treated cells with T4 decreases the levels of the cytosolic pro-apoptotic protein B-cell lymphoma extra-large (Bcl-X) and the formation of nuclear complexes between pERK and COX2. These effects lead to a blockage of p53 phosphorylation, thus inhibiting apoptosis [18]. However, others have demonstrated that high concentrations of T3 induce breast cancer cell apoptosis via the TRβ-dependent downregulation of the anti-apoptotic senescence marker protein-30 gene (*SMP30*) [137]. The involvement of TRβ in apoptotic pathways is further supported by studies showing that TRβ can act as a tumor suppressor, interfering with the recruitment of retinoblastoma protein and p53 via the SV40Tag oncoprotein through a protein-protein interaction [12]. TNF-related apoptosis-inducing ligand (TRAIL/Apo2L) is a potent effector of tumorigenesis that not only promotes apoptosis but also triggers non-apoptotic pathways [138]. T3 upregulates TRAIL expression at the transcriptional level in TR-overexpressing hepatoma cells, which in turn promotes cell migration and invasion [139].

Compilation of data supports the anti-apoptotic activity of THs in several tumor cells. TH action occurs mainly through physiological levels of T4 via genomic and nongenomic signaling modulating multiple components of the extrinsic and intrinsic apoptosis pathways.

The maintenance of telomere integrity and telomerase protect cells from apoptosis. Telomerase inhibition elicits an apoptotic response in cancer cells, while restoration of telomerase activity in somatic cells promotes resistance to apoptosis [140]. Thus far, no studies on the effect of THs on telomerase activity in cancer models have been reported. However, hypothyroidism leads to decreased telomerase activity in stem cells [141], an observation that should be further explored.

2.4. Tissue invasion and metastasis

The spread of cells from the primary lesion to distant organs is the most worrisome aspect of cancer. Alterations in cell shape and in their attachment to both other cells and the extracellular matrix (ECM) are essential for this process [142]. Tumor cells must invade the basement membrane and migrate through the ECM surrounding the tumor epithelium to spread, which occurs mainly via interactions between integrin receptors and ECM components. Matrix metalloproteinase-9 (MMP-9) is a pivotal matrix metalloproteinase that contributes to ECM degradation, thereby enhancing invasiveness [143]. THs contribute to the regulation of cell adhesion and migration in several tumor models [144; 145] For instance, THs induce MMP-9 via the αVβ3-MAPK pathway, promoting increased adhesion to fibronectin and enhancing cell migration in myeloma cells [146].

THs status might influence the spread of liver cell cancer. However, as already mentioned, the effects of THs on liver tumorigenesis are complex and depend on TR expression status, cancer stage and other co-effectors present in the tumor microenvironment [147]. Acting mostly through TRs, TH actions on HCC development may lead to the suppression or promotion of prometastatic mechanisms. T3 enhances HCC cell invasion *in vitro* and *in vivo* [147]. T3 treatment increases the invasive capacity of HepG2 cells expressing TRs, possibly due to the upregulation of furin, a calcium-dependent serine endoprotease, which increases the processing of MMP-2 and MMP-9. Moreover, T3 administration to mice inoculated with HepG2-TRα1 cells caused furin overexpression. Notably, these animals displayed greater tumor sizes and metastasis rates than euthyroid animals, supporting the metastasis-promoting effect of T3 in HCC [148]. Several members of the MMP family, including MMP-2, MMP-9, and MMP-7, are upregulated upon r-TRAIL stimulation in hepatoma cells, an effect confirmed by increased invasiveness in both *in vitro* and *in vivo* models [139]. Cathepsin H, a protease involved in the degradation of ECM components, leading to cancer cell migration and metastasis, is induced by T3 in HCC cells, enhancing the invasion potential of hepatoma cells *in vitro* and *in vivo* [149]. Likewise, T3 treatment in HCC cells also enhanced tumor cell

migration and invasion by stimulating the overexpression of brain-specific serine protease 4 protein levels, which was associated with ERK1/2-C/EBPβ-VEGF cascade activation [150]. Inversely, other studies have demonstrated that T3 treatment of the same cells leads to spondin 2 overexpression, which inhibits cell invasion and migration [144]. T3 treatment also upregulates the expression of DKK4 protein, an antagonist of Wnt, in HepG2 TR-expressing cells [151], suggesting that the T3-upregulation of the TR/DKK4/Wnt/β-catenin cascade inhibits the metastasis of hepatoma cells [121].

T3-induced cell migration in HCC is mediated in part to a reduction in miR-17 and miR-130b expression [152; 153] and the overexpression of miR-21 [154]. The overexpression of miR-17 markedly inhibits HCC cell migration and invasion *in vitro* and *in vivo* via the suppression of MMP-3 [152], whereas the effect of miR-130b involves the regulation of genes critical for metastasis, such as MMP-9, mTOR, ERK1/2, AKT and STAT-3 [153].

Breast cancer cell migration is also influenced by the nongenomic action of T3. The focal adhesion kinase (FAK) protein is an essential regulator of the actin cytoskeleton, thus modulating the steps involved in cell migration and invasion. T3, acting through integrin αVβ3, promotes the phosphorylation of FAK by activating the Src/FAK/PI3K pathway, thereby modulating cell adhesion and migration [155].

2.5. Induction of angiogenesis

Tumor growth, invasion, and metastasis are strongly dependent on angiogenesis [156]. The initiation and maintenance of a vascular supply involve the local release of angiogenic molecules, such as VEGF, FGF2, PDGF, TGFs and angiopoietins (Angs) [157]. The concept of TH-induced neovascularization was first described a decade ago in the chick chorioallantoic membrane assay of angiogenesis [17; 158]. TH pro-angiogenic effects seem to be mainly promoted by T4 binding to integrin αVβ3, followed by MAPK signal transduction. The TH-αvβ3 complex causes the transcription of several factors, such as TRβ1, ERs, TP53, and STATs, leading to the increased expression of angiogenic modulators, such as FGF2, VEGF, and Ang-2 [15; 43; 104; 159]. The addition of T3 to cultures of HCC, lung, and kidney carcinoma cells leads to HIF-1α induction and increases in VEGF levels [160]. T3 upregulates HIF-1α through the PI3K pathway, which in turn stimulates the secretion of HIF-responsive genes, such as VEGF, FGF2, interleukin-6, stromal cell-derived factor-1 and TGF-β1 [161]. T3 and T4 also regulate the differentiation and migration of mesenchymal stem cells (MSCs) via integrin αVβ3. This regulation affects not only indicators of tissue remodeling and invasion, such as tenascin-C (*THBS1*) and thrombospondin-1 (*TSP1*), but also proteins associated with

angiogenesis, such as α-smooth muscle actin (α-SMA), desmin, and VEGF, thus contributing to tumor stroma dysregulation [162].

Of note, tetrac reduces VEGF-A mRNA levels while increases the transcripts of the TSP-1 gene, an adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions, blocking the T4 proangiogenic effects [163; 164]. Indeed, tetrac administration to nude mice inoculated with FTC or medullary thyroid carcinoma (MTC) cells reduces the vascularization and growth of grafted tumors [135; 164]. The tetrac-associated inhibition of angiogenesis has been observed in a variety of tumor xenografts, indicating a therapeutic potential that merits exploration in clinical settings [165; 166; 167].

2.6. Genomic instability and cellular senescence

Genomic instability is a hallmark of most cancer cells. Failure in maintaining DNA integrity impairs cell proliferation and survival, resulting in senescence, a phenomenon in which normal cells cease to divide. Cells can be induced to senesce via DNA damage due to increased ROS levels [168]. T3, mediated by TRβ, induces senescence in mouse embryonic fibroblasts, promoting DNA damage secondary to oxidative stress. The effect is dependent on the activation of ataxia telangiectasia mutated (ATM)/adenosine monophosphate-activated protein kinase (PRKAA), proteins that play pivotal roles in detecting genomic damage [169]. Of note, TRβ1 and TRβ2 are highly expressed in retinoblastoma cells, and participate in maintaining genomic stability [170].

2.7. Dysregulation of cell bioenergetics/energy metabolism

The sustenance of cancer cells also depends on metabolic adaptations. Tumor cells are characterized by increased aerobic glycolysis and lactic acid production in normoxic conditions. This phenomenon, which has been a biochemical hallmark of cancer for decades, is known as the Warburg effect [171; 172]. Lately, some studies have established a connection between the mitochondrial and TH metabolisms in the context of modulating the Warburg phenomenon in breast cancer [173; 174]. The authors evaluated the effects of T3 in modulating the bioenergetics profiles by monitoring glucose uptake, lactate generation, and the mitochondrial oxygen consumption rate. Interestingly, they showed that T3 directly increases the mitochondrial metabolism in aggressive breast cancer cells and directly regulates one of the isoforms of pyruvate kinase that is vital for sustaining the Warburg effect [173].

Oxidative stress is known to disrupt the function of deiodinases [175], key enzymes for the regulation of the intracellular levels of active THs [9; 176]. Neoplastic cells are known to be

hypoxic, a condition that has been shown to upregulate D3 expression through HIF-1 in non-tumoral models [177; 178]. D3 reactivation in the neoplastic cells of solid tumors increases TH inactivation and reduces the metabolic rate, which may favor cell proliferation. This phenomenon has been associated with a poor therapeutic response and an increased risk of recurrence [179]. In a non-tumoral model of rat brain, D3 participates in the hypoxia-induced reduction in thyroid hormone signaling. Moreover, ischemia/hypoxia induces a heat-shock protein 40 (Hsp40)-mediated translocation of D3 to the nucleus, facilitating thyroid hormone inactivation proximal to the thyroid hormone receptors. [180; 181]. THs can directly protect or damage cells by modulating oxidative stress [182]. Thus, it is reasonable to consider that intracellular TH levels contribute to the disruption of tumoral bioenergetics. The effects of THs on glycolytic fueling require further exploration since common pathways appear to be activated in several tumors [183].

3. Intracellular microenvironment: deiodinase control over TH status

The intracellular TH status is highly dependent on the activation or inactivation of THs by deiodinases. Particularly, alterations in the balance between TH-activating and TH-inactivating deiodinases can be critical in modulating the balance between cell proliferation and differentiation [29; 30]. Indeed, changes in the expression levels of deiodinases are present in several malignant human neoplasias. *DIO1* downregulation occurs in renal, lung, hepatic, and prostate cancer tissues [184; 185; 186; 187]. Studies performed using human PTC samples found a consistent decrease in *DIO1* levels compared with the surrounding thyroid tissue, suggesting that diminished *DIO1* expression might be an early event in thyroid cell dedifferentiation. In contrast, *DIO1* and D1 activity levels are increased in follicular adenoma and FTC samples [188]. In renal clear cell cancer, miR-224 expression correlates negatively with the *DIO1* mRNA level and T3 concentration, suggesting that miR-224 induces intracellular hypothyroidism via reduced D1 function [189]. Interestingly, D1 activity does not differ significantly between benign and malignant tumors as compared with healthy liver parenchyma cells [190]. In contrast, D1 activity in non-cancerous breast tissues is very low or non-measurable, whereas it is increased in breast cancer, indicating a tissue-specific regulation of D1 expression [191].

Changes in *DIO2* expression have also been demonstrated in several human neoplasias. *DIO2* expression is induced in most brain tumors, including those derived from glial cells [192; 193; 194], FTC cells and MTC cells [195; 196]. In contrast, *DIO2* mRNA and activity are decreased in PTC cells as compared with normal follicular thyroid cells [188; 197].

Increased *DIO3* expression is observed in several human tumor types, including astrocytoma, oligodendroglioma, glioblastoma multiforme, and BCC [198]. Tumoral D3 activity is markedly elevated in vascular tumors, including infantile hemangioma and hemangioendothelioma in adults [199; 200; 201], even to the extent of inducing clinical hypothyroidism (consumptive hypothyroidism). Opposing regulation of *DIO3* and *DIO1/DIO2* expression has been reported in various human neoplasias, such as PTC, TSH tumors, BCC, and colon cancer [4; 100; 114; 188; 201]. Studies performed using 105 pituitary tumors demonstrated that *DIO2* and *DIO3* mRNA levels were significantly augmented in pituitary tumors compared with normal pituitary tissue. In the rare TSH-secreting pituitary tumor subtype, increased *DIO3* expression and *DIO2* mRNA downregulation were observed, which may explain the ‘resistance’ of these tumors to TH feedback [202]. In human BCC samples, upregulated *DIO3* expression correlated with the functional status of the SHH pathway described above, which is a critical oncogenic pathway [4]. Interestingly, co-expression of D3 and D2 was found in BCC, and manipulation of the expression of each enzyme, with consequent alteration of intracellular TH levels, dramatically modifies the proliferative potential of BCC [8]. This illustrates the critical regulatory role of THs on proliferation of certain tumors.

The induction of *DIO3* expression was also recently demonstrated in human PTC samples. Remarkably, D3 levels were positively associated with increased tumor size and increased rates of local and distant metastasis at diagnosis [113]. Most interesting, D3 upregulation in PTC samples is modulated by crosstalk between the MAPK and SHH pathways and varies according to the genetic alterations in this tumor type [114]. Increased *DIO3* expression was also observed in FTC but not in medullary or anaplastic thyroid carcinoma samples [113]. Higher levels of D3 were also detected in human intestinal adenoma and carcinoma compared with healthy intestinal tissue. However, *DIO3* expression was reduced in lesions with higher histological grades [100].

4. Tumor microenvironment

Increasing evidence indicates that what is occurring inside tumor cells depends on exogenous stimuli originating around the tumor cells [203; 204]. Specifically, surrounding tumor stroma and immune cells can be “activated,” thus influencing tumor behavior.

4.1. Evading immune destruction and promoting inflammation

The immune system antagonizes and enhances tumor development and progression. The tumor-associated inflammatory response has the paradoxical effects of promoting tumorigenesis and helping neoplastic cells acquire hallmark capabilities [205; 206]. The endocrine and immune systems are complexly interconnected, and THs affect immune cells, modulating their responses [207].

THs seem to enhance the antiviral action of interferon-ϒ via the MAPK pathway [208]. Moreover, T3 activates PI3K/AKT signaling, thus activating myeloid cell leukemia-1 (MCL1) [134] and the *HIF1A* gene [7], which are critical molecules that elicit the immune response.

In vitro models have shown that T3 promotes tumor growth through the modulation of soluble factors released by surrounding microglial cells [209]. In contrast, the T3-TRβ complex influences the antitumor responses of dendritic cells (DCs), the main antigen-presenting cells during tumor growth when activated by T cells [210]. This TH effect seems to depend on AKT activation [211], while AKT phosphorylation enhances DC survival [212]. In addition to the complex effects of THs on T lymphoma cell proliferation and death, Sterle's group has investigated thyroid status in the tumor microenvironment [116]. They found that THs have a substantial effect on the distribution of different immune cell populations and on lymphocyte infiltration, particularly on the prevalence of cytotoxic T cells. Together, these results highlight the importance of THs in modulating the immune response and related signaling in the tumor milieu through different pathways.

4.2. Cancer Stem Cells (CSCs)

CSCs may be involved in tumor initiation and may drive tumor progression. They carry oncogenic and tumor suppressor mutations that genetically define the disease. Both T3 and T4 increase the migration of MSCs toward tumor signals and increase the invasion of MSCs into tumor cell spheroids, thus impacting crucial steps of tumor stroma formation [162]. In a model of HCC CSCs, T4 was a potent promoter of CSC self-renewal. TH signaling in HCC occurs through the nuclear receptor TRα with the cooperation of NF-κB, inducing the expression of stem cell genes, such as CD44, BMI1, NOTCH1 and HIF-1α, thus enhancing the self-renewal of HCC CSCs [213]. However, evidence of TH influences on CSCs remains scarce.

CONCLUSION AND FUTURE DIRECTIONS

In conclusion, an extensive set of data has indicated that the status of THs plays a significant role in the carcinogenesis process. Changes in TH levels seem to occur due to a disruption in TR and/or deiodinase expression and via nongenomic signaling pathways that broadly contribute to the acquisition of steps necessary for cancer development. TH status alterations are known to contribute to cancer development and/or progression via direct effects on virtually all the hallmarks of cancer. Therefore, adjuvant therapies targeting TH actions might be considered alternative treatments for cancer cell proliferation, metastasis, and angiogenesis.

The genomic and nongenomic actions of THs overlap in the regulation of pro- and anti-tumoral cascades that lead to cancer growth. THs have a wide effect on tumoral progression, contributing to the acquisition of all hallmarks of cancer by predisposed cells. Moreover, intracellular TH changes due to a disruption in deiodinase status seem to be critical for modulating cell proliferation and differentiation. Accordingly, experimental and observational studies indicate TH status imbalance as a risk factor for several neoplasias. Furthermore, clinical trials have demonstrated that induced hypothyroidism leads to extended survival in different types of cancer [28; 214]. Targeting cancer pathways to control tumor dissemination has been studied through integrin αVβ3 blockade, in an effort to inhibit angiogenesis. Pharmacologically targeting the membrane receptor with tetrac and other derivatives inhibits the trophic effects of the hormone in some cancer cells [164; 215]. Moreover, targeting the SHH pathway in BCC inhibited proliferation in clinical settings [216; 217], although the direct effect on D3 activity was not analyzed. Theoretically, the pharmacological modulation of intracellular TH levels in a cell-specific manner could contribute to cancer treatments. In the same way, blocking pathways abnormally activated by THs, without interfering with the systemic balance of the TH metabolism, could lead to pro-apoptotic and anti-proliferative actions to control tumor growth or enhance the effectiveness of existing chemotherapeutic cancer drugs.

Figure 1.

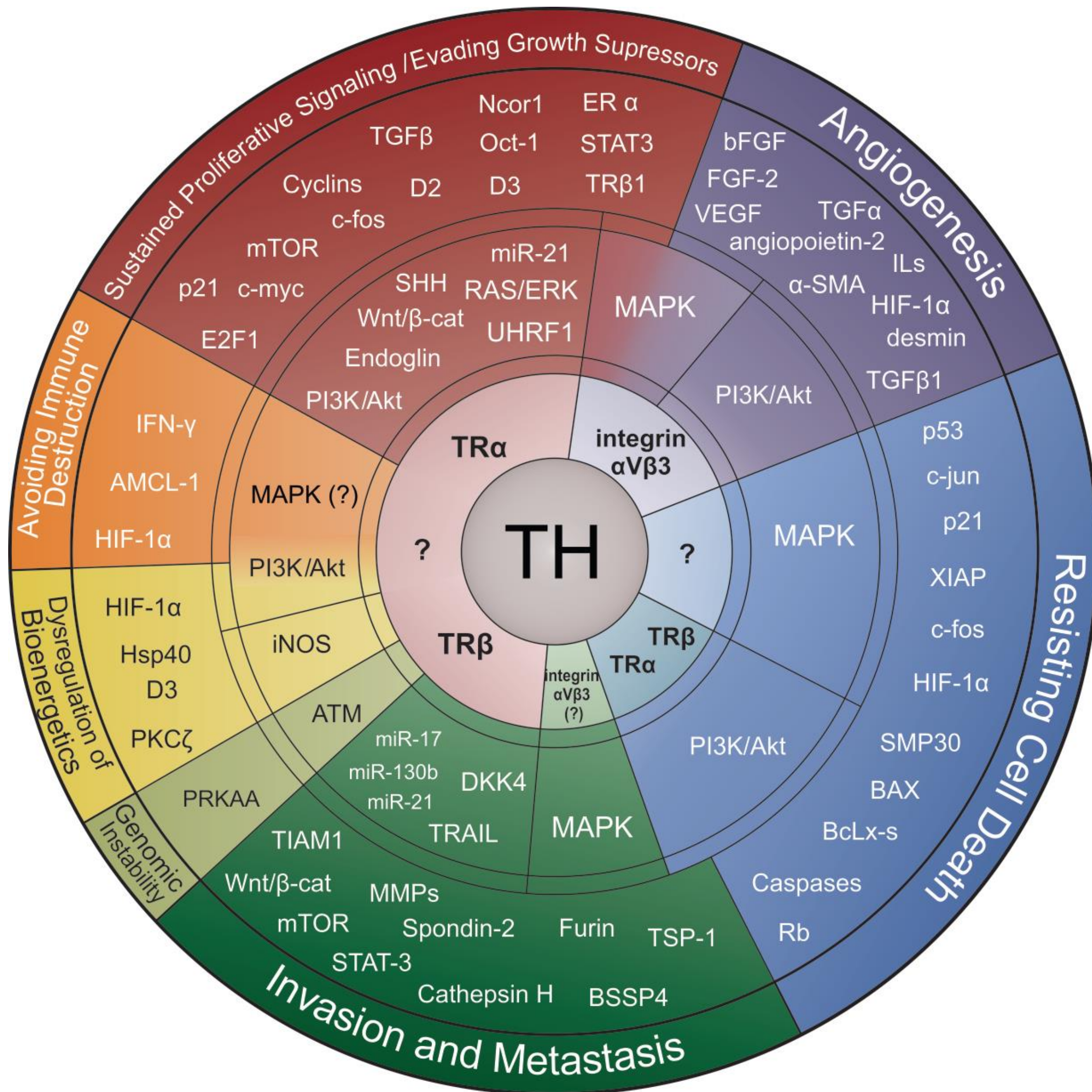

Figure 1. The effects of THs on the hallmarks of cancer involve several pathways and effectors. The THs (center) act via integrin αVβ3 or TRs (inner circle), modulating critical signaling pathways classically involved in carcinogenesis (middle circle). Note that for some nongenomically driven pathways, integrins have not been shown to be the membrane receptor mediators. Downstream targets of TH actions are represented in the outer circle.

Figure 2.

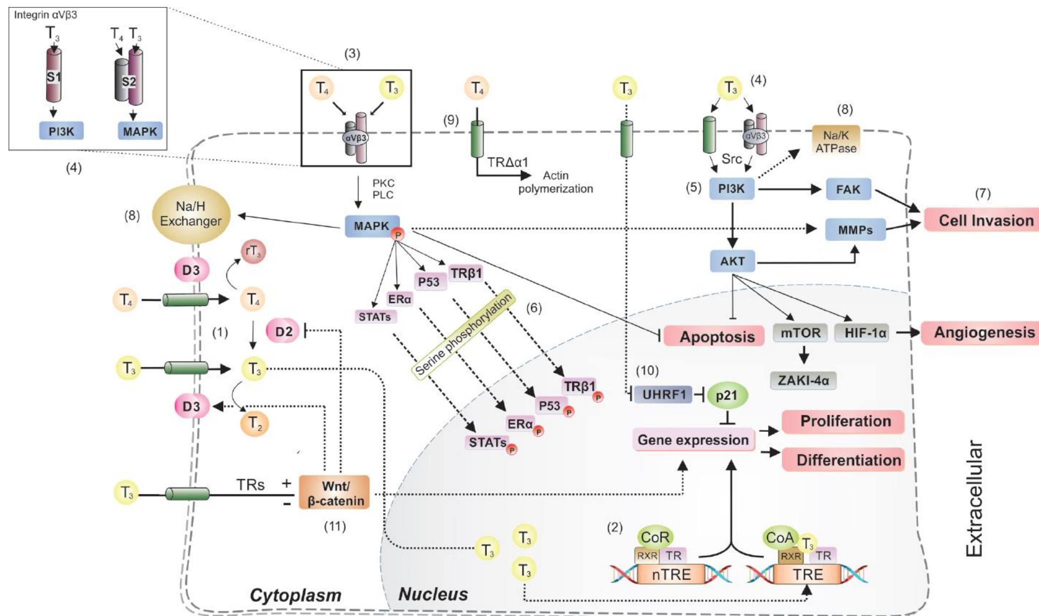

Figure 2. Proposed mechanism of genomic and nongenomic actions of THs in the neoplastic process. The actions of THs occur at the plasma membrane, in the cytoplasm, and within the cell nucleus. To exert their genomic effects, T4 and T3 enter the cell through transporter proteins, such as monocarboxylate transporter (MCT) 8 and 10 or organic anion-transporting polypeptides. Inside the cells, D2 convert T4 to the active form T3, while D3 inactivates both THs, producing rT3 and T2 (1). T3 binds to nuclear TRs that activate transcription by binding TREs located in the regulatory regions of the target genes. Activity is regulated by an exchange of corepressor (CoR) and coactivator (CoA) complexes. Negative TREs (nTREs) can mediate ligand-dependent transcriptional repression; however, in this case, the roles of CoAs and CoRs are not well defined (2). THs can also regulate genes that do not contain a TRE by nongenomic effects. These “rapid effects” are initiated by THs binding to integrin αVβ3 (3), leading to the activation of different signaling pathways and resulting in distinct cellular events, such as cell proliferation, migration, angiogenesis and apoptosis inhibition. One site of the integrin αVβ3 (4) binds T3 exclusively, activating PI3K via Src kinase (5), stimulating FAK, HIF-1α, and mTOR, while also increasing the activity of the sodium pump (Na/K ATPase). The second site (4) binds T4 and T3, stimulating MAPK-dependent proliferation via phospholipase C (PLC) and protein kinase C (PKC), promoting the phosphorylation of several effectors (ERα, TRβ1, STAT1α, P52, and STAT-3, among others) (6). THs can induce the expression of matrix metalloproteinases (MMPs) nongenomically via MAPK and PI3K, thereby enhancing invasiveness (7). Another action THs initiate at the cell surface is modulation of the activity of the Na+/H+- exchanger and Na/K ATPase (8). Furthermore, T4 also interacts with a TRα variant in the cytoplasm to cause a modification of intracellular actin that contributes to cell migration (9). T3 negatively regulates UHRF1 through TRα1, leading to inhibition of cancer growth, by promoting stability of a cyclin-dependent kinase inhibitor (p21)(10). While T3 negatively or positively regulates Wnt/β-catenin expression, depending on the TR that is active, Wnt/β-catenin regulates the intracellular levels of T3 by modulating *DIO2* and *DIO3* expression. The D2 level is downregulated by β-catenin while D3 is induced, illustrating the complex crosstalk between THs and the Wnt/β-catenin pathway (11). Note that for some nongenomically driven pathways, integrin αVβ3 has not been demonstrated as the membrane receptor mediator.

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Parte II

Current concepts and challenges to unravel the role of iodothyronine deiodinases in human neoplasias

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TITLE: Current concepts and challenges to unravel the role of iodothyronine deiodinases in human neoplasias

SHORT TITLE: DEIODINASES AND CANCER

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ABSTRACT

Thyroid hormones (THs) are essential for the regulation of several metabolic processes and the energy consumption of the organism. Their action is exerted primarily through interaction with nuclear receptors controlling the transcription of thyroid hormone-responsive genes. Proper regulation of TH levels in different tissues is extremely important for the equilibrium between normal cellular proliferation and differentiation. The iodothyronine deiodinases types 1, 2 and 3 are key enzymes that perform activation and inactivation of THs, thus controlling TH homeostasis in a cell-specific manner. As THs seem to exert their effects in all hallmarks of the neoplastic process, dysregulation of deiodinases in the tumoral context can be critical to the neoplastic development. Here, we aim at reviewing the deiodinases expression in different neoplasias and exploit the mechanisms by which they play an essential role in human carcinogenesis. TH modulation by deiodinases and other classical pathways may represent important targets with potential to oppose the neoplastic process.

INTRODUCTION

Thyroid hormones (THs) are essential modulators of several physiological processes, including organ development, cell differentiation, and tissue growth. Since the description of 3,3',5-triiodothyronine (T_3) in human plasma by Gross & Pitt-Rivers [1], numerous studies have demonstrated that it is mainly derived from the peripheral deiodination of 3,3',5,5'-tetraiodothyronine, or thyroxine (T_4) [2; 3]. Monodeiodination of T_4 yields T_3 by enzymatic outer ring deiodination (ORD) of T_4 in the peripheral tissues such as the liver and the kidney, whereas the inactive form 3,3',5'-tri-iodothyronine (reverse tri-iodothyronine, rT_3), is formed by inner ring deiodination (IRD)(Fig. 1). Both triiodothyronines are further degraded by a cascade of deiodination steps [2; 4; 5].

Despite an initial hypothesis that sequential deiodination was performed by two distinct enzymes acting either in the phenolic or the tyrosyl ring, evidence soon demonstrated that a single enzyme, type 1 deiodinase (*DIO1*, DIO1), was responsible for both ORD and IRD [6; 7; 8]. This process was classically studied in the liver, kidney and the thyroid, and was subjected to 6-propyl-2-thiouracil (PTU) inhibition [9; 10; 11]. However, PTU did not inhibit the local deiodination of T_4 to T_3 the in brain and pituitary tissues, suggesting the existence of two separate pathways of enzymatic ORD in these tissues. Investigation of the distinct biochemical properties of a possible second enzyme led to the identification of type 2 deiodinase (*DIO2*, DIO2) [12]. DIO2 has a K_m for T_4 that is approximately three orders of magnitude lower than that of DIO1 in *in vitro* conditions. The observations that higher rates

of IRD occur in neonatal tissues and that high levels of rT_3 are present in fetal serum, led to the identification of a specific enzyme responsible for IRD, generating rT_3 from T_4 and 3,3'-diiodothyronine (3,3'-T_2) from T_3. This enzyme was subsequently demonstrated to be type 3 deiodinase (*DIO3*, DIO3) (Fig. 1) [13; 14]. DIO3 has a much lower K_m for T_4 than DIO1 and is the main enzyme involved in TH inactivation. This enzyme controls TH homeostasis locally, protecting the tissues, such as the brain and fetal tissues, from an excess of THs [15].

Deiodinases are selenoproteins, meaning they contain a single selenocysteine residue (SeC) in the catalytic center, which is highly conserved between the three enzymes. To incorporate the SeC into the amino acid chain, the cell must recognize the UGA as a Sec codon rather than a STOP translation signal. This is performed by a stem-loop structure in the 3' untranslated region (UTR) called the Sec insertion sequence (SECIS) element. The SECIS element is the signal that recodes the UGA from a STOP to a Sec codon [16; 17]. The three enzymes depend on an yet unidentified physiologic thiol cofactor that is substituted during *in vitro* reactions by reduced dithiols such as dithiothreitol (DTT). The group of selenoproteins still intrigues us due to their peculiar characteristics and mechanisms of action [18]. The mechanism of reductive deiodination of iodothyronines is not yet fully understood. Recently, the crystal structure of the type 3 deiodinase catalytic domain was identified, and it was shown to resemble the family of peroxiredoxin(s) (Prx). These findings can explain some previously enigmatic features of deiodinase biochemistry and confirms its thioredoxin (Trx) scaffold, suggesting that dimerization is mediated by the catalytic domain and primarily by the N-terminal region of the protein. Moreover, dimerization activates the enzyme by relaxing an autoinhibitory loop, providing access to the binding site. Analysis of Dio3 structure further reveals deiodinase-specific features classifying them as evolutionarily related to atypical 2-Cys Prx. Structure and biochemical data suggest that oxidized enzyme can be directly reduced by exogenous thiols *in vitro*. These data suggest an evolutionary pathway with Prx as an ancestor of iodothyronine deiodinase [19].

Deiodinases are Trx fold-containing dimeric enzymes with a molecular weight that varies between 29 and 33 kDa (each monomer) that are located in the plasma membrane (DIO1 and DIO3) and in the endoplasmatic reticulum (ER) (DIO2) [20]. All three deiodinase enzymes are integral membrane proteins and are subject to dimerization [21]. While DIO1 and DIO3 expression are known to be controlled mainly through pretranscriptional mechanisms, DIO2 is uniquely known for its post-transcriptional activity-induced inactivation. The inactivation process involves ubiquitination of the active enzyme by WD repeat and SOCS box-containing protein 1 (WSB-1), which leads to an inactive DIO2 conformation, followed

by proteasomal degradation [22; 23; 24]. However, DIO2 can also be reactivated through deubiquitination by ubiquitin specific peptidase 33 (USP33) [25]. DIO1 activity is also regulated by rT3 in a post-translational level through a mechanism that possibly involves post-catalytic structural changes in the DIO1 homodimer inactivating the enzyme [26]. The mechanism of substrate-induced inactivation of DIO2 and DIO1 suggests that this regulation might be applicable to all three deiodinases [18]. There is also evidence of post-transcriptional regulation of DIO3. Drug-induced hepatotoxicity decreased DIO3 protein levels in rat liver, although *DIO3* mRNA levels were not changed [27]. Moreover, whole-cell deiodination assays with Peroxiredoxin 3 (Prx3) knockdown strongly indicate that this DIO3-associated protein plays a specific role in DIO3 regeneration, contributing to the post-translational regulation of the enzyme [28].

In humans, DIO1 is mainly expressed in the liver, the kidney, and the thyroid gland [29]. DIO2 expression, however, is more widely distributed. *DIO2* mRNA and/or DIO2 activity are found in the human thyroid, esophagus, heart, brain, pituitary, skeletal muscle, skin, brown adipose tissue and reproductive organs (Fig. 2) [30; 31; 32; 33; 34; 35]. The administration of PTU (which inhibits DIO1 activity) to hypothyroid individuals receiving levothyroxine supplementation, reduces T_3 production by only approximately 25% [36], supporting *in vitro* studies that show that PTU-insensitive deiodination by DIO2 is a major source of T_3 in humans [37]. DIO2 plays an essential role in different organs and systems regulating local T_3 production. In a system that transiently coexpresses DIO1 and DIO2, analysis of deiodination at physiologic free T_4 levels demonstrates that DIO2 has a much higher catalytic efficiency than DIO1, and is the primary source of extrathyroid-produced T_3 in the euthyroid state [37]. DIO3, which is translated from a paternally imprinted gene and is located in the *DLK-DIO3* genomic region, is significantly increased in several tissues during embryogenesis, such as the embryonic liver, cerebral cortex, gonads, intestine, and skin. It is critical for TH homeostasis in this context, as exposure of the embryo to high TH levels can be detrimental to proper development [15; 38]. It is also expressed in the placenta, where it broadly protects the fetus from excessive TH exposure [39; 40; 41]. Importantly, DIO3 is reexpressed in normal and pathological hyperproliferative conditions. DIO3 reactivation has been demonstrated in the pathological context of cardiac hypertrophy, myocardial infarction, critical illness and several types of cancer [42; 43; 44].

Several signaling pathways and hormonal stimuli regulate deiodinase expression and activity in normal tissue. The human *DIO1* gene is under the control of GC-rich SP1 promoters and contains two thyroid hormone response elements (TREs) that contribute to the

T_3 responsiveness of the *DIO1* promoter [45; 46; 47]. The most potent modulator of DIO1 activity is T_3. T_3 promotes transcriptional activation of the *dio1* gene in the rat and the mouse in a process that does not require protein synthesis [48; 49; 50]. Although studies of developmental changes with embryonic chickens showed a causal relationship between the increase in plasma growth hormone (GH) and T_3 levels, no changes in *DIO1* mRNA were observed [51; 52]. However, GH (and also dexamethasone) decreased DIO3 activity by acting at the pre-translational level, which could explain the increased levels of T_3 in this model [51]. DIO1 is also positively regulated by the adenylyl cyclase (cAMP) cascade, hepatocyte nuclear factor 4 alpha (HNF4A), liver X receptor alpha (LXRA), thyroid stimulating hormone (TSH), prolactin and beta-adrenergic stimulation [53; 54; 55; 56]. Forkhead box (FOX) transcription factors play a key role in the regulation of crucial biological processes, including cell proliferation and metabolism [57]. Interestingly, forkhead box A1 (FOXA1) and forkhead box A2 (FOXA2) regulate *DIO1* expression in liver. *DIO1* is positively regulated by FOXA1 and negatively regulated by FOXA2 [58]. DIO1 has been shown to be negatively regulated by cytokines, such as interleukin-1 beta and tumor necrosis factor alpha. However, stimulatory or inhibitory effects of these molecules depend on the type of cytokine, the species and the organ studied [59; 60; 61; 62; 63].

DIO2 mRNA and DIO2 activity levels are upregulated by epidermal growth factor (EGF), cAMP, nuclear factor kappa B, forkhead box O3, peroxisome proliferator-activated receptor gamma, forskolin, bile acids and beta-adrenergic agonists [64; 65; 66; 67; 68]. In contrast, DIO2 is negatively regulated at both the pre-translational and post-translational level by THs and at the pre-translational level by tumor necrosis factor alpha (TNFA), dexamethasone, forkhead box protein O1 and liver X receptor/retinoid X-receptor pathway [64; 69; 70; 71; 72; 73; 74]. Platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF), which are essential mitogens for vascular smooth muscle cells, cause the induction of DIO2, which is mediated at least partially by the extracellular signal-regulated kinases (ERK)1/2 pathway [75]. GATA2, a transcription factor that determines thyrotroph differentiation, also stimulates *DIO2* promoter, as do GATA4 and Homeobox protein Nkx-2.5, central regulators of tissue-specific transcription in cardiomyocytes [76; 77].

The Hedgehog signaling pathway transmits the required information to embryonic cells for appropriate cell differentiation and is considered to be one of the critical regulators of vertebrate development [78]. Among the Hedgehog homolog proteins, Sonic hedgehog (Shh) is the most studied and is involved in the development of the brain, skeleton, musculature, gastrointestinal tract and lungs [79]. The Shh pathway has also been implied in neoplastic

processes [80] and importantly modulates both DIO2 and DIO3 expression. DIO2 is downregulated post-transcriptionally primarily by ubiquitination [23] while DIO3 is subjected to Shh upregulation through the transcription regulator zinc finger protein GLI2 (GLI2) in normal keratinocytes [81]. T_3, retinoic acid, 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and bFGF induce *DIO3* expression and DIO3 activity in rat astrocytes. The effects of TPA and bFGF seem to be mediated by the mitogen-activated protein kinase (MAPK) signaling pathway [82]. The *DIO3* gene is also transcriptionally induced by transforming growth factor beta (TGFB) via a Smad and MAPK-dependent pathway, by hypoxia-inducible factor 1 alpha (HIF1A) and by Wnt/beta-catenin pathway [83; 84; 85; 86].

Thyroid hormone levels and the neoplastic process

Depletion of THs or their excess promotes modifications in tumoral growth and development. These changes correspond to the ability of THs to promote or inhibit cell proliferation in a cell type-dependent manner, as well as to induce differentiation, in a process linked to growth arrest and exit from the cell cycle. Indeed, THs seem to exert their effects in all hallmarks of the neoplastic process, which include sustained proliferation signaling, resistance to growth suppressors, evasion of programmed cell death, replicative immortality, sustained angiogenesis and promotion of invasion and metastasis [87].

In plasma, the amount of total T_4 exceeds the amount of T_3 by two orders of magnitude [88]. Both T_4 and T_3 enter the cell via transporters, including the monocarboxylate transporter 8 (MCT8) and the organic anion transporting polypeptide C1 [89; 90]. T_4 can be deiodinated to T_3 in the intracellular environment by DIO2. In contrast, DIO3 acts locally to decrease cellular T_3 concentrations. Thereby, the deiodinases are critical for the regulation of intracellular T_3 levels and therefore contribute to hormone nuclear concentration and saturation of thyroid hormone receptors (TRs) [91].

It is widely accepted that T_4, which comprises the main secretory product of the thyroid gland, is a prohormone and must be converted to the active form T_3 by DIO1 or DIO2 to promote TH metabolic effects. However, increasing evidence suggests that T_4 can promote nongenomic effects through direct interactions with several pathways, particularly in the context of neoplasia. This broad issue has been recently reviewed by our group and others and will not be discussed here [87; 92]. It should also be noted that rT_3, which is generally regarded as an inactive metabolite, seems to be relevant to the structure of both normal cells and tumor cells, by supporting the integrity of the actin cytoskeleton [93]. Critical intracellular signaling pathways, such as MAPK, Wnt, and Shh are dysregulated in tumoral cells, which may lead to upregulation or downregulation of the deiodinase enzymes depending on the context.

Moreover, the theory of the stem cell origin of neoplastic cells, and the relevant role of DIO3 in stemness, suggests an essential role of DIO3 in tumor development [15]. The yet unidentified reducing cofactor of deiodinases might be subject to alterations in redox and oxidative stress, which are well-known characteristics of the neoplastic microenvironment [94]. Thus, the catalytic efficiency of deiodinases can be impaired by neoplastic conditions [43].

Changes in deiodinase expression have been reported in several neoplasias (Table 1) [95; 96; 97; 98]. One of the best examples of how changes in deiodinases might alter TH concentrations is the clinical condition of consumptive hypothyroidism, a severe form of hypothyroidism due to high levels of DIO3 activity in the neoplastic tissues. It was first described in infantile liver hemangiomas (Huang, et al. 2000). Subsequently, pediatric and adult liver vascular tumors were also associated with increased expression and activity of DIO3 (Huang, et al. 2002; Weber Pasa, et al. 2017). Indeed, large vascular tumors can express enough DIO3 sufficient to inactivate a significant amount of plasma T_3 and cause overt hypothyroidism. DIO3 upregulation occurs through the Shh pathway and the MAPK signaling cascade in these types of tumors (Aw, et al. 2014). However, infantile hemangiomas origin is not fully elucidated. Current evidence suggests that hemangiomas are clonal proliferation of fetal endothelial cells, not hepatocytes. Another hypothesis speculates that these cells are derived from the placenta (Boye, et al. 2001; Chen, et al. 2013). Interestingly, either type of cells express a significant amount of DIO3 protein (Huang et al. 2003), what corroborates the suggestion that these tumors arise from other cells than hepatocytes.

As deiodinases control TH levels, they also contribute to the balance between proliferation and differentiation within the cell. Few studies have actually evaluated both deiodinase expression and intracellular TH concentrations at the same time (see the data below regarding basal cell carcinoma, glioblastoma and clear cell renal cell carcinoma) (Table 1) [81; 99; 100]. Nevertheless, it is important to keep in mind that disturbed deiodinase expression can go beyond the regulation of intracellular levels of T_4 and T_3 in the tumoral context. The upregulation or downregulation of deiodinases can reflect the overactivation or suppression of critical signaling pathways involved in carcinogenesis. Moreover, the expression of deiodinases may be a marker of hypermethylation or hypomethylation of the DNA regions where they are located, indicating that they may just represent a small portion of a bigger picture of aberrant cell function. Indeed, several tumor-related aberrations in the chromosomal regions of *DIO1*, *DIO2,* and *DIO3* have been described [96]. Thus, since the neoplastic process has distinct tissue-related features, it is reasonable to speculate that examining the role of

deiodinases in specific tumoral contexts can render a better understanding whether they are a cause or a consequence of neoplastic cellular imbalance.

The thyroid: a classical model

Significant amounts of ORD are found in normal thyroid tissue due to the high expression of DIO1 and DIO2 [11; 31; 101; 102]. DIO1 is the main enzyme responsible for T_3 production within the gland. DIO3 activity is regarded as being absent in the thyroid, but traces of *DIO3* mRNA transcripts have been found in human thyroid tissue samples [103; 104] (Fig. 2). When evaluating thyroid nodules, an increased 5' deiodination was observed in toxic and also follicular adenomas, while decreased activity was found in cold nodules when compared to healthy tissue [102; 105].

Differentiated thyroid cancer from follicular cells is the most common malignant neoplasia of the endocrine system. Papillary thyroid carcinoma (PTC) is the most prevalent histologic type accounting for more than 90% of cases, while follicular thyroid carcinoma (FTC) is responsible for the remaining cases (https://seer.cancer.gov/statfacts/html/thyro.html, Accessed on 02/09/2018) [106]. Genetic activation of the MAPK signaling pathway is a hallmark of PTC [107]. *DIO1* and *DIO2* seem to be underexpressed in PTC [103; 108; 109; 110; 111]. Earlier studies performed in human PTC samples have shown that *DIO1* mRNA levels were reduced in all the samples that were analyzed (n=14) when compared to the normal surrounding tissue. This was paralleled by a decrease in DIO1 activity with only one exception of a follicular variant of PTC [112]. Arnaldi et al. also reported significant *DIO1* and *DIO2* underexpression in most but not all PTC samples that were matched to normal tissue [113]. On the other hand, increased levels of *DIO3* mRNA and DIO3 activity have been demonstrated in human PTC samples. Of interest, PTC tumors carrying the $BRAF^{V600E}$ mutation had the highest levels of DIO3 activity. Moreover, a positive correlation between tumor size and DIO3 activity, as well as an increased DIO3 activity was demonstrated in thyroid tumor samples from patients advanced disease at diagnosis [103].

Taken together, one could speculate that changes in deiodinase expression in PTC could lead to decreased intracellular hormone levels and favor tumor proliferation. The increase in DIO3 and the decrease in DIO1 and possibly DIO2 that lead to diminishing T_3 concentrations in the microenvironment could provide an advantage for tumor cell proliferation since THs can block the oncogenic Ras-mediated proliferation that interferes specifically with the activity of the MAPK pathway [114]. Recently, crosstalk between the MAPK and SHH pathways leading to *DIO3* upregulation has been demonstrated in human

PTC cell lines [86; 103]. In support of this line of reasoning, the inhibition of *DIO3* mRNA expression through small interfering RNA (siRNA) decreases cyclin D1 expression and induces a partial G1 phase cell cycle arrest, thereby downregulating cell proliferation [86]. These observations indicate that SHH/Gli2 pathway contributes to DIO3 overexpression, suggesting that the consequent decrease in intracellular T_3 levels may be a critical factor for tumor proliferation in PTC. MAPK canonical signaling pathway is activated by the $BRAF^{V600E}$, the most commonly detected BRAF mutation in human PTC. Interestingly, mice expressing the $BRAF^{V600E}$ mutation in thyroid follicular cells developed rapid clinical hypothyroidism (within 48 hours) [115]. This might indicate that the reactivation of DIO3 in PTCs that harbor the $BRAF^{V600E}$ mutation through MAPK pathway. However, DIO3 expression was not evaluated in this model. Of note, increased immuno-stained DIO3 protein has been observed in FTC but not in medullary or anaplastic thyroid carcinoma samples [103].

In FTC samples we observed a significant increase in *DIO1* mRNA levels compared with nontumoral tissue, while others found comparable levels between the tumoral and the normal tissues or even decreased DIO1 activity [112; 113; 116; 117]. DIO1 activity was significantly higher in samples of metastases from follicular carcinoma [112]. Higher DIO2 activity was found in samples of larger metastasis of FTC. However, no significant changes in *DIO2* mRNA levels were observed, which suggests that DIO2 upregulation occurs mainly by post-transcriptional regulatory mechanisms [112; 117; 118].

Although DIO2 expression has not been evaluated in normal C cells, we described detectable DIO2 activity in medullary thyroid cancer (MTC) samples, which was comparable to the amounts in the surrounding normal follicular tissue. *DIO2* mRNA and DIO2 activity levels have also been demonstrated in the TT cell line (derived from MTC), which might suggest a potential role of intracellular T_3 in this neoplastic tissue [119].

We performed an analysis of The Cancer Genome Atlas (TCGA) database (http://cancergenome.nih.gov/) through Gene Expression Profiling Interactive Analysis (GEPIA) (http://gepia.cancer-pku.cn) [120], which is a bioinformatics research platform for the profiling and interactive analysis of cancerous gene expression based on TCGA and Genotype-Tissue Expression (GTEx) public databases [121]. Intriguingly, analysis of TCGA database showed that *DIO1* and *DIO3* genes are downregulated in thyroid carcinoma (77% PTC, 21% FTC) ($n = 512$, $p<0.01$ for *DIO1* and *DIO3*) compared to matched TCGA and GTEx data of normal tissue. *DIO2* expression in tumors is comparable to that in normal tissue (Fig. 3).

Basal cell carcinoma (BCC): insights on the imbalance of the proliferation/differentiation equilibrium

DIO2 and *DIO3* mRNA transcripts, as well as DIO2 and DIO3 activities, are present in normal human skin [33; 122; 123]. BCC, which is the most common cutaneous malignancy, is a non-melanocytic skin cancer that arises from basal cells (the lower layer of the epidermis) [124]. Dysregulated Hedgehog (Hh) signaling is a hallmark of this neoplasia, due to inactivation of Protein Patched Homolog 1 (PTCH1), which is an inhibitor of Hh signaling [125]. Most of the research regarding deiodinases and BCC tumorigenesis has been performed by Dentice et al. [81; 126; 127; 128]. They demonstrated that sonic hedgehog (Shh), through Gli2, directly induces DIO3 in human BCCs, reducing intracellular T_3 and thus increasing cyclin D1 and proliferation. Shh also mediates DIO2 reduction through post-transcriptional mechanisms. Thus, DIO3 knockdown blocks proliferation and reduces the oncogenic potential of BCC tumor cells. Indeed, the growth of BCC cells implanted in DIO3 knockdown mice was dramatically reduced, suggesting that T_3 reduces BCC proliferation and tumorigenic potential [81]. Moreover, in G2N2c keratinocyte cells, DIO3 depletion led to the arrest of the cell cycle in G_1 and to decreases in cyclin D1 levels, further demonstrating that cell proliferation is drastically reduced by DIO3 inhibition. Inversely, T_3 treatment decreases Gli2 protein levels through upregulation of cAMP/PKA signaling. Whether or not T_3 directly affects DIO3 levels in this context is unknown [126]. However, T_3 modulates DIO3 expression indirectly through another pathway. T_3 has a suppressive effect on the oncogenic microRNA (miRNA) miR21, which in turn induces DIO3 expression through downregulation of the Grainyhead-like protein 3 homolog (GRHL3). GRHL3 is tumor suppressor factor that is expressed in the skin and is essential for epidermal differentiation. Therefore, the existence of a miR21/GRHL3/DIO3 axis critically contributes to the intracellular TH imbalance in the context of BCC [127]; thus, BCC is an excellent model to study the TH role in the delicate balance between cell proliferation and differentiation. *DIO3* mRNA and DIO3 activity have been reported in BCC cells, while only *DIO2* mRNA has been demonstrated in these cells. The authors infer the presence of DIO2 activity in BCC cells by the fact that genetically-induced depletion of *DIO2* gene using CRISPR/Cas9 technology leads to decreased levels of T3-responsive targets. Therefore artificial modulation of these enzymes can alter the local TH levels, and their effects on tumor growth can be evaluated [128]. Interestingly, TH activity, as evaluated by a T_3-dependent artificial promoter that drives the luciferase gene, is reduced in BCC DIO2KO cells and enhanced in BCC DIO3KO cells. DIO2KO-BCC (low intracellular T_3) cells are characterized by a high proliferation rate, a high proportion of S-phase cells and

decreased apoptosis. On the other hand, DIO3KO-BCC cells have decreased proliferation and low levels of cyclin expression. This interesting model could be expanded to other neoplasias aiming for a better understanding of the effects of THs on cancer [128].

Colorectal cancer: deiodinases mediate TH changes as differentiation agents

Colorectal cancer (CRC) is characterized by a complex array of genetic alterations, among which the mutation in the adenomatous polyposis coli (APC) gene is the most frequent (85% of cases). This mutation leads to a constitutively active Wnt pathway due to inadequate degradation of beta-catenin by the APC protein [129]. Dentice et al. provided the first evidence suggesting an interplay between the Wnt/beta-catenin pathway and the TH signaling pathway in the balance between proliferation and differentiation in colorectal cancer [85]. The authors elegantly showed that exogenous T_3 treatment reduced proliferation and increased differentiation *in vitro* in CRC-derived cell lines. Moreover, they demonstrated that the activation of Wnt/beta-catenin induced the expression of *DIO3* mRNA while decreasing *DIO2* mRNA. This dual mechanism could result in intracellular hypothyroidism, favoring proliferation over differentiation. Additionally, they showed that DIO3 depletion had T_3-like effects and that xenografts of DIO3-depleted cells exhibited reduced tumor growth.

Dentice and colleagues have also shown DIO3 expression was associated with neoplastic transformation in CRC. Using immunohistochemistry in 105 human paraffin-fixed samples, they observed that DIO3-positivity was found in 10% of the normal tissues, whereas 80% of carcinomas and 90% of adenomas were DIO3-positive, suggesting that DIO3 is a Wnt/beta-catenin target in the context of colon carcinoma proliferation [85]. Moreover, T_3 treatment or DIO3 inhibition could be used to promote CRC-stem cell (CRC-SC) differentiation via Wnt and BMP4 signaling. Interestingly, TH-induced differentiation increased CRC-SC sensitivity to traditional chemotherapy (oxaliplatin and 5-FU), raising the possibility of combination therapy in the future [130].

The analysis of the public TCGA database trough GEPIA demonstrated low expression of *DIO1* in CRC and detectable levels of *DIO3* mRNA that were similar in tumor tissue and normal tissue (Fig. 3). These findings are in contrast with the findings of Dentice et al. [85] who found that DIO3 protein levels were increased as evaluated through immunohistochemistry in 22 out of 24 tumors tissue samples compared with normal surrounding tissue. These differences could be attributed to post-translational mechanisms that regulate protein expression as well as to differences in the controls (normal predisposed surrounding tissue vs. tissue from healthy individuals). *DIO2* was overexpressed in CRC

tissue when compared to non-paired tissue (n=275, $p<0.01$) and was found to be a marker of good prognosis in this database (5-year survival: 66% in the high expression group vs. 46% in the low expression group; $p<0.01$). This is a finding that should be further confirmed.

The glioma model: the adverse effects of T_3

Brain cells are uniquely sensitive to the effects of THs; therefore they require even tighter control of TH homeostasis when compared to other organs. THs stimulate the processes of myelination, the proliferation of glial cells as well as axon growth and formation. The necessity for strict control of TH levels might explain the detectable activity of DIO2 and DIO3 in glial cells [12; 15; 131; 132; 133]. Indeed, studies performed in rat cerebral cortex demonstrate that approximately 80% of the T_3 bound to nuclear receptors is produced locally by monodeiodination of T_4 [131; 134]. DIO3 activity is present in adult human brain tissue, and high DIO3 levels in placental tissue play an essential role in protecting the developing brain of the fetus from excessive T_3 concentrations [135; 136].

Gliomas are tumors arising from the brain parenchyma, with a broad range of aggressiveness. Grades I and II gliomas are referred to as low-grade gliomas, while the more rapidly progressive tumors are referred to as high-grade gliomas (grades III and IV) [137]. The modulation of intracellular T_3 and T_4 levels by deiodinases in glioblastoma is of great importance, since the PI3K, Src kinase, and ERK1/2 signaling cascades are parallel pathways that are stimulated by T_3 in U-87MG cells, a commonly studied human glioblastoma cell line [138]. These are among the most commonly dysregulated pathways in this type of cancer. *DIO2* expression and DIO2 activity were found in glioblastoma and were the highest in a tissue sample from an anaplastic oligodendroglioma [139]. Nauman et al. made an effort to correlate deiodinase activity and the T_4/ T_3 concentrations of gliomas and normal surrounding tissue. They found that T_4 and T_3 concentrations in the tumor tissues were lower than those in the non-tumor tissues in the majority of the patients, whereas DIO2 activity was higher in tumor tissues than in normal tissues [99]. In one study, DIO3 activity was detected in samples of gliomas, although no comparison to healthy tissues was performed [140]. In another study, DIO3 was detected in a heterogeneous manner. In two samples, the DIO3 activity was lower than in the tumor tissue, but in all the high-grade gliomas (IV), the DIO3 activity was considerably higher in the tumoral tissue. These findings suggest that the expression of deiodinases and the metabolism of THs are altered in human brain tumors, and these changes might be related essential factors that contribute to tumorigenesis or tumor growth [99]. Finally, it is worth

mentioning that limited clinical data suggest that medically induced hypothyroidism may increase patient survival in high-grade glioblastoma [141; 142].

TCGA database analysis through GEPIA demonstrated that *DIO2* expression was significantly downregulated in glioblastoma samples (n=163) when compared with non-paired normal tissue. Similarly, *DIO3* tended to be underexpressed in glioblastoma tumors when compared to healthy tissue (Fig. 3B and 3C). However, post-transcriptional factors, as well as substrates and cofactor availability, could explain the discrepancies between these data and the results of previous studies that showed more heterogeneous patterns of enzyme activity. Moreover, TCGA data that were analyzed included only glioblastoma multiforme samples, and does not comprises more differentiated tumor subtypes. It is of great importance to evaluate if altered deiodinase expression is modulated by signaling pathways that are often dysregulated in this type of cancer, such as the PI3K, Src kinase, and ERK1/2 signaling pathways, and to what extent this contributes to tumor aggressiveness.

Clear cell renal carcinoma: *TRB1* and *DIO1* as tumorigenesis protagonists

Clear cell renal cell carcinoma (ccRCC) is the most common type of kidney cancer, accounting for approximately 75% of all cases. One of the first reports of the dysregulation of THs in cancer involved ccRCC, and there have been multiple and consistent reports supporting decreased expression of *DIO1* mRNA and DIO1 protein in ccRCC, as well as decreased expression of thyroid hormone receptor beta (*THRB)*1 [143; 144]. Studies indicate that T_3 acts through *THRB1* as the primary controller of *DIO1* expression in the kidney, unlike in other tissues where T_3 acts through both thyroid hormone receptor alpha (THRA) and THRB [145].

Dysregulation of the splicing mechanisms in ccRCC and the existence of multiple splicing variants of *DIO1* mRNA and *THRB1* have been consistently reported, suggesting a cause for DIO1 disturbances [100; 146; 147]. Additionally, microRNAs targeting the 3'UTR region of *DIO1* mRNA (miR-224 and miRNA-383) were reported to be upregulated in ccRCC. The transfection of pre-miRNA-224 or pre-miRNA-383 reduced *DIO1* mRNA expression *in vitro*, confirming the suppressive effect of these miRs in *DIO1* expression. Consistent with the downregulation of *DIO1*, intratumoral T_3 levels were 58% lower than in control tissue [148]. More recently, Poplawski et al. provided clear evidence of DIO1 involvement in tumorigenesis by demonstrating that induction of *DIO1* inhibits proliferation and migration and improves adhesion to laminin in ccRCC-derived cell lines (KIJ265T and KIJ308T) [149]. Moreover, they noted decreased mRNA expression the of genes involved in the G1-to-S

transition (cyclin D1, cyclin E2, Cdk2, E2F2) in the KIJ 265T cell line. These findings are in accordance with the previously reported *DIO1* downregulation in ccRCC, showing that a lower DIO1 activity could increase proliferation, promote migration and allow cells to detach from ECM proteins more easily. Indeed, *DIO1* expression is significantly down-regulated in ccRCC when compared to normal samples based on TCGA data analysis through GEPIA (n=523, p<0.01) (Fig. 3A). However, no significant changes in *DIO2* and *DIO3* expression were found (Fig 3B and 3C). Whether these effects are the result of intracellular hypothyroidism due to the insufficient conversion of T_4 to T_3 remains unclear. Supplementation of cells with T_3 did not reverse the effects of the diminished DIO1 activity. However, this finding might also be due to the inefficient transport of T_3 into the cell [149]. Restoration of *DIO1* expression in ccRCC 'downregulates' oncoproteins that promote proliferation, migration, and invasion while triggers proteins involved in regulation of anti-oxidative processes. Together, these results suggest that loss of *DIO1* expression could be an adaptive mechanism, protecting the cells against overstimulation of cancer metabolism and induction of apoptosis [150].

Liver neoplasias: complex effects of THs, but few data on deiodinase expression

Classically, significant T_4 ORD is found in human liver homogenates [151; 152]. When evaluating liver microsomes, high DIO1 and DIO3 activities are detected in fetal liver but only DIO1 and mostly none DIO3 activities are found in adult liver. DIO2 activity is virtually absent in both fetal and adult tissues [153]. Both human hepatocytes as well as human hepatoblastoma-derived cells HepG2 show an approximately 10-fold lower rate of iodothyronine metabolism when compared to rat hepatocytes [154; 155; 156]. However, deiodinase data in human liver tumors is scarce, and the influence of TH in hepatocellular carcinoma (HCC) is controversial. While THs seem to reduce growth, they also promote cell migration in hepatoma cell lines [157; 158; 159] THs can also induce cell self-renewal and promote drug resistance of HCC CSCs [160].

In an analysis of 13 benign lesions and 7 samples of hepatocellular carcinoma that were compared to normal tissue, there was no difference in DIO1 activity [161]; however, DIO1 activity was decreased in hepatic hemangiomas [152]. HepG2 express functional DIO1 [156; 162] but neither DIO2 or DIO3 activity has been observed in these cells [163].

Indirect clinical and basic data also point to a possibility that *DIO3* upregulation might occur in HCC. In a case-control trial, the high prevalence of hypothyroidism among patients with HCC (11.7%) suggested that long-term hypothyroidism was associated with HCC [164].

Studies in mouse HCC models have identified a cluster of microRNAs (miRNA) that are involved in the upregulation of the *DLK1-DIO3* genomic imprinted region, where the *DIO3* gene is located. More interestingly, overexpression of the *DLK1-DIO3* miRNA cluster was positively correlated with HCC stem cell markers and associated with a high level of serum α-fetoprotein, which is a conventional biomarker for liver cancer, and for reduced survival rates in HCC patients [165]. No differences in deiodinase expression between tumor tissue and normal tissue were observed in TCGA data (Fig. 3).

Breast cancer: deiodinases as markers or effectors?

THs are essential for mammary gland growth and development[166]. Studies in rats demonstrate that the mammary glands express significant amounts of functional DIO1 only during the functional stages (lactation) or during differentiation (puberty) [55; 167; 168]. In contrast, DIO2 activity is found in non-stimulated mouse mammary glands, and its expression decreases substantially in the lactating stage [169]. In human non-lactating tissue, low or undetectable DIO1 activity has been demonstrated [170] while no difference in *DIO1* mRNA expression was observed between normal and lactating tissue [171]. We were unable to find data on DIO2 and DIO3 expression in human normal breast tissue.

The first studies of TH metabolism in breast cancer were performed in rat mammary adenocarcinoma. The R3230AC mammary adenocarcinoma is an estrogen-responsive autonomous tumor that has been maintained by serial transplantation in female Fischer rats since 1963 [172]. ORD has been demonstrated in this tumor and is insensitive to PTU, which would be compatible with DIO2 activity, as well as IRD, generating rT_3 from T_4, suggesting that the deiodination pathways were preserved in this tumor model [173]. More recently, abundant activity of DIO1 and high *DIO1* mRNA levels have been shown in human breast cancer, particularly in the most differentiated subtypes [170; 171]. DIO1 activity was also found in the breast cancer cell line MCF-7 (differentiated epithelial carcinoma). However, the more dedifferentiated MDA-MB-231 (estrogen receptor negative) cell line did not express any deiodinase activity [174]. Despite these negative findings, upregulation of *DIO1*, *DIO2,* and *DIO3* mRNAs has been shown in MCF-7 cells. On the other hand, only *DIO2* is upregulated in MDA-MB-231 when these cell lines were compared to non-tumoral human breast cells MCF-10A [175]. The differences between deiodinase mRNA expression and protein activity could be explained by different subtypes of breast tumor as well as posttranscriptional regulatory mechanisms. Taken together, these results may suggest a role for DIO1 as a marker of differentiation in breast neoplasias. It is interesting to correlate these data with the effects

of T_3 treatment on cell proliferation. T_3 leads to increased proliferation in MCF-7 cells but does not interfere with the growth of MDA-MB-231 cells [176; 177; 178].

Analysis of TCGA public data demonstrates that *DIO2* expression is upregulated in breast cancer when compared to normal tissue (n=1085, $p<0.01$) (Fig. 3B), and the other deiodinases expression levels in breast cancer are comparable to normal tissue (Fig. 3A and 3C). As THs influence the proliferation of breast cancer cells, it is worthwhile to consider that DIO3 expression in mammary neoplasias may play a role in modulating intracellular T_3 levels and thus contribute to tumor progression [179]. We observed moderate immunostaining of DIO3 in normal human mammary gland tissue and a significant expression of DIO3 staining in samples of invasive ductal carcinoma (I.M. Goemann, V. Marczk, A.L. Maia, unpublished observations). *DIO3* has also been shown to be expressed in the MCF-7 cell line [163; 180]. To what extent these alterations contribute to tumor growth due to the modulation of intracellular T_3 or represent markers of altered signaling pathways has yet to be demonstrated.

Deiodinase expression in other neoplasias

DIO2 expression is induced in most brain tumors derived from glial cells [99; 139; 140]. Studies performed in 105 pituitary tumors demonstrated that *DIO2* and *DIO3* mRNA were significantly augmented in pituitary tumors when compared with normal pituitary tissue. However, in the rare TSH-secreting pituitary tumor subtype, the *DIO3* mRNA was strongly induced, while reduced *DIO2* mRNA levels were detected. Interestingly, in the case of TSH-secreting pituitary adenomas, the observed pattern of deiodinase mRNA expression may explain the 'resistance' of these tumors to TH feedback (Tannahill, et al. 2002). When evaluating enzyme function in pituitary adenomas, Baur et al. found both DIO1 and DIO2 activity in normal and tumoral tissue. Of note, highest activities of both enzymes were found in TSH- and PRL-producing adenomas [181]. These sets of deiodinase abnormalities may have functional consequences on pituitary tumor growth.

The clinical picture of consumptive hypothyroidism is not restricted to liver hemangiomas. A recent systematic review by our group revealed that among children, 97% had vascular tumors, with hepatic vascular tumors representing 88% of the cases, parotid hemangiomas 5%, cutaneous hemangiomatosis 2%, and fibrosarcomas 2%. Because there is a high risk of bleeding associated with vascular tumor biopsy, only three patients underwent tissue sampling. High DIO3 activity was confirmed in all tumor specimens. Tumor histology in the adult population differs from that in pediatric patients. Hepatic vascular tumors represented only 33% of the cases. Gastrointestinal stromal tumors (GIST) and fibrous tumors

each accounted for 33% of the cases. Functional assays confirmed high DIO3 activity levels in all the adult patients [180; 182].

The analysis of deiodination in lung cancer tissue demonstrated that the activity of DIO1 is significantly lower in tumor tissue when compared to the peripheral normal lung tissue, while DIO2 activity is similar in peripheral lung and lung cancer tissue [183]. Interestingly, *DIO3* was found to be hypermethylated in a subset of hematological malignancies, as assessed by microarray-based methylation analysis, suggesting that aberrant epigenetic modifications may confer *DIO3* tumor-associated properties [184]. This is also supported by data that demonstrates *DIO3* hypermethylation in lung cancer, which was associated with lower levels of *DIO3* mRNA expression as compared to normal tissue [185]. Therefore, silencing of DIO3 gene by hypermethylation might be an epigenetic pattern common to different types of human cancer.

FUTURE DIRECTIONS AND CONCLUSION

The set of data summarized here clearly indicates a potential role of alterations in deiodinase-related TH levels on the promotion of human carcinogenesis. In addition to the importance of this evidence, it is critical to keep in mind that these studies mostly show an association between changes in the levels of deiodinases and cancer, demonstrating that there is still a lack of knowledge regarding the direct effect of these enzymes on oncogenic processes. Indeed, the majority of data available so far have been obtained by studies performed in normal and tumor tissues from adult patients, human cancer cell lines and *in vivo* models of carcinogenesis. By using these models, the most relevant data that specifically analyzed the effect disruption of deiodinases on carcinogenesis were obtained by chemical inhibition or gene knockout/knockdown. Such approaches may imply the effects of enzyme reactivation on already established tumors, and highlight the advantages of such inhibition on tumor behaviors. However, they add limited information to the knowledge of cell transformation and cancer development. Comparative analysis of TCGA and GTEx database can now provide further insight into deiodinases mRNA expression in different types of tumors. Likewise, this approach has also intrinsic limitations. TCGA and GTEx data were not collected in a single experiment. This may especially affect measurements of expression and correlation across different samples. Moreover, GTEx (normal tissue database) RNA are extracted from all tissues of postmortem donors with variable ischemic time (what could compromise RNA quality). On the other hand, TCGA comprises one of the largest and most comprehensive cancer genomics datasets in the world, providing analyses of high-throughput

RNA sequencing data of 33 types of cancer, describing tumor tissue and matched normal tissues from more than 11,000 patients. This data is well validated and contributed to more than a thousand studies of cancer by different research groups (https://cancergenome.nih.gov/). Using the Human Proteome Map project and RNA-Seq measurements from the GTEx project, a comprehensive tissue- and gene-specific analysis of 16,561 genes and the corresponding proteins revealed that across the 14 tissues, the correlation between mRNA and protein expression was positive and ranged from 0.36 to 0.5 (Spearman correlation value) [186]. As deiodinases are subject to posttranslational regulation as previously described, analysis confined to mRNA expression through public databases should be interpreted carefully, since it may not represent final protein levels and consequent TH changes within the tumor. TCGA also provides additional proteomics data, though only of a limited number of proteins, amongst which deiodinases are not included [187].

Thus, *in vivo* and *in vitro* functional models are needed to fully understand how specific drivers, such as deiodinases, impact tumor initiation and maintenance. Genetically engineered mouse models (GEMMs) have been recognized as powerful tools to investigate the impact of gene function on tumorigenesis [188; 189]. Over the last several years, genome engineering and stem cell technologies have allowed the production of strains in which specific genes can be expressed in a tissue-specific manner. With the advent of Cre-lox and CRISPR/Cas9 technologies, conditional knockout and/or knockin alleles can precisely model events associated with human carcinomas. Furthermore, conditional gene expression systems based on the tetracycline (tet) response system or estrogen–receptor (ERT2) fusions allow temporal analysis of gene function. In addition, the insertion of fluorescent reporters enables lineage tracing and examination of activity [190; 191]. These new approaches in combination allowed the generation of genetically and histopathologically accurate *in vitro* and *in vivo* models of various human cancers that in turn can be applied to explore the role of the disruption of deiodinases (reactivation and/or downregulation) on cancer initiation and behavior.

In the context of the present review, most of the tumors mentioned above have already been modeled using stem cell technology which has emerged as a great tool for this kind of research. With regard to the role of genes on the initiation and progression of thyroid cancer, the generation of 3D functional thyroid models derived from mouse embryonic stem cells (mESC) [192] constitutes a major breakthrough in the field of thyroid research and raises opportunities for addressing questions related to thyroid organogenesis and diseases. By the induction of thyroid transcriptional factors [192] or by induction of specific pathways with

chemical timing in mESCs [193], these protocols allow us to obtain thyroid cells at different stages of differentiation. In addition, they provide the advantage of generating 3D functional follicles, which is a more sophisticated model to address gene effects of thyroid cell transformation and can be easily manipulated using estrogen–receptor fusions, Cre-lox and/or CRISPR/Cas9 technologies [192; 194].

Human pluripotent stem cells (hPSCs) have been used as a valuable model for studying the development and progression of gliomas. In addition, neural differentiation protocols allow the derivation of relevant early neural stem cells that are often inaccessible. Thus, early tumorigenesis can be studied in the proper cellular context [195; 196]. Gene function tests can be evaluated by mutational models, such as lentiviruses encoding constitutively active forms of mutated genes; and knockdown studies can be performed by using shRNA [196]. Similarly, human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) have been used to model human diseases of the large intestine. Through modulation of signaling pathways that are known to regulate normal mouse embryonic development, a stepwise strategy was designed for the progressive generation of definitive endoderm (DE), hindgut endoderm (HE), and subsequently the formation of colonic organoids (COs). These 3D structures constitute the best models that resemble *in vivo* organ function and are one of the best tools for disease modeling and drug discovery for some types of cancer [197]. Therefore, we can make use of this technology to evaluate deiodinase dysregulation and consequent TH imbalance in the fine equilibrium of cellular proliferation and differentiation.

The expression of deiodinases in the neoplastic context is cancer-specific and dependent on several clinical and tumoral characteristics. Despite the challenges in studying selenoenzymes *in vitro* an *in vivo*, the role of DIO1, DIO2 and DIO3 in each tumoral context is beginning to unravel (Table 1). Deiodinases can function as markers of disease and cell differentiation or play essential roles as intracellular TH regulators, though we still lack data on the potential concomitant function of all enzymes in each neoplastic context. Moreover, deiodinases can participate in signaling pathways through "TH-independent" mechanisms that need to be further explored. In summary, the understanding of the myriad of mechanisms underlying the balance between tumor cell proliferation and differentiation promoted by THs through deiodinase regulation is critical for the development of new treatment strategies for cancer, inducing tissue-specific or even intracellular changes in TH status that could block excessive proliferation and/or induce tumor redifferentiation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

Table 1.

Table 1. Expression of deiodinases in different types of cancer in humans.

Tumor	DIO1	DIO2	DIO3	Overall effect on intracellular T_3 levels	Potential effect of T_3 on hallmarks of cancer	References
Basal cell carcinoma	N/A	increased *DIO2* mRNA levels, presence of DIO2 activity levels	increased *DIO3* mRNA, protein and enzymatic activity levels	decreased*	promotes of proliferation and decreases apoptosis	(Dentice, et al. 2007; Miro, et al. 2017)
Breast cancer	increased mRNA and enzyme activity levels, particularly in the most differentiated subtypes	*DIO2* mRNA upregulated in MDA-MB-231 cell line but not in MCF-7 cell line	*DIO3* mRNA upregulation is found in MCF-7 cells, and DIO3 protein is present in breast cancer samples	changes in deiodinases status involve T_3 activation and inactivation and its "consequence" on intracellular TH concentrations are unclear	influences cell proliferation	(Debski, et al. 2007; Garcia-Solis and Aceves 2003; Rusolo, et al. 2017; IM Goemann et al., unpublished observations)
Clear cell renal carcinoma	decreased mRNA and enzyme activity levels	N/A	N/A	decreased (measured)	stimulates proliferation and invasion, contributes to oxidative stress response	(Pachucki, et al. 2001; Poplawski and Nauman 2008; Poplawski, et al. 2017a; Poplawski, et al. 2017b)
Colorectal cancer	N/A	N/A	upregulation of *DIO3* mRNA, higher protein expression in cancer samples when compared to normal tissue	decreased*	induces proliferation	(Dentice, et al. 2012)
Glioma	N/A	increased *DIO2* mRNA and DIO2 activity levels	variable levels of *DIO3* mRNA and DIO3 activity	decreased (measured)	induces proliferation	(Mori, et al. 1993; Murakami, et al. 2000; Nauman, et al. 2004)
Hemangioma	decreased DIO1 activity levels	N/A	increased *DIO3* mRNA and DIO3 activity	decreased (measured)	?	(Huang, et al. 2000; Kornasiewicz, et al. 2014; Kornasiewicz, et al. 2010)
Lung cancer	decreased DIO1 activity levels	DIO2 activity similar to normal lung tissue	decreased *DIO3* mRNA levels	changes in deiodinases status involve T_3 activation and inactivation and its "consequence" on intracellular TH concentrations are unclear	?	(Molina-Pinelo, et al. 2018; Wawrzynska, et al. 2003)
Papillary thyroid cancer	decreased *DIO1* mRNA and DIO1 activity levels	decreased *DIO2* mRNA and DIO2 activity levels	increased *DIO3* mRNA and DIO3 activity levels	decreased*	induces proliferation and invasion	(Ambroziak, et al. 2005; de Souza Meyer, et al. 2005; Köhrle, et al. 1993; Murakami, et al. 2001; Romitti, et al. 2012; Toyoda, et al. 1992)
Pituitary tumors	both DIO1 and DIO2 activity is detected in tumoral and normal tissues in variable levels		increased *DIO3* mRNA, variable DIO3 activity levels	changes in deiodinases status involve T_3 activation and inactivation and its "consequence" on intracellular TH concentrations are unclear	?	(Baur, et al. 2002; Tannahill, et al. 2002)

*Intracellular T_3 levels were evaluated indirectly by intracellular T_3-responsive reporters or inferred according to changes in deiodinases expression. DIO1: deiodinase type 1, DIO2: deiodinase type 2, DIO3: deiodinase type 3. N/A: not available.

Figure 1.

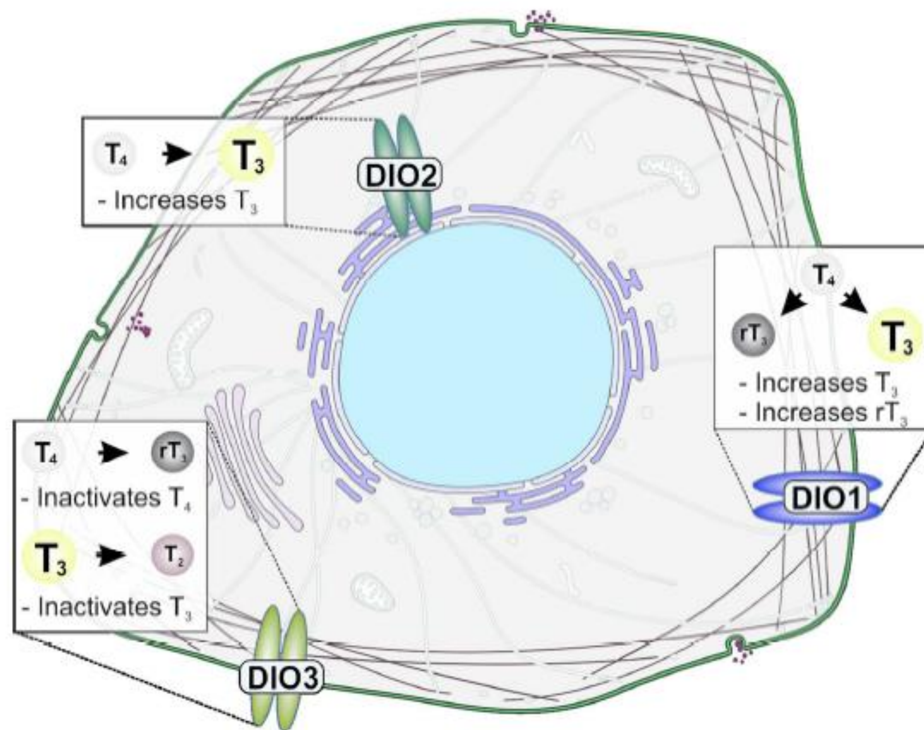

Figure 1: Schematic representation of the localization of deiodinases within the cell as well as the pathways of deiodination by which iodothyronines are generated. D1 and D3 are located in the plasma membrane, while D2 is located in the endoplasmatic reticulum. D1 catalyses both ORD and IRD, promoting both TH activation (generating T3 from T4) and inactivation (generating rT3 from T4). D2 is responsible for exclusive ORD, yelding T3 form T4 and T2 from rT3. D3 is an exclusive TH inactivating enzyme, generating T2 from T3 and rT3 from T4. Cell graphic representation adapted from the Human Protein Atlas (https://www.proteinatlas.org/images_static/cell.svg).

Figure 2.

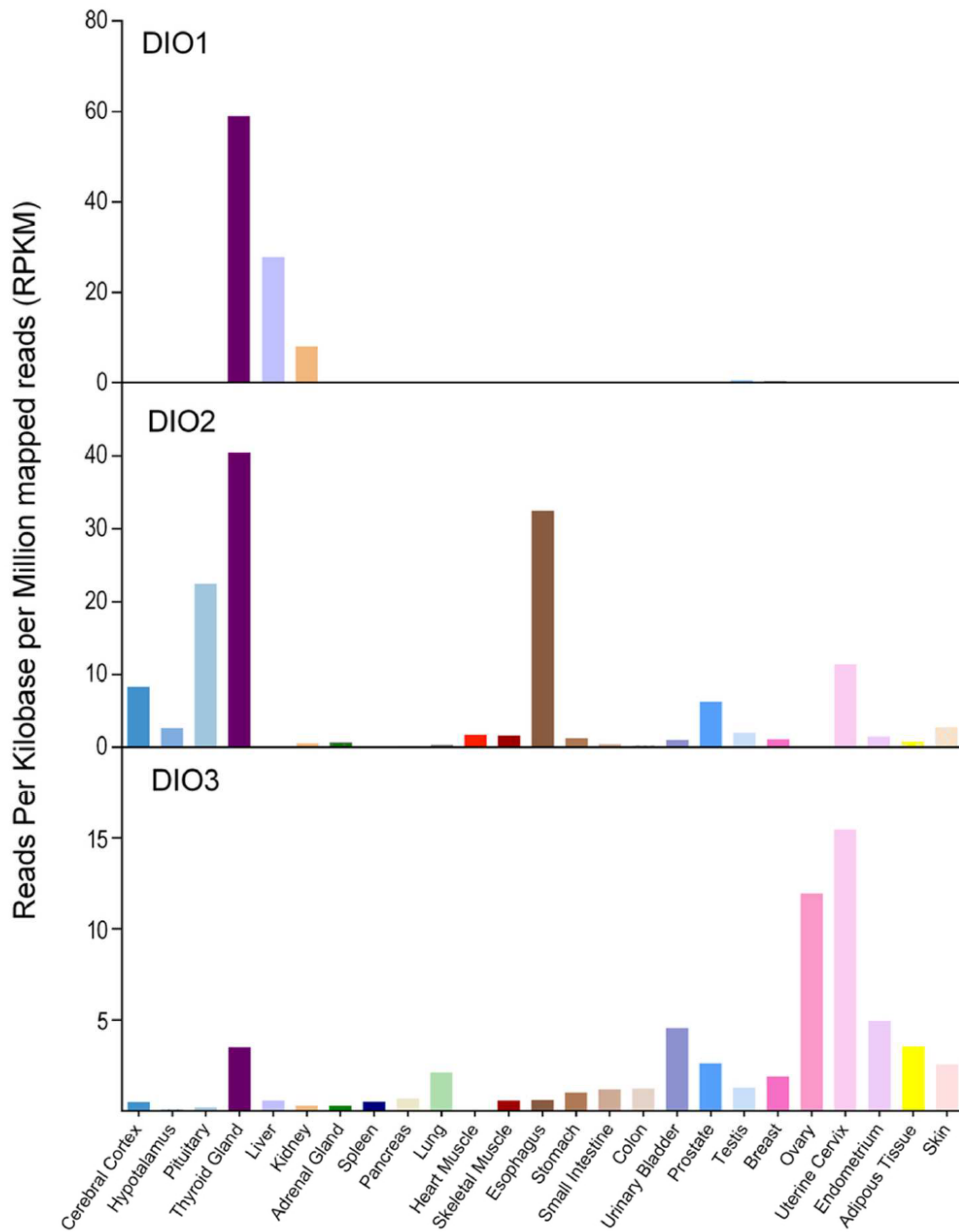

Figure 2: Graphic representation of mean mRNA levels of *DIO1*, *DIO2* and *DIO3* in human tissues. RNA-Seq data are reported as the median reads per kilobase per million mapped reads (RPKM) generated by the Genotype-Tissue Expression (GTEx) project[121]. Data were downloaded from the Human Protein Atlas available at v18.proteinatlas.org (www.proteinatlas.org)[104].

Figure 3.

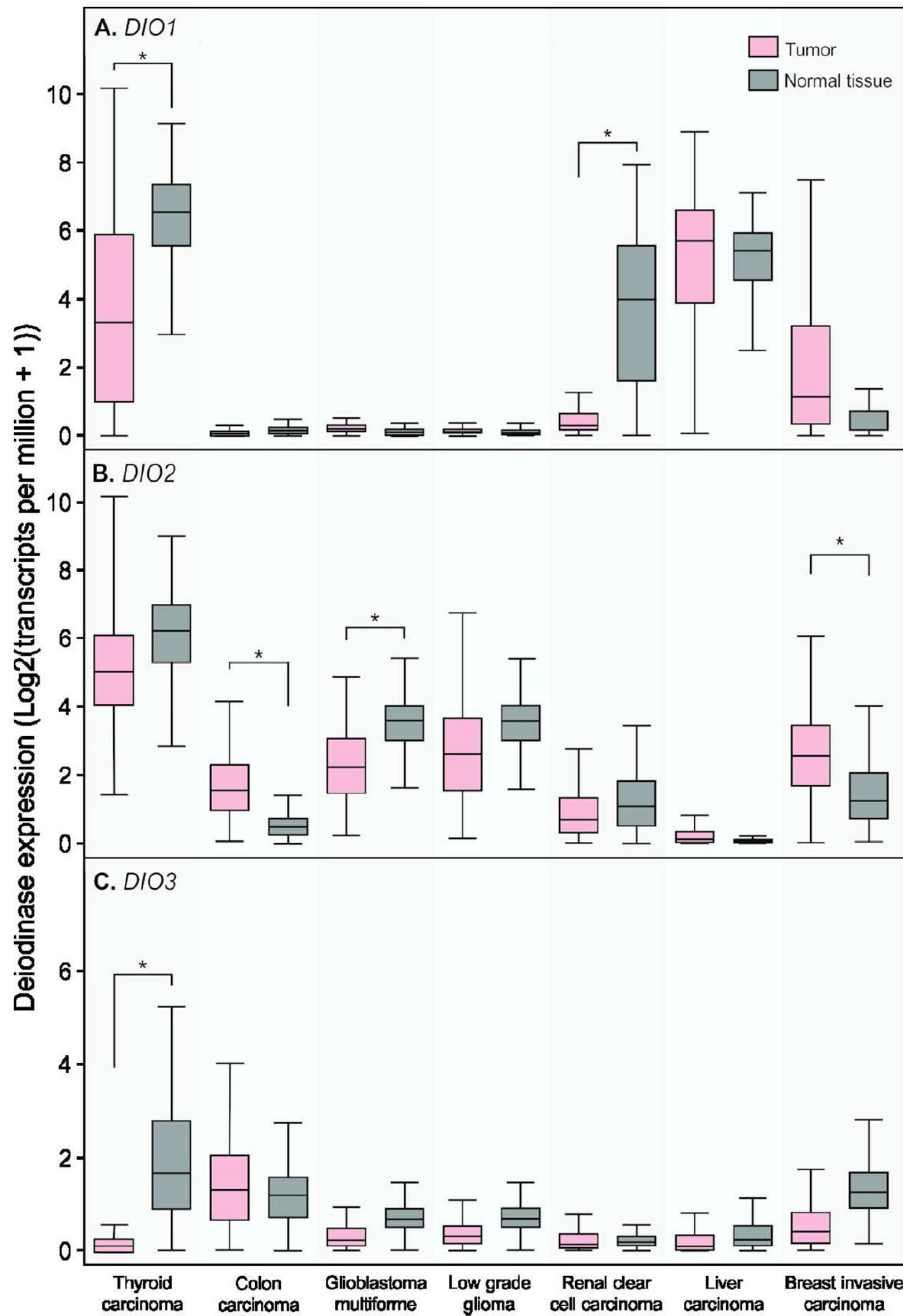

Figure 3. Expression of *DIO1*, *DIO2,* and *DIO3* in different neoplasias (red) compared to matched TCGA and GTEx data from normal tissue (gray). Expression values are presented in log-scale (log_2[Transcripts per million(TPM)] + 1). Data were obtained from TCGA and GTEx databases and processed and analyzed with GEPIA. *$p<0.01$.

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Parte III

Decreased expression of the thyroid hormone-inactivating enzyme type 3 deiodinase is associated with lower survival rates in breast cancer

Decreased expression of the thyroid hormone-inactivating enzyme type 3 deiodinase is associated with lower survival rates in breast cancer

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ABSTRACT

Breast cancer is a highly heterogeneous disease, diverse in its behavior and responsiveness to the different modalities of treatment. The identification of biomarkers that predict tumor biological behavior is warranted in improving patient survival. Thyroid hormones (THs) are critical regulators of several cellular processes, and alterations in TH status are known to contribute to cancer progression through virtually all the hallmarks of cancer. Clinical studies associate THs levels with breast cancer mortality, while THs have been shown to influence breast cancer proliferation, apoptosis and migration in *in vitro* models. Type 3 deiodinase (DIO3) is the main enzyme responsible for TH inactivation, and its expression profile in breast cancer is unknown. We aimed to evaluate expression patterns and the prognostic significance of DIO3 in breast cancer. Methods: The expression of DIO3 was investigated through immunohistochemistry (IHQ) in a primary cohort of 53 samples of breast tissue and was validated in a second cohort using the RNA-Seq data of 1094 patients from TCGA database (BRCA study). We assessed DIO3 expression in both populations according to retrieved clinicopathological information. For gene methylation analysis, DNA methylation and clinical data for 890 samples from the TCGA-BRCA study were obtained. Regulation of *DIO3* gene was investigated in MCF-7 and MDA-MB-231 cell lines. Results: We observed significant expression of *DIO3* mRNA and DIO3 protein in normal breast tissue. In the primary cohort, DIO3 expression in FFPE tissues of breast cancer was positive in 35/39 (89.7%) of Invasive Ductal Carcinoma (IDC), with a mean H-Score of 104.9 ± 55, and only in 1 of 3 samples of invasive lobular carcinoma (ILC) (H-Score=86). Moreover, loss of DIO3 expression was associated with reduced overall survival (OS) (HR 4.29 [95% CI, 1.24-14.7] P=0.021). We then validated these findings in the second cohort. *DIO3* mRNA expression was reduced in tumor samples when compared to normal tissue (logFC=-1.54, p<0.0001) and was increased in ER-positive when compared to ER-negative samples (logFC=0.428; P=0.013). When patients were divided by median mRNA expression, low DIO3 expression was associated with worse overall survival in a univariate model (HR 1.6 [95% CI, 1.18-2.26]; P=0.003) and remained as an independent prognostic factor in a multivariate model adjusted for age, tumor size, lymph node and distant metastasis, ER and PR status (HR 1.55 [95% CI, 1.07-2.24]; P=0.02). The estimated rate of overall survival at five years was 90.4% (95% CI, 86.4% - 94.5%) in the high DIO3 group and 77.4% (95% CI, 71.3% - 84.1%) in the low DIO3 group. DNA methylation analysis in this population revealed that DIO3 gene promoter is hypermethylated in tumors when compared to healthy tissue (p <0.0001). In the

breast cancer cell line MCF-7, *DIO3* was subject to T_3 stimulation (2.2 fold increase in mRNA levels), and was under the regulatory control of MAPK pathway, as MEK inhibition (U0126) resulted in a reduction of 50% of *DIO3* mRNA levels (p=0.004).

Conclusions: Our data demonstrate that DIO3 is expressed in healthy breast tissue and breast cancer. Here, we demonstrate that reduced DIO3 expression distinguishes breast cancer patients with poor prognosis, suggesting a role for DIO3 as a prognostic marker in breast cancer. *DIO3* gene regulation by MAPK pathway and hypermethylation of its promoter region could have therapeutic implications and should be further studied.

INTRODUCTION

Breast cancer is the most common cancer in women worldwide, accounting for more than two million new cases and 14.9% of all cancer-related deaths in women in 2018[1]. Despite remarkable advances in the treatment of breast cancer during recent decades, not all patients benefit from current therapeutic options and will experience relapse[2; 3]. Breast cancer has been characterized based on receptor and gene expression profiles that, together with clinicopathological variables such as tumor size, tumor grade, nodal involvement and histologic type, guide treatment, follow up and estimate the risk of recurrence[3; 4; 5]. Gene-expression profiling studies have established at least four molecularly distinct types of breast cancer that can be expanded to the "intrinsic" subtypes luminal A (LumA), luminal B (LumB), HER2-enriched, basal-like, and normal-like[5; 6; 7; 8; 9]. Furthermore, genomic tests have been developed to improve clinical prediction of patients outcomes and to determine the necessity of adjuvant chemotherapy to endocrine therapy[3; 4] However, breast cancer is a highly heterogeneous disease, which is diverse in its behavior and responsiveness to the different modalities of treatment[10; 11]. Thus, the identification of biomarkers that predict tumor biological behavior or are targets of directed therapies is warranted in improving patient survival.

The association between thyroid hormones (TH) and breast cancer was reported as early as 1896, when thyroid extract was used as a potential treatment for the disease[12]. Since then, numerous studies have established THs as critical regulators of multiple cellular processes in normal and tumoral cells. They contribute to cellular proliferation and differentiation during development and adulthood and are conditioned to a fine-tune tissue-specific control[13; 14]. Clinical studies associate THs levels with breast cancer risk and

mortality[15; 16], while *in vitro* models demonstrate that THs influence breast cancer cell proliferation, apoptosis and migration[17; 18; 19].

Modulation of THs concentrations is orchestrated by a group of selenoproteins called iodothyronine deiodinases, which can activate and inactivate thyroid hormones[20]. Briefly, the type 1 deiodinase (DIO1) is responsible for both activation and inactivation of thyroxine (T_4), generating triiodothyronine (T_3), and reverse triiodothyronine (rT_3), respectively. Type 2 deiodinase (DIO2) acts locally converting the prohormone T_4 into the active T_3. Meanwhile, type 3 deiodinase (DIO3) degrades T_4 and T_3 to inactive metabolites (rT_3 and diiodothyronine, respectively), and thus is the main inactivator of THs[21]. The *DIO3* gene belongs to the *DLK1-DIO3* genomic region, which is located on human chromosome 14q32[22]. *DIO3* gene is subject to genomic imprinting, an uncommon epigenetic phenomenon that results in the preferential expression of one of the alleles (paternal allele in the case) [23; 24]. *DIO3* gene expression is increased in several tissues during embryogenesis[25], but it decreases in most tissues in adulthood[26]. Notably, DIO3 is reexpressed in normal and pathological hyperproliferative conditions, where it has been implicated in cell proliferation and differentiation[24; 25; 27; 28]. In particular, studies have demonstrated that the local control of THs signaling provided by the regulation of DIO3 activity is associated with cancer development, progression, and recurrence [27; 29; 30]. We have previously reported that DIO3 expression and activity levels are increased in papillary thyroid cancer (PTC), and are associated with tumor size and advanced disease at diagnosis[30]. Others have described hyperexpression of this enzyme in basal cell carcinoma (BCC), modulating intracellular T_3 concentrations and thus contributing to the cell tumorigenic potential[31]. It exerts a similar function in colon cancer, which suggests that attenuating the TH signal is part of a neoplastic program, at least in some types of cancer[27]. Of interest, the *DLK1-DIO3* cluster represents a specific genomic region that is associated with the acquisition of pluripotency capacity of embryonic cells[32], and its silencing, a unique event of reprogramming[33; 34]. The disturbed expression of genes or altered hypermethylation patterns of this region are involved in the pathogenesis of different types of cancer [35; 36; 37; 38].

Considering the significant role of *DIO3* gene in human carcinogenesis, and the potential effect of TH in breast carcinogenesis[15; 17; 18], we sought to study the patterns of DIO3 in normal breast tissue and breast cancer. We demonstrate that DIO3 is expressed in breast tissue and breast cancer tissue. In breast cancer, reduced DIO3 expression is associated with reduced overall survival in two different populations. Interestingly, loss of expression in explained, at least partially, by gene hypermethylation in neoplastic tissues.

MATERIAL AND METHODS

Patients and tissues: primary cohort

Neoplastic tissue from 44 patients diagnosed with breast cancer was retrospectively collected from a consecutive series of unselected patients in the pathology department of Hospital de Clínicas de Porto Alegre. Tissue samples of normal breast (n=5) and fibroadenomas (n=4) were also collected. Haematoxylin-eosin (HE) slides of breast cancer cases were re-assessed by an experienced pathologist (MG) to confirm the tumor type. Histopathological reports containing information on tumor type, grade and immunohistochemistry were obtained; clinical data were retrospectively reviewed in medical records. Tumors were histologically classified according to the 8th edition of AJCC recommendations (anatomic tumor staging system)[39]. The study was approved by the Institutional Review Board and Research Ethics Committee from the Hospital de Clínicas de Porto Alegre (HCPA; protocol number 16-0246).

Tumor Classification

Treatment decisions regarding breast cancer depend mainly on the status of estrogen receptor (estrogen receptor alpha, ER), progesterone (progesterone receptor, PR) and human epidermal growth factor receptor 2 (HER2). Samples from the primary cohort were classified concerning the presence or absence of these receptors and the level of Ki-67 expression into the following groups: Luminal A (LumA), luminal B (LumB), triple negative and HER2. A Ki-67 index cut point of 14% was defined to distinguish HER2 negative lumB from lumA tumors [40; 41; 42; 43].

Immunohistochemistry studies and DIO3 staining assessment

DIO3 protein expression was evaluated by immunohistochemistry studies in formalin-fixed paraffin-embedded (FFPE) tissue blocks from normal breast tissue, fibroadenomas and primary breast cancer. Immunohistochemistry analysis was performed on 6-mm sections of FFPE. The immunohistochemical technique consists of tissue deparaffinization and rehydration, antigenic recovery, inactivation of endogenous peroxidase and blockage of unspecific reactions. Anti-DIO3 rabbit polyclonal antibody (Abcam 102926, Cambridge, UK) was incubated overnight at a temperature of 4 ºC, at dilution of 1:50, followed by subsequent incubation with biotinylated secondary antibody, streptavidin–HRP conjugate (LSAB; Dako, Carpinteria, CA, USA) and diaminobenzidine tetrahydrochloride (Kit DAB; Dako). The slides were examined using an Olympus BX51 microscope. The QCapturePro software was

used to capture the images. The expression of DIO3 was evaluated by an experienced pathologist that was blind to the molecular profile and TNM staging of each patient. The immunohistochemical results of DIO3 staining were assessed dichotomically (negative and positive) and semi-quantitatively by the H-score method as described previously[44; 45]. The H-score combines the percentage of positive cells and staining intensity level (weak 1+, moderate 2+, strong 3+) and is calculated using the following formula: [1 × (% cells 1+) + 2 × (% cells 2+) + 3 × (% cells 3+)], with results ranging from 0 to 300. Positive and negative internal controls were assessed for all the evaluated cases. Staining of epidermis, placenta, and epidermal nevus were used as positive control, while the staining of other structures such as connective tissue and adipose tissue were used as a negative control.

Differential Gene Expression (DGE) and Methylation Analysis using TCGA breast cancer study (BRCA) data

For the validation cohort, RNA sequencing (RNA-Seq) RSEM gene expression data for The Cancer Genome Atlas (TCGA) breast cancer (BRCA) study were obtained from the Genomic Data Commons (GDC) Data Portal (https://gdc-portal.nci.nih.gov/) using TCGABiolinks R/Bioconductor package[46]. Raw expression signals were normalized and analyzed for differential expression of *DIO3* using the limma-Voom pipeline from the limma R/Bioconductor package[47]. P-values were adjusted for multiple comparisons using the false discovery rate (FDR) procedure of Benjamini and Hochberg[48]. Clinicopathological information for TCGA-BRCA samples, including ER status, tumor stage, and overall survival time, were downloaded through TCGABiolinks and the Broad GDAC Firehose (http://gdac.broadinstitute.org/) (merged level 1 clinical data). For tumors of the TCGA-BRCA cohort, data retrieved from PAM50 classification were used to define tumor subtype classification[7]. Overall survival (OS) was estimated by the Kaplan-Meier method and compared by the log-rank test using functions provided by TCGABiolinks. For the methylation analysis, we used TCGAbiolinks R/Bioconductor package[46] to obtain and analyze Illumina 450K methylation and clinical data for 890 samples from the TCGA-BRCA study, including 97 samples from healthy tissue and 793 from the primary solid tumor. Differentially methylated CpG sites for *DIO3* were screened with the TCGAanalyze_DMR function, adopting an FDR-adjusted Wilcoxon rank-sum P-value < 0.05 and a minimum absolute difference among groups' average beta values (Δ *beta*) of 0.15.

Cell culture studies

Breast cancer cell lines MCF-7 (BCRJ code 0162) and MDA-MB-231(BCRJ code 0392) and thyroid cancer cell line K1 (BCRJ code 0292), which carries the $BRAF^{V600E}$ mutation and expresses DIO3[49] were obtained from Banco de Células do Rio de Janeiro (RJ, Brazil). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)(MDA-MB-231) or Roswell Park Memorial Institute (RPMI)-1640 (MCF-7) medium supplemented with 10% fetal bovine serum and antibiotics (100 ug/ml ampicillin and 100 µg/mL streptomycin). The cells were incubated at 37ºC and 5% CO_2 in a humidified atmosphere. For the studies of regulation, cells in 6-well plates were cultured for 24 hours in 1 ml serum-free 0.5% BSA in DMEM or RPMI plus 2-100 nMol T_3 (resulting in a free T_3 fraction of 3.5%)[50], or 10-100 nMol of dexamethasone. To evaluate the effect of MAPK pathway signaling on DIO3 expression, we incubated cells with the specific inhibitor to the signaling effector MEK (U0126: 10 uM; Sigma–Aldrich) for 24h. All assays were performed in triplicate in at least two independent experiments.

SiRNA transfection and cell proliferation assays

SiRNA studies were performed to evaluate the effects of DIO3 inhibition on cell proliferation. The following siRNA was used as positive control: Silencer Select GAPDH siRNA (#4390849, Life Technologies, Ambion Inc., Austin, TX, USA); Silencer Select Negative Control (#4390843, Life Technologies, Ambion Inc.) and Silencer Pre-designed DIO3 siRNA (#AM16708, Life Technologies, Ambion Inc.) were used in the experiments. Transfection studies were performed using Lipofectamine RNAiMAX reagent, according to the manufacturers' instructions (Life Technologies, Invitrogen). A total of 2×10^5 cells/well (MCF-7) 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. Cell proliferation was evaluated by absolute cell number count by using a CCK-8 kit (Dojindo Mol Technologies, Rockville, MD USA). Briefly, cells were plated at 5×10^3 cells/well in 96-well plates and cultured for 6 days. After every 24 hours, the CCK-8 reagent was added and the absorbance value at 450 nm of every well was obtained using a spectrophotometer. Proliferation assays were performed for three times in triplicate.

Western Blotting

Cultured cells were lysed and prepared for Western Blotting analysis, as previously described[51]. Briefly, 30–50 milligrams of protein of each sample was fractionated by 8–12% SDS–PAGE and blotted onto an Immobilon PVDF membrane (Millipore, Billerica, MA,

USA). Non-specific binding sites were blocked with nonfat dry milk in Tris-buffered saline 0.1% Tween-20. The following primary antibodies were used: anti-DIO3 (1:200; Novus Biologicals), anti-ERK1/2 (1:200; Santa Cruz Biotechnology), anti-phospho-ERK1/2 (1:200; Santa Cruz Biotechnology), and anti-beta-actin (1:25 000; Sigma–Aldrich). The antigen-antibody complexes were visualized using HRP-conjugated secondary antibody and an enhanced chemiluminescence system (GE Healthcare, Pittsburgh, PA, USA). Expression was quantified using image densitometry with ImageJ analysis software (NIH, Bethesda, MD, USA).

Real-time polymerase chain reaction

Total RNA was extracted from cells using RNeasy mini-kit (Qiagen, Germantown, US). One microgram of RNA was reverse transcribed into cDNA using the SuperScript VILO cDNA synthesis kit (Invitrogen) as recommended by the manufacturer. The cDNAs generated were used in a real-time polymerase chain reaction (PCR) with an SYBR Green PCR Master Mix (Applied Biosystems) in a 7500 Fast Real-Time PCR System Thermal Cycler. Experiments were performed by real-time monitoring of the increase in fluorescence of SYBR Green dye. Each sample was assayed in triplicate, and a negative control was included in each experiment. Quantification of *DIO3* cDNA was performed by the ΔΔCT method and expressed relative to the the reference gene (cyclophilin A). Changes in gene expression were expressed as relative fold difference (n-fold change) or as arbitrary units (AUs). The oligonucleotides used were the following: human DIO3 gene, 5'-AACTCCGAGGTGGTTCTGC-3' and 5'TTGCGCGTAGTCGAGGAT -3'; cyclophilin A (internal control), 5'-GCCGATGACGAGCCCTTG-3' and 5'-TGCCGCCAGTGCCATTATG-3'.

Statistical analysis

Clinicopathological data are reported as mean and standard deviation or median with percentiles 25 and 75 for the continuous variables, and frequency and percentages were reported for categorical variables. Student's t-test or chi-square test were used to compare clinicopathological variables among different groups as appropriate. Student's t-test or one-way ANOVA were used to compare H-Score among different groups. Cox's Proportional Hazard Method was used to test univariable and multivariable statistical effects of DIO3 protein and *DIO3* mRNA expression on the patient survival. Time-to-event analysis was performed with overall survival (OS) as the primary outcome and was evaluated with a log-

rank analysis using Kaplan-Meier curves and both unadjusted and multivariable Cox regression analyses. All tests were two-tailed, and all analyses were performed using Statistical Package for Social Science Professional software version 20.0 (SPSS, Chicago, IL USA). A two-tailed $P < 0.05$ was considered statistically significant.

RESULTS

DIO3 expression in normal breast and fibroadenoma

The breast is composed of glandular structures surrounded by fibrovascular and adipose tissue. A network of ducts connects the acini, where the milk production occurs, to the nipple. Both ducts and acini are composed by a layer of epithelial cells lining the lumen and more deeply, an external layer of myoepithelial cells around the basal membrane [52]. We detected anti-DIO3 staining by immunohistochemistry in all samples of normal breast tissue (n=5) in an overall moderate intensity (H-Score 160 ± 63). DIO3 staining was predominantly cytoplasmatic and more pronounced in the apical extremity in luminal cells in both ducts and acini (Fig. 1A). DIO3 was markedly positive in myoepithelial cells (Fig. 1A, bottom). Benign fibroadenoma lesions (n=4) were also positive for DIO3 staining, with an intensity comparable to healthy tissue (H-Score 153 ± 41 vs. 160 ± 63, P=0.75).

Patient and tumor characteristics

To study DIO3 expression in breast cancer, we analyzed our cohort (primary cohort, n=44) and validated the results in the TCGA-BRCA cohort (validation cohort, n=1094). The clinical and pathological characteristics of the patients with breast cancer from both cohorts are summarized in Table 1. In the primary cohort, all patients were female except one, with a median age at diagnosis of 52 years. They were followed by a mean of 81.9 ± 32.7 months. Most patients had Invasive Ductal Carcinoma (IDC) (90.9%). Tumors were evaluated for hormone-receptor status, HER2 status, levels of Ki-67 expression and classified accordingly. Of these, 18.2% were LumA, 38.6% were LumB, 15.9% were HER2, and 22.7% were triple negative. Regarding tumoral staging, median tumor size was 20 (13-30) millimeters, lymph node metastasis was present in 17 (39%) patients, and distant metastasis was observed in 4 (9.3%) patients. According to the TNM/AJCC anatomic staging, most patients were classified either as stages I (34.9%) or II (34,9%), 18.6% were stage III and 9.3% were stage IV. One patient had a ductal carcinoma *in situ* (DCIS), which is considered stage 0. Only one

patient had prior hypothyroidism, and two developed hypothyroidism after treatment. During the follow-up period, mean survival time was 115.7 months (95% CI, 102.2-129.2), with death occurring in 11/43 (25.5%) patients.

For patients in the validation cohort (TCGA-BRCA), mean age at diagnosis was 58.6 ± 13.2 years. Regarding node status, 52% were confirmed to have nodal involvement, and 1.7% had distant metastasis. After excluding missing data, 807 (77%) subjects were ER positive, 698 (67%) were PR positive and 114 (15%) were HER2 positive. During a mean follow up of 22.2 months days, 152 (13.9%) patients died (Table 1).

Expression of DIO3 protein in breast cancer: primary cohort

Patterns of DIO3 expression evaluated through immunohistochemistry are exemplified in Figure 1B-D. DIO3 expression in FFPE tissues of breast cancer was positive in 35/39 (89.7%) of IDC, with a mean H-Score of 104.9 ± 55. When evaluating invasive lobular carcinoma (ILC), only in 1 of 3 samples was positive for DIO3 (H-Score=86). A sample of DCIS was also positive for DIO3 expression (H-Score=100). A graph comparing H-score in non-malignant tissue and malignant types breast cancer is presented in Figure 2A, with a marginal decrease in DIO3 expression in invasive lobular carcinoma (ILC) when compared to normal tissue (P=0.05). H-score of invasive ductal carcinoma was similar to normal tissue (P=0.78). No differences in DIO3 expression were observed between the molecular subtypes of breast cancer (P=0.8) (data not shown). We found no association of DIO3 positivity (negative or positive) with tumor size (P=0.18). There was no difference on H-score between tumors with ER status positive vs. negative (93.5 ± 62.8 vs. 111.7 ± 48.9, P=0.31) (Fig. 2B), or between tumors with HER2 status positive vs. negative (104.5 ± 66 vs. 99.7 ± 55.9; P=0.81)(Fig. 2C). There was also no correlation of Ki-67 score with H-score (P=0.9), but a marginal association of DIO3 positivity with histological grade (Nottingham Histologic Score) (P=0.052). H-score in patients with or without lymph node metastasis (Fig. 2D) and with or without distant metastasis (Fig. 2E) was similar. Moreover, there were no differences in expression when comparing patients with stages I/II vs. III/IV of anatomic stages of TNM/ AJCC classification (P=0.41) (Fig. 2F). We obtain both primary and lymph node tissue from 5 patients. In this subset of patients, there was no difference in DIO3 expression when comparing the primary tumor to the lymph node metastasis (P=0.36).

The analyses of correlations between DIO3 expression and clinicopathological characteristics in both populations are summarized in Table 2. In the primary cohort, patients were categorized with positive or negative staining for DIO3 and according to H-score (H-

Score ≤100 or >100). There were no significant differences between groups concerning age or clinicopathological characteristics in this population.

In a univariate analysis, variables associated with increased risk for death are shown in Table 3 and include tumor size, lymph node or distant metastasis, and advanced TNM stage. Interestingly, negative DIO3 expression was an indicator of poor prognosis in this analysis (HR: 4.29; 95% CI, 1.24-14.7; P=0.021). As DIO3 expression was associated with overall survival (OS), we evaluated the prognostic value of the DIO3 expression using Kaplan–Meier analysis and the log-rank test. Patients with negative DIO3 expression had worse OS than those with positive DIO3. (Fig. 2G, log-rank: P=0.012). The mean OS was 73.3 months (95% CI, 41 to 105) in the negative DIO3 group and 122 months (95% CI, 109 to 135) in the positive DIO3 group.

DIO3 expression correlates with poor prognosis in breast cancer patients

We have previously demonstrated that DIO3 protein levels and activity correlate with *DIO3* mRNA levels in different contexts[49; 53]. Therefore, to further validate whether DIO3 could predict the prognosis of patients with breast cancer, we performed an analysis of *DIO3* mRNA expression in the validation cohort. In this second population, *DIO3* expression was found to be reduced in tumor samples when compared to normal sample tissues (logFC = -1.54, adjusted P-value <0.00001, Fig. 3A) and also when only tumors with matched normal tissue where evaluated (logFC = -1.800 adjusted P-value <0.00001, Fig. 3B). When we compared the different tumor subtypes, classified according to PAM50 classification[7], all subtypes demonstrated reduced *DIO3* expression when compared to normal tissue (P<0.00001). *DIO3* mRNA was also reduced in lumB when compared to lumA subtypes (logFC = -0.915 adjusted P-value = 0.0009), and also in basal-like tumors when compared to lumA (logFC = -1.024; adjusted P-value =0.0003) (Fig. 3C). *DIO3* expression was increased in ER-positive samples when compared to ER-negative samples (logFC = 0.428; P= 0.013, Fig. 3D). Among ER-positive patients, there was no difference of expression between patients who were PR-positive or PR-negative (logFC = 0.207; adjusted P-value = 0.498). There was no significant difference when comparing gene expression among patients with or without lymph node disease (logFC = 0.0359 adjusted P-value = 0.914)(data not shown) or the presence or absence of distant metastasis (logFC = -0.190, adjusted P-value= 0.971, Fig. 3E). Regarding tumoral staging, all tumor stages had decreased *DIO3* mRNA expression when compared to normal tissue (P<0.01). However, there was no difference in expression between stages (Fig. 3F).

We analyzed the association of clinicopathological characteristics and *DIO3* expression in this second population (Table 2). Interestingly, low DIO3 expression was associated with greater tumor size (P=0.019) and estrogen receptor negativity (P=0.022), while there was a marginal association of DIO3 negativity with HER2 status (P=0.058). There were no other significant associations between DIO3 and the characteristics that were analyzed.

We then evaluated the prognostic association of *DIO3* mRNA expression and overall survival. We considered high *DIO3* expression values of logCPM above the median and low *DIO3* expression values of logCPM below the median. Low *DIO3* expression was associated with reduced survival with a HR 1.6 (95% CI, 1.18-2.26; P=0.003) in a univariate model (Table 4). An additional analysis using a multivariate model adjusted for all variables with P<0.1 in the univariate analysis and vital status as the dependent variable, demonstrated that low *DIO3* was maintained as an independent prognostic factor for death (HR 1.55; 95% IC 1.07-2.24; P=0.02 (Table 4, Fig. 4A). The median overall survival was 113.5 months (95% CI, 92.5 to 134.4) in the low *DIO3* group and was not reached in the high *DIO3* group. The estimated rate of OS at 5 years in the Kaplan–Meier analysis was 90.4% (95% CI, 86.4% - 94.5%) in the high *DIO3* group and 77.4% (95% CI, 71.3% - 84.1%) in the low *DIO3* group (Fig. 4A). Survival probability between patients with low or high *DIO3* levels did not differ when each tumoral subtype was analyzed individually (data not shown).

In a subgroup analysis, it is notable that low *DIO3* expression was associated with worse OS among patients with ER-positive tumors (P=0.0012) but not among those with ER-negative tumors (P=0.89)(data not shown). More interestingly, despite a low number of patients with advanced disease (stage IV) or distant metastasis, *DIO3* was an important prognostic marker in these subgroup of patients. Among patients with stage IV disease, patients with low *DIO3* expression had reduced OS when compared to patients with high *DIO3* expression (P=0.011)(Fig. 4B). Similarly, low *DIO3* expression was associated with reduced survival in patients with metastasis. While 67% metastatic patients with high *DIO3* expression were alive after five years of follow up, all had died in the low *DIO3* group(log-rank P=0.038).

***DIO3* gene promoter is hypermethylated in breast cancer**

To further investigate possible factors that could lead to decreased *DIO3* expression in breast cancer when compared to normal tissue, we performed DNA methylation analysis of a subgroup of patients from TCGA database from whom DNA methylation data were available

(n=890). Our analysis demonstrates that global DNA methylation levels of breast cancer samples are similar to the levels of healthy breast tissue (Fig. 5A). However, methylation levels of CpG sites of *DIO3* gene region are increased when compared to healthy tissue (Fig. 5B) (p <0.0001). Figure 5 details CpG sites that were analyzed and that are hypermethylated (*) within *DIO3* gene region. The first 1.5 kb of 5' flanking region (red) are known to be extremely G+C rich (80% of the sequence) and this region is highly conserved between mouse and human genome[54]. Promoter region (~-250 bp of the 5' flanking region) is composed by several promoter elements (Fig. 5C, enhanced), including a TATA box, two CAAT boxes and CG rich regions[55]. We can observe a significant increase in DNA methylation levels in CpG sites that are located both at the promoter region and in the 5' flanking 1.5 kbp conserved region of the gene (Fig 5C-D).

***DIO3* is expressed in breast cancer cell lines**

To evaluate *DIO3* gene regulation in breast cancer, experimental studies were performed using MCF-7 and MDA-MB-231 cell lines. We observed detectable levels of *DIO3* mRNA in both cell lines, although *DIO3* mRNA levels were significantly higher in MCF-7 cell lines (200-fold) (Fig. 6A). MAPK cascade is often dysregulated in breast cancer and has been demonstrated to modulate DIO3 expression in other conditions[49; 56; 57; 58]. Incubation of cells with a MEK inhibitor (U0126, 10 mM) for 3 hours resulted in a reduction of ERK phosphorylation (Fig. 6B) in both cell lines and a 50% *DIO3* mRNA in MCF-7 cells (P=0.004) and MDA-MB-231 cells (P=0.126). *DIO3* is known to be induced by T_3 treatment in different tissues[59; 60; 61]. Therefore, we incubated MCF-7 and MDA-MB-231 cells with 2 nM to 100 nM of T_3 for 24h to evaluate T_3 effect on *DIO3* expression. T_3 at 10 nM caused a 5 fold increase in *DIO3* mRNA in K1 cells (positive control), a ~2 fold increase in *DIO3* mRNA levels in MCF-7 cells, and a ~3 fold increase in MDA-MD-231 cells (Fig. 6C-E). No further effect was observed with increased doses of 100 nM of T_3. Interestingly, the cytotoxic compound dimethyl sulfoxide (DMSO) caused a dose-dependent increase in *DIO3* mRNA levels (Fig. 6F) in MCF-7. Dexamethasone treatment caused suppression of *DIO3* mRNA levels in MCF-7 (Fig. 6G) as expected for normal regulation of *DIO3* gene, an effect that was not observed in MDA-MB-231 (Fig. 6H).

DIO3 inhibition is not associated with increased proliferation of MCF-7 cells

Considering that loss of DIO3 expression in breast cancer samples is associated with a worse prognosis and that this association is more evident in ER-positive patients, we tested the

hypothesis that *DIO3* inhibition could increase proliferation in an ER-positive cell line. We could not observe changes in MCF-7 cells proliferation when *DIO3* was transiently inhibited through five day period (data not shown).

DISCUSSION

THs alterations contribute to cancer development and progression through virtually all the hallmarks of cancer[14]. Such hormonal changes can occur due to the disruption of deiodinases expression, rendering to the neoplastic cells the acquisition of necessary steps for cancer development[62]. Here, we demonstrate that the TH-inactivating enzyme DIO3 is expressed in normal breast tissue, and its expression highly prevalent in breast cancer. More interestingly, our results suggest that low DIO3 expression is an independent prognostic factor associated with reduced overall survival in patients with breast cancer.

DIO3 activity and *DIO3* mRNA have been detected in MCF-7 cells previously[63; 64], but to our knowledge, this is the first study that describes the presence of DIO3 expression in normal human breast tissue. This finding is compatible with the presence of DIO3 in other tissues of ectodermal origin such as the skin and the nervous system[65; 66]. Meanwhile, DIO1 levels have been shown to be low or undetectable in normal and lactating breast tissue, but increased in cancer samples[67; 68], while DIO2 has not been studied in human breast tissue. We demonstrate expression of DIO3 in breast cancer both at mRNA and protein level in patient samples and cancer cell lines. DIO3 protein was detected in the majority of the cases in a primary cohort. In a larger population, *DIO3* mRNA expression was detected in breast cancer tissue and demonstrated to be reduced in tumors when compared to healthy tissue. DIO3 was not associated to adverse clinicopathological characteristics such tumor size, the presence of lymph node or distant metastasis in the exploratory cohort, but loss of its expression was associated with greater tumor size (P=0.019) and absence of ER expression (P=0.022) in the validation cohort.

We demonstrate for the first time a role for DIO3 expression as a prognostic marker in breast cancer, with low levels of expression correlating with reduced patient survival. In a multivariate model, low *DIO3* expression was an independent prognostic factor for death (HR 1.55; 95% IC 1.07-2.24; P=0.02). Moreover, *DIO3* expression was a particularly important prognostic factor in the subgroup of patients with advanced disease. Among patients with Stage IV disease, low *DIO3* expression was associated with reduced OS (P=0.011). The existence of a correlation between DIO3 expression and tumor behavior is not exclusive to

breast tissue. Interestingly, loss of DIO3 expression was associated with tumor aggressiveness in colon cancer[69], and this association is also suggested in thyroid cancer, since DIO3 is found in papillary and follicular subtypes, but its expression is lost in the most aggressive and dedifferentiated anaplastic subtype[30]. These findings suggest that loss of DIO3 expression might be a common hallmark of dedifferentiation in the neoplastic process. However, to what extent the presence of this enzyme is needed in order to maintain low intracellular levels of T_3 and consequent tissue differentiation remains to be explored. The development of tumoral models of aggressive cancer with artificial reexpression of the enzyme could provide further insight into this question. Intriguingly, a recent population-based case-control study found that T_3 levels were lower in cases of breast cancer in comparison to controls[70]. These findings might reflect the existence of levels of tumoral DIO3 high enough to impact on plasma levels of THs. These are in agreement with previous studies that reported lower levels of T_3 in breast cancer patients when compared to controls[71; 72; 73], and, even more interestingly, to the fact that this correlation was lost in more advanced tumors[72].

When we performed subgroup survival analysis, we observed that the difference in survival between groups with distinct DIO3 expression is significant in ER-positive patients but not differs in ER-negative patients. The observation that the prognostic value of DIO3 is useful in those women with ER-positive tumors is intriguing. This may potentially be explained by a cross-talk between TH and estrogen-mediated signaling. Previous studies indicate the existence of a cross-talk between estrogen and TH dependent regulatory pathways in breast cancer[17; 74; 75; 76]. T_3 regulates cell cycle progression and proliferation of breast cancer cells *in vitro* by a common mechanism involving ER and T_3 receptor-mediated pathways[74]. Moreover, T_4 can phosphorylate nuclear ER-alpha in MCF-7 cells by a MAPK-dependent pathway promoting proliferation[17]. Therefore, loss of DIO3 expression and the consequent increase of intracellular T_3 levels could be specifically detrimental in tumors that express ER, as our results suggest. Also contributing to this interplay, previous studies demonstrate that estrogen and progesterone are known to increase DIO3 activity in uteri of ovariectomized rats[77]. DIO3 is also highly expressed in rat decidua, where it is induced by estrogen and progesterone[78], and is dependent on the presence of PR. This might also occur in breast cancer. Therefore, we cannot rule out that in the breast, DIO3 benefits from the presence of functional estrogen and progesterone receptors to remain expressed.

Considering the reduced levels of DIO3 in tumors when compared to normal tissue, we hypothesized that loss of DIO3 expression could be consequential to gene hypermethylation in the tumoral context. We analyzed DNA methylation patterns of breast

cancer patients and observed that although mean global methylation in breast tumor is comparable to normal tissue, the *DIO3* genomic region is significantly hypermethylated in tumors when compared to normal tissue. The promoter region of this gene is highly conserved among species and comprises a TATA box, two CAAT boxes and several GC boxes within the first 150 bp of 5′-flanking region. Methylation probes directed to this region demonstrated that several CpG sites located here are found to be hypermethylated in breast cancer when compared to normal tissue a fact that can, at least in part, explain the loss of expression of the enzyme. This is in agreement with data found in B-cell, T-cell and myeloid malignancies, and lung cancer, where *DIO3* gene was also found to be hypermethylated[36; 37]. Epigenetic silencing of genes within the *DLK1-DIO3* region is also a common event in Giant cell tumors, mediated at least in part by hypermethylation[38].

We expanded our evaluation to breast cancer cell lines with positive (MCF-7) and negative (MDA-MB-231) ER status, demonstrating that *DIO3* is also expressed in these cell lines. In MCF-7 cells, *DIO3* is expressed in comparable levels to the thyroid cancer cell line K1, but is found in much lower levels in MDA-MD-231 cells. *DIO3* expression is known to be regulated by several factors such as T_3, MAPK pathway, dexamethasone, transforming growth factor β, sonic hedgehog, HIF and microRNAs[31; 61; 79; 80; 81; 82]. In the present study, we demonstrated a stimulatory effect of T_3 over *DIO3* gene in two different breast cancer cell lines. Moreover, treatment of MCF-7 cells with dexamethasone and with MAPK pathway inhibitor U0126 was shown to inhibit *DIO3* expression, an effect that did not occur in MDA-MB-231 cells. The absence of inhibition of *DIO3* in these cells could be attributed to low basal levels of gene expression (200-fold lower than MCF-7 cells), and the consequent lack of sensibility to detect further inhibition. However, we cannot rule out the enzyme lacks the capacity to be regulated by this MAPK pathway in MDA-MB-231 cells. Therefore, in an ER-positive cell line, the MAPK pathway is involved in *DIO3* regulation, as also observed in fibroblasts and papillary thyroid cancer[49; 83].

Local T_3 concentrations and DIO3 activity modulate tissue proliferation and differentiation in several contexts[27; 31; 84; 85]. Since T_3 induces proliferation of ER-positive breast cancer cells[76; 86], DIO3-mediated T_3 inactivation could have a “protective effect” over cell proliferation in this context. However, we could not demonstrate changes in cell proliferation in an ER-positive cell line (MCF-7) that expresses high amounts of DIO3 protein when we transiently silenced *DIO3 in vitro*. This suggests that alterations in DIO3 might be consequential, and not cause, of disturbed oncogenic regulation in this context.

In conclusion, we demonstrate that DIO3 is expressed in breast tissue and breast cancer. In breast cancer, decreased DIO3 expression distinguishes patients with reduced overall survival, suggesting a role for DIO3 as an independent prognostic marker in this neoplasia. Reduced DIO3 expression in breast cancer can be explained at least in part by gene hypermethylation. DIO3 gene regulation by MAPK pathway, interplay with estrogen receptors and hypermethylation of its promoter region could have therapeutic implications and should be further investigated.

Table 1: Baseline characteristics of patients with breast cancer included in the primary cohort and in the validation cohort

Characteristic	Primary cohort (N=44)	Validation cohort (=1094)	P value
Median age at diagnosis (range) - yr	52 (26-71)	59 (26-90)	0.07
Tumor size in the largest dimension - mm			
Median (IQR)	20 (13-30)	N/A	
Mean (+- SD)	31.15±29.1	N/A	
Histological grade of tumor – no (%)			
Grade I	6 (13.6%)	N/A	
Grade II	16 (36.4%)		
Grade III	19 (43.2%)		
Missing	3 (6.8%)		
Estrogen Receptor – no (%)			
Positive	25 (58.1%)	807 (73.7%)	0.006
Negative	18 (41.9%)	237 (21.6%)	
Missing	0	50 (4,6%)	
Progesterone Receptor – no (%)			
Positive	24 (55.8%)	698 (63.8%)	0.14
Negative	19 (44.2%)	343 (31.3%)	
missing	0	53 (4.8%)	
HER2 status – no (%)			
Positive	12 (27.9%)	114 (13.2%)	0.03
Negative	30 (69.8%)	649 (59%)	
missing	1 (2.3)	331 (30%)	
Histological type of tumor – no (%)			
Invasive Ductal Carcinoma (IDC)	40 (90.9%)	813 (79.7%)	0.03
Invasive Lobular Carcinoma (ILC)	3 (6.8%)	207 (20.3%)	
Ductal Carcinoma *in situ* (DCIS)	1 (2.3%)	0	
Clinical-pathological subtype – no (%)	AJCC 2018	PAM50[a]	
Luminal A	8/44 (18.2%)	231 (45%)	0.01
Luminal B	17/44 (38.6 %)	127 (24.7%)	
HER2	7/44 (15.9%)	58 (11.3%)	
Triple Negative	10/44 (22.7%)	97 (18.9%)	
Non classified	2 (4.5%)		
Lymph node metastasis – no (%)			
yes	17 (39%)	558 (52%)	0.08
no	26 (61%)	516 (48%)	
Distant metastasis – no (%)			
yes	4 (9.3%)	14 (1.8%)	0.001
no	39 (91.7%)	768 (98.2%)	
Tumor staging – no (%)	Anatomic Tumor Staging		
Stage I	15/44 (34,9%)	182 (16.6%)	<0.001
Stage II	15/44 (34,9%)	619 (56.5%)	
Stage III	8/44 (18.6%)	249 (22.7%)	
Stage IV	4/44 (9.3%)	20 (1.8%)	
Stage 0	1/44 (2.3%)	0	
missing	1 (2.3%)	24 (2.1%)	
Neoadjuvant therapy – no (%)			
Yes	4	13	<0.001
No	40	1079	
Pre-treatment hypothyroidism – no (%)	1/43 (2.3%)	N/A	
Post-treatment hypothyroidism – no (%)	3/43 (7%)	N/A	
Follow-up (mean ± SD) - months	81.9 +- 32.7	22.2 (12.9-47.5)	0.8
All cause mortality - no (%)	11/43 (25.5%)	152/1094 (13.9%)	0.03
Mean survival months (95% CI)	115.7 (102.2-129.2)	153.7 (136.8-170.6)	0.20

Table 2. Associations between DIO3 protein and *DIO3* mRNA expression and clinicopathological characteristics in breast cancer in two different populations

Characteristics	DIO3 expression			DIO3 H-Score			*DIO3* mRNA expression		
	negative (N=6) %	positive (N=37) %	P value	H-Score ≤100 (N=20) %	H-Score >100 (N=24) %	P value	low DIO3 mRNA N=546 %	high DIO3 mRNA N=546 %	P value
Age (years)									
≤50	50	43	0.75	55	35	0.18	29	31	0.332
>50	50	57		44	65		71	69	
Tumor size (milimeters)									
≤20 mm	50	57	0.81	50	58	0.58	23	29	**0.019**
>20 mm	50	43		50	42		77	71	
Estrogen receptor status									
positive	83	54	0.18	63	54	0.55	74	80	**0.022**
negative	17	46		37	46		26	20	
HER2 receptor status									
positive	33	28	0.78	26	30	0.80	23	18	0.058
negative	77	72		74	70		77	82	
Lymph node status									
positive	77	35	0.13	50	29	0.16	55	49	0.086
negative	33	65		50	71		45	51	
Distant metastasis									
positive	0	11	0.40	10	9	0.88	3	2	0.509
negative	100	89		90	91		97	98	
Ki67 positivity									
<14%	40	28	0.57	28	30	0.85	N/A		
≥14%	60	72		72	70				
TNM Staging									
I/II	77	72	0.78	63	78	0.28	75	74	0.751
III/IV	33	28		27	22		25	26	
Histological Grade									
I/II	83	48	0.115	60	47	0.43	N/A		
III	17	52		40	53				

N/A: not available

Table 3. Univariate Cox regression analysis of overall survival in breast cancer patients in the primary cohort

Variable	HR (95% CI)	P value
Age at diagnosis (years)	1 (0.95-1.06)	0.74
Tumor size (mm)	**1.027(1.01-1.04)**	**0.002**
Lymph node metastasis (neg vs. pos)	**4.5 (1.2-17)**	**0.026**
Distant metastasis (neg vs. pos)	**4.4 (1.1-17.2)**	**0.032**
E2 status (neg vs. pos)	0.54 (0.16-1.79)	0.32
P status (neg vs. pos)	0.4(0.12-1.38)	0.15
HER2 positivity (neg vs. pos)	1.8 (0.49-6.4)	0.38
TNM staging (I/II vs III/IV)	**6.54 (1.83-23)**	**0.003**
DIO3 status (pos vs. neg)	**4.29 (1.24-14.7)**	**0.021**

Table 4: Univariate and multivariate Cox regression and for overall survival in the validation cohort.

	Univariate Analysis		Multivariate Anaylsis*	
Variables	HR (95% CI)	P-value	HR (95% CI)	P-value
Age at diagnosis (years)	**1.032 (1.02-1.04)**	**<0.001**	**1.039 (1.02-1.05)**	**<0.001**
Tumor size (<=2cm vs > 2 cm)	**1.48 (1.00-2.18)**	**0.045**	**1.31 (0.83-2.08)**	**0.25**
Lymph node metastasis (neg vs. pos)	**2.13 (1.49-3.05)**	**<0.001**	**1.87 (1.24-2.81)**	**0.003**
Distant metastasis (neg vs. pos)	**4.3 (2.57-7.20)**	**<0.001**	**2.92 (1.61-5.30)**	**<0.001**
E2 status (neg vs. pos)	0.7 (0.48-1.00)	0.056	0.66 (0.36-1.22)	0.187
P status (neg vs. pos)	0.3 (0.52-1.02)	0.066	0.31 (0.42-1.31)	0.309
HER2 positivity (neg vs. pos)	1.43 (0.89-2.28)	0.13		
TNM staging (I/II vs III/IV)	**2.49 (1.78-3.48)**	**<0.001**		
DIO3 status (high vs. low)	**1.6 (1.18-2.26)**	**0.003**	**1.55 (1.07-2.24)**	**0.02**

*All variables with P<0.1 were included in the multivariate model. TNM is not included as it is derived from variables already present in the model.

Figure 1.

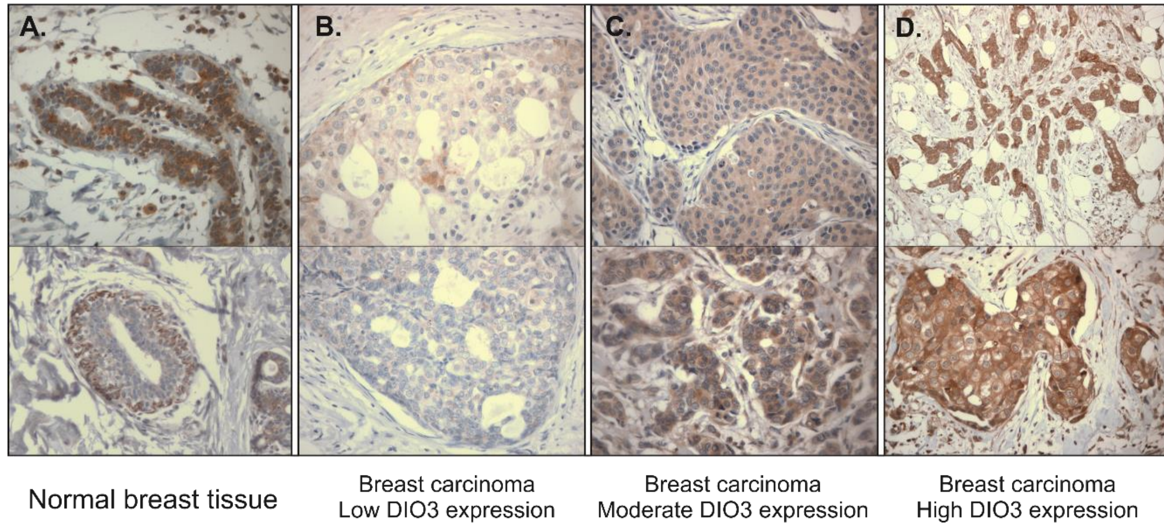

Figure 1. Patterns of expression of DIO3 in breast samples. Immunostaining was performed as described in Materials and methods. From left to right: (A) Normal glandular breast tissue, (B) breast carcinoma with low expression (overall intensity 1+), (C) breast cancer with moderate expression (overall intensity 2+) and (D) breast cancer with high expression (overall intensity 3+) of DIO3 protein evaluated through immunohistochemistry. Staining intensity level is used to calculate H-score, combined with the percentage of positive cells (see Materials and methods).

Figure 2.

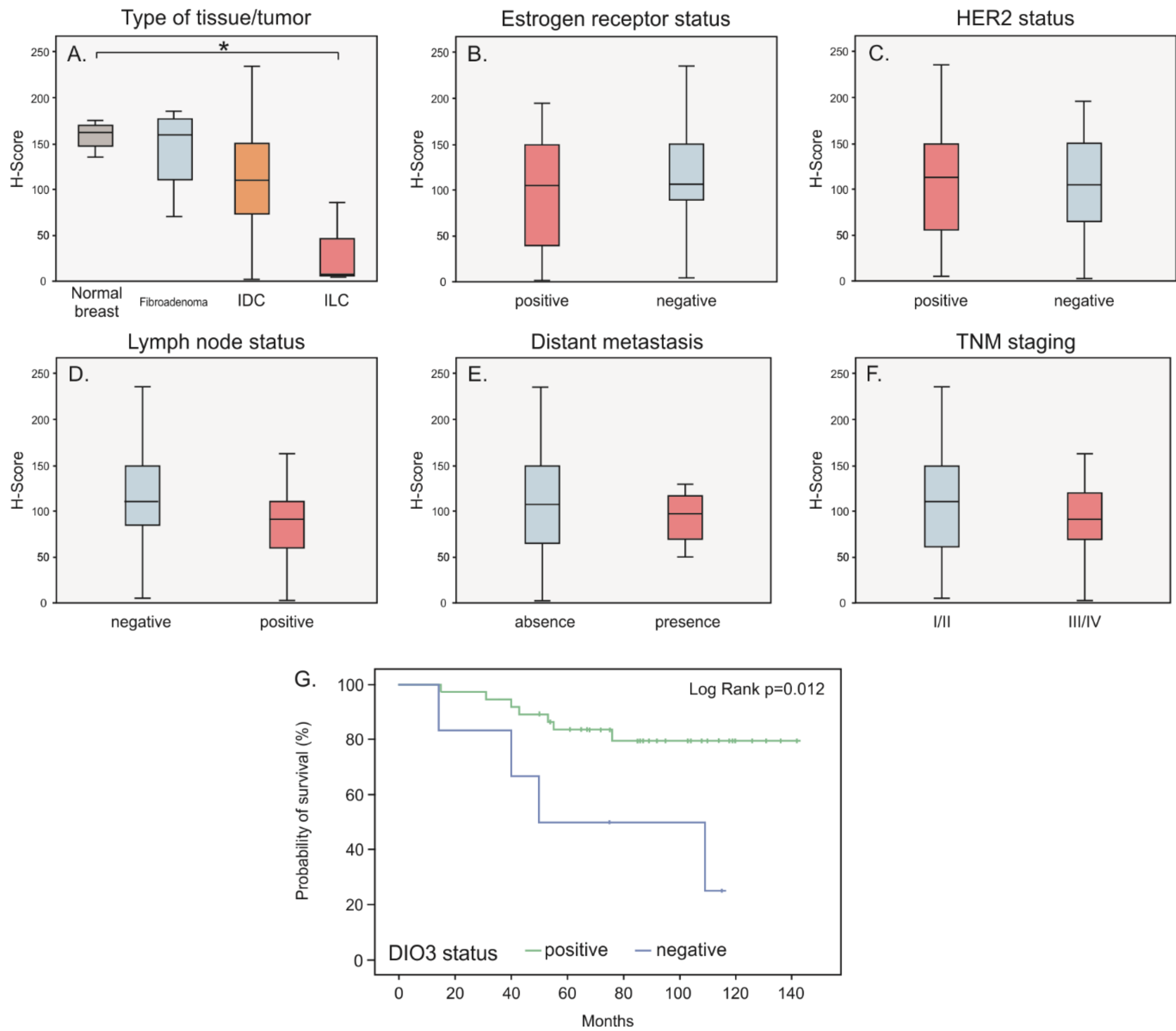

Figure 2. DIO3 expression and clinicopathological characteristics of patients with breast cancer in the primary cohort. A-F: Box plots of DIO3 expression in breast samples evaluated through immunohistochemistry and quantified by H-Score. Samples were divided according to clinicopathological data as follows: (A) type of tissue analyzed, (B) ER status, (C) HER2 status, (D) lymph node status, (E) distant metastasis and (F) TNM anatomic staging. (G) Kaplan-Meier plot of overall survival in patients with the presence (green) or absence (blue) of DIO3 expression in breast cancer evaluated through immunohistochemistry.*P=0.05

Figure 3.

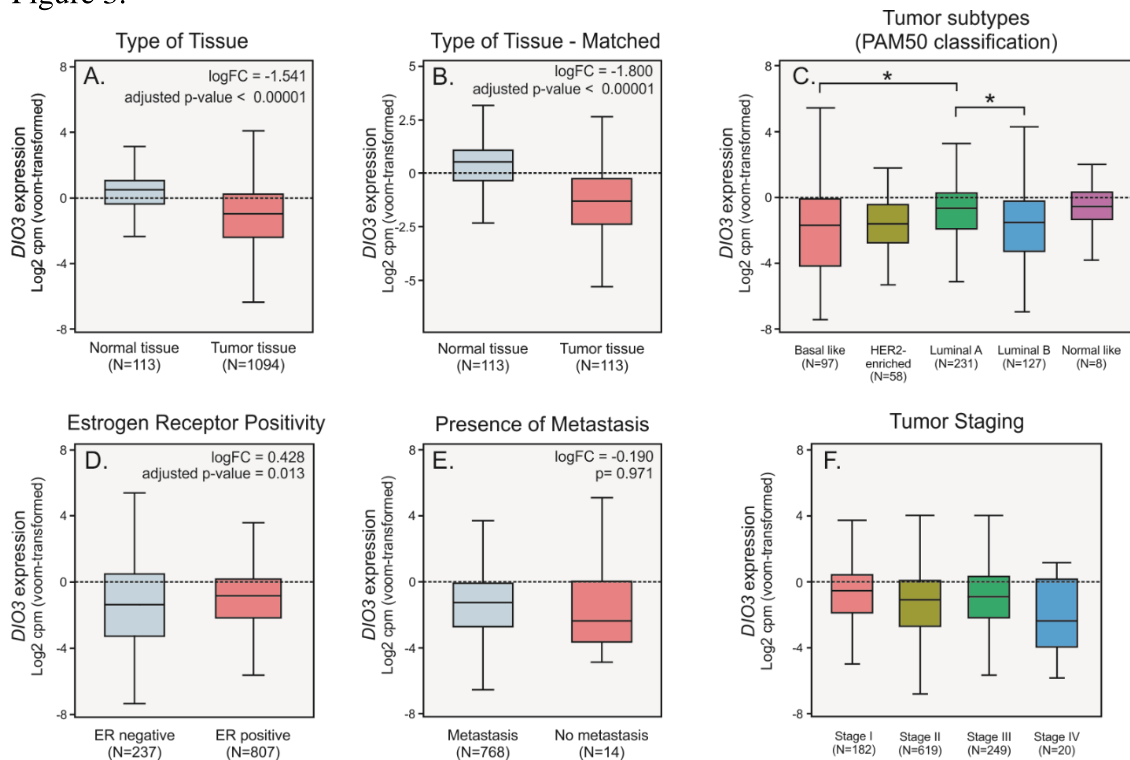

Figure 3. The relationship between *DIO3* mRNA expression and clinicopathological parameters in breast cancer samples of patients from TCGA-BRCA cohort expressed in Log2 counts per million (voom-transformed). Comparative expression demonstrates that *DIO3* mRNA is decreased in tumoral tissue when compared to normal tissue when analyzing (A) all samples or (B) only matched samples. (C) All tumor subtypes have decreased expression of *DIO3* mRNA when compared to normal tissue. Expression in LumA tumors is increased compared to basal-like tumors and compared to LumB tumors. (D) *DIO3* expression is increased in ER-positive samples when compared to ER-negative samples. (E). *DIO3* expression in similar in patients with or without metastasis. (F) When samples were separated according to tumoral staging, all tumoral stages had decreased *DIO3* expression when compared to normal tissue, but there was no difference of expression between stages. *P<0.0001.

Figure 4.

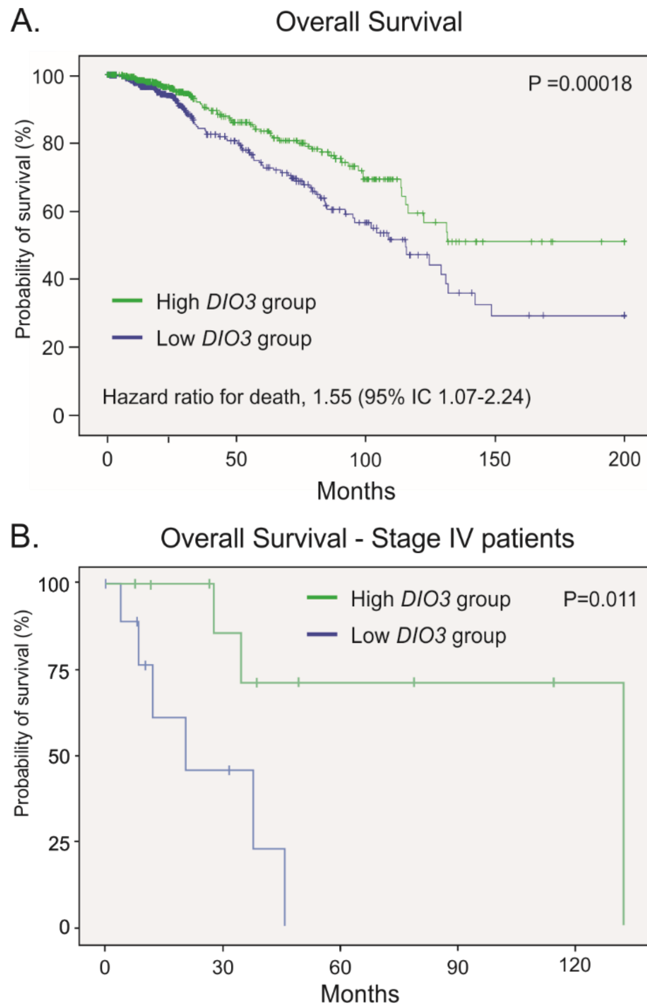

Figure 4. Kaplan-Meier estimates of overall survival in patients of the TCGA-BRCA cohort according to *DIO3* mRNA expression, where green lines refer to patients high *DIO3* expression and blue lines refer to patients with low *DIO3* expression. Groups are divided according to the median of *DIO3* expression in the population. Plot A demonstrates overall survival in the entire cohort. Plot B refers only to patients with stage IV disease (N=7 in each group).

Figure 5.

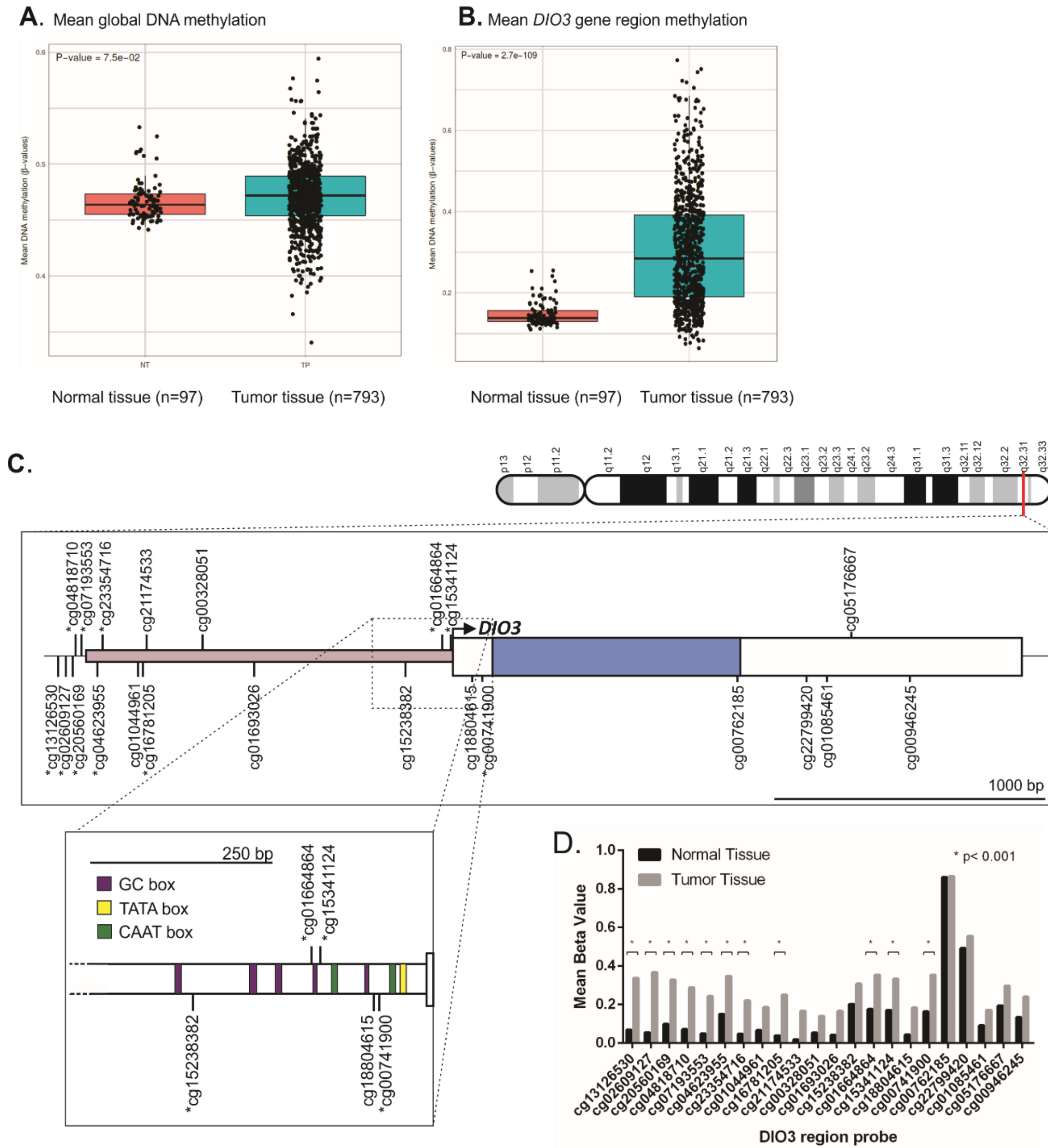

Figure 5. Panel A demonstrates mean global DNA methylation levels (β-values) in breast cancer tissue compared to healthy breast tissue. Panel B demonstrates that the mean DNA methylation of *DIO3* gene region is increased in tumor tissue when compared to normal tissue (P<0.001). Panel C is a schematic representation of the location of *DIO3* gene in chromosome 13 and the regions that were evaluated by CpG probes (red= ~1.5 kbp conserved region, blue= coding region). The promoter region of DIO3 gene is displayed below (enhanced), showing significant hypermethylation in several CpG sites (*). Panel D presents mean β-values of CpG sites mapped in *DIO3* gene region comparing normal and tumoral tissue.

Figure 6.

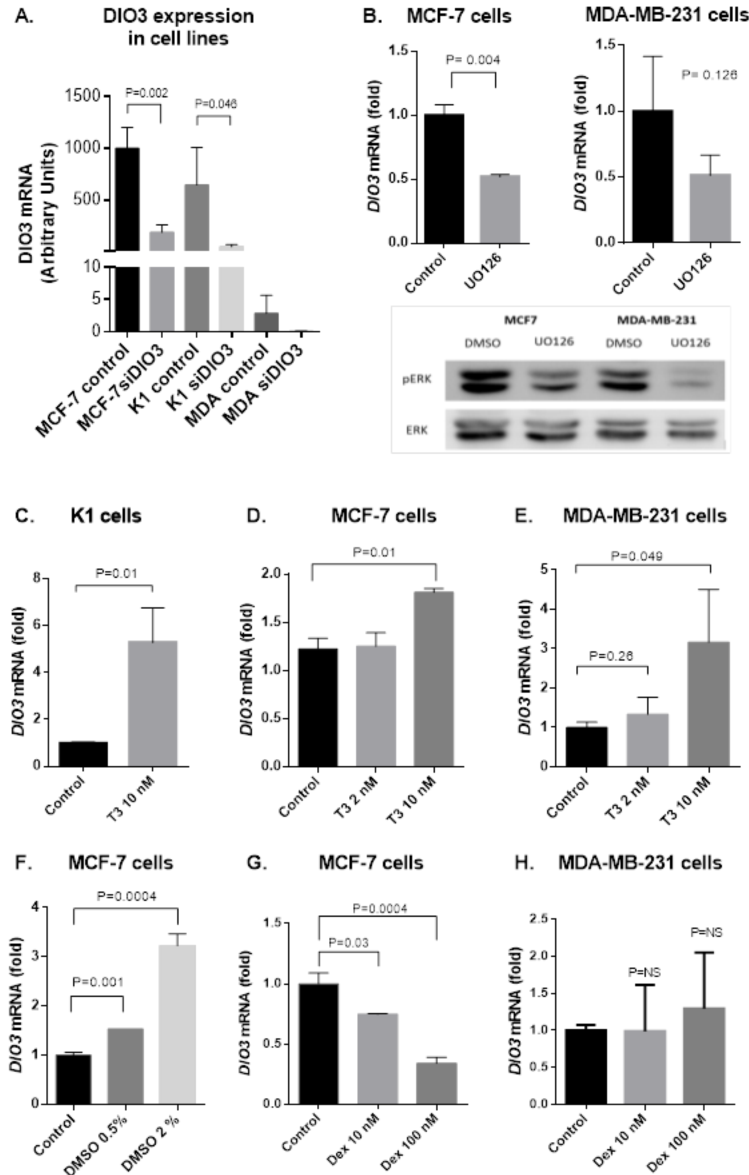

Figure 6. Regulation of *DIO3* gene in cell lines derived from breast cancer (MCF-7 and MDA-MB-231). (A) *DIO3* is expressed in MCF-7 at comparable levels to K1 thyroid cancer-derived cells (positive control), and approximately 200 fold more than in MDA-MD-231 cells. Transfection with *siDIO3* decreased *DIO3* mRNA levels by more than 80% in both MCF-7 and K1 cells. (B) Treatment with specific MEK1 inhibitor U0126 (10 uM) caused a 50% reduction in *DIO3* mRNA levels in MCF-7 cells but non-significant suppression in MDA-MB-231 (see discussion). Below we confirm significant blocked of the ERK phosphorylation in MCF-7 and MDA-MB-231 cells when incubated with U0126 (MEK inhibitor), evaluated through western blotting. (C-E): T_3 treatment at 10 nM stimulates *DIO3* expression in K1, MCF-7 and MDA-MD-231 cells. (F) Dose-dependent effect of the cytotoxic compound dimethyl sulfoxide (DMSO) on *DIO3* expression in MCF-7 cells. (G-H) Treatment with dexamethasone (Dex) in different concentrations decreases *DIO3* mRNA levels in MCF-7 cells but not in MDA-MB-231 cells. *MCF control vs MCF siDIO3, P=0.002; **K1 control vs K1 siDIO3, P=0.046; ***MCF-7 control vs MDA-MB-231 control, P=0.0066.

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CONCLUSÃO

Diante dos trabalhos expostos, concluímos que alterações hormônios tireoidianos (HTs) - como reguladores de processo celulares essenciais - contribuem para a progressão tumoral através de virtualmente todos os "*hallmarks*" do câncer. Além disso, alterações na expressão das enzimas que ativam e inativam os HTs ocorrem em diversos tipos tumorais contribuindo para o processo neoplásico. A enzima desiodase tipo 3 (DIO3) é a principal enzima responsável pela inativação dos HTs, e nossos resultados indicam que a DIO3 encontra-se expressa em tecido mamário normal e em câncer de mama, sendo sua baixa expressão associada a pior prognóstico em pacientes com esta neoplasia. Esses resultados apontam para a DIO3 como um novo marcador prognóstico em câncer de mama, sendo a redução de sua expressão associada a pior sobrevida. Diminuição da expressão da DIO3 em câncer de mama pode ser explicada ao menos em parte por hipermetilação gênica neste tipo tumoral.