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Papel das ecto-nucleotidases em células foliculares de tireoide e no carcinoma papilar de tireoide

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Papel das ecto-nucleotidases em células foliculares de tireoide e no carcinoma papilar de tireoide

Dissertação submetida ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Mestre

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"A verdadeira viagem de descobrimento não consiste em procurar novas paisagens, e sim em ter novos olhos".

Marcel Proust

DEDICATÓRIA

Dedico esta Dissertação ao meu marido, Fabiano e à minha filha, Maria Fernanda, aos meus pais Arcangêlo e Rita Santin e aos meus irmãos Bruno, Caroline e Ricardo, obrigada por sempre me apoiarem e ficarem na torcida nas minhas conquistas! Amo vocês

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SUMÁRIO

RESUMO

A prevalência de câncer diferenciado de tireoide, especialmente o carcinoma papilar de tireoide (PTC), tem aumentado ao longo das últimas decadas e os mecanismos moleculares envolvidos nesta patologia não são bem compreendidos. Nos últimos anos, a investigação centrou-se em avaliar o microambiente tumoral, e nucleotideos extracelulares (por exemplo, ATP) e nucleosideos (por exemplo, adenosina) têm emergido como importantes moduladores deste microambiente tumoral. O ATP extracelular pode ser hidrolisado pela NTPDase1/CD39 e 2 e, o AMP produzido, é hidrolisado a adenosina pela ecto-5'-nucleotidase (CD73), a qual é considerada um promotor de crescimento tumoral e de metástases. Não há estudos que tenham avaliado atividade ou a expressão gênica das ectonucleotidases em células normais ou tumorais da tireoide. Ainda, o papel de CD73 em PTC é pouco estudado e controverso. No primeiro estudo, demonstrou-se que as linhagens de células derivadas de tireoide normal apresentam uma maior capacidade de hidrolisar ATP e que esta, encontra-se de acordo com uma maior expressão nos níveis de *ENTD1-2*, enquanto as células tumorais apresentam uma maior capacidade de hidrolisar AMP e apresentam níveis mais elevados mRNA para CD73. No segundo estudo, nossos resultados demonstram que a atividade de CD73 é *up*-regulada em células foliculares derivadas de PTCs, quando comparadas às células normais de tireoide humana e, ainda que os níveis de mRNA para a CD73 são significativamente

mais elevados em PTCs quando comparados a seus tecidos adjacentes não tumorais Além disso, verificou-se que a razão dos níveis de mRNA de CD73 do tecido tumoral em relação ao seu respectivo adajacente não tumoral, apresentou-se maior na presença de hiperplasia nodular no parênquima tireoidiano adjacente quando comparado com tireoidite normal ou linfocítica, na presença de linfonodos com mestástases, na presença de microinvação do local, na maior classificação de risco *ATA* e com maior tamanho tumoral. Dessa forma nós sugerimos que a elevada expressão da CD73 induz um o acúmulo de adenosina extracelular no microambiente tumoral, sugerindo que haja um *crosstalk* entre a célula normal e tumoral, podendo favorecer nichos de acumulação de ATP e/ou adenosina, o que pode promover um microambiente favorável para a progressão tumoral. Assim, a sinalização purinérgica pode ser considerada como um potencial alvo futuro para o manejo/tratamento do carcinoma papilar de tireoide.

Palavras chaves: Tireoide; Cultura Primária; Célula Folicular; ATP; Adenosina; CD73; CD39; Carcimona Papilar de Tireoide; Sinalização Purinérgica.

ABSTRACT

The prevalence of differentiated thyroid cancer, especially papillary thyroid cancer (PTC), has been increasing over the time and molecular mechanisms involved in this pathology are not well understood. In recent years, research focused on the niche within the tumor microenvironment, and extracellular nucleotides (e.g. ATP) and nucleosides (e.g. adenosine) have emerged as important modulators of tumor microenvironment. Extracellular ATP is hydrolyzed by NTPDase1/CD39 and 2 and the AMP produced is hydrolyzed to adenosine by CD73 which is considered a promoter of tumor growth and metastasis. There are no studies evaluating ectonucleotidase activity or gene expression on thyroid normal or tumors cells and the role of CD73 in PTC remains understudied and controversial. In the first study, we showed that thyroid cells derived from normal thyroid were shown to have a higher capacity to hydrolyze ATP, and in according with higher mRNA levels for *ENTD1-2* while tumor cells had a higher ability to hydrolyze AMP and showed the highest levels of CD73 mRNA. In the second study, our results show that CD73 activity is upregulated in thyroid cells from human PTCs, when compared to normal thyroid cells, in primary culture and CD73 mRNA levels is significantly higher in PTCs with respect to its matched adjacent normal tissue specimens. Also, we showed that CD73 mRNA tumor/non-tumor levels ratio means were higher when there was nodular hyperplasia and in adjacent thyroid parenchyma when compared to normal or lymphocytic thyroiditis, with metastatic

lymph nodes, microinvasion in thyroid bed, ATA risk classification and tumor size. Together, these results showed that components of the purinergic system are present and are functional in the thyroid follicular cells and that the high expression of CD73 leads to the accumulation of extracellular adenosine in the tumor microenvironment, suggesting that normal and tumor cell crosstalk may favor niches of ATP and/or adenosine accumulation, which may promote a favorable microenvironment for tumor progression. Therefore, purinergic signaling could be considered as a potential target to thyroid cancer management/treatment in the future.

Keywords: Thyroid; Primary Culture; Follicular Cell; ATP; Adenosine; CD73; CD39; Thyroid Papillary Carcinoma; Purinergic Signaling.

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LISTA DE ABREVIATURAS

ADO - Adenosina

ADP - Adenosina di-fosfato

AMP - Adenosina mono-fosfato

ATP - Adenosina tri-fosfato

cDNA - ácido desoxirribonucleico complementar

Ecto-5'-nucleotidase/CD73 – Enzima extracelular Ecto5'-nucleotidase

NTPDase - Nucleosídeo Trifosfato Difosfohidrolase (NTPDase1-8)

PTC - Carcimona Papilar de Tireoide

SFB - Soro Fetal Bovino

UTP - Uridina tri-fosfato

INTRODUÇÃO

A tireoide é uma glândula endócrina formada por folículos esféricos típicos, revestidos por uma monocamada de células epiteliais (Hoyes and Kershaw 1985, Policeni, Smoker et al. 2012). As células foliculares representam cerca de 80% das células formadoras da glândula e estão dispostas ao redor do coloide, exibindo uma superfície basal voltada para o espaço intersticial e capilares. Há também uma superfície apical, a qual contém numerosas microvilosidades, sendo a região de interface entre célula folicular e o coloide. As junções comunicantes presentes na membrana plasmática permitem um metabolismo coordenado entre as células foliculares adjacentes. Em cultura primária, as células foliculares apresentam uma disposição em monocamada. São representativas do seu respectivo funcionamento *in vivo*, pois são sensíveis e dependentes do hormônio TSH, sendo capazes de gerar iodação de resíduos de tirosina, além de produzir e secretar os hormônios (Roger and Dumont 1984, Rasmussen, Kayser et al. 1996, Mruck, Pfahlberg et al. 2002). A ligação do TSH ao seu receptor de membrana nestas células, de um modo geral, acaba por aumentar os níveis de AMPc, o qual é reponsável por induzir proliferação (Kimura, Van Keymeulen et al. 2001, Dremier, Coulonval et al. 2002), bem como a diferenciação tanto morfológica quanto funcional das célula foliculares (Passareiro, Roger et al. 1985, Roger, Taton et al. 1988, Kupperman, Wen et al. 1993).

As condições anormais da tireóide estão associadas à liberação excessiva de seus hormônios (hipertireoidismo), à deficiência desses hormônios (hipotireoidismo) e a bócios e tumores. Segundo o Ministério da Saúde, para o Brasil, estima-se no ano de 2016, 1.090 casos novos de câncer de tireoide para o sexo masculino e 5.870 para o sexo feminino, com um risco estimado de 1,08 casos a cada 100 mil homens e 5,70 casos a cada 100 mil mulheres (INCA, 2016).

A importância da sinalização purinérgica tanto na fisiologia normal como em alterações patológicas, tem sido progressivamente reconhecida nas últimas décadas. Ainda, múltiplos estudos *in vitro* e *in vivo* demonstraram uma vigorosa participação das ecto-nucleotidases no crescimento e progressão tumoral. Novos *insights* sobre os mecanismos que determinam a imunossupressão associada ao tumor permitiram apostar no desenvolvimento de estudos clínicos, utilizando a enzima CD73 e bloqueadores de receptores de adenosina como agentes anti-tumorais.

REVISÃO BIBLIOGRÁFICA

Carcinoma Papilar de Tireoide

O câncer de tireoide é o tumor maligno mais comum do sistema endócrino e são provenientes de dois grupos celulares, que possuem origens embriológicas distintas: os originários das células foliculares – Carcinomas Papilífero, Folicular e Anaplásico – e o originário das células C neuroendócrinas – o Carcinoma Medular. Dentre eles, destaca-se o carcinoma papilífero (PTC), uma vez que é o mais frequente, correspondendo a 80% dos casos. Os carcinomas papilares e foliculares são chamados de carcinomas diferenciados de tireoide, significando que ambas se assemelham fisicamente a células foliculares normais de tireoide. Apesar do conhecimento e da associação do carcinoma papilífero com a prevalência aumentada em mulheres, idade e da presença de mutações de ponto e rearranjos cromossômicos, pouco se sabe sobre os mecanismos que determinam e ou desencadeiam a patofisiologia destes carcinomas. A exposição a radiação ionizante é o fator de risco mais bem esclarecido até o momento para o desenvolvimento de carcimona de tireoide (White, Cipriani et al. 2016).

Histologicamente, o carcinoma papilífero caracteriza-se pela presença de núcleos aglomerados, grandes, ovoides, em aspecto de “vidro despolido” e podem apresentar fendas nucleares características. Outro achado é a pseudoinclusão nuclear, que se refere a herniações citoplasmáticas intranucleares, sendo as figuras mitóticas geralmente ausentes ou raras (LiVolsi 2011). Em alguns carcinomas papilíferos, as características nucleares citadas não são tão bem desenvolvidas ou ocorrem apenas focalmente. Geralmente, o carcinoma papilífero é infiltrativo, porém, alguns podem ser

circunscritos ou até encapsulados. As papilas usualmente são arboriformes com um delicado aglomerado fibrovascular. No entanto, elas podem ser amplas, com ninhos formados por um tecido fibrocelular, edematoso ou hialinizado. Os folículos estão frequentemente presentes e variam em tamanho e contorno, mas comumente são alongados e irregulares, contendo colóide espesso (LiVolsi 2011).

Embora o carcimona papilar de tireoide seja caracterizado por um crescimento lento e de bom prognóstico, recentemente foi demostrado que o risco de recorrência (local e à distância) da doença é mais freqüentemente analisado em relação ao prognóstico, com uma estimativa de cerca 30% de recorrência (Omry-Orbach 2016). Além das características clínico-patológicas consideradas como contribuindo para um maior ou menor risco de recorrência da doença, foram sugeridos elementos adicionais no processo de estratificação de risco nos guidelines publicados pela Associação Americana de Tireoide (ATA), sendo eles: presença ou ausência e grau de invasão vascular, multifocalidade, número e tamanho de linfonodos cervicais com mestástases e presença ou não de invasão capsular (Haugen 2016). Neste sentido, a busca por novos fatores que possam auxiliar o entedimento dos riscos que levam a recorrência é de suma importância para melhorar a abordagem terapêutica.

Devido a heterogeinidade dos PTCs (Nikiforov, Seethala et al. 2016), tem-se dado uma atenção especial à possibilidade de selecionar pacientes com PTC em grupos de risco adequados, a fim de otimizar a abordagem terapêutica e avaliação do prognóstico da doença (Omry-Orbach 2016). A classificação do estadiamento pós-operatório recomendado pela ATA é através do sistema *TNM* (abreviatura de tamanho do tumor primário (*T*), a presença ou ausência de metástase para linfonodo regional (*N*) e a presença ou ausência de metástase a distância (*M*)), o qual é baseado nos achados cirúrgicos e na faixa etária (abaixo de 45 anos ou 45 anos ou mais). Este sistema permite

selecionar quatro estágios de avanço clínico (I, II, III e IV) e tem como objetivo analisar o prognóstico, além de auxiliar na decisão de como proceder a terapia em relação a um determinado paciente (Omry-Orbach 2016).

Sistema Purinérgico

Existem muitas evidências na literatura que demonstram que inúmeros receptores e vias de sinalização são alvos-chave para a terapia de vários tumores. Desde 1972, quando Burnstock propôs a molécula de ATP como uma molécula neurotransmissora e introduziu o termo "purinérgico", a sinalização purinérgica tem-se demonstrada participante tanto em funções celulares normais como em condições patológicas. Os componentes essenciais que estão envolvidos na sinalização purinérgica são ectoenzimas que promovem a degradação do ATP liberado (ADP, AMP, adenosina, inosina e hipoxantina) e receptores específicos para esses transmissores moleculares (receptores P1 para adenosina e receptores P2 para ATP e ADP).

Classicamente, conforme esquematizado na Figura 1, o ATP e os demais nucleotídeos púricos e pirimidinicos podem ser degradados por diversas ecto-nucleotidases, entre elas membros da família das NTPDases (*ecto-nucleosídeo trifosfato difosfoidrolases*), compostas até o presente momento, por oito membros (NTPDases1-8) clonados e caracterizados que diferem funcionalmente entre si, por hidrolisarem as purinas e pirimidinas com diferentes afinidades (Zimmermann 2001). A ecto-5'-nucleotidase (*CD73*) transforma o AMP em adenosina que, por sua vez, pode ser captada no meio extracelular (Baldwin, Mackey et al. 1999). A participação das ecto-nucleotidases constitui uma via altamente sofisticada, capaz de controlar os níveis extracelulares de ATP e adenosina, que modulam uma série de processos fundamentais a nível celular em diversos órgãos e tecidos (Di Virgilio and Adinolfi 2016).

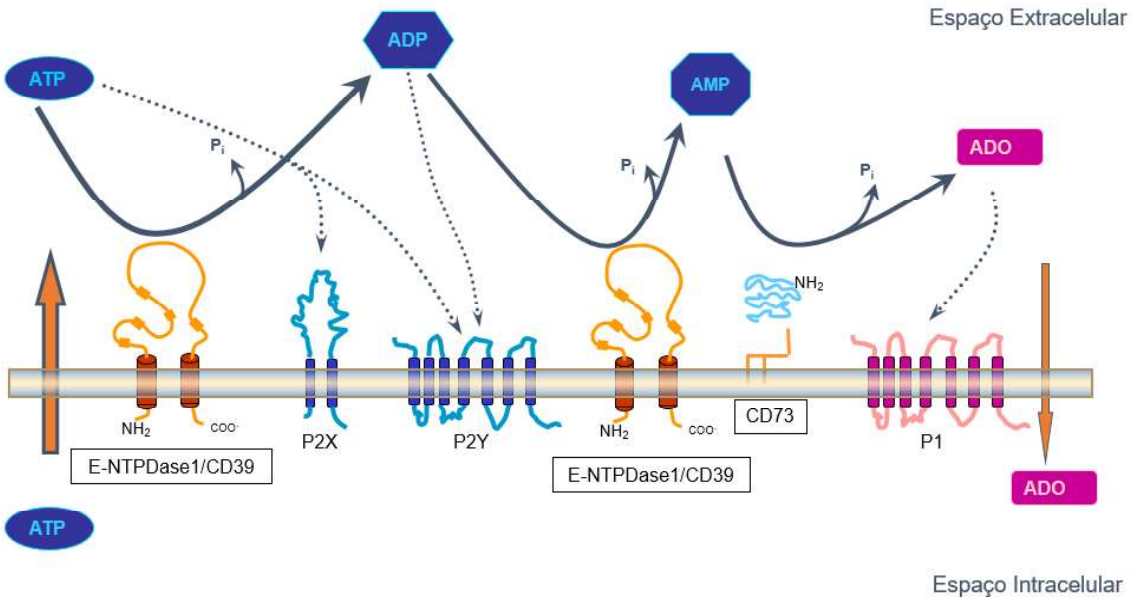

Figura 1. Esquema geral do sistema purinérgico. Neste esquema é possível visualizar a liberação do ATP intracelular, sua degradação pela E-NTPDase1/CD39 com liberação de fosfato inorgânico (Pi) até adenosina (ADO), bem como a ativação dos receptores purinérgicos P2X, P2Y (receptores para o ATP/ADP) e P1 (receptores da adenosina). A adenosina gerada pode ser captada para o meio intracelular e metabolizada até seu produto final ácido úrico. Figura gentilmente cedida por Sevigny e colaboradores.

Sistema Purinérgico em modelos tireoideanos

A função e os efeitos dos componentes purinérgicos têm sido mostrados em diversas linhagens de células da tireoide e, basicamente, parecem atuar na regulação da atividade dos canais de íons presentes na membrana celular e em algumas vias de sinalização intracelular. Contudo, não há evidências a respeito da funcionalidade e do *status* dos componentes do sistema purinérgico no microambiente normal e tumoral da glândula tireoideana bem como de suas respectivas células. Em seguida, descrevemos

uma breve revisão da literatura sobre o conhecimento atual de cada componente do sistema purinérgico em modelos de tireoide *in vitro* e *in vivo*.

ATP e adenosina

De um modo geral, conforme revisado abaixo, o ATP extracelular parece estar envolvido na regulação da fisiologia normal de transporte de íons, enquanto a adenosina parece estar envolvida na proliferação e diferenciação de linhagens de células tireoideanas, derivadas tanto de tecido normal, como anormal.

A maioria dos estudos das ações extracelulares do ATP foram avaliados na linhagem celular imortalizada, originária de tireoide de rato Fischer, FRTL-5. Nestas células, o ATP estimula o tráfico de íons na membrana plasmática, que parece ser independente da formação de poros (Kochukov and Ritchie 2004), bem como a estimulação de exocitose de diversos íons (Kochukov and Ritchie 2005). Outras evidências sugerem que o ATP extracelular aumenta os níveis de cálcio intracelular em tireócitos isolados de nódulos hipofuncionais de tireoide humana e em linhagens diferenciadas de câncer de tireoide. Entretanto, este efeito não é observado em linhagens provenientes de carcinoma indiferenciado da tireoide (Schofl, Rossig et al. 1997). Em células foliculares primárias isoladas de cães (Rani, Schilling et al. 1989) e nas linhagens PCCL3 (linhagem celular de tiroide normal de rato) (Elia, Muscella et al. 2003) e em FRTL-5 (Okajima, Sato et al. 1989, Tornquist 1991, Tornquist 1991, Aloj, Liguoro et al. 1993, Smallridge and Gist 1994, Ekokoski, Dugue et al. 2000, Ekokoski, Webb et al. 2001) também foi observado que o ATP leva a um aumento na concentração de cálcio intracelular. Os nucletídeos ATP e UTP demonstraram ser equipotentes na mobilização de cálcio em células FRTL-5 (Ekokoski, Webb et al. 2001). Além disso foram capazes de inibir a absorção de sódio em células epiteliais isoladas de tiroide normal de

suínos (Bourke, Abel et al. 1999). Ainda, outros efeitos do ATP extracelular nas células da tireoide proporcionam alguns cenários mais complexos que incluem a produção e liberação de interleucina-6 (Caraccio, Monzani et al. 2005), ativação da cascata do ácido araquidônico (Okajima, Sato et al. 1989, Raspe, Laurent et al. 1991, Ekokoski, Dugue et al. 2000), aumento da síntese de DNA e de expressão dos protooncogenes c-Fos e c-Jun (Ekokoski, Webb et al. 2001), efluxo de iodo (Iosco, Cosentino et al. 2014) e de cloro (Martin 1992), bem como aumento na produção de H_2O_2 (Bjorkman and Ekholm 1992, Bjorkman and Ekholm 1994, Kimura, Okajima et al. 1995). Vale ressaltar que a produção de H_2O_2 é um dos passos limitantes da taxa de organificação e da biossíntese dos hormônios tireoideanos (Schofl, Rossig et al. 1997). O ATP, do mesmo modo que o ADP, foi demostrado ser fracamente hidrolisado em homogenatos de tireoide de rato normal, suprimida ou estimulada (Bastomsky, Zakarija et al. 1971).

A adenosina tem sido demonstrada em diversos modelos diminuir os níveis de monofosfato de adenosina cíclico (AMPc) intracelular, através da inibição da adenilato ciclase em células estimuladas por TSH (Frauman and Moses 1989, Moses, Tramontano et al. 1989, Laezza, Migliaro et al. 1997), tendo como principais consequências a inibição do crescimento celular (Tramontano, Moses et al. 1988, Laezza, Migliaro et al. 1997, Vainio, Saarinen et al. 1997). *Per se*, a adenosina parece não apresentar qualquer dos efeitos citados acima, na células tireoideanas (Moses, Tramontano et al. 1989, Vainio, Saarinen et al. 1997, Harii, Endo et al. 1999). Sugerindo que a adenosina poderia atuar como um fator auxiliar na regulação de fatores mitógenos clássicos. Em relação a funcionalidade, há dois trabalhos conflitantes na literatura, sendo que um demonstra que a presença de adenosina regula positivamente a expressão de co-transportador de sódio/iodeto (NIS) e leva a captação de iodo (Harii, Endo et al. 1999) e outro,

demonstrando que o análogo da adenosina, N6-Isopentenyladenosine, o regula negativamente a captação de iodo pelas células FRTL-5 (Laezza, Migliaro et al. 1997).

Ecto-enzimas

Não há estudos realizados avaliando a expressão proteica ou gênica e a atividade enzimática das NTPDases na tireoide normal ou anormal, embora no Atlas de Proteína Humana tenha sido descrita a expressão do mRNA dos genes *ENTPD1, 3, 5* e *6* em tecido de tiroide humano normal (Uhlen, Fagerberg et al. 2015). Outro estudo também demonstrou a presença gene *ENTPD3* que codifica a NTDPase3 no tecido tireoideano normal (Zimmermann, Zebisch et al. 2012).

Em relação a CD73, há trabalhos demonstrando, superficialmente, sua expressão e atividade em diversos modelos: tecido tireoideano humano (Stanbury, Wicken et al. 1969, Mizukami 1983, Amano, Watanabe et al. 1985, Cohen, Miller et al. 1986), suíno (Franc, Hovsepian et al. 1984, Niedzwiecka and Jaroszewicz 1996), bovino (Stanbury, Wicken et al. 1969, Matsuzaki, Pochet et al. 1973, Peeters, de Wolf et al. 1988) e em ratos (Matsuzaki 1976). Um estudo monstrou um aumento da atividade da CD73 em homogenatos de tecido tireoideano de ratos submetidos à estimulação crônica com dieta deficiente de iodo (desenvolvimento de hipertireoidismo) ou tratados com propiltiouracil (desenvolvimento de hipotireoidismo) (Bastomsky, Zakarija et al. 1971). Estudos do nosso grupo demonstraram que os hormônios tireoidianos geraram uma resposta dose dependente no aumento da atividade e expressão da CD73 em células de músculo liso isoladas da aorta de ratos (Tamajusuku, Carrillo-Sepulveda et al. 2006) e em células de glioma de rato C6 (Wink, Tamajusuku et al. 2003).

Considerando-se a literatura atual, há dois estudos avaliando o *status* da CD73 em tecido tireoidiano de pacientes com alterações malignas e benignas. Cohen e

colaboradores (1886) demostraram ausência da CD73 em carcinoma medular e uma baixa expressão em células foliculares de tireoide normal, bócio multinodular, tireoidite, doença de Graves, adenoma folicular benigno, bem como em carcimonas papilares e foliculares (Cohen, Miller et al. 1986). Contudo, um segundo estudo mais recente, demonstrou uma superexpressão e uma alta atividade da CD73 em células foliculares de carcinoma papilar, e uma baixa expressão e atividade em células foliculares de tireoide normal, bócio nodular e de adenoma folicular. Neste trabalho ainda, os autores sugerem que o aumento da adenosina extracelular, gerado pela alta atividade da CD73, no carcinoma papilar pode estar relacionado à transformação neoplásica (Cohen, Miller et al. 1986).

Diante do exposto, notamos que o sistema purinérgico é expresso e parece ser funcional tanto na glândula normal como anormal da tireoide, contudo, não há dados suficientes disponíveis na literatura para realizamos uma hipótese da atuação da sinalização purinérgica em células tireoidenas normais ou anormais da tireoide.

JUSTIFICATIVA

Diante da relevância da sinalização purinérgica no microambiante tumoral e considerando a ausência de dados sobre o papel da sinalização purinérgica em células normais e tumorais da tireoide, bem como em pacientes com carcimona papilar de tireoide, os dados deste estudo podem contribuir para um melhor conhecimento da fisiopatologia normal das células foliculares e, possivelmente, da etiopatogênese de nódulos malignos da glândula tireoide.

OBJETIVOS

Objetivo Geral

Avaliar a hidrólise dos nucleotídeos ATP, ADP e AMP e a expressão das ecto-nucleotidases em células foliculares da tireoide normais e tumorais, bem como avaliar a relação da expressão gênica da CD73 com aspectos clínicos de pacientes com PTC.

Objetivos Específicos

1. Caracterizar a hidrólise dos nucleotídeos ATP, ADP e AMP e a expressão gênica das E-NTPDases1-8 e CD73 nas linhagens de células tireoidianas FRTL-5, PCCL3, K1 e TPC1;
2. Caracterizar a hidrólise dos nucleotídeos ATP e AMP e a expressão gênica da E-NTPDase1/CD39 e CD73 em células foliculares em cultura primária derivadas de tecido tireoideano não nodular e com carcinoma papilar;
3. Comparar os níveis de mRNA da CD73 em tecido tireoideano com carcinoma papilar e seu respectivo tecido adjacente não neoplásico e
4. Correlacionar os níveis de mRNA da CD73 com aspectos clínicos de pacientes com PTC.

CAPÍTULO I

Extracellular nucleotides are differentially metabolized in cell lines derived from papillary thyroid carcinoma and normal thyroid

Este artigo será submetido em forma de “original research” à revista

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Extracellular nucleotides are differentially metabolized in cell lines derived from papillary thyroid carcinoma and normal thyroid

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Short title: Extracellular nucleotide metabolism in normal and in cancer thyroid cells

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Abstract

The prevalence of differentiated thyroid cancer, has been increasing andits molecular mechanisms are not well understood. In recent years, extracellular nucleotides and nucleosides have emerged as important modulators of tumor microenvironment. Extracellular ATP is hydrolyzed by NTPDase1/CD39 and 2 and the AMP produced is hydrolyzed to adenosine by CD73 which is considered a promoter of tumor growth and metastasis. There are no studies evaluating ectonucleotidase activity or gene expression on thyroid normal or tumors cells. In these study, we showed that thyroid cells derived from normal thyroid were shown to have a higher capacity to hydrolyze ATP, and in according with its higher mRNA levels for *ENTD1-2* while thyroid cells tumor cells had a higher ability to hydrolyze AMP and showed the highest levels of CD73 mRNA. Together, these results showed that components of the purinergic system are present and are functional in the thyroid follicular cells and that the high expression of CD73 leads to the accumulation of extracellular adenosine in the tumor microenvironment, suggesting that normal and tumor cell crosstalk may favor niches of ATP and/or adenosine accumulation, which may promote a favorable microenvironment for tumor progression. Therefore, purinergic signaling could be considered as a potential target to thyroid cancer management/treatment in the future.

Introduction

The incidence of papillary thyroid carcinoma (PTC) has been increasing over time (Omry-Orbach 2016). Nevertheless, its molecular mechanisms are not well understood.

In recent years, research focused on the niche within the tumor microenvironment, in attempt to make possible target-directed therapeutic approaches (Chen, Zhuang et al. 2015), and extracellular nucleotides (e.g. ATP) and nucleosides (e.g. adenosine) have emerged as important modulators of tumor microenvironment (Wink, Lenz et al. 2003, Morrone, Oliveira et al. 2006, Braganhol, Morrone et al. 2009). The signaling events induced by these molecules are controlled by ectonucleotidases, an important group of ectoenzymes that control the status of ubiquitous nucleosides and nucleotides present at low concentrations in physiological conditions, and at high concentrations in tumor microenvironment, where they might contribute to tumor development and progression (Bergamin, Braganhol et al. 2012).

The major members of ectonucleotidases are ectonucleoside triphosphate diphosphohydrolase (NTPDase;EC 3.6.1.5) and ecto-5'-nucleotidase (e'NT/CD73; E.C.3.1.3.5) (Zimmermann, Zebisch et al. 2012). The molecular characterization and distribution of eight different NTPDase have already been described: NTPDase1, 2, 3 and 8 are cell surface-located enzymes with an extracellularly facing catalytic site, while NTPDase4, 5, 6 and 7 are intracellular and only NTPDase5 and 6 have been shown to be secreted after expression. Extracellular ATP is hydrolyzed by NTPDase1 and 2 and the AMP resulting is hydrolyzed to adenosine by ecto-5'-nucleotidase (CD73). There are no studies evaluating ectonucleotidase activity or gene expression on thyroid cells, although CD73 expression and activity have been shown to be increased in PTCs (Kondo, Nakazawa et al. 2006).

In this study, we analyzed the ATP, ADP and AMP catabolism on surface of thyroid cell lines, as well as, the level of the ectonucleotidases expressed by normal and tumor cells. Based on the different but complementary ectoenzyme status of normal and tumor thyroid cells, we showed that upregulation of CD73 can contribute to the understanding of the molecular mechanism involved in transformed thyroid cells and thus might be a potential target to thyroid disease.

Material and Methods

Ethics statement

This project was submitted and approved by the Research Ethics Committee of the Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil (Nº 15-0950).

Chemical agents

Cell culture supplies, nucleotide standards (ATP, ADP, AMP and adenosine), and mono-basic potassium phosphate (KH_2PO_4) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tetrabutylammonium choride ($C_{16}H_{36}ClN$) was purchased from Sigma-Aldrich (Steinheim, Switzerland), and HPLC grade methanol was obtained from Panreac ITW Companies (Barcelona, Spain). All other solvents and reagents were analytical grade and used as received.

Thyroid cell cultures

Human thyroid cell lines TPC-1 (Tanaka, Ogura et al. 1987) and K1 (Challeton, Branea et al. 1997) derived, respectively, from papillary thyroid carcinoma (PTC) and from metastasis of a well-differentiated PTC, were grown in DMEM containing 5% fetal bovine serum (FBS), and ampicillin/streptomycin in a 5% CO_2 atmosphere. PCCL-3 (Fusco, Berlingieri et al. 1987) and FRTL-5 (Ambesi-Impiombato, Parks et al. 1980) which are representative of normal rat cell lines were cultured in Ham's F-12 Coon's modification medium supplemented with 10% FBS, 10 µg/mL insulin, 5 µg/mL transferrin, 1 mU/mL TSH, 100 U/mL kanamycin at 37°C with 5% CO_2. TPC-1 and K1 cell lines were kindly supplied by Dr Ana Luisa Maia (Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil) and PCCL3 and FRTL-5 cell lines were kindly supplied by Dr. Denise Pires de Carvalho (Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil).

RNA isolation, cDNA synthesis and qPCR

Cellular total RNA was extracted with Trizol® Reagent (Macherey Nagel, Düren, Germany) and reverse transcribed with the SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). mRNA was detected by real-time quantitative PCR using Fast SYBRGreen Master Mix (Applied Biosystems, Foster City, CA, USA) for genes described in Table 1S and 2S. Ectonucleotidase mRNA levels were calculated with the standard curve method using a serial five-fold dilution of samples and ACTB mRNA levels were used as control.

Ectonucleotidase assay

Enzymatic activities of thyroid cells were determined by using ATP and AMP as substrates as described already (Wink, Lenz et al. 2003). Briefly, thyroid cell lines were seeded in 24 multi-well plates (5.000 cels/well) and cultivated until reach 90-95% confluence. ATPDase activities were measured by adding 1 mM ATP or ADP to reaction medium (2 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM Hepes – pH 7.4) at 37°C for increasing incubation times (10, 20, 30 or 60 min). For AMP hydrolysis, the same incubation conditions were applied, with the exception that 2 mM $MgCl_2$ was used instead of $CaCl_2$ in the reaction medium. Chemical competitive inhibitor of CD73, a non-hydrolysable ADP analog adenosine 5′-(α,β-methylene) diphosphate (APCP), was used to confirm AMP hydrolysis through CD73. Cell cultures were exposed to APCP (10 μM) and following 15 min of preincubation, 1 mM AMP was added to reaction medium in presence of APCP for 10, 20, 30 or 60 min. The reaction was stopped by removing an aliquot of incubation medium and transferring it to a pre-chilled tube containing trichloro-acetic acid (5% w/v). The release of inorganic phosphate (Pi) was measured by the malachite green method [13] with KH_2PO_4 as a Pi standard. Specific activity was expressed as nmol of Pi released per min per mg of protein. Protein concentration of samples was determined by Bradford protein assay [14].

Chromatographic separation and analysis of extracellular ATP metabolism

FRTL-5 and TPC-1, which are representative of normal and cancer thyroid cell lines, respectively, were seeded in 24 multi-well plates (5.000 cells/well) and confluent

cells were exposed to 100 μM ATP or 100 μM AMP in absence or in presence of 1 μM APCP, which was preincubated with cells for 15 min, in incubation medium (pH 7.4) as described above. Following 0, 10, 20, 30, 60, 90 and 120 min, the reaction was stopped by removing an aliquot of incubation medium and transferring it to a pre-chilled tube. Samples were centrifuged at 12.000*g* for 15 min and stored at -80°C until analysis. The HPLC analysis of nucleotides was performed on LC-20A HPLC instrument (Shimadzu, Kyoto, Japan) equipped with the following modules: SIL-20A HT autosampler; LC-20AD HPLC pump; CTO-20A column; SPD-M20A photodiode (PDA) array detector. The chromatographic separation was performed on a Shimadzu Shim-pack CLC (M) C18 column (5μm, 150 × 4.6 mm) equipped with a guard column (5μm, 150 × 4.6 mm) Shimadzu Shim-pack GVP-ODS (4.6 × 10 mm) at 32°C. The injection volume was 10 μL and the total run time was 45 min. Data were acquired using the LC Solution Software (Shimadzu). The mobile phase consisted of buffer A, 60 mM KH_2PO_4 5.0 mM $C_{16}H_{36}ClN$, pH 5.9; and buffer B, 60 mM KH_2PO_4, 5.0 mM $C_{16}H_{36}ClN$, 30% methanol, pH 5.9. Buffer solutions were filtered through a 0.22 μm pore membrane (Millipore, Bedford, USA). The mobile phase was eluted at a flow rate of 1.2 mL/min along the gradient that was proposed by Vallé et al. (1998). The analyte was detected by retention time at 254 nm and compared with the nucleotide/nucleoside standards at 100 μM. Data were expressed as μM.

Results

Extracellular nucleotide hydrolysis in thyroid cell lines

Firstly, we tested the ability of the cell to hydrolyze extracellular ATP, ADP and AMP. PTC-derived cell lines (TPC-1 and K1) hydrolyzed ATP in a rate lower than normal thyroid-derived cell lines, FRTL-5 and PCCL3. However, the amount of Pi produced from AMP by TPC-1 and K1 was higher than by FRTL-5 and PCCL3 cells (Fig. 1). The mean ratio of AMP:ATP hydrolysis was 0.125:1 for normal thyroid-derived lines and 2.43:1 for PTC-derived cell lines. In these cells, AMP hydrolysis was ~20x higher than in normal thyroid-derived cells. ADP and ATP hydrolysis were similar in FRLT-5, K1 and TPC-1 cells; in PCCL3, ADP hydrolysis was similar to AMP hydrolysis (Table 1).

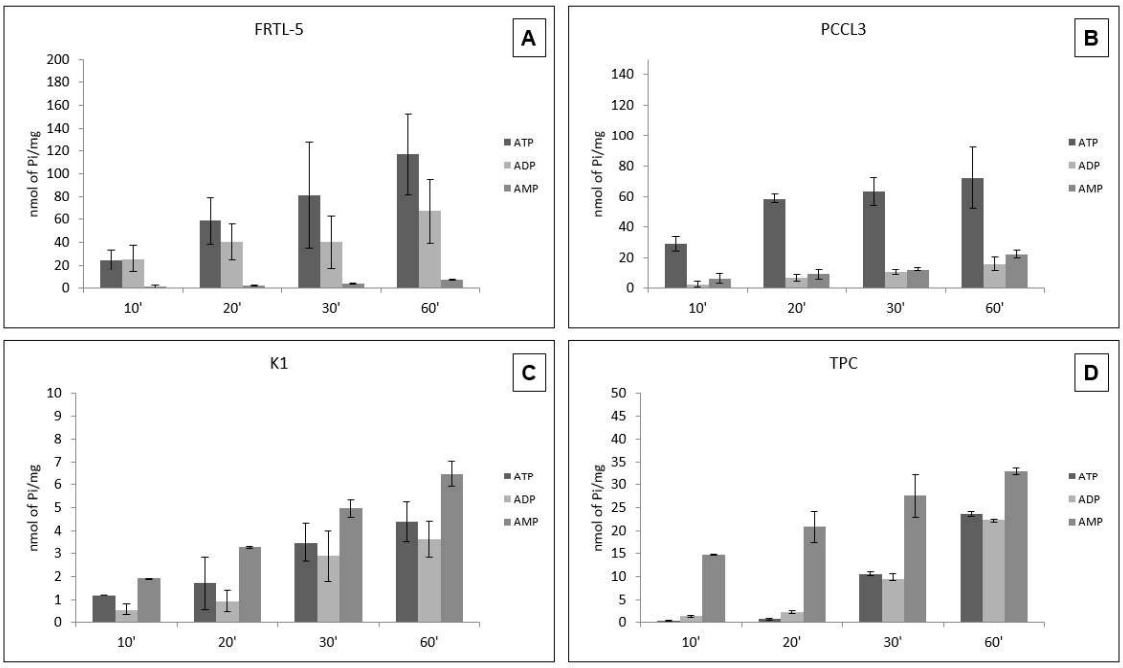

Figure 1. ATP, ADP and AMP hydrolysis on thyroid cell line surface. Thyroid cells were incubated in a phosphate-free buffer containing 1 mM nucleotides at 37°C for 10, 20, 30 and 60 minutes as described in material and methods. In normal thyroid-derived cells, FRTL-5 **(A)** and PCCL3 **(B)**, ATP hydrolysis was higher than AMP, while, in papillary thyroid carcinoma derived cells, K1 **(C)** and TPC-1 **(D)**, AMP hydrolysis was higher than ATP and ADP. Data are expressed as nmol Pi/mg of protein and represent the mean ± SD of three experiments.

Table 1. Specific activities for ATP, ADP and AMP hydrolysis and rates in normal and cancer thyroid cells.

Cell Line	ATP	ADP	AMP	ATP/ADP	ATP/AMP	AMP/ATP
FRTL-5	2.62± 0.41	1.94 ± 0.79	0.14 ± 0.01	8.40	5.32	0.19
PCCL3	2.66 ± 0.82	0.32 ± 0.05	0.52 ± 0.11	1.26	17.87	0.06
TPC-1	0.35 ± 0.03	0.32 ± 0.04	1.15 ± 0.38	1.12	0.31	3.24
K1	0.11± 0.02	0.07± 0.02	0.17± 0.03	1.59	0.62	1.62

Nucleotide hydrolysis was measured in rat normal thyroid (FRTL-5 and PCCL3) and in human PTC cancer cell lines (TPC-1 and K1). Ectonucleotidase activities of intact cells were determined at 37ºC following 30 min incubation with 1 mM ATP, ADP or AMP. Nucleotide hydrolysis are expressed as nmol of Pi liberated/min/mg of protein and represent mean ± SD of three experiments.

Extracellular ATP metabolism by HPLC analysis

To better investigate the pattern of extracellular ATP hydrolysis on FRTL-5 and TPC-1, which are representative of normal and cancer thyroid cell lines, respectively, cell cultures were exposed to medium containing 100 µM ATP for increasing times (0-120 min) and ATP degradation metabolites were measured by HPLC (Fig. 2).

FRTL-5 cells metabolized ATP gradually along 120 min of incubation with subsequent production of ADP and AMP, which were the main products accumulated at the end of reaction (27.32 and 41.13 µM, respectively). In opposite, ATP was poorly metabolized by TPC-1 cells and small amounts of ADP and AMP were detected in the extracellular medium. After 120 min of incubation, the amount of ATP remaining in FRTL-5 and TPC-1 cells were, respectively, 10.36 ± 0.25 and 84.86 ± 4.3 µM. Interestingly, the incubation with ATP led to an accumulation of AMP in normal FRTL5 cells but not in TPC-1 cells, confirming the previous results that show the increased CD73 activity exhibited by cancer cells (Fig. 1). In FRTL-5 normal thyroid cell line, ATP seemed not to be directly dephosphorylated to AMP, since there was detectable ADP levels in the extracellular medium, which was smaller inTPC-1 cells.

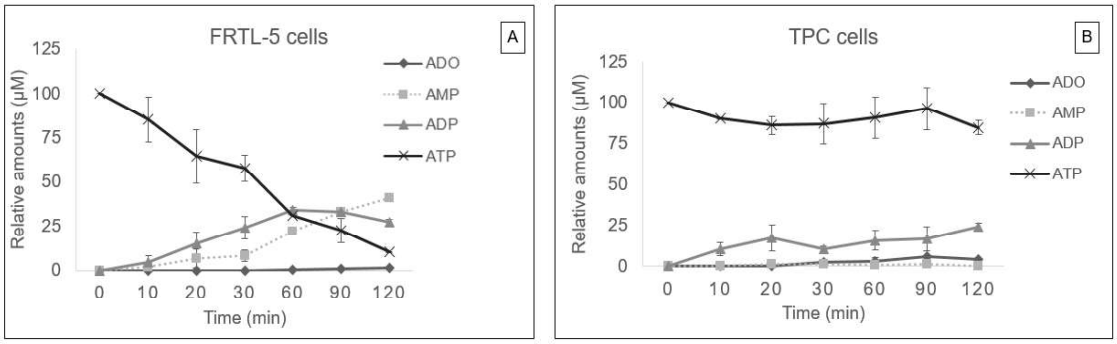

Figure 2. Metabolism of extracellular ATP by FRTL-5 and TPC-1 cell lines. **(A)** FRTL-5 and **(B)** TPC-1 cells were incubated with 100 µM ATP. Supernatant aliquots were

analyzed following 0, 10, 20, 30, 60, 90 and 120 min of incubation. ATP, ADP, AMP and adenosine (ADO) were identified and quantified by HPLC comparing with reference standards. Data are shown as mean ± standard deviation (bars) of triplicates. The experiments were repeated twice with similar results.

Inhibition of CD73 enzyme activity by APCP in TPC-1 cells

In order to confirm the participation of CD73 on AMP metabolism in TPC-1 cells, these cells were incubated with AMP in presence of APCP, and AMP hydrolysis was evaluated by malachite green and HPLC assays. As shown in Figure 3 (panel A), APCP (10 μM) inhibited AMPase activity in ~30% when compared to control. HPLC experiments (Fig. 3B; triangle *"solid line*") confirm that TPC-1 efficiently hydrolyzes extracellular AMP to adenosine. Indeed, AMP (~60 %) was metabolized by cancer cells within 180 min incubation. The addition of APCP (1 μM) to reaction medium promoted a decrease of AMP metabolism (Fig. 3B; *"dashed line*") and a consequent reduction of extracellular adenosine generation (~20%; dot *"dotted line"*) following 180 min incubation. These findings suggest that APCP, which prevents the binding of AMP to CD73, decreased the ability of cells to hydrolyze AMP, suggesting that CD73 plays an important role converting AMP to adenosine in these cells.

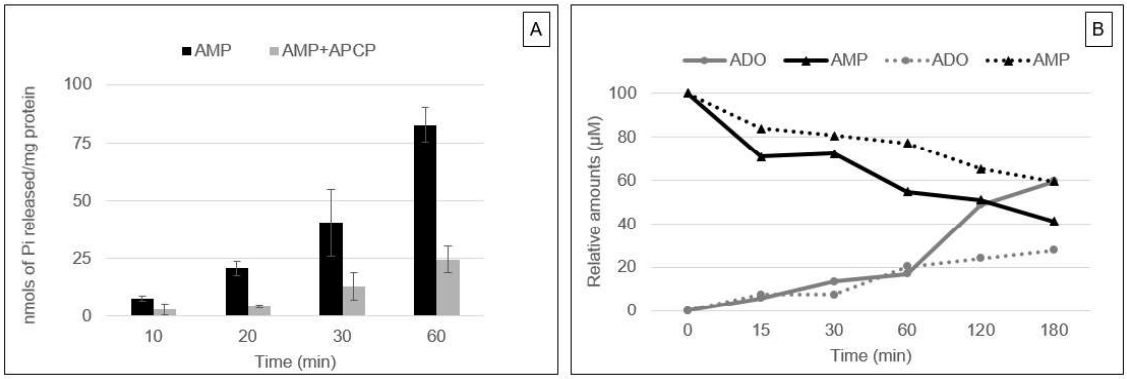

Figure 3. APCP inhibits AMP hydrolysis in thyroid cancer TPC-1 cells. **(A)** TPC-1 cells were incubated with 1 mM AMP in absence (black bars) or in presence (gray bars) of 10 μM APCP and the AMPase activity was determined by malachite green assay. Data were expressed as nmol Pi released/mg of protein and represent the mean ± SD of three experiments. **(B)** HPLC analysis showing the extracellular AMP metabolism in cancer cells following incubation with 100 μM of AMP in absence (dotted line) or in presence

(dashed line) of 1 μM APCP. Note the decrease of adenosine (ADO) formation promoted by APCP. AMP and ADO were identified and quantified by HPLC comparing with reference standards. Data are shown as mean ± standard deviation (bars) of triplicates. The experiments were repeated twice with similar results.

Ecto-nucleotidase mRNA expression in thyroid cell lines

RT-qPCR was performed to evaluated mRNA expression of *ENTPD1, 2, 3, 5, 6, 8* and CD73 in thyroid cell lines (Fig. 4). In FRTL-5 and PCCL-3 cells, *ENTPD1*, *2*, *5* and CD73 were moderately expressed, while *ENTPD*3 could not be detected. *ENTPD*6 mRNA levels were detected in PCCL-3 but not in FRTL-5 cells. *ENTPD1*, *ENTPD2*, *ENTPD*5 and *ENTPD6* were expressed in very low or undetectable levels in K1 and TPC-1 cells. *ENTPD*3 was moderately expressed in K1 and highly expressed in TPC-1 cells (5.9-fold increase). *ENTPD8* mRNA could not be detected in any of the four thyroid cell lines studied. TPC-1 and K1 cells showed the highest levels of CD73 mRNA expression, in about 5 times, in agreement with the enzymatic activity results.

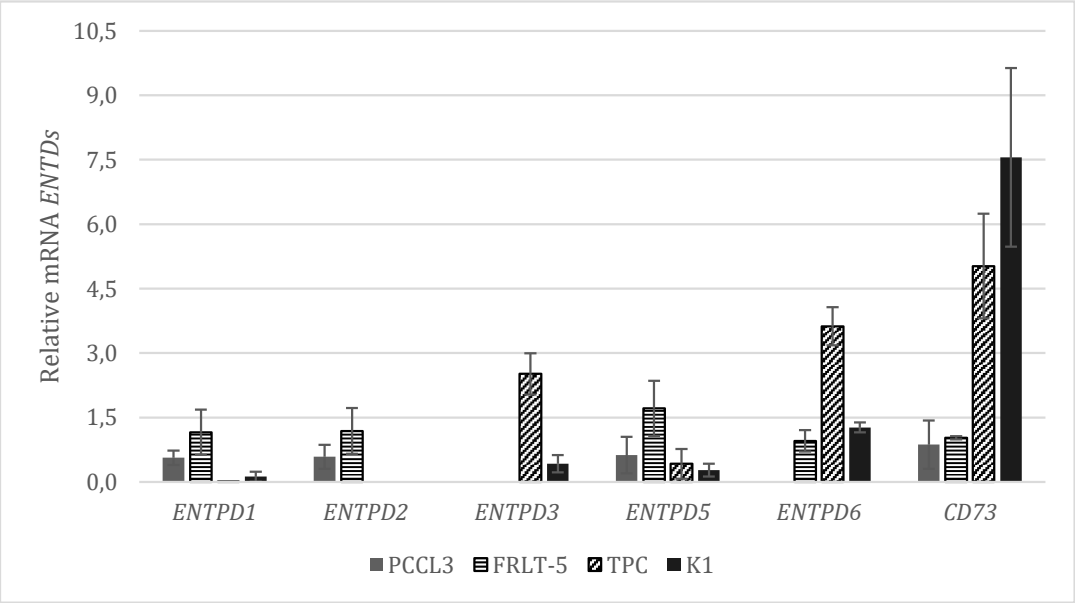

Figure 4. Ectonucleotidase mRNA expression in normal and cancer thyroid cell lines by RT-qPCR. The levels of mRNA were normalized to those of ACTB. Ectonucleotidase mRNA relative expression are presented as mean ± SD of at least three samples. *ENTPD*2 mRNA expression was present only in cells derived from normal thyroid, while *ENTPD3* mRNA was present only in tumor-derived thyroid cells. CD73

mRNA expression was higher (~6.5 fold) in tumor derived thyroid cells, when compared to cells derived from normal thyroid.

Discussion

The present study demonstrates that genes which encoding NTPDases are expressed in thyroid cell lines derived from normal and papillary thyroid cancer, and its profile differ according to the origin of the cell line in two aspects: (*i*) the ability to hydrolyze ATP and AMP and (*ii*) in the mRNA levels for these enzymes. Cells derived from normal thyroid were shown to have a higher capacity to hydrolyze ATP, and in according expressed higher mRNA levels for *ENTD2*. However, tumor cells had a higher ability to hydrolyze AMP and showed the highest levels of CD73 mRNA.

The overexpression of CD73 in PTC and a negative immunoreactivity for CD73 in normal thyroid epithelial cells was described previously (Kondo, Nakazawa et al. 2006). Our results showing a higher CD73 mRNA levels in two human PTC derived cells (K1 and TPC-1), when compared to normal thyroid derived cells (FRTL-5 and PCCL3) are in line with these observations. The same authors measured the CD73 activity in thyroid tissues using Wachstein and Meisel's methods, showing that its activity was faint in normal thyroid cells and strong in PTC cells (Kondo, Nakazawa et al. 2006). Similarly, our results obtained from HPLC analysis showed that more than 60% of AMP applied was hydrolyzed by TPC-1 cells after 180 min, with the expected increasing accumulation of adenosine.

By the other hand, FRTL-5 cells showed a progressive accumulation of AMP and very low adenosine production when ATP was given as substrate. The profile of low ATPase and high AMPase activities in tumor thyroid cells can be explained by the low to absent levels of *ENTPD1* and *ENTPD2* and high CD73 gene expression in these cells. Previously, our group showed a similar profile of high ATP and low AMP degradation in normal cells, and low ATP and high AMP degradation in glioma cancer cells (Wink, Lenz et al. 2003, Morrone, Oliveira et al. 2006, Braganhol, Morrone et al. 2009). Primary rat astrocytes hydrolyze higher rates of ATP (~1,000 nmol/min/mg of protein) and express *ENTD*2 mRNA at higher levels than rat C6 glioma cells (less than 10 nmol/min/mg of protein). In opposite, CD73 activity was increased in C6 glioma cells, when compared to

astrocytes (~100 *versus* 15 nmol Pi/min/mg of protein, respectively) (Wink, Lenz et al. 2003, Wink, Braganhol et al. 2006). In addition, our group have shown that a proportional increase in CD73 activity and mRNA expression occurred in dose-response fashion after triiodothyronine (T3) treatment in C6 rat glioma cells (Wink, Tamajusuku et al. 2003), suggesting that its status could be affect by thyroid follicular cell hyperfunction, which is responsible for the production and secretion of thyroid hormones.

We hypothesize that within thyroid lesions, where co-exist 'normal' follicular cells with benign or malignant lesions in thyroid gland, these 'normal' thyroid cells hydrolyze ATP and ADP to AMP and tumor thyroid cells hydrolyze AMP, producing adenosine (Fig. 5).

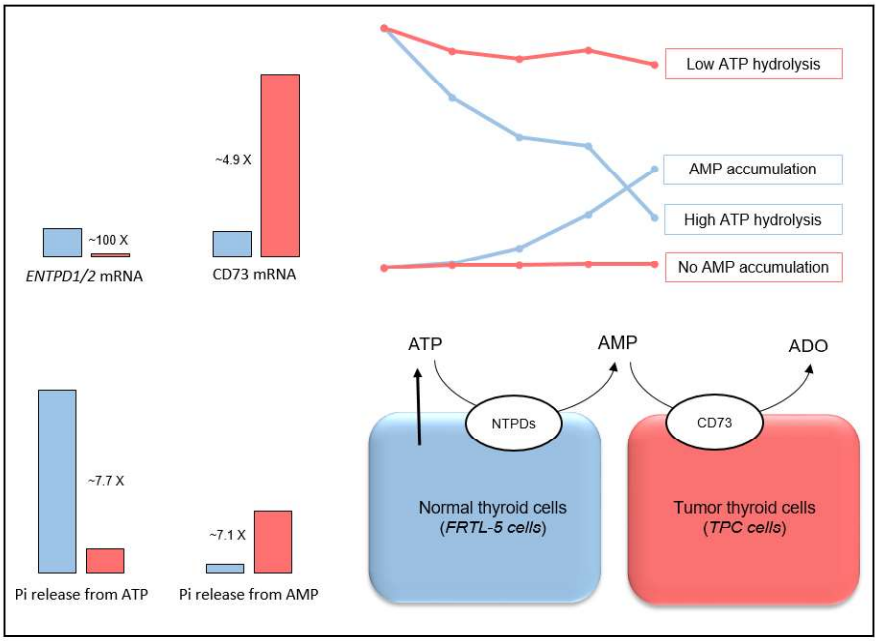

Figure 5. Schematic illustration summarizing nucleotide metabolism and ectonucleotidase expression profile in normal (*blue*) and tumor (*red*) thyroid cells. Normal thyroid cells express high levels of NTPDase1 and 2 when compared to tumor cells, leading to an efficient extracellular ATP hydrolysis with consequent accumulation of ADP and AMP. Conversely, tumor thyroid cells express NTPDase3 while NTPDase1 and 2 are absent, which result in a poor ATP metabolism when compared to normal cells, resulting in accumulation of this nucleotide in tumor microenvironment. By other side, CD73 is highly expressed in TPC-1 and K1 cancer cells in comparison with normal cells, what explains the fast degradation of AMP and adenosine production. Then, in thyroid gland, might have a collaboration between the 'normal' cells that hydrolyze ATP and ADP,

providing AMP that will be dephosphorylated to adenosine by tumor cells. The adenosine accumulated can be implicated in several pro-tumorigenic features, as immunosuppression and angiogenesis, in tumor microenvironment.

Studies previous suggested that adenosine might have a positive modulatory role in the growth regulation of FRTL-5 cells in normal conditions (presence of thyroid stimulating hormone (TSH) and insulin) but interestingly, act as an inhibitor of cell growth in absence of classical mitogenic factors (Tramontano, Moses et al. 1988, Frauman and Moses 1989, Vainio, Saarinen et al. 1997), suggesting that adenosine *per se* did not modulate thyroid cells growth. In addition, after stimulation with CGS21680, an adenosine A2a-specific agonist, vascular endothelial growth factor (VEGF) expression was increase in FRTL-5 cells (Zhang, Xu et al. 2013) and also human thyroid cancers express more VEGF mRNA than normal thyroid tissues (Soh, Duh et al. 1997). The effects of adenosine produced into the extracellular space of human normal or tumor thyroid gland as well as its effects in the tumor microenvironment need to be further investigated.

As a result of low ATP/ADP metabolism by cancer cells, ATP accumulation within and surrounding tumor may sensitizes P2 receptors both in tumor cells, inducing cell proliferation, as well as in immune cells (and normal thyroid cells), promoting its recruitment to tumor area with consequent release of protumor cytokines. By other side, the high ATP/ADP metabolism of normal cells may provide AMP to CD73 highly expressed by tumor cells produce adenosine, which via P1 receptor activation plays an important role in angiogenesis and in immune suppression. Therefore, the orchestrated extracellular adenine nucleotide metabolism by normal and cancer cells may promote differential P1/P2R sensitization on both normal and tumor cells, generating a “proliferative” advantage to cancer cells. Since both ATP and adenosine accumulation is described in tumor microenvironment, we suggest that normal and tumor cell crosstalk may favor niches of ATP and/or adenosine accumulation, which may promote a favorable microenvironment for tumor progression. Therefore, purinergic signaling could be considered as a potential target to thyroid cancer management/treatment in the future.

Disclosures

No conflicts of interest, financial or otherwise, are declared by the author(s).

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Supplementary Table 1: Primer sequences used for genes amplification of human thyroid cell lines

Human Gene	Primer Sequence	Product length (bp)
ENTPD1	Forward: 5'-CCTCTCTCACGGAGACGGA-3' Reverse: 5'-TCTTGGAGCAAAATGTCTTCACG-3'	131
ENTPD2	Forward: 5'-CTCCTACTGCTGTGCGTCC-3' Reverse: 5'-TGTCGTTCTCCTTGTCTGCC-3'	133
ENTPD3	Forward: 5'-GCTAGTCGCCTTCTCCGAAT-3' Reverse: 5'-GCAAGACCACCAAGGCAATG-3'	136
ENTPD5	Forward: 5'-GGAGTGTCTTGGCTGAATCCT-3' Reverse: 5'-CACACCTGCAGAGGCAATTT-3'	150
ENTPD6	Forward: 5'-TCGCCTTTCCTTGTAGGGGA-3' Reverse: 5'-GCTCCCTCCTGGAGTTTTCA-3'	117
CD73	Forward: 5'-GAAGGCCTTTGAGCATAGCG-3' Reverse: 5'-CGACACTTGGTGCAAAGAACA-3'	144
ACTB	Forward: 5'-AAGACAGTGTTGTGGGTGTAGG-3' Reverse: 5'-TGGGATGGGGAGTCTGTTCA-3'	126

Supplementary Table 2: Primer sequences used for genes amplification of rat thyroid cell lines

Rat Gene	Primer Sequence	Product length (bp)
Entpd1	Forward: 5'-CTGCCCTTACTCCCAGTGTG-3' Reverse: 5'-GACACTGTCGTTCGCCATCT-3'	124
Entpd2	Forward: 5'-TCTGCTACTTTGCGTCCCTAC-3' Reverse: 5'-TGTCATTCTCCTTGTCCGCTG-3'	131
Entpd3	Forward: 5'-GGAGTGGTCAGCCAAACCTT-3' Reverse: 5'-TCGTGGAGATGCTTTGGGAC-3'	140
Entpd5	Forward: 5'-TGCCGGCACCTTTTATGGAA-3' Reverse: 5'-GAAAGTCCCGGCTTCACAGA-3'	141
Entpd6	Forward: 5'-CACCTTGACCCACGAAACCT-3' Reverse: 5'-GGAATGTGTTGCTTGGCGAC-3'	120
CD73	Reverse: 5'-CCCAATCCAAGCGACACGAT-3' Forward: 5'-TTTGGATGCTGGCGATCAGT-3' Reverse: 5'-TCAATCAGTCCTTCCACACCG-3'	147
Actb	Forward: 5'-CAGGATGCAGAAGGAGATTAC-3' Reverse: 5'-CAGTGAGGCCAGGATAGA-3'	115

CAPÍTULO II

Gene expression profiling of CD73 in papillary thyroid carcinoma may predict a risk of recurrence

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Gene expression profiling of CD73 in papillary thyroid carcinoma may predict a risk of recurrence

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Abstract

The incidence of papillary thyroid cancer (PTC) has been increasing in the last years and a large number of studies have been performed to better understand its molecular pathways. The ectoenzyme CD73 is considered a promotes of tumor progression and notably, is frequently overexpressed in several tumors. However, the role of CD73 in PTC remains understudied and controversial. Here, we investigated the nucleotides hydrolysis in human normal thyroid and PTC cells in primary culture and also evaluate the association of CD73 expression to clinical aspects of PTC diagnosed patients. Our results show that CD73 activity is upregulated in thyroid cells from human PTCs, when compared to normal thyroid cells, in primary culture and CD73 mRNA levels is significantly higher in PTCs with respect to its matched adjacent normal tissue specimens (n = 32; p=0.004). Also, we showed that CD73 mRNA tumor/non-tumor levels ratio means were higher when there was nodular hyperplasia and in adjacent thyroid parenchyma when compared to normal or lymphocytic thyroiditis (p=0.0042), with metastatic lymph nodes (p=0.0002), microinvasion in thyroid bed (p=0.0141), ATA risk classification (p=0.0005) and tumor size (p=0.0493) but no significant with age (p=0.9143) and multifocality (p= 0.1929), bilaterally (p=0.8203) or AJCC stages (p=0.6853). Then, our results support the hypothesis that adenosine is a key regulator of the tumor microenvironment, raising the interesting possibility that CD73 might be a potential therapeutic target in PTC.

Introduction

The prevalence of papillary thyroid cancer (PTC), the most common tumor derived from thyroid follicular cells, has been increasing in the last years (Omry-Orbach 2016). Since around 15% of patients with PTC have not been cured with available treatment, a large number of studies have been performed to better understand its molecular pathways, which could allow the development of novel therapies (Hu, Wang et al. 2016). Furthermore, it is known that PTCs show different behaviors according to its subtypes (Nikiforov, Seethala et al. 2016).

Extracellular ATP and adenosine accumulation has been related in tumor microenvironment (TME) of several tumors . Increased amounts of extracellular adenosine in TME promotes immunessupression (Di Virgilio and Adinolfi 2016), angiogenesis, enhances tumor cell proliferation and prevents tumor cell apoptosis (Ohta 2016). In contrast to adenosine, extracellular ATP is related to proinflammatory functions, by inducing immune cells recruitment and by modulating the secretion of proinflammatory cytokines in TME (Trautmann 2009). In normal tissues, extracellular ATP concentration is at nanomolar range, whereas in TME its concentration is in order of hundreds of micromolar, or even greater than that (Pellegatti, Raffaghello et al. 2008). Simultaneous activities of both ectonucleotidases, CD39 (EC 3.6.1.5, ecto-nucleoside triphosphate diphosphohydrolase 1, E-NTPDase1) and CD73 (EC 3.1.3.5, ecto-5'-nucleotidase, 5'NT), have been reported as the most important extracellular pathways for catalyze the hydrolysis of ATP to adenosine in healthy or neoplastic tissues (REF).

Consistent with the involvement of purinergic signaling in cancer progression, our group has shown that in contrast to normal cells, tumor cells exhibits a downregulation of ATPDase activities and an upregulation of AMPase activity, which may promote extracellular accumulation of ATP and adenosine (Wink, Braganhol et al. 2003, Wink, Lenz et al. 2003, Morrone, Oliveira et al. 2006). In human thyroid cells, either normal or tumor, there is a paucity of studies about the profile of ectonucleotidase enzymatic activity and expression. In one study, CD73 activity and expression was elevated in PTC when compared to normal thyroid cells (Kondo, Nakazawa et al. 2006). The purpose of this study was to investigate the ATP and AMP hydrolysis in human normal thyroid and

PTC cells in primary culture and also evaluate the association of CD73 expression to clinical aspects of PTC diagnosed patients.

Material and Methods

Ethics statement

For obtaining primary cells from human thyroid, the project was submitted and approved by Research Ethics Committee of Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil (Nº 15-0950). For expression of CD73 and comparison of patient's outcomes with PTC, the study was performed with approval by Research Ethics Committee of Santa Casa de Misericórdia de Porto Alegre (Nº 331.061) and Research Ethics Committee of the Universidade Federal de Ciências da Saúde de Porto Alegre (Nº 362.887).

Thyroid tissue acquisition and primary cell culture

Tissue samples were obtained from patients submitted to thyroidectomy as part of the treatment for differentiated thyroid cancer. Four specimens of PTC and one normal contralateral lobe were collected after evaluation by a pathologist. Cell culture supplies and nucleotide standards (ATP, AMP) were purchased from Sigma-Aldrich (St. Louis, MO), unless stated otherwise. Tissue fragments were kept in Hank's solution at about 4ºC until processing. Human thyroid primary cultures were performed as described previously by our group (Santin, Souza et al. 2013). Briefly, thyroid tissue was cut in fragments of about 1 mm^3 and digested with 1.5 mg/mL of collagenase Type I (GIBCO, Grand Island NY., USA). The resulting suspension was filtered through nylon meshes with 60 µm pore size and epithelial thyroid cells were cultured in Ham's F-12 Coon's modification medium supplemented with 10% fetal bovine serum (FBS), 10 µg/mL insulin, 5 µg/mL transferrin, 1 mU/mL TSH, and 100 U/mL kanamycin. Cell cultures were maintained in incubator at 37°C with 5% CO_2.

Human thyroid tissue samples for gene expression

Thirty two samples of PTC and the respective non-neoplastic adjacent tissue were provided by Thyroid section, Endocrine Division, Irmandade Santa Casa de Misericórdia de Porto Alegre from 2013 to 2016.

Immunohistochemistry

All human tissue specimens were cut with a microtome at 4 μm thickness and placed on slides coated with silane. Embedded sections were initially heated for 30 min at 80ºC, deparaffinized in xylene, rehydrated through a graded series of alcohols and water. The sections were submitted to heat-induced epitope retrieval in steamer for 20 min with citrate buffer (pH 6.0) and endogenous peroxidase activity was blocked with hydrogen peroxide 5% solution in methanol. CD73 immunostaining was performed using a rabbit monoclonal anti-human CD73 antibody (dilution 1:400; D7F9A - Cell Signaling, Danvers, MA, USA) overnight at 4ºC, followed by a goat anti-rabbit IgG HRP conjugated secondary antibody (dilution 1:400; AP307P – Chemicon, DA, Germany) for 90 min, visualized with diaminobenzidine (Liquid Dab, Dako, K3468). After visualization, the slices were counterstained with Harris's haematoxylin, differentiated in ammoniac water and mounted using cover slips with *Entellan* resin (Merck, Germany). Samples of human liver and thyroid tissues not exposed to primary antibody were applied as positive and negative controls, respectively.

RNA isolation, cDNA synthesis and qPCR

Total RNA was extracted with Trizol® Reagent (Macherey Nagel, Düren, Germany) and reverse transcribed with SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). For human samples, total RNA was obtained using RNeasy® mini kit (QIAGEN GmbH, Hilden, Germany) and reverse transcribed with the RT²PCR Array First Strand Kit (QIAGEN Sciences, Maryland, USA). Gene expression was detected by real-time quantitative PCR using Fast SYBRGreen Master Mix (Applied Biosystems, Foster City, CA, USA) for TBP, ACTB, B2M, HPRT-1 and CD73 for human samples.

The expression of CD73 mRNA levels were calculated with a standard curve method using a serial dilution of samples. TBP mRNA level was used as control (Table S1; see section on supplementary data).

Ectonucleotidase assays

Enzymatic activities of ectonucleotidases in thyroid cells were determined by using ATP and AMP as substrates as already described (Wink, Lenz et al. 2003). Briefly, ATPase activity was measured by adding 1 mM ATP to reaction medium (2 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM Hepes – pH 7.4) at 37°C. For AMP hydrolysis, the same incubation medium was used with the exception that 2 mM $MgCl_2$ was used instead of $CaCl_2$ and the final nucleotide concentration was 1mM. Following 60 min incubation, the reaction was stopped by removing an aliquot of the incubation medium and transferring it to a pre-chilled tube containing trichloro-acetic acid (5% w/v). The release of inorganic phosphate (Pi) was measured by the malachite green method (Chan, Delfert et al. 1986) with KH_2PO_4 as a Pi standard. Specific activity was expressed as nmol of Pi released per min per mg of protein. Protein concentration of samples was determined by Bradford protein assay (Bradford 1976).

Statistical analysis

Statistical analysis was performed using SAS software (Version. 6.1, SAS Institute Inc., Cary, N.C.). Generalized linear model (GLM) with a log-link and gamma distribution was used to analyze the effects of clinical variables on the mean CD73 tumor/non-tumor expression ratio. Spearman's correlation coefficients were used to analyze the strength of association between tumor size and CD73 mRNA expression in tumor/non-tumor ratio, and between age and CD73 mRNA expression in tumor/non tumor ratio. *P* values of less than 0.05 indicated statistical significance.

Results

General characteristics of primary cell cultures of normal and thyroid cancer tissues

Primary cell cultures were performed from three classical PTC, one follicular variant PTC, one normal thyroid lobe after evaluation by an experienced pathologist (BMAB).

The efficacy of isolation of the epithelial thyroid cells and its typical phenotype was assessed by phase-contrast microscopy (Fig. 1; D-F) and all experiments were performed with at least ~80% confluence. In addition, cells were not expanded to avoid changes in growth rate and morphology.

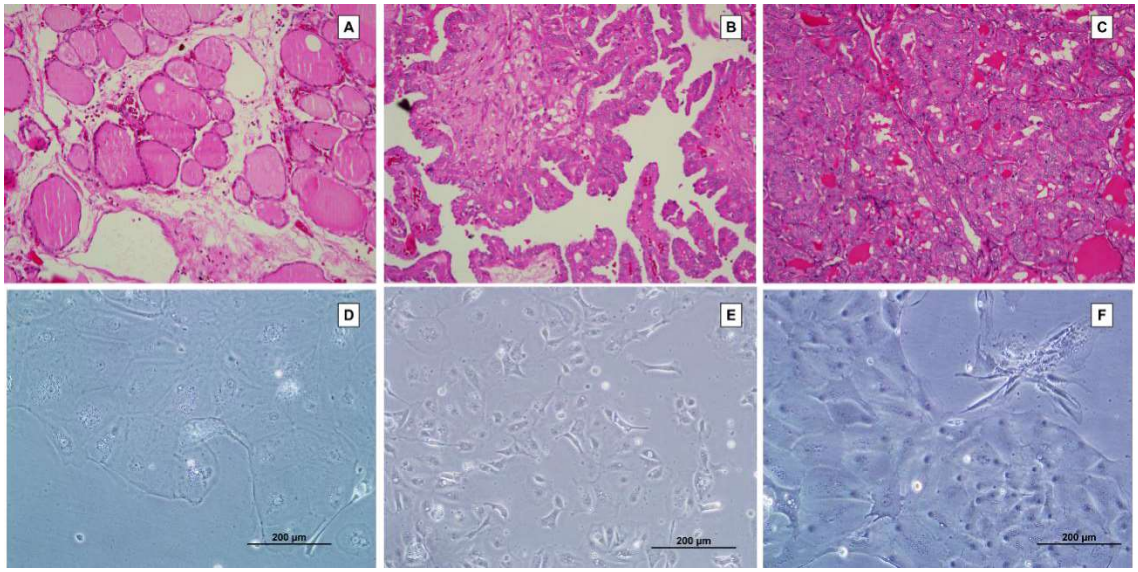

Figure 1. Morphology of epithelial cells in primary culture and their respective original tissues stained with hematoxylin and eosin (HE): normal thyroid (A and D), classical papillary thyroid carcinoma (B and E) and follicular variant of papillary thyroid carcinoma (C and F). Original magnification x200 (A, B, and C).

ATPase and AMPase activities in normal and thyroid cancer primary cultures

To compare the ATP and AMP metabolism in normal and PTC thyroid cells, cell cultures from normal lobe and cancer thyroid tissues were incubated with ATP or AMP and the inorganic phosphate (Pi) released to supernatant was measured following 60 min incubation by Malachite Green assay, as described in Methods. As shown in Figure 2,

normal thyroid cells exhibited a slightly higher ATP than AMP hydrolysis with an ATP:AMP ratio of 2.4:1. To evaluate this profile in PTC cells, thyrocyte cultures from four classical PTCs and one follicular variant of PTC (FVPTC) were examined. Interestingly, thyroid cells isolated from classic PTC exhibited higher AMPase than ATPase activity, with an 1455.4:1(*TPC1*); 9.8:1 (*TPC2*); 2.8:1 (*TPC3*) and 1.6:1 (*TPC4*) AMP:ATP ratio (Fig. 2). Thyroid cells isolated from FVPTC exhibited the same hydrolysis for both nucleotides (Fig. 2). The variability observed is not surprising, considering the biological diversity of tumors, the microenvironment of these epithelial cells and other factors. Taken together, data indicate that ATPase activity was higher in normal thyroid cells, while AMPase activity was higher in tumoral thyroid cells, characterizing an inversion of preference by substrate.

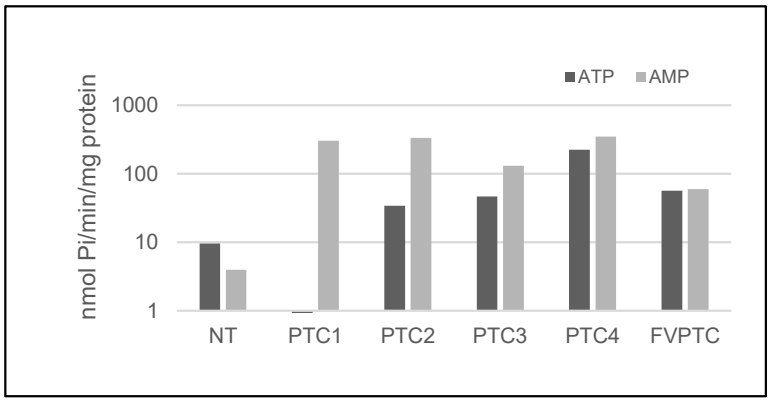

Figure 2. ATPase and AMPase activities of human thyroid cells in primary cultures derived from normal thyroid (n=1; NT) and classic papillary thyroid carcinoma (n=4; PTC1 to PTC4), follicular variant papillary thyroid carcinoma (n=1; FVPTC). Ectonucleotidase activities were determined in confluent cells (~80%) using ATP and AMP as substrate. The released inorganic phosphate was measured by the Malachite Green assay.

CD73 immunohistochemistry

The expression of CD73 was examined by immunohistochemistry (IHC) on tissues, which originated the primary cell cultures. Figure 3 shows representative examples of normal thyroid, PTC, and FVPTC.

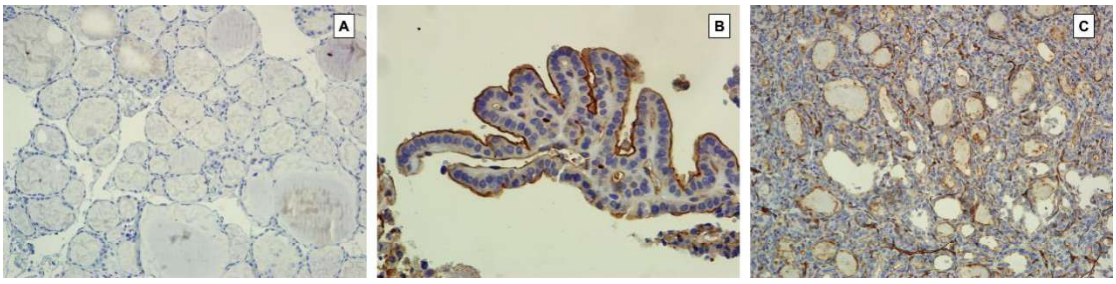

Figure 3. CD73 immunostaining in human thyroid tissue specimens. In normal thyroid tissue, there was no staining (A; x200), while in classical PTC (B; x400), and follicular variant of PTC (C; x200), there was a strong staining for CD73, which was more intense in the apical region.

Characteristics of clinical cases

To further investigate the role of CD73 on thyroid cancer malignity/progression/prognosis, a comparative analysis of CD73 mRNA expression in tumor (T) and non-tumor (NT) areas from patient samples was performed by qPCR. The experimental group was composed by 15 (46.9%) classic PTC, 6 (18.8%) FVPTC, 5 (15.6%) encapsulated follicular variant of PTC, 4 (12.5%) encapsulated papillary variant, 1 (3.1%) classic encapsulated papillary, and 1 (3.1%) Warthin-like variant of PTC. There were 4 males and 28 females, aged 22–76 years (mean age 40.2 $\pm$ 14.4 years). The mean tumor size was 2.38±1.43 cm (ranging from 0.7 to 6.3 cm). According to AJCC tumor-node-metastasis-based staging system, 25 patients (78.2%) were staged as I, 2 (6.2%) as II, and 5 (15.6%) as III. The presence of lymphocytic thyroiditis was observed in 8 (25%) cases, and tumor multifocality was observed in 24 cases (75%). Metastatic lymph nodes were identified in 13 cases (40.6%), and none case of local invasion was observed (Table 1).

Comparative CD73 mRNA expression

CD73 mRNA levels were analyzed in 32-paired tumor and non-tumor margin tissue specimens from PTC patients. Overall, CD73 expression was found in all tested samples, displaying a significantly higher expression of CD73 mRNA in PTC tumors (1.18± 0.87), relatively to paired adjacent non-tumor tissues (0.71 ± 0.39) (p=0.0088; Fig. 4A).

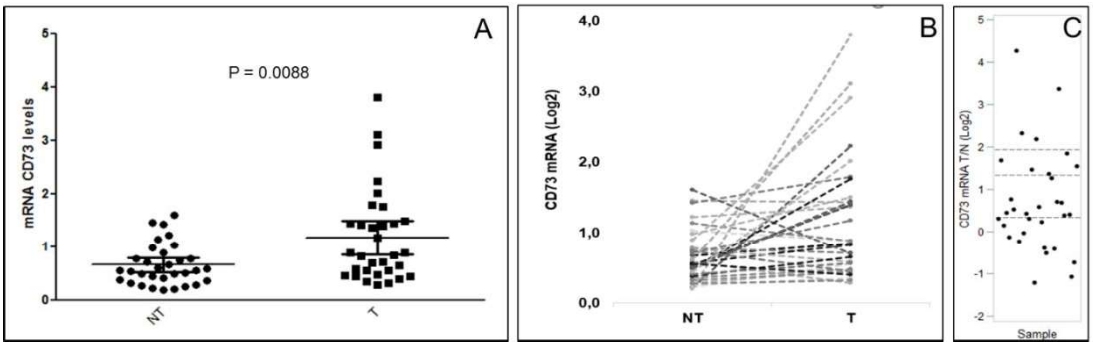

Figure 4. CD73 mRNA expression in thyroid tissues. CD73 mRNA levels were analyzed by RT-qPCR in **(A)** Adjacent non-tumor tissue (NT) and tumor (T) for each patient; **(B)** Paired NT and T for each patient; **(C)** T/NT ratio. CD73 mRNA was up-regulated in PTC tissues in comparison with non-tumor tissues (P=0.0088, Wilcoxon matched-pairs signed-rank test). In A, the larger lines represent means, and the small lines the SD; In C, dashed lines represent the mean and 95% confidence interval.

Association of CD73 expression with clinical aspects of patients with PTC

CD73 mRNA tumor/non-tumor levels ratio means were higher when there was nodular hyperplasia and in adjacent thyroid parenchyma when compared to normal or lymphocytic thyroiditis (p=0.0042). Metastatic lymph nodes (p=0.0002), microinvasion in thyroid bed (p=0.0141), ATA risk classification (p=0.0005) and tumor size (p=0.0493) were also associated to higher CD73 mRNA tumor/non-tumor level ratio means (Table 1). There was no significant association of CD73 expression with age (p=0.9143) and multifocality (p= 0.1929), bilaterally (p=0.8203) or AJCC stages (p=0.6853).

Table 1. Association of CD73 mRNA expression in tumor and CD73 mRNA expression in tumor/non-tumor ratio (T/NT) with clinical features.

Features	n (%)	CD73 mRNA (T/NT)	
		Mean (± SD)	*p* value
Adjacent thyroid parenchyma			
Normal	16 (50.0)	1.70 (1.18)	0.0042*
Lymphocytic thyroiditis	8 (25.0)	2.01 (1.33)	
Nodular hyperplasia	8 (25.0)	4.81 (6.70)	
Multifocality			
Yes	8 (25.0)	1.76 (1.32)	0.1929
No	24 (75.0)	2.81 (4.08)	
Metastatic lymph nodes			
Yes	13 (40.6)	4.10 (5.31)	0.0002*
No	19 (59.4)	1.50 (0.78)	
Bilaterally			
Yes	3 (9.4)	2.85 (1.70)	0.8203
No	29 (90.6)	2.52 (3.76)	
Local minimal invasion			
Yes	20 (62.5)	3.18 (4.39)	0.0141*
No	12 (37.5)	1.51 (1.20)	
ATA Risk			
1	13 (40.6)	1.29 (0.80)	0.0005*
2	19 (59.4)	3.42 (4.47)	
AJCC stages			
I	25 (78.2)	2.39 (3.71)	0.6853
II	2 (6.2)	2.36 (1.26)	
III	5 (15.6)	3.46 (4.02)	
Age			
> 45	22 (68.7)	2.58 (1.21)	0.9143
< 45	10 (31.3)	2.49 (1.32)	
Tumor size			
>= 2 cm	18 (56.2)	1.42 (1.17)	0.0493*
< 2 cm	14 (43.8)	0.911 (1.18)	

Data is given as number (n) and percentage (in parentheses). Expression of CD73 mRNA and CD73 mRNA tumor/non-tumor ratio is given as mean and standard deviation (SD). *P* values were determined by generalized linear model (GLM) with a gamma distribution for clinical variables and Spearman's correlation coefficients were used to analyze tumor size and age. AJCC: American Joint Committee on Cancer; ATA: American Thyroid Association; PTC: papillary thyroid carcinoma.

Discussion

This is the first study demonstrating that CD73 activity is upregulated in thyroid cells from human PTCs, when compared to normal thyroid cells, in primary culture. Also, we showed that CD73 expression is significantly higher in PTCs, when compared to adjacent non-tumor tissue, in larger tumors, and in tumors with metastatic lymph nodes.

In primary culture, we were able to demonstrate that normal and PTC cells had detectable CD73 mRNA, which is in line with our previous studies in thyroid cell lines (unpublished results). Nevertheless, AMPase activity was higher in tumor cells in primary culture, which corroborate with the data obtained analyzing thyroid cell lines (*unpublished results*). Also, our results are in accordance with previously published study by Kondo *et al* showing that CD73 is overexpressed and present strong activity in slices of PTC, when compared to normal thyroid (Kondo, Nakazawa et al. 2006). Another study showed that CD73 was absent in epithelial cells in medullary carcinomas and weakly expressed in normal thyroid, multinodular goiters, thyroiditis, Graves' disease, benign follicular adenoma, or papillary and follicular carcinoma (Cohen, Miller et al. 1986). Likewise, the higher rate of ATP hydrolysis, in comparison to AMP observed in normal primary cells, also agrees with our previous findings in normal thyroid cell lines (*unpublished results).* The increased AMPase activity in malignant tumors was already described in glioma cell lines (Wink, Lenz et al. 2003) and in medulloblastoma cells (Cappellari, Rockenbach et al. 2012).

Although the tissue surrounding tumors can be affected by tumor microenvironment, a recent study showed that gene expression in paired samples allows to verify relevant information about intermediate state between normal and tumor tissues. Also, this comparison provides insights on disease etiology and disease progression (Huang, Stern et al. 2016).

We observed that overexpression of CD73 mRNA in tumor, relatively to the paired normal thyroid tissue, is associated to tumor size, lymph node metastasis, and ATA risk. Both, lymph node metastasis and ATA risk classification are important predictor factors of recurrent/persistent disease in PTCs (Wada, Suganuma et al. 2007, Tuttle, Tala et al.

2010). It is known that overexpression of CD73 leads to accumulation of extracellular adenosine that has been connected to tumor growth promotion and metastasis (Zhang 2012). We also had shown that tumor-derived thyroid cell lines produced far greater amounts of adenosine than thyroid cell lines derived from thyroid cells (*unpublished results*). One unexpected finding was the overexpression of CD73, when the tumor surrounding tissue was nodular thyroid hyperplasia, as compared to normal thyroid or lymphocytic thyroiditis. Then, our results support the hypothesis that adenosine is a key regulator of the tumor microenvironment, raising the interesting possibility that CD73 might be a potential therapeutic target in PTC. Then, this reinforce the importance of clinical trials to confirm if anti-CD73 therapies might inhibit tumor growth and metastasis.

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CONSIDERAÇÕES FINAIS

O carcinoma papilífero da tireoide é a variante mais comum dentre os tumores malignos que acometem a glândula tireoide e representam cerca de 80% dos casos. Embora este tipo de carcinoma represente um bom prognóstico, estudos atuais demonstram que o risco de recorrência da doença é mais freqüentemente analisado em relação ao prognóstico, com uma estimativa de cerca 30% de recorrência (Schreinemakers, Vriens et al. 2012). Outro fato relevante é o caráter heterogêneo desta neoplasia, demonstrado pelas diferenças nas características histopatológicas, fatores genéticos e nas diferenças de prognóstico. Essa heterogeneidade acaba afetando diretamente o padrão de escolha de tratamento, o qual apresenta divergências entre protocolos empregados e além de dados conflitantes na literatura. Recentes diretrizes da *American Thyroid Association* e da Sociedade Brasileira de Endocrinologia e Metabologia recomendam atenção na estratificação de risco de algumas variantes de PTC, a fim de diminuir procedimentos cirugicos desnecessários e tratamentos mais agressivos como radioterapia, os quais levam a um aumento do potencial de morbidade, além de aumento dos custos financeiros. Por outro lado, pacientes inicialmente considerados de baixo risco de recorrência, com base na sua classificação, podem apresentar uma resposta incompleta ao tratamento que, sem uma vigilância adequada, podem não ser devidamente tratados. Assim, evidenciamos que pouco se conhece sobre os mecanismos que determinam uma maior ou menor agressividade no

carcinoma papilar. Sendo que esta base nos proporciona a buscar um melhor entendimento dos mecanismos moleculares e alvos chaves que atuam nos padrões distintos de expressão gênica, sinalização e características clínicas e que possam estar favorecendo ou não a progressão tumoral.

Neste contexto, e diante da disponibilidade de técnicas de genética molecular, sucederam-se inúmeros estudos nas ultimas décadas, mostrando um painel diversificado e crescente de marcadores moleculares de diagnóstico e de prognóstico a diversos tipos tumorais. Estudos recentes, complexos e bem delineados têm demonstrado que a presença de altas concentrações de adenosina no microambiente tumoral, possa atuar como um importante agente imunossupressor. Além de estimular a progressão tumoral, por promover a proliferação celular, angiogenêse e prevenir eventos apoptóticos (Allard, Beavis et al. 2016, Ohta 2016). Por outro lado, o acúmulo do ATP extracelular tem sido considerado um promotor de respostas inflamatórias no microambiente tumoral e um importante indutor de morte celular (Trautmann 2009).

Nossos resultados inéditos - apresentados no capítulo I – demonstram que as células derivadas de carcinoma papilar de tireoide, K1 e PTC-1, apresentam uma atividade AMPásica elevada em relação a células derivadas de tireoide normal, FRTL-5 e PCCL3. Esse padrão de hidrólise foi corroborado pelo perfil molecular destas linhagens, demostrando que as linhagens turmorais analisadas apresentam uma expressão cerca de 5 vezes maior da enzima CD73, quando comparadas as linhagens normais. Ainda, os baixíssimos níveis ou ausência dos genes que codificam as E-NTPDases 1 e 2 em ambas linhagens tumorais, porém presentes nas linhagens normais, sugerem que o processo de transformação maligna levou a perda da expressão destas enzimas e que por tal, podemos inferir que ambas E-NTPDases não

são enzimas cruciais para o metabolismo das células tumorais. Seguindo este raciocínio, podemos sugerir que acúmulo de adenosina observado pela degradação de AMP (Figura 3, Capítulo I) também é um mecanismo de malignização celular, uma vez que a presença de altas concentrações de adenosina pode agir de forma a auxiliar a progressão tumoral e na evasão do sistema imune. Esses dados corroboram com trabalhos prévios publicados por nosso grupo, os quais atividade AMPásica apresenta-se elevada em células tumorais quando comparadas a células normais (Wink, Braganhol et al. 2003, Wink, Lenz et al. 2003, Morrone, Oliveira et al. 2006).

Nossos resultados apresentados no Capítulo II foram obtidos a partir de células de tireoide humana em cultura primária, provenientes de tecido tireoideano normal e de carcinoma papilar de tireoide. Embora as células foliculares em cultura primária apresentem limitações, principalmente em relação a impossibilidade de expansão e a heterogeneidade entre os isolamentos, temos a grande vantagem destas células mantêrem as características do tecido de origem.

A histologia característica dos fragmentos utilizados para o isolamento das células foliculares confirmou a normalidade, bem como a presença de carcimona papilar os tecidos tireoideanos. Entre as células foliculares isoladas de carcimona papilar, podemos observar uma maior atividade AMPásica quando comparada a atividade ATPásica, igualmente observamos nas linhagens tumorais K1 e TPC-1. Ainda, podemos observar que a atividade específica variou entre as células isoladas de carcinoma folicular, refletindo a variabilidade comumente descrita nesta neoplasia.

A forte imunoreatividade da CD73 nos fragmentos utilizados para os cultivos primários (Figura 3; Capítulo II), mostram que a localização da CD73 está na porção apical da membrana plasmática da célula folicular, a qual é voltada para o colóide. Onde

também estão localizadas diversas enzimas importantes nos processos de iodação de resíduos de tirosina da tireoglobulina, síntese e secreção de hormônios tireoideanos e transporte intracelular de iodo. Trabalhos de nosso grupo demonstraram que a exposição a hormônios tireoideanos em células de glioma C6 e em células de músculo liso isoladas da aorta, levou a um aumento da atividade e expressão da CD73 (Wink, Tamajusuku et al. 2003, Tamajusuku, Carrillo-Sepulveda et al. 2006). Da mesma forma, outro estudo demonstrou um aumento da atividade da CD73 em homogenatos de tecido tireoideano submetidos a hiperplasia (Bastomsky, Zakarija et al. 1971). Baseando-se nestes achados, podemos sugerir uma correlação positiva entre a expressão da CD73 e hiperfunciomento das células foliculares. Ainda, podemos sugerir que devido a sua localização na região apical, a expressão da CD73 seja modulada pelo painel de enzimas reponsáveis pela produção dos hormônios tireoideanos.

A relevância da CD73 na patogênese do carcinoma papilar de tireoide é pobremente explorada por dois estudos que são conflitantes entre si. Cohen em sua publicação de 1986, demonstra uma baixa expressão da CD73 em carcimonas papilares e foliculares (Cohen, Miller et al. 1986). Enquanto Kondo, em uma breve comunicação publicada em 2006, demonstra uma superexpressão e uma alta atividade da CD73 em células foliculares de carcinoma papilar.

No Capítulo II, a nossa análise dos dados de expressão gênica, em espécimes de PTCs, revelam uma expressão altamente significativa, quando comparadas ao tecido adjacente não tumoral (Figura 4; Capítulo II).

Quando emparelhamos a expressão nas amostras de PTCs com seu respectivo tecido adjacente não tumoral, revelamos uma correlação positiva com a presença de

linfonodos com metástases, maior tamanho tumoral, maior risco segundo classificação sugerido pela ATA e presença de invasão local.

A presença de metástases é fator de malignidade tumoral e representa uma das principais causas de morte em pacientes com câncer. Desta forma, moléculas que estejam associadas a metástases, apresentam um alvo importante para o desenvolvimento de novas drogas. A correlação da alta expressão da CD73 em tipos tumorais como melanomas (Sadej, Spychala et al. 2006), carcinoma gástrico (Durak, Cetin et al. 1994), câncer colorretal (Eroglu, Canbolat et al. 2000), câncer de ovário (Turcotte, Spring et al. 2015) e carcinoma de mama (Wang, Zhou et al. 2008), nos quais também se observou uma relação da expressão com presença de metástases, estimulou o desenvolvimento de ensaios clínicos. A base desses ensaios foram drogas que bloqueiam a atividade da CD73. bem como o bloqueio de repcetores de adenosina, a qual é gerada através hidrólise de AMP e se encontra elevada no microambiente tumoral (Allard, Beavis et al. 2016). Os principais mecanismos sugeridos para explicar a relação da CD73 na aquisição deste fenótipo invasivo foi através da promoção da transição epitelial-mesenquimal induzida (EMT) nas células tumorais (Xiong, Wen et al. 2014), pela estimulação de receptores de adenosina (A2a) (Stagg, Divisekera et al. 2010, Beavis, Divisekera et al. 2013), pela promoção de angiogênese (Wang, Tang et al. 2013) e por promover a evasão do sistema imune (Zhang 2012). Estes mecanismos citados, seriam os mesmos pelo quais a adenosina, produzida pela superexpressão da CD73, levaria a um aumento do crescimento tumoral (Zhang 2012).

Outro achado importante em nosso trabalho foi associação da CD73 com o risco de recorrência que é predito pela classificação maior (II), sugerido pela ATA (Tabela 1; Capítulo II). Vale ressaltar que a presença de linfonodos com metástases de PTC

também é um importante fator de risco para recorrência da doença. Uma das principais discussões apresentadas na literatura sobre o risco de recidiva em PTCs, é justamente quando há presença de linfonodos metastáticos, porém uma ausência de outros fatores que determinam um pior prognóstico. Interessantemente, a expressão a CD73 não esteve associada a maior grau de malignidade, segundo estadiamento sugerido pela AJCC, ou uma maior idade (Tabela 1; Capítulo II), sendo que estes dois fatores estão associados a um pior prognóstico. Contudo, o baixo número de pacientes analisados e a discrepância de pacientes em cada categoria podem ter influenciado a análise dessas associações. A correlação de maior expressão da CD73 no tecido adjacente que apresentava hiperplasia nodular, em comparação com tecido adjacente normal ou com presença tiroidite linfocítica, também precisa ser melhor analisado, uma vez que há trabalhos na literatura associando uma maior atividade da CD73 com modelos induzidos de hiperplasia tireoideana (Bastomsky, Zakarija et al. 1971).

Por fim, com base nos achados desta dissertação, entedemos que os componentes do sistema purinérgico estão presentes e são funcionais nas células foliculares da tireoide. A alta expressão de CD73 promovendo a hidrólise de AMP e levando ao acúmulo adenosinafaz com que o carcinoma papilar seja mais um dos tipos tumorais que possa entrar para lista dos tumores que possam responder positivamente a terapias anti-CD73, afim de inibir o crescimento tumoral e prevenir metástases.

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