Evidence that polymorphisms in detoxification genes modulate the susceptibility for sporadic medullary thyroid carcinoma

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Abstract

Aim: Polymorphic low-penetration genes have been consistently associated with the susceptibility to a series of human tumors, including differentiated thyroid cancer.

Methods: To determine their role in medullary thyroid cancer (MTC), we used TaqMan SNP method to genotype 47 sporadic MTC (s-MTC) and a control group of 578 healthy individuals for CYP1A2*F, CYP1A1m1, GSTP1, NAT2 and 72TP53. A logistic regression analysis showed that NAT2/C/C (OR = 3.87; 95% CI = 2.11–7.10; P = 2.2 × 10⁻³) and TP53/C/C genotypes (OR = 3.87; 95% CI = 1.78–6.10; P = 2.8 × 10⁻⁴) inheritance increased the risk of s-MTC. A stepwise regression analysis indicated that TP53/C/C genotype contributes with 8.07% of the s-MTC risk.

Results: We were unable to identify any relationship between NAT2 and TP53 polymorphisms suggesting they are independent factors of risk to s-MTC. In addition, there was no association between the investigated genes and clinical or pathological features of aggressiveness of the tumors or the outcome of MTC patients.

Conclusion: In conclusion, we demonstrated that detoxification genes and apoptotic and cell cycle control genes are involved in the susceptibility of s-MTC and may modulate the susceptibility to the disease.

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Introduction

The prevalence of medullary thyroid carcinoma (MTC) among thyroid cancers varies from 3% to no more than 8% in different series of patients, but MTC are responsible for 13.4% of the deaths caused by thyroid tumors (¹–²). The identification of germline RET mutations differentiates the sporadic from the hereditary or familial form of the disease, a form that accounts for only 20–25% of the cases, occurring in a ratio of one case for every 30 000 individuals (¹–²).

Currently available genetic tests for identification of RET mutations offer a prospective successful therapy, even before C-cell hyperplasia evolves to medullary thyroid carcinoma, and disease prognostic accuracy. In addition, the genetic screening of patients with apparent sporadic MTC (s-MTC) allows the identification of a relevant percentage of hidden familial MTC, preclinical diagnosis and prompt treatment of unsuspected affected family members. In effect, somatic RET mutations have been documented in 40–50% of MTC cases (³). Modern guidelines determine the timing and extent of surgery according to aggressiveness and age of onset of inherited MTC, which differ depending on the specific genetic mutation. However, even in patients with the same RET mutation, there is considerable phenotypic variability (⁴–⁶). Some studies have suggested that polymorphisms in the RET gene, perhaps combined with other low-penetration genes, could explain this variability. Recently, the CDKN1B V109G polymorphism was associated with a more favorable s-MTC progression than the wild-type allele, suggesting an additional role for low-penetration genes in the prognosis of MTC patients (⁷).

In fact, polymorphic low-penetration genes have been consistently associated with the phenotype of a series of human tumors, including differentiated thyroid cancer (⁸–¹¹). A growing number of genes encoding enzymes involved in the biotransformation of toxicants has been identified and cloned, leading to increased knowledge of allelic variants in genes and genetic defects that may result in a differential susceptibility towards poisonous environmental elements. Moreover, low-penetrating polymorphisms in metabolism genes tend to be much more common in the population than allelic variants of high-penetrating cancer genes. For that reason, these genes are of considerable importance from a public health point of view.

Most carcinogens we are exposed to during our lifetime are metabolized by the cytochrome P450 family...
of enzymes, including CYP1A2*F, CYP1A1m1 and others. The products of these enzymes are further yielded to the action of enzymes such as GSTP1 and arylamine N-acetyltransferase 2 (NAT2), which can promote detoxification and excretion of these compounds in bile or urine. If this detoxification does not happen or is incomplete, these toxicants may cause genetic damage by different processes of DNA interaction, triggering a carcinogenesis process. However, a series of genes involved in cell cycle control and apoptosis, such as TP53, can repair the damaged cell or lead it to death, preventing uncontrolled cell growth and a consequent cancer. Polymorphisms in these genes can lead to an aberrant protein activity, affecting cell function.

Different inherited genetic profiles can provide specific protection or determine a risk for the development of a particular cancer type. Along with environmental factors, which probably serve as triggers of diseases, this inherited profile of detoxifying and repairing systems may modulate the specific phenotype of each patient (12). In addition, polymorphisms of relevant xenobiotic metabolizing enzymes may be used as toxicological susceptibility markers, as our group and others have demonstrated in differentiated thyroid carcinomas and many other human tumors (12).

This study aimed to investigate the role and outcome of germline inheritance of polymorphisms in some of the most important genes related to differentiated thyroid carcinomas susceptibility such as CYP1A2*F, CYP1A1m1, GSTP1 codon 105, NAT2 C282T and TP53 codon 72 genes in patients with s-MTC.

**Materials and methods**

**Patients**

This study was approved by the Ethics and Research Committees of both Federal University of São Paulo (UNIFESP) and University of Campinas (UNICAMP) and was in agreement with the 1975 Declaration of Helsinki revised in 1983. A signed letter of informed consent was obtained from each individual included in this analysis.

We studied a total of 625 individuals, including 47 patients with s-MTC, and 578 individuals without MTC selected from an iodine-sufficient area. All patients were sequenced for the complete RET gene and familial MTC was excluded. None of the s-MTC patients had any other type of malignant tumor. Individuals with a history of past thyroid disease, radiation exposure, specific environmental risk or occupational exposure risks and antecedents of malignancy were excluded. Data regarding: lifetime occupational history; dietary habits; alcohol, coffee and drug consumption; medical history with an emphasis on previous and/or current thyroid diseases; use of exogenous hormones and concomitant medications; reproductive history and family history of cancer and other anamnestic data were obtained using a structured questionnaire. Owing to the limited reliable data obtained as to the duration in years of smoking, at what age smoking started, quantity smoked and number of years since smoking stopped, patients and controls were grouped into the categories never-smoked and ever-smoked. This last group comprised individuals who consumed at least 20 packages, 20 cigarettes-per-pack during 1 year for the prior 5 years. All data, including nodule size, tumor histological features and laboratory examinations, were confirmed in patient records.

**RET gene sequencing**

Genomic DNA was isolated from peripheral blood leucocytes by standard phenol techniques. PCR products of exons 8, 10, 11, 13, 14, 15 and 16 of the RET gene were purified using the Concert Rapid PCR Purification System (Invitrogen) and submitted to direct sequencing using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) as described previously (4). Each sample was sequenced at least twice and in both directions.

**Polymorphisms genotyping**

Five polymorphisms were genotyped: CYP1A2*F (rs762551), CYP1A1m1 (rs 4646903), NAT2 C282T (rs1041983), GSTP1 codon 105 (rs 1695) and TP53 codon 72 (rs 1042522) using TaqMan method (Applied Biosystems). Fluorescence signals were detected by the 7500 system sequence detection software (Applied Biosystems). The sequence-specific primers were: CYP1A2*F (C_8881221_40), NAT2 C282T (C_8684085_20), GSTP1 codon 105 (C_3237198_20) and TP53 codon 72 (C_2403545_10). The gene CYP1A1m1 primer was designed according to the following information: Forward 5′-GCACCTGTAC- CATTTCGTTACT-3′; Reverse 5′-GCTGAGTGCGGA- GAATCGT-3′. The corresponding sequences of fluorescence signals were VIC – CACCTCCTGGGCTCA and FAM – ACCTCCCGGGCTCA.

TaqMan PCR and genotyping analyses were performed on Applied Biosystems 9600 Emulation System (Applied Biosystems). The reaction mixtures were amplified in 2 μl of genomic DNA (10 ng/ml), 2.5 μl of 2× TaqMan Universal Master Mix, 0.25 ml of 40× primer/probe mix and 0.25 μl of ddH2O in a volume of 5 μl. PCR cycling conditions were as follows: one cycle at 60 °C for 1 min as initial step; one cycle at 95 °C for 20 min; 40 cycles at 92 °C for 3 min and at 60 °C for 30 s; and one cycle at 60 °C for 1 min as the annealing step. The results were analyzed on Applied Biosystems 9600 Emulation System using the allelic discrimination assay program.
Statistical analysis

We used t-test and Fisher’s exact test to evaluate differences between the groups concerning age, gender and smoking, using R environment (13). Hardy–Weinberg equilibrium (HWE) was analyzed from genotypes by ARLEQUIN Software (14). These analyses were performed by SAS Statistical Software (Statistical Analysis System, version 9.1.3, 2002–2003). Logistic and stepwise regressions were used to analyze association between polymorphisms and MTC by R Statistical Software (13). Post-hoc statistical power of the sample was evaluated using GPower Software (15), with a statistical significant level of 0.05.

Results

As shown in Table 1, patients and controls were similar regarding age ($P=0.8576$), gender ($P=0.7869$) and smoking habits ($P=0.3546$). Our sample presented 97.05% of statistical power to detect genetic association between polymorphisms and MTC by logistic regression analysis.

Among the polymorphisms studied, GSTP1 was not in HWE. This fact may be due to the relatively small size of the groups studied and the high genetic heterogeneity of the Brazilian population, composed of relatively recent groups of immigrants from Europe, Africa, Asia, all mixed to the indigenous populations (16). Therefore, although GSTP1 was associated with the risk of s-MTC development, as shown in Table 2, this polymorphism was not included in further statistical analysis.

Table 3 depicts the results of the logistic regression analysis that demonstrates an association between s-MTC and NAT2/C genotypes ($P=2.2 \times 10^{-5}$; OR $=3.87$; 95% CI = 2.11–7.10); NAT2/C/T ($P=3 \times 10^{-7}$; OR $=0.15$; 95% CI = 0.06–0.36); TP53/C/C ($P=2.8 \times 10^{-4}$; OR $=3.87$; 95% CI = 1.78–6.10) and TP53/G/G genotypes ($P=2.8 \times 10^{-4}$; OR $=0.28$; 95% CI = 0.13–0.62). A stepwise regression analysis demonstrated that TP53/C/C genotype contributes with 8.07% of the s-MTC phenotype, whereas 1.53% is due to the age of tumor onset as represented in Fig. 1.

We were unable to establish any relationship between the profile of the studied genes and patients’ clinical characteristics, lifetime occupational history; dietary habits; alcohol, coffee and drug consumption or outcome. A sample size calculation indicated that a much larger cohort of patients would be needed to reach the statistical power to detect main effects or interactions or the precision to quantify them.

Discussion

Germline RET mutations confer a high risk of developing MTC, but they do not account for all cases; neither do they explain the variability in presentation and outcome in clinical patients. A sizeable fraction of the remaining heritability that still needs to be identified is thought to be due to cumulative effects of susceptibility alleles associated with low-to moderate-penetrance genes, in accordance with a model of polygenic inheritance. A series of detoxifying and repairing genes that may contribute to this model have been identified in differentiated thyroid cancer and other tumors (8–11). In fact, differences in the cellular mechanisms of activation and detoxification of carcinogenic chemicals may confer different degrees of susceptibility to cancer to each individual, accounting for the observed phenotypic variations (9–11).

This study demonstrates, for the first time to our knowledge, an association between NAT2 and TP53 detoxifying genes and s-MTC phenotype. In fact, the

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inheritance of a C homozygous allele for NAT2 C282T gene increases the risk for the development of s-MTC by more than three times. Epidemiological studies have shown that a significant part of all cancers are related to environmental factors, considering tobacco smoke and diet as the main attributable exposures in a long and increasing list of carcinogenic elements (17). NAT gene acts as an inactivating enzyme, catalyzing the conjugation of carcinogenic substances such as ionising radiation and tobacco (18). The enzyme is responsible for the N-acetylation of arylamine and hydrazine xenobiotics, and has been implicated in the susceptibility to various cancers including differentiated thyroid carcinomas (11).

Furthermore, the presence of C homozygous allele for TP53 gene also increases by more than three times the susceptibility to s-MTC. Cell cycle and apoptosis regulators directly involved in the initiation of malignant cell proliferation have long been preferred targets as cancer risk markers (19). The Pro allele was also associated with an increased risk of differentiated thyroid cancer in previous reports (10).

Although the genetic risks of these polymorphisms are relatively modest, their high frequency in the population suggests that they may have a considerable impact on the incidence of s-MTC. In fact, polymorphisms of genes that codify enzymes involved in the detoxification of toxicants may contribute to the variable susceptibility to a series of carcinogenic compounds. The identification of a profile of susceptibility to s-MTC might allow the recognition of a group of individuals at risk, hence deserving a more careful observation.

A sizeable fraction of the remaining heritability that still needs to be identified is thought to be due to cumulative effects of susceptibility alleles associated with low- to moderate-penetrance genes, in accordance with a polygenic model of inheritance. A series of detoxifying and repairing genes that may contribute to this model have been identified in differentiated thyroid cancer and other tumors (8–11).

Unfortunately, pathways of carcinogen metabolism are complex and mediated by the activity of multiple genes. In addition, these polymorphisms may have additive or opposite effects; likewise, different toxicants may produce composite and complex effects. Therefore, definite conclusions depend on studies with larger sample sizes that determine the risk estimates associated with other variants, gene–gene and gene–environment interactions. Biological microchips designed to identify polymorphisms in a large series of xenobiotic-metabolizing, apoptotic and cell cycle control genes may allow the investigation of multiple contributing factors to the susceptibility to s-MTC and help understand their relationship (20).

### Table 3

Logistic regression analysis of the association among different genotypes and the OR for a sporadic medullary thyroid carcinoma development risk.

<table>
<thead>
<tr>
<th>SNP/genotype</th>
<th>β</th>
<th>P value</th>
<th>OR Low</th>
<th>OR High</th>
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<tr>
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<td></td>
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<td>0.63</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>0.4042537</td>
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<td></td>
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</tr>
<tr>
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**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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**References**


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