Association between inflammatory cytokine polymorphisms and papillary thyroid carcinoma

Associação entre polimorfismos de citocinas inflamatórias com o carcinoma papilífero de tireóide

Asociación entre polimorfismos de citoquinas inflamatorias y carcinoma papilar de tiroides

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How to cite: Barros AR, Souza JN, Sobrinho AB, Morais RM, Oliveira JR, Silva ICR. Association between inflammatory cytokine polymorphisms and papillary thyroid carcinoma. REVISA. 2020;9(1): 13-23. Doi: https://doi.org/10.36239/revisa.v9.n1.p13a23

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Received: 12/10/2019
Accepted: 15/12/2019

RESUMO

Objetivo: Analisar a associação entre os polimorfismos dos genes IFNG e IL4 com o CPT, e suas características clínicas **Método:** Foram coletados o sangue de 30 pacientes portadores de CPT, e de 82 controles saudáveis. A genotipagem se deu através da técnica de PCR qualitativa. Os resultados foram cruzados com os níveis de TSH e Tiroglobulina dos pacientes com CPT e analisados com o programa SPSS 25.0. O estudo teve aprovação no comitê de ética sob CAAE 57382416.6.0000. 0023. **Resultados:** O genótipo AA do +874 A/T IFNG apresentou frequência de 60% nos participantes com CPT, nos controles o genótipo TA apareceu em 55,6%, o valor de significância foi p=0,0038. Em relação ao IL4, o genótipo B2/B2 foi o mais comum em ambos os grupos com significância de p=0,271. As amostras estavam em equilíbrio HW. Em relação as medianas de Tiroglobulina (ng/mL) e TSH (uUI/mL), foram observados os seguintes valores de significância respectivamente: p=0,612 e p=0,419 em relação ao IFNG e p= 0,431 e p=0,655, em relação ao IL4. **Conclusão:** Houve associação estatística com o polimorfismo +874 A/T IFNG e o CPT, entretanto não houve associação entre os níveis de TSH e tiroglobulina em pacientes com CPT. Em relação ao gene IL4 não foram observados significância entre a frequência genotípica e o CPT e os níveis de TSH e Tiroglobulina. O presente trabalho reforça a necessidade da produção de mais estudos acerca do tema a fim de estabelecer-se se de fato é possível afirmar se tais associações (ou ausência de associação) são de fato realidade no contexto do CPT.

Descritores: Neoplasias da Glândula Tireoide; Polimorfismo Genético; Citocinas.

ABSTRACT

Objective: To assess the association between IFNG and IL4 gene polymorphisms and CPT and their clinical characteristics Method: Blood was collected from 30 patients with CPT and 82 healthy controls. **Method:** Blood was collected from 30 patients with TLC and 82 healthy controls. Genotyping was performed by the qualitative PCR technique. Results were crossed with TSH and Thyroglobulin levels of patients with CPT and analyzed using the SPSS 25.0 program. The study was approved by the ethics committee under CAAE 57382416.6.0000. 0023. **Results:** The +874 A / T IFNG AA genotype showed a frequency of 60% in participants with CPT, in controls the genotype TA appeared in 55.6%, the significance value was p = 0.0038. Regarding IL4, the B2 / B2 genotype was the most common in both groups with significance of p = 0.271. The samples were in HW equilibrium. Regarding the median Thyroglobulin (ng / mL) and TSH (uUI / mL), the following significance values were observed respectively: p = 0.612 and p = 0.419 for IFNG and p = 0.431 and p = 0.655 for IL4. **Conclusion:** There was a statistical association with +874 A / T IFNG polymorphism and CPT, however there was no association between TSH and thyroglobulin levels in patients with CPT. Regarding the IL4 gene, no significance was observed between genotypic frequency and CPT and TSH and Thyroglobulin levels. The present work reinforces the need to produce more studies on the subject in order to establish if it is in fact possible to affirm if such associations (or absence of association) are in fact in the context of the CPT.

Descriptors: Thyroid Neoplasms; Polymorphism, Genetic; Cytokines.

RESUMEN

Objetivo: analizar la asociación entre IFNG y polimorfismos del gen IL4 con CPT y sus características clínicas **Método:** Se recogió sangre de 30 pacientes con CPT y 82 controles sanos. El genotipado se realizó mediante la técnica cualitativa de PCR. Los resultados se cruzaron con niveles de TSH y tiroglobulina de pacientes con CPT y se analizaron utilizando el programa SPSS 25.0. El estudio fue aprobado por el comité de ética bajo CAAE 57382416.6.0000. 0023. **Resultados:** El genotipo AAA IFNG +874 A / T presentó una frecuencia del 60% en los participantes con CPT, en los controles el genotipo TA apareció en el 55,6%, el valor de significación fue p = 0,0038. Con respecto a IL4, el genotipo B2 / B2 fue el más común en ambos grupos con un significado de p = 0.271. Las muestras estaban en equilibrio HW. Con respecto a las medianas de tiroglobulina (ng / ml) y TSH (uUI / ml), se observaron los siguientes valores de significancia respectivamente: p = 0.612 y p = 0.419 para IFNG y p = 0.431 y p = 0.655 para IL4. **Conclusión:** hubo asociación estadística con +874 A / T IFNG polimorfismo y CPT, pero no hubo asociación entre TSH y niveles de tiroglobulina en pacientes con CPT. Sobre el gen IL4, no se observó significación entre la frecuencia genotípica y los niveles de CPT y TSH y tiroglobulina. Eso trabajo refuerza la necesidad de producir más estudios sobre el tema para establecer si de hecho es posible afirmar si tales asociaciones (o ausencia de asociación) están de hecho en el contexto del CPT.

Descriptores: Neoplasias de la Tiroides; Polimorfismo Genético; Citocinas.

ORIGINAL

Introduction

Thyroid is an endocrine gland that has essential functions in maintaining the metabolic activity of the human body. It is formed by parenchymal cells, such as parafolicular and follicular.[1] Follicular cells are grouped into functional units called follicles, located around colloid structures formed by thyroglobulin, which will serve as a substrate for the synthesis of thyroid hormones T3 (triiodothyronine) and T4 (thyroxine). Parafolicular cells are responsible for the synthesis of calcitonin hormone.[2]

The release of T3 and T4 is mediated through a harmonious feedback mechanism involving TSH (Thyroid Stimulating Hormone) and TRH (Thyrotropin Stimulating Hormone). TSH is a pituitary-secreted glycoprotein that stimulates the production and release of T3 and T4. TSH release is regulated by HRT which in turn is produced in the hypothalamus.[3]

Several pathologies may compromise thyroid function, among them thyroid cancer, which is the most prevalent endocrine neoplasia. There are four main types of thyroid, papillary, follicular, medullary and anaplastic tumors.[4] The overall incidence of these tumors has reported an increase over the years in both men and women, although the vast majority of thyroid cancers are more prevalent in females.[5] The most prevalent tumor is papillary type, originating from follicular cells, this type compared to the others has the best prognosis.[1,5-6] Papillary Thyroid Carcinoma (TCC) accounts for 80 to 85% of cases of neoplasms affecting this gland.[1] In Brazil, it is estimated that between 2016 and 2017 there would be 6,960 new cases, with a higher incidence in females.[7]

Consolidated literature data suggest that CPT as well as other thyroid tumors are closely related to variations in the human genome.[6] This genetic basis would be related to processes that would favor the regulation of cell proliferation.[6]

Among the existing genetic variations we can highlight the genetic polymorphisms, focus of this work, which can be defined as being a genetic variation that occurs in at least 1% of the population, the most common types are those of Single Nucleotide (SNP) and Variable Number Tandem Repeat (VNTR), such changes may increase susceptibility to some pathologies such as breast cancer.[8-9]

Cytokines are proteins known for their properties related to modulation of the immune system, having the ability to influence the activation, differentiation and proliferation of target cells, mainly T and B lymphocytes, thus regulating the inflammatory activity of the organism. These molecules play a crucial role in several pathologies, including malignant thyroid tumors. Genes encoding cytokines are extremely polymorphic, these genetic variants are often associated with susceptibility to disease and / or prognostic factors.[10]

Gamma interferon (IFN-γ) is a cytokine produced by Th1 lymphocytes, which plays an important role in viral and bacterial processes, however its properties related to antiproliferative effects have been drawing attention in recent years.[11] This cytokine is able to stimulate a chemokine called CXCL10 which in turn has angiogenesis inhibiting properties, a process that consists in the formation of new blood vessels that are essential for cell proliferation.[12-13] IFN-γ is encoded by a gene called IFNG, located on the long arm of chromosome12 (12q15).[14] The SNP polymorphism at position +874 where there

is an exchange of adenine for thymine has been widely studied due to its functional characteristic and data suggest that the T allele would be associated with higher IFN-y production and the allele A the lowest. production of this cytokine.[11,14]

IFN-γ function is antagonized by the Th2-type lymphocyte pool, the function of these lymphocytes is associated with various types of cancers such as colon, breast and kidney.[15-16] Interleukin 4 (IL-4) cytokine is produced by Th2 lymphocyte-activated CD4 + T cells, as it has both inflammatory and anti-inflammatory character and its role in the cancer pathway is bilateral.[16] IL-4 is able to alter tumor process-related autoimmunity by regulating the immune response of apoptotic activity-related lymphocytes.[8,16]

IL-4 is encoded by the IL4 gene located on the long arm of chromosome 5 (5q31.1). The intron 3 VNTR-type polymorphisms are composed of a 70 bp repetition, such variation is capable of influencing IL-4 expression, and RP2 allele (three repeats) is associated with decreased IL-4 expression.[8,16]

Considering the relevance of the theme and the absence of studies that focus on the study of these polymorphisms with CPT, the present work aims to investigate the association between IFNG +874 A / T (rs2430561) and IL4 VNTR intron 3 genetic polymorphisms with CPT and TSH and thyroglobulin levels.

Method

For conducting this work, a cross-sectional study design- case and control- was chosen.

Research participants

The number of participants in the case group was estimated taking into account the prevalence of 1% of TLC in the adult population, 5% sampling error and 95% confidence interval (CI), in a sample universe of 8,450 patients. to the total of 12 participants. This study consisted of 30 patients with TLC who underwent thyroidectomy surgery. These participants were recruited from the company Medical Images of Brasilia (IMEB) where they would undergo iodotherapy and had an average age of 47.5 ± 12.3 years (19 women and 11 men).

For the control group, 81 individuals with no history of malignant thyroid cancer or other neoplasms were recruited from the University of Brasilia Laboratory for Clinical Analysis Laboratory, Ceilândia campus, and the average age of the participants in the control group was 52.3 ± 5. , 7 years old (43 women and 38 men)

The Informed Consent Form (ICF) was obtained from all participants in this research. This study was approved by the UniCeub Ethics Committee, CAAE No. 57382416.6.0000. 0023.

Genotyping

From both groups about 5 mL of peripheral venous blood was collected in EDTA tubes. DNA was extracted from this material using the PureLink® Genomic DNA Mini Kit from Invitrogen (catalog # K1820-02, lot # 19339891). The DNA concentrations obtained were determined with spectrophotometry

using the NanoDrop® equipment (Thermo Fisher Scientific Inc.). The average yield achieved was 20 ng / µl.

The DNA extracted from the participants was submitted to qualitative PCR technique for genotyping. For each PCR reaction, 8.0 µL of genomic DNA was used at the final concentration of 2.5 ng / µL; 12.5 µl 10x buffer (10mM Tris and 50mM KCl); 6.25 µL 50 mM MgCl2 (Ludwig Biotec, Alvorada, Rio Grande do Sul, Brazil), 10 µL deoxyribonucleotideostrifoast (dNTPs); 2.5 mM; (Ludwig Biotec, Dawn, Rio Grande do Sul, Brazil); 2 µL Taq Polymerase, (Ludwig Biotec, Alvorada, Rio Grande do Sul, Brazil), 10 U / µL; 1.5 µL of each oligonucleotide (10 µM, IDT technologies); supplementing with Milli-Q water to a final volume of 25 µL per reaction.

For IFNG +874 A / T gene polymorphism (rs2430561) the following oligonucleotide sequences were used: CP 5'-TCA ACA AAG CTG ATA CTC CA-3 '; T: 5´-TTC TTA CAA CAC AAA ATC AAA TCT-3´; A: 5'-TTC TTA CAA CAC AAA ATC AAA TCA-3´.14 The following thermocycling conditions were used: initial denaturation at 95ºC for 5 minutes followed by 35 cycles of denaturation at 95ºC for 1 minute, annealing at 60ºC for 1 minute, extension at 70 ° C for 2 minutes and a final extension at 72 ° C for 10 minutes. The PCR products were electrophoretically run on 2% agarose gel with ethidium bromide for 1 hour at 100W.

For IL4 gene intron 3 VNTR genotyping the following oligonucleotide sequences were used: F: 5'AGGCTGAAAGGGGGAAAGC-3 ', R: 5'CTGTTCACCTCAACTGCTCC-3'.[15] Thermocycling conditions were: initial denaturation at 94 ° C for 5 minutes, followed by 30 cycles of denaturation at 94 ° C for 50 seconds, annealing at 62 ° C for 30 seconds, extension at 72 ° C for 30 seconds and a final extension at 72 ° C for 5 minutes. PCR products were electrophoretically run on 3% agarose gel with ethidium bromide for 1 hour at 100W.

The PCR products of both genetic polymorphisms studied were visualized in a transluminator (L-PIX Touch) with ultraviolet source, and the genotypic frequency was after direct amplicon counting. The size of the fragments visualized in the polymorphic analysis of the IFNG gene (rs2430561) was 262bp. For IL4 gene polymorphism, the size of the fragments visualized was 183bp and 253bp.

Statistical analysis

Data were analyzed using the SPPS version 25.0 statistical program. Hardy-Weinberg equilibrium (HWE) was evaluated using the chi-square test. Comparison between genotypes was also performed using the same test. To compare the different genotype groups and clinical characteristics, the Kruskall-Wallis nonparametric H test was used. For all analyzes, a significance level of 5% ($p < 0.05$) was considered.

Results

HWE equilibrium analysis revealed that the distribution of genotypes in the control group of both polymorphisms analyzed were in equilibrium (INFG: $p = 0.129$; IL4: $p = 0.344$).

Data analysis regarding genotypic frequency of IFNG +874 A / T gene

polymorphism (rs2430561) showed a higher frequency of AA genotype (60%) in the group of participants with CPT, the other genotypes presented a frequency of 20% (TA and TT). In the group composed of individuals without CPT, the most frequent genotype TA (55.6%) followed by genotype AA (33.3%) and TT (11.1%), after analysis with chi-square test it was observed that the differences were statistically significant (p = 0.0038). Such data are shown from Table 1.

As shown in Table 1, after genotype dichotomization, it was observed that AA genotype confers a risk factor (OR = 3.0; 95% CI = 1.26-7.11]; p = 0.011) for susceptibility to CPT However, the allelic distribution analysis did not show significant statistical values between case and control groups (p = 0.272).

Table 1- Distribution of genotypic and allelic frequencies of the +874 A / T polymorphism of the IFNG gene (rs2430561) in the case and control groups.

IFNG +874	Groups					
	CPT		Control			
A/T	N	%	N	%	P	OR (ICI 95%)
TT	6	20,0	9	11,1	0,0038*	N/A
TA	6	20,0	45	55,6		
AA	18	60,0	27	33,3		
Total	30	100,0	81	100,0		
AA	18	60,0	27	33,3	0,011*	3,0(1,26-7,11)
TT+TA	12	40,0	54	66,7		
Total	30	100,0	81	100,0		
T	18	30,0	63	38,9	0,272	0,67(0,35-1,27)
A	42	70,0	99	61,1		
Total	60	100,0	162	100,0		

* p> 0.05. CPT: Papillary Thyroid Carcinoma. N / A: Not applicable. OR: Odds Ratio. Test applied: Chi-Square.

As shown in table 2, in relation to the IL4 VNTR intron 3 gene polymorphism, it can be observed that the RP2 / RP2 genotype was the most frequent in both groups (CPT = 73.3%; control = 71%) followed by genotypes. RP1 / RP2 (CPT = 20.0%; control = 29.0%) and RP1 / RP1 (CPT = 6.7%; control: absent), such differences between groups were not significant (p = 0.271). Dichotomization of genotypes showed no statistically significant value (p = 0.841). After analysis of the allelic distribution it was observed that the RP2 allele (three 70 bp repetitions) was the most frequent in both groups (TLC = 83.3%; control = 85.5%). The RP1 allele (two 70 bp repetitions) was present in the minority of individuals in both groups (CPT = 16.7% and control = 14.5%), however the statistical analysis showed no significant differences (p = 0.740).

Table 2- Distribution of genotype and allelic frequencies of IL4 gene intron 3 VNTR polymorphism in participants with CPT and healthy controls.

	Groups					
	CPT		Control			
IL4 VNTR	N	%	N	%	P	OR (CI 95%)
RP1/RP2	6	20,0	9	29,0	0,271	N/A
RP1/RP1	2	6,7	0	0,0		
RP2/RP2	22	73,3	22	71,0		
Total	30	100,0	31	100,0		
RP2/RP2	22	73,3	22	71,0	0,841	1,12(0,36-3,45)
RP1/RP2+RP1/RP1	8	26,7	9	29,0		
Total	30	100,0	31	100,0		
RP1	10	16,7	9	14,5	0,740	1,11(0,44-3,13)
RP2	50	83,3	53	85,5		
Total	60	100,0	62	100,0		

CPT: Papillary Thyroid Carcinoma. N / A: Not applicable. OR: Odds Ratio.RP1: Two 70bp repetitions; RP2: Three 70bp repeats. Test applied: Chi-Square.

As shown in Table 3, the variables related to the clinical characteristics (Thyroglobulin and TSH levels) of the patients with TLC were crossed with the genotypic distribution of the polymorphisms analyzed. Nonparametric statistical analysis showed that the differences are not significant for both clinical characteristics.

Table 3- Medians, median intervals and P-values of Thyroglobulin and TSH measurements in participants with TLC according to IFNG and IL4 gene genotype.

		[Thyroglobulin] ng/mL			[TSH] uUI/mL		
		CL lower than 95,0% for median	Median	CL upper than 95,0% for median	CL lower than 95,0% for median	Median	CL upper than 95,0% for median
+874 IFNG	AA	1,02	2,88	5,43	7,46	76,65	121,00
A/T	TA	1,63	8,09	500,00	0,05	35,09	130,07
	TT	0,52	6,12	31,90	65,91	97,57	118,08
P value			0,612			0,419	
IL4 (VNTR	RP1/RP1	5,58	18,79	31,99	77,90	99,45	121,00
Íntron 3)	RP1/RP2	1,06	2,88	3,08	0,72	95,42	143,92
	RP2/RP2	0,77	3,00	8,09	17,65	68,94	103,36
P value			0,431			0,655	

Kruskall-Wallis H Test.

Discussion

The etiology of thyroid cancer so far is unknown, but there is solid evidence that genetic changes, such as polymorphisms, contribute significantly to the increased risk of thyroid cancer susceptibility.[17] When it comes to the analysis of genetic polymorphisms and their association with differentiated thyroid tumors, most publications focus on the study of classical oncogenes, such as P53.[18-19]

However in a literature review conducted by Lumachi F; Basso SMM and Orlando R., (2010) demonstrated the close relationship between cytokines and thyroid cancer, including highlighting their role as serum biomarkers in the follow-up of patients with thyroid cancer.[10] When it comes to the analysis of these polymorphisms (IFNG +874 A / T and IL4 VNTR intron 3) and their association with CPT and a Brazilian sample, this present study is pioneering.

The +874 A / T IFNG polymorphism over the years has been associated with infectious processes such as chagas disease and tuberculosis; however, over the years, evidence has pointed to the role of cytokine IFN-y in the progression of malignant neoplasms.[20] However, although the +874 A / T polymorphism has a functional character, where its alleles are associated with IFN-γ levels, the association of this polymorphism with the risk of developing cancer seems uncertain.[21] A meta-analysis by Yu-Zheng Ge et al. (2014) about the + 874 A / T polymorphism and cancer risk found no statistically significant association.[22] However, in a more recent study conducted by Karakus N. et al., (2019) found a statistically significant association between TA + AA genotypes and breast cancer risk.[11] Similar result to that found in this study, where AA genotype was more frequent in patients with TLC. One hypothesis that explains this correlation is genotypes that have the low IFN-γ-producing allele A, which activate less angiogenesis inhibition pathways, favoring cell proliferation.[12-13]

About the second polymorphism analyzed in this work, Duan Y et al. (2014) performed a meta-analysis about VNTR intron 3 IL4 polymorphism and cancer risk, and find that this polymorphism may influence cancer risk, being the RP2 allele. (three repetitions) associated with lower cancer risk.[16] Our study showed no statistical relationship between any of the alleles or genotypes of this polymorphism and susceptibility to CPT, a result similar to that found by Al-Eitan et al. (2019) and Konwar R et al. (2009) when analyzing this genetic variant. and the risk for breast cancer.[8,23]

Regarding TSH and TLC, diagnosis of this pathology begins with the evaluation of thyroid nodules normally found in routine or radiological examinations. At this moment of initial evaluation, TSH values> 5.5 mU / mL represent a risk 11 times. malignancy compared to patients with nodules and TSH values <0.4mU / mL[24-25], therefore the TSH value is a useful tool in the diagnostic evaluation of TLC.[25] Post-thyroidectomy endogenous (hypothyroid) or exogenous TSH stimulation increases detection sensitivity of ultrasensitive thyroglobulin for detection of residual tumors.[26] Regarding TSH levels, the high median values found in our sample, in the different genotypes of both polymorphisms, were within the expected, considering the characteristics (post surgery) of the participants.[27]

Thyroglobulin is the best serum marker for residual disease assessment, as studies indicate that there appears to be an association between marker levels and remaining thyroid tissue mass.[26] Thyroglobulin values above 10 ng

/ mL indicate residual tumor, and worse prognosis, values above 70 ng / mL indicate high likelihood of metastasis.[28] In our study, regarding thyroglobulin levels, we observed that homozygous patients with RP1 allele (two repetitions) of IL4 intron 3 VNTR polymorphism had the highest medians (18.79 ng / mL), indicating a worse prognosis, however. Only 2 individuals were carriers of this genotype. When Ibrahimi, M. et al. (2018) analyzed this polymorphism and its association with breast cancer in Iranian patients, they observed that the RP2 allele (3 repeats) constituted a protective factor for susceptibility.15 In a recent Laith study N AL-Eitan et al. (2019) found an association between predictive clinical characteristics of breast cancer prognosis and intron 3 IL4 VNTR polymorphism, suggesting the RP2 allele associated with better prognostic features.[8]

Conclusion

The literature review necessary for the construction of this article showed that there is a lack of publications about the association of these polymorphisms with the CPT, which limited the comparison of our results with the findings in the literature.

Our findings conclude that IFNG +874 A / T polymorphism is associated with susceptibility to CPT in a sample of Brazilian participants, but is not associated with TSH and thyroglobulin levels presented by these patients. The role of this polymorphism in several strains of cancer is still controversial.

IL4 VNTR intron 3 genetic polymorphism does not appear to be associated with susceptibility to CPT in our sample, nor with the clinical features explored here, although there is a certain tendency for the RP1 / RP1 genotype to be associated with higher levels of thyroglobulin (worst prognostic factor). This difference is not statistically significant.

However, it is noteworthy that although our sample size is representative, the small number of participants prevented us from further exploring the relationship between TSH and thyroglobulin levels and the different genotypes, which is a limitation of this study. Investigations on the association of inflammatory cytokine polymorphism with CPT need to be continued through studies with a larger sample number covering different ethnic populations.

Acknowledgment

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001

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