UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS

AVALIAÇÃO DA MODULAÇÃO DO RECEPTOR GPER1 EM TIREÓCITOS

PATRÍCIA DE ARAUJO MANFROI

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“Sem suor, sem trabalho, sem muita força de vontade, sem persistência e sem determinação você não chega a lugar nenhum”.

Gilmar Manfroi

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RESUMO

Os mecanismos etiopatogênicos que levam ao desenvolvimento de nódulos e tumores da tireoide ainda não são bem conhecidos. Está bem estabelecido que a prevalência dessas lesões é maior em mulheres e que o estradiol tem efeitos proliferativos nas células da tireoide. Sabe-se também que existem receptores clássicos de estrogênio (ERα e ERβ) nessas células, mas novos receptores estão sendo descritos ao longo do tempo. Assim, em 2005, dois grupos independentes descreveram um receptor de estrogênio acoplado à proteína G (GPER1/GPR30/GPER) como um novo receptor de estrogênio não clássico. Poucos estudos foram realizados para avaliar a modulação desse receptor na tireoide, portanto, o objetivo deste estudo foi avaliar a expressão gênica e proteica do GPER1 em carcinoma papilífero de tireoide e sua localização, em tecidos não neoplásicos e em tumores desse tipo histológico, bem como a associação da expressão gênica do GPER1 e características clínicopatológicas do CPT. As metodologias utilizadas para avaliar a expressão gênica e proteica de GPER1 foram: Reação em Cadeia da Polimerase quantitativa a partir de Transcrição Reversa (RT-qPCR) e imunohistoquímica (IHC), respectivamente. As bases de dados TCGA e GEO foram utilizadas para as análises *in silico*. A expressão de GPER1 foi menor no CPT em comparação aos tecidos tireoidianos não malignos adjacentes, tanto na análise *in silico*, quanto em amostras frescas de CPT. A análise multivariada dos casos descritos na base TCGA mostrou associação independente de níveis mais baixos do gene GPER1 com linfonodos metastáticos, sexo feminino e mutação do gene BRAF. Esses resultados apoiam a hipótese de que o GPER1 tem um papel no CPT e pode ser um alvo potencial para seu tratamento. Mais estudos são necessários para determinar a funcionalidade desses receptores na tireoide, bem como seu papel em condições normais e anormais.

Palavras chave: “GPER1”, “GPR30”, “GPER’, ‘Câncer de Tireoide, ‘Tireoide’, “G1’ e ‘G15’.

ABSTRACT

The etiopathogenic mechanisms that lead to the development of thyroid nodules and tumors are not yet well known. It is well established that the prevalence of these lesions is higher in women and that estradiol has proliferative effects on thyroid cells. It is also known that there are classic estrogen receptors (ERα and ERβ) in these cells, but new receptors are being described over time. Thus, in 2005, two independent groups described a protein G-coupled estrogen receptor (GPER1/GPR30/GPER) as a new non-classical estrogen receptor. Few studies have been done to evaluate the modulation of this receptor in the thyroid, so the aim of this study was to evaluate the gene and protein GPER1 expression in papillary thyroid carcinoma (PTC) and its localization in non-neoplastic thyroid tissues and in PTC, as well as the association of GPER1 gene expression and clinicopathological characteristics of PTC. . The methodologies used to evaluate GPER1 gene and protein expression were quantitative Polymerase Chain Reaction from Reverse Transcription (RT-qPCR) and immunohistochemistry (IHC), respectively. TCGA and GEO databases were used for in silico analyses. GPER1 gene expression was lower in PTC as compared to paired non-malignant thyroid tissues in both *in silico* analysis, as well as in fresh samples of PTC. Multivariate analysis of PTC cases described in the TCGA database revealed an independent association of lower GPER1 gene expression with metastatic lymph nodes, female gender, and BRAF mutation. These results support the hypothesis that GPER1 have a role in PTC and might be a potential target for PTC therapy. Further studies are needed to determine the functionality of these receptors in the thyroid as well as its role in normal and abnormal conditions.

Keywords: "GPER1", "GPR30", "GPER", "Thyroid Cancer", "Thyroid", "G1" and "G15".

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

AMPc	Adenosina Monofosfato cíclico
CPT	Carcinoma Papilífero de Tireoide
EGFR	Receptor do Fator de Crescimento Epidermal
ERα	Receptor de Estrogênio Alfa
ERβ	Receptor de Estrogênio Beta
ERK 1/2	Proteína Quinase Regulada por Sinais Extracelulares 1 e 2
E2	17-β-estradiol
G1	Agonista do GPER1
G15	Antagonista do GPER1
G36	Antagonista do GPER1
GEO	Expressão Genética Omnibus
GPCR	Receptor Acoplado à Proteína G
GPER	Receptor de Estrogênio Acoplado à Proteína G
GPER1	Receptor de Estrogênio Acoplado à Proteína G
GPR30	Receptor Acoplado à Proteína G 30
IHC	Imunohistoquímica
LNM	Metástase Linfonodal
MAPK	Proteína Quinase Ativada por Mitógeno
PCR	Reação em Cadeia da Polimerase
TCGA	O Atlas do Genoma do Câncer
RT-PCR	Reação em Cadeia da Polimerase por Transcriptase Reversa

SUMÁRIO

1. INTRODUÇÃO

Os mecanismos etiopatogênicos que levam ao desenvolvimento dos nódulos e tumores da tireoide ainda não são bem conhecidos. Porém, é fato estabelecido que a prevalência dessas lesões é maior em mulheres, e que nestas as taxas de pico ocorrem mais cedo (Henderson et al, 1982; Enewold et al, 2009). Além disso, sabe-se que o estradiol tem efeitos proliferativos nas células tireoidianas e foi postulado que estes efeitos seriam mediados por receptores clássicos de estrogênio (ERα e ERβ), como por exemplo na Figura 1 (Kavanagh et al, 2010; Santin & Furlanetto, 2011).

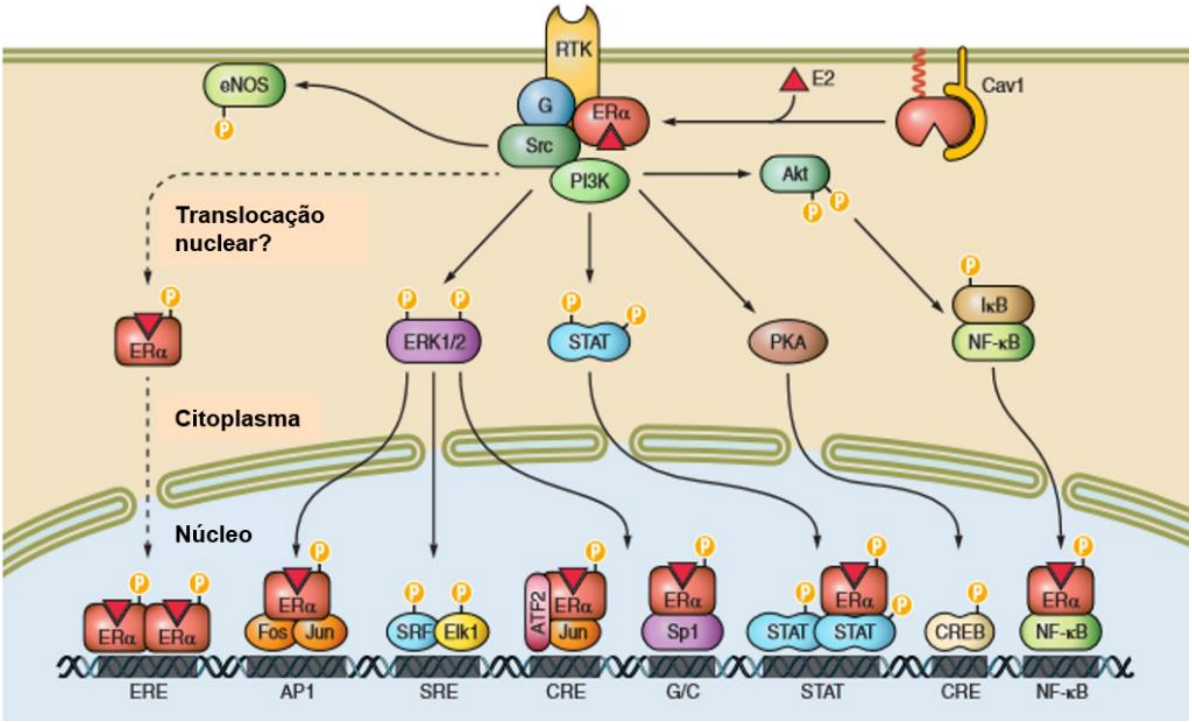

Figura 1: **Mecanismo de ação do estrogênio, mediado por ERα, genômico ou não**. eNOS: óxido nítrico sintetase endotelial; RTK: receptor tirosina quinase; G: proteína acoplada ao G; Src: co-ativador do receptor esteroide; ERα: receptor de estrogênio alfa; E2: 17-β-estradiol; Cav1: caveolina-1; PI3K: fosfatidilinositol 3-quinase; Akt: proteína quinase B; P: fosforilação; ERK: proteína quinase regulada por sinal extracelular 1 e 2; STAT: transdutor de sinal e ativador da transcrição; PKA: proteína quinase; IkB: proteína inibitória kappa b; NF-kB: fator nuclear kappa b; ERE: elementos de resposta ao estrogênio; Fos, Jun, SRF, Elk1, ATF2, Sp1, CREB, AP1, SRE, CRE, G/C: fatores de transcrição. Adaptado de Arnal & Fuentes, 2017.

Contudo, evidências sugerem que efeitos não genômicos também podem ser mediados por receptores não clássicos, como o receptor de estrogênio acoplado a proteína G, chamado GPER1 (Revankar et al, 2005; Thomas et al, 2005). Os primeiros estudos sobre o GPER1 foram publicados entre 1996 a 1998, quando pesquisadores independentes isolaram este receptor órfão em alguns tecidos (McCoy & Permutter, 1996; Owman et al, 1996; Bonini et al, 1997; Carmeci et al, 1997; Feng & Gregor, 1997; Kvingedal & Smeland, 1997; Takada et al, 1997; O´Dowd et al, 1998). A designação GPR30 foi adotada (Carmeci et al, 1997; Feng & Gregor, 1997; Kvingedal & Smeland, 1997; Takada et al, 1997; O´Dowd et al, 1998) após a numeração consecutiva de órfãos GPCR relatados na literatura.

Filardo e colaboradores foram os primeiros a identificar o GPER1 como um potencial receptor de estrogênio de membrana com base em sua pesquisa sobre estimulação de estrogênio da adenililciclase/AMPc e sinalização positiva do GPER1 para EGFR/MAPK (proteínas quinases ativadas por mitógenos) em células de câncer de mama (Filardo et al, 2000; Filardo et al, 2002). Sendo assim, na figura 2 podemos observar que o GPER1, quando ativado, desencadeia vias específicas que podem induzir a proliferação celular, a expressão gênica e o crescimento celular (Jung et al, 2019).

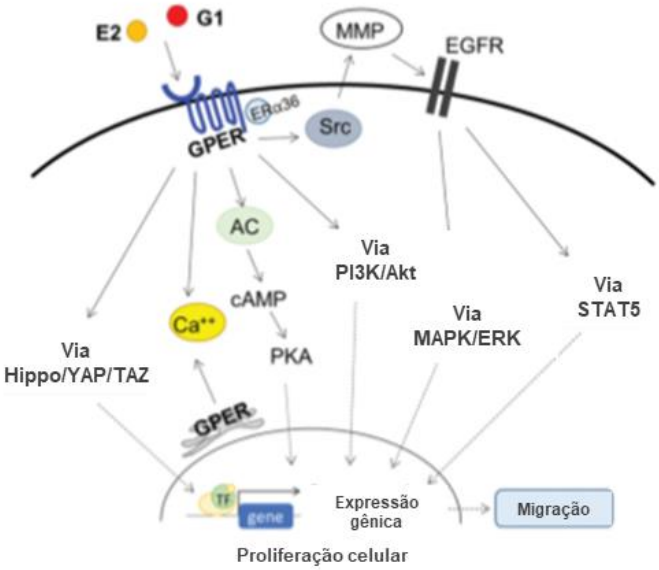

Figura 2: **Mecanismo de ativação do GPER1**. MMP: metaloproteinase de matriz; EGFR: receptor do fator de crescimento epidermal; Src: co-ativador do receptor esteroide; ERα36: receptor de estrogênio alfa 36; Hippo/YAP/TAZ: hippo/proteína associada Sim/coativador transcricional com domínio de ligação a PDZ; Ca^{++}: íon cálcio;

AC: adenilato ciclase: cAMP: adenosina monofosfato cíclico; PKA: proteína quinase; PI3K: fosfatidilinositol 3-quinase; Akt: proteína quinase B; MAPK: proteína quinase ativada por mitógeno; ERK: proteína quinase regulada por sinal extracelular; STAT5: transdutor de sinal e ativador da transcrição 5; TF: fator de transcrição. Adaptado de Jung et al, 2019.

Em 2001, quatro anos antes do GPER1 ser caracterizado como receptor de estrogênio, Manole descreveu que, nas células foliculares de adenoma, bócio e células de linhagem derivadas de câncer de tireoide, o 17β-estradiol ativava efeitos não genômicos, das vias MAPK e ERK 1/2 (Manole et al, 2001), que poderiam ser mediadas por ERα perto de membrana ou por outro receptor (Arnal et al, 2017; Fuentes & Silveyra, 2019). Então, em 2005, dois grupos independentes caracterizaram o GPER1 como um receptor intracelular transmembrana não clássico de estrogênio (Revankar et al, 2005; Thomas et al, 2005).

Logo após, em 2006, o grupo de Eric Prossnitz identificou o composto G1, que foi o primeiro agonista seletivo do GPER1 (Bologa et al, 2006). Em 2009, o mesmo grupo identificou o composto G15 como o antagonista seletivo do GPER1 usando uma combinação de triagem virtual e biomolecular. De estrutura similar ao agonista G1, esse composto se liga ao GPER1, mas não ao ERα ou ERβ (Dennis et al, 2009). Devido a esses estudos, observou-se que outros compostos podem atuar como agonistas do GPER1, devido as suas semelhanças estruturais com o estradiol, como o 4,4',4-[4-propil-(1H)-pirazol-1,3,5-triyl]tris-fenol (PPT), 17β-estradiol, genisteína, bisfenol A, diclorodifeniltricloroetano (DDT), tamoxifeno, raloxifeno, ICI182,780. Não obstante, observou-se que, além do G15, outro ligante poderia atuar como antagonista do GPER1, o G36, como vemos na figura 3 (Prossnitz & Barton, 2014).

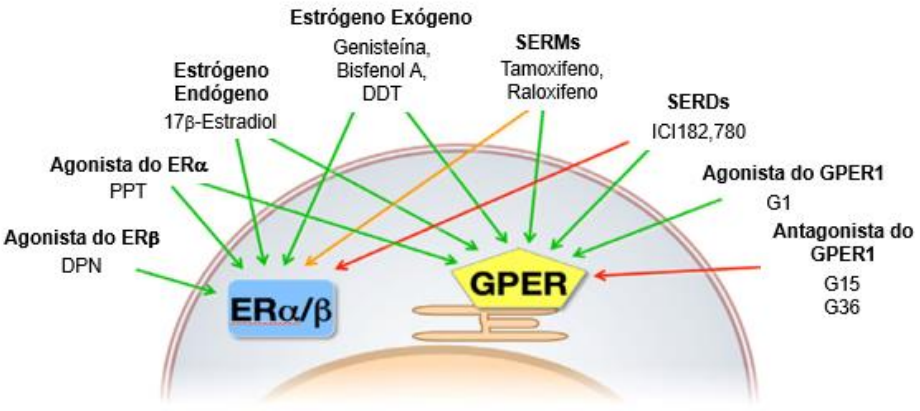

Figura 3: **Ligantes do GPER1 e do ERα/β.** As flechas verdes indicam que os ligantes são agonistas, as flechas vermelhas indicam que os ligantes são antagonistas e as flechas laranjas indicam que os ligantes são agonistas parciais. DPN: 2,3-Bis (4-hidroxifenil)-propionitrila; PPT: 4,4',4-[4-propil-(1H)-pirazol-1,3,5-triyl]tris-fenol; BPA: bisfenol A; DDT: diclorodifeniltricloroetano; SERMs: moduladores seletivos do receptor de estrogênio; SERDs: sub-reguladores seletivos do receptor de estrogênio. Adaptada de Prossnitz & Barton, 2014.

No entanto, ainda não há um consenso quanto à localização celular do GPER1, pois, nos estudos de Eric Prossnitz os autores concluíram que este é expresso e ativado intracelularmente (Revankar et al, 2005; Revankar et al, 2007), em contraste com estudos que demonstraram que ele é ativado na superfície celular (Thomas et al, 2005; Filardo et al, 2007).

Dados publicados pelo nosso grupo demonstraram uma redução da expressão gênica e da proteína deste receptor em bócio multinodular (Weber et al, 2015). Porém, há poucos estudos sobre a modulação e efeitos da ativação do GPER1 em carcinoma papilífero de tireoide (CPT) (Tang et al, 2014) ou em linhagens celulares derivadas de CPT (Kumar et al, 2010; Zhang et al, 2017).

Sendo assim, os objetivos desse estudo foram avaliar a influência da modulação do GPER1 e sua localização em amostras de CPT e tecidos não malignos circunjacentes, bem como a associação da expressão gênica do GPER1 no CPT com suas caraterísticas clinicopatológicas, contribuindo para esclarecer mecanismos envolvidos na patogênese do CPT e/ou possibilitar novas abordagens terapêuticas ou de valor prognóstico.

2. REVISÃO DA LITERATURA

A revisão da literatura se encontra no artigo “GPER1 in the thyroid: a systematic review”.

2.1 Estratégias para localizar e selecionar as informações

Artigos em inglês foram pesquisados nas bases de dados PubMed, Scielo e Cochrane Library utilizando os Medical Subjective Heading (MeSH) termos "GPER1”OR “GPR30” OR “GPER" AND "thyroid" no título ou resumo. Foram incluídos artigos publicados desde o início até junho de 2019 e revisados por dois dos autores. Outras fontes de obtenção de artigos foram utilizadas, como textos de referência cruzada.

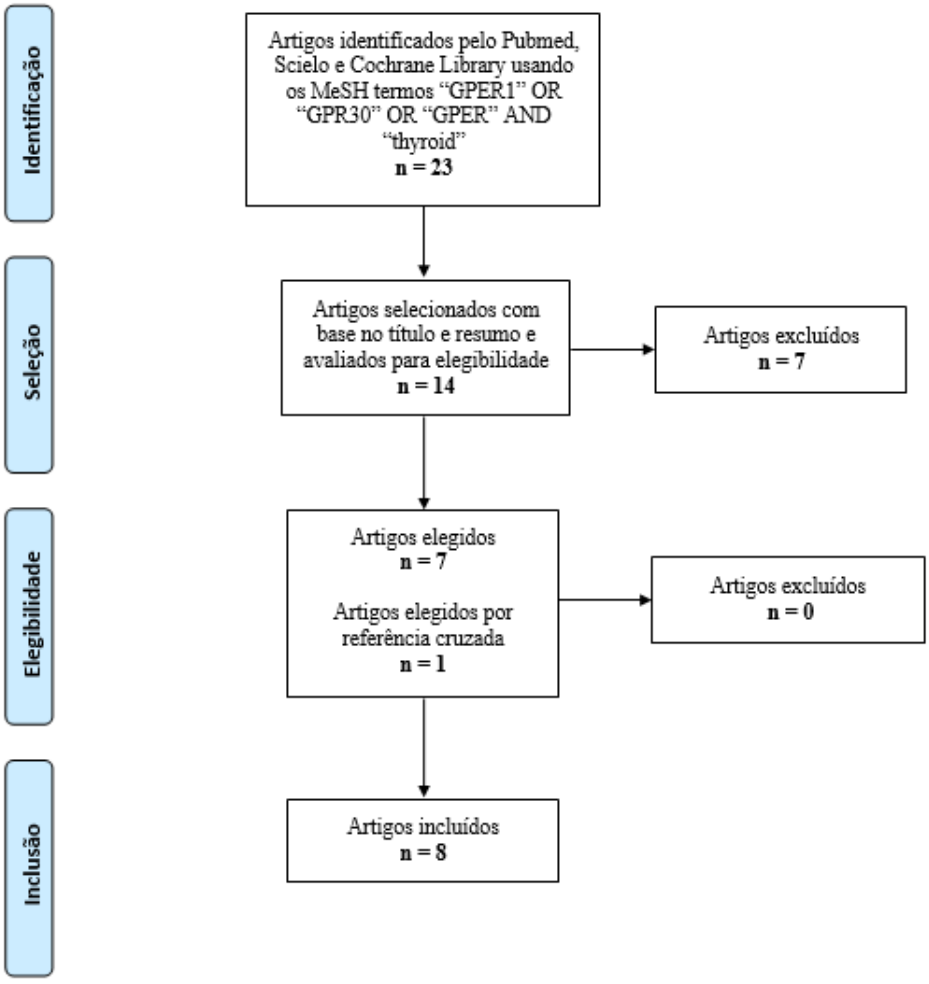

Figura 4: Processo de seleção dos estudos para a revisão sistemática.

3. MARCO CONCEITUAL

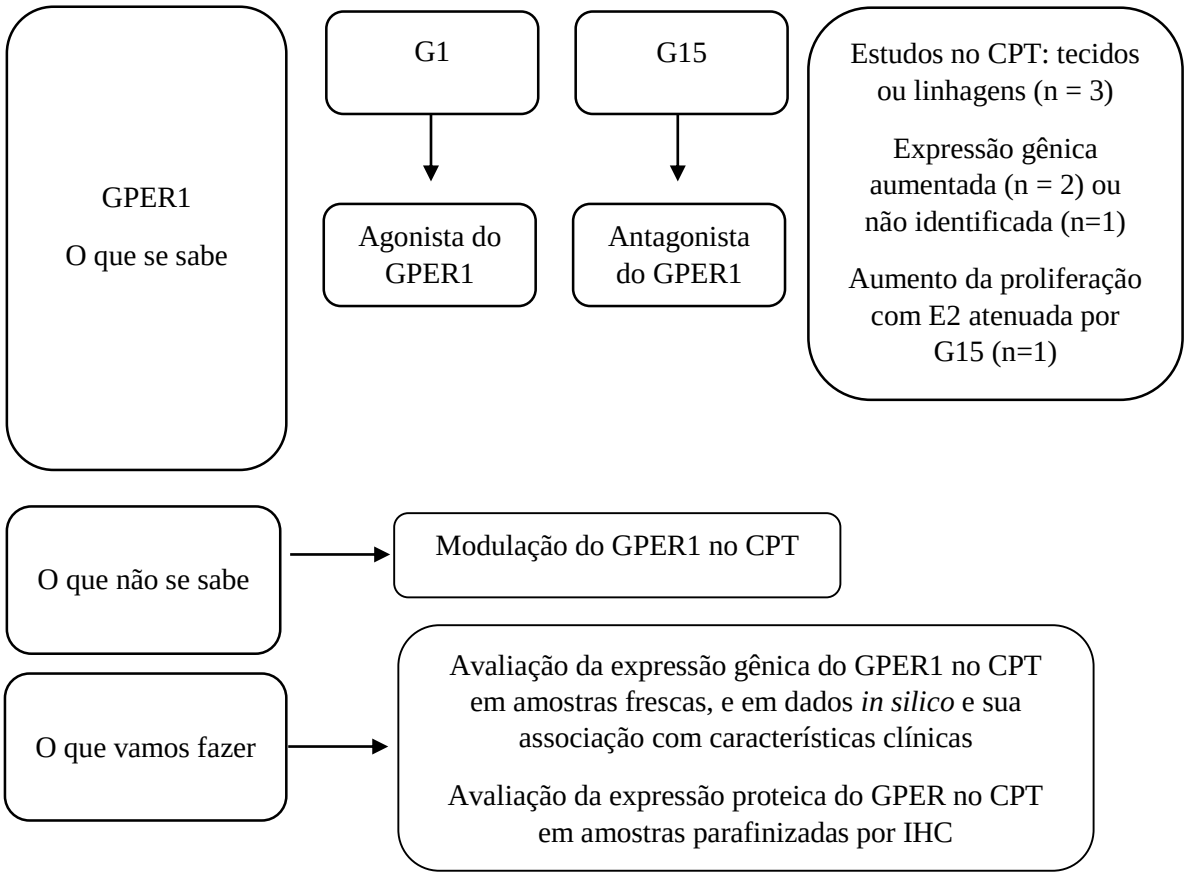

Figura 5: Marco conceitual do GPER1.

Expressão do gene GPER1 na tireoide

A expressão do gene GPER1 foi avaliada em seis estudos em tecidos tireoidianos normais e anormais. Feng & Gregor, 1997 descreveram uma expressão mais alta de GPER1 em amostras comerciais de tireoide e outros tecidos. Tang et al, 2014 e Zhao et al, 2015 em dois artigos diferentes do mesmo grupo, encontraram essa expressão semelhante em bócio nodular e tireoide normal. Curiosamente, o número de amostras de tireoide normal e bócio nodular foram os mesmos em ambos os artigos. Weber et al. foram capazes de amplificar a expressão de GPER1 em 100% das amostras normais da tireoide, o que foi maior do que no bócio (Weber et al, 2015). Essas diferenças provavelmente estão relacionadas a detalhes metodológicos. Tang et al, 2014 e Zhao et al, 2015 encontraram uma expressão mais alta de GPER1, respectivamente, em PTC e FTC, do

que no tecido tireoidiano normal. Além disso, o estudo de Tang et al. mostrou que a expressão do gene GPER1 era maior em PTC com metástase linfonodal (LNM), sugerindo maior expressão de GPER1 em PTC como fator de risco para LNM (Tang et al, 2014). Dois outros estudos investigaram a expressão de GPER1 em linhagens celulares de câncer de tireoide: não houve expressão nas células KAT5 e NPA87, derivadas de CPT, mas houve expressão nas células WRO, derivadas de (Kumar et al, 2010) e nas células BHP 10-3, derivadas de CPT (Zhang et al, 2017).

Expressão da proteína GPER1

A expressão da proteína GPER1 foi avaliada em cinco estudos em tecidos tireoidianos normais e anormais e não há consenso sobre sua expressão em tireoide normal e bócio. Quando avaliado por IHC, foi semelhante no bócio nodular e na tireoide normal (Tang et al, 2014; Zhao et al, 2015). No entanto, a expressão da proteína GPER1 por Western blot foi encontrada em 100% das amostras normais da tireoide e em 72% das amostras de bócio (Weber et al, 2015). Quando estudado em amostras de câncer ou linhagens, foi maior no CPT, FTC e adenoma da tireoide, em comparação com a tireoide normal (Tang et al, 2014; Zhao et al, 2015) e a expressão foi positiva nas células BHP 10-3 (Zhang et al, 2017), WRO e FRO (Zhu et al, 2017).

Proliferação

Três artigos avaliaram a proliferação celular induzida por estradiol e mediada por GPER1. Em um deles, Vivacqua et al., não encontraram ERα nas células ARO e uma variante de ERα não ativada por E2, nas células WRO e FRO (Vivacqua et al, 2006). Todas essas três linhagens celulares, quando tratadas com E2, proliferaram e mostraram um aumento na expressão do gene e proteína c-fos. Como estes efeitos foram impedidos pelo silenciamento de GPER1, eles foram atribuídos à ativação mediada por GPER1 da via da proteína quinase ativada por mitogênio (MAPK). Zhang et al. trataram BPH-10-3 com E2, induzindo concentração e proliferação dependente do tempo, e rápido aumento na fosforilação de AKT/mTOR, que foram revertidos por G15, de modo que atribuíram esses efeitos à sinalização mediada por GPER1 (Zhang et al, 2017). Zhu et al. relatou que E2 e G1 induziram proliferação, invasão e migração de células de câncer de tireoide WRO e FRO humanas, que possuem GPER1 endógeno, levando a uma rápida ativação de ERK/AKT e, depois, a translocação nuclear de NF-κB, aumentaram a expressão da

ciclina A e D1 e secreção de IL-8 (Zhu et al, 2017). A proliferação, invasão e migração foram suprimidas por inibidores específicos para GPER1, ERK, AKT e NF-κB, ou por silenciamento de GPER1. Os resultados desses estudos sugeriram que E2 poderia induzir o crescimento celular, por vias mediadas pelo GPER1, e concordaram com estudos em outros tipos de câncer, como mama, pulmão, cabeça, pescoço, neuroblastoma, próstata e carcinomas do ovário (Meng et al, 2016; Liu et al, 2016). No entanto, nas células H295R, derivadas de carcinoma adrenocortical, o G1 teve um efeito inibidor do crescimento *in vitro* e em um modelo in vivo de xenoenxerto. Esses eventos exigiram ativação sustentada do ERK 1/2. O silenciamento de GPER1 por um shRNA específico reverteu parcialmente os efeitos inibitórios do crescimento de células mediadas por G1, sem afetar a ativação de ERK (Chimento et al, 2015). Efeitos semelhantes foram observados em 3 linhagens de câncer de próstata (PC): G1 inibiu o crescimento das células PC-3, DU-145 e LNCaP *in vitro* e em modelos de xenoenxerto de células PC-3, via ativação ERK 1/2 e p21 (Chan et al, 2010). Além disso, a expressão de GPER1 foi maior em células PC resistentes à castração, que tiveram seu crescimento inibido por G1 via necrose, do que em células PC sensíveis a andrógenos (Lam et al, 2014). A partir desses dados, a ativação do GPER1 pode ter ações opostas em células diferentes.

Localização

Outro ponto de discussão na literatura tem sido a localização desse receptor. Como a sinalização mediada por GPER1 foi alterada em muitos estados patológicos (Prossnitz & Barton, 2011), esse receptor representa um alvo importante para o desenvolvimento de novos medicamentos (Prossnitz et al, 2008). Em dois estudos desta revisão, o GPER1 foi identificado na membrana celular e no citoplasma das células da tireoide por IHC (Tang et al, 2014; Zhao et al, 2015); outro estudo usando coloração por imunofluorescência foi capaz de identificar a proteína GPER1 apenas na membrana celular, na presença ou ausência de E2 (Zhang et al, 2017). Em outros tecidos, o GPER1 foi relatado na membrana plasmática (Thomas et al, 2005; Revankar et al, 2005; Lucas et al, 2010; Wang et al, 2010; Funakoshi et al, 2006; Sanden et al, 2011), no retículo endoplasmático (Revankar et al, 2005; Kleuser et al, 2008; Lin et al, 2009; Otto et al, 2008; Revankar et al, 2007) e no aparelho de Golgi (Sakamoto et al, 2007). Outra teoria altamente apoiada, o GPER1, passa por um processo de endocitose após sua estimulação, conforme revisão (Gaudet et al, 2015). Não há estudos sobre a localização e modulação do GPER1 na

cultura primária de tireócitos normais, o que poderia esclarecer seu papel na tireoide normal. Recentemente, esses estudos foram sugeridos para diminuir as contradições, considerando a variação do fenótipo nas linhagens celulares, mesmo sob condições fisiológicas ideais (Barton, 2012).

4. JUSTIFICATIVA

Considerando a heterogeneidade do carcinoma papilar de tireoide, bem como de outros tipos tumorais, sabe-se que quanto maior a disponibilidade de fatores prognósticos, melhor será a estratificação dos níveis de risco tumoral, reduzindo potencial de morbidade e diminuição dos custos financeiros relacionados a tratamentos. Por outro lado, pacientes inicialmente considerados com baixo risco, podem apresentar uma resposta incompleta ao tratamento que, sem um tratamento personalizado, podem não ser devidamente tratados. Este é o cenário que nos estimula a buscar um melhor entendimento dos mecanismos moleculares que possam estar favorecendo ou não à progressão tumoral bem como a busca de potenciais alvos terapêuticos e com valor prognóstico.

5. OBJETIVO GERAL

Avaliar a expressão gênica do GPER1 em amostras de CPT e tecidos circunjacentes e nos bancos de dados TCGA e GEO *in silico* e sua associação com dados clinico-patológicos.

5.1 Objetivos específicos

a) Avaliar *in silico* a expressão do receptor GPER1 em CPT e em seus respectivos tecidos circunjacentes não malignos;
b) Avaliar a expressão gênica e proteica do receptor GPER1 em CPT e em seus respectivos tecidos circunjacentes não malignos em amostras de tecidos frescos por RT-qPCR;
c) Avaliar a correlação da expressão gênica do GPER1 com dados clinico-patológicos em amostras de CPT;
d) Revisar sistematicamente a literatura avaliando dados sobre a expressão, localização e atividade do GPER1, bem como mecanismos mediadores de seus efeitos na tireoide.

6. REFERÊNCIAS BIBLIOGRÁFICAS

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7. ARTIGOS

7.1 GPER1 in the thyroid: a systematic review

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Title page

GPER1 in the thyroid: a systematic review

Short title: GPER1 in the thyroid

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Abstract

Background: Thyroid cancer incidence has been increasing, acquiring a greater importance in health, especially of women, who are more frequently affected. As 17-β-estradiol (E2) has been shown to have a proliferative effect on benign and malignant thyroid cells, G protein-coupled estrogen receptor (GPER1) could have a role on the pathogenesis of thyroid cancer. *Objective:* To evaluate data on GPER1 in the thyroid. *Data sources:* PubMed, Scielo and Cochrane Library databases were searched, using the keywords *GPER1 or GPR30 or GPER* and *thyroid*, since the inception until Jun, 2019. Other sources were used, as cross-referencing.

Study Selection: All studies which evaluated GPER1 *GPER1 or GPR30 or GPER* in the thyroid. *Data Extraction:* From 23 articles identified, eight studies were included: one in commercial samples of human thyroid, four in human thyroid cancer cell lines, and three in human samples of benign and/or malignant thyroid diseases. *Data Synthesis:* GPER1 gene and protein expression were described, respectively, in six and five studies, and the results varied according to the study. In three studies, increased proliferation of four thyroid cancer cell lines were induced by E2, with evidences suggesting that GPER1 at least partially mediated growth in these cells. GPER1 was identified in the cell membrane, in three studies, and in the cytoplasm in two studies. *Conclusions:* The paucity of studies about GPER1 in the thyroid, as well as methodological differences between them, precludes firm conclusions about GPER1 role in the thyroid, although there are some evidences of GPER1-induced proliferation of thyroid cancer cells.

Key words: GPER1, GPR30, GPER, thyroid cancer, thyroid

Introduction

The incidence of thyroid cancer has been increasing, acquiring a greater importance in health, especially of women, who are more frequently affected [1-6]. Differentiated thyroid cancers are derived from thyroid follicular cells and are more commonly papillary thyroid carcinomas (PTC) [7].

As 17-β-estradiol (E2) has been shown to have a strong proliferative effect on primary culture of thyroid cells from goiter and adenomas [8], in a normal thyroid transformed cell line (Nthy-ori 3-1)[9], and in several cell lines derived from thyroid carcinoma: BCPAP [9], HTC-TSHr [8], KAT5, WRO and NPA87 [10,11], as reviewed by Santin in 2011 [12], it could have a role on the pathogenesis of thyroid cancer. E2 classically acts through its nuclear receptors (ERα and ERβ) causing genomic effects; nevertheless, non-genomic effects of nuclear receptors, through rapid signaling events, have been described [13].

These rapid E2 effects led to the search for non-nuclear receptors, which could explain non-genomic signaling mediated by E2. In 1997, an orphan G protein coupled receptor (GPCR), called constitutively expressed peptide-like (CPER), was described [14] and started a new line of research; in 2000, Filardo et al. published that this receptor, now called GPR30, was able to induce proliferation in ERα negative breast cancer [15]. In 2005, GPR30 was shown to bind estrogen, mediating rapid cell signaling, by two independent groups, which characterized a non-classical ER [16,17]. In consequence, in 2007, it was renamed G protein-coupled estrogen receptor, GPER1, by IUPHAR (International Union of Basic and Clinical Pharmacology) [18].

Since then, GPER1 alterations have been described in cancer of several organs, including thyroid (Barton et al. 2018). Therefore, studying the role of GPER1 in the thyroid could be useful to understand the regulation of thyroid follicular cells in health and disease.

Objectives

Evaluate data on the expression, location, and activity of GPER1, as well as mechanisms mediating its effects in the thyroid.

Methods

This review followed the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) guidelines [19]. The Population, Intervention, Comparison, Outcomes, and Study Design (PICOS) criteria, described in Table 1, were used to formulate and narrow the focus of the following research questions: Is GPER1 present in the thyroid, and where? How its regulation is affected by disease? What are its actions, and which mechanisms mediate it?

Table 1: Population, Intervention, Comparison, Outcomes, and Study Design (PICOS) criteria

Population	Thyroid cells or tissue
Intervention	None or effects of agonists and/or antagonists of GPER1
Comparison	Presence or modulation of GPER1
Outcomes	Localization of GPER1, expression, and actions of GPER1
Study	Systematic review

Information Sources and Search

Articles in English language were collected from PubMed, Scielo, and Cochrane Library using the keywords *GPER1 or GPR30 or GPER* and *thyroid*, since the inception until Jun, 2019, and reviewed by at least two of the authors. Other sources were used, as cross-referencing.

Criteria for inclusion and exclusion of studies

All studies about *GPER1 or GPR30 or GPER* in the thyroid were included. Studies were excluded if they did not contain information about *GPER1 or GPR30 or GPER* in the thyroid.

Data collection process

At least two authors screened the titles and abstracts identified from the electronic search. In the event the reviewers disagreed about an abstract, the abstract was reevaluated, and disagreement was resolved by discussion and consensus. Full texts of selected articles were obtained and were again evaluated for eligibility by two authors. Additional papers were identified by cross-referencing. Study quality was not rated because of differences in study designs. No meta-analysis was performed.

Results

Study selection

From 23 titles and abstracts, 14 articles were selected for full-text review. Of these, seven were excluded for not containing information about GPER1 in the thyroid. Thus, seven studies were included, as well as one other obtained by cross-referencing (Figure 1).

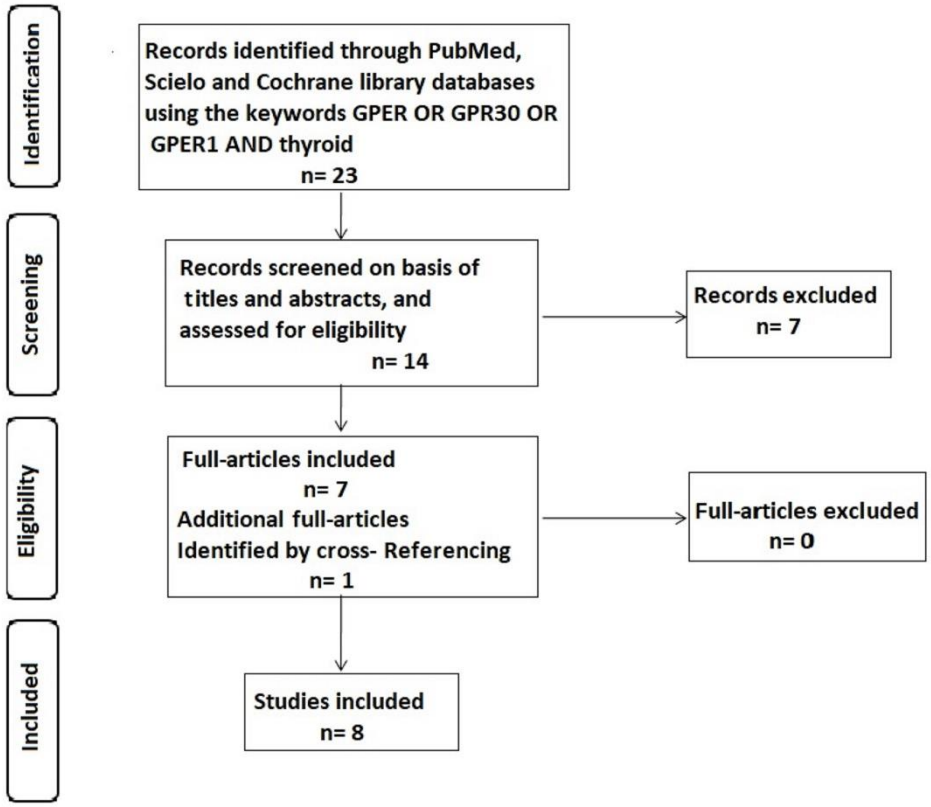

Figure 1: Process of study selection for the systematic review.

Study characteristics

All 8 studies were classified into the following groups: 1 study in commercial samples of human tissues, 4 studies in human thyroid cancer cell lines, and 3 studies in human samples of benign or malignant thyroid diseases.

Results of individual studies

In 1997, Feng & Gregor firstly described higher expression levels of a new gene, called CEPR, in the thyroid, as compared to other body tissues, in commercial samples of human tissues, measured by PCR, Southern blot, and Northern blot [14].

Vivacqua et al. described estradiol-induced proliferation and *c-fos* expression in thyroid carcinoma cell lines, devoid of ERα (ARO: derived from anaplastic carcinoma) or with ERα unable to be activated by E2 (WRO: derived from follicular carcinoma, and FRO: derived from anaplastic carcinoma), which was prevented by silencing GPER1 by transfection of GPER1/AS [20].

Kumar et al. studied GPER1 expression with RT-qPCR and were not able to identify it in NPA87 and KAT5 cells, which are PTC derived cell lines, and found low expression of GPER1 in WRO cells, as compared to MCF-7 cells, a breast cancer cell line [10].

On the other hand, another group was able to identify GPER1 protein expression by immunohistochemistry (IHC) in about half of the analyzed samples of normal thyroid and nodular thyroid hyperplasia, which was higher in PTC ($p<0.001$). When comparing GPER1 protein expression in PTC, it was higher in larger tumors ($p=0.049$). According to the IHC pattern, GPER1 protein was located in the cell membrane and cytoplasm. They also evaluated GPER1 gene expression, by RT-qPCR, and found increased expression in PTC ($p<0.001$), as compared to normal thyroid or goiter, which were similar ($p=0.106$). PTC with lymph node metastasis had higher GPER1 gene expression ($p<0.001$) (Tang, 2014). In the next year, the same group published a paper describing the results of GPER1 protein identified by IHC staining in normal and diseased thyroid: follicular thyroid carcinoma (FTC) had higher expression than follicular thyroid adenoma (FTA) ($p<0.001$), which was higher than in goiter and normal thyroid ($p<0.001$). The GPER1 gene expression by RT-qPCR was higher in FTC ($n=15$), as compared to FTA ($n=10$) ($p<0.001$), which was higher than in goiter ($p<0.001$). Results were similar, when comparing goiter and normal thyroid ($p=0.123$), and when comparing widely invasive FTC with minimally invasive FTC ($p=0.179$). The GPER1 location was also found in cell membrane and cytoplasm [21].

In addition, in 2015, our group found GPER1 gene expression in all studied samples of normal thyroid ($n=16$) and goiter ($n=19$), although lower in goiter. Similarly, GPER1 protein expression, by Western blot, was lower in goiter ($p=0.002$), being detected in 9/13 and 15/15, respectively, of goiter and normal thyroid, suggesting that its decrease could be involved in the pathogenesis of goiter [22].

Zhang et al. observed an E2-induced increase in the proliferation of BHP10-3 cells, a PTC derived cell line, which was dependent on concentration and time, with rapid phosphorylation of protein kinase B (AKT)/mTOR; GPER1 gene and protein expression were enhanced by E2, G1, and ICI, all agonists of GPER1. Importantly, G-15, antagonist of GPER1, significantly decreased E2 effects. They identified, by immunofluorescence assay, GPER1 protein on the cell membrane, which increased after exposure to E2 [23].

Zhu et al. identified GPER1 protein, by Western blot, in two thyroid cancer cell lines, WRO and FRO. They were able to demonstrate stimulatory effects of estradiol and G-1 on the proliferation, invasion and migration of WRO and FRO cells. Extracellular regulated kinase (ERK) and AKT effects were attenuated by G-15, which suggested that these effects were mediated by GPER1 rapid activation of ERK/AKT, leading to nuclear translocation of NF-κB and, then, increasing cyclin A and D1 expression, and IL-8 secretion [24].

Table 2: Studies of G protein-coupled estrogen receptor 1 (GPER1) on human thyroid cells.

Author year	Focus of investigations	Methods	Samples	Outcomes
Feng 1997	To clone novel members of the GPCR superfamily	PCR Southern blot Northern blot	Commercial samples of human tissues	**CEPR gene expression** Thyroid>other body tissues
Vivacqua 2006	Study proliferation and protein expression in estrogen target genes of cancer cells	Western blot RT-qPCR Proliferation assay Transfection of GPR30/AS	Cell lines ARO, FRO, WRO	**Erα** ARO: no; WRO, and FRO: variant not activated by E2 **Proliferation, and c-fos protein and gene expression** E2 induced all the above, which was prevented by silencing GPR30
Kumar 2010	Study the presence of GPER1 in thyroid cancer cells lines	RT-qPCR	Cell lines WRO NPA87 KAT5 MCF-7	**GPER1 gene expression** KAT5 and NPA87: none MCF-7 > WRO
Tang 2014	Analyze GPER1 protein and gene expression in the thyroid and evaluate its association with clinicopathological indicators in cancer	IHC	129 PTC 61 nodular goiter 118 normal thyroid	**GPER1 protein expression** ~50% of nodular goiter and normal thyroid in PTC PTC > nodular goiter = normal thyroid Associated with larger size in PTC **GPER1 location**: cell membrane and cytoplasm
		RT-qPCR	30 PTC 10 nodular goiter 10 normal thyroid	**GPER1 gene expression** PTC > nodular goiter = normal thyroid Associated with lymph node metastasis in PTC
Weber 2015	Investigate GPER1 protein and gene expression in normal thyroid and goiter	Western blot	15 normal thyroid 13 goiter	**GPER1 protein expression** Detected in 100% and 72%, respectively, of normal thyroid and goiter samples

				Normal thyroid > goiter
		RT-qPCR	16 normal thyroid 19 goiter	**GPER1 gene expression** Detected in all samples of normal thyroid and goiter Normal thyroid > goiter
Zhao 2015	Analyze GPER1 protein and gene expression in FTC, FTA, nodular goiter and normal thyroid	IHC	128 FTA 71 MI-FTC 35 WI-FTC 115 nodular goiter 90 normal thyroid	**GPER1 protein expression** FTC > FTA > nodular goiter = normal thyroid MI-FTC = WI-FTC **GPER1 location**: cell membrane and cytoplasm
		RT-qPCR	10 FTA 10 MI-FTC 5 WI-FTC 10 nodular goiter 10 normal thyroid	**GPER1 gene expression** FTC > FTA > nodular goiter = normal thyroid MI-FTC = WI-FTC
Zhang 2017	Evaluate the effect of E_2 on ER expression, cell proliferation and AKT/mTOR signaling in cancer cells	CCK-8 proliferation assay Western blot RT-qPCR	Cell line: BHP10-3	**Proliferation** Increased by E2 and dependent on concentration and time E2 quickly phosphorylated proteins AKT/mTOR, which was increased by G1 and reverted by G15 **GPER1 protein and gene expression** Enhanced by E2, G1 and ICI182780 G15 reverted E2 effect
Zhu 2017	Evaluate the role of GPER1 signaling in the proliferation, invasion, and migration of cancer cells	Immuno fluorescence assay Western blot Invasion assay Migration assay	Cell lines WRO FRO	**GPER1 location**: cell membrane, increased with E2 **GPER1 protein expression** Positive in WRO and FRO **Proliferation and Signaling** E2 and G1 → Rapid activation of ERK and AK →Nuclear translocation of NF-κB

		BrdU proliferation assay		→ Increased expression of cyclins A and D1 → Secretion of IL-8 → Proliferation, invasion, and migration of cells, attenuated by G15

Abbreviations: GPCR: G protein-coupled receptor; CEPR: constitutively expressed peptide-like receptor, and GPR30: G protein coupled receptor 30, former names of GPER1; GPER1/AS: antisense GPR30/GPER1; PCR: polymerase chain reaction; RT-qPCR: quantitative reverse transcription PCR; Human cancer derived cell lines: ARO and FRO: anaplastic thyroid carcinoma; WRO: follicular thyroid carcinoma; KAT5, NPA87 and BHP10-3: papillary thyroid carcinoma (PTC), and MCF-7: breast cancer. E2: 17 β-estradiol; ERα: estrogen receptor alpha; IHC: immunohistochemistry; FTC: follicular thyroid carcinoma; FTA: follicular thyroid adenoma; MI-FTC: minimally invasive FTC; WI-FTC: widely invasive FTC; CCK-8: cell counting kit-8; G15: GPER1 antagonist; ICI182780: ER antagonist and GPER1 agonist; G1: GPER1 agonist; IL-8: interleukin-8.

Discussion

GPER1 gene expression

GPER1 gene expression was evaluated in six studies in normal and abnormal thyroid tissues. Feng & Gregor described a higher expression of GPER1 in commercial samples of thyroid and other body tissues. Tang et al. and Zhao et al., in two different articles of the same group, found this expression to be similar in nodular goiter and normal thyroid [25,21]. Interestingly, the number of samples of normal thyroid and nodular goiter were the same in both papers. Weber et al. were able to amplify GPER1 expression in 100% of normal thyroid samples, which was higher than in goiter. These differences are probably related to methodological details, such as the internal calibrator for gene normalization, the primers sequences for GPER1 amplification or the method used to quantify GPER1 expression. Tang et al. and Zhao et al. found a higher expression of GPER1, respectively, in PTC and FTC, than in normal thyroid tissue. In addition, the study of Tang et al. showed that GPER1 gene expression was higher in PTC with lymph node metastasis (LNM), suggesting higher GPER1 expression in PTC as a risk factor for LNM [25]. Two other studies investigated GPER1 expression in thyroid cancer cell lines: it was negative in KAT5 and NPA87 cells, and positive in WRO cells [10] and BHP 10-3 cells [23].

GPER1 protein expression

GPER1 protein expression was evaluated in five studies in normal and abnormal thyroid tissues and there is no consensus about its expression in normal thyroid and goiter. When evaluated by IHC, it was similar in nodular goiter and normal thyroid [25,21]. Nevertheless, GPER1 protein expression by Western blot was found in 100% of normal thyroid samples, and in 72% of goiter samples [21]. Whe studied in cancer samples or cancer cell lines, it was higher

in PTC, FTC, and thyroid adenoma, as compared to normal thyroid [25,21] and it was positive in BHP 10-3 cells [23], WRO, and FRO cells [24].

Proliferation

Three articles evaluated cell proliferation induced by E2 and mediated by GPER1. In one of them, Vivacqua et al., found no ERα in ARO cells, and a variant of ERα not activated by E2, in WRO and FRO cells [20]. All these three cells lines, when treated with E2, proliferated, and showed an increase in *c-fos* gene and protein expression. As these effects were prevented by silencing GPER1, they were attributed to GPER1 mediated activation of the mitogen-activated protein kinase (MAPK) pathway. Zhang et al. treated BPH-10-3 with E2, inducing concentration and time-dependent proliferation, and rapid increase in phosphorylation of AKT/mTOR, which were reverted by G15, so they attributed these effects to GPER1-mediated signaling [23]. Zhu et al. reported that E2 and G1 induced proliferation, invasion and migration of human WRO and FRO thyroid cancer cells, which have endogenous GPER1, leading to a rapid activation of ERK/AKT, and then nuclear translocation of NF-κB, increased expression of cyclin A and D1, and secretion of IL-8 [24]. Proliferation, invasion and migration were suppressed either by specific inhibitors for GPER1, ERK, AKT, and NF-κB, or by knock-down of GPER1. The results of these studies suggested that E2 could induce cell growth, through GPER1 mediated pathways, and agreed with studies in other cancer types, as breast, lung, head, neck, neuroblastoma, prostate and ovarian carcinomas [26,27]. Nevertheless, in H295R cells, derived from adrenocortical carcinoma, G1 had a growth inhibitory effect both *in vitro* and in a xenograft model. These events required sustained ERK 1/2 activation. Silencing of GPER1 by a specific shRNA partially reversed G1-mediated cell growth inhibitory effects, without affecting ERK activation [28]. Similar effects were observed in prostate cancer: G1 inhibited the growth of prostate cancer cell lines, PC-3, DU-145, and LNCaP, and xenograft models of PC-3 cells, via ERK1/2 and p21 activation [29]. Also, GPER1 expression was higher in castration resistant PC cells, which had their growth inhibited by G1 via necrosis, than in androgen-sensitive PC cells [30]. From these data, GPER1 activation could have opposing actions in different cells.

Location

Another point of discussion in the literature has been the localization of this receptor. As GPER1-mediated signaling has been altered in many pathological states [31], this receptor represents an important target for the development of new drugs [32]. In two studies of this review, GPER1 was identified in the cell membrane and cytoplasm of thyroid cells by IHC [25,21]; another study using immunofluorescence staining was able to identify GPER1 protein only in the cell membrane, in the presence or absence of E2 [23]. In other tissues, GPER1 has

been reported in the plasma membrane [17,16,33-36], in the endoplasmic reticulum [16,37-40], and in the Golgi apparatus [41]. Another highly supported theory, GPER1 goes through an endocytosis process after its stimulation, as reviewed by Gauted et al [42]. There are no studies about the location and modulation of GPER1 in primary culture of normal thyrocytes, which could shed light about its role in normal thyroid. These studies have been suggested recently to decrease contradictions, considering the phenotype variation in cell lines, even under ideal physiological conditions [43]. Although, the key words were carefully selected, some information, not described by them, could have been lost.

Conclusions

The paucity of studies about GPER1 in the thyroid, as well as methodological differences between them, precludes firm conclusions about GPER1 role in the thyroid, although suggested an increase in proliferation of cancer cells mediated through GPER1.

The authors declare that there are no conflicts of interest.

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7.2 The gene expression of GPER1 is low in papillary thyroid carcinoma (PTC) in fresh human samples, and *in silico* analysis

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The gene expression of GPER1 is low in papillary thyroid carcinoma (PTC) in fresh human samples, and *in silico* analysis

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Abstract

Thyroid nodules and differentiated thyroid cancer are more common in women, and the incidence of the last has been increasing, which could be due to estrogen effects in thyroid cells. Estrogen responses are typically mediated through classical estrogen receptors, the α and β isoforms, which have been described in normal and abnormal human thyroid tissue, although effects mediated through G protein estrogen receptor 1 (GPR30/GPER/GPER1), described in some thyroid cancer cell lines, could be responsible for regulation of growth in normal cells. In this study, GPER1 gene and protein expression are described in non-malignant and in papillary thyroid cancer (PTC), as well as its association with clinical features of patients with PTC. The GPER1 expression was lower in PTC as compared to paired non-malignant thyroid tissues in both *in silico* analysis of GEO and TCGA databases, and in fresh samples of PTC. In PTC cases of TCGA database, Multivariate analysis showed that low GPER1 mRNA expression was independently associated with metastatic lymph nodes, female gender, BRAF mutation. These results support the hypothesis that GPER1 have a role in PTC and might be a potential target for PTC therapy. Further studies are needed to determine the functionality of these receptors in normal and diseased thyroid.

Keywords: GPR30/GPER1; Thyroid carcinoma; metastatic lymph nodes; tumor microenvironment; 17β-estradiol.

Introduction

Differentiated thyroid cancer, whose incidence has been increasing in the last years, occurs more frequently in women [1].17β-estradiol (E2) could have a role in this epidemiological observation due to its ability to increase proliferation and dedifferentiation in thyroid follicular cells[2-4]. Genomic effects of E2 are mediated through two classical estrogen receptors (ER) isoforms, α and β (ERα and ERβ), which have been described in normal and abnormal human thyroid tissue as well as in several thyroid cancer cell lines [5]. Besides, two estrogen receptors have been described in the plasma membrane, ERα [6] and GPER1, which was characterized and cloned in 2005 by two independent groups [7, 8]. Since then, GPER1 has been studied in several normal and abnormal cells, as reviewed recently [9].

In 2001, Manole described the activation of the ERK1/2 pathway in thyroid follicular goiter cells induced by E2 and abolished by fulvestrant, suggesting that this hormone was capable of triggering non-genomic effects in these cells, mediated by ERa [10]. Nevertheless, as recently reviewed by our group (*Manfroi, 2019*), proliferative effects of E2 on thyroid cells has been attributed to GPER1-mediated signaling [11, 12].

The characterization of this receptor in physiological and pathological conditions on the thyroid is not well established, which justified the elaboration of this study. Our aims were to assess the gene and protein expression of GPER1 in PTC, and its association with clinical characteristics of patients and in non-malignant thyroid tissue.

2. Materials and Methods

2.1 Search of in silico data of GPER1 in PTC

We searched the profile of GPER1 in PTC tissues in The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) datasets.

The TCGA dataset was obtained through the UCSC Cancer Genome Browser platform (www.genome-cancer.ucsc.edu), accessed on March 24th, 2018. The PTC data "PTC gene expression (AffyU133a)" was used. Tumor gene expression data, which had no clinical information of the patients, as well as the follicular variant of PTC, were excluded, leading to a final number of 385 cases included in the present study. The GPER1 mRNA paired expression levels in 45 cases was available and analyzed.

From NCBI-GEO (https://www.ncbi.nlm.nih.gov/gds/), accessed on June, 2018, two gene expression datasets, GSE33630 and GSE3467, which contained data on fresh frozen tissue samples of the classical PTC subtype and paired non-malignant thyroid tissue samples were selected. The GSE33630 dataset included 44 paired samples from Ukraine via the Chernobyl Tissue Bank [13, 14] and the GSE3467 dataset consisted of 9 samples from Finland [15].

2.2 Human thyroid tissue samples for GPER1 expression, as measured by RT-qPCR

Thyroid samples of PTC and its respective adjacent non-neoplastic tissue were provided by the Thyroid Unit, Endocrine Division, Irmandade da Santa Casa de Misericórdia de Porto Alegre. Samples were frozen in liquid nitrogen after surgical resection and stored at -80°C. Histological classification was confirmed by an experienced pathologist. Total RNA was extracted from about 20 mg of frozen tumors and surrounding thyroid tissues by RNeasy® mini kit (QIAGEN GmbH, Hilden, Germany). Samples were disrupted in a rotor-stator tissue homogenizer for simultaneous disruption and homogenization of the samples in TRIZOL LS reagent (Life Technologies, Carlsbad, USA). cDNA was synthesized from 1 μg of total RNA in a 20 μL reaction, using oligo-dT primers and the SuperScript II Reverse Transcriptase (Invitrogen), according to the manufacturer's instructions and stored at −20°C until use. RT-qPCR was performed to analyze GPER1 and TATA binding protein (TBP) mRNA using Fast SYBRGreen Master Mix (Applied Biosystems, California, USA) and primer pairs, which were described previously by Maggiolini [16] and Naasani [17], respectively. GPER1 mRNA expression levels were calculated with the standard curve method using a serial dilution of samples, and TBP mRNA levels were used as control, as previously standardized for paired normal and PTC thyroid samples [18].

2.3 *Immunohistochemistry for GPER1 protein*

GPER1 immunostaining of human tissue specimens, after deparaffinization, was performed using a polyclonal anti-human GPER1 antibody (SC48254 - Santa Cruz Biotechnology, Texas, USA) followed by an anti-rabbit secondary antibody (AP132P - Millipore, Massachusetts, USA), and DAB staining. Samples of human breast cancer were used as positive control and thyroid tissues not exposed to primary antibody were used as negative control. The following parameters were used: pH 6, 1:200 primary and secondary antibody dilution and one minute secondary antibody exposure time to DAB. Global immunoreactivity was classified by a semi-quantitative score for intensity of staining, as follows: 0 (none), 1 (weak), 2 (moderate), 3 (strong) or 4 (very strong) [19]. The intensity score was assigned as 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining), and 4 (very strong). Staining was evaluated as absent, diffuse or non-diffuse.

2.4 Statistical analysis

Data distribution was evaluated by the Kolmogorov–Smirnov test. The paired Student's t-test was used to compare means of GPER1 expression in GEO and TCGA datasets, and in fresh thyroid tissue. In the TCGA database, the z-score positive or negative was used to analyze the relative expression level of GPER1. The association of GPER1 expression with clinical factors was adjusted by a model of multivariable regression analysis, using SPSS software, version 21.

Non-parametric Kolmogorov–Smirnov Z test was used to compare the tumor size between groups through GraphPad Prism, version 7. Pearson's correlation coefficient (r) was used to assess relationships between mRNA expression levels of GPER1 and thyroid iodine-handling genes. Immunohistochemical expression patterns of GPER1 were compared using Chi-square test in assessment of the categorical data. Differences were considered statistically significant when p values were < 0.05.

3. Results

3.1 In silico analysis of GPER1 expression in tissue from PTC and normal thyroid

Data obtained from the GSE33630 database showed lower mean expression of GPER1 ($p<0.0001$) in PTC (4.216 ± 0.4069; n=44) than in adjacent non-tumor tissues (4.858 ± 0.4632; n=44). Similar results were obtained when evaluating GPER1 expression data from the GSE3467 database, which were lower ($p<0.0001$) in PTC (4.581 ± 0.2503; n=9), as compared to adjoining non-malignant tissues (5.344 ± 0.4355; n=9), as shown in Figure 1A and 1B. As observed in GSE33630 and GSE3467 datasets analyzes, mean GPER1 expression was lower in PTC [$1.174\pm$ 1.120 (95% CI: -0.8926_4.778); n=49], when compared to paired adjacent non-malignant tissues [2.161+0.696 (95% CI: 0.3878-3.385); n=49]; ($p<0.0001$), by paired Wilcoxon Signed Rank test, in the TCGA database, as shown in Figure 1C.

3.2 GPER1 mRNA expression in fresh samples of PTC and adjacent thyroid tissue

The *in silico* approach was validated by RT-qPCR evaluation of GPER1 mRNA levels, which were analyzed in 19-paired PTC and adjacent tissue. GPER1 expression was identified in all samples, with a lower mean expression in PTC [0.648 ± 0.273 (95%CI: 0.319-0.8804)], when compared to paired adjacent non-malignant tissues [1.67 ± 0.706 (95%CI: 1.124-3.480)] ($p=0.0028$). These results are shown in Figure 1D.

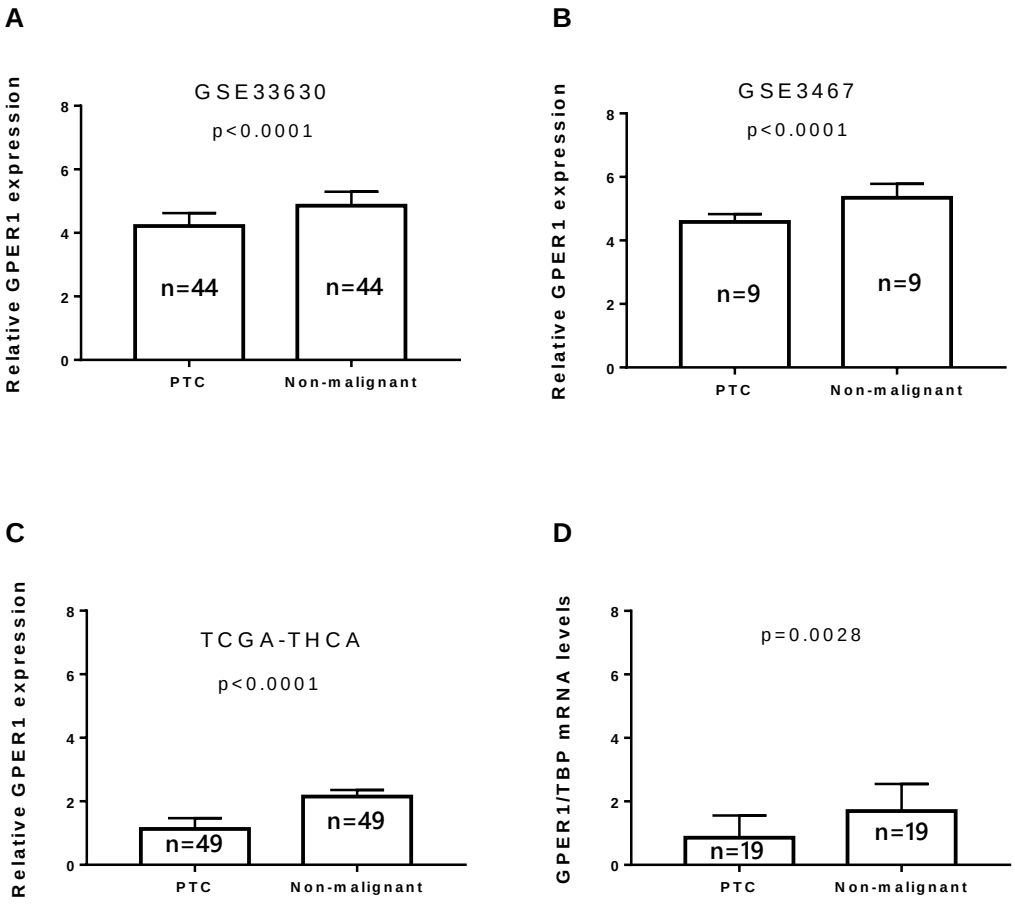

Figure 1: GPER1 gene expression is lower in papillary thyroid carcinoma (PTC), as compared to paired non-malignant thyroid. Gene expression was analyzed by microarray in A and B, respectively, from GSE33630, and GSE3467 datasets, by RNA-Seq in C from the Cancer Genome Atlas (TCGA)-Thyroid Cancer (THCA), and in D by RT-qPCR, adjusted to TATA binding protein (TBP) gene, in fresh samples from our patients. Box plots show mean and standard deviation of GPER1 expression. Means were compared by the paired Student's t-test.

3.3 Clinicopathological features associated with GPER1 expression in non-follicular variant PTC patients from TCGA and GSE33630 datasets

To substantiate better our findings, we analyzed the profile of the GPER1 expression and its association with clinical data of 370 PTC patients with histologic subtype of classical or tall cell variant from the TCGA dataset. The cases of follicular variant of PTC were excluded to avoid possible inclusion of Noninvasive Follicular Thyroid Neoplasm with Papillary-Like Nuclear Features (*NIFTP*), due to its very low risk of adverse outcomes [20].

The association of the relative expression of the GPER1 mRNA with the PTC clinical data in the TCGA database is shown in Table 1. According to the eighth edition of the AJCC/TNM staging system for DTC [21], the cut off for age was 55 years-old.

Table 1: Association of GPER1 expression levels (z-scores) with clinical characteristics in patients with non-follicular variant PTC from the TCGA database (n=375).

		Univariate analysis				**Multivariate analysis**			
Feature	***N* (%)**	**Mean**	**95% confidence limits**		***P* value**	**B**	**SE**	**beta**	***P* value**
Classification									
Classical	340 (90.7)	1.18	1.07	1.29	0.013	0.105	0.177	0.030	0.553
Tall Cell	35 (9.3)	0.87	0.65	1.10					
Extrathyroidal extension									
No	235 (63.5)	1.23	1.09	1.36	0.031	0.110	0.110	0.052	0.317
Yes	135 (36.5)	0.99	0.82	1.15					
Metastatic lymph nodes									
No	184 (49.7)	1.39	1.24	1.54	<0.0001	-0.532	0.101	-0.261	<0.0001
Yes	186 (50.3)	0.89	0.75	1.02					
Gender									
Female	268 (72.4)	1.08	0.96	1.20	0.074	-0.239	0.112	-0.105	0.034
Male	102 (27.6)	1.29	1.09	1.50					
Age (years)									
< 55	251 (67.8)	1.11	0.98	1.23	0.398	0.005	0.003	0.075	0.140
≥ 55	119 (32.2)	1.20	1.02	1.39					
BRAF									
No	147	1.516	1.328	1.705	<0.001	-0.585	0.101	-0.281	0.000
Yes	222	0.915	0.8038	1.025					
AJCC stages*									
I and II	236 (64.13)	1.20	1.06	1.33					
III and IVA/IVB/IVC	132 (35.87)	1.03	0.87	1.19	0.126	NA			

Data are given as number (n) and percentage (%) or mean and 95% confidence limits. AJCC: The American Joint Committee on Cancer7th edition. NA: not included for colinearity. SE: standard error.

In univariate analysis, GPER1 expression was not associated with gender (p=0.074), age ≥ 55years-old (p=0.398) or between stages I/II vs III/IVA/IVC AJCC stages of the 7th edition of the American Joint Committee on Cancer. (p=0.126); on the other hand, it was lower in tumors presenting with lymph node metastases (P<0.0001) and extrathyroidal extension (p=0.031), in tumors with tall-cell variant subtype, as compared to the classical variant of PTC (p=0.013), and in tumors with BRAF mutation (p<0.001). In a multivariable model of regression analysis, there was no association of GPER1 expression with PTC subtype (p=0.553), extrathyroidal extension (p=0.317), and age (p=0.140); low expression of GPER1 was independently associated with lymph node metastases (p<0.0001), female sex (p=0.034), and BRAF mutation (p=0.000), as shown in Table 1.

In addition, in the GSE33630 dataset, lower levels of GPER1 were associated with PTC ≥2cm, as compared with PTC <2cm (p=0.0331), respectively, 4.130±0.4142 (n=22) and 4.350±0.4116 (n=27).

3.4 Correlation of GPER1 and thyroid iodine-handling genes in TCGA

In addition, we compared the Pearson correlation between the expression of GPER1 and the thyroid iodine-handling genes: thyroid peroxidase (TPO), thyroglobulin (TG), sodium/iodide symporter (SLC5A5), pendrin (SLC26A4), and thyroid-stimulating hormone receptor (TSHR), which were down-regulated in PTC [22] in the TCGA database. This analyses revealed a positive correlation between expression of GPER1 and TG (r = 0.523, n=375, p< 0.001), TPO (r = 0.429, n=375, p< 0.001), SLC26A4 (r = 0.395, n=375, p< 0.001), TSHR (r = 0.278, n=375, p< 0.001) and SlC5A5 (r = 0.230, n=375, p< 0.001).

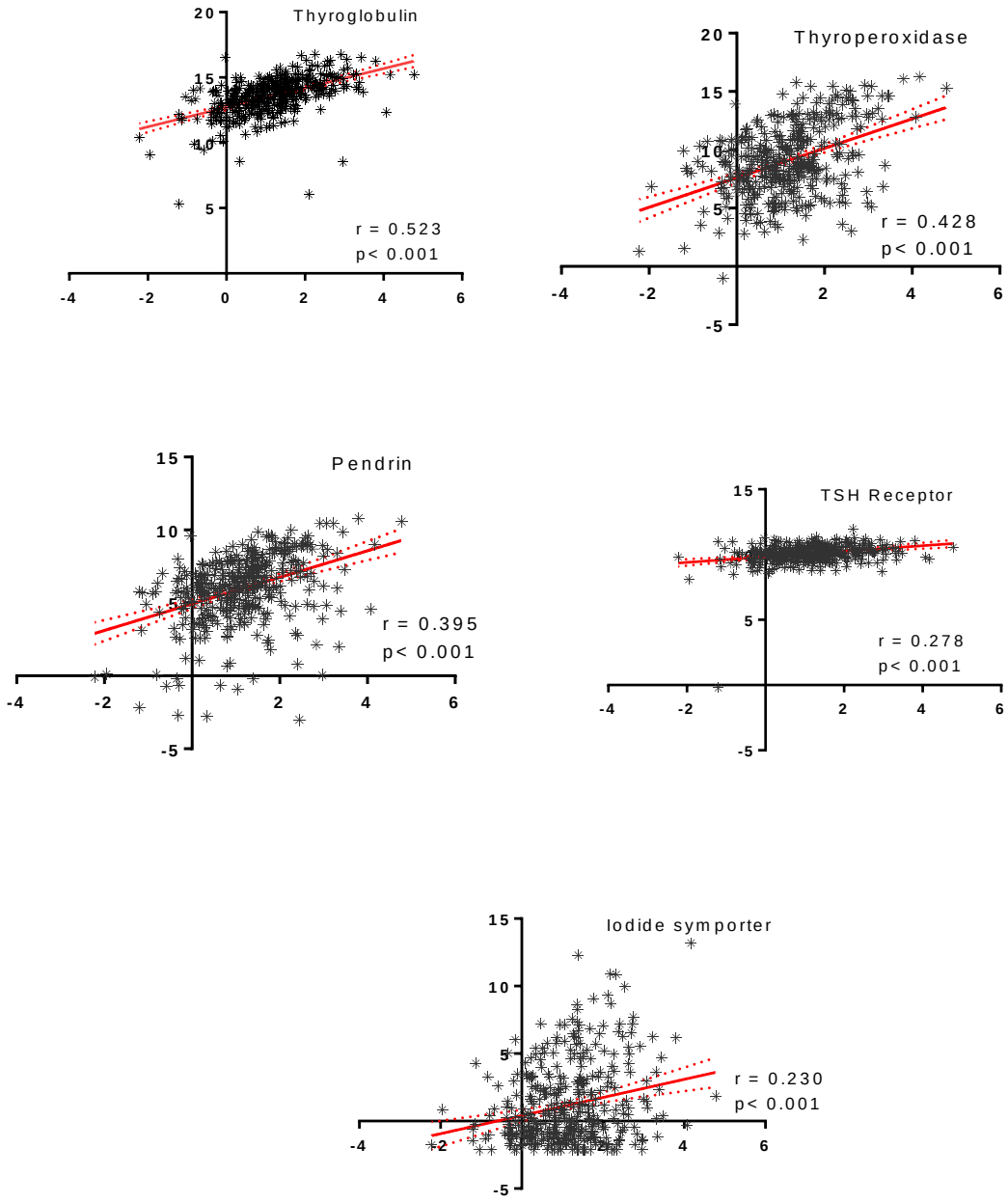

Figure 1: Iodine-handling genes expression were positively associated with GPER1 gene expression in TCGA dataset, as evaluated by the Pearson correlation test

3.5 Expression of GPER1 and clinicopathological features of PTC in our patients (n=28)

Of all 28 cases of PTC, 67.8% of cases were classified as classical PTC, while follicular variant of PTC comprised 32.2%. Our analysis revealed that expression of GPER1 was significantly downregulated (p=0.0362) in classical PTC [0.68 (0.092 - 3.48)], when compared with the follicular variant [1.37 (0.22 - 3.541)] (Figure 2A). Thus, considering this difference in the GPER1 profile according to PTC variant, we used only cases classified as classical PTC

(n=19) to evaluate the relationship of GPER1 expression with clinicopathological features that are shown in Figure 2. No significant association was observed between the expression ratio of GPER1 expression and the following clinical features: normal or lymphocytic thyroiditis or nodular hyperplasia in the adjacent non-tumor thyroid parenchyma, multifocality, metastatic lymph nodes, minimal local invasion, BRAF mutation, age higher than 45 years and tumor size larger than 2 cm. These data are shown in Table 2.

Table 2: Association of the tumor/paired-non-malignant tissue expression ratios of GPER1 mRNA (PTC/adjacent paired) with clinical features.

		GPER1 mRNA (tumor/normal)	
Feature	n (%)	Mean	p
Histology			
Classical	19 (67.8)	0.68 (0.092 - 3.48)	0.0362
Var Fol	9 (32.2)	1.37 (0.22 - 3.541)	
Adjacent Thyroid Parenchyma			
Normal	6 (31.6)	0.70 (0.28 - 10.6)	
Lymphocytic Thyroiditis	6 (31.6)	0.89 (0.09 - 3.48)	0.4761
Nodular Hyperplasia	7 (36.8)	0.47 (0.21 - 0.89)	0.3189
Multifocality			
Yes	4 (21.1)	0.55 (0.28 - 1.06)	0.8968
No	15 (78.9)	0.72 (0.092 - 3.48)	
Lymph Node			
Yes	8 (50.0)	0.61(0.358 – 0.865)	0.2700
No	10 (50.0)	0.46 (0.28 – 0.64)	
Minimal Local Invasion			
Yes	15 (78.9)	0.50 (0.092 - 1.06)	0.2528
No	4 (21.0)	1.35 (0.51 - 3.48)	
BRAF			
Positive	13 (68.4)	0.53 (0.092 -1.06)	0.3759
Negative	6 (31.6)	1.014 (0.24 - 3.48)	
Age			
≤ 45	14 (73.7)	0.77 (0.092 - 3.48)	0.3259

> 45	5 (26.3)	0.42 (0.21 - 0.71)	
Tumor Size			
≤ 2 cm	13 (68.4)	0.74 (0.21 - 3.48)	0.7621
> 2 cm	6 (31.6)	0.42 (0.092 - 0.89)	

Data are given as number (n) and percentage (%). GPER1 gene expression are shown as the least square means (95% confidence interval). P values were determined by the non-parametric Kolmogorov–Smirnov test.

3.6 GPER1 protein expression, as measured by immunohistochemistry, in patients with PTC (n=16)

On the thyroid normal and tumoral tissue, we found GPER1 to be expressed in the cytoplasm in all analyzed samples. In a recent revision of our group, we showed that there is no consensus about location of GPER1 in thyroid tissues which was found in the cell membrane or in the cytoplasm (*Manfroi, 2019*).

The GPER1 IHC staining pattern was different (Chi-square value: 24.889, $p<0.0001$), when comparing PTC, which had a diffuse pattern in 16/16 cases (100%), with adjacent thyroid tissue, which showed non-diffuse staining in 14/16 cases (87.5%). Representative cases are shown in Figure 2. Interestingly, in the two of three adjacent thyroiditis cases, a diffuse staining pattern was observed for GPER1.

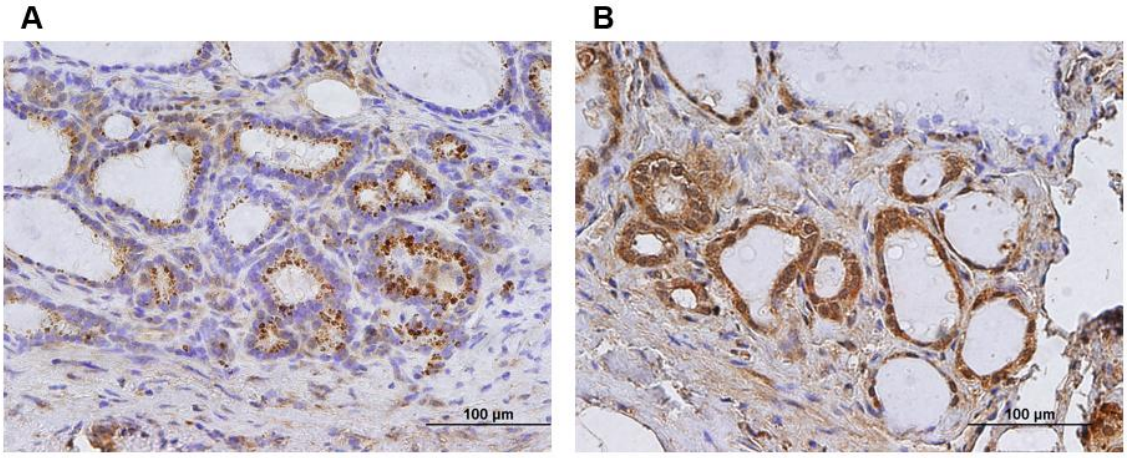

Figure 2: Expression of GPER1 in human thyroid tissues. Immunohistochemical staining of normal thyroid tissue for GPER1 was present in the cytoplasm of (A) normal and (B) PTC thyroid cells.

Chi-square analysis showed no statistical differences in the intensity of GPER1 expression between adjacent non-malignant and PTC thyroid tissues (p=0.2439; Table 3).

Table 3: Distribution of immunohistochemical expression of GPER1 in adjacent non-malignant and PTC thyroid tissues.

	Non-Malignant N (%)	PTC N (%)	Chi-square, p value
SCORE 0	0	0	
SCORE1	0	0	
SCORE2	1 (6.3)	3 (18.8)	2.822, 0.2439
SCORE3	8 (50)	10 (62.5)	
SCORE4	7 (43.8)	3 (18.8)	

Score grade: 0 (negative), 1 (low intensity), 2 (moderate intensity), 3 (strong intensity) and 4 (very strong intensity).

4. Discussion

In the present study GPER1 transcripts were downregulated in PTC as compared to paired non-malignant thyroid tissues in both *in silico* analysis of publicly available datasets from GEO and TCGA database, as well as in fresh samples of PTC. Analysis of TCGA database showed an independent association of low GPER1 levels with metastatic lymph nodes, BRAFV600E mutation, and female sex. Tumor size larger than 2 cm was associated with lower expression of GPER1 in GEO database. The positive correlation observed between GPER1 and iodine handling genes is noteworthy, because their decrease could be associated with cell dedifferentiation.

GPER1 gene encodes a widely distributed G protein-coupled estrogen receptor in human tissues, whose activation affects several signaling pathways in normal and abnormal conditions. Conflicting data have been reported on the role of GPER1 in cancer, which was recently reviewed [23]. In gastric cancer, it has been reported that loss of GPER1 was associated with reduced patient overall survival and disease-free survival; and its reduced protein expression was associated with a higher grade and stage [24]. In the same line, low expression of GPER1 mRNA predicted poor prognosis and was more common in metastatic lesions compared with primary lesions in ERα-positive endometrial cancer [25].

Also, in the non-tumor mammary tissue, the GPER1 mRNA levels were significantly higher than those in breast cancer tissue and associated with poor overall outcomes [26-29]. On the other

hand, some articles described association of higher expression of GPER1 , with high-grade, and lower survival rates in lung [30], breast [31], endometrium [32], and ovary cancer [33, 34]. Together with our findings, the GPER1 transcriptional silencing or overexpression during tumorigenesis appear to vary according to origin, stage, and/or environment [23].

In strong contrast to our findings, immunohistochemistry and genic studies by Tang and coworkers suggested that GPER1 was overexpressed in PTC and associated with LNM [19]. This discrepancy probably is due to study design and methodological aspects. First, in the qPCR analyses there are some methodological aspects to note: (a) Tang et al. used amplicons larger than 150 base pairs which could have caused rapid fluorescence saturation, since larger amplicons can bind more SYBRGreen molecules [35], and also (b) the choice of the gene to normalize GPER1 levels could have influenced the results. Recently, we found better stability of TBP gene, as compared to β-actin (ACTB), β-2-microglobulin (B2M), or hypoxanthine guanine phosphoribosyl transferase 1 (HPRT-1), when samples of paired normal and PTC thyroid samples were analyzed [18]. Second, to avoid bias caused by handling during processing, we used only paired data (non-tumor and PTC) to evaluate mean GPER1 gene expression in fresh samples, as well as *in silico* analysis.

Our immunohistochemical analysis using an antibody polyclonal for GPER1 revealed a diffuse pattern of staining in PTC in contrast to a non-diffuse pattern in adjacent tissues. Besides, there was no difference in intensity of GPER1 protein expression in PTC, as compared to adjacent non-malignant tissue. These are preliminary data, due to sample size, and there are sparse data in PTC to compare to. Tang et al found increased protein expression in PTC, as compared to normal thyroid, using a score, which included intensity and proportion of stained cells [19].

5. Conclusion

In this study, mRNA levels of GPER1 were down-regulated in PTC, when compared with their respective adjacent non-tumor tissue. Further studies are needed to determine the functionality of these receptors in the thyroid as well as its role in normal and abnormal conditions.

Conflicts of interest

The authors declare no conflict of interest.

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8. CONSIDERAÇÕES FINAIS

Conforme os dados apresentados no primeiro artigo, concluímos que ainda não existe um consenso sobre a localização do GPER1 em amostras normais e tumorais de CPT. Além disso, existem poucos estudos que avaliam a expressão gênica e proteica, e a proliferação do GPER1 no CPT.

No segundo artigo, demonstramos que o GPER1 apresenta uma expressão gênica diminuída no CPT em relação aos tecidos pareados não malignos e que há uma associação desta baixa expressão com a presença de linfonodos metastáticos, sugerindo que a perda de expressão do GPER1 possa estar envolvida no aumento da capacidade de migração das células tireoidianas. Neste sentido, estudos funcionais do receptor GPER1 são necessários para elucidar esta hipótese.

9. PERSPECTIVAS FUTURAS

Conforme revisados no primeiro artigo, não há consenso sobre a modulação do GPER1 na tireoide. Considerando os achados promissores do segundo artigo, temos como perspectiva principal avaliar a funcionalidade desse receptor nas células tireoidianas normais e anormais, expondo-as ao agonista (G1) e antagonista (G15), os quais são específicos para o receptor GPER1. Após a exposição, a proposta é avaliar alterações celulares como crescimento, migração e invasão, bem como avaliar o perfil molecular dos genes específicos, que marcam a diferenciação das células tireoidianas e, ainda, avaliar a atividade da enzima tireoperoxidase em células expostas às moléculas G1 e G15.

Em relação à localização, temos interesse de realizar imunomarcação do GPER1 por fluorescência confocal em tecidos tireoidianos normais e anormais e em células tireoidianas expostas às moléculas G1 e G15.

10. ANEXOS E/OU APÊNDICES

HOSPITAL DE CLÍNICAS DE PORTO ALEGRE

Grupo de Pesquisa e Pós Graduação

Carta de Aprovação

Projeto

2018/0331

Pesquisadores:

TANIA WEBER FURLANETTO

ANA PAULA SANTIN BERTONI PATRICIA DE ARAUJO MANFROI

Número de Participantes: 3

Título: Avaliação da modulação do receptor GPE30/GPER em tireócitos humanos em cultura primária

Este projeto foi APROVADO em seus aspectos éticos, metodológicos, logísticos e financeiros para ser realizado no Hospital de Clínicas de Porto Alegre.
Esta aprovação está baseada nos pareceres dos respectivos Comitês de Ética e do Serviço de Gestão em Pesquisa.

- Os pesquisadores vinculados ao projeto não participaram de qualquer etapa do processo de avalição de seus projetos.

- O pesquisador deverá apresentar relatórios semestrais de acompanhamento e relatório final ao Grupo de Pesquisa e Pós-Graduação (GPPG).

PATRICIA ASHTON PROLLA

Grupo de Pesquisa e Pós-graduação

Impresso do sistema AGHU-Pesquisa por RAFAEL LEAL ZIMMER em 13/11/2018 14:28:05