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Involvement of *CYP1A1*, *GST*, *72TP53* polymorphisms in the pathogenesis of thyroid nodules

A.A.S. Reis[1,2], D.M. Silva[2,5], M.P. Curado[3,4] and A.D. da Cruz[1,2,5]

[1]Programa de Pós-Graduação em Biologia Celular e Molecular, Universidade Federal de Goiás, Goiânia, GO, Brasil
[2]Departamento de Biologia, Núcleo de Pesquisas Replicon, Pontifícia Universidade Católica de Goiás, Goiânia, GO, Brasil
[3]Serviço de Cabeça e Pescoço, Hospital Araújo Jorge da Associação de Combate ao Câncer em Goiás, Goiânia, GO, Brasil
[4]Descriptive Epidemiology Production Group, International Agency for Research on Cancer, Lyon, France
[5]Laboratório de Citogenética Humana e Genética Molecular, Superintendência Leide das Neves Ferreira, Secretaria de Estado da Saúde de Goiás, Goiânia, GO, Brasil

Corresponding author: A.A.S. Reis
E-mail: angeladamski@gmail.com

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ABSTRACT. Specific genotypes appear to be related to the development of thyroid disease. We examined whether polymorphisms of the genes *CYP1A1*, *GSTM1*, *GSTT1*, and *TP53* at codon 72 are associated with increased risk for thyroid nodules. Blood samples were obtained from 122 thyroid patients with nodules and from 134 healthy control individuals from Goiânia city, GO, Brazil. We found no significant association of *CYP1A1m1* and *CYP1A1m2* genotypes with thyroid diseases ($P > 0.05$). The null genotypes of *GSTM1* and *GSTT1* genes were predominant in patients with nodules, indicating that individuals that possess these genotypes have a predisposition for thyroid disease. The genotype *p53Arg Arg* was associated with a low risk for thyroid cancer (OR = 0.15; $P < 0.0001$), indicating that the arginine allele in homozygosis could have

a protective effect against carcinogenesis. On the other hand, the *p53ArgPro* genotype was significantly associated with malignant neoplastic nodules (OR = 3.65; P = 0.001). Interindividual variation in susceptibility to thyroid diseases could provide new perspectives for early diagnosis, prognosis and treatment, indicating which patients with thyroid nodules will benefit from treatment, depending on specific polymorphic profiles.

Key words: Polymorphism; Thyroid disease; Susceptibility; p53; CYP; GST

INTRODUCTION

Thyroid nodules are common findings in clinical practice and their prevalence depends mainly on the method of screening, population evaluation, increasing age, female gender, iodine deficiency, and history of head and neck radiation (Dean and Gharib, 2008). Ultrasonography is unquestionably the most sensitive thyroid-imaging test that is able to detect thyroid nodules in 19-67% of randomly selected individuals, with higher frequencies in women and elderly (Hegedüs et al., 2003; Hegedüs, 2004). The incidence of thyroid cancer has also increased during the past 30 years probably due to the earlier detection of nodules (Mazzaferri, 2006; Dean and Gharib, 2008), and currently, genetic susceptibility, with regard to specific genotypes, suggests the combination of some genetic alleles in the etiology of this kind of disease (Hegedüs, 2004; Dean and Gharib, 2008).

Cytochrome P450 1A1 (*CYP1A1*) is a key enzyme in phase I bioactivation (Nebert et al., 1996), which activates procarcinogens to genotoxic electrophilic intermediates. It contributes to aryl hydrocarbon hydroxylase activity, catalyzing the first step in the metabolism of a number of polycyclic aromatic hydrocarbons (PAHs). It is also involved in estrogen metabolism, catalyzing the hydroxylation of 17β-estradiol at the C-2 position (Masson et al., 2005).

Following the action of phase I enzymes on xenobiotics, phase II enzymes, such as glutathione S-transferase (GST), is characterized by the conjugation of endogenous water-soluble compounds to lipophilic substrates (Sweeney et al., 2000; Stankov et al., 2006). *GSTM1* is involved in the detoxification of PAH and other mutagens, and cells from *GSTM1* null individuals are more susceptible to DNA damage caused by these agents (Rossini et al., 2002). *GSTT1* wild-type enzyme detoxifies smaller reactive hydrocarbon intermediates, such as ethylene oxide, and the wild-type alleles do not appear to be associated with the presence of PAH or DNA adducts (Agundez, 2004).

The tumor protein *p53* gene (*TP53*) is usually recognized as a tumor-suppressor gene because it encodes protein p53, and it participates in the processes of cell-cycle arrest and apoptosis. Codon 72 polymorphism on the 4th exon of *TP53* is involved in multiple steps of carcinogenesis and may also account for genetic differences in susceptibility to cancer (Boltze et al., 2002; Aral et al., 2007; Almeida et al., 2008). It has been demonstrated that the *TP53* polymorphism varies according to ethnic and geographical distribution, like most human genetic polymorphisms. *72TP53* works independent of *CYP1A1*, *GSTM1* and *GSTT1* polymorphisms, which encode carcinogen-metabolizing enzymes.

Thus, we focused on the role of genetic susceptibility with regard to specific genotypes that could be related to the development of thyroid diseases. We conducted a study at Araújo Jorge Hospital in 122 untreated Brazilian patients, and we investigated the association between genetic polymorphisms of *CYP1A1m1*, *CYP1A1m2*, *GSTM1*, *GSTT1*, and *72TP53* to clarify individual differences in genetic susceptibility to thyroid nodule pathogenesis.

MATERIAL AND METHODS

Samples

We used a prospective blood sample collection from 122 patients with thyroid nodules and 134 healthy individuals. All samples were collected at Araújo Jorge Hospital in the Head and Neck Service before any kind of treatment. The patient samples were collected after the diagnosis was confirmed by fine-needle aspiration. All patients were submitted to thyroidectomy.

The case group (18-83 years old) was composed of 35 malignant neoplastic nodules (MNN), including 30 papillary carcinoma (PTC), 2 follicular carcinoma, 2 medullar carcinoma, and 1 Hurthle cell carcinoma; 20 benign neoplastic nodules (BNN), including 11 follicular adenoma and 9 Hurthle cell adenoma, and 67 non-neoplastic nodules (NNN), including 62 goiters and 5 Hashimoto's thyroiditis. The control group (21-65 years old) was randomly selected from the general population of Goiânia, GO, and the individuals were considered to have a normal iodine intake. The simplified characteristics of the group are summarized in Table 1. The genomic DNA of patients and controls was isolated from peripheral blood samples according to a standard commercial kit (GE Healthcare®, USA). The study was approved by the Ethics Committee of the Araújo Jorge Hospital of the Associação de Combate ao Câncer em Goiás (ACCG) in accordance with the Declaration of Helsinki (2000) and all subjects gave informed consent.

Table 1. Characteristics of the study subjects.

Variable	MNN	BNN	NNN	Controls
Age (years)				
≤40	16 (45.7%)	6 (30.0%)	24 (35.8%)	83 (61.9%)
41-60	14 (40.0%)	14 (70.0%)	33 (49.3%)	47 (35.1%)
≥60	5 (14.3%)	0 (0.0%)	10 (14.9%)	4 (3.0%)
Gender				
Male	8 (22.9%)	3 (15.0%)	8 (11.9%)	98 (73.2%)
Female	27 (77.1%)	17 (85.0%)	58 (88.1%)	36 (26.8%)

Data are reported as number of subjects with percent in parentheses. MNN = malignant neoplastic nodules; BNN = benign neoplastic nodules; NNN = non-neoplastic nodules.

Polymerase chain reaction

Genotypes *CYP1A1m1* (*Msp*I) and *CYP1A1m2* (*Bse*MI) were identified by polymerase chain reaction (PCR)-restriction fragment length polymorphism. The primers for *CYP1A1m1* sites were M1F: (5' CAG TGA AGA GGT GTA GCC GCT 3') and M1R: (5' TAG GAG TCT TGT CTC ATG CCT 3'), which produce a 340-bp fragment. The primers for *CYP1A1m2* sites were M2F: (5' TTC CAC CCG TTG CAG CAG GAT AGC C 3') and M2R: (5' CTG TCT CCC TCT GGT TAC AGG AAG 3'), which generate a 204-bp fragment, according to Song et al. (2001). The restriction enzyme *Msp*I (Fermentas UAB, Lithuania) was used to distinguish the *CYP1A1m1* polymorphism, since the wild-type allele shows a single band representing the entire 340-bp fragment and since the variant allele results in two fragments of 200 and 140 bp.

The restriction enzyme *Bse*MI (Fermentas UAB) digested the 204-bp *CYP1A1m2* product. The cleavage site was lost in the case of the mutation and gave a single band of 204 bp, whereas the wild-type alleles generate 149- and 55-bp fragments. The restriction products were analyzed by electrophoresis on 8% silver-stained polyacrylamide gels.

A multiplex PCR was performed in which *GSTM1* and *GSTT1* were co-amplified using the primers M1F: (5' GAA CTC CCT GAA AAG CTA AAG C 3') and M1R: (5' GTT GGG CTA AAT ATA CGG TGG 3') and T1F: (5' TTC CTT ACT GGT CCT CAC ATC TC 3') and T1R: (5' TCA CCG GAT CAT GGC CAG CA 3'), generating fragments of 215 and 480 bp, respectively. The PCR products were analyzed by electrophoresis on a 2% ethidium bromide-stained agarose gel. *TP53* codon 72 genotyping was carried out by PCR. Samples were submitted to PCR using two different primer sets for the amplification of the *p53Arg* AF: (5' TCC CCC TTC CCG TCC CAA 3') and AR: (5' CTG GTG CAG GGG CCA CGC 3'), and *p53Pro* PF: (5' GCC AGA GGC TGC TCC CCC 3') and PR: (5' CGT GCA AGT CAC AGA CTT 3'), according to Sourvinos et al. (2001). PCR products were analyzed on 8% silver-stained polyacrylamide gels. A single band of approximately 177 bp indicated an individual homozygous for *p53Pro*; the presence of a band with 144 bp characterized an individual homozygous for *p53Arg*. Heterozygous samples, *p53Arg/Pro*, displayed two fragments with 144 and 177 bp.

Statistical analysis

The genotype and allelic frequencies for all the individuals from the thyroid nodule group were separated and compared statistically with the corresponding data for the group. For this purpose, we used the χ^2 test with BioEstat 5.0®. Results were considered to be statistically significant when the P value was less than 5% ($P < 0.05$). Odds ratios (OR) were calculated for disease susceptibility associated with specific genotypes.

RESULTS

Genotyping results showed that the predominant polymorphic types were the wild type in *CYP1A1m1* and *CYP1A1m2* among the cases and controls. The results demonstrated no statistically significant associations of *CYP1A1m1* genotypes with thyroid diseases.

On the other hand, in the *CYP1A1m2* gene, both allele or genotype frequencies showed a statistically significant difference ($P < 0.05$). Such results could be explained since the BNN group showed only the wild-type genotype, and the OR revealed a lack of association between the development of non-neoplastic and neoplastic thyroid nodules and *CYP1A1m2*.

The *GSTM1* and *GSTT1* null genotypes were observed to be more frequent among cases than among controls [$\chi^2 = 22.9$; $P < 0.0001$ and $\chi^2 = 33.72$; $P < 0.0001$, respectively]. A significant difference in genotype frequencies of *GSTM1* and *GSTT1* was observed ($P > 0.05$). *GSTM1* and *GSTT1* null genotypes were predominant in all types of nodules when compared to the control group, indicating a predisposition to benign thyroid diseases ($OR = 4.53$; $P < 0.001$).

Therefore, either in BNN or NNN, the risk is higher when compared to the lack of the *GSTT1* genotype. The *p53Arg* allele was significantly higher in both patients and controls. We also demonstrated that the genotype *p53Arg/Arg* showed a lower risk of MNN, indicating that the arginine allele in homozygosis could have a protective effect against carcinogenesis ($OR = 0.15$; $P < 0.0001$).

On the other hand, the *p53ArgPro* genotype demonstrated a statistically significant difference in the MNN group, which showed an increased risk of developing malignant neoplastic nodules ($OR = 3.65$; $P = 0.001$) when compared to other genotypes. Thus, the heterozygous genotype increased the risk of malignant nodules more than 3.6 times compared to other genotypes. The *pro/pro* genotype frequencies in both groups did not show any significant differences ($P > 0.05$). The statistical analyses are summarized in Table 2.

Table 2. *CYP1A1m1*, *CYP1A1m2*, *GSTM1*, *GSTT1*, and *p53* frequencies in case and control groups.

Polymorphism	Types	MNN	OR (95%CI)	P	BNN	OR (95%CI)	P	NNN	OR (95%CI)	P	Control
CYP1A1m1	T/T	17 (48.5%)	-	-	13 (65.0%)	-	-	41 (61.2%)	-	-	65 (48.5%)
	T/C	14 (40.0%)	0.57 (0.30-1.082)	0.11	6 (30.0%)	0.51 (0.18-1.44)	0.30	21 (31.3%)	0.92 (0.41-2.03)	0.99	58 (43.2%)
	C/C	4 (11.5%)	0.72 (0.23-2.22)	0.76	1 (5.0%)	0.45 (0.05-3.83)	0.75	5 (7.5%)	1.39 (0.39-4.91)	0.86	11 (8.3%)
CYP1A1m2	A/A	23 (65.7%)	-	-	20 (100.0%)	-	-	44 (65.6%)	-	-	80 (59.7%)
	A/G	9 (25.7%)	0.70 (0.36-1.34)	0.36	0 (0.0%)	0	0	19 (28.3%)	0.63 (0.27-1.49)	0.40	49 (36.5%)
	G/G	3 (8.6%)	1.45 (0.37-5.69)	0.85	0 (0.0%)	0	0	4 (6.1%)	2.08 (0.46-9.39)	0.59	5 (3.8%)
GSTM1	*positive*	12 (34.3%)	-	-	6 (30.0%)	-	-	14 (20.9%)	-	-	73 (54.9%)
	null	23 (65.7%)	2.29 (1.05-4.98)	0.05	14 (70.0%)	2.79 (1.10-7.70)	0.07	53 (79.1%)	4.53 (2.29-8.94)	<0.0001	61 (45.1%)
GSTT1	*positive*	22 (62.9%)	-	-	1 (5.0%)	-	-	10 (14.9%)	-	-	54 (40.3%)
	null	13 (37.1%)	0.39 (0.18-0.85)	0.02	19 (95.0%)	12.82 (1.66-98.66)	0.004	57 (85.1%)	3.84 (1.80-8.19)	0.0005	80 (59.7%)
TP53	*Arg Arg*	6 (17.14%)	0.15 (0.06-0.39)	<0.0001	7 (35.0%)	0.40 (0.15-1.06)	0.1	31 (46.6%)	0.64 (0.35-1.15)	0.17	77 (57.0%)
	Arg Pro	21 (60.0%)	3.65 (1.69-7.91)	0.001	9 (45.0%)	1.99 (0.77-5.19)	0.24	30 (44.7%)	1.98 (1.07-3.63)	0.04	39 (30.0%)
	Pro Pro	8 (22.86%)	1.91 (0.75-4.85)	0.26	4 (20.0%)	1.61 (0.48-5.36)	0.65	6 (8.7%)	0.63 (0.24-1.68)	0.48	18 (13.0%)

Data are reported as number of subjects with percent in parentheses or unless otherwise indicated. MNN = malignant neoplastic nodules; BNN = benign neoplastic nodules; NNN = non-neoplastic nodules; OD = odds ratio; 95%CI = confidence interval at 95%.

DISCUSSION

Although in this study the *CYP1A1 m1* and *CYP1A1m2* genotypes were not associated with an increased risk of thyroid diseases, the influence of the *CYP1A1* gene in the development of thyroid nodules cannot be excluded, since haplotypes involving many metabolic genes could contribute in a distinct manner to carcinogenesis.

A recent study (Siraj et al., 2008) demonstrated that the wild-type *CYP1A1m1* homozygous allele is more frequent either in thyroid nodules or in papillary carcinomas compared to the control group, indicating that cigarette smoke and other metabolites dependent on *CYP1A1* activation are not implicated in the risk of goiter development, or in the process of thyroid tumorigenesis. The cited authors also found no statistical significance for *CYP1A1m2* when comparing the control group with patients showing thyroid nodules.

Siraj et al. (2008) also demonstrated that the hormone levels could be affected by genetic variability, and that polymorphisms could influence estrogen production, which contribute to thyroid carcinogenesis, due to the strong association of the *CYP1A1m4* mutant homozygote (AA) with the risk of developing PTC (OR = 3.5; $P < 0.001$). There are some important studies that have evaluated thyroid nodules associated with *CYP1A1m1* and *CYP1A1m2* polymorphisms (Gaspar et al., 2004; Bufalo et al., 2006; Bozina et al., 2009).

CYP1A1 is the only one of many genes involved in xenobiotic metabolism that acts on different substrates, being tissue-specific. The relationship between genotype and phenotype in *CYP1A1m1* and *CYP1A1m2* is not yet fully understood. Our study also demonstrated that the *GSTM1* and *GSTT1* null genotypes were distinct between patients and controls, which is consistent with some studies (Morari et al., 2002; Hernández et al., 2003; Gaspar et al., 2004; Bufalo et al., 2006; Ho et al., 2006; Gonçalves et al., 2007; Lemos et al., 2008; Siraj et al., 2008) involving these genotypes and the risk of developin benign and malignant thyroid diseases.

The appearance of malignant lesions in the thyroid is related to the deletion of the GST *theta* and *mu* genes *GSTT1* and *GSTM1*, respectively. GST consists of a large multigenic group of detoxification enzymes.

GSTs catalyze the conjugation of toxic compounds with glutathione (GSH - γ-glutamyl cysteinyl glycine, which is a tripeptide), thereby blocking hazardous effects in the cell. These protein dimers bind electrophilic molecules and free radicals in order to reduce them to products that are nontoxic to the organism. According to previous studies (Morari et al., 2002; Gaspar et al., 2004; Ho et al., 2006) individuals with the null genotypes for *GSTM1* and *GSTT1* could have an increased susceptibility to thyroid cancer. In addition, it was suggested that the combination of *GSTM1*, *GSTT1* and *GSTP1* polymorphisms promoted a moderate increased risk of thyroid cancer, especially PTC (Siraj et al., 2008). On the other hand, another study demonstrated that *GSTM1* and *GSTT1* are weakly involved in the susceptibility to thyroid carcinogenesis (Chen et al., 2008).

The authors of some studies (Morari et al., 2002; Bufalo et al., 2006; Chen et al., 2008; Siraj et al., 2008) have suggested that genotypic analysis could indicate the metabolic pathways of specific carcinogens. However, discrepancies are found among the different studies involving such metabolic genes. Longitudinal studies that evaluate the impact of phase II enzymes associated with thyroid cancer are essential and constitute important tools to identify patients with a potential risk of future carcinomas.

The human *TP53* tumor suppressor gene plays a central role in many cellular processes

via DNA repair and apoptosis (GBC). The allele of *TP53Arg/Pro* polymorphism reduces the ability of *p53*, and this type of mutation has been reported in more than 50% of cancer patients.

Therefore, the *TP53Arg/Pro* polymorphism involved in multiple steps of the metabolism of carcinogens may account for genetic differences in MNN susceptibility. In this study, a relationship between the presence of *Arg/Pro* genotype and increased risk of MNN, involved multiple steps of carcinogenesis, rather than a pathway associated with a particular carcinogen-metabolizing enzyme (GBC).

In summary, women with *GSTM1* and *GSTT1* null genotypes and *Arg/Pro* genotype were found to be at high risk of developing benign and malignant thyroid nodules in Goiânia, respectively. Future research efforts will allow a better understanding of the pathogenesis of thyroid nodules. Interindividual variation in the susceptibility to thyroid diseases could indicate new perspectives into early diagnosis, prognosis and even treatments, indicating which patients with thyroid nodules will benefit according to specific polymorphic profiles.

Genotype analysis provides information concerned with the metabolic pathways related to specific carcinogens. However, discrepancies can be found among different studies involving metabolic genes and *TP53* polymorphism. Longitudinal studies that evaluated the impact of the genetic polymorphisms associated with thyroid cancer are essential for screening patients with a potential risk to future carcinomas.

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