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Marcadores Prognósticos nas Neoplasias de Tireoide

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Tese de Doutorado

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- Revisão breve sobre o assunto.

- **Artigo 1 (original):** Serum TSH levels as a predictor of malignancy in thyroid nodules: A prospective study; publicado em PLoS One, 2017. Impact Factor: 2.776.

- **Artigo 2 (original):** Role of vascular endothelial growth factor polymorphisms in the pathogenesis of medullary thyroid carcinoma.

Além dos artigos que fazem parte da presente tese, ao longo do período do doutorado, participei de outros projetos que resultaram nos seguintes manuscritos:

- Neoadjuvant multikinase inhibitor in patients with locally advanced unresectable thyroid carcinoma**.** Nava C; Scheffel R, **Cristo AP**; Ferreira CV; Weber S; Zanella AB; Paixão FC; Migliavaca A; Guimaraes JR; Graudenz MS; Maia AL. Front Endocrinol (Lausanne) 2019. Impact Factor: 3.519

- Impact of Serum TSH and Anti-Thyroglobulin Antibody Levels on Lymph Node Fine-Needle Aspiration Thyroglobulin Measurements in Differentiated Thyroid Cancer Patients. Duval MADS, Zanella AB, **Cristo AP**, Faccin CS, Graudenz MS, Maia AL. Eur Thyroid J. 2017. Impact Factor: 3.025

- Increasing diagnostic effectiveness of thyroid nodule evaluation by implementation of cell block preparation in routine US-FNA analysis. **Cristo AP**, Goldstein HF, Faccin CS, Maia AL, Graudenz MS. Arch Endocrinol Metab. 2016. Impact Factor: 1.571

LISTA DE ABREVIATURAS E SIGLAS

ATC - Anaplastic Thyroid Carcinoma

BRAF - Serine/threonine-protein kinase B-Raf

CAT - Carcinoma Anáplasico de Tireoide

CDT - Carcinoma Diferenciado de Tireoide

CEA - antígeno carcinoembrionário

CFT - Carcinoma Folicular de Tireoide

CMT - Carcinoma Medular de Tireoide

CPT - Carcinoma Papilar de Tireoide

DTC - Differentiated Thyroid Carcinoma

FTC - Follicular Thyroid Carcinoma

INCA - Instituto Nacional do Câncer

MAPK - Mitogen-activated protein kinase

miRNA- MicroRNA

MTC - Medullary Thyroid Carcinoma

PAAF - Punção aspirativa por agulha fina

PAX8 - Paired box gene 8

PI3K - Phosphatidylinositol 3-kinase

PPARγ – Peroxisome proliferator-activated receptor γ

PTC - Papillary Thyroid Carcinoma

RET (REarranged during Transfection) - proto-oncogene RET

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

SNP - polimorfismos de nucleotídeo único

TKR - receptores tirosina-cinase

TSH - hormônio tireotrófico

VEGF - Vascular endothelial growth factor

SUMÁRIO

RESUMO

Nódulos tireoidianos são achados clínicos comuns. O câncer de tireoide, em contraste, é raro, embora consista na neoplasia endócrina maligna mais frequente. Um dos principais desafios no manejo de pacientes com neoplasias de tireoide é identificar, além dos parâmetros clínicos, características morfológicas e alterações moleculares capazes de diferenciar tumores que se comportarão de forma mais agressiva. A identificação de marcadores prognósticos confiáveis que determinem já ao diagnóstico, o comportamente biológico do tumor poderá evitar que pacientes de baixo risco sejam expostos a condutas agressivas desnecessárias, e distinguir estes pacientes daqueles que, por apresentarem evolução menos favorável, necessitem de cirurgias mais extensas e acompanhamento mais intenso.

Com os avanços no conhecimento da patogênese das neoplasias tireoidianas, inúmeros estudos vêm investigando marcadores preditivos e prognósticos em relação aos tumores da tireoide, possibilitando o desenvolvimento de novos agentes antineoplásicos. Dessa forma, a tendência na atualidade é de que o tratamento seja feito de forma mais individualizada, considerando o quadro clínico e marcadores, morfológicos, imunohistoquímicos e moleculares do paciente e do tumor.

A primeira parte desta tese compreende uma avaliação prospectiva do papel dos níveis de hormônio tireotrófico (TSH) como preditores de malignidade em nódulos da tireoide em uma amostra de 615 pacientes submetidos à punção aspirativa por agulha fina (PAAF) guiada por ultrassom. O TSH é um fator de crescimento essencial para as células da tireoide e sua via de sinalização é necessária para a expressão de outros fatores de crescimento, receptores e proto-oncogenes. Consequentemente, a supressão do TSH é importante para o manejo clínico do câncer de tireoide. De modo interessante, observamos que níveis séricos mais altos de TSH foram associados a um risco aumentado de malignidade. Pacientes com nódulos malignos apresentaram TSH sérico mais elevado que pacientes com nódulos benignos nos dois ensaios analisados (2.25 vs. 1.50; P = 0.04 e 2.33 vs. 1.27; P = 0.03) e o risco de malignidade foi aproximadamente três vezes maior em pacientes com níveis de TSH $\geq$ 2.26μU/mL do que em pacientes com níveis mais baixos de TSH. Nossos resultados corroboram estudos prévios que sugerem o uso do TSH como ferramenta diagnóstica auxiliar na estratificação do risco de malignidade associado a nódulos tireoidianos bem como na tomada de decisão da conduta terapêutica.

A segunda parte deste trabalho, por sua vez, avaliou em uma coorte de 420 pacientes com carcinoma medular de tireoide (CMT), o papel de polimorfismos de nucleotídeo único (SNPs) do gene VEGF-A na patogênese da doença através da correlação das frequências das variantes com dados clínicos, laboratoriais e prognóstico. Sabe-se que em diversos tumores, incluindo o CMT, há uma superexpressão do VEGF-A e seus receptores, o que possibilita utilizá-los como alvos moleculares para terapias com inibidores multiquinase (MKI) e também como marcadores prognósticos. Os SNPs do VEGF-A rs699947 (C> A), rs833061 (T <C) e rs2010963 (G> C) foram genotipados usando ensaio de TaqMan e os haplótipos foram inferidos usando o programa Phase. 202 (48%) pacientes apresentaram a forma hereditária e 218 (52%) apresentaram a forma esporádica do CMT. A idade média do diagnóstico foi de 40 ± 19.7 anos e 61% eram do sexo feminino. As freqüências alélicas dos SNPs do VEGF-A foram: rs699947 (37.2%), rs833061 (45.6%) e rs2010963 (34.4%). No CTM hereditário, observamos uma associação independente do VEGF-A rs833061 com menor idade ao diagnóstico (genótipo TT) e menor tamanho do tumor (genótipo CC). Além disso, o VEGF-A rs2010963 (genótipo GG) foi associado com menor tamanho tumoral. Para pacientes com CMT esporádico, não foram observadas associações independentes. Nossos resultados evidenciaram relação entre SNPs do gene VEGF-A e fatores prognósticos em CMT sugerindo que essas variantes podem impactar o comportamento tumoral e a apresentação da doença.

Em conjunto, nossos resultados indicam que o conhecimento mais aprofundado de marcadores clinicopatológicos ou moleculares específicos podem melhorar a determinação do prognóstico e comportamento tumoral, auxiliando no diagnóstico e no direcionamento para formas de tratamentos mais adequadas.

ABSTRACT

Thyroid nodules are common clinical findings. Thyroid cancer, in contrast, is rare and consists of the most frequent malignant endocrine neoplasia. One of the main challenges in the management of patients with thyroid neoplasms is to identify in addition to clinical parameters, morphological characteristics and molecular changes that are able to differentiate certain tumors that will behave more aggressively than others. The finding of accurate prognostic markers that determine the severity of the case at diagnosis may avoid low-risk patients from being exposed to unnecessary aggressive medical conduct and distinguish these patients from those who, due to their less favorable evolution, require more extensive surgery and follow-up more intense.

With advances in the knowledge of the pathogenesis of thyroid neoplasms, numerous studies have been investigating predictive and prognostic markers in relation to thyroid tumors, enabling the development of new antineoplastic agents. Thus, the current trend is that the treatment should be more individualized, considering the clinical condition and morphological, immunohistochemical and molecular markers of the patient and the tumor.

The first part of this research work comprises a prospective evaluation in a sample of 615 patients submitted to fine-needle aspiration biopsy (FNAB) guided by ultrasound, the usefulness of thyroid-stimulating hormone (TSH) levels as a predictor of malignancy in thyroid nodules. TSH is a major thyroid cell growth factor, while TSH signaling pathway activation may berequired for the expression of other growth factors, receptors, and proto-oncogenes. Accordingly, TSH suppression is an important therapeutic tool of clinical thyroid cancer management. Interestingly, we observed that higher serum TSH levels were associated with an increased risk of malignancy since patients with malignant nodules presented higher TSH levels than patients with benign nodules in two TSH assays (2.25 vs. 1.50; $P = 0.04$ and 2.33 vs. 1.27; $P = 0.03$) and the risk of malignancy was approximately 3-fold higher in patients with TSH levels ≥ 2.26 μU/mL than in patients with lower TSH levels. Our results corroborate previous studies that suggest the use of TSH as an auxiliary diagnostic tool in the stratification of the risk of malignancy associated with thyroid nodules as well as in the decision of therapeutic conduct.

The second part of this work, in turn, evaluated in a cohort of 420 patients with medullary thyroid carcinoma (MTC), the role of single nucleotide polymorphisms (SNPs) of the *VEGF-A* gene in the pathogenesis of the disease through the correlation of the frequencies with clinical, laboratory and prognostic data. It is known that in several tumors, including MTC, there is an overexpression of VEGF-A and its receptors, which makes it possible to use as molecular targets for therapies with multiquinase inhibitors (MKI) and also as prognostic markers. The VEGF-A SNPs rs699947(C>A), rs833061(T<C) and rs2010963(G>C) were genotyped using TaqMan Genotyping Assays. Haplotypes were inferred using Phase program. Of the 420 MTC patients analyzed, 202 (48%) presented hereditary and 218 (52%) had the sporadic form of disease. The mean age of diagnosis was 40 ± 19.7 years and 61% were female. The minor allele frequencies of VEGF-A SNPs were: rs699947 (37.2%), rs833061 (45.6%), and rs2010963 (34.4%). In hereditary MTC, we observed an independent association of the VEGF-A rs833061 with younger age at diagnosis (TT genotype) and smaller tumor size (CC genotype). Additionally, VEGF-A rs2010963 (GG genotype) was correlated with smaller tumor size.Our results showed a relationship between SNPs of the *VEGF-A* gene and prognostic factors in MTC, suggesting that these variants may impact tumor behavior and disease presentation.

These data suggest that further knowledge of clinicopathological or specific molecular markers that may improve prognostic determination and tumor behavior, may assist in the correct definition of the diagnosis as well as targeting more appropriate forms of treatment.

INTRODUÇÃO

Nódulos tireoidianos são achados clínicos comuns, sendo a prevalência de nódulos palpáveis de tireoide de, aproximadamente, 5% em mulheres e 1% em homens que vivem em áreas iodo-suficientes (1). A grande importância clínica dos nódulos está relacionada à necessidade de exclusão de malignidade (5-10% dos casos). Atualmente, a punção aspirativa por agulha fina (PAAF) guiada por ultrassom é a melhor ferramenta para avaliação e diagnóstico nos nódulos tireoidianos. Nos últimos anos, nódulos incidentais de tireoide vêm sendo diagnosticados com uma frequência crescente através da utilização de técnicas de imagem altamente sensíveis (2, 3). Mais recentemente, o Colégio Americano dos Radiologistas (ACR) também publicou uma classificação ultrassonográfica, conhecida como Thyroid Imaging, Reporting and Data System (TI-RADS). O risco de malignidade é similar em nódulos únicos e múltiplos, portanto, frente a uma tireoide multinodular, devem-se selecionar para punção, nódulos que apresentam características ultrassonográficas de maior risco de malignidade (4 ,5).

O câncer de tireoide é a neoplasia endócrina mais comum, representando aproximadamente 1-1.5% de todos os novos cânceres diagnosticados nos EUA e na Europa, com aumento continuado da incidência nas últimas décadas (6, 7). No Brasil, o Instituto Nacional do Câncer (INCA) estima para cada ano do biênio 2018/2019, 9.610 novos casos de câncer de tireoide (1.570 em homens e 8.040 em mulheres). Esses valores correspondem a um risco estimado de 1.49 casos a cada 100 mil homens e 7.57 casos a cada 100 mil mulheres, sendo o 5º tumor maligno mais frequente em mulheres (INCA; http://www.inca.gov.br).

As neoplasias da tireoide abrangem um grupo heterogêneo de tumores com comportamento clínico, características histológicas, fenotípicas e padrões de disseminação metastáticos distintos (8, 9). O carcinoma diferenciado de tireoide (CDT) é o tipo mais frequente de câncer de tireoide, correspondendo a 80-90% dos casos enquanto as formas mais raras são o carcinoma medular da tireoide (CMT) e o carcinoma anaplásico de tireoide (CAT) (10).

O CDT desenvolve-se a partir das células foliculares da glândula e compreende o carcinoma papilar de tireoide (CPT) e o carcinoma folicular de tireoide (CFT) (11, 12). Em sua maioria, o CDT mantém a capacidade de captar iodo e de responder a

estímulos fisiológicos como o hormônio tireotrófico (TSH). O CDT apresenta excelente prognóstico, mesmo nos casos de doença metastática. A taxa média de sobrevida em 10 anos para pacientes com CPT é de aproximadamente 95% nos casos de doença restrita à tireoide. Em pacientes com doença metastática, a sobrevida média é de 70% e 64% em 10 e 15 anos, respectivamente (13, 14).

O CMT tem origem nas células C tireoidianas e representa em torno de 3-4% dos cânceres da tireoide, podendo ocorrer de forma esporádica (75% dos casos) ou hereditária (25%), como parte da síndrome da neoplasia endócrina múltipla tipo 2 (NEM2). A NEM2 é causada por mutações de linhagem germinativa no proto-oncogene *RET*, sendo o padrão de herança autossômico dominante, sendo subdividida em NEM2A e NEM2B. A NEM2A é caracterizada pela presença de CMT (95%), hiperparatireoidismo (10-20%) e feocromocitoma (30-50%) enquanto indivíduos com NEM2B apresentam CMT (>90%) e feocromocitoma (45%), acompanhados por ganglioneuromas (100%) e habitus marfanoide (65%) (15).

O curso clínico do CMT pode variar de um tumor indolente que permanece inalterado por anos para uma forma agressiva associada a uma alta taxa de mortalidade. A doença metastática progressiva constitui o principal desafio no manejo de pacientes com CMT uma vez que as opções terapêuticas disponíveis apresentam resultados pouco expressivos. A terapia inicial para o CMT é a tireoidectomia com ou sem dissecção de linfonodos por compartimentos. Já na doença metastática avançada, os objetivos da cirurgia são paliativos com vistas a minimizar complicações e comorbidades levando-se em consideração a expectativa e a qualidade de vida do paciente. Os tratamentos para o CMT metastático avançado consistem na possível ressecção do tumor, na radioterapia por feixe externo (EBRT) ou na administração de terapia sistêmica (16). De modo geral, o prognóstico do CMT é considerado menos favorável do que o prognóstico de tumores derivados de células foliculares diferenciados. De fato, apesar de representar de 3 a 4% dos tumores tireoidianos, o CMT é responsável por uma taxa de mortalidade de 13% (15-18).

O CAT ou carcinoma indiferenciado é uma forma extremamente rara e agressiva, representando entre 1% e 2% dos tumores tireoidianos. O CAT pode se originar de novo ou ser resultado da progressão e/ou desdiferenciação dos carcinomas diferenciados. Aproximadamente 50% dos pacientes com CAT tiveram carcinoma diferenciado prévio ou coexistente. Em contraste com as células do CDT, as células no

CAT não preservam as características e funções biológicas das células foliculares normais da glândula como captação de iodo, síntese de tireoglobulina e dependência de TSH. O curso clínico do CAT é caracterizado por doença local agressiva, altas taxas de metástases e rápido desfecho fatal (20-50% de todas as mortes por câncer de tireoide). O tempo médio de sobrevida é de aproximadamente 5 a 6 meses e apenas 10 a 15% dos pacientes sobrevivem 2 anos após a apresentação da doença (19-21).

Marcadores Clínico-Patológicos e Moleculares de Prognóstico

A presença de nódulos na tireoide é bastante comum. A etiologia da doença nodular da tireoide é multifatorial, compreendendo um espectro que vai do pequeno nódulo achado de forma incidental a um grande bócio multinodular intra-torácico. Fatores de risco para malignidade vêm sendo estabelecidos para direcionar o diagnóstico, prognóstico e manejo do paciente com nódulo tireoidiano (22).

Marcadores de risco de malignidade e prognósticos mais ou menos favoráveis incluem idade, sexo, história familiar de câncer de tireoide, exposição à radiação ionizante, nível de TSH sérico, entre outros (23). Características ultrassonográficas como nódulos sólidos, hipoecogênicos, com microcalcificações, mal delimitados e com aumento de fluxo nodular central ao Doppler podem ser consideradas suspeitas para malignidade, apesar de não ser possível diferenciar lesões benignas de malignas somente pelo exame ultrassonográfico (3, 24, 25).

A prevalência de nódulos tireoidianos aumenta com o avanço da idade. Em indivíduos mais velhos o achado de um fenótipo histológico de maior risco é mais provável, como variantes mais agressivas de CPT, carcinomas pouco diferenciados ou CAT (26). Outro parâmetro clínico objeto de estudos relacionado com risco de malignidade é o TSH sérico. O TSH é um fator de crescimento essencial das células da tireoide e sua via de sinalização é necessária para a expressão de outros fatores de crescimento, receptores e proto-oncogenes. Estudos prévios sugerem o uso do TSH como ferramenta diagnóstica auxiliar na estratificação do risco de malignidade associado a nódulos tireoidianos e também no direcionamento da conduta terapêutica (27, 28).

Dessa forma, o reconhecimento de marcadores prognósticos é essencial não apenas para a exclusão da malignidade em nódulos mas, principalmente, para

complementar o diagnóstico nos casos de câncer de tireoide. Os tumores tireoidianos apesar de apresentarem histologia e características clínicas bastante diversas, são indolentes na maioria dos casos, com resultados satisfatórios através da terapia convencional disponível. No entanto, alguns tumores podem ocorrer sob formas agressivas associadas a altas taxas de mortalidade e prognósticos desfavoráveis uma vez que as opções de tratamento para estes casos são restritas tornando a eficácia terapêutica limitada e incerta (29).

Apesar das controvérsias e mudanças nos últimos anos acerca da classificação morfológica, é irrefutável que o câncer de tireoide manifesta um espectro de morfologias que se correlacionam com o comportamento clínico (23, 30). Informações sobre o significado dos vários componentes histológicos e suas características incluindo arquitetura, padrão de crescimento, citologia e padrões de invasão têm impactado a determinação do diagnóstico e manejo dos pacientes. Além disso, marcadores expressos pelo tumor e identificados por imunohistoquímica podem colaborar em conjunto com outras características morfológicas na determinação do prognóstico (31).

A abordagem terapêutica atual inclui vigilância ativa, ressecção cirúrgica que pode ser parcial ou total, com ou sem dissecção de linfonodos, radioterapia, radioiodo ou ainda, terapias moleculares alvo-específicas. As novas estratificações de risco apontam para a necessidade da análise do perfil prognóstico do paciente, características do tumor e uso racional dos recursos considerando os riscos de subtratamento ou tratamento exagerado (30, 32).

Atualmente, a identificação de biomarcadores que tenham a capacidade de fornecer características específicas do tipo tumoral e da sua progressão, além de informações sobre o comportamento biológico do tumor, como agressividade e capacidade de disseminação, constitui um desafio importante no tratamento das neoplasias tireoidianas (30, 33).

Neste contexto, a angiogênese tem sido relacionada à taxa de crescimento tumoral e prognóstico através da expressão de marcadores associados ao comportamento do tumor. Esse mecanismo complexo fornece nutrientes essenciais para o desenvolvimento tumoral como oxigênio, enzimas proteolíticas, hormônios e fatores de crescimento e tem como um de seus principais reguladores o fator de crescimento endotelial vascular (VEGF) (34).

Estudo realizado pelo nosso grupo demonstrou que o VEGF e seus receptores estão superexpressos e associados a prognóstico menos favorável em CMT, sugerindo

que essas moléculas podem ser consideradas biomarcadores e também possíveis alvos terapêuticos para tumores não ressecáveis ou metastáticos (35). Em relação ao *VEGF-A*, um estudo multicêntrico observou uma correlação entre alguns polimorfismos de nucleotídeo único (SNPs) comuns ao gene e risco de recorrência estrutural doença ou sobrevida livre de doença em pacientes com câncer de tireoide diferenciado não-avançado (papilar e folicular) (36).

Nas últimas décadas, observou-se um progresso considerável na compreensão dos mecanismos moleculares envolvidos na patogênese do câncer de tireoide (10). As anormalidades genéticas mais comuns no CPT são a ativação aberrante da via de sinalização celular mitogen-activated protein kinase (MAPK), mutações pontuais no gene *BRAF* ou *RAS* e rearranjos RET/PTC. O CFT está frequentemente associado à ativação das vias fosfatidilinositol-3-cinase (PI3K) e MAPK, mutações de *NRAS*, rearranjos como PAX8/PPARγ, e outros eventos (37-39).

No CMT, mutações na linhagem germinativa no proto-oncogene *RET* (REarranged during Transfection) são responsáveis pela forma hereditária da doença e mutações somáticas no *RET* são observadas em casos esporádicos (15, 16). A presença de mutação somática do códon M918T do *RET*, os níveis séricos de calcitonina e antígeno carcinoembrionário (CEA) e os respectivos tempos de duplicação e o número de linfonodos e compartimentos acometidos são os principais indicadores prognósticos nesses pacientes (1, 15, 16).

O CAT compreende tumores mais complexos geneticamente com múltiplas alterações em genes que codificam receptores tirosina-cinase (TKR) e vias como PI3K/AKT/mTor (19).

Dessa forma, mutações, rearranjos genéticos, alterações nas vias de sinalização e outros potenciais fatores moleculares como os microRNAs (miRNA) e os SNPs estão sendo estudados como marcadores prognósticos e podem proporcionar terapias direcionadas visto que estão associados com progressão, metástases e risco de recorrência da doença (37).

A avaliação de possíveis indicadores prognósticos é fundamental para que terapias mais adequadas sejam oferecidas para cada caso visto que esse conjunto de fatores é individual e determinante para a maior ou menor eficácia do tratamento, além de identificar pacientes com maiores riscos de recorrência e morbidades associadas. Em vista do exposto, o objetivo deste estudo foi avaliar o impacto no curso clínico das neoplasias de tireoide de alguns fatores de risco e marcadores prognósticos já bem

estabelecidos como idade, sexo, características ultrassonográficas, entre outros, assim como marcadores ainda controversos na literatura como o TSH e os polimorfismos no gene *VEGF*, envolvidos no processo de angiogênese.

Parte 1

Serum TSH levels as a predictor of malignancy in thyroid nodules: A prospective study.

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PLOS ONE

RESEARCH ARTICLE

Serum TSH levels as a predictor of malignancy in thyroid nodules: A prospective study

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Abstract

Background

The role of serum TSH concentrations as a predictor of malignancy of thyroid nodule remains unclear.

Objective

To prospectively evaluate the usefulness of serum TSH levels as a predictor of malignancy in thyroid nodules.

Methods

Patients with thyroid nodule(s) who underwent fine-needle aspiration biopsy under ultrasonographic guidance in a tertiary, university-based hospital were consecutively evaluated. Patients with known thyroid cancer and/or patients receiving thyroid medication were excluded. Serum TSH levels were measured by two differents methodologies, chemiluminescent (CLIA) and electrochemiluminscent immunoassay (ECLIA). Anatomopathological exam of tissue samples obtained at thyroidectomy was considered the gold standard for the diagnosis of thyroid cancer.

Results

A total of 615 patients participated in the study. The mean age was 55.9±14.7 years, and 544 (88.5%) were female. The median TSH values were 1.48 and 1.55 μU/mL, using CLIA and ECLIA, respectively. One-hundred-sixty patients underwent thyroidectomy and the final diagnoses were malignant in 47(29.4%) patients. TSH levels were higher in patients with malignant than in those with benign nodules in both TSH assays: 2.25 vs. 1.50; P = 0.04 (CLIA) and 2.33 vs. 1.27; P = 0.03 (ECLIA). Further analysis using binary logistic regression identified elevated TSH levels, a family history of thyroid cancer, the presence of microcalcifications, and

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solitary nodule on US as independent risk factors for malignancy in patients with thyroid nodules. Additional analyses using TSH levels as a categorical variable, defined by ROC curve analysis, showed that the risk of malignancy was approximately 3-fold higher in patients with TSH levels ≥2.26 μU/mL than in patients with lower TSH levels (P = 0.00).

Conclusions

Higher serum TSH levels are associated with an increased risk of thyroid cancer in patients with thyroid nodules. Using TSH levels as an adjunctive diagnostic test for stratifying the risk of malignancy associated with a thyroid nodule may help on defining the best therapeutic approaches.

Introduction

Palpable thyroid nodules are a common disorder detected in 4–7% of adults in the general population and in 19–67% of patients who undergo high-resolution ultrasound [1–5]. In contrast, thyroid cancer is rare. However, in most cases, thyroid carcinoma presents clinically as a nodule (solitary or in a multinodular gland) that is indistinguishable from benign neoplasia. The challenge for clinicians, therefore, is to distinguish malignant (5–10%) from benign thyroid nodules[4,5]. Of note, the last National Cancer Institute State Cancer Profile has shown that the incidence of thyroid cancer is rising faster than that of any other malignant neoplasia [6].

Fine-needle aspiration biopsy (FNAB) is the gold standard for evaluating patients with thyroid nodules, and it is currently the most reliable diagnostic technique for evaluating thyroid nodules under ultrasound guidance[5,7]. Diagnostic results are obtained in most cases, as inadequate specimens (nondiagnostic or unsatisfactory) occur in only 5–10% of cases. However, FNAB cannot reliably rule out cancer in 20–30% of nodules, reported as indeterminate for malignancy [8–10] The Bethesda classification [11] recognizes three specific cytological diagnoses characterized by indeterminate cytology. The predicted probability of cancer is 5–10% in Bethesda III patients, 20–30% in Bethesda IV patients, and 50–75% in Bethesda V patients, but variability has been noted at different centers [8,10] and most patients with indeterminate cytology undergo surgery to establish the histopathologic diagnosis. However, only 10–20% of the thyroid nodules with indeterminate cytology (Bethesda III and IV) are malignant[8,12–14]. New diagnostic approaches based on thyroid cancer molecular biomarkers have recently been studied, and some are already introduced in clinical settings. Currently, the most successful panels testing for mutations in thyroid FNAB samples are those testing for BRAF and RAS point mutations and RET/PTC and PAX8/PPARγ rearrangements, as well as TRK rearrangements [15, 16]. Although the use of these molecular tools have been validated in some studies, these tests are expensive (and not cost-effectivity depending of thyroid cancer prevalence), and their impact on patient management remains debatable[17–20].

The role of TSH as a predictor of thyroid nodule malignancy has been evaluated by several studies in the last years. Since Boelaert has reported parallel increases in malignancy risk and serum TSH levels[21], several other authors have investigated the relationship between serum TSH levels and thyroid cancer with conflicting results[22–24]. A recent meta-analysis found a positive association between higher serum TSH levels and increased risk of thyroid cancer[25]. However, the analysis had some limitations, as all of its included studies were cross-sectional, and the vast majority was retrospective (only two prospective studies). In contrast, the EPIC

study, which is based on a huge European cohort, demonstrated a negative association between TSH levels and thyroid cancer risk[26]. Although the EPIC study is a large prospective study, it is a case-control study featuring control subjects who were chosen from a group of cancer-free participants constituting the EPIC cohort (healthy population), thus, which may limit its conclusions.

Here, we aimed to evaluate the role of serum TSH levels as predictor of thyroid nodule malignancy in a cohort of patients with thyroid nodules in a tertiary, university-based hospital.

Materials and methods

Consecutive patients with thyroid nodule(s) who underwent FNAB under ultrasonographic guidance (US-FNAB) between 2012 and March of 2016 in the Thyroid Unit of the Hospital de Clínicas de Porto Alegre, a tertiary, university-based hospital, were prospectively evaluated. The geographical area of the study is iodine sufficient[27]. All patients were referred for evaluation of thyroid nodules and underwent a complete history and physical examination. Patients with known thyroid cancer and/or patients receiving thyroid-related medication were excluded. TSH levels, ultrasound characteristics of the nodules, demographic and clinical data were compared in patients with benign and malignant thyroid nodule. The project was approved by the Research Ethics Committee of the Hospital de Clínicas de Porto Alegre (GPPG 140538). The study was conducted in accordance with the ethical standards of the local institutional, national research committee and with the Helsinki declaration. The local institutional and national research committee did not request informed consent, as the data were analyzed anonymously and the assistance of patients follow routine clinical indications.

Laboratory evaluation

Serum TSH levels were measured by two different methodologies, chemiluminescent immunoassay (CLIA) (ADVIA Centaur XP; Siemens, Tarrytown, NY), with interassay coefficient of variation of 5.3% over the range 0.35–5.50 μU/mL, were used until November 2014. After that, TSH levels were measured by electrochemiluminscent immunoassay (ECLIA) (Roche Diagnóstica, São Paulo, Brasil), with interassay coefficient of variation of 3.11%, reference values: 0.27–4.20 uUI/mL. The thyroid peroxidase antibodies (TPO-Ab) was evaluated by chemiluminescent immunoassay (IMMULITE Systems, Siemens Healthcare Diagnostics, Tarrytown, NY), reference value inferior of 34.0UI/mL.

Ultrasound examination

All patients underwent thyroid US-FNAB, which was performed by the same radiologist using a high-resolution ALOKA ultrasound device with a 7.5 MHz linear transducer (Tokyo, Japan). The number of thyroid nodules, as well as sizes, US characteristics (echogenicity, the presence of microcalcification, halos, contours, and shape), and the presence of cervical lymphadenopathy were documented in the medical records of all patients.

FNAB, cytological and histological diagnosis

FNA and cyto-cell block testing. US-FNA was performed with a disposable needle (21G) connected to a 10 ml disposable syringe[14]. Multidirectional aspiration was performed in dominant nodules in patients with multinodular goiters and/or suspicious nodules on ultrasonography[5,7]. Rapid on-site evaluations of all fine-needle aspiration specimens were performed to determine the adequacy of each specimen. A thyroid FNA specimen was considered satisfactory if at least 6 groups of follicular cells were present, and each group comprised

at least 10 cells. Immediate on-site reaspiration was performed in cases in which specimens considered inadequate for diagnosis were obtained. Six cytology slides were prepared for each patient, four of which were air-dried and immediately stained via the May Grümwald Giemsa technique. The other two slides were immediately fixed in ethanol 96° and subsequently stained via the Papanicolaou technique. The residual hemorrhagic aspirated in the syringe and needle was fixed in 10% formalin. Then, the aspirated material was centrifuged, paraffin-embedded (cell block), sectioned and stained with hematoxylin and eosin to serve as a complementary diagnostic specimen to the FNA specimen. A sample was considered viable if it contained a sufficient number of cells with intact morphology (60 cells from at least 6 groups of 10 follicular cells).

Two independent pathologists reviewed the cytological and histological slides of each case together and assigned the samples to a final diagnostic category. Cytological aspirate adequacy was defined according to the recommendations of the *Papanicolaou Society of Cytopathology Task Force on Standards of Practice* (1996), and the cytological results were classified into the following 6 diagnostic categories according to the criteria of the Bethesda System for Cytological Classification of Thyroid Nodules: 1) non-diagnostic or unsatisfactory, 2) benign, 3) atypia of undetermined significance, 4) a follicular neoplasm or suspicious for a follicular neoplasm, 5) suspicious for malignancy and 6) malignant.

Cell-block slides were reviewed for the presence of cellular elements and classified into the following two categories: 1) diagnostic and 2) non-diagnostic. Cell-block US-FNBA specimens were used as adjunctive diagnostic specimens, and the results were described as cyto-cell blocks[14].

Anatomopathological examinations of tissue samples obtained at thyroidectomy were carried out according to the World Health Organization Guidelines, and the pathology reports pertaining to these samples were considered the gold standard for the diagnosis of thyroid cancer. The pathology reports provided information regarding AJCC/UICC TNM staging and the presence of invasion. The risk of recurrence was evaluated according to the ATA thyroid cancer guidelines[5,7].

Statistical analysis

The clinical, laboratory, ultrasonography and cytological data, which are reported as the mean–standard deviation (SD) values, or as the median with percentiles 25 and 75 (continuous variables), or as absolute numbers and percentages (categorical variables), were compared using an unpaired Student's t-test, Mann–Whitney U-test, or chi-square test, as appropriate. The influence of clinical factors on the risk of thyroid cancer was investigated using binary logistic regression analysis. The differences in the cancer rates between groups with determined TSH levels, considering ROC curve cutoff value, were calculated using odds ratios and 95 percent confidence intervals. Statistical analysis of the results was performed with SPSS software (Statistical Package for Social Sciences) version 18.0.

Results

Patient characteristics

A total of 886 patients with thyroid nodules were consecutively evaluated. Of them, 234 were excluded due to thyroid hormone replacement therapy and/or a previous diagnosis of thyroid cancer, and 37 patients because they did not have available TSH measurement. Thus, 615 patients participated in the study. The mean of age of the participants was 55.9 ±14.7 years, and 544 (88.5%) were female. Solitary nodules were noted in 226 patients (36.7%). As mentioned in the Materials and Methods section, two different assays were used to measure the

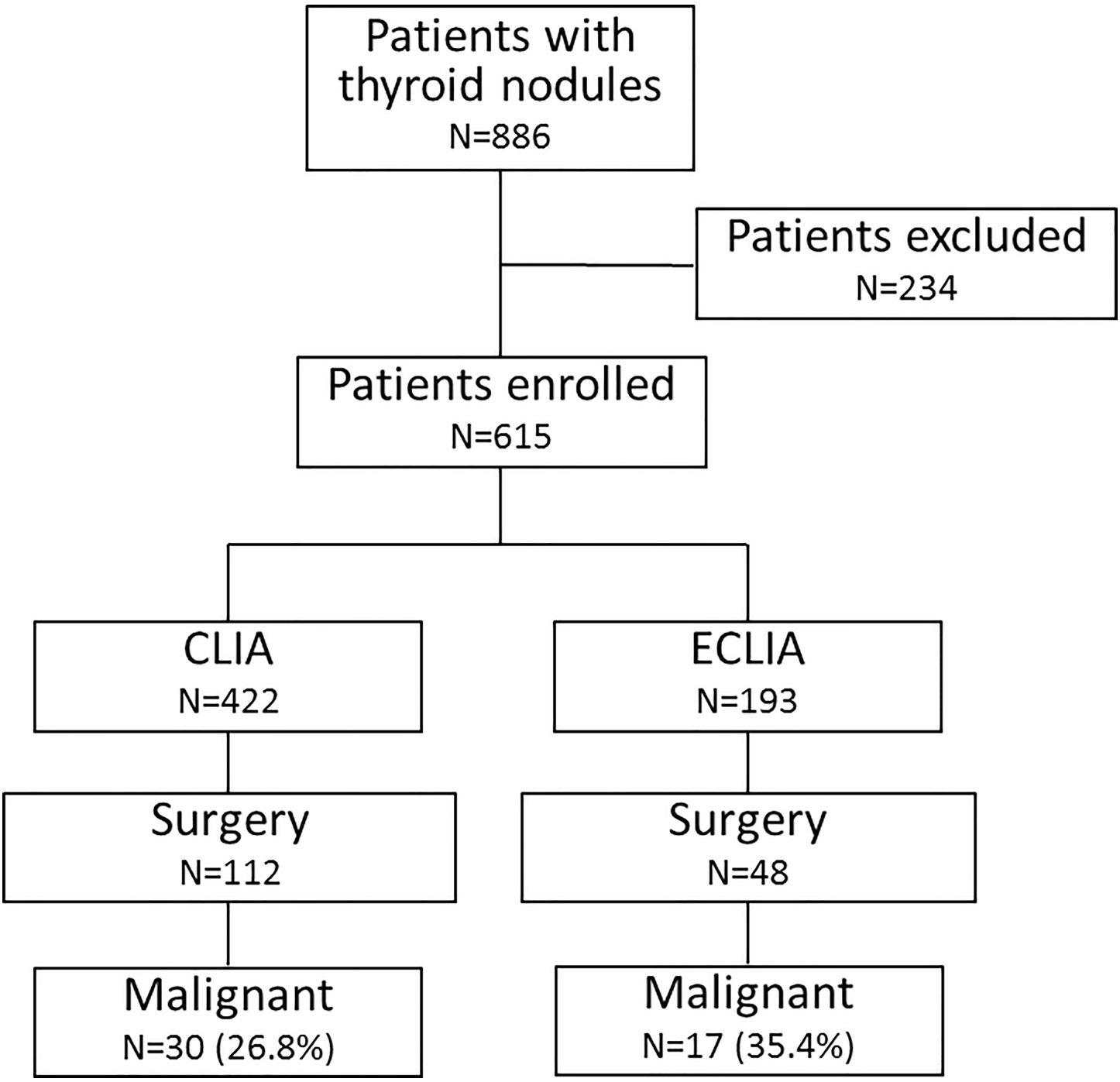

Fig 1. Flow chart of patients who met inclusion/exclusion criteria for the study population. CLIA, Chemiluminescent immunoassay; ECLIA, electrochemiluminscent immunoassay.

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TSH levels during the study period. Since the reference ranges were slightly different between these assays, we have analyzed the patients separately (Fig 1). The median TSH in 422 patients evaluated by CLIA was 1.48 μU/mL (P25: 0.93 and P75: 2.31) while the median TSH was 1.55 μU/mL in 193 patients analyzed by ECLIA, (P25: 0.95 and P75: 2.80). There was no

Table 1. Clinical and oncology features of patients included in the study.

	All Samples	CLIA	ECLIA	P value
	(N = 615)	(N = 422)	(N = 193)	
Age (yr.)	55.9 ± 14.7	56.4 ± 14.0	54.9 ± 16.0	0.25
Female gender (%)	544 (88.5)	378 (89.6)	166 (86)	0.20
Family history of thyroid cancer (%)	37/602 (6.1)	29/409 (7.1)	8/193 (4.1)	0.16
Personal history of other neoplasia (%)	68/604 (11.3)	48/411 (11.7)	20/193 (10.4)	0.63
Exposure to radiation (%)	28/601 (4.7)	23/408 (5.6)	5/188 (2.7)	0.98
Serum TSH (μU/mL)		1.48 (0.93–2.31)	1.55 (0.95–2.80)	0.32
Solitary nodule (%)	226 (36.7)	155 (36.7)	71 (36.8)	0.99
Microcalcifications (%)	106/483 (21.9)	80/292 (19)	26(13.5)	0.00
FNA cyto-cell block (%)				0.00
I—Nondiagnostic	35 (5.7)	20 (4.7)	15 (7.8)	
II—Benign	434 (70.6)	315 (74.6)	119 (61.7)	
III—Indeterminate	56 (9.1)	28 (6.6)	28 (14.5)	
IV—Follicular lesion	50 (8.1)	30 (7.1)	20 (10.4)	
V/VI—Malignant	40 (6.5)	29 (6.9)	11 (5.7)	

Values are the mean ± SD or median (P25-P75). CLIA, chemiluminescent immunoassay; ECLIA, electrochemiluminscent immunoassay. The TSH reference range is 0.35–5.50μU/mL by CLIA and 0.27–4.20μUI/mL by ECLIA. Solitary nodules and microcalcifications were evaluated by ultrasonography.

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significant differences in median TSH values (P = 0.32). Indeed, the correlation between the two different TSH assays showed a strong correlation (r = 0.99). Forty-three out of 261 (16.5%) patients analyzed presented positivity to TPO-Ab.

All patients underwent US-FNBA and had their sample analyzed by cytologic and cell block diagnostic testing. The combined cytological and cell block (cyto-cell) interpretations were as follows: 5.7% (n = 35) unsatisfactory, 70.6% (n = 434) benign, 9.1% (n = 56) indeterminate, 8.1% (n = 50) follicular lesion and 6.5% (n = 40) malignant (Table 1). Surgery was indicated for 170 patients with the following cyto-cell results: 7 nondiagnostic cyto-cell blocks, 30 indeterminate lesions, 43 follicular lesions, 36 malignant lesions and 54 benign lesions, based on clinical (compressive symptoms, recent growing) and ultrasonographic characteristics (large nodules, microcalcification, irregular margin). Final histological data were available for 160 patients, 113 of whom had benign lesions (70.6%), and 47 of whom had malignant lesions (29.3.% of surgical cases and 7.6% of the sample). Of the subgroup evaluated by CLIA, 112 patients underwent surgery and 30 (26.8%) had malignant nodules while 48 out of 193 patients analyzed by ECLIA were thyroidectomized and 17 (35.4%) had thyroid cancer. The characteristics of all enrolled patients are shown in Table 1. The distribution of age, gender, and other clinical features were similar between both TSH assay groups. However, the presence of microcalcification were higher in CLIA group (19 vs. 13.5%; P = 0.00) and the distribution of cytological evaluation showed a higher prevalence of indeterminate category in ECLIA group (6.6 vs. 14.5%; $P<0.001$).

TSH and thyroid cancer

In the group evaluated by CLIA, 91.7% of patients showed serum TSH levels within the normal range; 23 patients (5.5%) presented TSH levels less than 0.35 μU/mL, and 12 (2.8%) had TSH levels greater than 5.5 μU/mL. In patients analyzed by ECLIA, TSH was within the normal range in 87%; 4 (2.1%) patients showed TSH levels less than 0.27 μU/mL and 21 patients (10.9%) have TSH levels greater than 4.20 μU/mL.

Noteworthy, patients with thyroid cancer exhibited higher median TSH levels than patients with benign nodules irrespectively of the assay methodology: 2.25 vs. 1.50 (CLIA;P = 0.04) and 2.33 vs. 1.27 (ECLIA; P = 0.03) (**Fig 2**;Table 2). Moreover, a family history of thyroid cancer, solitary nodules and the presence of microcalcifications were associated with malignancy (Table 2). Further analysis using binary logistic regression identified elevated TSH levels, a family history of thyroid cancer, the presence of microcalcifications and solitary nodule on ultrasonography as independent risk factors for malignancy in patients with thyroid nodules. There were no differences in age, gender, non-thyroid neoplasia history, presence of TPO-Ab positivity or previous radiation exposure history between patients with benign nodules and those with thyroid cancer.

To further explore the potential role of TSH levels as a predictor of thyroid cancer, we subdivided the sample into quartiles in accordance with the TSH level distribution of the study (≤0.93, 0.94–1.48, 1.49–2.31 and ≥2.32 μU/mL for CLIA and ≤0.95, 0.96–1.55, 1.56–2.80 and ≥2.81 μU/mL ECLIA). Interestingly, we observed that the frequencies of malignancy in each TSH quartile varies in accordance with TSH levels, for both assays (P = 0.02 and P = 0.04; respectively, Fig 3). For CLIA group, the prevalence of malignancy in patients with TSH levels in the first quartile (≤0.93 μU/mL) was 14.3%, while those in the upper quartile (≥2.32 μU/mL) exhibited a cancer prevalence of 48.3% (OR 5.6; CI 1.35–23.2)(P = 0,01). The prevalence of malignancy in patients evaluated by ECLIA with TSH levels in the first quartile (≤0.95 μU/mL) was 23%, while those in the upper quartile (≥2.81 μU/mL) exhibited a cancer prevalence of 64% (OR 5.83; CI 1.00–34.6)(P = 0,04). Of note, even within normal range of TSH, there was a significantly increased risk of thyroid cancer as TSH increased.

We subsequently performed ROC curve analysis to identify the best TSH cutoff for predicting malignancy. Remarkable, the TSH value of 2.26μU/mL was identified to both assays. Then, we perform additional analyses using TSH levels as a categorical variable, the prevalence of malignancy was higher in those patients with TSH levels >2.26 μU/mL than in patients with lower TSH levels (<2.26 μU/mL) (OR 3.87; CI 1.87–8.01)(P = 0.00). Similar results were

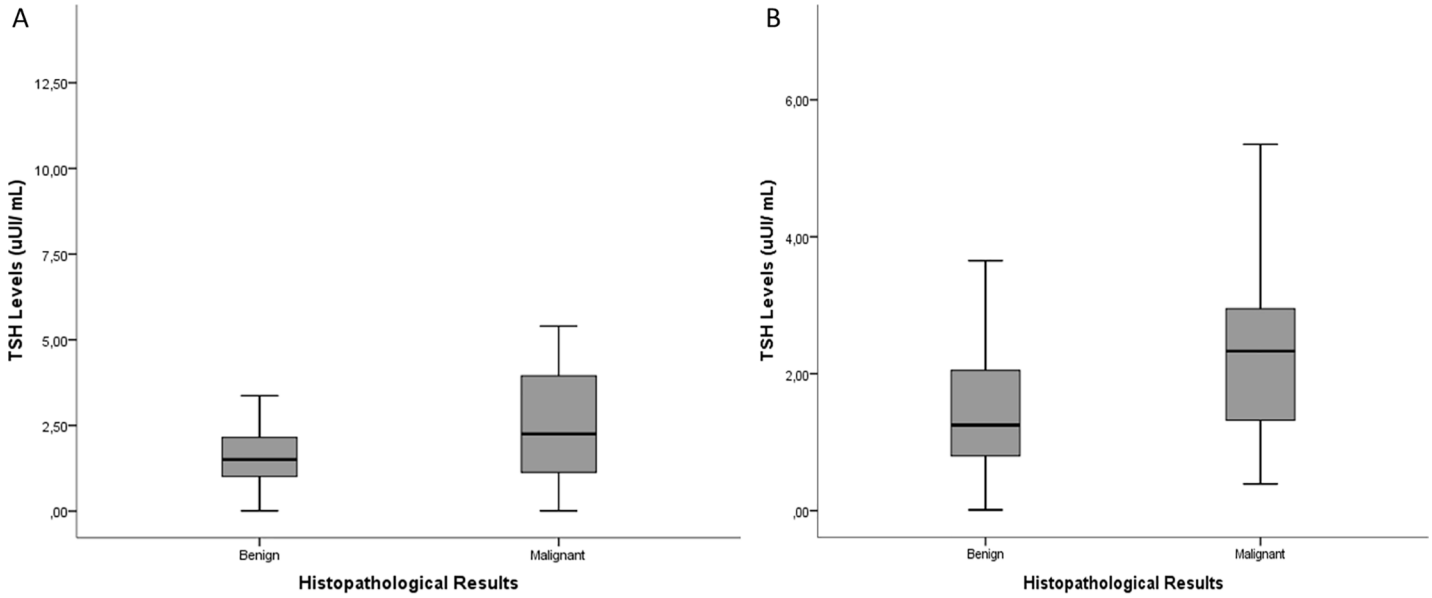

Fig 2. TSH level in malignant and benign thyroid nodules. CLIA, Chemiluminescent immunoassay; ECLIA, electrochemiluminscent immunoassay. Box plot illustrating the median TSH level and the TSH level quartiles and ranges in malignant and benign thyroid nodules for CLIA(A) and ECLIA (B). Patients with thyroid cancer exhibited higher median TSH levels than patients with benign nodules for both methodology. P = 0.04 (A) and P = 0.03 (B).

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Table 2. Characteristics of patients with benign and malignant nodules.

	Malignant nodule	Benign nodule	P value
	(N = 47)	(N = 113)	
Age (yr.)	47.9 ± 13.6	50.9 ± 14.8	0.24
Male gender (%)	10 (21.3)	12 (10.6)	0.07
Family history of thyroid cancer (%)	7 (14.9)	5 (4.4)	0.03*
Personal history of other neoplasia (%)	4 (8.5)	8 (7.1)	0.79
Exposure to radiation (%)	2 (4.2)	4 (3.5)	0.84
Serum TSH (μU/mL)			
CLIA	2.25 (1.12–4.07)	1.50 (0.99–2.17)	0.04
ECLIA	2.33 (1.30–3.06)	1.25 (0.79–2.10)	0.03
Microcalcifications (%)	21 (44.7)	16 (14.2)	0.00*
Solitary nodule (%)	26 (55.3)	42 (37.2)	0.03*
TPO-Ab positively (%)	4/22 (18.2)	6/55 (10.9)	0.39

Values are the mean ± sd or median (P25-P75). CLIA, chemiluminescent immunoassay; ECLIA, electrochemiluminscent immunoassay.The TSH reference range is 0.35–5.50μU/mL by chemiluminescent and 0.27–4.20μU/mL by electrochemiluminscent. Solitary nodules and microcalcifications were evaluated by ultrasonography (during US-FNA).

*Independent variable by binary logistic regression.

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obtained when only patients with normal TSH levels were analyzed (OR 3.42 CI 1.62–7.24) (P = 0.00).

Among the 47 patients with thyroid cancer, three cases harbor medullary thyroid carcinoma and 44 differentiated thyroid carcinoma (DTC). Among patients with DTC, 11 cases were microcarcinoma (25.0%). There were no differences in prevalence of TSH >2.26 μU/mL or distribution of TSH quartile between patients with or without microcarcinoma.

Discussion

In the present prospective study, we demonstrated that patients with malignant thyroid nodules presented higher serum TSH levels than patients with benign nodules.

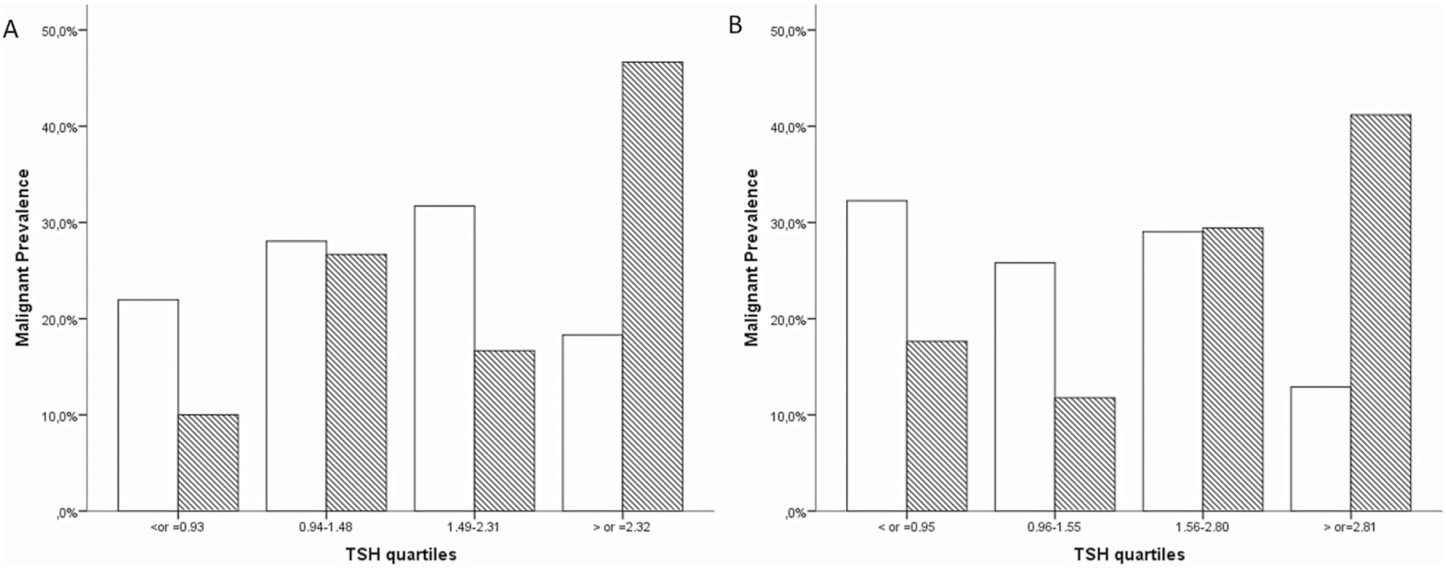

Fig 3. Frequencies of malignant and benign nodules according to TSH quartile. CLIA, Chemiluminescent immunoassay; ECLIA, electrochemiluminscent immunoassay. Frequencies of malignant (dashed bars) and benign (white bars) nodules according to TSH quartile for CLIA(A) and ECLIA (B). A significant increase in the prevalence of malignancy was noted in higher TSH quartiles. P<0.01.

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Accordingly, the prevalence of malignancy was higher in subjects with TSH levels >2.26 μU/mL, as defined via ROC curve analyses. Remarkably, additional analyses using TSH levels as a categorical variable show that the risk of malignancy was higher in patients with TSH levels in upper quartile as compared with patients with lower TSH levels in two different methodologies.

TSH is a major thyroid cell growth factor, while TSH signaling pathway activation may be required for the expression of other growth factors, receptors, and proto-oncogenes[28–30]. Accordingly, TSH suppression is an important therapeutic tool of clinical thyroid cancer management[5,7]. In the last years, several studies have addressed the role of TSH as a predictor of thyroid nodule malignancy but the results are still open to discussion. Here, we have demonstrated that patients with higher TSH levels have increased risk for malignancy. Remarkable, as TSH quartiles increased, the likelihood of malignancy rose, and the odds ratio of thyroid cancer in patients with TSH levels in the upper quartile was 5-fold higher than in those patients with TSH levels in the first quartile (Fig 3). Similar results were observed using the ROC analyses to define the TSH levels. Our observations are in agreement with previous studies [21–25] although contrast with the data from the EPIC study[26]. Noteworthy, however, the EPIC was a case-control study that includes only cancer-free subjects which might be a limiting when compared with our study.

Despite the consistent association between higher TSH levels and malignant nodules shown in most series, including this one, an optimal TSH cutoff value for predicting the risk of cancer has not been yet identified. Indeed, the lack of previous studies validating nomograms or equations intended to determine an optimal TSH cutoff value has limited the use of serum TSH levels as a malignancy predictor. Here, we found the TSH cutoff value (≥2.26 μU/mL) using the best point of a ROC curve for the two different TSH assays used during the study period. Some authors have suggested that while no consensus exists regarding a TSH cutoff value, it may be practical to preferentially perform US-FNAB in nodules less than 1.5 or 2.0 cm exhibiting patterns arousing low or very low suspicion for malignancy on ultrasonography[2,25]. Haymart et al. suggested TSH may play a key role in optimizing surgical interventions when aspirates are suspicious for malignancy[23]. These recommendations might support an adjunctive role for TSH levels when evaluating thyroid nodules.

This study has several strengths, as it included a large number of patients with thyroid nodules who were evaluated at a single institution and did not exclude patients with abnormal TSH levels, which enhances the external validity of its findings and increases the clinical applicability of its data. Also, two TSH methodologies were analyzed with consistent results. Thus, the results presented here may have important clinical implications, since its indicate that TSH levels may help on the diagnosis strategy, in conjunction with clinical, ultrasonographic and cytological features. However, as noted above, TSH should not be used for diagnostic decision-making in isolation. Also, although we demonstrated a prospective study data, the design of our study was not delineated for evaluate the TSH as a causative role in thyroid cancer pathogenesis. In this view, we do not recommend screening for thyroid cancer in patients with chronic TSH elevations nor suppressive treatment for subclinical hypothyroidism and for benign nodular disease.

Conclusions

Higher serum TSH levels are associated with an increased risk of thyroid cancer in patients with thyroid nodules. The use of TSH as an adjunctive diagnostic test for stratifying the risk of malignancy associated with thyroid nodules may have value to decision-making on diagnostic approaches.

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Visualization: Lenara Golbert.

Writing – original draft: Lenara Golbert.

Writing – review & editing: Lenara Golbert, Marcia Silveira Graudenz, Ana Luiza Maia.

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Parte 2

Role of vascular endothelial growth factor polymorphisms in the pathogenesis of medullary thyroid carcinoma

Artigo em preparação

Role of vascular endothelial growth factor polymorphisms in the pathogenesis of medullary thyroid carcinoma

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Abstract

Background: The increased expression of vascular endothelial growth factor (VEGF-A) and its receptors is reported in several tumor types and stimulates cell proliferation, survival and migration. Overexpression of VEGF-A is observed in medullary thyroid carcinoma (MTC) staining tissue and VEGF receptors are molecular targets for multikinase inhibitor (MKI) therapies. Of interest, studies have reported association between some *VEGF-A* polymorphisms (SNPs) and tumor aggressiveness, suggesting that these variants might have an impact on tumor behavior.

Objective: To evaluate *VEGF-A* SNPs and correlate their frequencies with laboratory and clinical data, outcome, and prognosis in MTC patients.

Design and methods: Four-hundred-and-twenty MTC patients were included. Clinical data were retrospectively reviewed in medical records. The *VEGF-A* SNPs rs699947(C>A), rs833061(T<C) and rs2010963(G>C) were genotyped using Custom TaqMan Genotyping Assays. Haplotypes were inferred using phase 2.1 program.

Results: Of the 420 MTC patients analyzed, 202 (48%) presented hereditary and 218 (52%) had the sporadic form of disease. The mean age of diagnosis was 40 ± 19.7 years and 61% were female. The minor allele frequencies of *VEGF-A* SNPs were: rs699947 (37.2%), rs833061 (45.6%), and rs2010963 (34.4%). In hereditary MTC, we observed an independent association of the *VEGF-A* rs833061 with younger age at diagnosis (TT genotype) and smaller tumor size (CC genotype). Additionally, *VEGF-A* rs2010963 (GG genotype) was correlated with smaller tumor size. For patients with sporadic MTC, no independent associations were observed. No association was found between *VEGF-A* SNPs and tissue expression of VEGF-A and its receptors (VEGFR-1 and VEGFR-2).

Conclusions: Our results demonstrate an association between the *VEGF-A* SNPs and prognostic factors in MTC patients, suggesting that these variants might play a role in disease presentation.

Introduction

Thyroid cancer is the most common malignant neoplasm in the endocrine system and the incidence has been increasing over the last few decades (1-3). Medullary thyroid carcinoma (MTC) originates from the parafollicular C cells and accounts for 3-4% of all types of thyroid cancer. MTC may occur sporadically in 75% of cases or as part of the hereditary tumor syndrome multiple endocrine neoplasia type 2, caused by germline mutations in the *RET* proto-oncogene (4, 5). At the time of diagnosis, about half of patients presented cervical lymph nodes involvement, while a significant number of patients have already distant metastases to the liver, lungs, bones and, less frequently, brain and skin (6). Surgery is the only curative treatment for MTC. In advanced metastatic disease, the goals of surgery are palliative in order to minimize complications and comorbidities considering the patient's quality of life. Patients with tumors confined to the thyroid gland have a 10-year survival rate of 95.6%, whereas patients with regional stage disease or distant metastases at diagnosis have an overall survival rate of 75.5% and 40 %, respectively (7-9).

Angiogenesis is a hallmark of tumor pathogenesis (10). The angiogenesis mediated complex mechanism to provide essential nutrients for tumor development such as oxygen, proteolytic enzymes, hormones and growth factors. Tumor growth and early metastasis to cervical and mediastinal lymph node chains in MTC is dependent on the angiogenesis process (11). Vascular endothelial growth factor (VEGF-A) and its receptors (VEGFR-1 and VEGFR-2) represents the main mediators of angiogenesis causing increased vascular permeability, stimulating neovascularization and represent a key event in cancer development. In addition, it plays an important role in neoplastic evolution, leading to growth, local invasion and metastasization in different tumors (11, 12). VEGF-A and its receptors, the main regulators of tumoral angiogenesis, are overexpressed in MTC lesions and might be implicated in less favorable prognosis (13).

VEGF-A is a highly polymorphic gene, and a number of single-nucleotide polymorphisms (SNPs) have been reported to be associated with increased risk of susceptibility and aggressiveness behavior of several disorders, including thyroid cancer (14-16). SNPs with significant prognostic impact were all located in regulatory regions, thus suggesting that the effect on cancer evolution was related to transcriptional or post-transcriptional regulation (17). Among them, those located in the 5´ untranslated region

(5´UTR) of the *VEGF-A* gene are believed to affect gene expression through the elimination/creation of transcription-factors binding sites (TFBS), whereas those identified in the 3´UTR region likely act as the post-transcriptional level through the modulation of mRNA stability (18).

Indeed, *VEGF-A* SNPs in the promoter, 5'UTR and 3'UTR regions affect potential hypoxia-sensitive regulators and contribute to the high variability of VEGF-A production between tissues (18-20). The SNPs -2578 C>A (rs699947) and -460 T>C (rs833061), present in the promoter region of the gene, may influence transcription activity, altered expression of VEGF-A and are associated with more aggressive phenotypes in breast cancer (10, 21-23). SNP +405 G>C (rs2010963), located in the 5'-UTR region, affects protein translation efficiency and correlates with the production of VEGF-A from peripheral blood mononucleated cells (18) (Figure 1). Marotta *et al*., in a multicenter study, reported a correlation between common *VEGF-A* SNPs -2578 C>A (rs699947), -460 T>C (rs833061) and +405 G>C (rs2010963) and risk of recurrent structural disease and disease-free survival in patient with non-advanced differentiated thyroid cancer (papillary and follicular) (17).

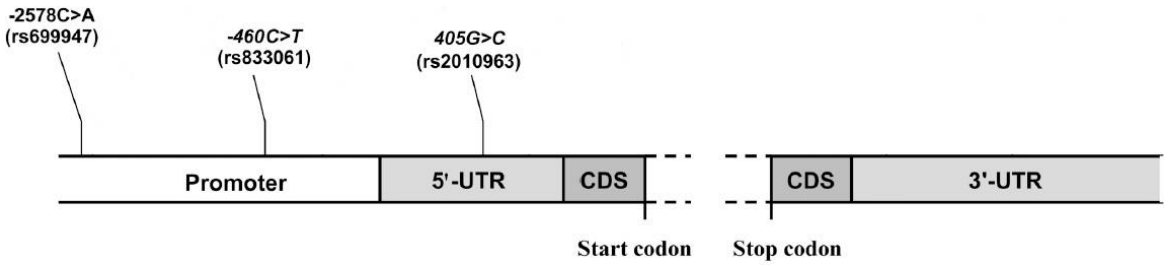

Figure. 1: (adapted of Jain *et al*. 2009). Structure of *VEGF* gene and position of *VEGF* SNPs relative to translation start sites. Dashed lines indicate the region consisting of coding sequence (CDS) and seven introns. UTR: untranslated region.

SNPs in *VEGF-A* might affect VEGF-A protein concentrations, influence the process of angiogenesis and has been associated with interindividual variation in the risk and progression of tumors. Despite that, little is known about the role of *VEGF-A* SNPs in the pathogenesis or clinical evolution of MTC. Thus, this study aimed to evaluate three common SNPs of *VEGF-A* gene -2578 C>A (rs699947), -460 T>C (rs833061) and +405 G>C (rs2010963) in a cohort of MTC patients followed at our

institution. In addition, we have examined the impact the presence of these sequence variants on clinical presentation and tumor behavior.

Materials and Methods

Patients and study design

Patients with a diagnosis of MTC attending the Thyroid Unit at Hospital de Clínicas de Porto Alegre (a tertiary care, university-based teaching hospital) were invited to participate in the study. From 1997, our division has been a reference center for RET mutations testing in Brazil, and therefore, patients referred to us by other Brazilian centers were also invited to participate. Our study included 420 consecutive patients with MTC and the control group was composed of 187 unrelated cancer volunteers attending the blood donation facility of our Institution.

Clinical and histopathological data

The diagnosis of MTC was based on histopathological/immunohistochemistry findings and clinical and laboratory data were collected for each individual, including the clinical characteristics of family members, association of other endocrine neoplasias, the type of *RET* mutations, and information on atypical features noted, such cutaneous lichen amyloidosis (CLA). Total thyroidectomy was performed in all patients with varying cervical neck dissection procedures. The diagnosis of lymph node metastasis was based on histological examination. Patients with suspicious distant metastasis underwent imaging exams (cervical, thoracic and abdominal CT (or liver magnetic resonance imaging), and bone scintigraphy.

Genotyping assay

SNPs of *VEGF-A* gene -2578 C>A (rs699947), -460 T>C (rs833061) and +405 G>C (rs2010963) were analysed. Genomic DNA was obtained from peripheral blood leukocytes by standardized procedures. Genotype analysis was performed using Human Custom TaqMan SNP Genotyping Assays 40 X (Applied Biosystems, Foster City, CA, USA). The reactions were conducted in a 96-well plate, in a total 5 µl reaction volume using 10ng genomic DNA, TaqMan Genotyping Master Mix 1 X (Applied Biosystems),

and Custom TaqMan Genotyping Assay 1 X. The plates were then positioned in a real-time PCR thermal cycler (7500 Fast Real PCR System; Applied Biosystems) and heated for 10 min at 95°C, followed by 50 cycles of 95°C for 15s and 60°C for 1 min. Fluorescence data files from each plate were analyzed using automated allele calling software (SDS 2.1; Applied Biosystems).

Immunohistochemistry (IHC) analysis

Our sample comprised 73 specimens with histopathological/immunohistochemistry findings of MTC obtained from patients attending the Endocrine or Head Neck Divisions at Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil, from 1997 to 2008. IHC was performed on thin sections (3µm) of previously formalin-fixed and paraffin-embedded tissues. The antibodies used were polyclonal rabbit anti-human VEGF-A (clone VG1; M7273 Dako Cytomation, Carpinteria, CA), monoclonal rabbit anti-human VEGFR-1 (VEGFR-1: #1303-1; Epitomics, Burlingame, CA), and monoclonal mouse anti-human VEGFR-2 (A-3: SC-6251; Santa Cruz Biotechnology, Santa Cruz, CA). Sections representing MTC were submitted to routine immunohistochemical technique, which comprises deparaffinizationand rehydration, antigenic recovery, inactivation of endogenous peroxidase, and blockage of unspecific reactions. Primary antibodies were incubated overnight at a temperature of 4°C, at dilutions of 1:400 (VEGF-A), 1:100 (VEGFR-1), and 1:200 (VEGFR-2), followed by application of streptavidin horseradish peroxidase conjugate (LSAB; Dako Cytomation), and diaminobenzidine tetrahydrochloride (Kit DAB; Dako Cytomation). Positive controls were human tissues, skeletal muscle tissue for VEGF-A, human placenta for VEGFR-1, and intestinal tumor for VEGFR- 2; negative control was obtained by omission of the primary antibody. The intensity of VEGF-A, VEGFR-1, and VEGFR-2 staining in each lesion was determined and quantified as grade 0 (absent, -), grade 1 (weak, +), grade 2 (moderate, ++), and grade 3 (strong, +++) based on the staining characteristics of most of the tumor. The slides were read independently by two blinded and experienced pathologists who were not aware of the respective clinicopathological data. When the two experts differed in their interpretations, they consulted together and reached a consensus.

Statistical analysis

Results are expressed as mean ± S.D. or median (IQ 25–75) unless otherwise specified. Hardy–Weinberg equilibrium for each polymorphism was assessed by X2 tests. Baseline characteristics were compared using X2 tests or Fisher's exact test for qualitative variables. Quantitative variables were compared between groups using Student's t-test, ANOVA, or Kruskal–Wallis tests. Differences in cumulative lymph node and/or distant metastases between groups were tested by Kaplan–Meier curves, and comparisons between curves were performed using the log rank test. The Statistical Package for the Social Sciences 18.0 (PASW, Inc., Chicago, IL, USA) software was used for all analyses, and $P<0.05$ was considered statistically significant.

The haplotypes were constructed based on the combination of allelic variants and their frequencies were inferred using the phase 2.1 program, which implements a Bayesian statistical method (24). Between all pairs of biallelic loci, we examined widely used measures of linkage disequilibrium (LD), Lewontin's D´|D´| and r^2 (25).

Ethics Statement

The information obtained during the study did not affect the patient's diagnosis or treatment. No additional procedures were performed as a result of this study.The protocol was approved by the Committee on Research Ethics from Hospital de Clínicas de Porto Alegre (GPPG 2018-0021 and CAAE 88468817500005327), and all patients and/or their legal representatives provided written informed consent for the study. Clinical investigation was conducted according to the principles expressed in the Declaration of Helsinki.

Results

Clinical and oncological features of the included MTC patients are described in Table 1. Our sample comprised 420 patients, 202 with hereditary MTC and 218 with the sporadic form of disease. The mean age at the time of diagnosis was 40 (±19.7) years, and 256 (61%) were women. The median levels of calcitonin and CEA were 298 pg/ml (33.67 – 1296) and 17.2 ng/ml (3.07 – 104.8), respectively. The median tumor size was 2 (1.2 – 3.4) cm; 162 (50.5%) patients had lymph node metastases, and 51 (16.4%) had distant metastases. The control group comprises 187 blood donor volunteers. The ethnic background (95% Caucasian) and the mean age (40 ±19.7 vs 43.3 ±8.0) were similar

between MTC patients and control group (P= 0.06). However, the frequency of females was higher in MTC patients (61% vs 32.6%, P= 0.00).

Frequency of VEGF-A SNPs in MTC patients and controls

The minor allele frequencies observed in MTC patients were as follows: rs699947 (37.2%), rs833061 (45.6%) and rs2010963 (34.4%). There were no differences in the frequency of *VEGF-A* polymorphisms between patients and controls (Table 2; all P>0.05).

Haplotype Construction and Linkage Disequilibrium Analysis

We used a Bayesian statistical method to estimate the frequency of different haplotypes produced by the combination of the -2578 C> A (rs699947), -460 T> C (rs833061) and +405 G> C (rs2010963) polymorphisms in MTC patients and control group. A total of seven haplotypes were inferred in our study population. The haplotype frequencies were shown in Table 3. We found no significant differences in haplotype distributions between cases and controls (*P*= 0.56).

Interestingly, we observed a partial linkage disequilibrium (|D´|= - 0.98 and r^2= 0.651) between variants in the promoter region of the *VEGF-A* gene -2578 C> A (rs699947) and -460 T> C (rs833061).

VEGF-A SNPs genotypes and disease presentation

No association was observed between the three polymorphisms and clinical features or laboratorial data when the whole group of MTC patients were analysed (n=420, all P>0.05). Since the pathogenesis of hereditary and sporadic form of thyroid medullary tumors differ, we also analyzed both groups separately.

Hereditary Medullary Thyroid Carcinoma

A total of 202 patients presented the hereditary form of the disease. The mean age at diagnosis was 28.83 ± 18.2 years and 59.4% were women (Table 1). The frequency of lymph node and distant metastasis were 38.9 and 9.3%, respectively (Table 1).

In relation to the genetic profile, the most commonly identified mutations were as follows: C634Y (44.1%), C634R (12.4%) and M918T (7.4%). The others mutations (E768D, S891A, V804M, C620G, C634W, C609Y, C620R, C618R, L790F, S649L,

C611Y, C618G, S904F, C634S, DEL631) had lower frequencies. There was no association between the frequency and/or type of *mutations and VEGF-A SNPs (P>0.05).*

The clinical and oncological features of patients with hereditary MTC according to polymorphic alleles are shown in Table 4. No association was observed between rs699947 polymorphism and clinical features (all P>0.05).

Patients with homozygous TT genotype for rs833061 were younger age at diagnosis (P=0.00) while patients with homozygous CC allele had smaller tumor size (P=0.02). The associations remained significant when adjusted to the presence of germline mutations (P= 0.003 and 0.027, respectively). No differences were observed in serum calcitonin, CEA levels and lymph node or distant metastasis (all P>0.05).

Interestingly, for *VEGF-A* SNP rs2010963, patients with homozygous CC genotype presented younger age at diagnosis (P=0.01) and highest CEA levels (P=0.04) while those with GG genotype presented smaller tumor size (P=0.00). When adjusted for the presence of germline mutations, the association with tumor size remains significant (P= 0.001) and, although not statistically significant, the difference of age at diagnosis is 9.6 years between groups (P=0.056). No differences were observed for lymph node or distant metastasis (P=0.31 and P=0.86, respectively).

Moreover, Kaplan-Meier survival curves indicated no significant differences in the occurrence of lymph node metastases, although distinct curves may be observed in individuals harboring rs833061 TT genotype (P= 0.056; Figure 2A) and rs2010963 CC genotype (P= 0.071; Figure 3A). No differences were observed for distant metastases (all P>0.05, Figures 2B and 3B).

Sporadic Medullary Thyroid Carcinoma

The clinical and oncological characteristics of the 218 patients with sporadic MTC are summarized in Table 1. The mean age at diagnosis was 50 ± 15.1 years and 62.4% are women. The frequency of lymph node and distant metastasis were 58.4 and 21.4%, respectively. The clinical and oncological features of patients with sporadic MTC according to polymorphic alleles are shown in Table 5.

There were no association between genotypes of SNPs of *VEGF-A* rs699947 and rs833061 and clinical features (all P>0.05). Patients carrying the *VEGF-A* rs2010963 CC genotype presented smaller tumor size (P=0.03) than those who carried GG or CG genotypes, however, this result does not remain significant when corrected for presence

of M918T somatic mutation (P=0.571). No significant differences were observed in age at diagnosis, serum calcitonin, CEA levels and lymph node or distant metastasis.

Expression of VEGF-A and VEGFRs in hereditary and sporadic MTC tumors

We evaluated expression of VEGF-A in 73 MTC samples with immunohistochemical staining detected in 71 (97%) of the cases. VEGFR-1 immunoreactivity was detected in 33 of 34 (97%) while VEGFR-2 in 57 of 67 (85%) of the MTC samples. No significant associations were found between positive immunoreactions of VEGF-A, VEGFR- 1 and VEGFR-2 and variant alleles of the *VEGF-A*.

Discussion

In the present study, we investigated the potential association between VEGF-A polymorphisms and clinical features in a cohort of patients with MTC. Here we show that SNPs rs833061 and rs2010963 are associated with prognostic factors such as age at diagnosis and tumor size.

Progressive metastatic disease is the main challenge in the management of patients with MTC since the conventional therapeutic options offer limited results for these individuals. In recent years, knowledge about the molecular events involved in the process of tumorigenesis has aided in the search for molecules that may serve as potential therapeutic targets. Antiangiogenic treatment strategies have been devised using agents alone or in combination of several gene therapy approaches such as tyrosine kinase inhibitors and monoclonal antibody-based (26). Nevertheless, these molecular targeted therapies are currently approved only for advancedMTC, while their use is limited by adverse effects on patient's quality of life, the intrinsic or secondary drug resistance, and their significant cost (21).

Many studies have investigated the prognostic significance of VEGF-A SNPs in several solid tumors, including breast, non-small cell lung, colorectal and prostate cancers. However, there is a great heterogeneity among the results. In breast cancer, for example, the rs833061 (TC + CC) was associated with high histological grade and the presence of lymph node metastases at diagnosis in a Brazilian cohort (27). These results are in opposition to those of Sa-Nguanraksa and cols. who reported lower lymphovascular invasion of breast tumors with the variant homozygous genotype CC

(28). Moreover, Rahoui et al., found no associations with these variants and histopathological features (29). A possible explanation for these results is the variability of the study population (ethnicity and tumor type) and small sample size. This make difficult to elucidate the role of these variants in cancer development and/or progression. In addition, the lack of consensus about the role of these SNPs could be because the presence of linkage with other unknown functional variants (30). In the thyroid field, haplotype analyses were performed in PTC patients. In this study, the presence of ACG homozygous haplotype (rs699947-A, rs833061-C, rs2010963-G) offered a protective effect against structural recurrence in ATA low-intermediate risk patients. In agreement, the CTG homozygous genotype (rs699947-C, rs833061-T, rs2010963-G) was significantly associated to higher rate of structural recurrence in stage I-II subjects. Furthermore, survival analysis has shown that the ACG and the CTG genotype were associated to higher and lower disease free survival, respectively (17).

Here, we found no differences in the frequency of the three examined SNPs between MTC patients and controls. In hereditary MTC, we observed an independent association of or the VEGF-A rs833061 with younger age at diagnosis (TT genotype) and smaller tumor size (CC genotype). Additionally, VEGF-A rs2010963 (GG genotype) was correlated with smaller tumor size. No independent associations were observed in patients with sporadic MTC.

Associations between SNPs in the *VEGF-A* gene and the promoter activity of the gene and protein concentrations of VEGF-A have been demonstrated in some studies (18, 31). The functional role of these SNPs is reported contradictive, showing both low and high VEGF levels (32, 33). One of the possible explanations for the conflicting results of functional studies may lie in the fact that it is not an individual SNP but a combined haplotype that is more important influencing the gene function and disease risk/susceptibility. Haplotypes might be more predictive and informative than individual SNPs (30). In this study, we are not able to demonstrate association between haplotype distributions and MTC predisposition or disease aggressiveness.

Primary solid tumours originate close to pre-existing tissue vasculature, initially growing along such tissue blood vessels, and this phenomenon is particularly important for the metastatic potential which frequently occurs in highly vascularized tissues (34). Thyroid tumors are more vascular than non-tumor thyroid tissue, and VEGF expression is upregulated. There is a clear correlation between increasing VEGF-A expression and more aggressive tumor behavior (35, 36). Here, we found tissue expression of VEGF-A

and its receptors (VEGFR-1 and VEGFR-2) in the vast majority of MTC samples evaluated; however, we did not show any association of the *VEGF-A* SNPs and tissue expression of these molecules.

Conclusion

Thus, these results suggest a possible involvement of the *VEGF-A* variants in the MTC pathogenesis. Moreover, our findings may be considered preliminary and suggestive for further studies with these observations support the biological and prognostic implications of *VEGF-A* SNPs.

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Table 1: Clinical and oncological features of MTC patients.

	Total patients	**Sporadic MTC**	**Hereditary MTC**
N	420	218	202
Female (%)	256 (61)	136 (62.4)	120 (59.4)
Age at diagnosis (yr) [a]	40 ± 19.7	50 ± 15.1	28.83 ± 18.2
Calcitonin (pg/ml) [b]	298 (33.67 – 1296)	536 (86.3 – 2000)	129.40 (20.8 – 972.9)
CEA (ng/ml) [b]	17.20 (3.07 – 104.8)	26.4 (4.8 – 150.3)	13.2 (2.1 – 52.6)
Size tumor (cm) [b]	2 (1.2 – 3.4)	2.3 (1.47 – 3.5)	1.85 (0.8 – 3.0)
N1 (%)	162 (50.5)	111 (58.4)	51 (38.9)
M1 (%)	51 (16.4)	39 (21.4)	12 (9.3)

CEA, carcinoembryonic antigen; N1, lymph node metastasis; M1, distant metastasis.

[a]Data expressed as mean ± S.D.
[b]Data expressed as median (IQ 25-75)

Table 2: Frequency of VEGF-A polymorphisms in MTC and control groups.

Sequence variant	Region	**Genotype distribution**			**Minor Allele Frequency MTC (%)**	**Minor Allele Frequency Controls (%)**	***P***
		Wild type (%)	Heterozygous (%)	Homozygous (%)			
-2578 C>A (rs699947)	Promoter	45.5	35.2	19.3	37.2	31.8	0.26 [a]
-460 T>C (rs833061)	Promoter	29.8	49.3	21.0	45.6	41.9	0.45 [a]
+405 G>C (rs2010963)	5´UTR	41.2	48.8	10.2	34.4	37.1	0.36 [a]

[a]Data compared using the X^2 test or Fisher's exact test.

Table 3: Frequency of VEGF-A haplotypes in MTC and control groups.

	Presence/absence of			Polymorphic alleles (n)	Haplotype (%)	
Haplotypes	-2578 C>A (rs699947)	-460 T>C (rs833061)	+405 G>C (rs2010963)		MTC n= 420	Controls n= 187
Hpt1	-	-	-	None	18 (4.3)	9 (4.8)
Hpt2	-	-	+	1	103 (24.5)	54 (28.9)
Hpt3	-	+	-	1	29 (6.9)	10 (5.3)
Hpt4	+	-	-	1	3 (0,7)	0
Hpt5	-	+	+	2	41 (9.8)	23 (12.3)
Hpt6	+	+	-	2	124 (29.5)	54 (28.9)
Hpt7	+	+	+	3	102 (24.3)	37 (19.8)

Hpt, haplotype; P = 0,56 (P value for the comparisons of haplotype frequencies between patients and controls; data compared using Fisher's exact test)

Table 4: Effect of VEGF-A polymorphic alleles on clinical features of patients with hereditary MTC.

-2578C>A (rs699947)	**CC (n= 89)**	**AA (n= 45)**	**AC (n= 68)**	***P***
Age at diagnosis (yr) [1]	27.3 ± 15.3	26.0 ± 19.6	34.5 ± 12.2	0.05
Calcitonin (pg/ml) [1]	186.4 (14.9 – 1685.5)	109.0 (26.1 – 2258)	930.1 (78.8 – 2956.5)	0.31
CEA (ng/ml) [1]	8.9 (2.3 – 143.0)	21.3 (2.7 – 73.5)	33.4 (14.7 – 72.6)	0.74
Tumor size (cm) [1]	2.5 (0.7 – 3.6)	1.0 (0.6– 1.8)	2.5 (1.0 – 4.2)	0.06
N1 (%) [2]	41.3	40.0	34.2	0.77
M1 (%) [2]	6.6	13.8	10.3	0.52
-460T>C (rs833061)	**TT (n= 52)**	**CC (n= 47)**	**CT (n= 103)**	***P***
Age at diagnosis (yr) [1]	23.7 ± 17.4	26.0 ± 19.6	32.6 ± 12.7	0.00[a]
Calcitonin (pg/ml) [1]	72.7 (10.6 – 3265)	109.0 (26.1 – 2258)	437.0 (44.9 – 2240)	0.22
CEA (ng/ml) [1]	4.3 (2.0 – 174.6)	21.3 (2.7 – 73.5)	28.3 (7.4 – 92.4)	0.46
Tumor size (cm) [1]	1.7 (0.6 – 4.5)	1.0 (0.6 – 1.8)	2.7 (1.0 – 3.6)	0.02[b]
N1 (%) [2]	44.4	40.0	35.4	0.66
M1 (%) [2]	8.3	13.8	7.8	0.63
+405G>C (rs2010963)	**GG (n= 82)**	**CC (n= 24)**	**CG (n= 96)**	***P***
Age at diagnosis (yr) [1]	25.8 ± 17.2	16.5 ± 8.5	34.4 ± 13.8	0.01[c]
Calcitonin (pg/ml) [1]	77.0 (14.4 – 497.2)	2647 (409.5 – 4216.7)	706.5 (55.8 – 2320)	0.40
CEA (ng/ml) [1]	8.71 (2.4 - 30.0)	136.0 (1.5 – 417)	38.3 (9.7 – 128.1)	0.04[d]
Tumor size (cm) [1]	1.0 (0.6 – 1.3)	3.0 (1.0 – 4.6)	3.0 (1.3 – 4.2)	0.00[e]
N1 (%) [2]	37.0	9.0	22.0	0.31
M1 (%) [2]	9.6	12.5	8.2	0.86

CEA, carcinoembryonic antigen; N1, lymph node metastasis; M1, distant metastasis. [1]Data compared using Kruskal–Wallis or [2] X^2 tests. P value adjusted for the presence of germline mutations: a) P=0.003; b) P=0.027; c) P=0.056; d) P=0.958; e) P=0.001.

Table 5: Effect of VEGF-A polymorphic alleles on clinical features of patients with sporadic MTC.

-2578C>A (rs699947)	**CC (n= 102)**	**AA (n= 36)**	**AC (n= 80)**	***P***
Age at diagnosis (yr) [1]	50.0 ± 16.0	53.5 ± 10.0	53.0 ± 14.0	0.31
Calcitonin (pg/ml) [1]	298.0 (16 – 2680)	8235.5 (612.5 – 40620.5)	472.0 (158 – 1530)	0.99
CEA (ng/ml) [1]	14.1 (2.8 – 162.5)	435.5 (94.3 – 632.2)	33.6 (8.5 – 117.1)	0.25
Tumor size (cm) [1]	1.8 (1.3 – 4.4)	3.6 (2.3 - 4.4)	2.5 (1.6 – 4.0)	0.82
N1 (%)[2]	55.6	60.0	61.4	0.74
M1 (%)[2]	22.1	24.1	19.4	0. 85
-460T>C (rs833061)	**TT (n= 73)**	**CC (n= 41)**	**CT (n= 104)**	***P***
Age at diagnosis (yr) [1]	52.2 ± 13.7	53.5 ± 10.0	50.8 ± 16.0	0.56
Calcitonin (pg/ml) [1]	126.7 (11.3 – 1385.7)	8235.5 (612.5 – 40620.5)	521.5 (204.7 – 1899.7)	0.50
CEA (ng/ml) [1]	11.1 (2.9 – 122.7)	435.5 (94.3 – 632.2)	30.0 (7.7 – 156)	0.23
Tumor size (cm) [1]	1.6 (1.1 – 3.5)	3.6 (2.3 – 4.4)	2.5 (1.5 – 4.5)	0.52
N1 (%) [2]	52.4	61.8	61. 3	0.49
M1 (%) [2]	20.7	21.2	22.0	0.98
+405G>C (rs2010963)	**GG (n= 91)**	**CC (n= 18)**	**CG (n= 109)**	***P***
Age at diagnosis (yr) [1]	45.9 ± 14.3	51.6 ± 10.2	55.8 ± 14.3	0.45
Calcitonin (pg/ml) [1]	400.0 (101.1 – 2000)	467.5 (63 – 13454.7)	489.5 (86 – 2528.7)	0.67
CEA (ng/ml) [1]	29.8 (6.4 – 225.7)	197.5 (3.7 – 780.5)	21.7 (3.8– 144.6)	0.59
Tumor size (cm) [1]	2.2 (1.8 – 4.4)	1.1 (0.7 – 2.8)	2.5 (1.5 – 4.5)	0.03 [a]
N1 (%) [2]	58.2	57.1	58.8	0.99
M1 (%) [2]	18.9	8.3	25.0	0.32

CEA, carcinoembryonic antigen; N1, lymph node metastasis; M1, distant metastasis. [1]Data compared using Kruskal–Wallis or [2] X^2 tests. [a] P value adjusted for the presence of somatic M918T mutation, P=0.571.

Figures

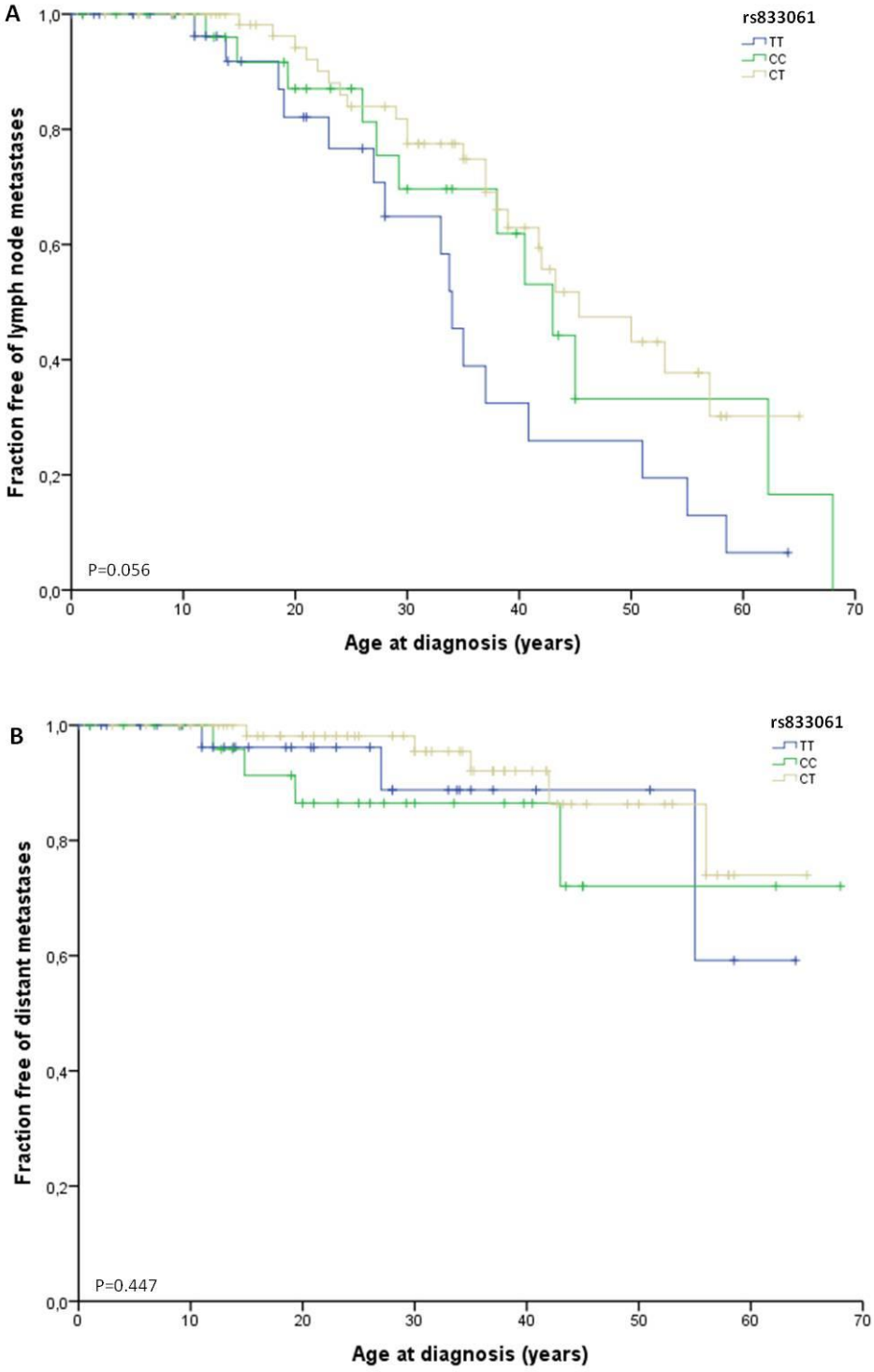

Figure 2. Kaplan–Meier estimates of the proportion of hereditary MTC patients with lymph node (A) or distant metastases (B) at diagnosis. The log rank test was used to compare curves.

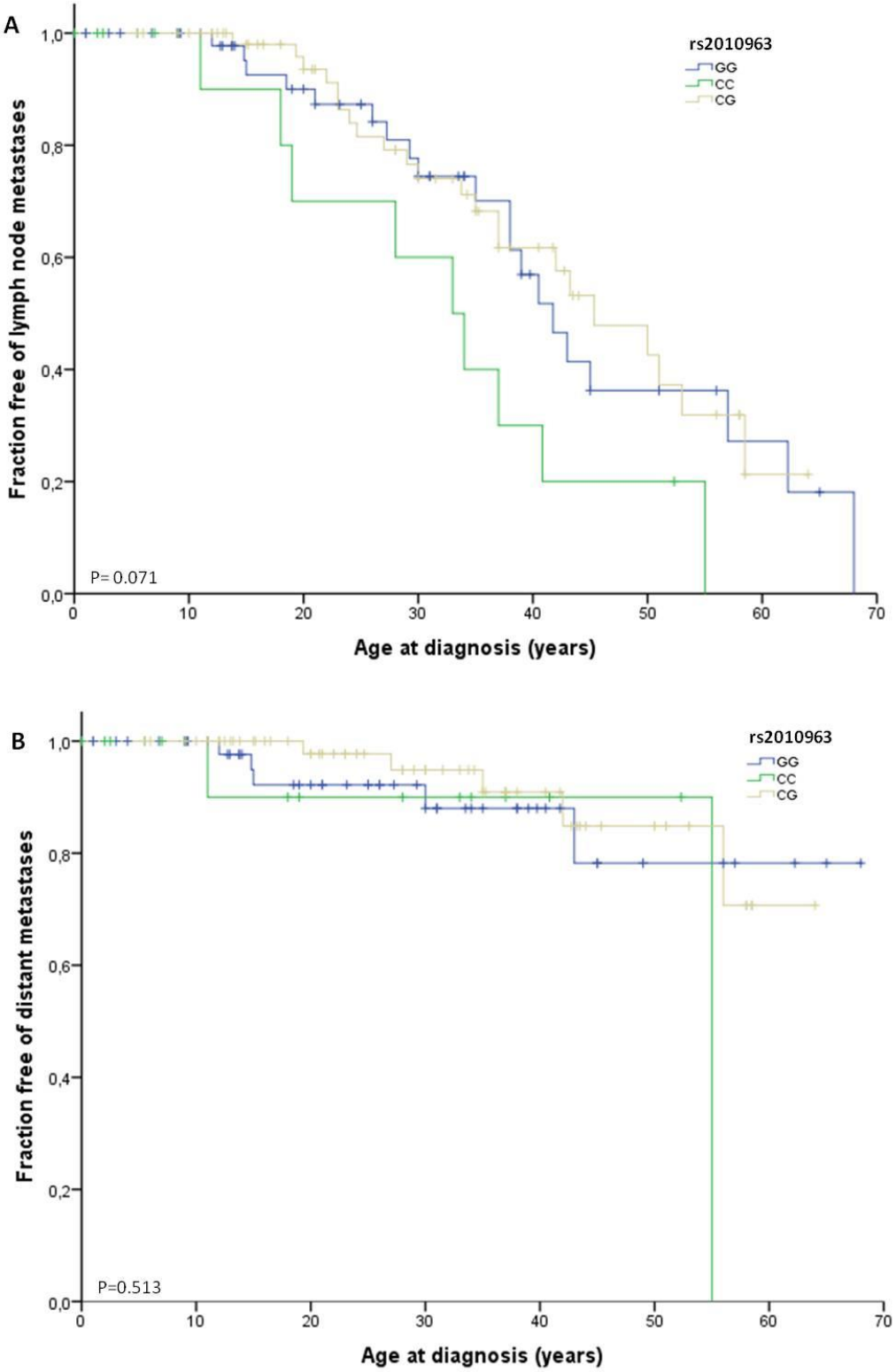

Figure 3. Kaplan–Meier estimates of the proportion of hereditary MTC patients with lymph node (A) or distant metastases (B) at diagnosis. The log rank test was used to compare curves.

Conclusão

No presente estudo demonstramos que níveis séricos mais altos de TSH estão associados a um risco aumentado de câncer de tireoide em pacientes com nódulos tireoidianos. Além disso, nossos resultados evidenciaram associações entre polimorfismos do gene *VEGF-A* e fatores prognósticos em CMT, como idade ao diagnóstico, tamanho do tumor, entre outros, sugerindo que essas variantes podem desempenhar um papel importante na patogênese da doença.

Esses dados sugerem que a identificação de marcadores clínicos, morfológicos ou moleculares que tenham a capacidade de predizer malignidade em nódulos ou informar sobre o tipo de tumor que terá um comportamento mais agressivo é de muita relevância para a prática clínica. Dessa forma, esses resultados colaboram com a ampliação do conhecimento acerca do prognóstico e comportamento da doença tireoidiana.

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