Universidade Federal do Rio Grande Do Sul

Faculdade de Medicina

Programa de Pós-Graduação em Ciências Médicas: Endocrinologia

Mestrado e Doutorado

Expressão das Iodotironinas Desiodases nos Carcinomas da Tireóide

Erika Laurini de Souza Meyer

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

Porto Alegre, Novembro de 2007

Universidade Federal do Rio Grande Do Sul

Faculdade de Medicina

Programa de Pós-Graduação em Ciências Médicas: Endocrinologia

Doutorado

Expressão das Iodotironinas Desiodases nos Carcinomas da Tireóide

Erika Laurini de Souza Meyer

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

Tese apresentada ao Programa de Pós-Graduação em Ciências Médicas: Endocrinologia, para obtenção do título de Doutor

Porto Alegre, Novembro de 2007

Aos meus pais, Leuzinger (in memorian) e Marlene, pela minha existência.

Ao meu marido Bernard, meu primeiro e grande incentivador e colaborador.

À minha filha Eduarda que me proporcionou uma nova forma de amor .

Agradecimentos

À Prof[a]. Dr[a]. Ana Luiza Maia, os meus agradecimentos pela orientação, pela confiança, pela presença segura e estimulante. Agradeço a amizade que se consolidou nesses quase 7 anos de ótima convivência.

Às amigas e colegas do Laboratório de Biologia Molecular – Grupo de Tireóide: Márcia Punãles, Lenara Golbert, Simone Wajner, Clarissa Capp, Márcia Wagner, Andréia Possatti da Rocha, Débora Siqueira e Aline Estivallet, por proporcionarem tão agradável ambiente de trabalho.

Aos bolsistas e ex-bolsistas de iniciação científica, em especial ao residente Miguel Dora e ao acadêmico Iuri Goemann pelo auxílio na condução das pesquisas.

Aos Profs. Dr. Alceu Migliavacca e Dr. José Ricardo Guimarães, pela colaboração na coleta das amostras para o estudo.

Às Prof[as]. Dr[a]. Edna Kimura e Dr[a]. Mary Cleide Sogayar da Universidade de São Paulo pelo fornecimento da linhagem celular, fundamental para o seguimento dos experimentos.

Ao pessoal do Centro de Terapia Gênica do Hospital de Clínicas de Porto Alegre, representados pela Drª. Úrsula Matte, pelas orientações quanto à técnica de cultivo celular.

Aos preceptores do Serviço de Endocrinologia do Hospital São Lucas da Pontifícia Universidade Católica do Rio Grande do Sul, onde iniciei minha prática clínica em Endocrinologia.

Ao meu marido Bernard F Meyer, por todo o seu amor e carinho. Agradeço por seres um grande pai.

Aos meus pais, Leuzinger e Marlene, pelo amor incondicional, incentivo inicial, excelente convívio e carinho.

E a todos os meus amigos e colegas, que de certa forma, contribuíram para realização deste trabalho.

Esta Tese de Doutorado segue o formato proposto pelo Programa de Pós-Graduação em Ciências Médicas: Endocrinologia, Metabolismo e Nutrição, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, sendo apresentada na forma de 2 manuscritos sobre o tema da Tese:

- Artigo de revisão: Expressão das Iodotironinas Desiodases nas Neoplasias Tireoidianas; publicado nos Arquivos Brasileiros de Endocrinologia e Metabologia 2007;51/5:690-700.

- Artigo (1) original: Decreased type 1 iodothyronine deiodinase expression might be an early and discrete event in thyroid cell dedifferentation towards papillary carcinoma; publicado no Clinical Endocrinology 2005; 62:672-78.

- Artigo (2) original: Type 2 Iodothyronine Deiodinase is Highly Expressed in Medullary Thyroid Carcinoma; encaminhado para publicação em jornal científico de circulação internacional.

Conteúdo

Agradecimentos _____ 4

Resumo _____ 8

Parte I - Artigo de Revisão**:** Expressão das Iodotironinas Desiodases Nas Neoplasias Tireoidianas_____12

Parte II - Artigo Original: Decreased type 1 iodothyronine deiodinase expression might be an early and discrete event in thyroid cell dedifferentation towards papillary carcinoma _____50

Parte III - Artigo Original: Type 2 Iodothyronine Deiodinase is Highly Expressed in Medullary Thyroid Carcinoma _____79

Resumo

Os carcinomas da tireóide são as neoplasias malignas mais freqüentes do sistema endócrino. Classificam-se histologicamente em carcinomas papilar e folicular, derivados das células foliculares; carcinoma medular, derivado das células C ou para-foliculares, e carcinoma anaplásico**.** Os carcinomas diferenciados, papilar e folicular, representam mais do que 85% de todos os tipos histológicos. O carcinoma medular, compreende 5 a 10% e o anaplásico ou indiferenciado, 2% de todos os cânceres da tireóide.

Os carcinomas diferenciados da tireóide geralmente conservam as características bioquímicas da célula folicular tireoidiana. No entanto, vários estudos têm descrito anormalidades na expressão das principais proteínas envolvidas na biossíntese dos hormônios tireoidianos, tais como o receptor do TSH, o co-transportador de Na^+/I^- (NIS), a tireoglobulina (Tg) e a tireoperoxidase (TPO). As iodotironinas desiodases tipo 1 (D1) e tipo 2 (D2) são enzimas responsáveis pela ativação do T4 e produção do hormônio biologicamente ativo T3 na tireóide e nos tecidos periféricos. Poucos estudos analisaram a expressão das 5'-desiodases nos carcinomas diferenciados da tireóide. Estudos prévios demonstraram que a expressão da isoenzima D2 está reduzida no carcinoma papilar e aumentada no carcinoma folicular metastático. No entanto, uma expressão variável da D1 foi descrita em amostras de carcinomas papilares, sendo que não havia uma avaliação sistemática da expressão dessa isoenzima nos carcinomas foliculares.

O objetivo inicial do nosso estudo foi avaliar a expressão da D1 e da D2 nos tumores benignos e malignos da tireóide. De forma interessante, demonstramos que nos vários subtipos histológicos de carcinoma papilar, inclusive em microcarcinomas, os níveis de RNA mensageiro (RNAm) e de atividade da D1 estavam significativamente diminuídos em relação ao tecido tireoidiano normal correspondente (0,25 ± 0,24 *vs*. 1,09 ± 0,54 unidades arbitrárias (UA), *P*<0,001 e 0,08 ± 0,07 *vs*. 0,24 ± 0,15 pmol T4/min/mg proteína, *P*=0,045, respectivamente) . Por outro lado, observamos um aumento da expressão dessa enzima nos adenomas (1,9 ± 1,5 *vs.* 0,83 ± 0,58 UA, *P*= 0,028 e 2,67 ± 1,42 *vs.* 0,22 ± 0,06 pmol T4/min/mg proteína, *P*=0,044, respectivamente) e nos carcinomas foliculares (1,2 ± 0,46 *vs*. 0,67 ± 0,18 UA, *P*=0,038 e 1,20 ± 0,58 *vs.* 0,20 ± 0,10 pmol T4/min/mg proteína, *P*<0,001, respectivamente). Da mesma forma, confirmamos dados prévios sobre o aumento da atividade da D2 no carcinoma folicular metastático. A análise desses resultados permitiu concluir, originalmente, que a alteração na expressão da D1 parece ser um evento precoce e específico da desdiferenciação celular do carcinoma papilar (*Souza Meyer EL et al, Clinical Endocrinology 2005;62:672-78*).

O interesse pelo padrão de expressão das 5'-desiodases em outros tumores humanos e pelos mecanismos ou implicações do aumento ou da diminuição das desiodases na patogênese neoplásica, determinou um estudo da literatura que foi publicado em artigo de revisão *(Meyer ELS et al, Arquivos Brasileiros de Endocrinologia e Metabologia 2007;51/5:690-700).*

Uma observação inesperada durante as nossas pesquisas foi a presença da D2 no carcinoma medular da tireóide (CMT). A atividade da D2 em amostras de CMT estava comparável ao tecido folicular normal (0,41 ± 0,10 *vs.* 0,43 ± 0,41 fmol

T4/min/mg proteína, *P*=0,913). Para descartar a possibilidade de contaminação do tecido tumoral por tecido tireoidiano normal, a investigação foi ampliada para a análise dessa enzima nas células TT, uma linhagem celular humana de CMT. Os resultados confirmaram os achados nas amostras tumorais e evidenciaram a manutenção das propriedades bioquímicas da D2, tais como a inibição pelos hormônios tireoidianos, a insensibilidade à inibição pelo propiltiouracil e a baixa Constante de Michaelis-Menten, apesar da desdiferenciação celular. Sendo assim, os nossos dados propõem uma nova perspectiva sobre o papel dos hormônios tireoidianos nas células C da tireóide (*artigo submetido à publicação*).

Parte I

Expressão das Iodotironinas Desiodases nas Neoplasias Tireoidianas

Artigo publicado nos Arquivos Brasileiros de Endocrinologia e Metabologia 2007;51:690-700

Expressão das Iodotironinas Desiodases nas Neoplasias Tireoidianas

Erika L Souza Meyer, Márcia S Wagner, Ana Luiza Maia

Serviço de Endocrinologia, Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil

Título abreviado: Iodotironinas Desiodases nas Neoplasias

Suporte Financeiro: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brasil.

Correspondência: Profa. Dra. Ana Luiza Maia, Serviço de Endocrinologia, Hospital de Clínicas de Porto Alegre, Rua Ramiro Barcelos n° 2350, Porto Alegre, RS, Brasil, 90035-003. Fone: 51 3331-0207, fax: 51 3332-5188. E-mail: almaia@ufrgs.br

Resumo

Descritores: Iodotironinas desiodases; Neoplasias; Expressão gênica

As iodotironinas desiodases formam uma família de selenoenzimas com propriedades catalíticas distintas que ativam ou inativam os hormônios tireoidianos via desiodação do anel fenólico ou tirosínico da molécula do T4. As desiodases tipo I e II (D1 e D2) são as enzimas responsáveis pela geração do T3 e são amplamente expressas na tireóide normal. A transformação neoplásica benigna ou maligna da glândula tireóide está associada a alterações na expressão dessas isoenzimas, sugerindo um possível papel da D1 e da D2 como marcadores de diferenciação celular. Anormalidades na expressão de ambas enzimas e da desiodase tipo III (D3), inativadora dos hormônios tireoidianos, são também encontradas em outras neoplasias humanas. Os mecanismos ou as implicações do aumento ou diminuição das desiodases na patogênese neoplásica são pouco compreendidas. No entanto, é importante observar que a expressão anormal da D2 pode ser responsável por um quadro de tireotoxicose em pacientes com metastáses de carcinoma folicular de tireóide enquanto que o aumento da D3 em hemangiomas pode causar hipotireoidismo de difícil tratamento.

Abstract

Keywords: Iodothyronine deiodinases; Neoplasia; Gene Expression

The iodothyronine deiodinases constitute a family of selenoenzymes that catalyze the removal of iodine from the outer ring or inner ring of the thyroid hormones. The activating enzymes, deiodinases type I (D1) and type II (D2), are highly expressed in normal thyroid gland. Benign or malignant neoplastic transformation of the thyroid cells is associated with changes on the expression of these enzymes, suggesting that D1 or D2 can be markers of cellular differentiation. Abnormalities on the expression of both enzymes and also of the deiodinase type III (D3), that inactivates thyroid hormones, have been found in other human neoplasias. So far, the mechanism or implications of these findings on tumor pathogenesis are not well understood. Nevertheless, it's noteworthy that abnormal expression of D2 can cause thyrotoxicosis in patients with metastasis of follicular thyroid carcinoma and that increased D3 expression in large hemangiomas causes severe hypothyroidism.

Introdução

O hormônio tireoidiano é produzido pela glândula tireóide na forma de um precursor inativo, a tiroxina (3,5,3',5'-tetraiodo-L-tiroxina, T4). Em humanos, apenas 20% da forma biologicamente ativa do hormônio, a triiodotironina (3,5,3'-triiodo-L-tironina, T3), é secretada diretamente pela tireóide. Assim, a maior parte do T3 circulante é derivada da desiodação do anel externo da molécula de T4 nos tecidos periféricos, através da ação de enzimas denominadas iodotironinas desiodases (1).

O hormônio tireoidiano, dentre outras ações, estimula a diferenciação e proliferação celular. Alguns estudos indicam que, ao menos *in vitro*, os hormônios tireoidianos desempenhem um papel na transformação neoplásica induzida pela radiação ou por substâncias carcinógenas (2,3). Outros estudos têm associado o *status* tireoidiano com a tumorigênese e o crescimento de tumores sólidos (4,5). Uma das ações do T3 na proliferação de células neoplásicas foi documentada através de um estudo que demonstrou que concentrações fisiológicas de T3 bloqueiam a resposta transcricional sinalizada pela via RAS nas células de neuroblastoma (6). A repressão dos níveis de ciclina D1, proteína envolvida na progressão do ciclo celular, pelo T3, parece ser um importante componente do mecanismo que bloqueia a proliferação celular *ras*-dependente. O T3 pode inibir a resposta oncogênica das 3 isoformas N- K e H-*RAS* e ambos os receptores do hormônio tireoidiano (TRα e TRβ) parecem mediar essa ação (6).

O envolvimento do T3 na gênese tumoral também foi demonstrado em estudos com camundongos transgênicos, nos quais uma mutação na isoforma do receptor do hormônio tireoidiano TRβ, induziu a formação espontânea do

carcinoma folicular da tireóide (7). Nesses animais, uma mutação no éxon 10 em que ocorre uma inserção C no códon 448 produzindo um *frameshift* de 14 aminoácidos da cadeia carboxi-terminal do TRβ, impede a ligação do T3 ao seu receptor e age como um fator de transcrição dominante negativo nos elementos responsivos ao T3 (TREs). Essa mutação poderia alterar a transcrição de vários genes regulados por esse hormônio e que podem estar envolvidos na proliferação e diferenciação celular. Com base nessas observações, o gene do TRβ é questionado como um possível gene supressor tumoral. É interessante observar que alguns estudos prévios demonstraram anormalidades na expressão dos TRs, tanto ao nível do RNA mensageiro quanto da proteína, sendo que mutações somáticas no TRα e no TRβ foram identificadas em vários tumores humanos, tais como fígado, rim e tireóide (8-11).

Os carcinomas da tireóide constituem as neoplasias malignas mais freqüentes do sistema endócrino (12). Os carcinomas tireoidianos são classificados de acordo com o tipo histológico em papilar, folicular, medular e indiferenciado ou anaplásico, sendo que os carcinomas papilar e folicular, denominados carcinomas diferenciados, representam aproximadamente 85% dos casos (13). Os carcinomas diferenciados da tireóide na maioria das vezes mantêm as características bioquímicas da célula folicular normal. No entanto, várias anormalidades têm sido descritas na expressão gênica da tireoglobulina, da tireoperoxidase, do receptor do TSH e do co-transportador Na^+/I^- (14-16); proteínas responsáveis pela síntese dos hormônios da tireóide. A análise da expressão dessas proteínas em tecidos normais e neoplásicos da tireóide tem buscado a identificação de possíveis marcadores de diferenciação celular com

implicações no diagnóstico, tratamento e prognóstico dos carcinomas diferenciados da tireóide (17-19).

Recentemente, o padrão de expressão das desiodases nos tecidos neoplásicos também passou a despertar interesse. Anormalidades na expressão dessas enzimas são encontradas nos tecidos tumorais, sendo as alterações parecem ser específicas ao tipo histológico (20-25). Nas neoplasias tireoidianas, as desiodases têm sido questionadas como possíveis marcadores de diferenciação celular (20,21). Atualmente aceita-se que é necessário um suprimento adequado de T3 intratecidual, combinado com a sinalização mediada pelos TRs, para a manutenção das características diferenciadas das células, a apoptose e a prevenção do crescimento de clones tumorigênicos (26). O objetivo dessa revisão é descrever o padrão de expressão das desiodases nas neoplasias da tireóide e nos demais tumores humanos, bem como, discutir as possíveis implicações dessas alterações.

Neoplasias Benignas e Malignas da Glândula Tireóide: Aspectos Moleculares

A célula epitelial folicular da tireóide é responsável pela biossíntese dos hormônios tireoidianos. Quatro tipos distintos de neoplasias tireoidianas se originam dessa célula: os adenomas foliculares e os carcinomas papilar, folicular e anaplásico (13). Mutações ativadoras no gene do receptor do TSH e da proteína G são as anormalidades genéticas associadas aos adenomas foliculares hiperfuncionantes (27,28). A mutação no gene *BRAF (T1799A)* parece ser a alteração genética mais freqüente nos carcinomas papilares, sendo que essa

mutação não ocorre concomitante às alterações no *RAS* e *RET/PTC*, dois oncogenes também envolvidos na patogênese dos carcinomas diferenciados da tireóide (29,30). Ainda no carcinoma papilar destacam-se os rearranjos do oncogene *RET* com genes heterólogos dando origem ao gene quimérico *RET*/*PTC* (31). Nos adenomas e carcinomas foliculares, além das mutações ativadoras dos genes RAS, temos os rearranjos cromossomais entre o PAX-8, um fator de transcrição relevante para o desenvolvimento tireoidiano, e o receptor nuclear *PPARγ,* que está envolvido no controle da proliferação e diferenciação celular (32,33). Outras mutações de oncogenes e genes supressores tumorais contribuem para a desdiferenciação em carcinoma anaplásico (p53, Rb) (34). Uma leitura mais detalhada sobre a genética molecular dos carcinomas diferenciados da tireóide pode ser revista no artigo de Maciel e cols. (35).

Iodotironinas Desiodases

Os hormônios tireoidianos são pequenas moléculas ligantes hidrofóbicas não-peptídicas essenciais para vários processos biológicos como desenvolvimento, crescimento, diferenciação e metabolismo (36,37). Sua ação ocorre, fundamentalmente, através da ligação do hormônio a receptores nucleares (TRs) localizados em sequências específicas, os elementos responsivos ao hormônio tireoidiano (TREs), na região promotora dos genes-alvo. A formação do complexo hormônio-receptor resulta em alterações na composição do complexo transcricional e este processo pode tanto aumentar quanto reprimir a transcrição (38).

O T4, principal produto secretado pela tireóide, funciona primariamente como um pró-hormônio, sendo transformado no hormônio biologicamente ativo, o T3, que possui alta afinidade pelos TRs (39). As iodotironinas desiodases tipos I (D1), II (D2) e III (D3) formam uma família de selenoenzimas que, com propriedades catalíticas distintas, ativam ou inativam o hormônio tireoidiano via desiodação do anel fenólico ou tirosínico das iodotironinas (figura 1) (40). Embora as três desiodases apresentem em comum certas características estruturais importantes, essas enzimas diferem entre si em vários aspectos como no padrão de expressão tecidual, localização celular, papel fisiológico, preferência pelo substrato, sensibilidade a inibidores e na forma como são reguladas pelos hormônios tireoidianos. A tabela 1 sumariza as principais características das três desiodases humanas.

A atividade das desiodases é regulada por um processo complexo, e vários fatores e/ou situações fisiopatológicas que podem alterar a desiodação em um determinado tecido (1). De fundamental importância regulatória são as alterações no *status* dos hormônios tireoidianos que determinam mudanças significativas na atividade das desiodases. Do ponto de vista fisiológico, estas mudanças parecem coordenadas de forma a manter os níveis circulantes e intracelulares de T3 próximos do normal. Além de serem expressas em vários tecidos periféricos, as isoenzimas D1 e D2 são amplamente encontradas na tireóide, especialmente na célula folicular (41, 42). Aproximadamente 30 a 50% do T3 secretado diretamente pela tireóide deriva da desiodação intratireoidiana do T4 (42).

Desiodase Tipo 1

Em humanos, os níveis mais elevados de expressão da D1 são encontrados no fígado, rim, tireóide e hipófise (1). A D1 apresenta uma característica única entre as desiodases, pois pode catalisar a desiodação tanto do anel fenólico quanto do anel tirosínico das diversas iodotironinas e esses processos resultam, respectivamente, na ativação e inativação dos hormônios tireoidianos (Figura 1). Nos mamíferos em geral, a atividade da D1, particularmente da D1 hepática, é considerada uma importante fonte de T3 plasmático no estado eutireoideo (1, 43). Em humanos, apesar da D1 contribuir na produção do T3 plasmático, sua contribuição mais significativa ocorre nos pacientes com hipertireoidismo (44). Além de atuar na ativação do T4, a D1 também participa no rápido *cleareance* do rT3 e na inativação das iodotironinas sulfatadas (45). A expressão da D1 é regulada ao nível transcricional pelo hormônio tireoidiano. Vários estudos demonstraram que o T3 estimula a síntese da D1, através de um efeito direto sobre a taxa de transcrição gênica da enzima, induzindo aumentos nos níveis do RNA mensageiro e, consequentemente, da proteína (46). De fato, dois TREs funcionais foram identificados no promotor do gene da D1 humano (47, 48). Em particular na tireóide, a expressão da D1 é estimulada via TSH/AMP cíclico (AMPc) (49).

Expressão da D1 nas neoplasias tireoidianas

Vários estudos avaliaram a expressão da D1 nas neoplasias tireoidianas. Khörle *et al*.(20) demonstraram uma variabilidade nos níveis de expressão da D1

em 10 amostras não-pareadas de carcinoma papilar, sendo que foram observados desde níveis indetectáveis até níveis muito altos de expressão. Por outro lado, Toyoda *et al* (50) observaram uma redução da proteína de 27kDa com propriedades da D1, em 4 de 5 casos de carcinoma papilar. Em trabalho realizado pelo nosso grupo, observamos uma significativa redução nos níveis de RNA mensageiro e atividade da D1 em todas as 14 amostras de carcinoma papilar avaliadas (24). Esses resultados foram observados em todos os subtipos histológicos, que incluíam desde as formas clássica, variante folicular, variante esclerosante difusa até microcarcinomas; e nos diferentes estágios clínicos (Figura 2a,b). Em acordo com os nossos resultados, Arnaldi e cols.(25) também descreveram uma redução nos níveis de RNA mensageiro da D1 nos carcinomas papilares.

Em contraste com a significativa redução da D1 nos carcinomas papilares, nos adenomas e carcinomas foliculares da tireóide, os níveis da D1 estão aumentados em relação ao tecido normal (Figura 3a,b,c) (24). Achados semelhantes foram detectados em amostra de carcinoma de células de Hürthle (24). A análise desses resultados parece indicar que a desdiferenciação celular da tireóide promove alterações na expressão gênica da D1 através de mecanismos pré-transcricionais, um evento específico na progressão para o carcinoma papilar.

Atividade da D1 também foi descrita no carcinoma anaplásico. Estudos prévios relataram nível muito baixo ou indetectável nesse tipo de carcinoma, com resultados similares em linhagens celulares correspondentes (20,51). No entanto, nós detectamos níveis elevados de atividade em 1 amostra de carcinoma anaplásico misto (24). Com base nos achados da expressão da D1 nos carcinomas papilares e foliculares, esses resultados aparentemente conflitantes

podem, talvez, sugerir o tipo histológico a partir do qual ocorreu a desdiferenciação tumoral (52).

Alterações na regulação da D1 também foram documentadas nas células tireoidianas neoplásicas. Nos estudos realizados em linhagens de células humanas de carcinoma folicular de tireóide (FTC 133 e FTC 238), observou-se que a D1 perde a resposta ao estímulo fisiológico do TSH e do T3, mas mantém-se sensível ao ácido retinóico, um metabólito ativo da vitamina A que regula a taxa de crescimento e diferenciação de vários tipos celulares (51). Nessas células, o ácido retinóico estimula a atividade da D1 em 5 a 10 vezes. Nas linhagens humanas menos diferenciadas, ocorre também uma perda da resposta ao ácido retinóico (51).

Expressão da D1 em outras neoplasias

No carcinoma de células claras, o tipo mais freqüente de carcinoma renal, foram descritos níveis indetectáveis de RNA mensageiro e atividade da D1 em todas as 10 amostras analisadas (53). Os autores especulam que a perda da expressão da D1 seja em conseqüência da transformação neoplásica ou que a célula origem desse tumor seja tubular, que normalmente não expressa essa enzima. A redução na expressão da D1 também foi demonstrada em amostras de adenocarcinoma de fígado comparada ao tecido normal (54), sendo que nas células derivadas de carcinoma hepatocelular humano (Hep G2), um aumento de 2 vezes na atividade da D1 foi descrito com o uso do ácido retinóico (51). A atividade da D1 também está significativamente reduzida no adenocarcinoma e

carcinoma pulmonar de células escamosas (55). Achados semelhantes foram descritos no câncer de próstata (56).

Um resultado conflitante ao que foi evidenciado na maioria das neoplasias humanas, nas quais observamos uma hipoexpressão da D1, foi descrito em carcinoma de glândula mamária de ratas Sprague-Dawley induzido por N-metil-N-nitrouréia (MNU), em que a atividade enzimática da D1 estava pelo menos 2 vezes mais alta do que no tecido mamário normal não-lactante (57). No entanto, os níveis da D1 eram maiores nos tumores com 2 a 4 meses do que nos tumores com mais de 6 meses de crescimento (58), caracterizando uma perda da expressão da D1 com a evolução tumoral. Na glândula mamária de ratos, a D1 está aumentada na lactação principalmente no epitélio alveolar e sua expressão é regulada pela sucção através do estímulo β-adrenérgico (59).

Em relação à regulação da D1 nas células neoplásicas, foi demonstrado que nas células MCF-7, uma linhagem celular humana de carcinoma de mama ovariano-dependente, a D1 responde ao ácido retinóico, enquanto que está abolida a regulação pelo T3 e pelo agonista β-adrenérgico isoproterenol (60). Similarmente ao que ocorre em outras linhagens celulares neoplásicas, na linhagem menos diferenciada de carcinoma de mama (MDA-MB-231), não foi encontrada atividade da D1, tanto nas condições basais ou em tratamentos (60). Esses resultados reforçam a possibilidade da D1 ser um marcador de desdiferenciação celular como sugerido em outros estudos (20,21).

Ao contrário do sistema nervoso central (SNC) de ratos, nenhuma atividade da D1 foi documentada no SNC de humanos (61). No entanto, alguns estudos demonstraram que a D1 está presente tanto na hipófise normal quanto nos adenomas hipofisários humanos (62,63). Níveis muito baixos de RNA mensageiro

da D1, quase próximos ao limite da detecção do método, foram descritos em 50% das amostras de pituitária normal e tumoral. Outro estudo detectou atividade da D1 tanto na hipófise normal quanto nos adenomas hipofisários, sendo que níveis elevados de atividade da D1 foram descritos em algumas amostras de tecido normal e de tumores não-funcionantes ou produtores de prolactina (63). A tabela 2 sumariza os principais achados sobre a expressão da D1 nas neoplasias humanas.

Desiodase Tipo 2

A D2 é uma enzima que cataliza exclusivamente a desiodação do anel externo das iodotironinas desempenhando um papel crítico na ativação do T4 em diferentes tecidos. Em humanos, o RNA mensageiro e/ou a atividade da D2 estão presentes principalmente no cérebro, hipófise, tireóide, placenta e músculos cardíaco e esquelético (1). Embora o principal papel fisiológico atribuído à D2 seja a produção intracelular de T3, estudos recentes sugerem que a D2 presente no músculo esquelético contribui com uma parcela significativa da produção do T3 plasmático em humanos (44).

Em geral, a atividade da D2 apresenta uma correlação inversa com os níveis séricos dos hormônios tireoidianos, estando aumentada no hipotireoidismo e diminuída no hipertireoidismo (64). Mecanismos pré e pós-transcricionais estão envolvidos na regulação da expressão da D2 pelos hormônios tireoidianos com papéis distintos para o T3, T4 e rT3. O T3 atua principalmente ao nível transcricional diminuindo os níveis de RNA mensageiro da D2, enquanto o T4 e o rT3 rapidamente reduzem a atividade enzimática postranscricionalmente,

aumentando a taxa de ubiquitinação e posterior degradação da enzima via proteossomas (65). Vários fatores parecem capazes de modular a taxa de transcrição do gene da D2, como o AMPc, os fatores de transcrição TTF-1 e PAX-8, a proteína ativadora 1 (AP1) e agentes adrenérgicos (66). Na tireóide, a expressão da D2 está relacionada ao grau de estímulo tecidual via TSH/AMPc, com aumento da expressão encontrado na Doença de Graves e nos adenomas hiperfuncionantes (41).

Expressão da D2 nas neoplasias tireoidianas

No carcinoma papilar da tireóide foi descrito uma redução nos níveis de RNA mensageiro e na atividade enzimática da D2, um processo que segundo os autores, pode ser secundário à transformação neoplásica (23). Um dado interessante é que nas variantes foliculares dessa neoplasia, não se observa uma redução significativa nos níveis de expressão da enzima em relação ao tecido tireoidiano normal (25).

Semelhante ao que foi observado na expressão da D1 nas lesões foliculares, os níveis de RNA mensageiro da D2 nos adenomas e carcinomas foliculares são similares ao tecido não-neoplásico (25), enquanto que nos adenomas foliculares hiperfuncionantes é observada uma alta expressão da D2 (41). É interessante no entanto que, em metástases de carcinoma folicular foram descritos altos níveis de atividade da D2 que determinavam um persistente aumento da relação sérica de T3/T4 nos pacientes (24, 67,68). Em conseqüência do aumento da atividade da D2 no tumor, pacientes com grandes e/ou múltiplas metástases de carcinoma folicular podem apresentar um quadro clínico de T3-

tireotoxicose devido ao aumento da conversão do T4 exógeno pelo tecido tumoral (68).

Expressão da D2 em outras neoplasias

A D2 está normalmente expressa no tecido cerebral humano, sendo que em neoplasias cerebrais foram identificados receptores dos hormônios tireoidianos, indicando um possível envolvimento desses hormônios na proliferação celular desse tipo tumoral (61,69,70). A atividade da D2 foi identificada em astrocitomas, glioblastomas e oligodrendrogliomas, com forte correlação com os seus níveis de RNA mensageiro, sugerindo uma regulação pré-transcricional da expressão dessa enzima nos tumores cerebrais (71). Os maiores níveis de atividade foram encontrados nos gliosarcomas (72). Por outro lado, nenhuma atividade de desiodação do T4 foi detectada nos meningiomas, sendo a explicação mais provável a diferença na origem embriológica desses tumores (células da aracnóide), em contraste aos gliomas que se originam das células gliais (73).

Além do seu papel na regulação da secreção de TSH através do *feedback* negativo, os hormônios tireoidianos parecem estar envolvidos na regulação do crescimento e diferenciação da célula hipofisária (74,75). Com exceção dos tumores hipofisários secretores de ACTH e TSH, os demais tumores hipofisários apresentam um aumento significativo nos níveis de RNA mensageiro e atividade da D2 quando comparados com a pituitária normal (62). A maior atividade da D2 parece ocorrer nos subtipos produtores de TSH e prolactina, quando comparados aos tumores não-funcionantes, aos produtores de GH e ACTH (63). Segundo os autores, esses resultados demonstram que apesar da transformação tumoral, a D2

permanece ativa, mantendo o seu papel na função e regulação da hipófise anterior humana.

Várias linhagens celulares tumorais humanas apresentam atividade da D2. As células JEG3, uma linhagem de coriocarcinoma humano, mantém a expressão da D2 apesar da transformação tumoral e sua responsividade ao tratamento com AMPc (76). Foi demonstrado que as células MSTO-211H, uma linhagem de mesotelioma humano, expressam o gene da D2, apresentando níveis de RNA mensageiro 40 vezes mais elevados do que as células mesoteliais normais (77,78). Uma alta atividade da D2 foi encontrada nessas células, sendo aproximadamente 40% maior do que a encontrada em tecidos de pacientes com Doença de Graves (78). A D2 também foi identificada em células de osteossarcoma humano (SaOS-2), embora com expressão mais baixa em relação às células osteoblásticas normais (NHOst) (79). Nessas células, tanto os níveis de RNA mensageiro quanto a atividade enzimática rapidamente aumentam sob o estímulo do TSH. Além disso, as 3 isoformas de receptores do T3 foram encontradas, sugerindo um possível papel do T3 gerado localmente pela D2 na maturação e remodelamento do osso normal. A presença da D2 também foi descrita em carcinomas de pulmão, similares ao tecido circunjacente (55). A tabela 2 sumariza os principais achados sobre a expressão da D2 nas neoplasias humanas.

Desiodase Tipo 3

A D3 catalisa unicamente a desiodação do anel interno das moléculas de T4 e T3 resultando na formação dos metabólitos inativos rT3 e T2,

respectivamente (Figura 1). Os mais altos níveis de expressão da D3 são encontrados em tecidos fetais humanos e de roedores, na placenta, útero gravídico e vasos umbilicais (80,81). Na vida adulta, a D3 está expressa predomimantemente no sistema nervoso central e pele (61,82). A atividade da D3 não foi detectada na tireóide em nenhum estágio do desenvolvimento ou na vida adulta. O alto nível de expressão nos tecidos fetais, placenta e útero sugere que a D3 regule os níveis circulantes e teciduais do T3 de modo a proteger o feto em desenvolvimento de uma indevida exposição ao hormônio tireoidiano ativo (80). No cérebro e pele de diferentes espécies, a atividade e os níveis de mRNA da D3 estão aumentados no hipertireoidismo e diminuídos no hipotireoidismo, entretanto os mecanismos envolvidos nesta regulação ainda não foram determinados (83). Não existem dados na literatura sobre a expressão da D3 nos tumores tireoidianos.

Expressão da D3 em outras neoplasias

A existência de atividade da D3 no cérebro humano adulto parece ser responsável pela inativação dos hormônios tireoidianos no sistema nervoso central (61,73). Nauman *et al* (72) determinaram a concentração celular de T4 e T3 e a atividade enzimática em tecidos de gliomas humanos com vários graus de malignidade e em tecidos cerebrais normais. A atividade da D3 estava aumentada em todos os casos de gliosarcoma, em 9 de 10 casos de glioblastoma multiforme; e diminuída em todas as amostras de astrocitoma grau II e grau III quando comparada com a baixa atividade desta enzima encontrada no tecido cerebral

normal. Os autores sugerem que essas alterações podem estar relacionadas com a progressão maligna.

Estudos prévios não identificaram a expressão da D3 em linhagem celular derivada da hipófise anterior humana (84). No entanto, a análise de adenomas hipofisários demonstrou níveis de RNA mensageiro aproximadamente 7 vezes mais elevados do que no tecido hipofisário normal. Maior expressão foi encontrada nos tumores secretores de TSH (13 vezes), seguidos pelos produtores de ACTH (7 vezes), não-funcionantes (7 vezes) e pelos produtores de GH (6 vezes). No entanto, a atividade enzimática foi detectada somente em 3 de 16 tumores (18%), todos não-funcionantes (62).

A presença da D3 é relatada em anormalidades vasculares e em algumas linhagens de células malignas (83,85,86). Dados recentes indicam que a expressão dessa enzima pode ser reativada após o nascimento em tecidos normais durante enfermidades críticas e outras condições patológicas (87). Recentemente, o RNA mensageiro e a atividade da D3 foi identificado em hemangiomas infantis em níveis 7 vezes maiores do que na placenta, um órgão que normalmente expressa uma grande quantidade dessa enzima. Como a D3 inativa os hormônios tireoidianos, o crescimento tumoral ou a presença de grandes hemangiomas pode provocar hipotireoidismo severo e de dificil tratamento (85). O primeiro caso relatado foi de uma criança de 3 meses de idade com vários hemangiomas hepáticos e hipotireoidismo primário refratário à altas doses de L-tiroxina para restabelecimento do eutireoidismo e da secreção normal de tireotrofina (TSH). Os altos níveis de atividade da D3 no tumor, determinam uma maior degradação dos hormônios tireoidianos, sendo que o hipotireoidismo resolve com a ressecção ou involução do tumor. Os hemangiomas cutâneos também

podem expressar níveis elevados de D3 (87). Nos adultos a atividade aumentada de D3 em hemangiomas pode ser causa de hipotireoidismo subclínico (86). Dessa forma, uma alta suspeição para hipotireoidismo deve-se ter em crianças e adultos com grandes tumores vasculares.

Na busca de um maior entendimento do papel da D3 na regulação dos hormônios tireoidianos, alguns estudos têm avaliado a expressão dessa enzima em diferentes linhagens celulares humana. A atividade e o RNA mensageiro da D3 estão presentes e fortemente correlacionados nas células de carcinoma de endométrio (ECC-1), carcinoma de mama (MCF-7) e de neuroblastoma (SH-SY5Y) (83). A presença da atividade da D3 nas células MCF-7, segundo os autores, pode indicar que essa enzima pode estar expressa no tecido mamário somente em certas situações fisiológicas tais como no período de lactação. Os estudos regulatórios demonstraram que o estradiol aumentou a atividade da D3 somente nas células ECC-1, sugerindo uma contribuição desse hormônio na indução da D3 no útero gravídico. Além disso, a incubação com ácido retinóico determinou o aumento em 2 a 3 vezes na atividade da D3 nos tipos ECC-1 e MCF-7, mas a redução nas células SH-SY5Y, demonstrando com esses resultados, uma regulação específica conforme o tipo celular. A expressão da D3 não foi afetada pelos hormônios tireoidianos ou pelo AMPc em nenhum tipo celular(83). Esses resultados caracterizam a perda do estímulo fisiológico dos hormônios tireoidianos em decorrência do processo neoplásico. A tabela 2 sumariza os principais achados sobre a expressão da D3 nas neoplasias humanas.

Considerações finais

Em tumores humanos de origem epitelial provenientes de tecidos que normalmente expressam a D1, geralmente ocorre uma redução na expressão dessa enzima no tecido neoplásico. No entanto, a expressão da D2 se encontra inalterada ou mesmo aumentada, com exceção do carcinoma papilar da tireóide. Uma possível explicação, embora especulativa, é que a redução da expressão da D1 nos tumores possa decorrer da perda da resposta fisiológica ao T3. Já o mecanismo para as alterações na expressão da D2 parece ser mais complexo, em função dessa enzima apresentar regulação pré e pós-transcricional. De qualquer modo, é necessário determinar se as anormalidades na expressão das desiodases, principalmente da D2, são simplesmente uma conseqüência da transformação celular ou parte do processo de patogênese tumoral. Futuros estudos funcionais dessas enzimas em células tumorais auxiliarão no entendimento dessa questão. Até o momento, as repercussões da expressão anormal das desiodases nos tumores humanos são evidentes em metástases de carcinoma folicular da tireóide e em tumores de origem vascular.

Referências bibliográficas

1. Bianco AC, Salvatore D, Gereben B, Berry MJ, Larsen PR. Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. Endocr Rev 2002;23:38-89.
2. Borek C, Guernsey DL, Ong A, Endelman IS. Critical role played by thyroid hormone in induction of neoplastic transformation by chemical carcinogens in tissue culture. Proc Natl Acad Sci USA 1983;80:5749-52.
3. Guernsey DL, Borek C, Edelman IS. Crucial role of thyroid hormone in X-ray-induced neoplastic transformation in cell culture. Proc Natl Acad Sci USA 1981;78:5708-11.
4. Lemaire M, Bayens W, de Saint-Georges L, Baugnet-Mahieu L. Thyroid hormone influence on the growth of hepatoma HW-165 in Wistar rats. Biomedicine 1981;34:133-39.
5. Leuthauser SWC, Guernsey DL. Thyroid hormone affects the expression of neoplastic transformation induced by DNA-transfection. Cancer Lett 1987;35:321-26.
6. Garcia-Silva S, Aranda A. The thyroid hormone receptor is a suppressor of ras-mediated transcription, proliferation and transformation. Mol Cell Biol 2004;24:7514-23.
7. Kato Y, Ying H, Willingham MC, Cheng S-Y. A tumor suppressor role for thyroid hormone β receptor in a mouse model of thyroid carcinogenesis. Endocrinology 2004;145:4430-38.

8. Lin KH, Shieh HY, Chen SL, Hsu HC. Expression of mutant thyroid hormone nuclear receptors in human hepatocellular carcinoma cells. Mol Carcinog 1999;26:53-61.
9. Kamiya Y, Puzianowska-Kuznicka M, McPhie P, Nauman J, Cheng SY, Nauman A. Expression of mutant thyroid hormone nuclear receptors is associated with renal cell carcinoma. Carcinogenesis 2002;23:25-33.
10. Puzianowska-Kuznicka M, Krystyniak A, Madej A, Cheng SY, Nauman J. Functionally impaired TR mutants are present in thyroid papillary cancer. J Clin Endocrinol Metab 2002;87:1120-28
11. Cheng SY. Thyroid hormone receptor mutations in cancer. Mol Cel Endocrinol 2003;213:23-30.
12. Schlumberger M, Pacini F. Thyroid Tumors. Paris: Éditions Nucléon; 2003.
13. Hedinger C, Williams ED, Sobin LH. Histological typing of thyroid tumours. International histological classification of tumors. World Health Organization, vol.11, Second Edition, Springer-Verlag, Berlin, 1988.
14. Tanaka K, Otsuki T, Sonoo H, Yamamoto Y, Udagawa K, Kunisue H, et al. Semi-quantitative comparison of the differentiation markers and sodium iodide symporter messenger ribonucleic acids in papillary thyroid carcinomas using RT-PCR. Eur J Endocrinol 2000;142:340-6.
15. Arturi F, Russo D, Bidart JM, Scarpelli D, Schlumberger M, Filetti S. Expression pattern of the pendrin and sodium/iodide symporter genes in human thyroid carcinoma cell lines and human thyroid tumors. Eur J Endocrinol 2001;145:129-135.
16. Gerard AC, Daumerie C, Mestdagh C, Gohy S, De Burbure C, Costagliola S, et al. Correlation between the loss of thyroglobulin iodination and the

expression of thyroid-specific proteins involved in iodine metabolism in thyroid carcinomas. J Clin Endocrinol Metab 2003;88:4977-83.

17. Savin S, Cvejic D, Isic T, Petrovic I, Paunovic I, Tatic S, Havelka M. Thyroid peroxidase immunohistochemistry in differential diagnosis of thyroid tumors. Endocr Pathol 2006;17:53-60.
18. Spitzweg C, Morris JC. The sodium iodide symporter: its pathophysiological and therapeutic implications. Clin Endocrinol 2002;57:559-74.
19. Mirebeau-Prunier D, Guyetant S, Rodien P, Franc B, Baris O, Rohmer V, et al. Decreased expression of thyrotropin receptor gene suggests a high-risk subgroup for oncocytic adenoma. Eur J Endocrinol 2004;150:269-76.
20. Khörle J, Oertel M, Hoang-Vu C, Schnieders F, Brabant G. Type I 5'-deiodinase- a marker for differentiated thyroid carcinoma? Exp Clin Endocrinol 1993;101:60-72.
21. Khörle J. Thyroid carcinoma: interrelationships between local thyroid metabolism by the type I 5'- deiodinase and the expression of thyroid hormone receptors and other thyroid specific (de-)differentiation markers. Curr Top Pathol 1997;91:83-116.
22. Huang Y, Prasad M, Lemon WJ, Hampel H, Wright FA, Kornacker K, et al. Gene expression in papillary thyroid carcinoma reveals highly consistent profiles. Proc Natl Acad Sci USA 2001; 98:15004-049.
23. Murakami M, Araki O, Hosoi Y, Kamiya Y, Morimura T, Ogiwara T, et al. Expression and regulation of type II iodothyronine deiodinase in human thyroid gland. Endocrinology 2001;142: 2961-67.
24. Souza Meyer EL, Dora JM, Wagner MS, Maia AL. Decreased type 1 iodothyronine deiodinase expression might be an early and discrete event in

thyroid cell dedifferentiation towards papillary carcinoma. Clin Endocrinol 2005;62:672-78.

25. Arnaldi LAT, Borra RC, Maciel RMB, Cerutti JM. Gene expression profiles reveal that *DCN*, *DIO1*, and *DIO2* are underexpressed in benign and malignant thyroid tumors. Thyroid 2005;15:210-21.
26. Khörle J. Guard your master: thyroid hormones receptors protect their gland of origin from thyroid cancer. Endocrinology 2004;145:4427-29.
27. Parma J, Duprez L, Van Sande J, Hermans J, Rocmans J, Van Vliet G, et al. Diversity and prevalence of somatic mutations in the thyrotropin receptor and Gsα genes as a cause of toxic thyroid adenomas. J Clin Endocrinol Metab 1997; 82:2695-2701.
28. Paschke R, Krohn K. Clinical Review 133 - Progress in understanding the etiology of thyroid autonomy. J Clin Endocrinol Metab 2001; 86:3336-45.
29. Kimura ET, Nikiforova MN, Zhu Z, Knauf JA, Nikiforov YE, Fagin JA. High prevalence of BRAF mutations in thyroid cancer: genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma. Cancer Res 2003; 63:1454-7.
30. Fagin JA. Challenging dogma in thyroid cancer molecular genetic-role of *RET/PTC* and *BRAF* in tumor initiation. J Clin Endocrinol Metab 2004;89:4264-6.
31. Santoro M, Melillo M, Carlomagno F, Vecchio G, Fusco A. Minireview: *RET*: normal and abnormal functions. Endocrinology 2004;145:5448-51.
32. Cheung L, Messina M, Gill A, Clarkson A, Learoyd D, Delbridge L, et al. Detection of the PAX8-PPARg fusion oncogene in both follicular thyroid carcinomas and adenomas. J Clin Endocrinol Metab 2003;88:354-7.

33. Nikiforova MN, Lynch RA, Biddinger PW, Alexander EA, Dorn GW, Tallini G, et al. RAS point mutations and PAX8-PPARg rearrangement in thyroid tumors: evidence for distinct molecular pathways in thyroid follicular carcinoma. J Clin Endocrinol Metab 2003;88:2318-26.
34. Suarez H G. Genetic alterations in human epithelial tumors. Clin Endocrinol 1998; 48: 531-46.
35. Maciel RMB, Kimura ET, Cerutti JM. Patogênese dos tumores diferenciados da tireóide. Arq Bras Endocrinol Metab 2005;49:691-700.
36. Silva JE. Thyroid hormone control of thermogenesis and energy balance. Thyroid 1995;5:481-92.
37. St. Germain DL. Development effects of thyroid hormone: the role of deiodinases in regulatory control. Biochem Society Transactions 1999;27:83-8.
38. Wu, Y, Koening RJ. Gene regulation by thyroid hormone. Trends Endocrinol Metab 2000; 11:207-11.
39. St. Germain DL, Galton VA. The deiodinase family of selenoproteins. Thyroid 1997;7:655-68.
40. Kuiper GG, Kester MH, Peeters RP, Visser TJ. Biochemical mechanisms of thyroid hormone deiodination. Thyroid 2005; 15:787-98.
41. Salvatore D, Tu H, Harney JW, Larsen PR. Type 2 iodothyronine deiodinase is highly expressed in human thyroid. J Clin Invest 1996; 98:962-68.
42. Ishii H, Inada M, Tanaka K, Mashio Y, Naito K, Nishikawa M, Imura H. Triiodothyronine generation from thyroxine in human thyroid: enhanced conversion in Graves' thyroid tissue. J Clin Endocrinol Metab 1981; 52:1211-7.

43. Köhrle J. Local activation and inactivation of thyroid hormones: the deiodinase family. Mol Cell Endocrinol 1999;151:103-19.

44. Maia AL, Kim BW, Huang SA, Harney JW, Larsen PR. Type 2 iodothyronine Deiodinase is the major source of plasma T3 in euthyroid humans. J Clin Invest 2005;115:2524-33.

45. Visser TJ. Role of sulfation in thyroid hormone metabolism. Chem Biol Interact 1994; 92:293-303.

46. Maia AL, Kieffer JD, Harney JW, Larsen PR. Effect of 3,5,3' - Triiodothyronine (T3) administration on *dio1* gene expression and T_3 metabolism in normal and type 1 deiodinase-deficient mice. Endocrinology 1995;136:4842-9.

47. Jakobs TC, Schmutzler C, Meissner J, Kohrle J. The promoter of the human type I 5'-deiodinase gene-mapping of the transcription start site and identification of a DR+4 thyroid hormone-responsive element. Eur J Biochem 1997;247:88-97.

48. Zhang C, Kim S, Harney JW, Larsen PR. Further characterization of thyroid hormone response elements in the human type 1 iodothyronine deiodinase gene. Endocrinology 1998;247:288-97

49. Ishii H, Inada M, Tanaka K, Mashio Y, Naito K, Nishikawa M, et al. Induction of outer and inner ring monodeiodinases in human thyroid gland by thyrotropin. J Clin Endocrinol Metab 1983;57:500-5.

50. Toyoda N, Nishikawa M, Mori Y, Yoshimura M, Masaki H, Gondou A, et al. Identification of a 27-kilodalton protein with the properties of type I iodothyronine 5'-deiodinase in human thyroid gland. J Clin Endocrinol Metab 1992;74:533-8.

51. Schrek R, Schnieders F, Schmutzler C, Khörle J. Retinoids stimulate type I iodothyronine 5'-deiodinase activity in human follicular thyroid carcinoma cell lines. J Clin Endocrinol Metab 1994;79:791-8
52. Fagin JA: Molecular pathogenesis of tumors of thyroid follicular cells. In: Fagin JA (ed). Thyroid Cancer. Boston, Kluwer Academic Publishers, 1998; 59-83.
53. Pachucki J, Ambroziak M, Tanski J, Luczak J, Nauman J, Nauman A. Type I 5'-iodothyronine deiodinase activity and mRNA are remarkably reduced in renal clear cell carcinoma. J Endocrinol Invest 2001;24:253-61.
54. Sabatino L, Iervasi G, Ferrazzi P, Francesconi D, Chopra IJ. A study of iodothyronine 5'-monodeiodinase activities in normal and pathological tissues in man and their comparison with activities in rat tissues. Life Sci 2000;68:191-202.
55. Wawrzynska L, Sakowicz A, Rudzinski P, Langfort R, Kurzyna M. The conversion of thyroxine to triiodothyronine in the lung: comparison of activity of type I iodothyronine 5' deiodinase in lung cancer with peripheral lung tissues. Monaldi Arch Chest Dis 2003;59:140-5.
56. Dutkiewicz S, Witeska A, Nauman A. the deiodination of thyroxine to triiodothyronine in the testes of patients with prostate cancer. Int Urol Nephrol 1995;27:81-5.
57. Macejova D, Liska J, Brtko J. Mamary gland carcinoma-related increase of type I iodothyronine 5'-deiodinase activity in Sprague-Dawley rats. Gen Physiol Biophys 2001;20:293-302.
58. Aceves C, Gopainathrao G, Rajkumar L, Guzman R, Yang J, Nandi S, et al. Deiodinase type I (D1) in N-methyl-N-nitrosourea- induced rat mammary carcinomas: differential expression in early and late arising tumors. The

Endocrine Society 84th Annual Meeting, San Francisco, CA, 2002, p.299 (Abstract).

59. Aceves C, Rojas-Huidobro R. Effect of suckling and adrenergic stimulation on peripheral deiodination in lactating rats: differential expression of type 1 deiodinases mRNA forms. J Endocrinol 2001;171:533-40.
60. García-Solís P, Aceves C. 5'deiodinase in two breast cancer cell lines: effect of triiodothyronine, isoproterenol and retinoids. Mol Cell Endocrinol 2003;201:25-31.
61. Campos-Barros A, Hoell T, Musa A, Sampaolo S, Stoltenburg G, Pinna G, et al. Phenolic and tyrosil ring iodothyronine deiodination and thyroid hormone concentrations in the human central nervous system. J Clin Endocrinol Metab 1996;81:2179-85.
62. Tannahill LA, Visser TJ, McCabe CJ, Kachilele S, Boelaert K, Sheppard Mc, et al. Dysregulation of iodothyronine deiodinase enzyme expression and function in human pituitary tumours. Clin Endocrinol 2002;56:735-43.
63. Baur A, Buchfelder M, Kohrle J. Expression of 5'-deiodinase enzymes in normal pituitaries and in various human pituitary adenomas. Eur J Endocrinol 2002;147:263-8.
64. Silva JE, Larsen PR. Comparison of iodothyronine 5'-deiodinase and other thyroid-hormone-dependent enzyme activities in the cerebral cortex of hypothyroid neonatal rat. Evidence for adaptation to hypothyroidism. J Clin Invest 1982,70; 1110-23.
65. Burmeister LA, Pachucki J, St Germain DL. Thyroid hormones inhibit type 2 iodothyronine deiodinase in the rat cerebral cortex by both pre-and posttranslational mechanisms. Endocrinology 1997;138:5231-7.

66. Gereben B, Salvatore D. Pretranslational regulation of type 2 deiodinase. Thyroid 2005;15:855-64.

67. Kim BW, Daniels GH, Harrison BJ, Price A, Harney JW, Larsen PR, Weetman AP. Overexpression of type 2 iodothyronine deiodinase in follicular carcinoma as cause of low circulating free thyroxine levels. J Clin Endocrinol Metab 2003;88:594-8.

68. Takano T, Miyauchi A, Ito Y, Amino N. Thyroxine to triiodothyronine hyperconversion thyrotoxicosis in patients with large metastases of follicular thyroid carcinoma. Thyroid 2006;16:615-8.

69. Magrassi L, Butti G, Silini E, Bono F, Paoletti P, Milanesi G. The expression of genes of the steroid-thyroid hormone receptor superfamily in central nervous system tumors. Anticancer Res 1993;13:859-66.

70. Toms SA, Hercbergs A, Liu J, Kondo S, Barnett GH, Casey G, Barna BP. Thyroid hormone depletion inhibits astrocytoma proliferation via a p53-independent induction of p21 (WAF/1CIP1). Anticancer Res 1998;18:289-94.

71. Murakami M, Araki O, Morimura T, Hosoi Y, Mizuma H, Yamada M, et al. Expression of type II iodothyronine deiodinase in brain tumors. J Clin Endocrinol Metab 2000; 85:4403-6.

72. Nauman P, Bonicki W, Michalik R, Warzecha A, Czernicki Z. The concentration of thyroid hormones and activities of iodothyronine deiodinases are altered in human brain gliomas. Folia Neuropathol 2004;42:67-73.

73. Mori K, Yoshida K, Kayama T, Kaise N, Fukazama H, Kiso Y, et al. Thyroxine 5-deiodinase in human brain tumors. J Clin Endocrinol Metab 1993;77:1198-202.

74. Barrera-Hernandez G, Park KS, Dace A, Zhan Q, Cheng SY. Thyroid hormone-induced cell proliferation in GC cells is mediated by changes in G1 cyclin/cyclin-dependent kinase levels and activity. Endocrinology 1999;140:5267-74.

75. Stahl JH, Kendall SK, Brinkmeier ML, Greco TL, Watkins-Chow DE, Campos-Barros A, et al. Thyroid hormone is essencial for pituitary somatotropes and lactotropes. Endocrinology 1999;140:1884-92.

76. Canettieri G, Franchi A, Sibilla R, Guzman E, Centanni M. Functional characterization of the CRE/TATA box unit of type 2 deiodinase gene promoter in a human choriocarcinoma cell line. J Mol Endocrinol 2004;33:51-8.

77. Rihn B, Mohr S, McDowell SA, Binet S, Loubinox J, Galateau F,et al. Differential gene expression in mesothelioma. FEBS Lett 2000;480:95-100.

78. Curcio C, Baqui MM, Salvatore D, Rihn BH, Mohr S, Harney JW, et al. The human type 2 iodothyronine Deiodinase is a selenoprotein highly expressed in a mesothelioma cell line. J Biol Biochem 2001;276:30183-7.

79. Morimura T, Tsunekawa K, Kasahara T, Seki K, Ogiwara T, Mori M, Murakami M. Expression of type 2 iodothyronine deiodinase in human osteoblast is stimulated by thyrotropin. Endocrinology 2005;146:2077-84.

80. Huang SA, Dorfman DM, Genest DR, Salvatore D, Larsen PR. Type 3 iodothyronine deiodinase is highly expressed in the human uteroplacental unit and in fetal epithelium. J Clin Endocrinol 2003;88:1384-8.

81. Bates JM, St. Germain DL, Galton VA. Expression profiles of the three iodothyronine deiodinases, D1, D2, and D3, in developing rat. Endocrinology 1999;140:844-51.

82. Slominski A, Wortsman J, Khon L, Ain KB, Venkataraman GM, Pisarchik A, et al. Expression of hypothalamic-pituitary-thyroid axis related genes in the human skin. J Invest Dermatol 2002;119:1449-55.
83. Kester MHA, Kuiper GGJM, Versteeg R, Visser T. Regulation of type III iodothyronine deiodinase expression in human cell lines. Endocrinology 2006;147:5845-54.
84. Baur A, Khorle J. Type 1 deiodinase is stimulated by iodothyronines and involved in thyroid hormone metabolism in human somatomammotroph GX cells. Eur J Endocrinol 1999;140:367-70.
85. Huang, SA, Tu, H M, Harney, J W, Venihaki, M, Butte, A J, Kozakewich, H P W, Fishman, SJ, Larsen, PR. Severe hypothyroidism caused by type 3 iodothyronine deiodinase in infantile hemangiomas. New Eng J Med 2000;343:185-189.
86. Huang SA, Fish SA, Dorfman DM, Salvatore D, Kozakewich HP, Mandel SJ, Larsen PR. A 21-Year-Old Woman with consumptive hypothyroidism due to a vascular tumor expressing type 3 iodothyronine deiodinase. J Clin Endocrinol Metab 2002;87:4457-46.
87. Huang, A. Physiology and Pathophysiology of type 3 deiodinase in humans. Thyroid 2005;15:875-81.

Tabela 1: Principais características das iodotironinas desiodases

Características	D1	D2	D3
Localização do gene	Cr. 1p32-p33	Cr. 14q24.3	Cr. 14q32
Peso molecular (kDa)	27	30	31
Papel fisiológico	Fornece T3 para o plasma	Fornece T3 para o espaço intracelular	Inativa T3 e T4
Localização	Fígado, rim, tireóide	Hipófise, cérebro, tecido adiposo marrom, placenta, tireóide, músculos cardíaco e esquelético	Útero, placenta, tecidos fetais, pele, cérebro e hipófise
Preferência pelo substrato	rT3 >> T4 > T3	T4 > T3	T3 > T4
Sítio de desiodação	Anel interno e externo	Anel externo	Anel interno
Sensibilidade ao propiltiouracil (PTU)	Sensível	Resistente	Resistente
Efeito dos hormônios tireoidianos na atividade	Aumenta	Diminui	Aumenta

Tabela 2: Padrão de expressão das iodotironinas desiodases nas neoplasias humanas em geral

Tipo de tumor	D1		D2		D3		Refs.
	RNAm	**Atividade**	**RNAm**	**Atividade**	**RNAm**	**Atividade**	
Carcinoma papilar da tireóide	Diminuído	Diminuída	Diminuído	Diminuída	N/A	N/A	(23-25)
Carcinoma folicular da tireóide	Normal ou aumentado	Aumentada	Normal ou aumentado	Aumentada	N/A	N/A	(24,25)
Carcinoma renal de células claras	Diminuído ou indetectável	Diminuída ou indetectável	N/A	N/A	N/A	N/A	(53)
Adenocarcinoma de fígado	N/A	Diminuída	N/A	N/A	N/A	N/A	(54)
Adenocarcinoma e carcinoma de células escamosas de pulmão	N/A	Diminuída	N/A	Inalterada	N/A	N/A	(55)
Carcinoma de próstata	Diminuição da 5'-desiodação				N/A	N/A	(56)
Carcinoma de mama	N/A	Diminuída	N/A	N/A	N/A	N/A	(59)
Adenoma de hipófise	Diminuído	Diminuída*	Aumentado	Aumentada	Aumentado	Inalterada**	(62,63)
Mesotelioma	N/A	N/A	Aumentado	Aumentada	N/A	N/A	(78)
Osteossarcoma	N/A	N/A	Diminuído	Diminuída	N/A	N/A	(79)
Hemangioma	N/A	N/A	N/A	N/A	Aumentado	Aumentada	(85,86)

N/A: não avaliado

* detectável em 1 amostra de 16 adenomas não-funcionantes

** detectável em 3 amostras de 16 adenomas não-funcionantes

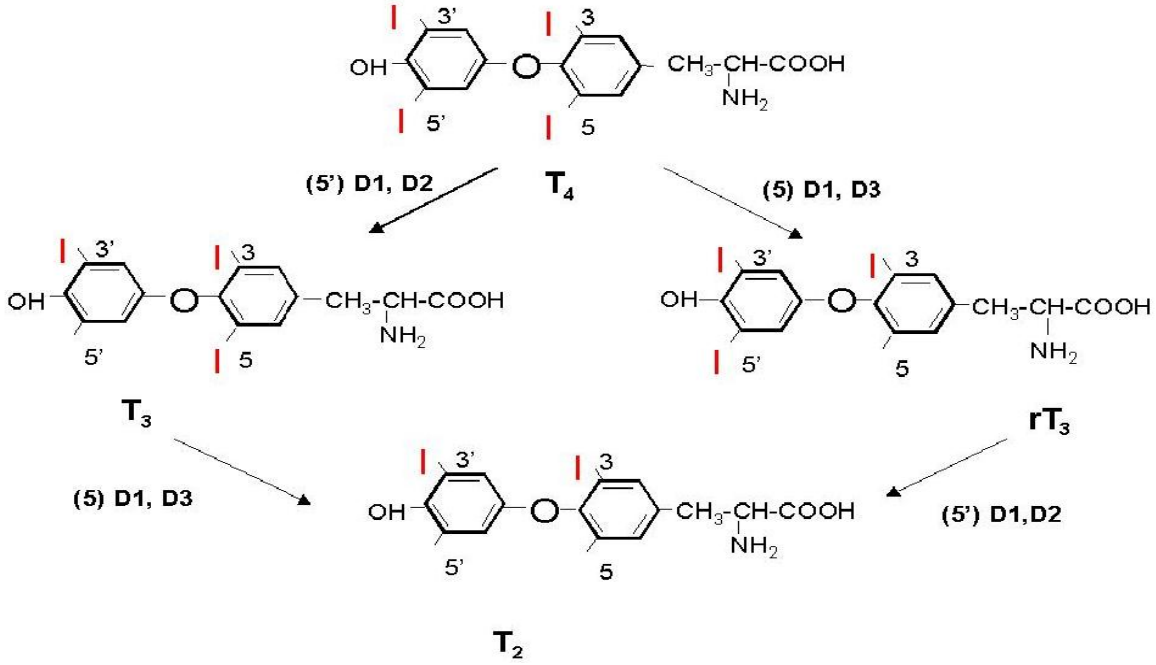


Figura 1

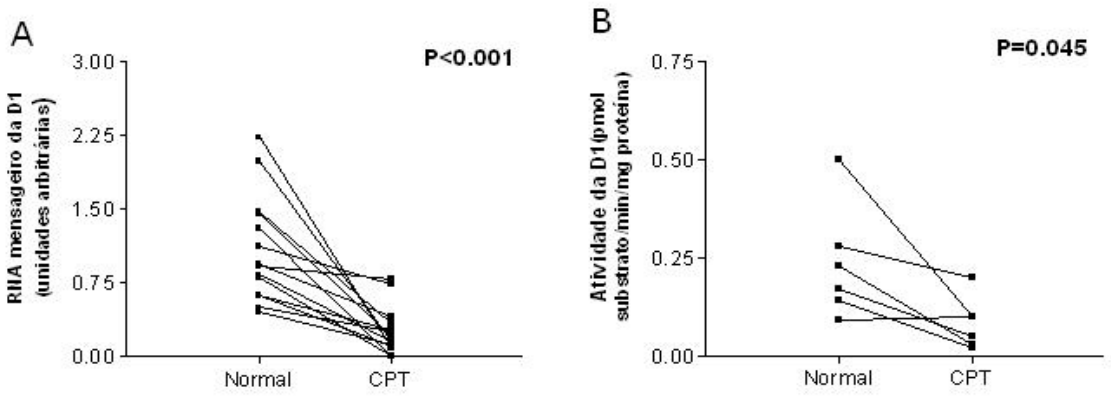

A
P<0.001
RNA mensageiro da D1 (unidades arbitrárias)
3.00
2.25
1.50
0.75
0.00
Normal
CPT
B
P=0.045
Atividade da D1(pmol substrato/min/mg proteína)
0.75
0.50
0.25
0.00
Normal
CPT

Figura 2

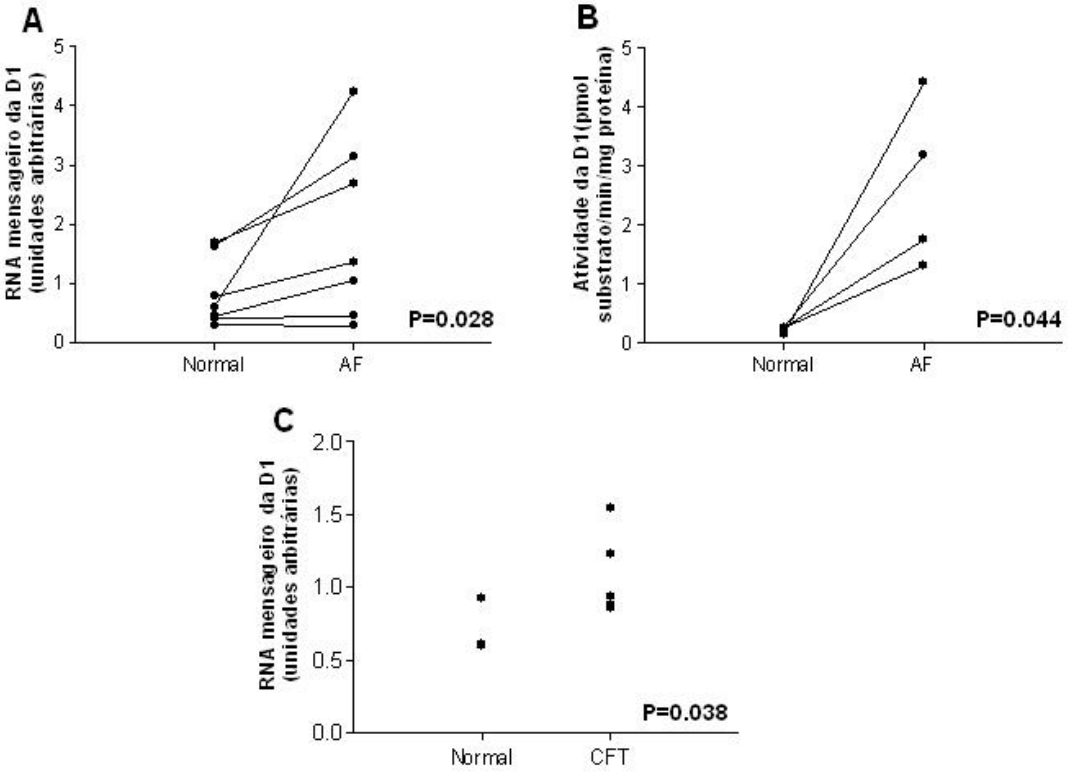

A
RNA mensageiro da D1 (unidades arbitrárias)
5
4
3
2
1
0
P=0.028
Normal
AF
B
Atividade da D1(pmol substrato/min/mg proteina)
5
4
3
2
1
0
P=0.044
Normal
AF
C
RNA mensageiro da D1 (unidades arbitrárias)
2.0
1.5
1.0
0.5
0.0
P=0.038
Normal
CFT

Figura 3

Legendas

Figura 1: Produtos da monodesiodação do T4.

Figura 2: (a) Níveis de RNA mensageiro da D1 em unidades arbitrárias no carcinoma papilar da tireóide (CPT) e tecido normal adjacente. (b) Atividade enzimática da D1 no CPT e tecido normal adjacente. A atividade da D1 foi realizada utilizando 1μM de T4 como substrato. Adaptada da ref.24.

Figura 3: (a) Níveis de RNA mensageiro da D1 em unidades arbitrárias no adenoma folicular da tireóide (AF) e tecido normal adjacente. (b) Atividade enzimática da D1 no AF e tecido normal adjacente. A atividade da D1 foi realizada utilizando 1μM de T4 como substrato. (c) Níveis de RNA mensageiro da D1 em unidades arbitrárias no carcinoma folicular da tireóide (CFT) e tecido normal. Adaptada da ref.24.

Parte II

Decreased type 1 iodothyronine deiodinase expression might be an early and discrete event in thyroid cell dedifferentiation towards papillary carcinoma

Artigo publicado no Clinical Endocrinology 2005; 62:672-78

Decreased type 1 iodothyronine deiodinase expression might be an early and discrete event in thyroid cell dedifferentiation towards papillary carcinoma

Erika L de Souza Meyer, M.D., José Miguel Dora, M.D. , Márcia s Wagner, PhD., and Ana Luiza Maia, M.D., PhD.

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

Running title: Iodothyronine deiodinase and papillary thyroid carcinoma

Grant support: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundo de Incentivo a Pesquisa do Hospital de Clínicas de Porto Alegre (FIPE), Brazil.

Correspondence: Ana Luiza Maia, M.D., Ph.D. Setor de Tireóide, Serviço de Endocrinologia, Hospital de Clínicas de Porto Alegre. Rua Ramiro Barcelos 2350, Porto Alegre, RS, Brazil, 90035-003. Phone: + 55 51 33310207; Fax: 55 51 3325188. E-mail: almaia@ufrgs.br

Summary

OBJECTIVE Type I iodothyronine deiodinase (D1) catalyzes the 5´monodeiodination of T_4 and is highly expressed in normal human thyroid gland. In the present study we investigated D1 expression in a series of benign and malignant differentiated thyroid neoplasias.

DESIGN Surgically isolated thyroid tumor fragments were used. The D1 expression was determined by RT-PCR and enzymatic assay.

PATIENTS Tumors and adjacent normal tissues were obtained from 28 unselected patients (papillary carcinoma, n = 14; follicular adenoma, n = 7; follicular carcinoma, n = 6; anaplastic carcinoma, n = 1).

MEASUREMENTS D1 mRNA levels were determined using specific primers for the human D1 gene and enzymatic assays were performed using T_4 as substrate.

RESULTS In papillary thyroid carcinoma, D1 mRNA and activity levels were decreased in tumor as compared with surrounding tissue (0.25 $\pm$ 0.24 *vs.* 1.09 $\pm$ 0.54 arbitrary units (AU), P<0.001 and 0.08 $\pm$ 0.07 *vs.* 0.24 $\pm$ 0.15 pmol T_4/min/mg protein, P=0.045, respectively). Decreased D1 expression was consistent and was observed in all histological subtypes and clinical stages analyzed, including microcarcinomas. In contrast, significantly higher D1 mRNA levels and enzyme activity were present in follicular adenoma (1.9 $\pm$ 1.5 *vs.* 0.83 $\pm$ 0.58 AU, P= 0.028 and 2.67 $\pm$ 1.42 *vs.* 0.22 $\pm$ 0.06 pmol T_4/min/mg protein, P= 0.044, respectively) and in follicular thyroid carcinoma (FTC) than in surrounding normal tissue (1.2 $\pm$ 0.46 *vs.* 0.67 $\pm$ 0.18 AU, P= 0.038 and 1.20 $\pm$ 0.58 *vs*. 0.20 $\pm$ 0.10 pmol T_4/min/mg protein, P<0.001, respectively).

Type II iodothyronine deiodinase (D2) activity was also significantly higher in metastatic FTC samples than in normal thyroid tissues (5.20 ± 0.81 *vs.* 0.30 ± 0.27 fmol T_4/min/mg protein, P< 0.001).

CONCLUSIONS These findings suggest that thyroid cell dedifferentiation promotes changes in D1 gene expression by pretranscriptional mechanisms and indicate that decreased D1 expression might be an early and discrete event in thyroid cell dedifferentiation towards papillary thyroid carcinoma.

Introduction

Type I (D1) and type II (D2) iodothyronine deiodinases catalyze 5´-monodeiodination of T_4, an essential step in thyroid hormone activation. D1 supplies a fraction of plasma T_3 and it is expressed mainly in liver, kidney, thyroid, and euthyroid anterior pituitary. D2 expression appears to be more widespread, with D2 transcripts or activity found in thyroid gland, brain, spinal cord, placenta and cardiac and skeletal muscles (Bianco *et al*., 2002). D1 and D2 are both expressed in normal and stimulated human thyroid gland. D1 transcripts seem to be constitutively expressed in human thyroid whereas D2 activity varies more closely with the degree of tissue stimulation (Salvatore *et al*., 1996). These two isoenzymes present rather different biochemical and regulatory characteristics, exhibiting different tissue distribution and developmental patterns of expression, and respond differently to thyroid status and inhibitors (Bianco *et al*., 2002).

Of the major forms of differentiated cancer derived from thyroid follicular cells, papillary thyroid carcinomas (PTC) are by far the most common. In contrast, follicular thyroid carcinomas (FTC) are now quite rare (Mazzaferri & Kloos, 2001). The genetics events towards PTC formation appear to be different from those of FTC (Fagin, 2002). RET/PTC rearrangements may occur as initiating events in papillary carcinoma, although it appears that RET/PTC itself is not sufficient to evoke malignant transformation (Mizuno et al., 1997; Fagin, 2004). Recent studies suggest that a somatic mutation of BRAF is the most common genetic change in PTC (Kimura et al., 2003; Namba et al., 2003) and may be an alternative tumor-initiating event in PTC (Fagin, 2004). These tumors generally retain many of the biochemical features of

normal epithelial thyroid cells, still various degrees of abnormalities have been demonstrated in the expression of major proteins involved in the complex machinery of thyroid hormone synthesis (Brabant *et al*., 1991; Otha *et al*., 1991; Arturi *et al*., 1998; Lazar *et al*., 1999). Interestingly, a recent study using microarray analysis has shown that the D1 and D2 genes are down regulated in PTC (Huang *et al*., 2001).

Few studies have focused on 5' deiodinase activity in differentiated thyroid carcinomas. The accumulated data show conflicting results: Köhrle *et al.* (1993) reported lower to elevated D1 levels in 10 samples of PTC whereas Toyoda *et al.* (1992), using N-Bromoacetyl-$^{[125I]}T_4$ as affinity label to identify D1 protein, found lower D1 levels in four out 5 cases of PTC. None of those studies used paired normal thyroid samples as controls. D1 expression has not been systematically evaluated in samples of FTC.

The present studies were performed to analyze the D1 gene expression in a series of papillary and follicular thyroid carcinomas. We observed a consistent decrease on D1 mRNA paralleled by a decreased on D1 activity in PTC samples. In contrast, increased D1 mRNA and activity levels were observed in both follicular lesions adenomas and carcinomas.

Materials and Methods

Patients

Samples of thyroid tumors (papillary carcinoma, n = 14; follicular adenoma, n = 7; follicular carcinoma, n = 6; anaplastic carcinoma, n = 1) were collected from 28 consecutive unselected patients attending the Endocrine or the Head and Neck

Surgery Divisions at Hospital de Clínicas de Porto Alegre. Surgery was indicated independently by attending physicians based on clinical indications and tissues obtained at surgery were immediately frozen and stored until analysis. Tumors were histologically classified according to WHO recommendations (Hedinger, Williams & Sobin, 1998). Clinical data were retrospectively reviewed in medical records. The clinical stage was determined by the TNM system (Beahrs *et al*., 1992). Serum TSH was measured by a double antibody–sensitive assay (Immulite, Diagnostic Products), serum T_4 by competitive immunoassay (Immulite, Diagnostic Products), and serum thyroglobulin was measured by an electrochemiluminescence immunoassay (Elecsys Systems, Roche). The information obtained from the study did not influence or affect the patients' diagnosis or treatment. The Ethics Committee at the Hospital approved the study protocol, and all patients gave informed consent.

Tissue preparation and RNA isolation

Thyroid tissues were obtained from both tumor and adjacent normal tissue at the time of surgery. Each tissue was immediately frozen in liquid nitrogen and stored at – 70° C until preparation. Total RNA was isolated from 50 - 100 mg of thyroid tumor and surrounding nontumor tissues using TRIzol® reagent (Invitrogen™ Life Technologies Inc., NY, USA) according to the manufacturer's instructions. The purity of the total RNA was assessed by UV spectrophotometry (GeneQuant II®, Amersham Pharmacia Biotech).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

A semiquantitative RT-PCR technique was used to determine the expression levels of D1, D2, Sodium iodide symporter (NIS), and TSH receptor (TSH-R) genes in RNA samples isolated from thyroid tissues. RT-PCR was performed using the Superscript Preamplification System for First Strand cDNA Synthesis (Invitrogen™ Life Technologies Inc., NY, USA) with 3µg of total RNA as template. Specific oligonucleotides derived from the coding region of published sequences of human D1 (5'-AAGAGGCTCTGGGTGCTCTTGG-3' and 5'-GGTTCTGGTGATTTCTGATGTC-3'), D2 (5'-ACTCGGTCATTCTGCTCAAG-3' and 5'-GAGAACTCTTCCACCAGTTTTG-3'), NIS (5'-GCTCTGAACTCGGTCCTCAC-3' and 5'-GTCCAGAATGTATAGCGGCTC-3') and TSH-R (5'-GTCCAGAATGTATAGCGGCTC-3' and 5'-GCTTTTCAGGGACTATGCAATGAA-3') genes were used to prime target cDNA, resulting in a predicted 498, 368, 481 and 239 bp products, respectively. A human β_2-microglobulin primer set (5'-ATCCAGCGTACTCCAAAGATTCAG-3' and 5'-AAATTGAAAGTTAACTTATGCACGC-3') that generated a 623 bp product was used as an internal control. β_2-microglobulin was co-amplified within the same reaction in order to evaluate inter-sample variation in cDNA contents and PCR efficiency. The PCR reactions included 0,5 - 1 µl of RT products, and were carried out with Taq DNA polymerase (Invitrogen™ Life Technologies Inc., NY, USA) in a final 50 µl volume. The amplification profile was an initial denaturation step at 94°C for 3 min, followed by 94°C for 1 min, annealing at 58°C (D1, NIS) or 60°C (D2, TSH-R), and extension at 72°C for 2 min. Twenty-five amplification cycles were used for D1, 30 cycles for D2, 32 for NIS and 28 for TSH-R, with a final additional extension step at 72°C for 4 min. The β_2-microglobulin primers were included after the 5th cycle for D1, 10th cycle for D2, 12th for hNIS and 8th cycle and TSH-R. RT-PCR reactions without cDNA samples

were carried out as negative controls. All reactions were performed in duplicate. After amplification, 10μl of the PCR products were analyzed on an 1.5% ethidium bromide agarose gel and the intensity of each band was determined by optic densitometry (arbitrary units) (ImageMaster® VDS, Amersham Pharmacia Biotech). Each gene band intensity was normalized against the corresponding values of the β_2-microglobulin band intensity.

Deiodinase assays

Deiodinase assays were performed as previously described (Wagner *et al*., 2003). Briefly, tissue samples obtained from normal and tumoral thyroid tissues were homogenized on ice in PE buffer (0.1M potassium phosphate and 1mM EDTA) containing 10 mM dithiothreitol (DTT) and 0.25 M sucrose (pH: 6.9). D1 assays were performed using 100-300 μg tissue protein and 1μM unlabeled T_4 in a total volume of 300μl PE buffer containing 10mM DTT, and approximately 100.000 c.p.m [^{125}I]T_4 (Amershan, Biosciences). D2 assays were performed under the same conditions using 20mM DTT, 1nM unlabeled T_4 and 1mM propylthiouracil (PTU). The apparent K_m and V_{max} for D1 and D2 enzymes were determined using various amounts of unlabeled T_4 (0.25, 0.4, 0.5, 1, and 5μM and 0.25, 0.4, 1, and 5nM, respectively). Incubations were carried out at 37°C for 60-120 minutes. The reaction was terminated by adding 200μl of horse serum and 100μl 50% trichloroacetic acid. Samples were analyzed in duplicate.

Statistical analysis

Results are expressed as mean ± SD or otherwise specified. For comparisons of D1 mRNA and activity levels in tumoral and corresponding surrounding tissue, a

paired Student's t-test or Wilcoxon signed ranks test was applied. Correlation between variables was assessed by linear regression analysis. $P \leq 0.05$ were considered statistically significant.

Results

Patients

Table 1 shows the clinical and pathological characteristics of all patients with thyroid tumors included in this study. Thyroid samples covered patients age 10 to 70 years and, different histological subtypes and clinical stages, including microcarcinomas. D1 activity was measured in six PTC samples (cases 7, 8, 11, 12, 13 and 14), four adenoma samples (cases 17, 18, 20 and 21), and three FTC (cases 25, 26 and 27). D2 expression was also analyzed in samples of follicular and anaplastic thyroid carcinomas. Patients 25 and 26 had metastatic spread of follicular thyroid carcinoma.

D1 expression in thyroid tumors

Figures 1A-B shows levels of D1 expression in samples from patients with PTC. The D1 mRNA levels were significantly decreased in tumor tissue as compared with corresponding normal surrounding tissue (0.25 ± 0.24 *vs.* 1.09 ± 0.54 AU, $P< 0.001$). Lower D1 mRNA levels were present in all samples. No D1 expression was found in 2 cases. The lower level of D1 mRNA was paralleled by a decrease on D1 activity (0.08 ± 0.07 *vs.* 0.24 ± 0.15 pmol T_4/min/mg protein, $P= 0.045$) (Figure 1C). The only exception for that was found in a follicular variant of PTC case. In this sample, the D1 activity levels were similar in tumor and adjacent tissue (0.1 vs. 0.09 pmols/min/mg

protein; Figure 1C) despite a slight decrease in mRNA levels (0.73 vs. 0.96 AU; Figure 1B). Hence, a strong correlation between D1 mRNA and activity was observed (R^2=0.72, P=0.008).

In contrast, we observed a significant increase in D1 mRNA levels in FTC as compared with levels in nontumoral tissue (1.2 ± 0.46 *vs.* 0.67 ± 0.18, P=0.038; Figure 2A). Accordingly, D1 activity was significantly higher in samples of metastases of follicular carcinoma (1.20 ± 0.58 *vs*. 0.20 ± 0.10; P< 0.001). Further studies performed to determine the D1 kinetics in the neoplastic tissue demonstrated that the apparent K_m (T_4) in follicular carcinoma samples were similar to that found in normal thyroid tissue (2.9 *vs*. 2.1 μM, respectively). Nevertheless, D1 V_{max} was markedly higher in follicular carcinoma samples than in normal tissue (2.38 *vs.* 0.40 pmol T_4/min/mg protein, respectively), indicating that differences in the enzyme concentration, rather than in catalytic capacity, account for the observed higher D1 activities in tumor homogenates. Of particular interest was the finding that D1 V_{max} was about six-fold higher in FTC metastasis than in normal thyroid tissue (3.43 *vs*. 0.46 pmol T_4/min/mg protein; Figure 2B).

D1 expression was also measured in samples of benign follicular lesions. We observed increased D1 mRNA levels (0.83 ± 0.58 *vs.* 1.9 ± 1.5 AU, P=0.028) paralleled by an increase in D1 activity (0.22 ± 0.06 *vs.* 2.67 ± 1.42 pmol T_4/min/mg protein, P= 0.044) in follicular adenomas (Figure 2C-D).

The D2 expression was also analyzed in FTC. The D2 mRNA levels in FTC samples were similar to those in surrounding normal tissue (1.24 ± 0.86 *vs.* 1.49 ± 0.87 AU, P=0.86). However, the D2 activity was significantly higher in tumor samples than in normal thyroid tissues (5.20 ± 0.81 *vs.* 0.30 ± 0.27 fmol/min/mg protein,

P<0.001). Interesting, D2 activity was also higher in a sample of a follicular variant of PTC than in adjacent normal tissue (0.53 vs. 0.15 fmols/min/mg protein). The D2 kinetics in FTC was determined by double-reciprocal plot. As expected, the apparent K_m (T_4) was similar to that found in normal thyroid tissue (~6nM) whereas the D2 V_{max} was about 25-fold higher in FTC samples than in normal thyroid tissue (25.5 *vs*.1.0 fmol T_4/min/mg protein, respectively).

D1 and D2 activities were also measured in one sample of anaplastic thyroid carcinoma. Surprisingly both D1 and D2 activities were significantly higher in tumor homogenates than in corresponding normal tissue (5.25 *vs.* 0.14 pmol T_4/min/mg protein and 7.64 *vs.* 0.33 fmol T_4/min/mg protein, respectively). The samples were obtained from a 53-year-old man who had undergone surgery for a recurrent neck mass with extension into the mediastinum. Thyroid function tests showed a TSH of 13.7 mUI/L (reference range, 0.4-4.0 mU/L) and T_4 of 2.74 μg/dL (reference range, 4.5-12.5 μg/dL). A high serum thyroglobulin of 1061 ng/mL (reference range, 1.4-78 ng/mL) was present. The weight of the tissue removed weighed an impressive 1050g. Histological examination demonstrated a multinodular thyroid with areas of normal thyroid tissue, Hürthle cell carcinoma and anaplastic carcinoma. There was invasion of blood vessels, local soft tissues and regional lymph nodes. Immunohistochemical analysis revealed thyroglobulin positivity only in differentiated areas. The patient died 3 months after surgery.

NIS and TSH-R gene expression in PTCs

Expression of the NIS and TSH-R genes were analyzed in 12 PTC samples and corresponding normal thyroid tissues. Figure 3A shows levels of NIS expression in samples of PTC. The NIS mRNA levels were significantly decreased in tumor than

normal surrounding tissue (0.20 ± 0.18 *vs*. 1.41 ± 0.91 AU, P=0.001). Lower NIS mRNA levels were present in all samples analysed. No NIS expression was found in 4 cases. In contrast, the levels of TSH-R mRNA in PTC samples were similar to those in surrounding normal tissue (0.79 ± 0.58 *vs*. 1.21 ± 1.18 AU, P=0.102; Figure 3B) and TSH-R transcripts were detected in all samples analysed. In the follicular variant of PTC sample, we observed a decrease in NIS mRNA levels (0.47 vs. 1.5 AU, Figure 3A) whereas no differences were observed in TSH-R mRNA levels (0.35 vs. 032 AU, Figure 3B).

Discussion

In the present study, the D1 expression was determined in tumor thyroid samples by semi-quantitative RT-PCR and enzymatic assay. The results show that the D1 mRNA and enzyme activity levels are significantly decreased in PTC as compared with corresponding surrounding nontumor tissue. Although PTC is heterogeneous, the D1 expression pattern was consistent, and expression was lower in all samples analyzed, including microcarcinomas. In contrast, D1 overexpression was found in adenoma and follicular carcinoma.

Changes in the level of expression of genes are common in many cancers and may result from mutations in transcriptional factors, loss of cell differentiation, or unregulated production due to disrupted control mechanisms (Latchman, 1996; Ivan et al., 1997). Here we demonstrated that the reduced levels of D1 mRNA are paralleled by a decrease in enzyme activity in a series of PTC samples that included different histological subtypes and tumor stages. Lower levels of D1 expression were detected even in small papillary cancer less than 1 cm in diameter. Although the reasons for the

decreased D1 remain speculative, it is striking that D1 underexpression has been described in virtually all tumors derived from parenchymal or epithelial tissues (Pachucki et al., 2001; Garcia-Solis et al., 2003; Wawrzynska et al., 2003).

The rate of D1 synthesis can be influenced by a number of substances or conditions, the most potent being thyroid hormones and TSH (Salvatore et al, 1996; Bianco et al, 2002). Thyroid hormones regulate the D1 gene via transcriptional mechanisms that require the interaction of ligand with the thyroid hormone receptor (TR) (Toyoda et al, 1995; Zhang et al., 1998). Interestingly, aberrant expression of TRs has been reported in PTC as well as in other human cancers (Lin et al., 1999; McCabe et al., 1999; Kamiya et al., 2002; Cheng, 2003). In addition, sequencing analysis demonstrates a high frequency of TR mutations in PTCs (Puzianowska-Kuznicka et al., 2002). Changes in TRs can led to an impairment or loss of T3 binding and, thus, could explain the underexpression of the D1 gene. Indeed, this hypothesis has been considered to explain the decreased levels of NIS expression in PTC (Puzianowska-Kuznicka et al., 2002), showed here and other studies (Arturi et al, 1998; Lazar et al, 1999; Park et al, 2000). The NIS gene also contains a thyroid response element (TRE) within the flanking region and it is up regulated by thyroid hormones (Ohmori et al, 1998).

The expression pattern of D1 and NIS genes contrasts with that described for other genes in PTC, such as the TSH-R gene, which shows significantly lower expression in advanced stages but not in early stages of disease (Tanaka et al., 1996; Tanaka et al., 1997). In agreement with previous studies (Brabant et al, 1991; Park et al, 2000), we observed a heterogeneous pattern of expression of the TSH-R gene in PTC. It is noteworthy that TSH-R mRNA levels were detected in all samples analyzed.

Two inferences arise from these observations. First, distinct mechanisms may be responsible for down regulation of specific thyroid related genes in PTC. Also, it is unlikely that decrease TSH-R levels could explain the lower D1m RNA levels in PTC.

Increased D1 and D2 mRNA expression have been described in hyper functioning adenoma (Salvatore et al., 1996; Murakami et al., 2001; Brtko et al., 2002). The explanation of this finding is that both genes are up-regulated by the cAMP regulatory cascade, which is constitutively activated in hyperfunctioning thyroid adenoma, which is due primarily to an activating mutation in the TSH receptor gene or Gsα gene (Bianco et al, 2002; Vassart, 1997). Here we found increased D1 expression in all samples of adenoma and metastases of follicular carcinoma that we analyzed, indicating other mechanisms for induction of D1 gene. Although a possible explanation is that the higher serum TSH levels up regulate the D1 gene, this does not explain the higher D1 expression in samples from patients with normal serum TSH levels. Recently, Kim et al. (2003) reported D2 overexpression in metastases of FTC suggesting that this overexpression could be responsible for the persistently increased serum ratio of T3 to T4 observed in these patients. Our results further demonstrated that higher D1 levels might also contribute to increase peripheral T4 conversion.

Higher D2 activity was found in samples of metastasis of FTC, in agreement with a previous report (Kim et al., 2003). But, unlike the D1 gene, no significant changes in D2 mRNA levels were observed. Although D2 regulation is a complex process involving both transcriptional and posttranscriptional mechanisms (Burmeister et al., 1997; Bianco et al, 2002), thyroid hormones are considered to be the most important regulators of D2 activity, which is rapidly and markedly reduced by exposure to T4 (Steinsapir et al., 2000). In one of the two cases analyzed, the patient had

elevated TSH levels, implying hypothyroid state with low serum T4 levels. Both high serum TSH and low T4 levels up regulate D2 activity, but since no changes were observed in the D2 mRNA levels, the increase in the D2 activity might occur primarily at post-transcriptional level.

An interesting observation of this study came out from the analysis of a follicular variant of PTC sample. In this tumor, the level of D1 activity was similar to surrounding normal tissue, contrasting with all other PTC samples. Furthermore, contrary to previous studies that showed decreased levels of D2 expression in PTC (Murakami et al., 2001), we found higher levels of D2 activity in tumor than in adjacent normal tissue. Taken together, these findings suggest preponderance of the follicular component and may possibly illustrate the distinct D1 and D2 expression patterns in PTC and follicular neoplasias.

High levels of D1 and D2 activity were also found in samples of a mixed anaplastic and Hürthle cell carcinoma. Previous studies have demonstrated low or undetectable D1 activity in anaplastic carcinoma samples (Köhrle et al., 1993; Schreck et al., 1994). Undifferentiated thyroid carcinomas are the end stage forms of thyroid cancer and are believed to arise from papillary or follicular carcinomas (Fagin, 2004). Based on our findings, it is tempting to speculate a follicular origin in this particular case.

In conclusion, we demonstrated marked differences in the D1 gene expression between follicular lesions, either adenoma or carcinoma, and PTC. These findings are in agreement with the current knowledge on the molecular events involved in epithelial thyroid cell dedifferentiation, which suggests different gene expression pathways towards papillary or follicular neoplasias. Furthermore, the results suggest that

decreased D1 expression might be an early and discrete event in thyroid cells towards papillary carcinoma dedifferentiation.

Acknowledgments

We thank Dr. P. Reed Larsen for his valuable discussion and thoughtful review of this manuscript. We also thank Drs Alceu Migliavacca and José Ricardo Guimarães for surgical management of our patients.

References

Arturi, F., Russo, D., Schlumberger, M., Villard, J.A., Caillou, B., Vigneri, P., Wicker, R., Chiefari, E., Suarez, H.G. & Filetti, S. (1998) Iodide symporter gene expression in human thyroid tumors. *Journal of Clinical Endocrinology and Metabolism,* 83, 2493-2496.

Bearhrs, O.H., Henson, D.E., Hutter, R.V.P. & Myers, M.H. (1992) American Joint Committee on Cancer: Head and neck tumors. Thyroid gland. In: *Manual for staging of cancer*, pp. 53-54. J.B. Lippincott, Philadelphia.

Bianco, A.C., Salvatore, D., Gereben, B., Berry, M.J. & Larsen, P.R. (2002) Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. *Endocrine Reviews,* 23, 38-89.

Brabant, G., Maenhaut, C., Köhrle, J., Scheumann, G., Dralle, H., Hoang-Vu, C., Hesch, R.D., Mühlen, A., Vassart, G. & Dumont, J.E. (1991) Human thyrotropin receptor gene: expression in thyroid tumors and correlation to markers of thyroid differentiation and dedifferentiation. *Molecular Cellular Endocrinology,* 82, R7-R12.

Brtko, J., Bobálová, J., Podoba, J., Schmutzler, C. & Köhrle, J. (2002) Thyroid hormone receptors and type I iodothyronine 5'-deiodinase activity of human thyroid toxic adenomas and benign cold nodules. *Experimental Clinical Endocrinology and Diabetes,* 110, 166-170.

Burmeister, L.A., Pachucki, J. & St.Germain, D.L. (1997) Thyroid hormones inhibit type 2 iodothyronine deiodinase in the rat cerebral cortex by both pre-and postranscriptional mechanisms. *Endocrinology,* 138, 5231-5237.

Cheng, SY. (2003) Thyroid hormone receptor mutations in cancer. *Molecular Cell Endocrinology*, 213, 23-30.

Fagin, J.A. (2002) Perspective: Lessons Learned from Molecular Genetic Studies of Thyroid Cancer—Insights into Pathogenesis and Tumor-Specific Therapeutic Targets. *Endocrinology*, 143, 2025-2028.

Fagin, F.A. (2004) How thyroid tumors start and why it matters: kinase mutants as targets for solid cancer pharmacotherapy. *Journal of Endocrinology*, 183, 249–256.

Garcia-Solis, P. & Aceves, C. (2003) 5'-Deiodinase in two breast cancer cell lines: effect of triiodothyronine, isoproterenol and retinoids. *Molecular Cell Endocrinology*, 28, 25-31.

Hedinger, C.E., Williams, E.D. & Sobin, L.H. (1988) Histological typing of thyroid tumors. In: *WHO, Internacional histological classification of tumours*. 2nd ed. pp.5-6. Springer-Verlag, Berlin.

Huang, Y., Prasad, M., Lemon, W.J., Hampel, H., Wright, F.A., Kornacker, K., LiVolsi, V., Frankel, W., Kloos, R.T., Eng, C., Pellegata, N.S. & Chapelle, A. (2001) Gene expression in papillary thyroid carcinoma reveals highly consistent profiles. *Proceedings of the National Academy of Sciences,* 98, 15004-15049.

Ivan, M., Bond, J.A., Prat, M., Comoglio, P.M. & Wynford-Thomas, D. (1997) Activated ras and ret oncogenes induce over-expression of c-met (hepatocyte growth factor receptor) in human thyroid epithelial cells. *Oncogene,* 14, 2417-2423.

Kamiya, Y., Puzianowska-Kuznicka, M., McPhie, P., Nauman, J., Cheng, SY. & Nauman, A. (2002) Expression of mutant thyroid hormone nuclear receptors is associated with renal clear cell carcinoma. *Carcinogenesis,* 87, 1120- 1128.

Kim, B.W., Daniels, G.H., Harrison, B.J., Price, A., Harney, J.W., Larsen, P.R. & Weetman, A.P. (2003) Overexpression of type 2 iodothyronine deiodinase in follicular carcinoma as a cause of low circulating free thyroxine levels. *Journal of Clinical Endocrinology and Metabolism,* 88, 594-598.

Kimura, E.T., Nikiforova, M.N., Zhu, Z., Knauf, J.A., Nikiforov, Y.E. & Fagin, J.A. (2003) High prevalence of *BRAF* mutations in thyroid cancer: genetic evidence for constitutive activation of RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma. *Cancer Research,* 63, 1454-1457.

Köhrle, J., Oertel, M., Hoang-Vu, C., Schnieders, F. & Brabant, G. (1993) Type I 5'-deiodinase - a marker for differentiated thyroid carcinoma? *Experimental Clinical Endocrinology,* 101, 60-72.

Latchman, D.S. (1996) Transcription-factor mutations and disease. *New England Journal of Medicine,* 334, 28-33.

Lazar, V., Bidart, J.M., Caillou, B., Mahé, C., Lacroix, L., Filetti, S. & Schlumberger, M. (1999) Expression of the Na^+ / I^- symporter gene in human thyroid tumors: a comparison study with other thyroid-specific genes. *Journal of Clinical Endocrinology and Metabolism,* 84, 3228-3234.

Lin, KH., Shieh, HY., Chen, SL. & Hsu, HC. (1999) Expression of mutant thyroid hormone nuclear receptors in human hepatocellular carcinoma cells. *Molecular Carcinogenesis*, 26, 53-61.

Mazzaferri, E.L. & Kloos, R.T. (2001) Current approaches to primary therapy for papillary and follicular thyroid cancer. *Journal of Clinical Endocrinology and Metabolism,* 86, 1447-1463.

McCabe, C.J., Gittoes, N.J., Sheppard, M.C. & Franklyn, J.A. (1999) Thyroid receptor alpha 1 and alpha 2 mutations in nonfunctioning pituitary tumors. *Journal of Clinical Endocrinology and Metabolism* , 84, 649-53.

Mizuno, T., Kyoizumi, S., Suzuki, T., Iwamoto, K.S. & Seyama, T. (1997) Continued expression of a tissue specific activated oncogene in the early steps of radiation-induced human thyroid carcinogenesis. *Oncogene*, 15, 1455-1460.

Murakami, M., Araki, O., Hosoi, Y., Kamiya, Y., Morimura, T., Ogiwara, T., Mizuma, H. & Mori, M. (2001) Expression and regulation of type II iodothyronine deiodinase in human thyroid gland. *Endocrinology,* 142, 2961-2967.

Namba, H., Nakashima, M., Hayashi, T., Hayashida, N., Maeda, S., Rogonovitch, T.I., Ohtsuru, A., Saenko, V.A., Kanematsu, T. & Yamashita, S. (2003) Clinical implication of hot spot *BRAF* mutation, V599E, in papillary thyroid cancers. *Journal of Clinical Endocrinology and Metabolism,* 88, 4393-4397.

Ohmori, M., Endo, T., Haari, N. & Onaya, T. (1998) A novel thyroid transcription factor is essential for thyrotropin-induced up-regulation of Na^+/I^- symporter gene expression. *Molecular Endocrinology*, 12, 727-736.

Otha, K., Endo, T. & Onaya, T. (1991) The mRNA levels of thyrotropin receptor, thyroglobulin and thyroid peroxidase in neoplastic human thyroid tissues. *Biochemical Biophysical Research Communications,* 174,1148-1153.

Pachucki, J., Ambroziak, Z., Tanski, Z., Luczak, J., Nauman, J. & Nauman, A. (2001) Type I 5'-iodothyronine deiodinase activity and mRNA are remarkably reduced in renal clear cell carcinoma. *Journal of Endocrinological Investigation,* 24, 253-261.

Park, H.J., Kim, J.Y., Park KY, Gong, G., Hong, S.J., Ahn, I.M. (2000) Expressions of human sodium iodide symporter mRNA in primary and metastatic papillary thyroid carcinomas. *Thyroid*, 10, 211-7.

Puzianowska-Kuznicka, M., Krystyniak, A., Madej, A., Cheng, S.Y. & Nauman J. (2002) Functionally impaired TR mutants are present in thyroid papillary cancer. *Journal of Clinical Endocrinology and Metabolism*, 87, 1120-1128.

Salvatore, D., Tu, H., Harney, J.W. & Larsen, P.R. (1996) Type 2 iodothyronine deiodinase is highly expressed in human thyroid. *Journal of Clinical Investigation,* 98, 962-968.

Schreck, R., Schnieders, F., Schmutzler, C. & Köhrle, J. (1994) Retinoids stimulate type I iodothyronine 5'-deiodinase activity in human follicular thyroid carcinoma cell lines. *Journal of Clinical Endocrinology and Metabolism,* 79, 791-798.

Steinsapir, J., Bianco, A.C., Buettner, C., Harney, J. & Larsen, P.R. (2000) Substrate-induced down-regulation of human type 2 deiodinase (hD2) is mediated through proteasomal degradation and requires interaction with the enzyme's active center. *Endocrinology*, 141, 1127-1135.

Tanaka, K., Inoue, H., Miki, H., Komaki, K. & Monden, Y. (1996) Heterogeneous distribution of thyrotropin receptor messenger ribonucleic acid (TSH-R mRNA) in papillary thyroid carcinomas detected by *in situ* hybridization. *Clinical Endocrinology,* 44, 259-267.

Tanaka, K., Inoue, H., Miki, H., Masuda, E., Kitaichi, M., Komaki, K., Uyama, T. & Monden, Y. (1997) Relationship between prognostic score and thyrotropin receptor (TSH-R) in papillary thyroid carcinoma: immunohistochemical detection of TSH-R. *British Journal of Cancer,* 76, 594-599.

Toyoda, N., Nishikawa, M., Mori, Y., Yoshimura, M., Masaki, H., Gondou, A., Yonemoto, T. & Inada, M. (1992) Identification of a 27-kilodalton protein with the properties of type I iodothyronine 5'-deiodinase in human thyroid gland. *Journal of Clinical Endocrinology and Metabolism,* 74, 533-538.

Toyoda, N., Zavacki, A.M., Maia, A.L., Harney, J.W. & Larsen, P.R. (1995) A novel retinoid X receptor-independent thyroid hormone response element is present in the human type 1 deiodinase gene. *Molecular Cell Biology*, 15, 5100-5112.

Vassart G. (1997) New pathophysiological mechanisms for hyperthyroidism. *Hormone Research*, 48, 47-50.

Wagner, M.S., Morimoto, R., Dora, J.M., Benneman, A., Pavan, R. & Maia, A.L. (2003) Hypothyroidism induces Type 2 iodothyronine deiodinase expression in mouse heart and testis. *Journal of Mollecular Endocrinology*, 31, 541-550.

Wawrzynska, L., Sakowicz, A. Rudzinski, P., Langfort, R. & Kurzyna, M. (2003) The conversion of thyroxine to triiodothyronine in the lung: comparison of activity of type I iodothyronine 5' deiodinase in lung cancer with peripheral lung tissues. *Monaldi Archives for Chest Disease*, 59, 140-145.

Zhang, C., Kim, S., Harney, JW. & Larsen, PR. (1998) Further characterization of thyroid hormone response elements in the human type 1 iodothyronine deiodinase gene. *Endocrinology*, 139, 1156-1163.

Table 1 – Clinical characteristics of patients with thyroid tumors.

Patient no.	Sex/age (yr.)	TSH[a]	Histology	Size (cm)	Stage[b]	Invaded organ
Papillary thyroid carcinoma						
1	M/56	0.44	Classic type	4.0	3	Conjuntive tissue and skeletal muscle of neck
2	M/35	1.76	Classic type; multifocal; bilateral	0.5	1	Cervical lymph nodes and fibroadipose tissue
3	F/30		Classic type	1.0	1	
4	F/29	0.93	Classic type	3.2	1	Cervical lymph nodes
5	F/52	0.02	Classic type	1.2	2	
6	F/22		Classic type	1.0	1	Cervical lymph nodes
7	F/10	3.14	Diffuse sclerosing variant		2	Cervical lymph nodes and pulmonary metastases
8	M/70	3.57	Classic type	6.5	3	Skeletal muscle of neck
9	F/21	3.55	Classic type	1.2	1	Cervical lymph nodes
10	F/43	0.74	Follicular variant	1.1	1	
11	F/38	2.49	Follicular variant	0.7	1	Cervical lymph nodes
12	M/37		Classic type	2.8	2	Cervical lymph nodes
13	F/45	0.90	Classic type	2.5	2	
14	F/61	1.45	Classic type	0.8	1	
Non-toxic follicular adenoma						
15	M/37			2.7		
16	F/41	1.00		2.0		
17	M/47	2.95		3.8		
18	F/69	1.56		3.0		
19	M/51	1.93		2.5		
20	F/55			2.5		
21	F/54	6.63		2.0		
Follicular Thyroid Carcinoma						
22	F/28	1.12		5.0	1	
23	F/23	0.83		2.5	1	
24	F/66	5.81		2.5	2	
25	F/31	26.8		3.5	2	Parapharyngeal left
26	F/69	0.50			4	Paramediastinal right
27	F/23	0.50		5.0	2	
Anaplastic Thyroid Carcinoma						
28	M/53	13.7	Mixed with focal Hürthle cell carcinoma			Soft tissues of neck and Cervical lymph nodes

[a] TSH reference: 0.4 - 4.0 mUI/L.
[b] Stage according to the TNM system.

Figure 1

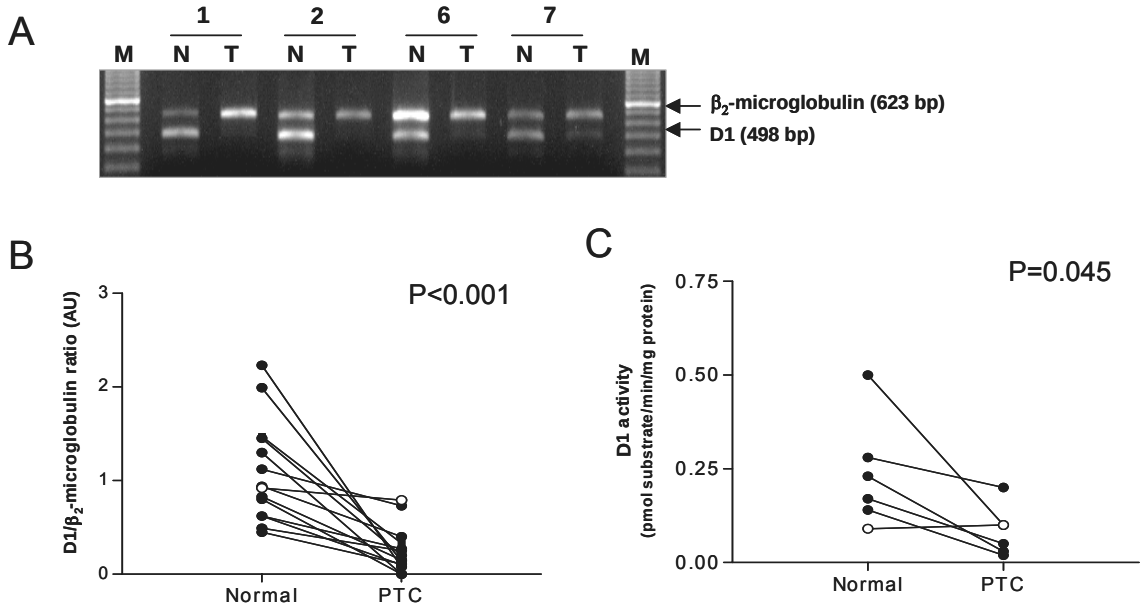

A
1
2
6
7
M
N
T
N
T
N
T
N
T
M
β2-microglobulin (623 bp)
D1 (498 bp)
B
P<0.001
D1/β2-microglobulin ratio (AU)
3
2
1
0
Normal
PTC
C
P=0.045
D1 activity
(pmol substrate/min/mg protein)
0.75
0.50
0.25
0.00
Normal
PTC

Figure 2

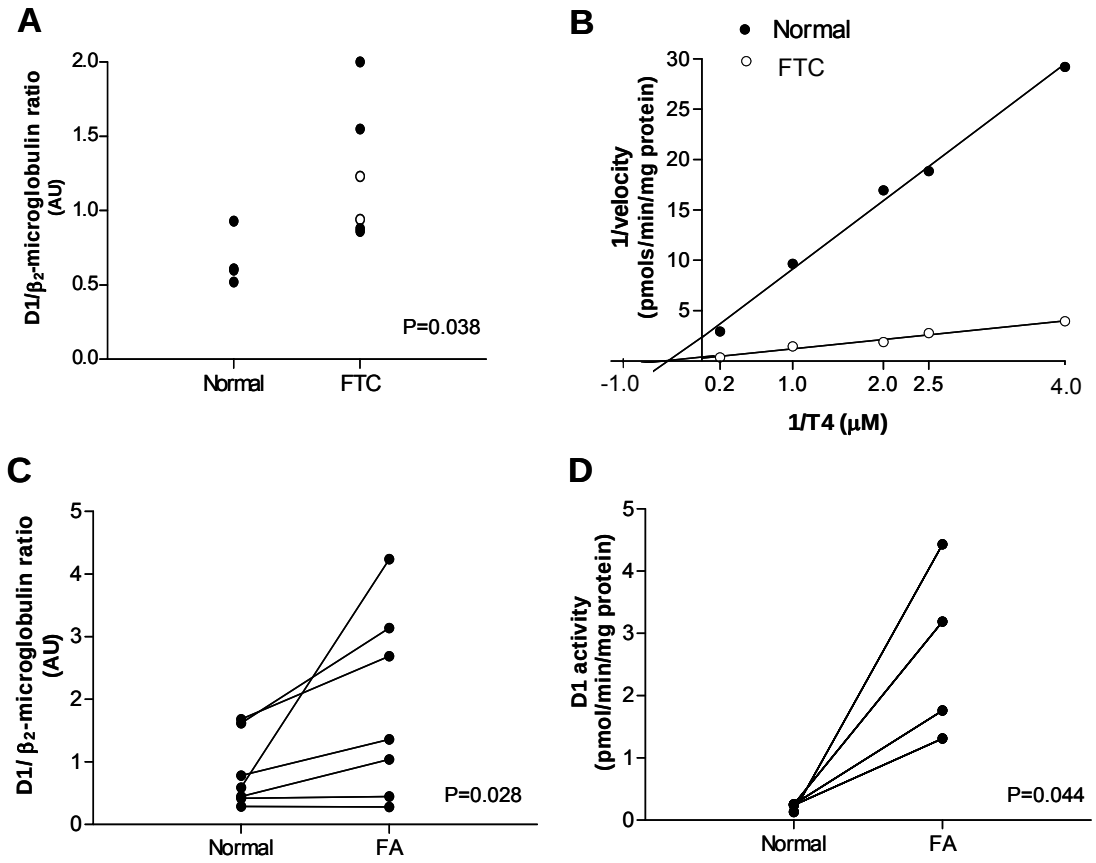
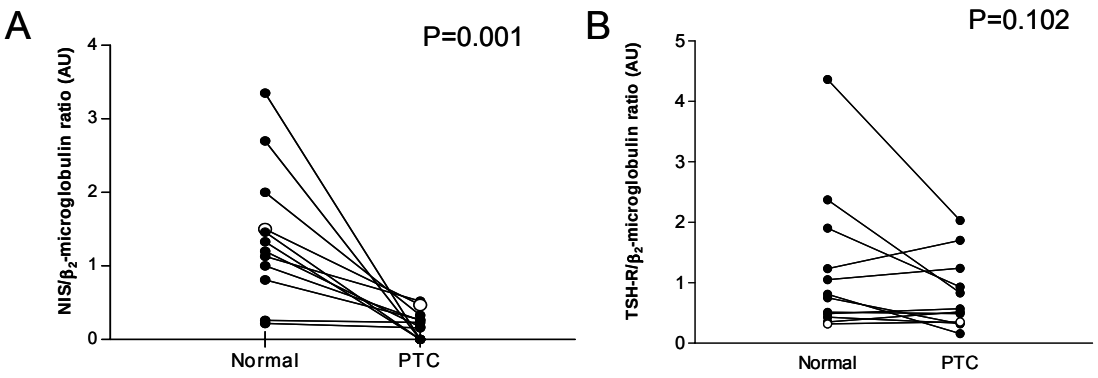

A
D1/β2-microglobulin ratio (AU)
2.0
1.5
1.0
0.5
0.0
P=0.038
Normal
FTC
B
Normal
FTC
1/velocity (pmols/min/mg protein)
30
25
20
15
10
5
-1.0
0.2
1.0
2.0
2.5
4.0
1/T4 (μM)
C
D1/ β2-microglobulin ratio (AU)
5
4
3
2
1
0
P=0.028
Normal
FA
D
D1 activity (pmol/min/mg protein)
5
4
3
2
1
0
P=0.044
Normal
FA

Figure 3


A
P=0.001
NIS/β2-microglobulin ratio (AU)
0
1
2
3
4
Normal
PTC
B
P=0.102
TSH-R/β2-microglobulin ratio (AU)
0
1
2
3
4
5
Normal
PTC

Legends

Figure 1: A. An illustrative semi-quantitative RT-PCR of D1 products in tumor (T) and surrounding normal tissue (N) from cases 1, 2, 6 and 7(Table1) on 1.5% agarose gel stained by ethidium bromide. **B.** D1 mRNA levels in papillary thyroid carcinoma (PTC) and corresponding normal tissue. **C.** D1 enzyme activity in PTC and corresponding normal tissues. Individual data points for each sample (cases 7,8,12,13 and 14) are depicted. D1 activity was measured as the release of I^- from 1μM T_4 as substrate. Results of mRNA levels are expressed in arbitrary units (AU). Individual data points for each 13 samples are depicted as *solid circles* and *open circles* represent a follicular variant of PTC (case 11).

Figure 2: A. D1 mRNA levels in follicular thyroid carcinoma (FTC) and normal thyroid tissues. Individual data points for each sample are depicted as *solid circles* and *open circles* represent samples of metastasis of FTC (cases 25 and 26). Results are expressed in arbitrary units (AU). **B.** Double reciprocal plot of type 1 iodothyronine deiodinase activity in normal tissue (solid circles) and follicular thyroid carcinoma (open circles). D1 activity was measured in randomly selected fragments from the thyroid tumor of patient 26 (Table 1) using different concentrations of ^{125}I-T_4 as described in *Materials and Methods*. **C.** D1 mRNA levels in samples of follicular adenoma (FA) and corresponding normal tissue. Results are expressed in arbitrary units (AU). **D.** D1 enzyme activity in FA (cases 17, 18, 20 and 21) and corresponding normal tissue. D1 activity was measured as the release of I^- from 1μM T_4 as substrate.

Figure 3. A. NIS mRNA levels in samples of papillary thyroid carcinoma (PTC) and corresponding normal tissue. **B.** TSH-R mRNA levels in samples of papillary thyroid carcinoma (PTC) and corresponding normal tissue. Individual data points for each 11 samples are depicted as *solid circles* and *open circles* represent a follicular variant of PTC (case 11).

Parte III

Type 2 Iodothyronine Deiodinase Is Highly Expressed in Medullary Thyroid Carcinoma

Artigo submetido à publicação

Type 2 Iodothyronine Deiodinase Is Highly Expressed in Medullary Thyroid Carcinoma

Erika L Souza Meyer, M.D., Iuri M Goemann, José Miguel Dora, M. D., and Ana Luiza Maia, M.D., Ph.D.

Endocrine Division, Hospital de Clínicas de Porto Alegre,

Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

Running title: Iodothyronine deiodinase and medullary thyroid carcinoma

Grant support: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundo de Incentivo a Pesquisa do Hospital de Clínicas de Porto Alegre (FIPE), Brazil.

Correspondence: Ana Luiza Maia, M.D., Ph.D. Serviço de Endocrinologia, Hospital de Clínicas de Porto Alegre. Rua Ramiro Barcelos 2350, 4° andar, Porto Alegre, RS, Brazil, 90035-003. Phone: + 55 51 33310207; Fax: 55 51 33325188. E-mail: almaia@ufrgs.br

Summary

Type II deiodinase (D2) plays a critical role in controlling intracellular T3 concentration and early studies indicated a follicular but not a parafollicular C-cell origin of D2 activity in the thyroid gland. Here, we show that D2 is highly expressed in human medullary thyroid carcinoma (MTC), a tumour that arises from the C-cells. D2 transcripts were detected in all MTC samples obtained from 12 unselected MTC patients and the levels of D2 activity were comparable to those found in surrounding normal follicular tissue (0.41±0.10 *vs* 0.43±0.41 fmol.min.mg.protein, *P*=0.91). Additional analysis in the TT cells, a human MTC cell line, demonstrated that the D2 expression is down regulated by thyroid hormones and enhanced by cAMP analogs and dexamethasone. The thyroid hormone receptor α1 and β isoforms were also detected in all MTC samples and in TT cells, thus suggesting a potential role of T3 locally produced by D2 in this neoplastic tissue.

Key words: type 2 deiodinase, medullary thyroid carcinoma, gene expression

Introduction

Medullary thyroid carcinoma (MTC) is an uncommon thyroid tumor that arises from the parafollicular C-cells of the thyroid, embryologically unrelated to the follicular epithelium. In addition to calcitonin gene products, MTC cells express several biochemical markers that typify secretory cells of the diffuse neuroendocrine system (Leboulleux et al., 2004). This tumor may be sporadic or may occur on a hereditary basis caused by germline mutations in the *RET* proto-oncogene (Ponder, 1999). The *RET* proto-oncogene is expressed in cells of neuronal and neuroepithelial origin and encodes a receptor tyrosine kinase (Mulligan et al., 1993). In patients with familial MTC (FMTC) only the thyroid is affected. Patients with multiple endocrine neoplasia (MEN) 2A develop MTC, pheochromocytoma (pheo) and/or primary hyperparathyroidism. In addition, MEN2B patients have MTC, pheo, ganglioneuromas of the digestive tract, mucosal neuromas, and/or skeletal abnormalities (Ponder, 1999).

The type 1 and type 2 iodothyronine deiodinases (D1 and D2) enzymes are responsible for catalyzing deiodination of T4 to T3. D2 plays a critical role in providing local T3 to regulate intracellular T3 concentration and recent studies suggest that this enzyme also contributes to a significant fraction of plasma T3 in rodents and humans (Bianco et al., 2002; Maia et al., 2005). D2 is expressed in normal and stimulated human thyroid gland and has been evaluated as a possible marker of thyroid follicular cell differentiation (Salvatore et al., 1996; Murakami et al., 2001a; Kim et al., 2003; De Souza Meyer et al., 2005; Arnaldi et al., 2005; Takano et al., 2006). Previous studies have described underexpression of D2 in papillary thyroid carcinoma (PTC) (Murakami et al., 2001a; Arnaldi et al., 2005). In contrast, D2 are

reported to be significant increased in follicular carcinoma (Kim et al., 2003; De Souza Meyer et al., 2005; Takano et al., 2006). Little is known about the metabolism of iodothyronine in MTC. Early studies have suggested that only differentiated thyroid neoplasias contain 5'-deiodinase activity, whereas medullary carcinomas do not, suggesting a follicular but not a C-cell origin of enzyme activity (Ishii et al., 1981; Boye et al., 1984). Here, we demonstrated that the D2 enzyme is also highly expressed in human MTC samples.

Materials and Methods

Patients and tissues

Samples of MCT were collected from 12 consecutive unselected patients attending the Endocrine or the Head and Neck Surgery Divisions at Hospital de Clínicas de Porto Alegre. Surgery was independently indicated by attending physicians based on clinical indications. Tumors were histologically classified according to WHO recommendations (Hedinger et al., 1988). Immunohistochemical staining for calcitonin was positive in all CMT samples. Identification of *RET* germline mutations was performed by standard procedures (Puñales et al., 2003). Clinical data were retrospectively reviewed in medical records. The clinical stage was determined by the Tumor/Node/Metastases (TMN) system (Greene and Sobin, 2002). Serum TSH was measured by a double antibody–sensitive assay (Immulite, Diagnostic Products Corporation, EUA). For determination of preoperative serum calcitonin levels, a sensitive immunoradiometric assay was performed (Calcitonin IRMA-DSL 7700, Diagnostic Systems Laboratories, Inc., Webster, TX ; reference range < 12 pg/ml).

Thyroid tissue was obtained from both tumor and adjacent normal tissue at the time of surgery. All tissues obtained at surgery were immediately frozen in liquid nitrogen and stored at – 70° C until analysis.

The information obtained from this study did not influence or affect the patients' diagnosis or treatment. The Ethics Committee at the Hospital approved the study protocol and all patients gave their informed consent.

Cell culture

The TT cells, a human MTC cell line, were cultured in F-12K medium supplemented with 10% fetal bovine serum (Life Technologies). Cells were maintained at 37° C in a humidified atmosphere of 5% CO_2 and 95% air, and the culture medium was changed twice a week. TT cells were seeded in six-well or in 60-mm plastic culture plates for RT-PCR and for measurement of deiodinase activity, respectively. The cells were cultured for 18-24 hours in serum-free 0.1% BSA in F-12K medium before harvesting, when the effects of thyroid hormones were tested. For other experiments, the cells were incubated in the usual medium containing compounds to be tested for the indicated period.

RNA isolation and reverse transcription

Total RNA was isolated from 50 - 100 mg of thyroid tumor and surrounding nontumor tissues using TRIzol® reagent (Invitrogen™ Life Technologies Inc., NY, USA) according to the manufacturer's instructions. For TT cells, the total RNA was isolated with the use of the Rneasy Mini Kit (Qiagen GmBH, Hilden, Germany) according to the manufacturer's protocol. The purity and integrity of the total RNA of all samples was assessed by UV spectrophotometry (GeneQuant II®, Amersham Pharmacia Biotech). RNA was reverse transcribed using the Superscript Preamplification System for First Strand cDNA Synthesis (Invitrogen™ Life Technologies Inc., NY, USA) with 3 μg of total RNA as template.

Reverse transcription polymerase chain reaction (RT-PCR)

A RT-PCR technique was used to determine the expression levels of D2, TSH receptor (TSH-R), thyroid hormone receptor TRα1 and TRβ genes in RNA samples isolated from thyroid tissues. Primer sequences are given in Table 1. PCR reactions included 1 μl of RT products, and were carried out with Taq DNA polymerase (Invitrogen^{TM} Life Technologies Inc., NY, USA) in a final 50 -μl volume. The amplification profile was an initial denaturation step at 94°C for 3 min, followed by 94°C for 1 min, annealing at 60°C (D2 and TSH-R) or 55°C (TRα1 and TRβ), and extension at 72°C for 2 min. Thirty amplification cycles were used for all genes, with a final additional extension step at 72°C for 4 min. The β_2-microglobulin primers were used as an internal control. RT-PCR reactions without cDNA samples were carried out as negative control. After amplification, 10 μl of the PCR products were analyzed on a 1.5 % ethidium bromide agarose gel and the intensity of each band was determined by optic densitometry (arbitrary units - AU) (ImageMaster® VDS, Amersham Pharmacia Biotech).

Quantitative PCR (qPCR)

Reactions for the quantification of D2 mRNA in TT cells experiments were performed in an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Warrington, UK) using the SYBR Green PCR Master Mix (Applied Biosystems) and cyclophilin as a housekeeping internal control. Primer sequences are given in Table 1. Samples were run in duplicate. The cycle conditions were 94°C x 5 min (Hot Start), 40 cycles of 94°C x 30 sec; 58°C x 30 sec; 72°C x 45 sec and a final 1 min extension period. Initially, standard curves representing 5-point serial dilution of mixed cDNAs

of the control and experimental groups were analyzed and used as calibrators to determine the relative quantification of product generated in the exponential phase of the amplification curve. Comparable efficiency was observed presenting r2 greater than 0.99. Sample quantification was calculated by the standard curve and corrected by the internal control cyclophilin in all experiments.

Iodothyronine deiodinase activity assays

Deiodinase assays were performed as described previously (Wagner et al., 2003). In brief, tissue samples were individually homogenized on ice in PE buffer (0.1 M potassium phosphate and 1 mM EDTA) containing 10 mM dithiothreitol (DTT) and 0.25 M sucrose (pH 6.9). Protein concentration was quantitated by Bradford assay using BSA as a standard. D2 assays were performed using 100-300 μg tissue protein and 1nM unlabeled T_4 in a total volume of 300 μl PE buffer containing 20 mM DTT, 1mM propylthiouracil (PTU), and approximately 100.000 cpm [^{125}I] T4 (Amersham, Biosciences). The apparent Km and Vmax for D2 enzymes were determined using various amounts of unlabelled T4 (0.25, 0.5, 1, 2 and 6 nM). Incubations were carried out at 37°C for 60-120 min. The reaction was terminated by adding 200 μl of horse serum and 100 μl of 50% trichloroacetic acid (TCA).

For measurements of activity in cell, the medium was removed and the cells washed twice with PBS, harvested, and sonicated in 0.25 M sucrose in PE buffer (0.1 M potassium phosphate and 1 mM EDTA) with 10 mM DTT. In deiodinase assays, we used 150–250 μg cell sonicate; 1nM unlabeled T4, and 20 mM DTT in a final volume of 300 μl PE. Incubation was for 60–120 minutes at 37°C, and ^{125}I– was separated from labeled T4 by TCA precipitation. Results are the mean of values derived from at least two separate experiments.

Statistical analysis

Results are presented as mean ± SD. Statistical differences were evaluated by *t* test. *P* values ≤0.05 were considered statistically significant.

Results

Patients

Table 2 shows the clinical and laboratorial characteristics of the 12 patients studied. Thyroid samples covered patients aged 15 – 65 years. Nine patients had hereditary MTC with *RET* germline mutations in codon 634 with bilateral disease and C-cell hyperplasia on pathological examination. Three patients had a sporadic form of MTC. All patients studied were on thyroid hormone replacement therapy and present serum TSH levels within the reference range (0.4 - 4.0 UI/L).

D2 expression in human medullary thyroid carcinoma

D2 mRNA was detected in all MTC samples (Fig.1A). Of the 12 samples analyzed, D2 activities were measured in 7 MTC samples (n° 3, 4, 6 -10 of table 2). Enzyme activities in MTC tissues were similar to those corresponding to normal thyroid tissues (0.41±0.10 *vs* 0.43±0.41 fmol.min.mg.protein, P=0.913) (Fig.1B). Further studies performed to determine the D2 kinectics in the neoplastic tissue demonstrated that the apparent Km (T4) values of 2.3 nM in the tumor homogenates, typical for this enzyme (Fig.1C). The Vmax for D2 was 0.86 fmol.min.mg.protein in tumor and was comparable to those of normal thyroid tissue (1.59 fmol.min.mg.protein). Interestingly, in a sample of a sporadic CMT (n° 3 of table 2), D2

activity was significantly higher than in surrounding normal follicular tissue (6.34 *vs*. 0.09 fmol min.mg.protein, respectively).

D2 expression in TT cell line

To assess whether the D2 expression found in MTC was truly expressed in this neoplastic tissue, we performed enzyme activities in TT cells, a human MTC cell line established from a specimen obtained by needle biopsy from a 77 year old female with MTC. The results obtained confirmed those observed in MTC samples (Fig. 2A). The Km (T4) for D2 in the TT cell sonicates was 2.59 nM and the D2 Vmax was 1.22 fmol.min.mg.protein

Effects of thyroid hormones on D2 expression in TT cells

To study the effects of thyroid hormones on D2 expression in TT cells, 10^{-7}M thyroid hormones were added to the culture medium for 16h before harvesting the cells. The addition of 10^{-7}M of T3 significantly decreases D2 mRNA levels (1.55± 0.38 *vs.* 0.89±0.32 AU, P=0.010). Addition of 10^{-7}M thyroid hormones to the incubation medium decreased the deiodinating activity in TT cells (0.12±0.02 *vs*. 0.03±0.03 fmol.min.mg.protein, P= 0.001 for T4; 0.06±0.02 fmol.min.mg.protein, P= 0.01 for rT3 and 0.12±0.02 fmol.min.mg.protein, P= 0.23 for T3). The potency of the inhibition effect was T4>rT3>T3 as shown in fig.2B.

Effects of cAMP and Dexametasona (DEX) on D2 expression in TT cells

The effects of dibutyril cAMP[$(Bu)_2$cAMP] and forskolin on D2 expression in TT cells were also evaluated. By 16h of incubation, 10^{-3} M $(Bu)_2$cAMP nearly doubled D2

mRNA levels (1.08±0.09 *vs*. 1.78±0.17 AU, P<0.001). In addition, $(Bu)_2$cAMP (10^{-3} M) and forskolin (10^{-5} M) significantly stimulated deiodinating activity in TT cells (0.06±0.03 *vs.* 0.13 ± 0.03 fmol.min.mg.protein, P=0.002 and 0.12±0.03 fmol.min.mg.protein, P=0.03, respectively) (Fig.2C). These results indicate that D2 expression in MTC is pretranslationally stimulated through a cAMP-mediated pathway

In the next experiment, the effects of DEX on stimulation of D2 expression were tested. Results demonstrated that 10^{-6} M DEX stimulated D2 mRNA (1.05± 0.09 *vs.* 2.18±1.34 AU, P=0.05) and D2 activity (0.12±0.02 *vs.* 0.21±0.02 fmol.min.mg.protein, P=0.001) (Fig.2D).

Thyroid hormone isoform transcripts in MTC samples and TT cell line

We evaluated TR α1 and β isoforms in MTC samples and TT cells by RT-PCR. The TR isoforms were observed in all 8 MTC samples (cases 3-10 of table 2) analyzed and in the TT cells (Fig. 3). We also evaluated the TSH-R expression in 7 MTC samples (cases 4-10 of table 2) and TSH-R transcripts were found only in one sample (case 5 of table 2).

Discussion

In the present study, we demonstrated that D2 enzyme is expressed in MTC tissues at levels comparable with normal human follicular thyroid cells. The biochemical and molecular properties of D2 enzyme in TT cells, a human MTC cell line, seem to be preserved despite C cell dedifferentiation. Furthermore, TRs were demonstrated in all MTC samples and in TT cells, suggesting a potential role of T3 locally produced by D2 in this neoplastic tissue.

The MTC arises from the C cells of the thyroid gland and represents 5% to 8% of all thyroid cancers. Unlike the endoderm-derived thyroid follicular epithelial cells, the C cells originate in embryonic neural crest and account for only 0.1% of thyroid cells. Several biochemical features including the production of calcitonin and other neuroendocrine markers such as chromogranin A, neuron-specific enolase, and the neural cell adhesion molecule (NCAM) characterize these cells (Leboulleux et al., 2004). Expression of thyroid-specific genes involved in the complex process of thyroid hormone synthesis, such as NIS, thyroglobulin, and TSH-R genes has been demonstrated in MTC samples (Pacini et al., 1991; Elisei et al., 1994; Dohán et al., 2001). However, little is known about the metabolism of iodothyronine in C- cells or MTC.

Previous data have indicated that microsomes prepared from thyroidal C-cell neoplasms did not contain 5'-deiodinases, suggesting that the source of thyroid deiodinases in the thyroid tissue was exclusive of the follicular cells (Boye et al., 1984). Here, we show that functional D2, an enzyme that catalyzes T4 activation, is expressed in both sporadic and hereditary MTC samples at levels comparable with normal follicular thyroid. D2 mRNA and activities were detected in all MTC samples analyzed. The potential explanation for this unexpected finding would be the presence of RNA and protein from follicular cells, contaminating the medullary tumor. However, the results obtained in MTC samples were further confirmed in the TT cells, a human MTC cell line that harbors a codon 634 mutation (Cys→Trp) in *RET* proto-oncogene which constitutively activates RET tyrosine kinase (Carlomagno et al., 1995).

The physiological importance of intracellular thyroid hormone activation by D2 has been clearly demonstrated in certain tissues. Adenohypophyseal T3 production by D2 plays an important role in feedback regulation of TSH secretion by thyroid hormones

and also regulates pituitary cell growth and differentiation (Barrera-Hernandez et al., 1999; Stahl et al., 1999; Bianco et al; 2002; Schneider et al., 2001). Interestingly, a variable expression of D2 has been described in a number of subtypes of pituitary adenomas, implying that this enzyme is still active in tumor tissues (Tannahill et al., 2002; Baur et al., 2002). It is well established that thyroid hormones play an important role at multiple steps in the development of glial cells that, like C-cells, is of neuroectodermic origin (Rodríguez-Peña et al., 1999) and high levels of D2 activity was noted in oligodendrogliomas, a tumor derived from the glial cells (Mori et al., 1993; Murakami et al., 2000). Although D2 expression has not been accessed in normal C cells, the presence of this enzyme in the TT cell line, considered a reliable model system for studies of human parafollicular cells, might also suggest a possible role of thyroid hormones in human C-cell metabolism (Zabel et al., 1995; Zabel and Grzeszkowiak, 1997). In agreement with this hypothesis, transcripts of both TR α1 and β isoforms were observed in all MTC samples analyzed and in TT cells. Further studies to evaluate the possible role of T3 locally produced by D2 in the regulation of C cell-specific gene expression are warranty.

Abnormal D2 expression and function have been described previously in other human neoplasms (Curcio et al., 2001; Morimura et al., 2005; Meyer et al., 2007; Dentice et al., 2007). Indeed, it is conceivable that thyroid status is linked to the development and growth of neoplastic tissues (Lemaire et al., 1981; Leuthauser et al., 1987). By activating T3, a differentiating agent, as well as blocking its production, changes in deiodination can influence the balance between cell proliferation and differentiation in tissue microenvironment. Interestingly, it was reported that D2 is down regulated and D3 is up regulated in human basal cell carcinomas, supporting a potential therapeutic application for T3 in this malignant neoplasia (Dentice et al.,

2007). On the other hand, high D2 activity has been demonstrated in the human mesothelioma cell line MSTO-211H (Curcio et al., 2001). This is an example of high levels of D2 in a human tumor cells derived from a tissue that normally does not express this enzyme.

D2 regulation in neoplastic cells is also of interest. D2 regulation is a complex process involving both transcriptional and post-transcriptional mechanisms. Thyroid hormones are the most important recognized regulators of deiodinase expression, with well-correlated changes in activities and mRNA levels (Bianco et al., 2002). In the TT cells, the D2 expression was inhibited by thyroid hormones. T4 and rT3 suppress D2 activity mainly at the posttranslational level through acceleration of the degradation rate D2 protein (Steinsapir et al., 1998). However, T3 was more potent than T4 to inhibit the synthesis of D2 mRNA, indicating that pretranscriptional mechanisms are also involved in the regulation of D2 expression in TT cells. On the other hand, D2 mRNA and activities levels were stimulated by $(Bu)_2$cAMP and forskolin in TT cells. These results indicate that D2 expression in C-cells is regulated by a cAMP-dependent mechanism at the pretranslational level, as previously reported in human thyroid follicular cells, human skeletal muscle cells, rat brown adipocytes, and rat pineal glands (Murakami et al., 2001a; Hosoi et al., 1999; Murakami et al., 2001b; Kamiya et al., 1999). In follicular thyroid cell, D2 gene is regulated via TSH-R-cAMP-mediated mechanism (Murakami et al., 2001a). Nevertheless, since MTC samples nor TT cells do not express TSH-R other factors might be involved in cAMP activation in these cells (Elisei et al., 2005).

Our results showed that glucocorticoid (DEX) significantly stimulated D2 expression in TT cells, in agreement with studies performed in GH4C1 and GH3 rat pituitary tumors cells (Kim et al., 1998; Araki et al., 2003). In contrast, DEX was

reported to decrease D2 activity in cultured mouse neuroblastoma cells (St Germain, 1986). Therefore, the effect of glucocorticoid on D2 expression seems to differ among the various neoplastic tissues.

An interesting observation in this study came from the analysis of a sporadic MTC sample. In this tumor, the level of D2 activity was much higher than the normal epithelial tissue of same patient. The possibility of a mixed follicular-medullary tumor was ruled out by negative immunohistochemical analysis for thyroglobulin (data not shown). Overexpression of D2 was also described in follicular carcinoma as a cause of low circulating free thyroxine levels (Kim et al., 2003). Both high serum TSH and/or low T4 levels up-regulate D2 activity, however, no changes were observed in thyroid function tests in this case.

In conclusion, the present results demonstrate that the D2 is expressed in samples of MTC, as well as in a human MTC derived cell line. Furthermore, TRs were demonstrated in MTC and in TT cells suggesting a potential role of T3 in this neoplastic tissue and presenting novel perspectives for thyroid hormone metabolism in human parafollicular C-cells.

Acknowledgments

We thank Dr. Alceu Migliavacca and Dr. José Ricardo Guimarães for surgical management of our patients. The authors are indebted to Mary Cleide Sogayar from Instituto de Ciências Básicas and Edna Kimura from Instituto de Ciências Biomédicas da Universidade de São Paulo (USP) for supplying cell line for the experiments. We are grateful to Gabriella Rejane dos Santos, Mateus Barbosa Vieira and Ursula Matte from Centro de Terapia Gênica do Hospital de Clínicas de Porto Alegre for their technical assistance in cell culture.

References

Araki, O., Morimura, T., Ogiwara, T., Mizuma, H., Mori, M. & Murakami, M. (2003). Expression of type 2 iodothyronine deiodinase in corticotropin-secreting mouse pituitary tumor cells is stimulated by glucocorticoid and corticotropin-releasing hormone. Endocrinology, 144, 4459-4465.

Arnaldi, L.A.T., Borra, R.C., Maciel, R.M.B. & Cerutti, J.M. (2005) Gene expression profiles reveal that *DCN*, *DIO1*, and *DIO2* are underexpressed in benign and malignant thyroid tumors. Thyroid, 15, 210-221.

Barrera-Hernandez, G., Soo Park, K., Dace, A., Zhan, Q. & Cheng, S. (1999) Thyroid hormone-induced cell proliferation in GC cells is mediated by changes **in G1** cyclin/cyclin-dependent kinase levels and activity. Endocrinology, 140, 5267-5274.

Baur, A., Buchfelder, M. & Köhrle, J. (2002) Expression of 5'-deiodinase enzyme in normal pituitaries and in various human pituitary adenomas. Eur. J. Endocrinol., 147, 263-268.

Bianco, A.C., Salvatore, D., Gereben, B., Berry, M.J. & Larsen, P.R. (2002) Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. Endocr. Rev., 23, 38-89.

Boye, N. & Laurberg, P. (1984) Deiodination of T4 to T3 and rT3 by microsomes from normal human thyroid tissue. Mol. Cell. Endocrinol., 37, 295-299.

Carlomagno, F., Salvatore, D., Santoro, M., de Franciscis, V., Quadro, L., Panariello, L., Colantuoni, V. & Fusco, A. (1995) Point mutation of the RET proto-oncogene in the TT human medullary thyroid carcinoma cell line. Biochem. Biophys. Res. Comm., 207, 1022-1028.

Curcio, C., Baqui, M.M.A., Salvatore, D., Rihn, B.H., Mohr, S., Harney, J.W., Larsen, P.R. & Bianco, A.C. (2001) The human type 2 iodothyronine deiodinase is a seleno protein highly expressed in a mesothelioma cell line. J. Biol. Chem., 276, 30183-30187.

Dentice, M., Luongo, C., Huang, S., Ambrosio, R., Elefante, A., Mirebeau-Prunier, D., Zavacki, A.M., Fenzi, G., Grachtchouk, M., Hutchin, M., Dlugosz, A.A., Bianco, A.C, Missero, C., Larsen, P.R., & Salvatore, D. (2007) Sonic hedgehog-induced type 3 deiodinase blocks thyroid hormone action enhancing proliferation of normal and malignant keratinocytes. Proc. Nat. Acad. Sci., 104, 14466-14471.

De Souza Meyer, E.L., Dora, J.M., Wagner, M.S. & Maia, A.L. (2005) Decreased type 1 iodothyronine deiodinase expression might be an early and discrete event in thyroid cell dedifferentiation towards papillary carcinoma. Clin. Endocrinol., 62, 672-678.

Dohán, O., Baloch, Z., Bánrévi, Z., Livolsi, V. & Carrasco, N. (2001) Rapid communication: predominant intracellular overexpression of the Na(+)/I(-) symporter (NIS) in a large sampling of thyroid cancer cases. J. Clin. Endocrinol. Metab., 86, 2697-2700.

Elisei, R., Pinchera, A., Romei, C., Gryczynska, M., Pohl, V., Maenhaut, C., Fugazzola, L. & Pacini, F. (1994) Expression of thyrotropin receptor (TSH-R), thyroglobulin, thyroperoxidase, and calcitonin messenger ribonucleic acids in thyroid carcinomas: evidence of TSH-R gene transcript in medullary histotype. J. Clin. Endocrinol. Metab., 78, 867-871.

Elisei, R., Vivaldi, A., Agate, L., Ciampi, R., Molinaro, E., Piampiani, P, Romei, C., Faviana, P., Basolo, F., Miccoli, P., Capodanno, A., Collecchi, P., Pacini, F. & Pinchera, A. (2005) All-trans-retinoic acid treatment inhibits the growth of retinoic acid receptor ß messenger ribonucleic acid expressing thyroid cancer cell lines but does

not reinduce the expression of thyroid-specific genes. J. Clin. Endocrinol. Metab., 90, 2403-2411.

Greene, F.L. & Sobin, L.H. (2002) The TNM system: our language for cancer care. J. Surg. Oncol., 80, 119-120.

Hedinger, C.E., Williams, E.D. & Sobin, L.H. (1988) Histological typing of thyroid tumors. In: *WHO* International Histological Classification of Tumours, 2nd edn, Springer-Verlag, Berlin, 5-6.

Hosoi, Y., Murakami, M., Mizuma, H., Ogiwara, T., Imamura, M. & Mori, M. (1999) Expression and regulation of type II iodothyronine deiodinase in cultured human skeletal muscle cells. J. Clin. Endocrinol. Metab., 84, 3293-3300.

Ishii, H., Inada, M., Tanaka, K., Mashio, Y., Naito, K., Nishikawa,M. & Imura, H. (1981) Triiodothyronine generation from thyroxine in human thyroid: enhanced conversion in Graves' thyroid tissue. J. Clin. Endocrinol. Metab., 52, 1211-1217.

Kamiya, Y., Murakami, M., Araki, O., Hosoi, Y., Ogiwara, T., Mizuma, H. & Mori, M. (1999) Pretranslational regulation of rhythmic type II iodothyronine deiodinase expression by β-adrenergic mechanism in the rat pineal gland. Endocrinology, 140, 1272-1278.

Kim, B. W., Harney, J.W. & Larsen, P.R. (1998) Studies of the hormonal regulation of type 2 5'-iodothyronine deiodinase messenger ribonucleic acid in pituitary tumor cells using semiquantitative reverse transcription-polymerase chain reaction. Endocrinology, 139, 4895-4905.

Kim, B.W., Daniels, G.H., Harrison, B.J., Price, A., Harney, J.W., Larsen, P.R. & Weetman, A.P. (2003) Overexpression of type 2 iodothyronine deiodinase in follicular carcinoma as a cause of low circulating free thyroxine levels. J. Clin. Endocrinol. Metab., 88, 594-598.

Leboulleux, S., Baudin, E., Travagli, J.P. & Schlumberger, M. (2004) Medullary thyroid carcinoma. Clin. Endocrinol., 61, 299-310.

Lemaire, M., Bayens, W., de Saint-Georges, L. & Baugnet-Mahieu, L. (1981) Thyroid hormone influence on the growth of hepatoma HW-165 in Wistar rats. Biomedicine, 34,133–139.

Leuthauser, S.W.C. & Guernsey, D.L. (1987) Thyroid hormone affects the expression neoplastic transformation induced by DNA-transfection. Cancer Lett, 35, 321–326.

Maia, A.L., Kim, B.W., Huang, S.A., Harney, J.W. & Larsen, P.R. (2005) Type 2 iodothyronine deiodinase is the major source of plasma T3 in euthyroid humans. J. Clin. Invest., 115, 2524-2533.

Meyer, E.L.S., Wagner, M.S. & Maia, A.L. (2007) Expressão das iodotironinas desiodases nas neoplasias tireoidianas. Arq. Bras. Endocrinol. Metabol., 51, 690-700.

Mori, K., Yoshida, K., Kayama, T., Kaise, N., Fukazawa, H., Kiso, Y., Kikuchi, K., Aizawa, Y & Abe, K. (1993) Thyroxine 5-deiodinase in human brain tumors. J. Clin. Endocrinol. Metab., 77, 1198-1202.

Morimura, T., Tsunekawa, K., Kasahara, T., Seki, K., Ogiwara, T., Mori, M. & Murakami, M. (2005) Expression of type 2 iodothyronine deiodinase in human osteoblast is stimulated by thyrotropin. Endocrinology, 146, 2077-2084.

Mulligan, L.M., Kwok, J.B., Healey, C.S., Elsdon, M.J., Eng, C., Gardner, E., Love, D.R., Mole, S.E., Moore, J.K., Papi, L., Ponder, M.A., Telenius, H., Tunnacliffe, A. & Ponder, B.A. (1993) Germ-line mutation of the *RET* proto-oncogene in multiple endocrine neoplasia type 2 A. Nature, 363, 458–460.

Murakami, M., Araki, O.,Morimura, T., Hosoi, Y., Mizuma, H., Yamada, M., Kurihara, H., Ishiuchi, S., Tamura, M., Sasaki, T. & Mori, M. (2000) Expression of Type II iodothyronine deiodinase in brain tumors. J. Clin. Endocrinol. Metab., 85, 4403-4406.

Murakami, M., Araki, O., Hosoi, Y., Kamiya, Y., Morimura, T., Ogiwara, T., Mizuma, H. & Mori, M. (2001a) Expression and regulation of type II iodothyronine deiodinase in human thyroid gland. Endocrinology, 142, 2961-2967.

Murakami, M., Kamiya, Y., Morimura, T., Araki, O., Imamura, M., Ogiwara, T., Mizuma, H. & Mori, M (2001b) Thyrotropin receptors in brown adipose tissue: thyrotropin stimulates type II iodothyronine deiodinase and uncoupling protein-1 in brown adipocytes. Endocrinology, 142, 1195-1201.

Pacini, F., Basolo, F., Elisei, R., Fugazzola, L., Cola, A. & Pinchera, A. (1991) Medullary thyroid cancer. An immunohistochemical and humoral study using six separate antigens. Am. J. Clin. Pathol., 95,300-308.

Ponder, B.A. (1999) The phenotypes associated with *RET* mutations in the multiple endocrine neoplasia type 2 syndromes. Cancer Res., 59, 1736–1742.

Puñales, M.K., Graf, H., Gross, J.L. & Maia, A.L. (2003) *RET* codon 634 mutations in multiple endocrine neoplasia type 2: variable clinical features and clinical outcome. J. Clin. Endocrinol. Metab., 88, 2644-2649.

Rodríguez-Peña, A. (1999) Oligodendrocyte development and thyroid hormone. J. Neurobiol., 40, 497-512.

Salvatore, D., Tu, H., Harney, J.W. & Larsen, P.R. (1996) Type 2 iodothyronine deiodinase is highly expressed in human thyroid. J. Clin. Invest., 98, 962-968.

Schneider, M.J., Fiering, S.N., Pallud, S.E., Parlow, A.F., St Germain, D.L. & Galton, V.A. (2001) Targeted disruption of the type 2 selenodeiodinase gene (DIO2) results in a phenotype of pituitary resistance to T4. Mol. Endocrinol., 15, 2137-2148.

Stahl, J.H., Kendall, S.K., Brinkmeier, M.L., Greco, T.L., Watkins-Chow, D.E., Campos-Barros, A., Lloyd, R.V. & Camper, S.A. (1999) Thyroid hormone is essential for pituitary somatotropes and lactotropes. Endocrinology, 140, 1884-1892.

Steinsapir, J., Harney, J. & Larsen, P.R. (1998) Type 2 iodothyronine deiodinase in rat pituitary tumor cells is inactivated in proteasomes. J. Clin. Invest., 102, 1895-1899.

St Germain, D.L. (1986) hormonal control of a low Km (typeII) iodothyronine 5'-deiodinase in cultured NB41A3 mouse neuroblastoma cells. Endocrinology, 119, 840-846.

Takano, T., Miyauchi, A., Ito, Y. & Amino, N. (2006) Thyroxine to triiodothyronine hyperconversion thyrotoxicosis in patients with large metastases of follicular thyroid carcinoma. Thyroid, 16, 615-618.

Tannahill, L.A., Visser, T.J., McCabe, C.J., Kachilele, S., Boelaert, K., Sheppard, J.A. Franklyn & Gittoes, N.J.L. (2002) Dysregulation of iodothyronine deiodinase enzyme expression and function in human pituitary tumours. Clin. Endocrinol., 56, 735-743.

Wagner, M.S., Morimoto, R., Dora, J.M., Benneman, A., Pavan, R. & Maia, A.L. (2003) Hypothyroidism induces type 2 iodothyronine deiodinase expression in mouse heart and testis. J. Mol. Endocrinol., 31, 541-550.

Zabel, M., Seidel, J., Kaczmarek, A., Surdyk-Zasada, J., Grzeszkowiak, J. & Gorny, A. (1995) Immunocytochemical characterization of two thyroid medullary carcinoma cell lines in vitro. Histochem. J., 27, 859-868.

Zabel, M. & Grzeszkowiak, J. (1997) Characterization of thyroid medullary carcinoma TT cell line. Histol. Histopathol., 12, 283-289.

Table 1 - Oligonucleotide sequences used for RT-PCR and qPCR reactions

Gene	Primers	PCR product size (bp)
DIO 2	F: 5'-ACTCGGTCATTCTGCTCAAG-3' R: 5'-GAGAACTCTTCCACCAGTTTTG-3'	368
TSH-R	F: 5'-GTCCAGAATGTATAGCGGCTC-3' R: 5'-GCTTTTCAGGGACTATGCAATGAA-3'	239
TRα1	F: 5'-TCGAGCACTACGTCAACCAC-3' R: 5'-TCGACTTTCATGTGGAGGAA-3'	127
TRβ	F: 5'-ACCAGAGTGGTGGATTTTGC R: 5'-AAGGGACATGATCTCCATGC-3'	105
β_2-microglobulin	F: 5'-ATCCAGCGTACTCCAAAGATTCAG-3' R: 5'- AAATTGAAAGTTAACTTATGCACGC-3'	623
DIO 2 *	F: 5'-ACTTCCTGCTGGTCTACATTGATG-3' R: 5'-CTTCCTGGTTCTGGTGCTTCTTC-3´	58
Cyclophilin *	F: 5'-GCCGATGACGAGCCCTTG-3 R: 5´TGCCGCCAGTGCCATTATG-3´	156

* primers used in quantitative polymerase chain reaction

Table 2 – Clinical characteristics of the patients with medullary thyroid carcinoma.

Patient No	Sex / age (yr.)	RET mutations	Preoperative basal calcitonin (pg/mL)	Tumor histology	Size (cm)	Stage [a]	Invaded organ
1	F/54	-	93	Unilateral	2.2	2	
2	F/50	-	50	Unilateral	5.0	2	
3	F/60	-	431	Unilateral	2.4	2	
4	F/65	C634Y	1100	Bilateral; C-cell hyperplasia	2.4	2	
5	M/15	C634Y	411	Bilateral; C-cell hyperplasia	1.2	2	
6	F/35	C634Y	43	Bilateral; C-cell hyperplasia	2.5	2	
7	F/37	C634Y	125	Bilateral; C-cell hyperplasia	3.0	3	Cervical lymph nodes
8	F/30	C634Y	882	Bilateral; C-cell hyperplasia	1.2	3	Cervical lymph nodes
9	F/62	C634Y	2258	Bilateral; C-cell hyperplasia	3.0	3	Cervical lymph nodes
10	F/35	C634R	1000	Bilateral; C-cell hyperplasia	1.3	2	
11	F/33	C634Y	3309	Bilateral; C-cell hyperplasia	1.3	2	
12	F/22	C634R	2200	Bilateral; C-cell hyperplasia	1.3	2	

[a] Stage according to the TNM system.

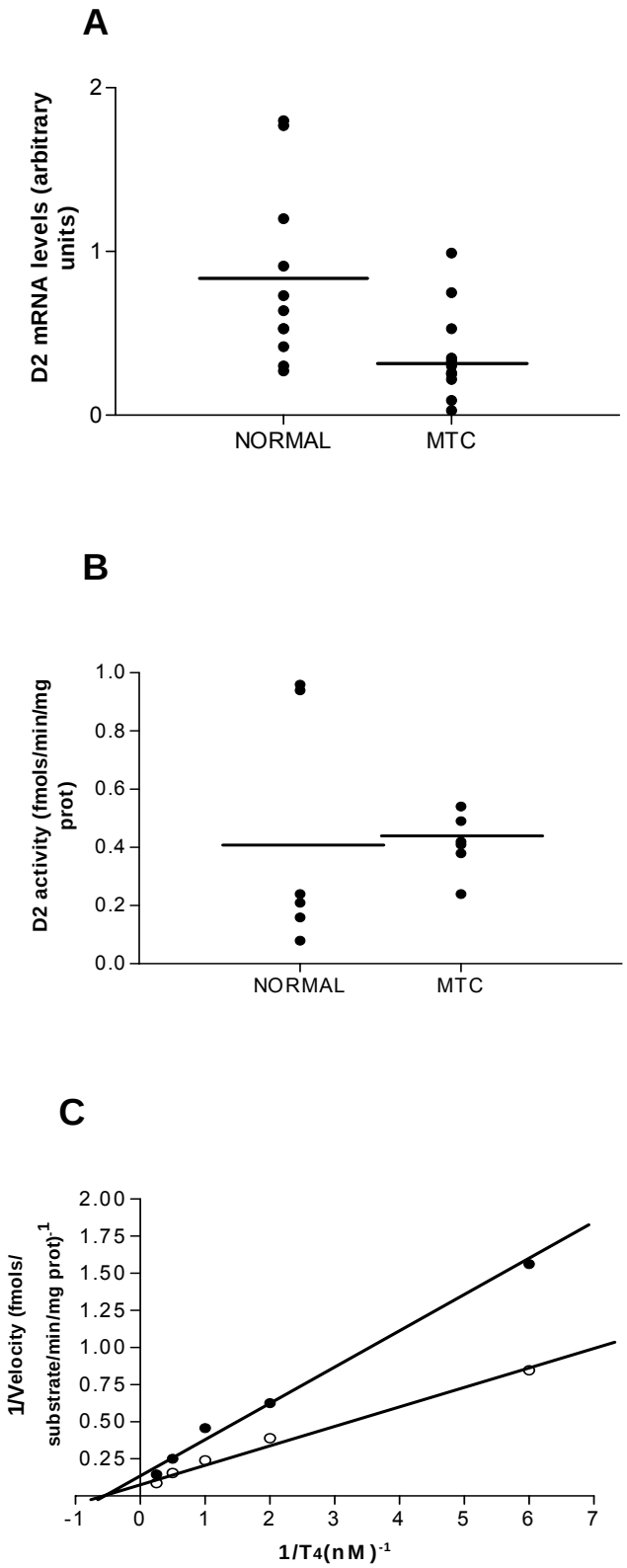

A
D2 mRNA levels (arbitrary units)
2
1
0
NORMAL
MTC
B
D2 activity (fmols/min/mg prot)
1.0
0.8
0.6
0.4
0.2
0.0
NORMAL
MTC
C
1/Velocity (fmols/substrate/min/mg prot)-1
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
-1
0
1
2
3
4
5
6
7
1/T4(nM)-1

FIGURE 1

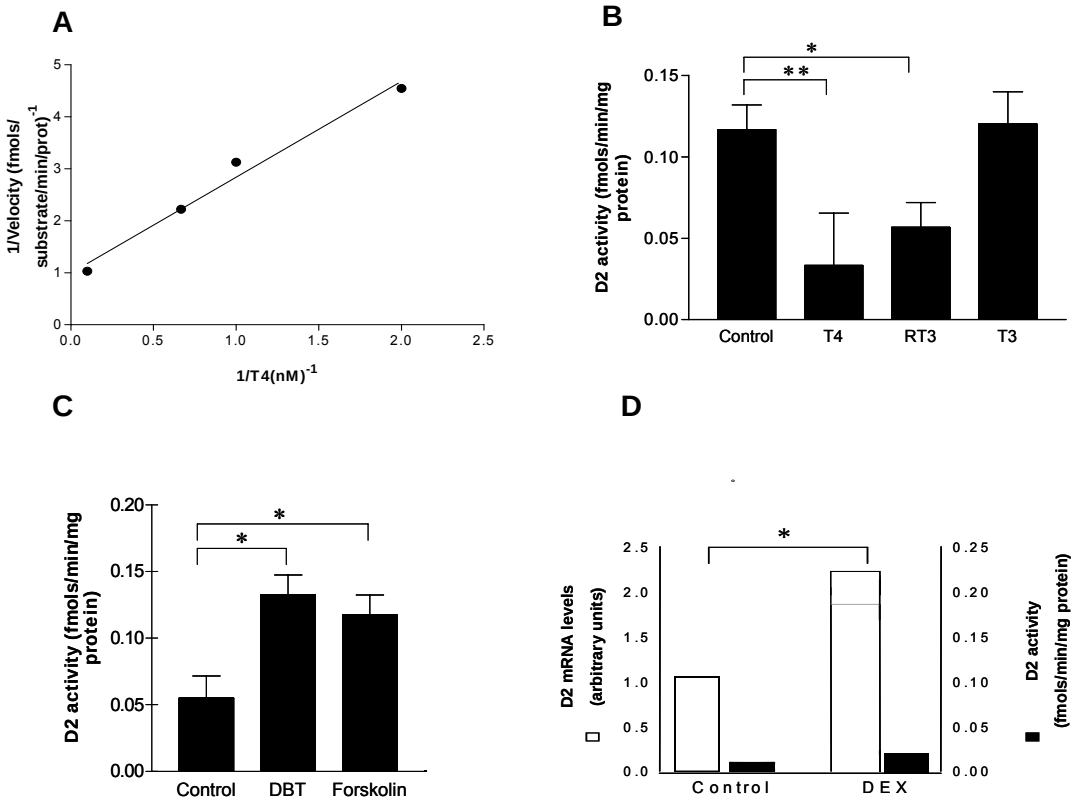

A
1/Velocity (fmols/substrate/min/prot)-1
1/T4(nM)-1
B
D2 activity (fmols/min/mg protein)
Control
T4
RT3
T3
C
D2 activity (fmols/min/mg protein)
Control
DBT
Forskolin
D
D2 mRNA levels (arbitrary units)
D2 activity (fmols/min/mg protein)
Control
DEX

FIGURE 2

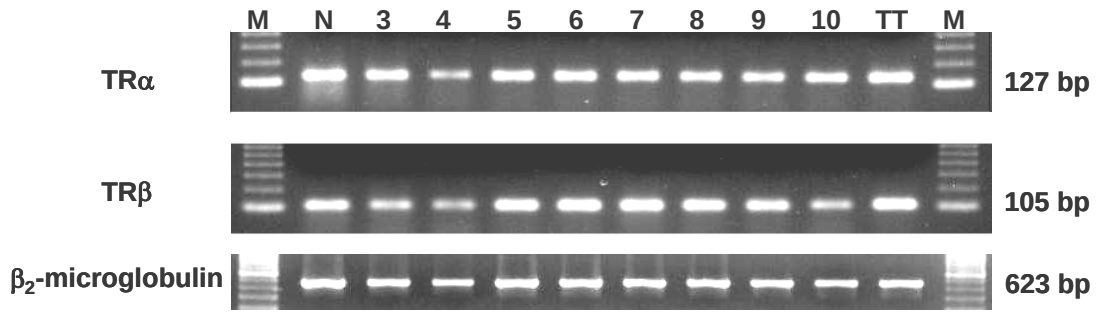

M
N
3
4
5
6
7
8
9
10
TT
M
TRα
127 bp
TRβ
105 bp
β2-microglobulin
623 bp

FIGURE 3

Legends

Fig. 1. D2 expression in human medullary thyroid carcinoma (MTC). **A.** D2 mRNA levels in MTC and corresponding normal tissue. Individual data points for each sample are depicted. Results are expressed in arbitrary units. **B.** D2 enzyme activity in MTC and corresponding normal tissue. Individual data points for each sample are depicted. D2 activity was measured as the release of I^- from 1nM T4 as substrate. **C.** Double-reciprocal plot of D2 activity in normal thyroid tissue (solid circles) and MTC (open circles). D2 activity was measured using different concentrations of ^{125}I-T4 as described in *Materials and Methods*.

Fig. 2. D2 expression in TT cells. **A.** Double-reciprocal plot of D2 activity in TT cells. D2 activity was measured using different concentrations of T4 as described in *Materials and Methods*. **B.** Effects of thyroid hormones on D2 activity in TT cells. TT cells were cultured with thyroid-hormone-depleted medium for 24 h, and then incubated with thyroid hormones for 16 h. **C.** Effects of $(Bu)_2$cAMP and forskolin on D2 activity in TT cells. TT cells were incubated with medium only (control), or medium containing $(Bu)_2$cAMP (10^{-3} M) and forskolin (10^{-5} M) for 16 h. **D.** Effects of DEX on D2 mRNA and D2 activity in TT cells. *, $P < 0.05$; **, $P < 0.01$.

Fig. 3. TRα and TRβ mRNA expression in MTC. RT-PCR analysis of PCR products from 8 samples of MTC (patients 3 – 10 of Table 2). M, Marker; N, Normal thyroid tissue (positive control); TT, TT cells.