NATÁSSIA ELENA BUFALO

ESTUDO DA RELAÇÃO ENTRE O PERFIL GENÉTICO DE DIFERENTES SISTEMAS DE DEFESA CONTRA XENOBIÓTICOS NAS DOENÇAS NEOPLÁSICA E AUTO-IMUNE DA TIRÓIDE

CAMPINAS

2007

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Dissertação de Mestrado apresentada à Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para obtenção do título de Mestre em Clínica Médica, área de concentração em Ciências Básicas.

ORIENTADORA: PROFª. DRª.LAURA STERIAN WARD

CAMPINAS

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FICHA CATALOGRÁFICA ELABORADA PELA
BIBLIOTECA DA FACULDADE DE CIÊNCIAS MÉDICAS DA UNICAMP
Bibliotecário: Sandra Lúcia Pereira – CRB-8ª / 6044

B863e

Bufalo, Natássia Elena
Estudo da relação entre o perfil genético de diferentes sistemas de defesa contra xenobióticos nas doenças neoplásica e auto-imune da tireóide. / Natássia Elena Bufalo. Campinas, SP : [s.n.], 2007.

Orientador : Laura Sterian Ward
Dissertação (Mestrado) Universidade Estadual de Campinas. Faculdade de Ciências Médicas.

1. Tireóide-câncer. 2. Gene P53. 3. Citocromo. 4. Glutationa transferase. I. Ward, Laura Sterian. II. Universidade Estadual de Campinas. Faculdade de Ciências Médicas. III. Título.

Título em ingles: Relationship study of the genetic profile of different xenobiotic systems in thyroid autoimmune and neoplastic diseases

Keywords:

- Thyroid-cancer
- Gene P53
- Cytochrome
- Glutathione transferase

Área de concentração : Clinica médica
Titulação: Mestrado em Ciências Básicas

Banca examinadora: Profª.Drª. Laura Sterian Ward
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Data da defesa: 30 - 08 - 2007

Banca Examinadora da Dissertação de Mestrado

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Data: 30/08/2007

DEDICATÓRIA

Aos meus queridos pais Walter e Elizabete, à minha irmã Sílvia e ao meu namorado Eduardo. Obrigada por todo o apoio.

AGRADECIMENTOS

À Deus, por ter me dado evidências de que é este o caminho profissional que eu devo seguir.

Aos meus pais, Walter e Elizabete, que acreditaram nos meus sonhos e na minha capacidade, contribuindo para que meus estudos pudessem ser realizados.

A minha irmã Silvia, que sempre me apoiou como pode.

A minha grande mestra, Dra. Laura, que confiou em mim dando oportunidades únicas na minha vida.

À Dra. Lígia pelas amostras de sangue periférico e a parte clínica dos pacientes com nódulos.

Ao Dr. Osmar Monte, Dr. Romaldini e Dr. Adriano pela ajuda com os pacientes portadores de Graves, tantos pelas amostras de sangue periférico quanto pela clínica.

Ao meu grande amigo Romildo, o qual me ensinou a ver a Biologia com outros olhos, dando a minha primeira oportunidade de estágio no Instituto Biológico de Campinas.

Ao meu namorado Eduardo, pela paciência e compreensão por esses dois anos de namoro (quase o mestrado inteiro). Obrigada pela ajuda em todos os momentos difíceis e felizes das nossas vidas. Muito obrigada por tudo, meu amor.

As minhas amigas e companheiras de bancada, Elaine, Janaína, Kika e Fabiana, que me acompanharam nas dificuldades da realização deste projeto e também pelas risadas que demos no laboratório.

Aos amigos de laboratório Renato, Hélio, Tiago, Aline, Carol, Juliana, Mariana, Maria, Joseane, por todos os momentos de descontração dentro do lab.

Às vizinhas de laboratório, Helen e Vivi. Por me emprestarem centrífugas, tubinhos de sangue, pelas fotos dos géis.

À Dra. Flávia e Dr. Roberto, muito obrigada pela paciência por me explicar toda a parte clínica deste trabalho.

Às minhas amigas de faculdade (impossível listar todas) por me ajudarem em todos os momentos, felizes ou tristes, pelos trabalhos, pelas baladas, pelas caronas, enfim, por tudo.

À todos vocês, muito obrigada.

"Diz-se que, mesmo antes de um rio cair no oceano ele treme de medo. Olha para trás, para toda a jornada, os cumes, as montanhas, o longo caminho sinuoso através das florestas, através dos povoados, e vê à sua frente um oceano tão vasto que entrar nele nada mais é do que desaparecer para sempre. Mas não há outra maneira. O rio não pode voltar. Ninguém pode voltar. Voltar é impossível na existência. Você pode apenas ir em frente. O rio precisa se arriscar e entrar no oceano. E somente quando ele entra no oceano é que o medo desaparece. Porque, apenas então, o rio saberá que não se trata de desaparecer no oceano. Mas tornar-se oceano. Por um lado é desaparecimento e por outro, renascimento."

(Autor desconhecido)

"A mente que se abre a uma nova idéia jamais voltará ao seu tamanho original".

Albert Einstein

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AcTg	Anticorpo tireoglobulina
AcTPO	Anticorpo tireoperoxidase
AMPc	Sistema adenil-ciclase
Arg	Aminoácido Arginina
ATP	Adenosina trifosfato
CDT	Carcinoma diferenciado da tiróide
CI	Índice de confiança
CYP	Citocromo P450
CYP1A1	Citocromo P4501A1
DAI	Doenças auto-imunes
DAT	Drogas antitiroidianas
DG	Doença de Graves
DNA	Ácido desoxirribonucléico
F	Teste exato de Fisher
G0	Fase G0 do ciclo celular
G1	Fase G1 do ciclo celular
GST	Sistema Glutationa S-transferase
GSTM1	Glutationa S-transferase Mu 1
GSTP1	Glutationa S-transferase Pi 1
GSTT1	Glutationa S-transferase Teta 1
GEMOCA	Laboratório de genética molecular do câncer
HT	Hormônios tiroidianos
IBGE	Instituto Brasileiro de Geografia e Estatística
Ile	Aminoácido Isoleucina

Ladder	Marcador de peso molecular
$MgCl_2$	Cloreto de magnésio
NATs	N-acetiltransferases
OG	Oftalmopatia de Graves
OMS	Organização Mundial da Saúde
OR	Teste de Odds Ratio
tp53	Proteína do gene *TP53*
HAPs	Hidrocarbonetos aromáticos policíclicos
PCR	Reação em cadeia da polimerase
Pro	Aminoácido Prolina
RFLP	Restrição do polimorfismo pelo tamanho do fragmento
RNA	Ácido ribonucléico
SUS	Sistema Único de Saúde
T3	Triiodotironina
T4	Tetraiodotironina
T4L	Tiroxina livre
Tg	Tireoglobulina
TgAb	Anticorpo antitireoglobulina
TH	Tiroidite de Hashimoto
TNM	Tumor, nódulo e metástase
TP53	Gene *TP53*
TPOAb	Anticorpo antiperoxidase
TSH	Hormônio estimulante da tiróide
TSNAs	Nitrosaminas específicas do tabaco
Val	Aminoácido Valina
X^2	Teste de qui-quadrado

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RESUMO

Tanto a doença de Graves como o câncer da tiróide são doenças de etiologia multifatorial e envolvem uma interação entre meio ambiente e fatores genéticos de predisposição. O hábito de fumar é um fator de risco reconhecido para o desenvolvimento da doença de Graves, particularmente para a oftalmopatia de Graves. Ao contrário, estudos epidemiológicos têm freqüentemente demonstrado a redução no risco ao carcinoma diferenciado da tiróide entre tabagistas. A herança de polimorfismos de genes relacionados com a metabolização e com a detoxificação de xenobióticos, assim como a herança de genes relacionados com a vida e a morte celular, desempenham um importante papel na suscetibilidade a doenças. Os objetivos foram determinar a influência dos polimorfismos dos genes *CYP1A1, GSTM1, GSTT1, GSTP1* e *72TP53* no risco para a doença de Graves e o papel do gene *CYP1A1* na tumorigênese tiroidiana. Para avaliar o papel destes genes na doença de Graves foi estudado um total de 400 pacientes com doença de Graves, comparados com 574 indivíduos-controle. Para analisar o papel destes genes no câncer da tiróide foi estudado 248 pacientes com nódulos tiroidianos, comparados com 277 indivíduos-controle, todos pareados para sexo, idade e etnia. As análises genotípicas foram feitas em DNA extraído de sangue periférico, através de amplificação por PCR, seguido de restrição enzimática para os genes *CYP1A1, GSTP1* e *72TP53* e PCR-duplex para os genes *GSTM1* e *GSTT1*. Não se encontrou relação entre os genótipos de *GSTM1* e *GSTT1* e a suscetibilidade à doença de Graves. Contudo, as variantes de *GSTP1* ($p<0.0001$), *CYP1A1 m1* ($p<0.0033$) e *Pro/ProTP53* ($p<0.0035$) foram mais freqüentes em pacientes com doença de Graves do que nos controles. A análise de regressão logística multivariada corrigida para sexo, idade e etnia indicou que o hábito de fumar e a herança das variantes dos genes *GSTP1, CYP1A1* e *Pro/ProTP53* são importantes fatores de risco para a doença de Graves. Em relação aos nódulos tiroidianos, o genótipo selvagem do gene *CYP1A1* foi mais freqüente entre pacientes com carcinoma papilífero (74.26%) do que na população-controle (62.45%) ($p= 0.0147$), diminuindo o risco para o desenvolvimento deste câncer (OR=0.564; 95% IC= 0.357 - 0.894). A análise de regressão logística multivariada corrigida para sexo, idade e etnia mostrou uma correlação inversa entre o hábito de fumar e a herança do gene *CYP1A1* e a suscetibilidade ao carcinoma papilífero. Conclui-se que os polimorfismos de *GSTP1, CYP1A1* e *TP53* podem estar associados à suscetibilidade relacionada com o tabagismo para a doença de Graves e que o genótipo de *CYP1A1* pode estar associado à redução no risco para o carcinoma papilífero entre fumantes.

ABSTRACT

Graves´s disease and differentiated thyroid cancer are multifactorial diseases with environmental and genetic interactions. Cigarette smoking is a well-recognized risk factor for Graves' disease and, particularly, for Graves' ophthalmopathy. Conversely, epidemiologic studies have consistently reported a reduced risk of differentiated thyroid cancer in tobacco consumers. Inheritance of germline polymorphisms genes related with metabolizing and detoxification of xenobioticos, besides genes involved in major DNA repair/apoptosis pathways, might have an important role in the susceptibility to these diseases. To assess the influence of the *GST, CYP* and *TP53* gene polymorphisms in the risk of Graves' disease and the *CYP1A1* role in thyroid tumorigenesis, we used a PCR strategy to genotype for *GSTT1, GSTM1, GSTP1, CYP1A1* and codon 72 of *TP53* a group of 400 Graves' disease patients compared to 574 control individuals with similar environmental exposure features and 248 patients with thyroid nodules and 277 controls with similar ethnic backgrounds. DNA was extracted from a blood sample and submitted to PCR-RFLP for *CYP1A1, GSTP1* and *72TP53* genes and PCR-duplex *GSTM1* and *GSTT1* genes assays. *GSTM1* and *GSTT1* genotypes were equally distributed in Graves´ disease and controls. However, *GSTP1* variants ($p<0.0001$), *CYP1A1* variants ($p<0.0033$), and *Pro/ProTP53* ($p<0.0035$) appeared more frequently in Graves' disease than in controls. A multivariate analysis indicated that cigarette smoking and the inheritance of GSTP1 variants, *CYP1A1* variants and *Pro/ProTP53* were important risk factors of risk for Graves' disease. Among the thyroid nodules, the wild-type *CYP1A1 m1* genotype was more frequent in papillary carcinomas patients (74.26%) than in the control population (62.45%) ($p= 0.0147$) indicating that the variants of these genes reduce the risk for this cancer (OR=0.564; 95% IC= 0.357 to 0.894). Multiple logistic regression analysis showed an inverse correlation between cigarette smoking and *CYP1A1* germline inheritance and the susceptibility to papillary carcinomas. We concluded that *GSTP1, CYP1A1* and *TP53* germline polymorphisms may be associated with smoking-related Graves' disease susceptibility and *CYP1A1* genotype might be associated to the reported reduced risk to papillary carcinomas among smokers.

1- INTRODUÇÃO

1.1- Doenças Tiroidianas

As doenças da tiróide manifestam-se através da disfunção hormonal, seja por excesso ou por deficiência de produção hormonal, ou por alterações anatômicas decorrentes do crescimento difuso ou nodular da glândula (Arber et al, 1995).

Os tumores da tiróide são classificados, de acordo com os critérios da Organização Mundial da Saúde (OMS), em benignos e malignos (Beveridge e Sobin, 1974; Sobin et al, 1997) (Quadro 1).

Quadro 1- Classificação simplificada dos tumores da tiróide.

Benignos	**Malignos**
1. Bócio endêmico 2. Bócio esporádico 3. Adenoma folicular 4. Outros	1. Carcinoma folicular 2. Carcinoma papilífero 3. Carcinoma medular 4. Carcinoma anaplásico 5. Outros

Os tumores diferenciados, que são a maioria absoluta (mais de 90%), são derivados das células foliculares e são subdivididos em dois grupos, os papilíferos e os foliculares, sendo também chamados de carcinomas diferenciados da tiróide (CDT). Os indiferenciados ou anaplásicos constituem apenas 5%-10% dos carcinomas tiroidianos. As células parafoliculares, produtoras de calcitonina, são responsáveis por cerca de 5% dos carcinomas tiroidianos, dando origem aos carcinomas medulares (Casella e Fusco, 2004).

As doenças auto-imunes da tiróide são representadas por um amplo espectro de manifestações clínico-laboratoriais em que se destacam dois extremos. De um lado, a produção excessiva de anticorpos estimuladores da glândula tiróide leva ao desenvolvimento da doença de Basedow-Graves, também denominada doença de Graves (DG). De outro lado, a produção de anticorpos destruidores da glândula tiróide leva à sua destruição progressiva, com características de infiltração linfocitária, conhecida como tiroidite crônica linfocitária ou doença de Hashimoto ou tiroidite de Hashimoto (TH).

O quadro 2 resume as principais doenças auto-imunes da tiróide e os respectivos anticorpos.

Quadro 2- Principais doenças auto-imunes da tiróide e seus anticorpos.

Doenças auto-imunes da tiróide	
Doenças	Autoanticorpos
Graves	Anti-TSHR (TRAB, TSAb)
Hashimoto	Anti-TPO, Anti-Tg
Tiroidite Sub-Aguda	Anti-TPO, Anti-Tg
Tiroidite Pós-Parto	Anti-TPO, Anti-Tg
Tiroidite Atrófica	Nenhum Anticorpo

O sexo feminino apresenta sempre maior prevalência de todas as doenças tiroidianas (Buescu e Grego Filho, 2001).

O grau de suficiência de iodo de uma região também é um fator que influi na prevalência de todas as doenças da tiróide. Assim, o bócio endêmico ocorre em regiões onde há carência de iodo, enquanto que as doenças auto-imunes têm sido relacionadas com um excesso de oferta de iodo na alimentação (Ward et al, 2007).

1.2- Câncer de Tiróide

O CDT representa cerca de 1% de todos os cânceres que acometem o ser humano (Ward, 2000). É o mais comum entre os cânceres de glândulas endócrinas, representando cerca de 90% destes e respondendo por 63% dos óbitos por neoplasias endócrinas (Ward, 2000).

A incidência do CDT vem aumentando no mundo todo (Steliarova-Foucher et al, 2006; Haselkorn et al, 2000; Liu et al, 2001; Burgess 2002; Coeli et al, 2005; Reynolds at al, 2005). Dados brasileiros também mostram elevada incidência em diversos estados do país (Coeli et al, 2005). Sem dúvida, parte deste aumento

deve estar relacionada com o melhor acesso ao Sistema Único de Saúde (SUS) e a melhores meios de diagnóstico, amplamente utilizados, como a ultrassonografia cervical. No entanto, a grande variação na prevalência do CDT em estados de nível socioeconômico, cultural e qualidade de serviços de atendimento em saúde similares, como mostram as figuras 1 e 2 abaixo, indica que outros fatores também devem contribuir para tal divergência na incidência (Coeli et al, 2005).

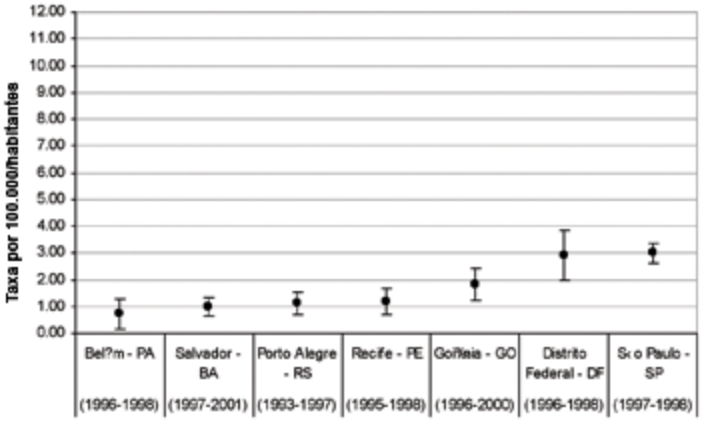

Figura 1. Taxa de incidência de câncer da glândula tireóide (IC 95%), ajustada por idade, segundo município de residência – Homens.

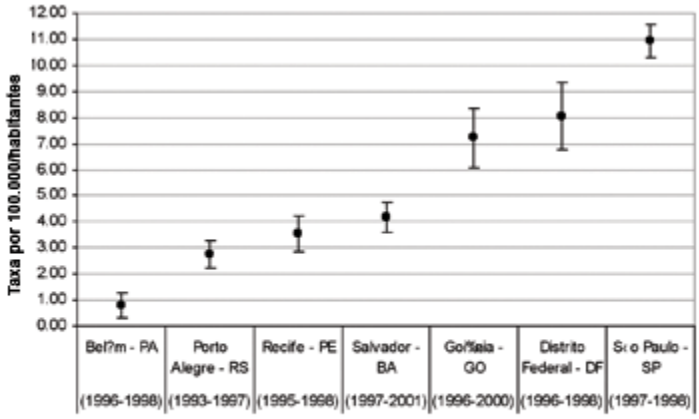

Figura 2. Taxa de incidência de câncer da glândula tireóide (IC 95%), ajustada por idade, segundo município de residência – Mulheres.

Dentre os fatores de risco para o CDT temos: radiação ionizante, predisposição familiar, ingestão de iodo, fatores hormonais e reprodutivos, fatores étnicos e geográficos, dieta e drogas (Ward et al, 2007).

Alguns dados sugerem que a presença de autoanticorpos seria um fator de risco para o CDT (Stocker et al, 2002; Zardo et al, 1999), porém não há evidências de associação entre anticorpos destruidores ou estimuladores de tiróide e o câncer. Ao contrário, mostramos que os autoanticorpos como AcTg e AcTPO podem proteger os pacientes com CDT, proporcionando boa evolução clínica (Souza et al, 2003).

O laboratório de genética molecular do câncer (GEMOCA) da FCM-UNICAMP tem demonstrado que o perfil genético para uma série de enzimas de detoxificação pode ser utilizado para delinear um perfil genético herdado de predisposição ao CDT. Morari et al (2002) demonstraram que a herança dos genótipos nulos combinados de *GSTM1* e *GSTT1* aumentam o risco para o desenvolvimento de câncer de tiróide em mais de duas vezes. Granja et al (2004, 2005) demonstraram que indivíduos que herdam as variantes dos genes *GSTP1* e *TP53* códon 72 possuem um risco aumentado para o desenvolvimento de câncer de tiróide em mais de sete vezes mas não encontrou relação entre o gene *GSTO1* e câncer de tiróide (Morari et al, 2002; Granja et al, 2004; Granja et al, 2004; Granja et al, 2005).

1.3- A Doença de Graves

A DG é o processo auto-imune responsável pela maioria dos casos de hipertireoidismo. É uma doença que afeta vários sistemas, caracterizada pelo aumento homogêneo da glândula tiróide, isto é, pela presença de bócio, hipertireoidismo, oftalmopatia infiltrativa, dermopatia (mixedema pré-tibial) e artropatia, podendo ou não faltar um ou mais destes elementos (Reiwen et al, 1993).

Nos EUA, acomete 0,5% das mulheres e 0,2% dos homens, predominando no sexo feminino (Mulheres: Homens = 3-10:1) e aparece preferencialmente entre a 3ª e a 5ª décadas de vida (Maciel, 1997). Apresenta uma incidência anual de 0,5 por 1.000 indivíduos/ano (Jacobson et al, 1997).

Do ponto de vista imunológico, verifica-se que os linfócitos B e T são sensibilizados por pelo menos quatro autoantígenos: o receptor do TSH (Kendler e Davies, 1993), a tireoglobulina, a peroxidase tiroidiana e o co-transportador de sódio/iodo (Weetman, 1991; Dai et al, 1996). O receptor do TSH parece ser o autoantígeno mais importante, enquanto os outros seriam envolvidos secundariamente na DG (Davies, 1996). Os anticorpos estimuladores do receptor do TSH exercem uma função similar à do TSH, ativando o sinal de transdução dos sistemas adenil-ciclase (AMPc) e a proteína kinase C, o que resulta no estímulo à produção e à liberação de hormônios tiroidianos, causando conseqüente estimulação da captação, organificação do iodo, síntese protéica e crescimento da célula folicular (Uyttersprot et al, 1997).

A figura 3 resume as principais etapas do processo fisiopatológico envolvidos no desenvolvimento da DG.

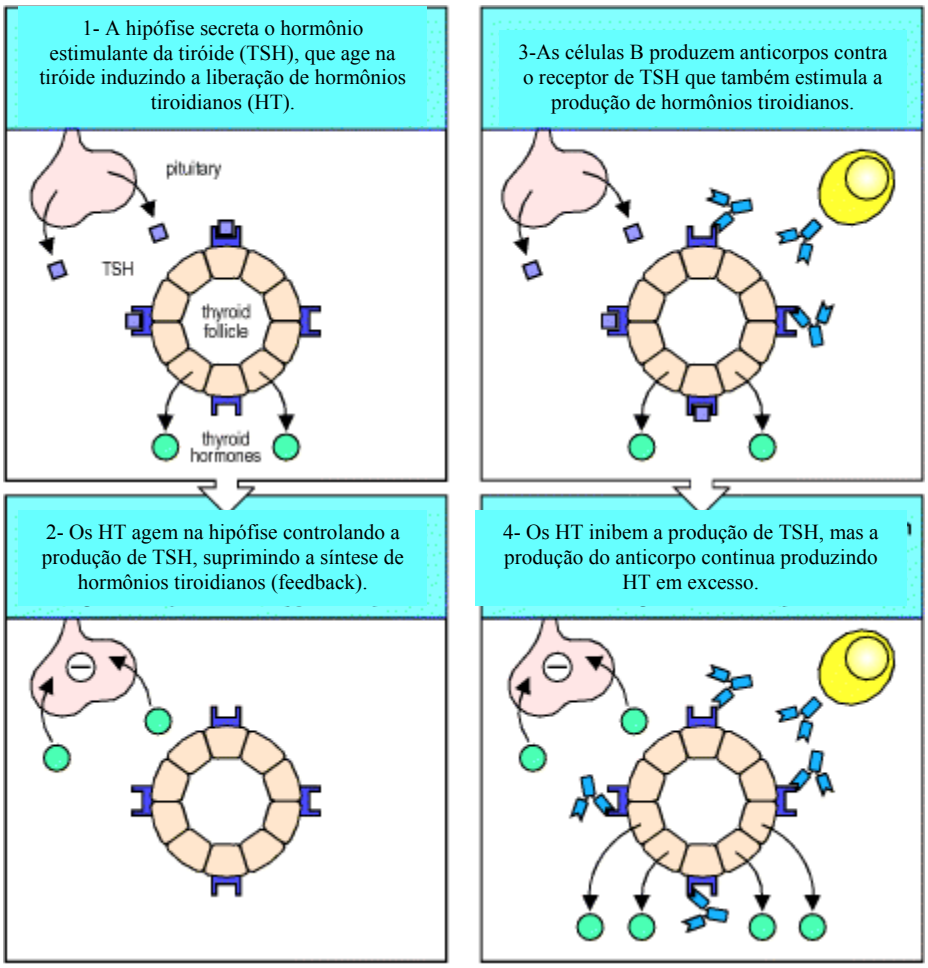

Figura 3- A regulação do feedback na produção de hormônios tiroidianos é interrompido na DG. A DG é causada por autoanticorpos específicos para o receptor do TSH. Normalmente, os hormônios tiroidianos são produzidos em resposta ao TSH hipofisário. Na DG, os autoanticorpos são agonistas para o receptor de TSH e, portanto, estimulam a produção de T3 e T4. Os hormônios tiroidianos inibem a produção normal de TSH, mas não afetam a de autoanticorpos; sua produção excessiva é a causa do hipertireoidismo (Janeway et al, 2001).

A causa da DG, ou seja, o evento inicial que deflagra a resposta auto-imune, bem como os fatores genéticos necessários para que ela ocorra ainda são desconhecidos (Carneiro et al, 2003). Como provavelmente em todas as desordens auto-imunes, a DG envolve uma complexa interação genética entre elementos ambientais exógenos e endógenos que, juntos, podem provocar uma desordem na auto-imunidade tiroidiana (Weetman, 2003; Prummel et al, 2004). Há fortes evidências da suscetibilidade genética nas doenças auto-imunes da tiróide, com risco calculado em 79 % para a DG (Brix et al, 2001).

Contudo, apenas alguns genes, listados no quadro 3, foram identificados como diretamente relacionados à DG, e raramente com um risco maior do que três no aumento da suscetibilidade à doença (Weetman, 2003; Prummel et al, 2004; Brix et al, 2001; Tait e Cough, 2003; Tomer e Davies, 2003).

Quadro 3- Genes relacionados a DG:

Gene	Localização	Desordens da Tiróide
HLA DR3	6p21	DG + TH
CTLA 4	2q33	DG + TH
D5S436	5q33	DG + TH
GD-1	14q31	DG
GD-2	20q11.2	DG
GD-3	Xq21.33	DG
IDDM6	18q12-22	DG
HT-1	13q22	TH
HT-2	12q22	TH

Por outro lado, estima-se que fatores ambientais como o hábito de fumar, estresse, infecções bacterianas e virais e uma grande variedade de drogas podem ser responsáveis por 21% da suscetibilidade no desenvolvimento da DG (Weetman, 2003; Prummel et al, 2004; Brix et al, 2001; Tait e Cough, 2003; Tomer e Davies, 2003). Iversen, em 1948, mostrou que a incidência da DG aumentou na Dinamarca no período de ocupação alemã, durante a Segunda Guerra Mundial (Iversen, 1948).

Uma série de drogas têm sido associadas ao desenvolvimento da DG, assim como infecções por bactérias e vírus e a exposição a diversos produtos químicos como aqueles presentes na fumaça do cigarro (Rekha et al, 2006; Coles et al, 1999; Krassas e Wiersinga, 2006; Matos-Santos et al, 2001; Iversen, 1948; Paunkovic et al, 1999).

Na terapêutica na DG existem três opções diferentes de tratamento: clínico, através de drogas anti-tiroidianas (DAT); radioiodoterapia, através do iodo 131 (^{131}I) e cirúrgico, através da tiroidectomia subtotal. Todos esses métodos são efetivos, mas a opção de tratamento varia consideravelmente entre países (Tominaga et al, 1997).

Uma das principais características da DG é a oftalmopatia de Graves (OG), que se refere às alterações orbitárias e de estruturas que ocorrem como conseqüência dos fenômenos de auto-imunidade relacionados à DG, podendo ocorrer com maior ou menor gravidade independentemente do estado de hiperfunção da tiróide (Carneiro et al, 2003).

OG pode ocorrer com maior freqüência e gravidade nos homens que apresentam DG do que nas mulheres e o tabagismo, a princípio mais difundido entre o sexo masculino, é um dos fatores predisponentes para essas características. Trabalhos realizados com habitantes de regiões onde há guerras mostram que mais importantes do que as próprias atribulações vividas, são as atitudes mentais negativas frente a elas que favorecem o aparecimento freqüente de oftalmopatia em suas formas severas (Banh, 2000).

1.4- Tabagismo e Doenças Tiroidianas

O tabaco industrializado contém mais de 3.000 componentes, incluindo 30 carcinógenos; já a fumaça do cigarro contém mais de 4.000 componentes, sendo 50 carcinogênicos. As três principais classes de carcinogênicos são: os hidrocarbonetos aromáticos policíclicos (HAPs), as aminas aromáticas e as nitrosaminas específicas do tabaco (TSNAs) (Bartsch et al, 2000).

Os mecanismos pelos quais as HAPs, como por exemplo o benzopireno, interagem com o DNA, ativam oncogenes e iniciam o processo carcinogênico envolvem a formação de carcinógenos. O benzopireno é convertido em metabólitos fenólicos mediados pela enzima CYP responsável pela metabolização de substâncias tóxicas. Um metabolismo

secundário, envolvendo outras formas de CYP, age na formação de reativos mais tóxicos. Inúmeros carcinógenos presentes na fumaça do tabaco são inativados pelas GSTs, como os genes *GSTM1* e *GSTP1,* responsáveis pela detoxificação dessas substâncias pelo organismo (Coles e Ketterer, 1990).

A fumaça do cigarro é responsável por aproximadamente 90% dos casos de câncer de pulmão (Levitz et al, 2004), além de estar associado aos cânceres de laringe, cavidade bucal, orofaringe, hipofaringe e aumenta o risco para leucemia, câncer sinonasal, nasofaringe e parte proximal da faringe, além de ser um grande risco para o desenvolvimento de câncer de estômago e pâncreas (International Agency for Research on Câncer, 2002). Entretanto, ao contrário de grande parte dos tumores no ser humano, tem-se descrito que o risco de desenvolver CDT tanto em homens quanto em mulheres fumantes é menor, tanto para o carcinoma papilífero quanto o folicular, em diferentes etnias, sugerindo-se que o cigarro exerce um efeito epidemiológico protetor contra o desenvolvimento do CDT (Mack et al, 2003).

Ao contrário, o cigarro tem sido associado a um aumento no risco para DG e para a OG (Bartalena et al, 1989; Berg et al, 1996; Chen et al, 1994; Winsa et al, 1993; Yoshiuchi et al, 1998).

1.5- Metabolização de Xenobióticos

A maioria dos agentes tóxicos requer ativação metabólica antes de se ligar ao DNA, ao RNA e às proteínas. Por isso, as variações nos processos de ativação e detoxificação de compostos químicos e drogas desempenham um importante papel na resposta orgânica (Bartsch e Hietanen, 1996). Distúrbios no equilíbrio desses processos podem explicar a variabilidade na resposta individual à exposição a tais compostos (Anwar et al, 1996).

Existem duas formas de metabolização de compostos tóxicos no organismo humano: a metabolização mediada pelas oxidases de função mista – ou de Fase I – e aquela mediada pelas enzimas de conjugação – ou de Fase II. Muitos compostos, tais como as

HAPs, as nitrosaminas e várias drogas medicamentosas são convertidos a metabólitos altamente reativos pelas enzimas oxidativas da Fase I, que são principalmente enzimas da família do citocromo P-450 (CYPs) (Bois et al, 1995). Assim, com a introdução de um ou mais grupamentos hidroxila ao substrato, um pró-carcinógeno pode tornar-se carcinogênico, como ocorre quando o benzopireno é convertido em epóxido de benzopireno, composto altamente reativo (Bois et al, 1995).

Já as reações da Fase II envolvem a conjugação com um substrato endógeno (glutationa, sulfato, glicose, acetato) através das glutationa-S-transferases (GSTs), UDP-glucoroniltransferases e N-acetiltransferases (NATs), que agem como inativadores dos produtos da Fase I, tornando os metabólitos mais hidrofílicos e, portanto, passíveis de excreção (Kromer et al, 1995).

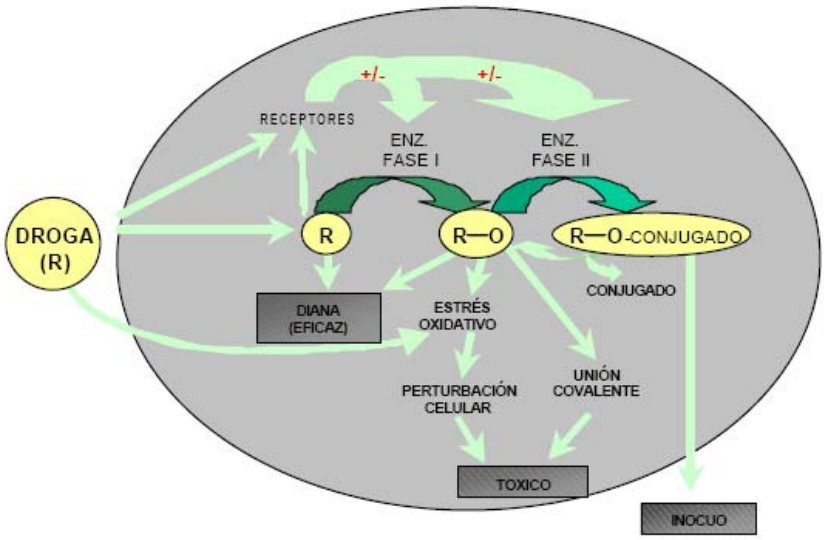

Figura 4- Esquema representativo do metabolismo de toxinas**:** assim que a toxina penetra na célula, tanto a droga metabolizada quanto seus metabólitos podem interferir no ciclo celular por formação de ligações covalentes com os ácidos nucléicos ou com proteínas (Santiago et al, 2002).

Portanto, a regulação e a expressão das enzimas de fase I e II, assim como seu equilíbrio metabólico na célula, podem ser importantes na determinação da suscetibilidade a doenças relacionadas à exposição a agentes tóxicos (Vineis, 2002).

1.6- Citocromo P-450

A família dos genes do citocromo P450 representa uma das principais classes de enzimas de biotransformação da Fase I, através de suas mais de 500 isoenzimas (Lange et al, 1999). Elas participam tanto da biossíntese como da degradação de esteróides, vitaminas, ácidos graxos, prostaglandinas, aminas, feromônios e metabólitos vegetais (Wolf, 1986). Metabolizam ainda inúmeras drogas, produtos químicos e poluentes ambientais denominados de xenobióticos (Guengerich, 2000). Tais reações metabólicas ocorrem principalmente no fígado, podendo ocorrer também por todo o organismo incluindo intestino, pulmão e rim, entre outros (Rogers, 1994).

Estima-se que no genoma humano existam em torno de 60 a 100 genes codificadores de enzimas P450, sendo que cerca de 20 deles estão envolvidos na codificação de enzimas que metabolizam compostos exógenos (Ingelman-Sundberg et al, 1999).

A importância desta classe de enzimas é ilustrada pelo fato de elas poderem ser encontradas em animais, plantas, leveduras, bactérias e mamíferos. Todos os genes que as codificam evoluíram a partir de um ancestral comum, em torno de 2,5 bilhões de anos atrás (Ingelman-Sundberg et al, 1999).

1.6.1- *CYP1A1*

O gene *CYP1A1*, localizado no cromossomo 15, na região 15q22-q24, codifica a enzima P4501A1, uma isoenzima que catalisa a oxidação de HAPs em produtos fenólicos e epóxidos (Gonzales, 1990).

A indução de *CYP1A1* ocorre via ligação do composto indutor ao receptor de "aryl hidrocarbon" (Ah), que ativa a transcrição na metabolização de xenobióticos (Kawajiri et al, 1993). A bioativação de vários HAPs é iniciada com estimulação do receptor Ah e este, por sua vez, ativa a transcrição da *CYP1A1*, do epóxido hidrolase e de outras enzimas (Hirvonen, 1995). Além dos HAPs, a *CYP1A1* é também induzida por xenobióticos encontrados em plantas da família das crucíferas (repolho e brócolis), tais como flavonas (Mcdonnel et al, 1992).

A atividade catalítica e o modo de indução de *CYP1A1* são conservados em animais superiores, confirmando sua importante função fisiológica. O padrão de atividade e a capacidade indutora desta enzima em humanos, relacionada à formação de metabólitos de HAPs capazes de reagir com a molécula de DNA é, portanto, de grande importância para a estimativa do risco de desenvolvimento de doenças. A enzima *CYP1A1* é considerada primariamente uma enzima extra-hepática em humanos, sendo encontrada no pulmão, nos linfócitos e na placenta após exposição à HAPs, incluindo aqueles presentes na fumaça do cigarro (Anttilla et al, 1991).

Através do uso da enzima de restrição *Msp1*, detectou-se um polimorfismo no gene codificador de *CYP1A1* originado pela mutação T6235→C, resultando no alelo polimórfico denominado *CYP1A1 m1*. Este polimorfismo ocorre de forma muito ligada à etnia, tendo sido observado em 31% de populações japonesas e 12% de populações caucasóides, respectivamente (Nebert et al, 1996).

Uma segunda mutação no gene codificador de *CYP1A1,* mutação de ponto (A4889→G), é responsável por outro polimorfismo no éxon 7 de *CYP1A1*, originando a variante *CYP1A1 m2* (Hayashi et al, 1991).

Os alelos variantes *CYP1A1 m1* e *CYP1A1 m2* foram correlacionados a um aumento na suscetibilidade ao câncer de pulmão em populações japonesas (Nakachi et al, 1995), porém, tal associação não foi verificada em populações caucasóides ou afro-americanas (Shields et al, 1993). Na população brasileira, um aumento no risco do câncer de pulmão foi significativamente associado à presença do alelo *m2* (Sugimura et al, 1995; Hamada et al, 1995). Não existem dados de literatura acerca da

presença destas variantes na DG, porém, há estudos que mostram a associação de *CYP1A1* com o lúpus eritematoso sistêmico (Yen et al, 2003).

1.7- Sistema Glutationa S-Transferase

A glutationa S-transferase (GST) é uma família de genes importantes na detoxificação de diferentes compostos endógenos e exógenos. As enzimas codificadas pelos genes do sistema GST possuem um papel importante no metabolismo celular e na modificação de compostos eletrofílicos reativos que são insolúveis em água ou lipofílicos. As GSTs catalisam reações que eliminam uma série de pré–carcinógenos como as HAPs presentes na fumaça do cigarro, drogas farmacológicas, incluindo paracetamol, agentes quimioterápicos e radicais livres gerados durante estresse oxidativo (Strange e Fryer, 2001; Their et al, 2003).

Estima-se que existam pelo menos 20 GSTs na espécie humana, assim como na maioria dos organismos vivos como as bactérias, leveduras, plantas e fungos (Buetler e Eaton, 1992). Seu mecanismo de ação baseia-se na conjugação com a glutationa que, além de diminuir a reatividade, aumenta a solubilidade dos xenobióticos hidrofóbicos, reduzindo, portanto, a meia-vida destes compostos no organismo. Desse modo, o conjugado é transportado para fora da célula por uma bomba de fluxo de glutationa S- transferase ATP- dependente e, a seguir, é eliminado pela urina e bile (Hayes et al, 1995).

Pode-se encontrar as GSTs em maior quantidade no fígado e em menores concentrações nos pulmões e intestino delgado (Shields, 1994). As GSTs são bem conhecidas por serem altamente polimórficas e a freqüência destes polimorfismos dependem da etnia (Bailey et al, 1998).

1.7.1- *GSTM1*, *GSTT1* e *GSTP1*

Polimorfismos dos genes *GSTM1*, *GSTT1* e *GSTP1* são intensamente estudados por estarem associados a diferentes tipos de câncer, particularmente causados por cigarro (Strange et al, 2000), resistência a tratamento quimioterápico e a drogas (Hayes e Pulford, 1995; Lear et al, 1996; Fryer et al, 2000).

Alguns dos genes que codificam as isoenzimas do sistema glutationa S-transferase, como, por exemplo, *GSTT1* e *GSTM1* possuem uma variante alélica nula na qual o gene inteiro está ausente e, portanto, não existe produção da respectiva enzima. Acredita-se que por não possuírem tais enzimas, indivíduos com genótipo nulo tornam-se mais susceptíveis para desenvolver doenças. Por outro lado, estes indivíduos respondem melhor a determinadas drogas quimioterápicas (Arai et al, 1999; Hayes e Pulford, 1995; Seidegard e Ekstrom, 1997; Pemble et al, 1994).

O gene *GSTM1* está localizado no cromossomo 1p13.3. Uma taxa de 20% a 50% dos indivíduos não expressam a enzima devido à deleção do gene em homozigose (Bailey et al, 1998; Roth et al, 2004). O gene *GSTM1* está envolvido na detoxificação de metabólitos químicos reativos derivados do cigarro, como benzopireno e aflatoxina B1, presentes no amendoim (Ketterer et al, 1992; Hayes e Pulford, 1995).

O gene *GSTT1* está localizado no cromossomo 22p11.2 e de 20% a 60% dos indivíduos não expressam a enzima correspondente (Pemble et al, 1994; Rebbeck et al, 1997). *GSTT1* metaboliza várias substâncias, como os agentes metilantes, pesticidas, solventes industriais e grande número de agentes químicos presentes no cigarro (Guengerich et al, 1997).

O gene *GSTP1* possui um polimorfismo envolvendo a troca do aminoácido isoleucina por valina no éxon 5 (I105V). Esta troca produz as variantes em homozigose *lle/lle* e *Val/Val* e a variante em heterozigose *lle/Val*. As variantes *lle/Val* e *Val/Val* possuem estabilidade e atividade específica menores do que as enzimas que contêm a isoforma *Ile/Ile* (Ali - Osmam et al, 1997). Assim, essas variantes enzimáticas possuem menor capacidade de detoxificação dos xenobióticos quando comparados com a enzima codificada pelo gene de tipo selvagem, denominado *lle/lle* (Johansson et al, 1998; Hu et al, 1997). Ao contrário, a hiperexpressão de *GSTP1* causa resistência a diversas drogas anti-neoplásicas e a presença do polimorfismo desse gene pode diminuir a eficácia terapêutica de quimioterápicos como o clorambucil e a ciclofosfamida (Henderson et al, 1998).

A expressão do gene *GSTP1* também vem sendo associada ao desenvolvimento de uma série extensa de neoplasias, particularmente relacionadas ao tabagismo, incluindo tumores de cabeça e pescoço, mama, pulmão e próstata (Stucker et al, 2002; Oude et al, 2003).

1.8- Gene *TP53*

O gene *TP53* está localizado no cromossomo 17, na posição p13.1. Codifica a proteína p53 que é uma fosfoproteína nuclear com papel fundamental na regulação do ciclo celular, principalmente na transição de G0 para G1. Essa proteína é encontrada em níveis muito baixos nas células normais, porém, em células danificadas, é expresso em grandes quantidades (Fridman e Lowe, 2003).

O *TP53* é considerado um gene supressor de tumores, pois se demonstrou que o tipo selvagem de *TP53* era capaz de inibir a transformação maligna de células e o crescimento de linhagens defeituosas, através de sua proteína normal (Finlay et al, 1989; Baker et al, 1990). É considerado o mais importante gene supressor de tumores nos seres humanos, por isso chamado de "guardião do genoma".

A proteína p53 está expressa em quase todos os tecidos (de Moura Gallo et al., 2005). Uma vez ativada, p53 regula a expressão de muitas classes de genes, através de seqüências específicas de ligação com DNA ou através de interações proteína-proteína. Sua regulação resulta em efeitos anti-proliferativos permitindo a preservação da integridade genômica (Cadwell e Zambetti, 2001).

O gene *TP53* evita a passagem de características vantajosas em termos de crescimento e proliferação a células filhas por dois mecanismos:

1- controla a parada do ciclo celular e permite reparos;

2- quando o reparo celular não é possível induz apoptose (Attardi, 2005).

A apoptose não só previne a geração do tumor como também regula a maturação e o controle da resposta auto-imune das células T- e B-, que são os componentes básicos de doenças auto-imunes da glândula tiróide (Lowe et al, 1993)

As alterações mais freqüentes no gene *TP53* são as mutações por alteração na seqüência das bases, resultando numa proteína defeituosa (DeWolf, 1995). A proteína normal do *TP53* está em concentração celular baixa, possuindo meia-vida de 20 minutos. Boa parte da proteína está sob forma latente, necessitando de mecanismos para sua ativação, como hipóxia e dano ao DNA (Levine, 1997; Guidos et al, 1996).

1.8.1- Polimorfismo do códon 72

As características estruturais do gene *TP53* têm sido bem preservadas por toda evolução. No códon 72, no entanto, foi reconhecido um polimorfismo de substituição do aminoácido arginina (*Arg*) pelo de prolina (*Pro*). Uma simples troca de base (de CGC para CCC) é responsável por tal mudança (Ara et al, 1990). O genótipo *Pro/Pro* possui até 15 vezes menos chance de provocar apoptose quando comparado ao *Arg/Arg* (Dumont et al, 2003).

A variante de *TP53* no códon 72 *Arg/Pro* tem sido associada, às vezes de forma controversa, a vários tipos de malignidades humanas, como câncer de mama (Buyru et al, 2003; Langerod et al, 2002), cervical (Storey et al, 1998; Arbel-Alon et al, 2002), pulmão (Papadakis et al, 2002) e em câncer esporádico de tiróide (Granja et al., 2004).

Estudos realizados no GEMOCA mostraram que indivíduos com genótipo *Pro/Pro* têm uma maior chance de desenvolverem câncer de tiróide (Granja et al, 2004), enquanto que a presença de um genótipo *Arg/Pro* aumenta em 2,5 vezes o risco de câncer de ovário (Morari et al, 2006).

Também se tem verificado uma associação do gene *72TP53* com algumas doenças auto-imunes, inclusive lúpus eritematoso sistêmico e artrite reumatóide (Lee et al, 2005, Lee et al, 2001). O gene está envolvido ainda na defesa do organismo contra agentes infecciosos ao impedir que células infectadas se dividam ou ao induzi-las à apoptose. Este

mecanismo de ação pode ser particularmente importante em situações de imunossupressão, como Leite et al (2006) demonstrou ao identificar maior risco de infecção viral em transplantados renais portadores de polimorfismos, que diminuem a ação anti-apoptótica de *TP53* (Leite et al, 2006).

TP53 faz parte dos mecanismos de defesa celular juntamente com as *CYPs* e as *GSTs*, como foi ilustrado na figura 5 (modificada de Stern et al, 2001).

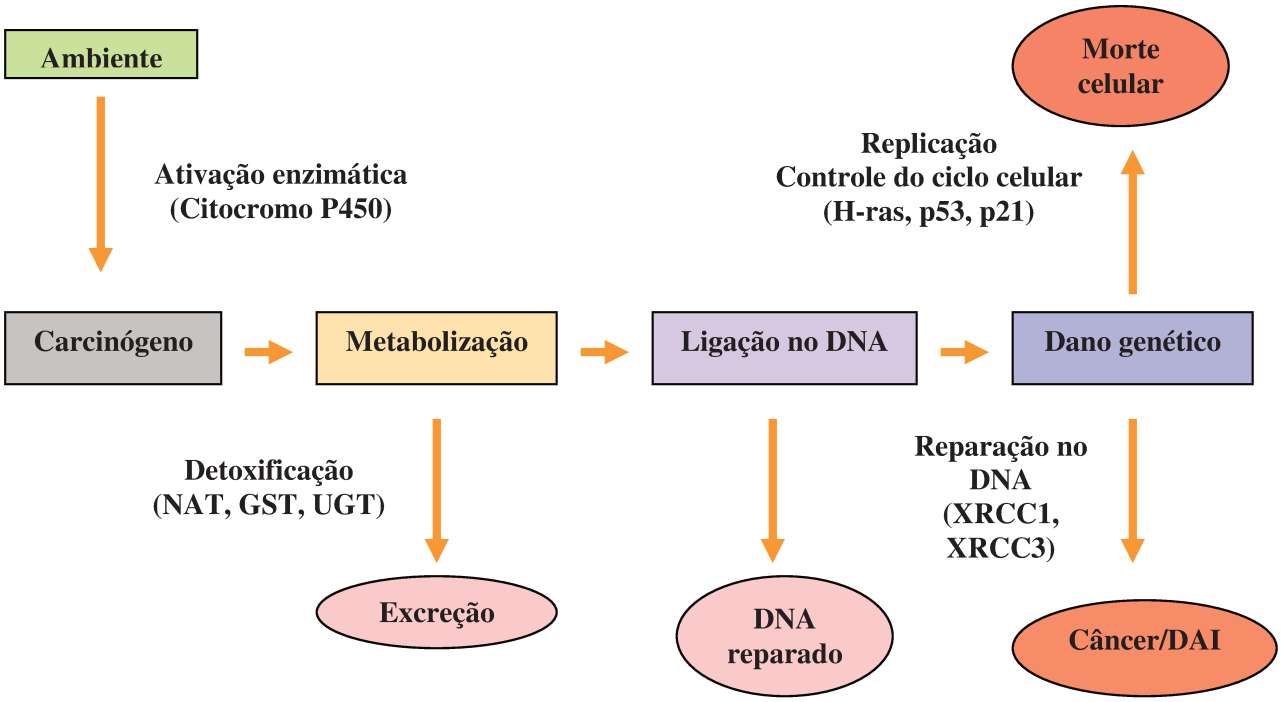

Figura 5- Esquema do mecanismo de interação entre os diversos sistemas de detoxificação e de reparo do DNA.

2- OBJETIVOS

1. Investigar a influência do gene *CYP1A1 m1* e *m2* na suscetibilidade ao câncer de tiróide.

2. Avaliar a associação entre os genes *CYP1A1 m1* e *m2* e os genes do Sistema Glutationa S-transferase em câncer de tiróide e sua resposta ao tratamento.

3. Analisar a influência da herança polimórfica dos genes *GSTT1*, *GSTM1*, *GSTP1*, *CYP1A1* e *72TP53* na doença de Graves.

4. Avaliar a associação entre os genes *GSTT1*, *GSTM1*, *GSTP1*, *CYP1A1* e *72TP53* em doença de Graves e a sua resposta ao tratamento.

3- MATERIAL E MÉTODOS

3.1- Pacientes com nódulos tiroidianos

Todos os participantes deste estudo retro-prospectivo do tipo caso-controle foram devidamente informados dos objetivos da investigação e assinaram o Termo de Consentimento Informado (anexo), conforme as determinações do Comitê de Ética em Pesquisa – FCM/ UNICAMP, que aprovou a linha desta pesquisa conforme Parecer CEP/FCM/UNICAMP #06/06/03- 072/98 (anexo).

Pacientes com nódulos tiroidianos foram atendidos na Disciplina de Endocrinologia do Hospital das Clínicas da Unicamp, durante o período de 1999 a 2005, no Ambulatório de Câncer de Tiróide. Todos esses pacientes seguiram um protocolo-padrão implantado no ambulatório há mais de 25 anos e possuem uma ficha na qual constam, além de dados de identificação, idade no diagnóstico, sexo, etnia, dados clínicos pré-cirúrgicos, exposição a agentes ambientais, dieta, tabagismo, uso de medicamentos e drogas, exames realizados (ultra-som, pesquisa de corpo inteiro com iodo^{131}, biópsia aspirativa), dados referentes à cirurgia e do exame anatomopatológico (medida do tumor, tipo histológico, grau de diferenciação e presença de linfonodos metastáticos).

Nenhum dos pacientes possuía histórico de exposição acidental ou médica à radiação ionizante ou de doença tiroidiana prévia e antecedentes de outras malignidades. Todos os dados, incluindo o diagnóstico de outras doenças concomitantes, foram confirmados nos prontuários dos pacientes.

Utilizou-se como critério para estadiamento a classificação proposta por De Groot (De Groot et al, 1995), que baseia-se no clássico método do TNM, isto é, no tamanho do tumor (T), acometimento de nódulos cervicais (N) e metástases à distância (M).

A população estudada foi composta por 80 casos com tumores benignos, incluindo 67 bócios multinodulares e 13 adenomas foliculares. Já nos tumores malignos, foram incluídos 168 casos, sendo 136 carcinomas papilíferos e 32 carcinomas foliculares, todos selecionados após resultado do anatomopatológico.

A etnia dos pacientes foi determinada através de entrevista, seguindo os critérios do Instituto Brasileiro de Geografia e Estatística (IBGE), mas por causa da dificuldade de classificação, pelo fato da população analisada ser altamente miscigenada,

decidiu-se agrupá-los em brancos e não-brancos. Em relação ao tabagismo, foram agrupados apenas em não-fumantes e fumantes, para fins estatísticos, porque os dados relativos ao número de cigarros fumados e tempo de uso de cigarro foram considerados pouco confiáveis.

3.1.1- Seguimento

Os pacientes com câncer foram acompanhados com pesquisa periódica de corpo inteiro com ^{131}I, TSH sérico e medidas de tireoglobulina (Tg) de acordo com o protocolo de seguimento. Essa pesquisa também incluiu Raio-X, ultra-sonografia, tomografia computadorizada e outros procedimentos para detectar metástase à distância, de acordo com cada caso. O período de seguimento foi de 12 meses a 341 meses (30±69 meses). Pacientes com altos valores de tireoglobulina (>2mg/dL) e/ou pesquisas de corpo inteiro suspeitas foram submetidos a uma busca através de exames de imagens. Os tumores foram definidos como recorrentes e/ou apresentando metástases à longa distância a partir do encontro de lesões nos exames de imagem e da persistência de valores ascendentes de Tg. Foram considerados pacientes assintomáticos e livres de doença aqueles que apresentavam Tg indetectável ou <1ng/dL.

3.1.2- Controles

O grupo-controle consistia de 277 indivíduos saudáveis selecionados da região de Campinas. O histórico obtido desses indivíduos incluiu fatores demográficos, étnicos, estilo de vida, hábitos alimentares, tabagismo, condições gerais de saúde e histórico de doenças.

Sua etnia foi classificada em brancos e não-brancos de acordo com a auto-classificação do paciente e segundo o IBGE.

Indivíduos com história prévia de doenças tiroidianas, exposição à radiação ou outros antecedentes de malignidade foram excluídos do estudo.

Foram selecionadas de duas a três mulheres no grupo-controle para cada homem visando parear esse grupo com o dos portadores de nódulo, cuja incidência é duas a três vezes maior em mulheres do que em homens.

Os pacientes foram agrupados apenas em não-fumantes e fumantes para fins estatísticos já que os dados relativos a números de cigarros fumados e tempo de uso foram considerados pouco confiáveis.

3.2- Pacientes com doença de Graves

Este estudo retro-prospectivo do tipo caso-controle também foi aprovado pelo Comitê de Ética em Pesquisa – FCM/ UNICAMP, Hospital da Santa Casa de São Paulo e Hospital da Pontifícia Universidade Católica de Campinas (FCM/ UNICAMP, CEP/FCM/UNICAMP #06/06/03- 072/98; PUC-Campinas, CEP#19/08/2005 – 332/04; Santa Casa São Paulo, CEP# 28/01/2005 – 036/04 – CONEP# 11012) (anexo).

Todos os 974 indivíduos foram devidamente esclarecidos dos objetivos deste trabalho e assinaram o Termo de Consentimento Informado.

Foram estudados 400 pacientes confirmados com a DG, sendo 300 mulheres e 100 homens, com média de idade de 35,5±13,7 anos. Estes pacientes foram selecionados pela FCM-UNICAMP, Santa Casa de São Paulo e PUC-Campinas.

Os pacientes com DG apresentavam os seguintes critérios diagnósticos: evidências clínicas e laboratoriais de tirotoxicose com TSH suprimido, valores elevados de T4 livre e T3, valores de captação de radioiodo de 24h ou de Tecnécio aumentados, com uma distribuição do traçador homogênea e difusa e/ou a positividade dos anticorpos contra o receptor do TSH (TRAb).

Os pacientes foram cuidadosamente examinados e tratados com drogas antitiroidianas, sendo 102 casos com metimazol e 56 casos com propiltiouracil. Duzentos e trinta e dois pacientes foram submetidos a radioiodoterapia e 10 casos a cirurgia. A terapia com drogas antitiroidianas foi mantida por pelo menos 12 meses, sendo os pacientes

encaminhados ao radioiodo ou a cirurgia quando apresentavam efeitos colaterais ou falta de adesão ao tratamento. Foram dosados TSH e T4 livre após o início de cada opção terapêutica a cada 30-60 dias e depois a cada três meses. Se evoluíssem para hipotiroidismo, era instituído o tratamento com levotiroxina.

Dos 400 pacientes apenas 169 apresentaram oftalmopatia. Sua avaliação foi baseada nos seguintes critérios:

a) Atividade da oftalmopatia quantificada através de um escore clínico "clinical activity score" que leva em consideração sete manifestações da doença: dor retro-bulbar espontânea, dor com o movimento ocular, eritema palpebral, edema palpebral, hiperemia conjuntival, quemose (edema de conjuntiva) e edema de carúncula. Um ponto é dado por cada manifestação, e a somatória varia de zero (sem atividade) até sete (atividade muito alta);

b) Medidas de proptose através do exoftalmômetro de Lind, sendo considerados os valores de normalidade de 18mm para asiáticos, 20 mm para caucasianos e 22mm para negróides.

A presença de oftalmopatia foi baseada na positividade do escore de atividade clínica (1-7) e/ou confirmação de proptose acima dos valores de normalidade.

3.2.1- Controles

O grupo controle foi selecionado da região de Campinas, sendo composto por 574 individuos saudáveis. Foram excluídos todos os indivíduos que apresentavam histórico familiar de doenças tiroidianas ou auto-imunes.

Como a DG é mais freqüente entre as mulheres, selecionamos três mulheres para cada homem no grupo-controle. Infelizmente, por questões éticas, não se conseguiu coletar amostras de sangue de crianças saudáveis.

Tanto os indivíduos do grupo-controle como os pacientes foram submetidos a exames físicos e responderam a um questionário que incluía ocupação, tabagismo, uso de drogas ilícitas e médicas, condições gerais da saúde e informações sobre doenças prévias.

Sua etnia foi classificada em brancos e não-brancos de acordo com a auto-classificação do paciente e pelo IBGE.

Os pacientes foram agrupados apenas em não fumantes e fumantes para fins estatísticos, já que os dados relativos a números de cigarros fumados e tempo de uso foram considerados pouco confiáveis.

3.2.2- Avaliação da função, auto-imunidade e captação tiroidiana

Os valores séricos de tirotrofina (TSH), T4 livre (T4l), triiodotironina (T3) total, anticorpos anti-tireoglobulina (AcTg) e anti-tiroperoxidase (AcTPO) foram avaliados em todos os pacientes. A captação de radioiodo foi avaliada 24h após administração de dose traçadora de 100 μCi de ^{131}I (valor de normalidade: 15%-41%).

3.3- Métodos

3.3.1- Extração de DNA

O DNA foi extraído de todas as amostras por meio de protocolo adaptado pelo GEMOCA, utilizando-se o método do fenol-clorofórmio. Após a extração, o DNA foi quantificado através de espectrofotometria e armazenado em congelador a –20º C.

3.3.2- Análise dos genes do sistema glutationa S-transferase

Para a análise das deleções de *GSTM1* e *GSTT1* utilizou-se a técnica PCR-duplex com a co-amplificação do gene da β-globina, que foi utilizado como controle. Os primers utilizados e as condições da PCR foram previamente descritos por Morari (2002) e são resumidos a seguir.

Tabela 1- Primers utilizados para amplificação dos fragmentos dos genes *GSTM1* e *GSTT1*.

Primers		**Seqüências**
GSTM1	**Sense**	**5' – CTGCCCTACTTGATTGATGGG - 3'**
	Anti-sense	**5' – CTGGATTGTAGCAGATCATGC – 3'**
Primers		**Seqüências**
GSTT1	**Sense**	**5' – TTCCTTACTGGTCCTCACATCTC - 3'**
	Anti-sense	**5' – TCACCGGATCATGGCCAGCA - 3'**

O volume utilizado na PCR foi de 25 µl, contendo 50 ng de DNA da amostra, 10µM de cada primer, 10X PCR Buffer (de acordo com o protocolo do fabricante), 25 mM de $MgCl_2$, 10 mM de dNTP e 1U Taq DNA polimerase. Os seguintes ciclos foram utilizados na PCR: a fase inicial de desnaturação foi de dois minutos a 94°C, seguida por 35 ciclos de 94°C por 30 segundos, 62°C por 50 segundos e 72°C por um minuto. A fase de extensão final foi de 10 minutos a 72°C. O sistema utilizado foi MJ PTC-200 PCR system.

Para a análise dos polimorfismos do gene *GSTP1* foi utilizada a PCR-RFLP. Os primers e as condições da PCR-RFLP foram previamente descritas por Morari (2006).

Tabela 2- Primers utilizados na PCR – RFLP do gene *GSTP1*.

Primer	**Seqüência**	
GSTP1	**Sense**	**5´ - CCAGGCTGGGGCTCACAGACAGC – 3´**
	Anti-sense	**5´- GGTCAGCCCAAGCCACCTGAGG – 3´**

Na reação de PCR utilizou-se 100 ng de DNA, 50nM de cada primer, 100 mM Tris-HCl (pH 8.0), 100µM de dNTP, 20 mM de $MgCl_2$ e 0.5 U Taq DNA polimerase, com um volume final de 20µl. A amplificação foi feita no termociclador MJ PTC-200 PCR system programado para realizar a desnaturação inicial a 94°C por cinco minutos, seguida de 35 ciclos de 94°C por 45 segundos, 65.6°C por 45 segundos e 72°C por um minuto e a extensão final foi de 72°C por sete minutos.

Os produtos obtidos na reação de amplificação por PCR para os genes *GSTM1*, *GSTT1* e *GSTP1* foram submetidos à eletroforese em gel de agarose a 2%, corado com brometo de etídeo (8 mg/ml) e a visualizados sob iluminação ultravioleta.

As figuras a seguir mostram o resultado da PCR-duplex dos genes *GSTT1* e *GSTM1*.

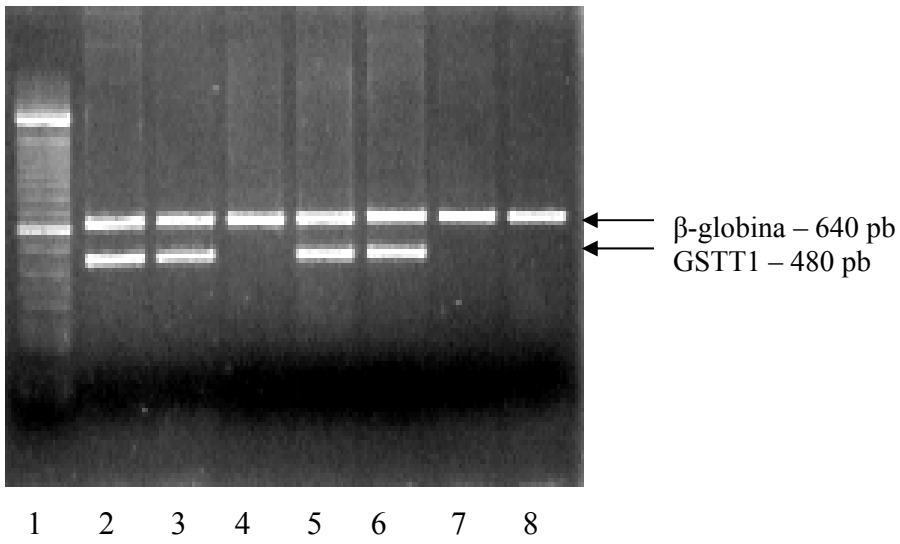

Figura 6- Resultado da PCR – duplex do gene *GSTT1* em gel de agarose corado com brometo de etídeo. 1: Ladder; 2, 3, 5 e 6: *GSTT1* presente; 4, 7 e 8: *GSTT1* ausente. Utilizou-se o primer da β-globina como controle positivo.

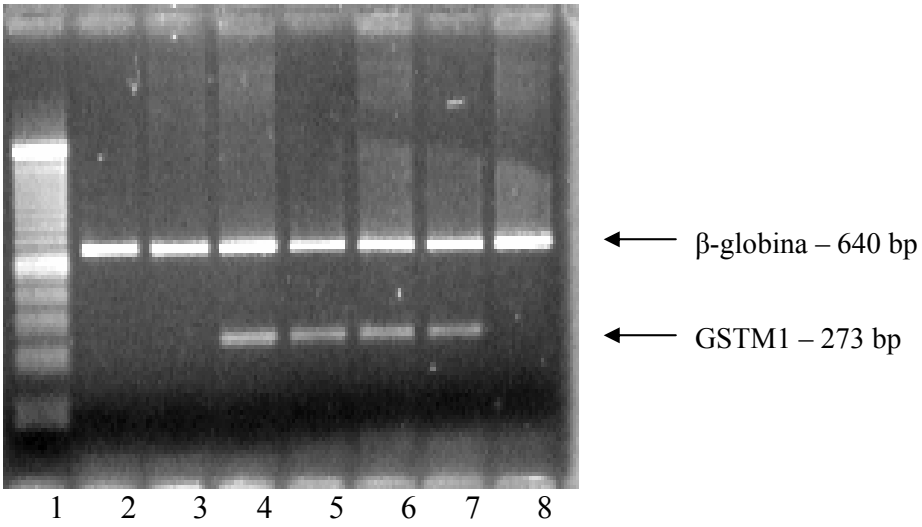

Figura 7- Resultado da PCR – duplex do gene *GSTM1* em gel de agarose corado com brometo de etídeo. 1: Ladder; 4, 5, 6 e 7: *GSTM1* presente; 2, 3 e 8: *GSTM1* ausente. Utilizou-se o primer da β-globina como controle positivo.

Já para a restrição enzimática do gene *GSTP1* foi utilizado um volume final de 15.5µl com a enzima de restrição *Alw26I* (Fermentas UAB, Lituânia), com processo de desnaturação a 37°C durante 16 horas, seguindo o protocolo do fabricante. Os resultados finais foram analisados em géis de agarose 3%, corados com brometo de etídeo (8mg/ml) e visualizados sob luz ultravioleta. Para o alelo homozigoto selvagem, encontrou-se um fragmento de 306 pb; o heterozigoto apresentou três bandas de 306 pb, 190 pb e 116 pb e o homozigoto mutante apenas duas bandas de 190 pb e 116 pb.

A figura a seguir mostra o resultado da PCR-RFLP do gene *GSTP1*.

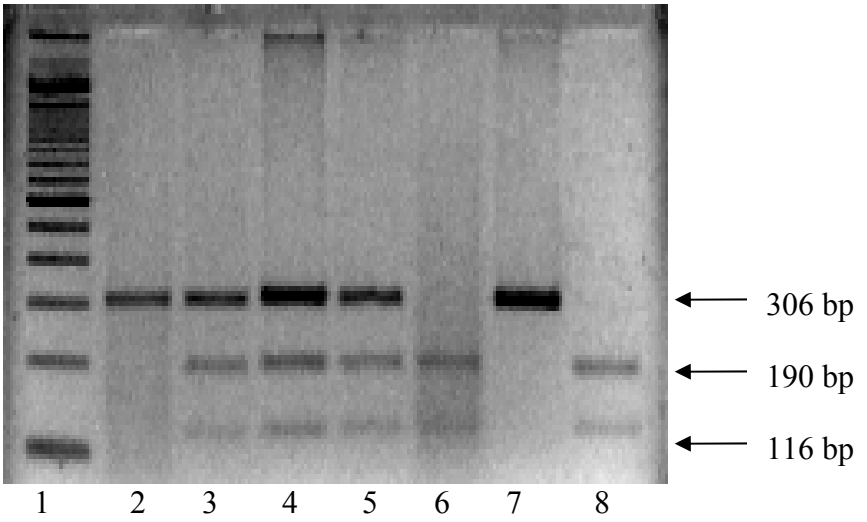

Figura 8- Resultado da restrição enzimática do gene *GSTP1* em gel de agarose corado com brometo de etídeo. 1: Ladder; 6 e 8: homozigoto mutante; 3, 4 e 5: heterozigoto; 2 e 7: homozigoto selvagem.

Seis amostras foram seqüenciadas diretamente em seqüenciador automático (ABI 377 Prism DNA Sequencer - Perkin Elmer) para confirmação dos resultados previstos na restrição enzimática.

3.3.3- Análise dos polimorfismos dos genes *CYP1A1 m1* e *CYP1A1 m2*

No caso do gene *CYP1A1 m1* e *m2* foram utilizados os primers descritos na tabela 3 para a realização PCR-RFLP.

Tabela 3- Primers utilizados para amplificação

Primers		**Seqüências**
CYP1A1 m1	**Sense**	**5' - CAGTGAAGAGGTGTAGCCGCT - 3'**
	Anti-sense	**5' – TAGGAGTCTTGTCTCATGCCT – 3'**
Primers		**Seqüências**
CYP1A1 m2	**Sense**	**5' – TTCCACCCGTTGCAGCAGCATAGCC - 3'**
	Anti-sense	**5' – CTGTCTCCCTCTGGTTACAGGAAG - 3'**

Os fragmentos gerados pelos primers de *CYP1A1 m1* e *CYP1A1 m2* foram respectivamente de 340 pb e 204 pb. Estes fragmentos foram amplificados separadamente em condições similares. O volume total utilizado na PCR foi de 25 µl, contendo 100 ng de DNA da amostra, 10µM de cada primer, 10mM Tris-HCl (pH 8.0), 20 mM de $MgCl_2$, 0.1 mM de dNTP e 0.5U Taq DNA polimerase.

Foram utilizados 35 ciclos na PCR de 94°C por 50 segundos, 60°C por 45 segundos para o gene *CYP1A1 m1*, 64°C por 45 segundos para o gene *CYP1A1 m2* e 72°C por um minuto, com a fase inicial de desnaturação de cinco minutos a 94°C e a fase final de extensão por 10 minutos a 72°C, usando o termociclador MJ PTC-200 PCR system. Os fragmentos da PCR foram visualizados em gel de agarose a 2%, corado com brometo de etídio, em um transiluminador de luz UV.

Para a análise dos alelos do gene *CYP1A1 m1* utilizou-se a enzima de restrição *MspI* de acordo com o protocolo do fabricante (Fermentas UAB, Lituânia). O alelo homozigoto selvagem apresentou um fragmento de 340 pb, o heterozigoto de 340 bp, 200 bp e 140 bp e o homozigoto apresentou apenas as bandas de 200 bp e 140 bp. Os produtos da restrição foram analisados em gel de agarose a 3%, corado com brometo de etídeo e visualizado no transiluminador de luz UV.

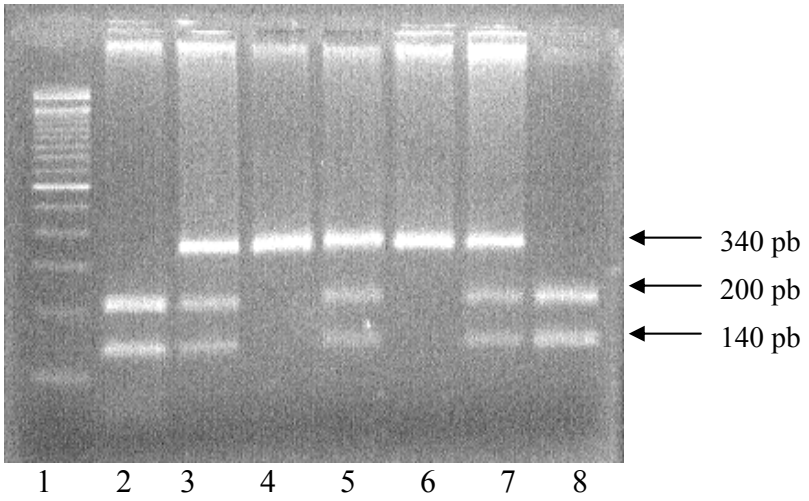

Figura 9- Exemplo de resultado obtido com a restrição enzimática de fragmento amplificado por PCR do gene *CYP1A1 m1,* em gel de agarose corado com brometo de etídeo. 1: Ladder; 2 e 8: homozigoto mutante; 3, 5 e 7: heterozigoto; 4 e 6: homozigoto selvagem.

Para identificar os polimorfismos do gene *CYP1A1 m2*, foi utilizada a enzima de restrição *BseMI* de acordo com o protocolo do fabricante (Fermentas UAB, Lituânia). O alelo homozigoto selvagem apresentou dois fragmentos de 149 bp e 55 pb; o heterozigoto de 204 bp, 149 bp e 55 pb e a variante homozigoto mutante apresentou apenas a banda de 204 pb. Os produtos da restrição foram analisados em gel de poliacrilamida a 12%, corado com brometo de etídeo e visualizado no transiluminador de luz UV. Seis amostras, tanto do gene *CYP1A1 m1* e *CYP1A1 m2,* foram seqüenciadas diretamente em seqüenciador automático (ABI 377 Prism DNA Sequencer - Perkin Elmer) para confirmação dos resultados previstos na restrição enzimática.

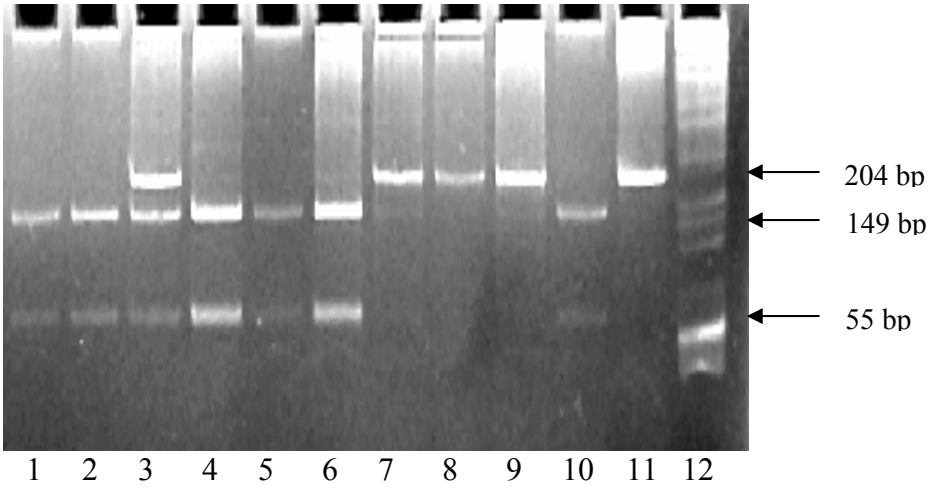

Figura 10- Exemplo de resultado obtido com a restrição enzimática em gel de poliacrilamida corado com brometo de etídeo. 7, 8, 9 e 11: indivíduos homozigotos mutantes; 3: indivíduo heterozigoto; 1, 2, 4, 5, 6 e 10: indivíduos homozigotos selvagens; 12: Marcador de peso molecular.

3.3.4- Identificação dos genótipos do gene *TP53* códon 72

Para a determinação dos polimorfismos do gene *TP53* códon 72 também foi utilizado o método de PCR-RFLP. Os primers utilizados estão descritos na tabela 2.

Tabela 4- Seqüências dos primers usados nas reações de PCR do gene *72TP53*

Primer	**Seqüência**	
***TP53* códon 72**	**Sense**	**5´ - ATCTCTACAGTCCCCCTCTTGCCG – 3´**
	Anti-sense	**5´- GCAACTCTGACCGTGACAGTCA – 3´**

O volume utilizado na PCR foi de 25 µl, contendo 100 ng de DNA, 50µM de cada primer, 10mM Tris-HCl (pH 8.0), 2.0 mM de $MgCl_2$, 100 µM de dNTP e 0.5U Taq DNA polimerase. Trinca e cinco ciclos foram utilizados na PCR de 94°C por 50 segundos, 65°C por 50 segundos e 72°C por um minuto, com a fase inicial de desnaturação de dois minutos a 94°C e a fase final extensão por 10 minutos a 72°C, usando o termociclador MJ

PTC-200 PCR system. Os fragmentos da PCR foram visualizados em gel de agarose a 2%, corados com brometo de etídio e visualizados sob luz ultravioleta.

A enzima de restrição utilizada foi a *Bsh1236I* para a análise dos alelos do gene *TP53* códon 72, de acordo com o protocolo do fabricante (Fermentas UAB, Lituânia). O alelo homozigoto selvagem apresentou dois fragmentos de 169 pb e 127 pb. A variante homozigota apresentou apenas um fragmento de 296 pb e o heterozigoto apresentou três bandas de 296 pb, 169 pb e 127 pb. O produto da restrição foi analisado por eletroforese em gel de agarose a 3% e corado com brometo de etídeo. Os resultados da RFLP foram confirmados através do seqüênciamento direto em seqüenciador automático (ABI 377 Prism DNA Sequencer - Perkin Elmer).

A figura a seguir mostra o resultado da PCR-RFLP do gene *TP53* códon 72.

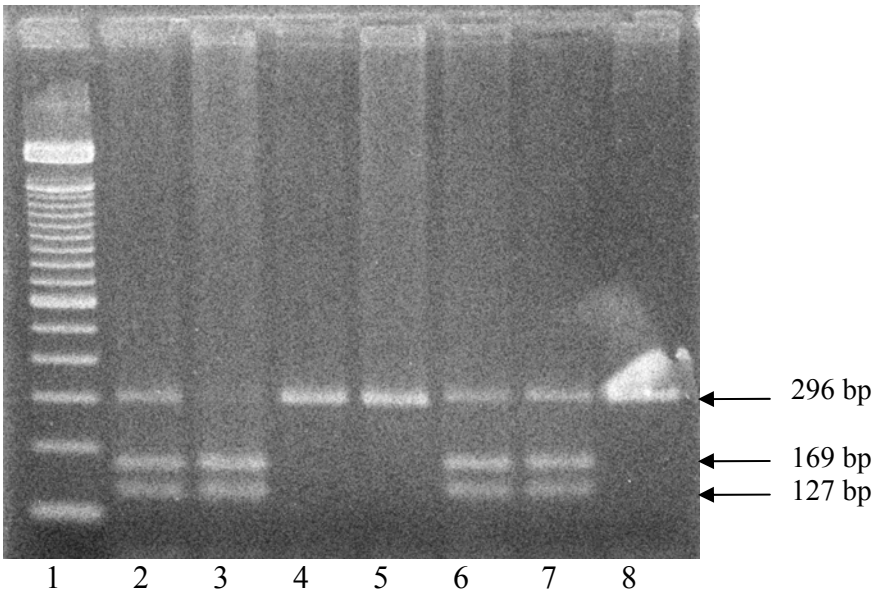

Figura 11- Gel de agarose corado com brometo de etídeo mostrando o resultado da PCR-RFLP do gene *TP53* códon 72. 1: Ladder; homozigoto selvagem (*Arg/Arg*); 2, 6 e 7: heterozigoto (*Arg/Pro*); 4, 5 e 8: homozigoto mutante (*Pro/Pro*).

3.4- Análise Estatística

Para a análise estatística realizada tanto com os pacientes com nódulos tiroidianos como os com DG foi utilizado o software SAS (Statistical Analyses System), versão 8.1, Cary, NC, USA, 1999-2000. Testes exatos de qui-quadrado (χ^2) e o teste exato de Fisher (F) foram usados para examinar a homogeneidade entre os casos e os controles com relação ao sexo, etnia, doenças tiroidianas prévias, tamanho do nódulo, uso de medicações, tabagismo e para os genótipos. O teste de Kruskal-Wallis (KW) foi usado para se comparar a idade entre os grupos. Os testes de Mann-Whitney ou Wilcoxon foram usados para se comparar a idade entre diferentes grupos genotípicos. O odds ratio (OR) e o coeficiente de intervalo de 95% "confidence interval-CI" foram usados para indicar o risco que um determinado genótipo de um determinado nódulo ou bócio possui em relação ao grupo controle. Para se analisar a relação entre os diferentes genótipos de *CYP1A1* e a idade, para os pacientes com nódulos tiroidianos, utilizou-se a analise de regressão logística univariada em pacientes acima ou abaixo de 45 anos. Fez-se o mesmo para os pacientes com DG e os genótipos *GSTM1, GSTT1, GSTP1, CYP1A1* e *72TP53*.

Utilizou-se uma análise de regressão logística multivariada para avaliar os efeitos dos genótipos, depois de ajustados para idade, sexo, etnia e fumo na determinação de risco, o diagnóstico de nódulos (maligno e benigno) e o tipo histológico do tumor (papilífero e folicular) para pacientes com nódulos tiroidianos e a doença de Graves na determinação da resposta terapêutica e evolução do tratamento a longo prazo. As interações entre as variáveis ambientais e os genótipos também foram avaliadas. A análise de regressão "stepwise" foi aplicada para identificar os fatores de risco tanto para a doença de Graves quanto para a oftalmopatia de Graves. Todos os testes foram realizados com p=0,05 como nível de significância. Todos os genes incluídos neste projeto foram testados quanto ao Equilíbrio de Hardy-Weinberg. Também foi utilizado o software PS para cálculo do tamanho amostral e do poder de cálculo de cada teste estatístico, em relação a cada um dos genes investigados.

RESULTADOS

Tabela 5- Proporções dos genótipos estudados tanto dos indivíduos-controle como dos pacientes com doenças tiroidianas benignas e malignas.

	Todos os casos				Papilíferos			Foliculares			Benignos		
	Controles	**Casos**	**OR***	**P**	**Casos**	**OR***	**P**	**Casos**	**OR***	**P**	**Casos**	**OR***	**P**
	N(%)	**N(%)**	**(95%CI)**		**N(%)**	**(95%CI)**		**N(%)**	**(95%CI)**		**N(%)**	**(95%CI)**	
CYP1A1 m1													
Homozigoto selvagem	173 (62.45)	179 (72.18)	0.629 (0.431;0.917)	**0.0160**	101 (74.26)	0.564 (0.357;0.894)	**0.0147**	19 (59.38)	1.148 (0.538;2.447)	0.7210	59 (73.75)	0.5921 (0.3401;1.031)	0.0832
Polimórficos	104 (37.55)	69 (27.82)			35 (25.74)			13 (40.63)			21 (26.25)		
CYP1A1 m2													
Homozigoto selvagem	180 (64.98)	161 (64.92)	1.033 (0.716;1.492)	0.8618	90 (66.18)	0.960 (0.620;1.487)	0.8545	16 (50.00)	2.026 (0.957;4.291)	0.0650	55 (68.75)	0.8435 (0.4948;1.438)	0.6226
Polimórficos	97 (35.02)	87 (35.08)			46 (33.82)			16 (50.00)			25 (31.25)		
GSTM1													
Positivo	114 (55.88)	124 (58.49)	1.046 (0.702;1.560)	0.8246	70 (60.87)	1.134 (0.703;1.830)	0.6055	17 (62.96)	1.282 (0.553;2.976)	0.5625	37 (52.86)	1.130 (0.6552;1.948)	0.7643
Negativo	90 (44.12)	88 (41.51)			45 (39.13)			10 (37.04)			33 (47.14)		
GSTT1													
Positivo	157 (76.96)	150 (78.53)	1.088 (0.670;1.767)	0.7346	77 (81.05)	1.218 (0.656;2.264)	0.5320	17 (77.27)	0.954 (0.329;2.765)	0.9315	56 (75.68)	1.074 (0.5757;2.002)	0.9494
Negativo	47 (23.04)	41 (21.47)			18 (18.95)			5 (22.73)			18 (24.32)		
GSTP1													
Homozigoto selvagem	121 (59.61)	113 (62.78)	0.766 (0.497;1.180)	0.2265	60 (64.52)	0.789 (0.466;1.335)	0.3769	5 (27.78)	3.200 (1.075;9.529)	0.0367	48 (69.57)	0.6456 (0.3598;1.158	0.1836
Polimórficos	82 (40.39)	67 (37.22)			33 (35.48)			13 (72.22)			21 (30.43)		
TP53 códon 72													
Arg/Arg	35 (30.17)	56 (51.85)	2.430 (1.297;4.552)	0.0056	37 (58.73)	3.522 (1.686;7.357)	0.0008	2 (25.00)	0.494 (0.029;4.248)	0.8278	17 (45.95)	0.5084 (0.2381;1.085)	0.1177
Pro/Pro+Arg/Pro	81 (69.83)	52 (48.15)			26 (41.27)			6 (75.00)			20 (54.05)		

4.1- Análise dos resultados

4.1.1- Análise de dados dos nódulos tiroidianos

Não houve diferença estatística entre os indivíduos-controle e os pacientes com nódulos tiroidianos em relação à idade (43±34 versus 46±94 anos), sexo (84 homens e 193 mulheres versus 57 homens e 191 mulheres) e etnia (230 brancos e 47 não-brancos versus 203 brancos e 45 não-brancos). O hábito de fumar também foi similar entre os indivíduos-controle e os pacientes (28% fumantes e 72% não-fumantes versus 33% fumantes e 67% não-fumantes).

Origens demográficas e características do estilo de vida tanto do grupo-controle quanto dos pacientes com nódulos tiroidianos foram similares.

4.1.1.1- Análise genética

Os genes *CYP1A1 m1* e *CYP1A1 m2,* estudados na população deste trabalho, se encontraram em equilíbrio de Hardy-Weinberg. Mas, infelizmente, tanto *CYP1A1 m1* quanto o *m2,* não possuem poder de cálculo, 62% e 59% respectivamente. O tamanho amostral calculado para que o poder de cálculo atingisse 80% seria de 584 pacientes.

Parte dos 277 indivíduos-controle e dos 248 pacientes com nódulos tiroidianos participaram de outros estudos no GEMOCA e já haviam sido genotipados para os genes *GSTM1*, *GSTT1*, *GSTP1* e *TP53* códon 72 (Morari et al, 2002, Granja et al, 2004, 2005). Todos estes genótipos e o gene *CYP1A1* dos indivíduos-controle e dos pacientes com doenças tiroidianas benignas e malignas estão apresentados na tabela 5.

4.1.1.2- Comparação entre pacientes com nódulos e controles

O genótipo homozigoto selvagem do gene *CYP1A1 m1* foi mais freqüente entre os pacientes com nódulos tiroidianos (72.18%) do que no grupo-controle (62.45%) (x^2; p= 0.0160) (OR: 0.629; IC95%= 0.431-0.917), indicando que os indivíduos que herdam o alelo variante possuem um risco 37% menor para desenvolvimento de nódulos tiroidianos do que aqueles que possuem o alelo selvagem.

Para o gene *CYP1A1 m2* não encontramos significância estatística quando comparamos o grupo-controle (64.98%) com os pacientes com nódulos tiroidianos (64.92%) (x^2; p= 0.8618).

Não houve associação entre os genótipos de *CYP1A1 m1* e *m2* e os genótipos de *GSTM1*, *GSTT1*, *GSTP1* e *TP53* códon 72.

4.1.1.3- Comparação entre pacientes com nódulos e controles levando-se em conta a idade

Analisando separadamente os pacientes com nódulos tiroidianos abaixo e acima de 45 anos observamos que somente *CYP1A1 m1* está associado aos pacientes com idade acima de 45 anos.

A presença das variantes de *CYP1A1 m1* diminui o risco para o desenvolvimento de nódulos tiroidianos em 47% (OR= 0.527; 95%CI= 0.306-0.907) em pacientes acima de 45 anos de idade (p= 0.0209).

A análise de regressão logística múltipla, ajustada para sexo, confirma o efeito protetor de *CYP1A1 m1* em indivíduos acima de 45 anos de idade (p= 0.0351; OR= 0.514; 95% CI= 0.277 to 0.954). Estes resultados são mostrados na tabela 6.

Tabela 6- Análise estatística da freqüência de variantes de *CYP1A1 m1* em indivíduos <45 anos de idade e >45 anos de idade com OR e intervalos com 95% de confiança obtidos pelo ajuste do modelo de regressão logística múltipla corrigido para sexo.

Variável	< 45 anos			≥ 45 anos		
	p-valor	OR	IC (95%)	p-valor	OR	IC (95%)
CYP1A1 m1	0.5338	0.810	(0.418-1.572)	0.0351	0.514	(0.277-0.954)

4.1.1.4- Comparação entre pacientes com câncer e controles

As características clínicas e os parâmetros de agressividade ao diagnóstico e o seguimento dos pacientes com câncer de tiróide estão apresentados na tabela 7.

Tabela 7- Distribuição de dados dos pacientes com carcinoma tiroidiano de acordo com a histologia e hábito de fumar comparando-os com características clínicas, incluindo idade (X±DP), sexo (F: feminino; M: masculino), etnia (B: branco; NB: não-branco); estadiamento (presença de linfonodos, metástase à distância) e seguimento.

			Carcinoma Papilífero		Carcinoma Folicular	
			Fumante	Não Fumante	Fumante	Não Fumante
Características Clínicas	Idade (X±DP)		42±12	45±16,4	54,4±16,5	45,7±21,2
	Sexo	F (%)	65	93	67	96
		M (%)	35	7	33	4
	Etnia	B (%)	89	82	83	82
		NB (%)	11	18	17	18
Diagnóstico	Linfonodos (%)		16	12	8	9
	Metástase à distância (%)		2	3	14	12
	Estadio	I+II (%)	67	66	51	53
		III+IV (%)	33	34	49	47
Seguimento	% de recorrência e/ou metástase à distância		14	10	23	21

Quando se analisa os diferentes nódulos tiroidianos, pôde-se observar correlação somente entre genótipo e carcinoma papilífero.

O genótipo selvagem de *CYP1A1 m1* foi mais freqüente entre os pacientes com carcinoma papilífero (74.26%) do que nos controles (62.45%) (p= 0.0147).

Portanto, a presença das variantes do gene *CYP1A1 m1* protege contra o desenvolvimento do carcinoma papilífero em 43% como mostra a tabela 8.

Tabela 8- OR e intervalos com 95% de confiança obtidos pelo ajuste do modelo de regressão logística univariada, corrigido para sexo e idade, considerando-se o grupo de carcinoma papilífero.

Variável	p-valor	OR	IC (95%)
CYP1A1 m1	0.0147	0.564	(0.357-0.894)

4.1.1.5- Comparação entre pacientes com câncer e controles levando-se em conta a idade

Considerando somente os pacientes com carcinoma papilífero abaixo e acima de 45 anos de idade, verificou-se que o genótipo selvagem de *CYP1A1 m1* foi mais freqüente entre os pacientes com o carcinoma papilífero acima de 45 anos de idade (78.04%) do que nos controles (60.52%) (p= 0.0128).

A presença das variantes do gene *CYP1A1 m1* protege contra o desenvolvimento do carcinoma papilífero em 40% (OR= 0.5936; 95% CI= 0.3869-0.9114).

4.1.1.6- Comparação entre pacientes com câncer e tabagismo

O cigarro não foi um fator de risco independente para o câncer de tiróide nesta casuística (p= 0.0941). A análise de regressão logística multivariada mostrou que nos pacientes com câncer havia uma associação entre o risco de desenvolvimento para o

carcinoma papilífero e o alelo homozigoto selvagem de *CYP1A1 m1* (p= 0.0063; OR= 2.243; 95% IC= 1.256-4.008).

Não se encontrou correlação entre o gene *CYP1A1* com os pacientes com carcinoma folicular.

4.1.1.7- Comparação entre *CYP1A1* e fatores de suscetibilidade

Para investigar a inter-relação entre os genótipos de *CYP1A1* e outros fatores de suscetibilidade, realizou-se a análise de regressão logística multivariada, corrigida para sexo e idade, considerando apenas os 77 pacientes com tumores tiroidianos e 66 controles que possuíam todas as avaliações clínicas e genotípicas completas, incluindo os genótipos *CYP1A1 m1*, *CYP1A1 m2*, *GSTM1*, *GSTT1*, *GSTP1* e *TP53* códon 72. Somente a herança do alelo homozigoto selvagem de *CYP1A1 m1* (p= 0.0373) e o hábito de fumar (p= 0.0348) apresentaram uma associação inversa significante no risco de câncer de tiróide. Essa associação se manteve tanto para *CYP1A1 m1* (p= 0.0237) quanto para o hábito de fumar (p= 0.0348) na análise dos 47 carcinomas papilíferos que possuíam todos os dados.

Não se encontrou correlação entre os carcinomas foliculares, provavelmente devido à insuficiência do tamanho amostral.

Não houve associação entre qualquer genótipo ou fator de risco para bócio ou para adenoma folicular, nem entre si.

Não se encontrou também, nenhuma relação entre os genótipos e os parâmetros de agressividade ao diagnóstico, histologia ou seguimento dos pacientes com câncer.

4.1.2- Análise de dados dos doentes de Graves

Não houve diferença entre os indivíduos-controle e os pacientes com DG em relação ao sexo (175 homens e 399 mulheres versus 100 homens e 300 mulheres - x^2; p= 0.0612), a hábitos dietéticos e atividade física; porém, houve uma diferença quanto à idade (43.3±15.7 versus 35.5±13.7 anos; p <0.0001) e etnia (89 não-brancos e 485 brancos

versus 125 não-brancos e 275 brancos; p <0.0001). O hábito de fumar foi também diferente em relação a indivíduos-controle e a pacientes (29,26% fumantes e 70,74% não fumantes versus 36.82% fumantes e 63,18% não fumantes) (p = 0.0397; OR= 1.409; 95% CI=1.023-1.940), como apresentado na tabela 9.

Tabela 9- Distribuição de dados clínicos dos pacientes que apresentam doença de Graves e indivíduos-controle incluindo idade (X±DP), sexo (F: feminino; M: masculino), etnia (B: branco; NB: não-branco) e tabagismo (P: positivo; N: negativo).

			Doença de Graves	**Controles**
Características Clínicas	Idade (X±DP)		35.5±13.7	43.3±15.7
	Sexo	F(%)	75	69.51
		M(%)	25	30.49
	Etnia	B(%)	68.75	84.50
		NB(%)	31.25	15.50
	Tabagismo	P(%)	36.82	29.26
		N(%)	63.18	70.74

Após um período mínimo de 12 meses de seguimento, 39 pacientes ainda se encontravam em tratamento, 274 (75.9%) indivíduos foram considerados curados e 87 pacientes ainda estavam em hipertiroidismo.

4.1.2.1- Análise genética

A tabela 10 resume os achados deste estudo, na genotipagem para *GSTM1*, *GSTT1*, *GSTP1*, *CYP1A1* e *72TP53* na população-controle e em pacientes de DG.

Tabela 10- Distribuição dos genótipos de *GSTM1* e *GSTT1* (presente= positivo; ausente= negativo); *GSTP1* e *CYP1A1* (homozigoto selvagem, heterozigoto e homozigoto mutante) e *TP53* códon 72 (*Arg/Arg, Arg/Pro e Pro/Pro*) entre os 400 pacientes com DG e os 574 indivíduos-controle.

Todos os Casos								
		Controles		**DG**		**OR***	**(95%CI)**	**P**
		N	**(%)**	**N**	**(%)**			
GSTM1	**Positivo**	275	56.35	239	59.75	0.895	0.675-1.186	0.4390
	Negativo	213	43.65	161	40.25			
GSTT1	**Positivo**	385	78.89	320	80.00	0.955	0.677-1.349	0.7951
	Negativo	103	21.11	80	20.00			
GSTP1	**Homozigoto Selvagem**	290	59.43	169	42.25	2.074	1.559-2.760	<0.0001
	Heterozigoto	153	31.35	186	46.50			
	Homozigoto Mutante	45	9.22	45	11.25			
CYP1A1 m1	**Homozigoto Selvagem**	173	62.45	52	44.44	1.971	1.254-3.0987	0.0033
	Heterozigoto	96	34.66	61	52.14			
	Homozigoto Mutante	8	2.89	4	3.42			
***TP53* códon 72**	***Arg/Arg***	139	44.98	121	44.65	4.685	1.707-12.861	0.0035
	Arg/Pro	158	51.13	115	42.44			
	Pro/Pro	12	3.88	35	12.92			

* ajustado para sexo, idade e etnia

4.1.2.2- Análise dos genes *GSTM1* e *GSTT1*

O gene *GSTM1* foi ausente em 40.25% dos pacientes com DG e 43.65% dos indivíduos-controle (p= 0.4390).

O gene *GSTT1* foi ausente em 20% dos pacientes com DG e 21.11% dos indivíduos-controle (p= 0.7951) como mostrado abaixo, na tabela 11. Infelizmente, o poder do cálculo dessas análises foi muito baixo (16% e 0.5%, respectivamente). Não se realizou o teste de Equilíbrio de Hardy-Weinberg para esses genes porque eles são herdados como polimorfismos de deleção e a teoria não se aplica a eles.

Tabela 11- OR e intervalos com 95% de **confiança** dos genótipos nulos de *GSTM1* e *GSTT1* corrigidos pelo sexo, idade e etnia.

Variável	p-valor	OR	IC(95%)
GSTM1	0.4390	0.895	(0.675-1.186)
GSTT1	0.7951	0.955	(0.677-1.349)

4.1.2.3- Análise do gene *GSTP1*

Os pacientes com DG possuem as variantes do genótipo *GSTP1* em número maior (homozigoto selvagem= 42.25%, heterozigoto= 46.50%, homozigoto mutante= 11.25%) do que o observado na população controle (homozigoto selvagem= 59.43%, heterozigoto= 31.35%, homozigoto mutante= 9.22%; p <0.0001).

A análise de regressão logística multivariada, corrigida para sexo, idade e etnia mostrou que a herança para as variantes alélicas de *GSTP1* aumentou o risco da DG em mais de duas vezes (OR= 2.074, 95%CI= 1.559-2.760) com um poder do cálculo de 99%. O grupo-controle não se encontra em equilíbrio de Hardy-Weinberg.

Quando os dados também foram corrigidos para o tabagismo a suscetibilidade de desenvolver a DG aumentou: OR= 2.125, 95%CI= 1.521-2.969 (p<0.0001), como mostra a tabela 12:

Tabela 12- OR e intervalos com 95% de confiança das variantes alélicas de *GSTP1*.

Variável	p-valor	OR	IC(95%)
GSTP1	<0.0001	2.074	(1.559-2.760)*
	<0.0001	2.125	(1.521-2.969)**

* ajustada para sexo, idade e etnia;

** ajustada para sexo, idade, etnia e tabagismo.

4.1.2.4- Análise do gene *CYP1A1 m1*

Também se observou que as variantes de *CYP1A1 m1* foram mais freqüentes em pacientes com DG (homozigoto selvagem= 44.44%, heterozigoto= 52.14%, homozigoto mutante= 3.42%) do que nos indivíduos-controle (homozigoto selvagem= 62.45%, heterozigoto= 34.66%, homozigoto mutante= 2.89%) (p<0.0033).

A análise de regressão logística multivarida indicou que a herança das variantes do gene *CYP1A1 m1* aumentou a suscetibilidade à DG em quase duas vezes (OR= 1.971, 95%CI= 1.254-3.098), com um poder do cálculo de 87%. O grupo-controle se encontrava em equilíbrio de Hardy-Weinberg.

A correção para o tabagismo reduziu ligeiramente o OR (OR= 1.884; 95%CI= 1.187-2.990; p = 0.0072), como mostra a tabela 13:

Tabela 13- OR e intervalos com 95% de confiança das variantes alélicas de *CYP1A1 m1*.

Variável	p-valor	OR	IC(95%)
CYP1A1 m1	<0.0033	1.971	(1.254-3.098)*
	<0.0072	1.884	(1.187-2.990)**

* ajustada para sexo, idade e etnia;

** ajustada para sexo, idade, etnia e tabagismo.

4.1.2.5- Análise do gene *TP53* códon 72

Em relação à análise das variantes do códon 72 de *TP53,* observou-se uma distribuição semelhante do alelo homozigoto selvagem em pacientes e controles.

Contudo, o alelo *Pro/Pro* foi mais freqüente na DG do que nos controles (p= 0.0007), indicando que a herança do alelo *Pro/Pro* aumenta o risco para a DG em quase três vezes (OR= 2.984; 95%IC= 1.433-6.217), com poder de cálculo de 97%. O grupo-controle não se encontrou em equilíbrio de Hardy-Weinberg.

Quando corrigimos para o tabagismo, o risco para a DG aumentou em mais de quatro vezes: OR= 4.685; 95%IC= 1.707-12.861; p = 0.0027, como mostra a tabela 14:

Tabela 14- OR e intervalos com 95% de confiança das variantes alélicas de *TP53* códon 72.

Variável	p-valor	OR	IC(95%)
72TP53	<0.0007	2.984	(1.433-6.217)*
	<0.0027	4.685	(1.707-12.861)**

* ajustada para sexo, idade e etnia;

** ajustada para sexo, idade, etnia e tabagismo.

4.1.2.6- Comparação entre os genótipos, a DG e levando-se em conta a idade

Como a DG tipicamente ocorre mais em adultos jovens, foi comparado os genótipos de pacientes abaixo e acima de 25 anos de idade.

A análise de regressão logística múltipla, ajustada para idade, sexo e etnia, mostrou que apenas as variantes de *GSTP1* são fatores de risco em indivíduos com menos de 25 anos de idade (OR= 17.798; 95%CI= 6.125-51.719; p <0.0001), enquanto que em indivíduos acima de 25 anos de idade as variantes dos genótipos de *GSTP1* (OR= 1.684; 95%CI= 1.244-2.279; p= 0.0007), *CYP1A1m1* (OR= 2.096; 95%CI= 1.283-3.424; p= 0.0031) e a variante *Pro/Pro 72TP53* (OR= 2.609; 95%CI= 1.207-5.638; p = 0.0147) apareceram como fatores de suscetibilidade à doença.

4.1.2.7- Comparação entre os genótipos, a DG e o tabagismo

A análise de regressão logística multivariada, corrigida para o sexo, identificou o hábito de fumar como fator de risco independente que aumenta a suscetibilidade para a oftalmopatia de Graves em mais de duas vezes (OR= 2.243; 95%CI= 1.325-3.797; p = 0.0026). Nenhum dos genótipos estudados está relacionado com o desenvolvimento da oftalmopatia de Graves.

4.1.2.8- Comparação entre os genótipos e fatores de suscetibilidade

Para examinar o papel dos genótipos investigados e outros fatores de suscetibilidade na DG aplicou-se uma análise de regressão "stepwise" (gradual) que confirmou a herança das variantes de *GSTP1* (OR= 1.881; 95%CI= 1.201-2.946; p= 0.0058) e do alelo *Pro/Pro* de *72TP53* (OR= 5.784; 95%CI= 1.688-19.816; p= 0.0052) como fatores de risco significantes.

Não houve nenhuma associação dos genótipos de *GSTM1*, *GSTT1*, *GSTP1*, *CYP1A1 m1* e *TP53* códon 72 entre si.

Também não se encontrou qualquer associação entre os genótipos investigados e as características clínicas dos pacientes, inclusive o tamanho de bócio, níveis de hormônio tiroidiano e a presença de anticorpos antitiroidianos.

Não houve relação entre qualquer genótipo e a evolução para cura ou não dos pacientes.

5- DISCUSSÃO

5.1- Epidemiologia Molecular

Knudson (1985) propôs uma teoria que permite classificar os indivíduos e as populações quanto às participações dos fatores ambientais e genéticos no desenvolvimento de doenças e diz que há mutações que conferem alto risco individual de um tipo particular de câncer, por exemplo, em algum estágio da vida, independente do ambiente. Os indivíduos dessa população são ditos predispostos e constituem minoria. Por outro lado, existe um grupo maior de pessoas que têm menores chances para o desenvolvimento de doenças, mas seus riscos ainda são mais elevados que os da população geral, pois respondem à exposição ambiental (Knudson, 1985).

Assim, a precisa identificação de marcadores de suscetibilidade é de fundamental importância na predisposição de riscos (Albertini, 1999). Por isso, faz-se necessário o uso de biomarcadores como "indicadores sinalizando eventos em amostras ou sistemas biológicos" (Nebert et al, 1996; Yuspa, 2000).

O princípio básico dos marcadores de suscetibilidade reside na diferença interindividual que confere graus de sensibilidade às doenças induzidas pelo ambiente. Esses marcadores podem incluir características genéticas, diferenças no metabolismo ou na capacidade diferencial de um órgão de se recuperar de agressões ambientais (Bartsch e Hietanen, 1996; Taningher et al, 1999).

Entre os marcadores de suscetibilidade mais comuns estão as diferenças genéticas na capacidade das células repararem lesões no DNA causadas por agentes ambientais (Spitz e Bondy, 1993; Kaderlik e Kadlubar, 1995). Um outro tipo de biomarcador de suscetibilidade baseia-se no fato de muitos xenobióticos não serem capazes de provocar os efeitos prejudiciais, sendo alterados por enzimas, cujas modificações podem aumentar ou diminuir a habilidade dessas substâncias interagirem com as biomoléculas informacionais (Guengerich, 2000).

5.2- Fatores ambientais e o câncer de tiróide

Um aumento contínuo da incidência de câncer de tiróide tem sido registrado durante as últimas décadas no Brasil e em todo o mundo (Steliarova-Foucher et al, 2006; Haselkorn et al, 2000; Liu et al, 2001; Burgess 2002; Coeli et al, 2005; Reynolds at al, 2005). A maior dificuldade é entender como ocorre o CDT. A exemplo de outros tumores, relata-se que a causa do câncer de tiróide está associada a um fator ambiental, principalmente pela exposição à radiação ionizante, além dos fatores físicos, químicos e ambientais que influenciam no desenvolvimento do câncer de tiróide (Jacob et al, 2006).

Inúmeros polimorfismos têm sido investigados com o objetivo de se delinearem modelos poligenéticos da suscetibilidade ao câncer. Tais modelos são particularmente interessantes para o câncer de tiróide. De fato, nódulos tiroidianos são detectados por ultrassonografia em aproximadamente 67% da população, sendo que poucos são malignos e requerem tratamento cirúrgico (Castro e Gharib, 2005). Por essa razão, um método de rastreamento capaz de identificar indivíduos que apresentam risco maior para desenvolver câncer de tiróide poderia selecionar indivíduos para tratamentos preventivos ou ainda para encaminhá-los a intervenção diagnóstica e terapêutica precoces, determinando quais pacientes com nódulos tiroidianos se beneficiariam de terapias específicas (Wogan et al, 2004).

Os genes envolvidos no metabolismo de xenobióticos podem ser interessantes para delinear modelos de risco. A habilidade individual de biotransformar substâncias potencialmente tóxicas tem sido associada com maior ou menor suscetibilidade a agentes tóxicos e ao risco para câncer. Indivíduos incapazes de detoxificar adequadamente substâncias agressivas ou metabólicos carcinogênicos podem sofrer um dano direto no DNA ou um dano celular, com a formação de elementos químicos e macromoléculas que podem causar instabilidade genômica (Wogan et al, 2004).

Inúmeros estudos realizados em diferentes populações têm correlacionado os polimorfismos de *CYP1A1* a diferentes tipos de câncer, geralmente relacionados à alta atividade oxidativa das enzimas de fase I (Vineis 2002; Agundes, 2004).

Os dados deste estudo demonstram uma associação inversa significante entre *CYP1A1 m1* e o hábito de fumar no risco para o desenvolvimento do câncer de tiróide. Demonstrou-se que o alelo homozigoto selvagem de *CYP1A1m1* é mais freqüente tanto em nódulos tiroidianos quanto em carcinomas papilíferos do que nos indivíduos-controle, sugerindo que a fumaça do cigarro e outros metabólitos dependentes da ativação de *CYP1A1* não estão implicados no risco para a formação de bócio nem no processo da malignidade tiroidiana.

Esta observação vai de encontro a vários estudos epidemiológicos que não são capazes de associar a fumaça do cigarro com o câncer tiroidiano e a dados que mostram um baixo risco de câncer de tiróide entre fumantes. Mack et al (2003), reunindo 13 trabalhos do tipo caso-controle que estudavam a associação entre a fumaça do cigarro e o câncer de tiróide, mostraram que o risco para desenvolver câncer de tiróide estava reduzido em 40% entre fumantes (Mack et al, 2003). Estudos realizados tanto na população canadense quanto na norte-americana não conseguiram demonstrar nenhuma associação entre o hábito do tabagismo e o risco para o câncer de tiróide (Iribarren et al, 2001; Navarro Silvera et al, 2005).

Por outro lado, os dados aqui achados demonstraram que *CYP1A1* influencia no risco para o desenvolvimento de nódulos tiroidianos somente em indivíduos acima de 45 anos de idade, sugerindo que a patogênese de tumores tiroidianos pode ser devida à contínua exposição a um ou mais produtos carcinogênicos metabolizados pelas enzimas oxidativas de Fase I.

É importante lembrar que *CYP1A1* deve ser apenas um de vários genes de detoxificação que atuam em diferentes substratos e são tecido-específicos, de forma que a sua ação em conjunto é que determina o desenvolvimento de tumores. Diferentes fatores ambientais podem estar implicados na patogênese do carcinoma folicular, explicando porque não se encontrou nenhuma relação entre os genótipos de *CYP*, cigarro e o risco para esse tumor. Aliado a isso, o resultado de nossos dados seguramente pode estar relacionado ao pequeno número relativo de carcinomas foliculares inclusos neste trabalho.

Não encontramos nenhuma correlação entre os genes estudados pertencentes às enzimas de Fase I e II no risco para o câncer de tiróide ou nos diagnósticos dos pacientes.

5.3- Doença de Graves e a suscetibilidade a fatores ambientais

Assim como o câncer, a DG é considerada uma doença multifatorial na qual o desenvolvimento da resposta auto-imune contra antígenos tiroidianos é facilitado por um determinado, mas ainda pouco conhecido, contexto poligênico. Os fatores ambientais provavelmente provocam ou desencadeiam o desenvolvimento da doença em indivíduos geneticamente suscetíveis, como indicado pelo índice baixo de concordância em gêmeos monozigóticos entre outras evidências (Weetman, 2003; Prummel et al, 2004; Brix et al, 2001; Tait e Cough, 2003; Tomer e Davies, 2003).

A associação de diversos polimorfismos específicos em um mesmo indivíduo seria necessária para o seu desenvolvimento. Entre os polimorfismos mais estudados estão o dos genes codificadores do sistema HLA, do receptor de linfócitos T-helper, do antígeno 4 dos linfócitos T citotóxicos (CTLA-4), e de várias citocinas (Wang e Crapo, 1997; Gough, 2000).

Tem sido demonstrado que alguns agentes químicos ambientais podem alterar o sistema imune em diferentes espécies (Gilbertson et al, 2003; Nichenametla et al, 2004). Em humanos, a exposição a xenobióticos ambientais, inclusive o tabagismo, foi proposta como um dos fatores de iniciação que levam à perda da tolerância à auto-proteína em indivíduos geneticamente suscetíveis a um número elevado de doenças, inclusive as doenças auto-imunes tiroidianas (Aune et al, 2004; Kita et al, 2004; Fourneau et al, 2004).

O tabagismo é um fator bem reconhecido do risco de desenvolvimento da DG e sobretudo para a OG (Vestergaard, 2002). Uma meta-análise que reuniu 435 casos e 777 controles mostrou que o risco para OG foi significativamente maior em pacientes tabagistas com a DG. O mesmo estudo mostrou que risco para a DG foi menor em homens do que em mulheres (Vestergaard, 2002). Os dados aqui apresentados confirmam a literatura atual que indica que o tabagismo desempenha um papel importante no risco à DG e maior ainda na OG (Vestergaard, 2002).

Winsa et al (1993) estudaram 208 pacientes com DG e encontraram que o número de pacientes tabagistas com DG foi significativamente maior do que nos indivíduos controle. Analisaram também indivíduos com DG e OG e encontraram que 64% dos pacientes tabagistas possuíam OG moderada e 71% com OG severa (Winsa et al, 1993).

Os resultados encontrados neste estudo sugerem que os indivíduos com variantes de *GSTP1* podem ser mais suscetíveis à indução da auto-imunidade, talvez pela ação de produtos ambientais ou endógenos que dependem da ação da enzima *GSTP1,* mas não da *GSTM1* ou *GSTT1* para sua detoxificação. As variantes de *GSTP1* possuem uma capacidade reduzida na detoxificação de substâncias envolvidas no estresse oxidativo e na resposta celular ao dano de DNA (Hayes e Strange, 2000; Cao et al, 2003). O fato de o tabagismo ter aumentado o risco na associação de *GSTP1* com a DG sugere que alguns dos mais de 4000 compostos tóxicos aspirados ou ingeridos ao se fumar possam estar implicados no desenvolvimento desta doença. As enzimas GST são sujeitas à regulação por hormônios tiroidianos, além de GH, insulina e hormônios sexuais (Coecke et al, 2000).

Tanto o T3 como T4 reduzem a atividade das GSTs, sugerindo que o estado de hipertiroidismo poderia exercer uma influência na resposta a drogas antitiroidianas ou até mesmo ao tratamento com radioiodo (Coecke et al, 2000). No entanto, não se encontrou evidência de qualquer relação entre o estado tiroidiano, a evolução dos pacientes e seu perfil genotípico para GSTs.

Este estudo demonstrou que as variantes de *CYP1A1 m1* estão relacionadas à suscetibilidade à DG. O sistema de enzimas P450 é um dos mais primitivos existentes, supondo-se que vem se desenvolvendo há mais de 3,5 bilhões de anos. Entretanto, durante o metabolismo oxidativo essas enzimas são capazes de gerar compostos tóxicos intermediários que podem estar envolvidos no desencadeamento da DG, como os dados aqui sugerem (Vineis, 2002). As enzimas de CYP, juntamente com o alelo *Pro/Pro* de 72*TP53*, talvez participem no metabolismo de algumas toxinas de efeitos cumulativos, já que parecem ser mais importantes em indivíduos acima de 25 anos.

O papel da variante homozigota *Pro* de *TP53* na suscetibilidade à DG é mais difícil de ser interpretado. Danos no DNA e alterações nos mecanismos de apoptose podem estar associados a doenças auto-imunes tiroidianas, como sugere o achado de anticorpos anti-p53, que podem ser detectados nos soros de aproximadamente 4 % de pacientes com suspeitas de doença autoimune tiroidiana (Fenton et al, 2000).

O papel da morte celular programada em doenças auto-imunes ainda é muito pouco entendido, mas é razoável assumir que a apoptose contribui, pelo menos parcialmente, na regulação da maturação e do controle da resposta imune mediada pelos linfócitos T e B (Duke et al, 1996). O papel das variantes de *TP53* em tumores relacionados com o tabagismo foi extensivamente investigado e os polimorfismos de *TP53* e *GSTs* foram até implicados em doenças vasculares como aterosclerose (Wang e Wang, 2005) e uma série de doenças auto-imunes como lúpus (Lee et al, 2005), artrite reumatóide (Lee et al, 2001; Morinobu et al, 2006), síndrome de Guillain-Barré (Kim et al, 2006; Stavropoulou et al, 2007; Kuwabara, 2007).

Os resultados deste trabalho indicam que a herança homozigota *ProTP53* também desempenha um papel importante no risco da DG, especialmente entre fumantes.

Vários estudos foram feitos até hoje na tentativa de se descobrir quais os fatores desencadeadores da DG e da OG e quais os mecanismos responsáveis pela perpetuação da ação auto-imune. Os polimorfismos de estruturas celulares das células foliculares e do sistema imunológico podem ser fatores importantes nestes fenômenos. Como a herança da doença é atribuída a múltiplos genes, a associação de diversos polimorfismos específicos em um mesmo indivíduo seria necessária para seu desenvolvimento (Carneiro et al, 2003).

Já que não foi encontrada qualquer associação entre características clínicas, oftalmopatia, cura e não cura e os genótipos estudados, supõe-se que esses polimorfismos não devem ser úteis como indicadores da gravidade da doença ou da sua resposta ao tratamento. Por outro lado, considerando que a DG é uma das doenças auto-imunes mais comuns, as conclusões obtidas podem contribuir para delinear um modelo poligenético da suscetibilidade a esta doença. No futuro, poderá servir de base a estudos para sua prevenção ou diagnóstico precoce. Além disso, a herança dessas enzimas pode ajudar a explicar a relação observada entre o hábito de fumar e esta doença auto-imune.

6- RESUMO DOS ACHADOS

Em resumo,

1. Investigamos a influência do gene *CYP1A1 m1* e *m2* na suscetibilidade ao câncer de tiróide;

2. Avaliamos a associação entre os genes *CYP1A1 m1* e *m2* e os genes do sistema glutationa s-transferase, já genotipados em nosso laboratório em câncer de tiróide, e a sua resposta ao tratamento;

3. Analisamos a influência da herança polimórfica dos genes *GSTT1*, *GSTM1*, *GSTP1*, *CYP1A1* e 72*TP53* na suscetibilidade à doença de Graves e na sua resposta ao tratamento.

A partir dos dados obtidos, podemos dizer que:

- A herança das variantes do gene *CYP1A1 m1* diminui o risco para o desenvolvimento de nódulos tiroidianos em 37%;

- O gene *CYP1A1 m2* não tem relação com nódulos tiroidianos;

- Não existe associação entre os genótipos de *CYP1A1 m1* ou *m2* e os genótipos de *GSTM1, GSTT1, GSTP1* e *TP53* códon 72;

- A presença das variantes de *CYP1A1 m1* diminui o risco para o desenvolvimento de nódulos tiroidianos em indivíduos acima de 45 anos de idade em 47% (OR= 0.527; 95%CI= 0.306-0.907) (p= 0.0209);

- A presença das variantes *CYP1A1 m1* protege contra o desenvolvimento do carcinoma papilífero em 43%, e em indivíduos acima de 45 anos de idade em 40%;

- O hábito de fumar não é um fator de risco independente para o câncer de tiróide;

- Não existe associação entre o gene *CYP1A1 m1* e *m2* e a resposta ao tratamento dos pacientes com nódulos tiroidianos;

- Não existe relação entre os genes *GSTM1* e *GSTT1* e a suscetibilidade à doença de Graves;

- A herança para as variantes alélicas de *GSTP1* aumenta o risco para a doença de Graves em mais de duas vezes. Este risco aumenta quando correlacionamos essas variantes ao tabagismo;

- A herança das variantes do gene *CYP1A1 m1* sozinhas ou quando associadas ao tabagismo aumentam a suscetibilidade à doença de Graves em quase duas vezes;

- A herança do alelo *Pro/Pro* de *TP53* aumenta o risco para a doença de Graves em quase três vezes e quando associamos ao hábito tabagista este risco aumenta em mais de 4 vezes;

- As variantes de *GSTP1* são fatores de risco em indivíduos com menos de 25 anos de idade enquanto que em indivíduos acima de 25 anos de idade apenas as variantes de *GSTP1*, *CYP1A1 m1 Pro72TP53* apareceram como fatores de suscetibilidade à doença;

- O hábito de fumar é um fator de risco independente que aumenta a suscetibilidade para a oftalmopatia de Graves em mais de duas vezes;

- Nenhum dos genótipos estudados está relacionado ao desenvolvimento da oftalmopatia de Graves e às características clínicas estudadas dos pacientes;

- Não há nenhuma associação entre os genótipos *GSTM1, GSTT1, GSTP1, CYP1A1 m1 e TP53* códon 72 na doença de Graves;

- Não houve relação entre qualquer genótipo e a evolução para cura ou não dos pacientes.

7- CONCLUSÃO

Foi demonstrado que o hábito de fumar se correlaciona inversa e negativamente com o perfil genotípico para *CYP1A1* no risco de desenvolvimento de nódulos e câncer da tiróide de tipo papilífero. Ao contrário, o hábito de fumar se correlaciona ao risco de desenvolvimento de doença de Graves.

Um perfil genotípico para genes relacionados à suscetibilidade a doenças tiroidianas poderá selecionar grupos que podem se beneficiar de medidas preventivas e/ou de intervenções diagnósticas e terapêuticas.

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9- ANEXOS

Faculdade de Ciências Médicas
COMITÊ DE ÉTICA EM PESQUISA
Caixa Postal 6111
13083-970 Campinas, SP
(0__19) 3788-8936
fax (0__19) 3788-8925
cep@head.fcm.unicamp.br

CEP, 06/06/03
(PARECER 072/98)

PARECER

I-IDENTIFICAÇÃO:

PROJETO: **"ESTUDO DO ENVOLVIMENTO DOS ONCOGENES E GENES SUPRESSORES TUMORAIS NA PATOGENIA DAS NEOPLASIAS DE PELE, GLÂNDULA MAMÁRIA, ADRENOCORTICAL E DA TIRÓIDE HUMANA"**

PESQUISADOR RESPONSÁVEL: Laura Sterian Ward

II - PARECER DO CEP

O Comitê de Ética em Pesquisa da Faculdade de Ciências Médicas da UNICAMP tomou ciência e aprovou a emenda que altera o título para **"GENES ENVOLVIDOS NA PATOGÊNIA DAS NEOPLASIAS"**, referente ao protocolo de pesquisa supracitado.

Recomendamos que a cada tipo de novo tipo câncer incluído na pesquisa seja informado ao CEP/FCM.

Aprovado "*ad referendun*" em 06 de junho de 2003.

Profa. Dra. Carmen Silvia Bertuzzo
PRESIDENTE DO COMITÊ DE ÉTICA EM PESQUISA
FCM / UNICAMP

PUC CAMPINAS
PONTIFÍCIA UNIVERSIDADE CATÓLICA

PONTIFÍCIA UNIVERSIDADE CATÓLICA DE CAMPINAS
CENTRO DE CIÊNCIAS DA VIDA
COMITÊ DE ÉTICA EM PESQUISA

Campinas, 19 de Agosto de 2005 Protocolo 332/04

Prezado Senhor João Hamilton Romaldini,

Parecer Projeto: APROVADO

I – Identificação:
Título do projeto (completo): "PERFIL GENOTÍPICO DE PACIENTES COM DOENÇA DE GRAVES E SUA INFLUÊNCIA NA RESPOSTA TERAPÊUTICA".
Pesquisador (a) responsável: JOÃO HAMILTON ROMALDINI, ROBERTO BERNARDO DOS SANTOS E LAURA STERIAN WARD.
Instituição onde se realizará: ENDOCRINOLOGIA – HOSPITAL E MATERNIDADE CELSO PIERRO – PUC-CAMPINAS.
Data de apresentação dos esclarecimentos solicitados pelo CEP: 19/08/05
Apresentar relatório: AO TÉRMINO DA PESQUISA.

II – Objetivos:
Determinar o perfil genotípico de pacientes com Doença de Graves com e sem oftalmologia.
Correlacionar tal perfil com a resposta às diferentes modalidades terapêuticas utilizadas no tratamento da Doença de Graves.

III - Sumário do projeto:
O estudo incluirá a avaliação clínica e ocular prospectiva em 150 pacientes com Doença de Graves hipertiroidianos. Serão avaliados também 150 indivíduos que não apresentem Doença de Graves (grupo controle) ou história familiar de doença tiroidiana. Estes pacientes serão submetidos a dosagens de TSH e anticorpo antiperoxidase para exclusão de doença auto-imune tiroidiana. Os pacientes serão divididos em 3 grupos:

- Pacientes em remissão anteriormente tratados com drogas antitiroidianas;
- Pacientes submetidos a radioiodoterapia que foram curados com uma única dose;
- Pacientes submetidos a radioiodoterapia que necessitam de 2 doses ou mais para serem curados.

Será analisa a influência do perfil genotípico (CTLA-4) na evolução (cura) dos diferentes tratamentos.
Serão avaliados de acordo com a evolução da oftalmopatia, e divididos novamente em 3 grupos:
G1 – Radioiodo associado com prednisona.
G2 – Somente radioiodo.
G3 – Uso de fármaco antitiroidiano.

IV - Parecer do CEP:
Após análise da mudança realizada no projeto (inclusão de um grupo controle), e dos esclarecimentos solicitados no parecer de 01.08.05, o parecer **ad referendum** do Comitê de Ética em Pesquisa é o que segue:

Av. John Boyd Dunlop, S/Nº Jardim Ipaussurama Campinas SP - CEP 13059-900
Telefone: (0XX19) 3729 6808 - Fax: (0XX19) 3729 6806 - e-mail: comitedeetica@puc-campinas.edu.br

Dessa forma, e considerando a Resolução no. 196/96 item VII.13.b, que ***define as atribuições dos CEPs e classifica os pareceres emitidos aos projetos de pesquisa envolvendo seres humanos,*** e, ainda que a documentação apresentada atende ao solicitado, emitiu-se o segundo parecer para a presente modificação feita no projeto: Aprovado.

V – Informações Complementares

O sujeito da pesquisa tem a liberdade de recusar-se a participar ou de retirar seu consentimento em qualquer fase da pesquisa, sem penalização alguma e sem prejuízo ao seu cuidado (Res. CNS 196/96 – Item IV.1.f) e deve receber uma cópia do Termo de Consentimento Livre e Esclarecido, na íntegra, por ele assinado (Item IV.2.d).

Incluir no Termo de Consentimento Livre e Esclarecido que o projeto foi avaliado por um Comitê de Ética em Pesquisa, assim como, incluir o telefone do mesmo.

Pesquisador deve desenvolver a pesquisa conforme delineada no protocolo aprovado e descontinuar o estudo somente após análise das razões da descontinuidade pelo CEP que o aprovou (Res. CNS Item III.1.z), exceto quando perceber risco ou dano não previsto ao sujeito participante ou quando constatar a superioridade do regime oferecido a um dos grupos de pesquisa (Item V.3.).

O CEP deve ser informado de todos os efeitos adversos ou fatos relevantes que alterem o curso normal do estudo (Res. CNS Item V.4.). É papel do pesquisador assegurar medidas imediatas adequadas frente a evento adverso grave ocorrido (mesmo que tenha sido em outro centro) e enviar notificação ao CEP e à Agência Nacional de Vigilância Sanitária – ANVISA – junto com seu posicionamento.

Eventuais modificações ou emendas ao protocolo devem ser apresentadas ao CEP de forma clara e sucinta, identificando a parte do protocolo a ser modificada e suas justificativas. Em caso de projeto do grupo I ou II apresentados anteriormente à ANVISA, o pesquisador ou patrocinador deve enviá-las também à mesma junto com o parecer aprovatório do CEP, para serem juntadas ao protocolo inicial (Res. 251/97, Item III.2.e.).

Relatórios parciais e final devem ser apresentados ao CEP, de acordo com os prazos estabelecidos na Resolução CNS-MS 196/96.

VI - Data da aprovação: 19/08/2005

Sendo só o que nos cumpre informar, aproveitamos da oportunidade para renovar votos de estima e consideração.

Atenciosamente.

Profa. Dra. Maria Luiza Cruz
Coordenadora do C.E.P.S.H.P
PUC-Campinas

Av. John Boyd Dunlop, S/Nº Jardim Ipaussurama Campinas SP - CEP 13059-900
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MINISTÉRIO DA SAÚDE
Conselho Nacional de Saúde
Comissão Nacional de Ética em Pesquisa - CONEP

PARECER Nº 163/2005

Registro CONEP: 11012 (Este nº deve ser citado nas correspondências referentes a este projeto)

Registro CEP: 036/04 **Processo nº 25000.151568/2004-51**
Projeto de Pesquisa: *"Mutações do Gene CTLA 4 e Associações com endocrinopatias auto-imunes"*
Pesquisador Responsável: Dr. Adriano Namo Cury (orientando)
Dr. Osmar Monte (orientador)
Instituição: Irmandade da Santa Casa de Misericórdia de São Paulo
Área Temática Especial: Genética Humana

Ao se proceder à análise do projeto de pesquisa em questão, em resposta ao Parecer nº 2411/04, cabem as seguintes considerações:

a) as informações enviadas relativas à adequação do Termo de Consentimento Livre e Esclarecido, atendem aos aspectos fundamentais da Res. CNS 196/96 sobre diretrizes e normas regulamentadoras de pesquisas envolvendo seres humanos;

b) o projeto foi aprovado pelo Comitê de Ética em Pesquisa – CEP da instituição supracitada.

Diante do exposto, a Comissão Nacional de Ética em Pesquisa – CONEP, de acordo com as atribuições definidas na Res. CNS 196/96, manifesta-se pela aprovação do projeto de pesquisa proposto.

Situação: Projeto aprovado.

Brasília, 28 de janeiro de 2005.

WILLIAM SAAD HOSSNE
Coordenador da CONEP/CNS/MS

LABORATÓRIO DE GENÉTICA MOLECULAR DO CÂNCER
UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Ciências Médicas
Departamento de Clínica Médica

TERMO DE CONSENTIMENTO

Projeto de Pesquisa em Doenças Crônicas da Tiróide
Orientadora: Profª Laura Sterian Ward

Paciente ou responsável pelo paciente
Sr(a).....
.....anos RG..... HC.....
Endereço.....
Telefone.....

Concordo em doar sangue para pesquisa de genes (contidos no DNA) que podem estar envolvidos em doenças tiroidianas. Sei que se trata de uma pesquisa científica e concordo que os dados de meu caso, registrados no meu prontuário médico, sejam usados para avaliar a importância dos genes, sabendo que meu nome assim como meus dados clínicos e de laboratório não serão individualmente citados e que nenhum momento meu diagnóstico ou tratamento serão prejudicados por tal doação. Também sei que esta pesquisa pode trazer benefícios para a cura ou o tratamento das doenças tiroidianas no futuro, mesmo que eu não me beneficie disso agora. Não terei nenhum gasto com a doação do meu material para esta pesquisa e sei que poderei cancelar minha decisão e deixar de participar em qualquer momento. Também não serei submetido a qualquer procedimento que não faça parte da rotina de meu tratamento normal, sob a orientação de meu médico habitual.

Estou consciente da importância de minha participação da qual posso desistir em qualquer momento. Fui informado de que este projeto está aprovado pelo Comitê de Ética em pesquisa e sei que poderei obter todas as informações que desejar e necessitar no fone: (19) 3521-8954.

Profª Dra. Laura Sterian Ward – Coordenação do GEMOCA – Medicina Interna
Cl Méd./ FCM – UNICAMP Cidade Universitária CEP: 13081-970 Campinas, São Paulo.
019 – 3521-8954 e-mail: ward@unicamp.br

Endocrine-Related Cancer (2006) **13** 1185–1193

Smoking and susceptibility to thyroid cancer: an inverse association with *CYP1A1* allelic variants

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Abstract

In contrast to most human malignancies, epidemiologic studies have frequently reported a reduced risk of differentiated thyroid cancer in tobacco consumers. Cytochrome P4501A1 (*CYP1A1*) gene variants may be related to an increased capacity to activate polycyclic aromatic hydrocarbons, producing highly reactive electrophilic intermediates that might damage DNA. Hence, the germline inheritance of a wild-type *CYP1A1* gene may decrease the susceptibility for thyroid cancer. The present study was designed to investigate *CYP1A1* (*m1* and *m2*) role in thyroid tumorigenesis and its connection with *GSTM1, GSTT1, GSTP1, GSTO1,* and *codon 72* of *p53* genotypes. A total of 248 patients with thyroid nodules, including 67 benign goiters, 13 follicular adenomas, 136 papillary carcinomas, and 32 follicular carcinomas, and 277 controls with similar ethnic backgrounds were interviewed on their lifetime dietary and occupational histories, smoking habit, previous diseases, and other anamnestic data. DNA was extracted from a blood sample and submitted to PCR-restriction fragment length polymorphism assays. The wild-type *CYP1A1m1* genotype was more frequent among papillary carcinoma patients (74.26%) than in the control population (62.45%; $P=0.0147$), reducing the risk for this type of cancer (odds ratio = 0.564; 95% confidence interval = 0.357–0.894). A multiple logistic regression analysis showed an inverse correlation between cigarette smoking ($P=0.0385$) and *CYP1A1* germline inheritance ($P=0.0237$) with the susceptibility to papillary carcinomas. We were not able to find any correlation between smoking, clinical features, parameters of aggressiveness at diagnosis or during follow-up, and any of the *GST* or *CYP* genotypes considered separately or in different combinations. We suggest that *CYP1A1* genotype might be associated with the reported reduced risk to papillary carcinomas among smokers.

Endocrine-Related Cancer (2006) **13** 1185–1193

Introduction

Epidemiologic studies show that 80–90% of all cancers are related to environmental factors, such as smoking, occupational, and dietary exposures (Doll & Peto 1981). It is well established that highly penetrant genes explain less than 5% of all cancers, but the proportion attributable to genetic polymorphisms of low penetrant genes, such as the ones involved in xenobiotic metabolism, and their interactions with environmental exposures are much less clear (Vineis 2002).

Cigarette smoking is directly responsible for approximately 90% of lung cancer cases, and is the leading cause of cancer-related deaths in the world (International Agency for Research on Cancer 2002, Levitz *et al.* 2004). Smoking is causally associated with oral cavity, laryngeal, oropharyngeal, and hypopharyngeal cancer and increases the risk of leukemia, sinonasal, nasopharyngeal, and esophageal cancer (International Agency for Research on Cancer 2002). Furthermore, cigarette smoking has been proved as a risk factor for developing cancer of the stomach and pancreas (International Agency for Research on Cancer 2002). Interestingly, the risk for thyroid cancer has been frequently reported as decreased in both men and women smokers, in the

DOI:10.1677/ERC-06-0002

Online version via http://www.endocrinology-journals.org

two major histological groups (papillary and follicular cancers) from all geographic regions (Mack *et al.* 2003).

Most procarcinogens in tobacco products require biotransformation and metabolic activation before they are able to react with DNA. This metabolic activation is generally initiated by phase I enzymes (Levitz *et al.* 2004). Biotransformation involves two stages: phase I, mainly controlled by enzymatic activity from cytochrome P-450 (CYP) family, and phase II, catalyzed by conjugation enzymes such as glutathione S-transferases (GST) and others. Phase I enzymes promote the activation of procarcinogens for the genotoxic electrophilic intermediaries. Phase II enzymes generally act as inactivating enzymes catalyzing the binding of intermediary metabolites into more hydrophilic products, thus facilitating their elimination (Vineis 2002). Therefore, the coordinated expression and regulation of both phases I and II enzymes and their metabolic equilibrium in the target organ cells can be important factors in determining the susceptibility to cancer as related to carcinogen exposure (Kawajiri *et al.* 1993, Vineis 2002).

CYP1A1 gene encodes for the enzyme aryl hydrocarbon hydroxylase (AHH), which plays a key role in phase I metabolism of estrogen and polycyclic aromatic hydrocarbons, such as those found in cigarette smoke, transforming them into carcinogens (Trell *et al.* 1985, Kawajiri *et al.* 1993). *CYP1A1* gene is located in chromosome 15q22–24 and various patterns of restriction fragment length polymorphism (RFLP) for this gene have been described. Two genetic polymorphisms of the *CYP1A1* gene have been reported to be associated with differences in the activity of the AHH enzyme activity – an isoleucine to valine substitution in exon 7 (m2 polymorphism) and a thymine/cytosine point mutation in the *MspI* restriction site (m1 polymorphism; Autrup 2000). Cytochrome P4501A1 (*CYP1A1*) m1 and m2 gene variants have been shown to be in close linkage disequilibrium and to be associated with a more inducible form of *CYP1A1* (Wu *et al.* 2002). The ensuing higher levels of the corresponding enzymes would result in an increased capacity to activate polycyclic aromatic hydrocarbons, producing highly reactive electrophilic intermediates that might damage DNA (Kawajiri *et al.* 1990).

We previously demonstrated that *GSTT1*, *GSTM1*, *GSTP1*, but not *GSTO1*, increased the risk of thyroid cancer (Morari *et al.* 2002, Granja *et al.* 2004*a*,*b*, 2005). We also showed that the Pro/Pro variant of *codon 72 of p53*, associated with a reduced p53 ability to activate apoptosis, could increase the risk for both papillary and follicular carcinomas (Granja *et al.* 2004*a*,*b*). We hypothesized that the combination of increased metabolic activation and decreased detoxification, together with an impaired cellular apoptotic function could lead to a high risk of thyroid carcinogenesis. Hence, the inverse epidemiologic association observed in the literature between smoking and thyroid cancer could be related to the germline inheritance of these genotypes. In addition, we aimed to further explore the influence of these genotypes on thyroid cancer patients' outcomes.

Material and methods

Patients

This case-control prospective study was approved by the Ethics Committee of the Medical Sciences School – State University of Campinas (FCM-Unicamp), and an informed written consent was obtained from all individuals. Patients consecutively referred to our Teaching Hospital – Medical Sciences School of the State University of Campinas for thyroid nodule evaluation between the years 1999 and 2005, were submitted to a careful clinical examination. The study population was composed of 80 cases of benign thyroid lesions, including 67 multinodular goiters and 13 follicular adenomas, as well as 168 cases of malignant thyroid tumors, including 136 papillary carcinomas and 32 follicular carcinomas. Differentiation stage and grade of the tumors were obtained from surgical and pathological records. Experienced pathologists of the Teaching Hospital confirmed all diagnoses. All cases were managed according to a standard protocol. The diagnosis of thyroid carcinoma was either established or suspected by fine-needle aspiration cytological study and/or by the histological analysis of thyroid tissues from patients who were referred to surgery due to thyroid nodules, presenting clinical or epidemiological suspicion of cancer. All patients were submitted to total or near-total thyroidectomy. Patients with preoperatively or intraoperatively palpable neck node metastases underwent regional neck dissection. Total body ^{131}I scans were performed, 4–6 weeks after the operations. All patients received 100 mCi ^{131}I. Long-term levothyroxine suppressive doses were administered following total body scan, in order to keep serum thyrotropin (thyroid-stimulating hormone; TSH) at low normal levels.

Data on lifetime occupational history, dietary habits, alcohol and drug consumption, medical history with emphasis on previous and/or present thyroid diseases, and other anamnestic data were obtained through interviews using a structured questionnaire.

Individuals with history of previous thyroid disease, accidental or medical radiation exposure, and antecedents of other malignancies were excluded. Skin color was determined by the interviewer, in accordance with the Brazilian Institute of Geography and Statistics (http://www.ibge.gov.br/english/), but, due to the difficulty in classifying our highly heterogeneous population, we further grouped individuals into whites and non-whites . Cigarette smoking habit was recorded but, due to the limited reliable data obtained on the duration of smoking, age started smoking, quantity smoked, and years since stopped smoking, the patients were grouped in never-smokers and ever-smokers categories. This last group included individuals who consumed at least 20 packages 20 cigarettes per pack for 1 year in the last 5 years. All data, including nodule size, tumor histological features, and laboratory examinations, were confirmed in the patients' records.

Follow-up

Cancer patients were followed with periodic total body scans, serum TSH, and thyroglobulin (Tg) measurements according to a routine follow-up protocol that included X-ray, ultrasonography, computer tomography scan, and other eventual procedures to detect distant metastasis for a period of 12–341 months (mean $\pm$ S.D. $=30\pm69$ months). Patients with high serum Tg levels (>2 mg/dl) and/or suspicious total body scans were submitted to a thorough image search. We defined tumors as recurrent and/or presenting long distance metastasis according to the above parameters.

Controls

A control group of 277 healthy individuals who were matched on the basis of gender, age, and ethnicity was selected from the general population of our region, considered to have a normal iodine intake. The history obtained from these subjects included demographic and ethnic background, diet routine, lifetime occupational history, smoking history, general health conditions, and previous diseases. Individuals with history of previous thyroid disease, radiation exposure, specific environment or occupational exposure risks, and antecedents of malignancy were excluded.

Identification of genotypes

Blood specimens were obtained from all patients and control individuals. Genomic DNA was extracted from frozen specimens and leukocytes were separated from whole blood using a standard proteinase K-phenol-chloroform protocol. *CYP1A1* genotypes at the m1 and m2 sites were analyzed by PCR followed by RFLP methods. Genotyping was conducted with blinding to case/control status. The primers for the m1 site 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 m2 were 5′-TTC CAC CCG TTG CAG CAG GAT AGC C-3′ and 5′-CTG TCT CCC TCT GGT TAC AGG AAG-3′, which generate a 204 bp fragment. These fragments were amplified separately but under similar conditions as follows: 25 μl volumes of a mixture containing 100 ng DNA, 10 μM of each primer, 10 mM Tris–HCl (pH 8.0), 0.1 mM of each diNTP, 2.0 mM $MgCl_2$, and 0.5 U Taq DNA polymerase. Amplifications were carried out for 35 cycles of 94 °C for 50 s, annealing temperatures of 60 °C for 45 s for m1 and 64 °C for m2 followed by 72 °C for 1 min, with an initial denaturation step of 94 °C for 5 min and a final extension step of 72 °C for 10 min using a Termocycler MJ PTC–200 PCR System. The PCR fragments were visualized in ethidium bromide-stained gels. The restriction enzyme MspI was used to identify the m1 polymorphism according to the manufacturer's protocol (Fermentas UAB, Vilnius, Lithuania). The wild-type allele has a single band representing the entire 340 bp fragment and the variant allele results in two fragments of 200 and 140 bp. The restriction enzyme BseMI was used to identify the m2 polymorphism. The wild-type allele has two fragments of 149 and 55 bp. The homozygote variant generates a single band representing the entire 204 bp fragment and the heterozygote variant presents the three bands, according to the manufacturer's protocol (Fermentas Life Sciences). The restricted products were analyzed by electrophoresis in 3% agarose gels containing ethidium bromide. RFLP results of *CYP1A1* m1 and m2 were confirmed by DNA sequencing of PCR products using ABI prism big dye sequencing kit (Perkin-Elmer, Warrington, Cheshire, UK) with an automated sequencer (ABI PRISM 377; Perkin-Elmer).

Part of the 277 control individuals and 248 thyroid nodule patients had participated in other studies previously carried out at our laboratory and were already genotyped for *GSTM1*, *GSTT1*, *GSTP1*, *GSTO1*, and *72 p53* (Morari *et al.* 2002, Granja *et al.* 2004*a*,*b*, 2005). Seventy-seven thyroid nodule patients and 66 control individuals had a complete evaluation of all the risk factors considered, including *CYP1A1*, *GSTT1*, *GSTM1*, *GSTP1*, *GSTO1*, and *72 p53* genotyping.

Statistical analysis

The statistical analysis was conducted using SAS statistical software (Statistical Analysis System, version 8.1, Cary, NC, USA, 1999–2000). Associations were assessed using 2X2 or 2Xn contingency table analysis and Chi-squared (χ^2) or Fisher's (F) exact tests were used to examine homogeneity between cases and controls regarding gender, color, previous thyroid disease, thyroid nodule size, use of medication, cigarette smoking, extent of the disease, and genotypes. The Kruskal–Wallis (KW) test was used to compare the ages among the groups. The Mann–Whitney or Wilcoxon tests were used to compare the age among the different genotype groups. The observed genotype frequencies were compared with those calculated using Hardy–Weinberg disequilibrium theory. The odds ratio (OR) and 95% CI provided a measure of the strength of association, e.g. indicating the increase in odds of a given thyroid nodule, demonstrating a particular genotype compared with the control population. In order to further explore the significance of *CYP1A1* genotypes in different ranges of age, we performed a univariate logistic regression analysis in patients under and over 45 years old after adjusting for gender. Logistic regression was used to evaluate the effect of all genotypes, after adjusting for other potential confounders such as age, gender, color, tobacco, and both alcohol and medication consumption. A multivariate logistic regression model was applied using the nodules diagnosis (malignant or benign) and the type of tumor (papillary or follicular carcinoma) as dependent variables and all genotypes and clinical risk factors, including gender, age, and cigarette smoking as explicative variables. All tests were conducted at the $P=0.05$ level of significance.

Results

Table 1 summarizes clinical characteristics and parameters of aggressiveness at diagnosis and during follow-up of the thyroid cancer patients. There were no differences between the control individuals and the thyroid disease patients regarding age (43 ± 34 vs 46 ± 94 years), gender (84 males and 193 females vs 57 males and 191 females) and color (230 white and 47 non-white versus 203 white and 45 non-white individuals). Also, cigarette-smoking habits were similar in control individuals and patients (28% ever smokers and 72% never smokers versus 33% ever smokers and 67% never smokers). The demographic and lifestyle characteristics of the subjects from both thyroid nodules and control groups, including alcohol consumption, red meat, vegetables and fat intake, education, and exercise were similar.

The proportion of *CYP1A1m1* and *CYP1A1m2* different genotypes Hardy–Weinberg equilibrium was tested in the population of 525 individuals genotyped in this study. Both variants were in equilibrium. The overall proportions of the *CYP1A1* genotypes in the control population and in the benign and malignant thyroid disease patients are presented in Table 2. The wild-type *CYP1A1m1* gene was present more frequently among patients with thyroid nodules (72.18%) than in the control individuals (62.45%) (χ^2; $P=0.0160$). There was no association between *CYP1A1m1* or *CYP1A1m2* genotypes and *GSTT1*, *GSTM1*, *GSTO1* or *72p53* genotypes, although a trend towards an association between *CYP1A1m2* and *GSTM1* null genotype was observed (χ^2; $P=0.0535$). There was no association of *CYP1A1* genotype and gender. When thyroid nodule patients under or over 45 years of age were analyzed separately, we observed that *CYP1A1* was associated to thyroid nodules only in patients over 45 years old. In these patients, *72p53* variants were also more frequent than in controls (χ^2; $P=0.0111$). The presence of a normal *CYP1A1m1* allele decreased the risk for a thyroid nodule by 47% (OR = 0.527; 95% CI = 0.306–0.907), while *Arg/Pro* or *Pro/Pro* variants of *p53* increased the risk for a thyroid nodule more than four times (OR = 4.287; 95% confidence interval (CI) = 1.395–13.176). Multiple logistic regression analysis adjusted for gender confirmed *CYP1A1m1* effect on individuals over 45 years old ($P=0.0351$; OR = 0.514; 95% CI = 0.277–0.954).

We observed that papillary carcinomas presented *CYP1A1* wild-type genotype more frequently (74.26%) than controls (62.45%) ($P=0.0147$). Indeed, the presence of a germline variant *CYP1A1m1* genotype protects against the risk to develop a papillary carcinoma (OR = 0.564; 95% CI = 0.357–0.894). Considering patients under and above 45 years of age, we found *CYP1A1m1* wild-type genotype to be more frequent among thyroid cancer older patients (78.04%) than in the controls (60.52%) ($P=0.0128$). The presence of a variant *CYP1A1m1* allele protects against thyroid cancer (OR = 0.5936; 95% CI = 0.3869–0.9114). The association between *CYP1A1m1* genotype and thyroid cancer risk is due to papillary carcinomas, as it disappeared in the relatively small group of follicular carcinomas. In addition, the association of *CYP1A1m1* to papillary carcinomas occurs only in patients over 45 years old, who presented *CYP1A1m1* wild-type allele in 82.53% of the cases, compared with only 60.52% of the controls

Table 1 Percentage distribution of thyroid carcinoma patients according to their histology and smoke habits comparing clinical features, including age (X ± S.D. in years), gender (F, female; M, male), color (W, white; NW, non-white); the presence of lymph node involvement, distant metastasis, and the stage at the time of the diagnosis; the diagnosis of recurrence and/or distant metastasis during the follow-up, and the molecular profile of papillary (PC) and follicular (FC) patients

		PC		FC	
		Smoker	Non-smoker	Smoker	Non-smoker
Clinical characteristics					
Age (X ± S.D.)		42 ± 12	45 ± 16.4	54.4 ± 16.5	45.7 ± 21.2
Sex	F (%)	65	93	67	96
	M (%)	35	7	33	4
Color	W (%)	89	82	83	82
	NW (%)	11	18	17	18
Diagnosis					
Lymph node (%)		16	12	8	9
Distant metastasis (%)		2	3	14	12
Stage	I + II (%)	67	66	51	53
	III + IV (%)	33	34	49	47
Follow-up					
% of recurrence and/or distant metastasis		14	10	23	21
Molecular date					
CYP1A1	M1 T/T (%)	73	76	67	54
	M1 T/C C/C (%)	27	24	33	46
CYP1A1	M2 Ile/Ile (%)	62	68	67	46
	M2 Ile/Val Val/Val (%)	38	32	33	54
GSTM1	Positive (%)	47	64	80	61
	Negative (%)	53	36	20	39
GSTT1	Positive (%)	87	82	90	73
	Negative (%)	13	18	10	27
GSTP1	Ile/Ile (%)	65	67	92	67
	Ile/Val Val/Val (%)	35	33	8	33
GSTO1	Ala/Ala (%)	81	84	76	87
	Ala/Asp Asp/Asp (%)	19	16	24	13
72p53	Arg/Arg (%)	50	55	67	25
	Arg/Pro Pro/Pro (%)	50	45	33	75

($P = 0.0025$). The chance of an individual over 45 years old harboring a variant *CYP1A1m1* allele to develop a papillary carcinoma is reduced by 64% (OR = 0.4571; 95% CI = 0.2589–0.8068). Also, *72p53* variants were overrepresented in the papillary carcinoma patients ($P = 0.0008$), increasing the risk for this malignancy over thrice (OR = 3.522; 95% CI = 1.686–7.357). A similar analysis revealed that *GSTP1* variants were overrepresented in the group of follicular carcinoma patients (72.22%) compared with control individuals (40.39%) ($P = 0.0367$), increasing the risk for this tumor 3.2 times (OR; 95% CI = 1.075–9.529).

The multivariate logistic regression analysis of the 249 thyroid nodules and 200 control individuals data corrected for gender and age showed a significant influence of *CYP1A1* genotypes ($P = 0.0078$; OR = 1.836; 95% CI = 1.173–2.872) on the risk for thyroid nodules. However, smoking was not an independent risk factor for thyroid cancer ($P = 0.0941$). The same multivariate logistic regression analysis model performed in the 168 malignant nodules revealed that *CYP1A1* genotype was associated with the risk of papillary carcinoma ($P = 0.0063$, OR = 2.243; 95% CI = 1.256–4.008) but not of follicular carcinoma. Again, smoking was neither a risk nor a protection factor against papillary ($P = 0.1570$) or follicular carcinomas ($P = 0.730$).

To further investigate the role of *CYP1A1* genotype and other susceptibility factors, we performed a multivariate logistic regression analysis corrected for gender and age, considering only the 77 thyroid tumors and 66 controls that had a complete evaluation of all clinical and pathologic risk factors and a complete genotyping analysis, including *CYP1A1m1*, *CYP1A1m2*, *GSTM1*, *GSTT1*, *GSTO1*, *GSTP1*, and *72p53*. Only *CYP1A1m1* ($P = 0.0373$) and smoking habits ($P = 0.0348$) had an inverse significant association with thyroid cancer risk. This association was maintained, with both *CYP1A1m1* ($P = 0.0237$) and smoking habits ($P = 0.0385$), in the analysis of the 47

Table 2 Distribution of *CYP1A1* (*m1* and *m2*), *GSTM1*, *GSTT1*, *GSTP1*, *GSTO1* and *p53codon72* wild-type and variant (homo and heterozygous) genotypes among all thyroid nodules, papillary carcinomas, follicular carcinomas, and benign nodule cases and control group individuals

	Control Individuals; ***N*** (%)	All thyroid nodules			Papillary			Follicular			Benign		
		Cases; *N* (%)	OR (95%CI)	*P*	Cases; *N* (%)	OR (95%CI)	*P*	Cases; *N* (%)	OR (95%CI)	*P*	Cases; *N* (%)	OR (95%CI)	*P*
CYP1A1m1													
Wild-type (T/T)	173 (62.45)	179 (72.18)	0.629 (0.431; 0.917)	0.0160	101 (74.26)	0.564 (0.357; 0.894)	0.0147	19 (59.38)	1.148 (0.538; 2.447)	0.7210	59 (73.75)	0.5921 (0.3401; 1.031)	0.0832
Polymorphic (T/C–C/C)	104 (37.55)	69 (27.82)			35 (25.74)			13 (40.63)			21 (26.25)		
CYP1A1m2													
Wild-type (Ile/Ile)	180 (64.98)	161 (64.92)	1.033 (0.716; 1.492)	0.8618	90 (66.18)	0.960 (0.620; 1.487)	0.8545	16 (50.00)	2.026 (0.957; 4.291)	0.0650	55 (68.75)	0.8435 (0.4948; 1.438)	0.6226
Polymorphic (Ile/Val—Val/Val)	97 (35.02)	87 (35.08)			46 (33.82)			16 (50.00)			25 (31.25)		
GSTM1													
Positive	114 (55.88)	124 (58.49)	1.046 (0.702; 1.560)	0.8246	70 (60.87)	1.134 (0.703; 1.830)	0.6055	17 (62.96)	1.282 (0.553; 2.976)	0.5625	37 (52.86)	1.130 (0.6552; 1.948)	0.7643
Negative	90 (44.12)	88 (41.51)			45 (39.13)			10 (37.04)			33 (47.14)		
GSTT1													
Positive	157 (76.96)	150 (78.53)	1.088 (0.670; 1.767)	0.7346	77 (81.05)	1.218 (0.656; 2.264)	0.5320	17 (77.27)	0.954 (0.329; 2.765)	0.9315	56 (75.68)	1.074 (0.5757; 2.002)	0.9494
Negative	47 (23.04)	41 (21.47)			18 (18.95)			5 (22.73)			18 (24.32)		
GSTP1													
Wild-type (Ile/Ile)	121 (59.61)	113 (62.78)	0.766 (0.497; 1.180)	0.2265	60 (64.52)	0.789 (0.466; 1.335)	0.3769	5 (27.78)	3.200 (1.075; 9.529)	0.0367	48 (69.57)	0.6456 (0.3598; 1.158)	0.1836
Polymorphic (Ile/Val—Val/Val)	82 (40.39)	67 (37.22)			33 (35.48)			13 (72.22)			21 (30.43)		
GSTO1													
Wild-type (Ala/Ala)	159 (86.41)	91 (82.73)	1.486 (0.737; 2.997)	0.2688	53 (84.13)	1.379 (0.595; 3.197)	0.4533	6 (75.00)	2.011 (0.175; 13.705)	0.7002	32 (82.05)	1.391 (0.5542; 3.492)	0.6496
Polymorphic Ala/Asp—Asp/Asp	25 (13.59)	19 (17.27)			10 (15.87)			4 (25.00)			7 (17.95)		
condon72 of p53													
Arg/Arg	35 (30.17)	56 (51.85)	2.430 (1.297; 4.552)	0.0056	37 (58.73)	3.522 (1.686; 7.357)	0.0008	2 (25.00)	0.494 (0.029; 4.248)	0.8278	17 (45.95)	0.5084 (0.2381; 1.085)	0.1177
Pro/Pro + Arg/Arg	81 (69.83)	52 (48.15)			26 (41.27)			6 (75.00)			20 (54.05)		

papillary carcinomas that had all genotypes studied, but not in the follicular carcinomas.

There were no differences between thyroid follicular adenomas and carcinomas concerning any of the studied risk factors. There was no association between any of the studied genotypes, either considered alone or in combination, and the histology or any parameter of aggressiveness at diagnosis or during follow-up.

Discussion

A continuous increase in the incidence of thyroid cancer has been registered during the past decades in Brazil as well all over the world (Parkin *et al.* 1997, Haselkorn *et al.* 2000, Liu *et al.* 2001, Burgess 2002, Coeli *et al.* 2005, Reynolds *et al.* 2005). This fact is certainly due in part to the larger use of better diagnostic tools such as the cytology obtained through fine-needle aspiration biopsy and ultrasonography. However, the incidence continues to increase in well-developed regions, and the major difficulty in understanding the reason is the fact that the causes for well-differentiated thyroid cancer are not yet fully known. Likewise, other tumors, thyroid cancer is related to environmental factors but, in addition to the exposure to ionizing radiation, no other physical, chemical, or biologic factor has been proven to cause thyroid cancer thus far (Ron *et al.* 1995, Jacob *et al.* 2006).

A series of polymorphisms in germline DNA have been investigated in an effort to delineate polygenic models of cancer susceptibility. Such models are particularly interesting in thyroid cancer. Indeed, thyroid nodules are detected by ultrasonography in up to 67% of the population, but few are malignant and require surgical treatment (Castro & Gharib 2005). Therefore, screening tools designed to identify individuals at risk for thyroid cancer, select persons for specific preventive or diagnostic interventions, and determine which patients with thyroid nodules are most likely to benefit from specific therapies, are an upmost necessity. Genes involved in xenobiotic metabolism may be interesting for this sort of screening. The interindividual variability in the ability to biotransform potentially toxic substances has been associated with greater or lesser susceptibility to toxicity or cancer risk. Individuals incapable of adequately detoxifying a toxic agent or a metabolic carcinogen would undergo greater DNA and cell damage, with the formation of adducts or chemical elements bound to the DNA and protein macromolecules, causing genomic instability. Consequently, these individuals would be at greater risk of developing tumors (Wogan *et al.* 2004).

A series of studies conducted in different populations have found a correlation between *CYP1A1* polymorphisms and different types of cancer. Consistent evidences for association between CYP polymorphisms and many human tumors have been reported, generally connecting a higher activity of phase I oxidative pathway to enhanced carcinogenesis (Vineis 2002, Agundez 2004). Controversial findings suggest that colorectal and prostate cancers may be associated with *CYP* polymorphisms, whereas no evidences for a relevant association with breast or bladder cancers have been reported (Vineis 2002, Agundez 2004). Our data demonstrated an inverse significant association between *CYP1A1*m1 and smoking habits in the risk of thyroid cancer. To our knowledge, this is the first report on *CYP1A1* influence on thyroid tumorigenesis. Interestingly, we found *CYP1A1* wild-type allele to be more frequent both in thyroid nodules and in papillary carcinomas than in the control population, suggesting that cigarette smoking and other metabolites that depend on *CYP1A1* activation are neither implicated in the risk of thyroid goitrogenesis nor in the following processes that lead to thyroid malignancy. This observation fits very well with the epidemiologic observations that were not able to associate cigarette smoking with thyroid cancer or which even found a lower risk for thyroid cancer among smokers. Indeed, 12 out of 13 case-control studies that examined the association between cigarette smoking and thyroid cancer found the risk of thyroid cancer to be decreased by 40% among smokers (Mack *et al.* 2003). Cohort studies, including a recent prospective Canadian one, were not able to demonstrate any association between smoking history and the risk of thyroid cancer (Iribarren *et al.* 2001, Navarro Silvera *et al.* 2005).

On the other hand, our data demonstrated that *CYP1A1* influenced thyroid nodules and papillary carcinoma risk only in individuals over 45 years old, suggesting that the pathogenesis of thyroid tumors could be related to the continuous exposure to one or more carcinogenic products metabolized by phase I oxidative enzymes. Since the enzymes encoded by detoxifying genes are substrate and tissue specific, and they act in conjunction, it is very difficult to explain the role each one plays. Different environmental factors could also be implicated in the pathogenesis of follicular carcinomas, explaining why we were not able to establish any significant association between *CYP* genotypes, smoking, and the risk for this type of tumor. However, the relatively small number of follicular carcinomas included in this study prevents any further consideration.

We did not find any additive or combined effect of the phase I and phase II genes studied in the

risk for thyroid cancer or in the patients outcome, but we observed a distinct profile of susceptibility genes in different ranges of ages, confirming our previous observations on the role of *72 p53* and GSTs in thyroid cancer risk (Morari *et al.* 2002, Granja *et al.* 2004*a*,*b*, 2005).

In conclusion, we demonstrated an inverse association between germline *CYP1A1* inheritance and smoking with the risk of thyroid nodules and papillary carcinomas that may help to explain the reduced risk of differentiated thyroid cancer in tobacco consumers observed in epidemiologic studies.

Acknowledgements

We are grateful to Professors Denise Wittman-Zantut and Elizabeth Pavin for helping us to obtain some of the patients with thyroid benign hyperplasia included in this study. We also acknowledge the skillful contribution of Helio Akinori Kitagaki Junior and Rafael Souza Queiroz to the genotyping assays. This study was supported by the State of São Paulo Research Foundation – FAPESP, under the grant numbers 03/02309-7 and 03/11026-9. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Identifying a Risk Profile for Thyroid Cancer

ABSTRACT

The large use of simple and effective diagnostic tools has significantly contributed to the increase in diagnosis of thyroid cancer over the past years. However, there is compelling evidence that most micropapillary carcinomas have an indolent behavior and may never evolve into clinical cancers. Therefore, there is an urgent need for new tools able to predict which thyroid cancers will remain silent, and which thyroid cancers will present an aggressive behavior. There are a number of well-established clinical predictors of malignancy and recent studies have suggested that some of the patient's laboratory data and image methods may be useful. Molecular markers have also been increasingly tested and some of them appear to be very promising, such as BRAF, a few GST genes and p53 polymorphisms. In addition, modern tools, such as immunocytochemical markers, and the measure of the fractal nature of chromatin organization may increase the specificity of the pathological diagnosis of malignancy and help ascertain the prognosis. Guidelines designed to select nodules for further evaluation, as well as new methods aimed at distinguishing carcinomas of higher aggressiveness among the usually indolent thyroid tumors are an utmost necessity. **(Arq Bras Endocrinol Metab 2007;51/5:713-722)**

Keywords: Predisposition factors; Environment; Susceptibility genes; Outcome

RESUMO

Identificando um Perfil de Risco para Câncer de Tiróide.
O uso cada vez mais freqüente de métodos diagnósticos simples e efetivos tem contribuído significativamente para um aumento no diagnóstico de câncer da tiróide nos últimos anos. Entretanto, existem importantes evidências de que muitos dos microcarcinomas papilíferos têm um comportamento indolente e podem nunca evoluir para cânceres clínicos. Existe, portanto, uma necessidade urgente de desenvolver novas ferramentas capazes de predizer quais os tumores tiroidianos que permanecerão silenciosos e quais desenvolverão comportamento agressivo. Há uma série de marcadores clínicos de evolução bem estabelecidos e alguns estudos recentes sugerem que dados laboratoriais e métodos de imagem podem ser úteis. Marcadores moleculares também vêm sendo ativamente investigados e alguns, como BRAF, os genes GST e polimorfismos de p53, parecem promissores. Além disso, marcadores imunocitoquímicos e a medida da natureza fractal da cromatina podem aumentar a especificidade do diagnóstico anatomopatológico e ajudar a predizer o prognóstico. Existe uma necessidade imperiosa de elaborarmos diretrizes destinadas a selecionar os nódulos que merecem prosseguimento em sua avaliação, assim como novos métodos capazes de identificar lesões mais agressivas entre os geralmente indolentes tumores tiroidianos. **(Arq Bras Endocrinol Metab 2007;51/5:713-722)**

Descritores: Fatores de predisposição; Meio ambiente; Genes de susceptibilidade; Evolução

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Recebido em 22/02/07
Aceito em 14/04/07

Recent improvements in medical technology have increased the detection of nodular thyroids, raising the incidence of incidental nodules to epidemic levels. Indeed, thyroid nodules may be detected in as much as 16% of imaging studies performed for other purposes on neck and chest computer

tomography scans, magnetic resonance, carotid Doppler studies and, especially, ultrasonography (1). The physician, encountering one of these lesions, is faced with a dilemma on which is the best course of management of this incidental finding (2). Fine-needle cytology has been more frequently indicated in view of the anxiety of most patients and the lack of reliable parameters of malignancy, this is certainly one of the causes of the increase in thyroid cancer detection we have been observing over the past decades (3). Aggressive evaluation of nodular thyroids has consequently contributed to a sharp increase in the number of small papillary carcinomas operated (3). However, there is compelling evidence that most of these tumors would never evolve clinically.

THYROID CANCER BIOLOGY

Microcarcinomas, defined by the World Health Organization as carcinomas 1.0 cm or less in diameter, have been described in 1% to 35.6% of autopsy studies and 5.5% to 10.5% of thyroid glands removed due to causes other than malignancy (4-11). Considering that thyroid cancer incidence in Brazil, as in most countries, presents in no more than 0.3% of men and 1% of women, a great part of microcarcinomas detected by ultrasonography in autopsies or surgical specimens will probably never evolve into clinical cancers (3,12). Searching for indicators of the clinical evolution of papillary microcarcinomas, we studied a total of 32 lesions identified during autopsy and in surgical specimen material. These lesions were found in 7.8% of the 166 consecutive autopsies examined and 7.2% of 261 thyroids that were surgically removed due to thyroid diseases in general, with a higher incidence between the ages of 30 and 49 years (13). Both genders were similarly affected: 9.3% of men and 8.8% of women in autopsy series, and 6.2% of men and 7.3% of women in surgical series, suggesting that hormonal factors may favor the subsequent development of clinical lesions in women (14). Indeed, the higher incidence of thyroid carcinoma in women during reproductive years compared to men, and the increased risk associated with the therapeutic use of estrogens have suggested a pathogenetic role exerted by these steroids in the development of thyroid cancer (14). In addition, gender is a significant prognostic marker, since women with differentiated thyroid carcinomas show a better survival than men in our own data and that of others (15,16). Recent studies have provided a new insight into the molecular mechanisms through which estrogens may induce the progression of thyroid cancer, demonstrating that the G protein-coupled receptor 30 (GPR30) and the mitogen-activated protein kinase (MAPK) pathway mediate both the up-regulation of c-fos and the growth response to 17β-estradiol (E2), genistein (G), and 4-hydroxyta-moxifen (OHT) in thyroid cancer cell cultures (17).

In addition to estrogens, other factors may certainly take part in the thyroid cancer pathogenetic process. It is reasonable to think that risk factors of thyroid cancer may also define tumor behavior and, in consequence, their outcome. The American Joint Committee on Cancer and International Union Against Cancer, together with the National Comprehensive Network (AJCC/UICC and NCCN), summarized the risk factors for metastases, recurrence and fatal outcome, taking into account patient and tumor data, as demonstrated in table 1 (18).

Unfortunately, most prognostic factors depend on tumor examination, preventing any other action before surgical intervention. Prognostic classification and risk group stratification have improved during the last two decades with a deeper understanding of the biology of well-differentiated thyroid tumors. However, level 1 evidence is not yet available, there are no prospective randomized trials based on a variety of treatments, mainly total versus less than total thyroidectomy and surgery versus observation for papillary microcarcinomas. Decisions on thyroid cancer management are based mainly on retrospective studies used to define staging systems. The most common

Table 1. Prognostic factors for metastasis, recurrence and death risk from differentiated thyroid carcinomas (18).

Patients	Tumor
Age < 15 years or age > 45 years	Tumor: large (> 2 cm)
Male gender	Localization: multifocal, bilateral
History of familial thyroid cancer	Local tumor invasion: beyond the capsule
	Subtypes: tall, columnar and Hürthle cell
	Nuclear atypia and tumor necrosis: accentuated
	Vascular invasion: present
	Cervical or mediastinal lymph nodes
	Distant metastases
	Low or no iodide uptake by the tumor and/or the metastases

staging system, TNM (tumor, node, metastasis), which adheres to the biology of tumors, has been extensively reviewed over the years (19), and has been successfully used for over 65 years in thyroid cancer patients (20). However, the literature still lacks an analysis of the TNM and other scoring systems (OSU, ACES, AMES, etc.), as well as accuracy and utility concerning papillary microcarcinomas. Our data indicated that extrathyroidal extension and lymph node metastases, which are important TNM elements, do not correlate with outcomes of patients with papillary microcarcinomas (21). In a cohort of 68 patients with papillary microcarcinomas followed-up for 103 months (15 to 289 months), we observed no deaths but a relatively high number (19.1% of the cases) of unfavorable events, such as local recurrence and even long-distance metastasis (21). These patients responded well to the therapeutic measures: 50% of TNM III or IV PMC patients remained free of disease during the follow-up. Therefore, TNM alone was not able to predict favorable or unfavorable outcome of these patients, as demonstrated in figure 1.

In addition, the independent value for each parameter needs to be determined in a multivariate analysis of all risk factors for each individual patient (22). Moreover, the influence of good number risk factors for differentiated thyroid cancer on the prognostics of these tumors has not been investigated. A population-based nested case-control study of the 5,554 differentiated thyroid cancer patients diagnosed in Sweden from 1958 to 1978 investigated the cause of death of thyroid cancer patients matched by age at diagnosis, gender, and period calendar controls (23). The authors aimed to investigate how factors such as smoking, number of children, previous thyroid disorders, previous radiotherapy toward the neck, family history of thyroid diseases and malignancies influenced survival. The analysis of the 595 cases and controls showed that smokers had a borderline significant increased risk of dying from differentiated thyroid cancer. Previous radiotherapy towards the neck region had no prognostic implication. A family history of differentiated thyroid cancer influenced prognostics although not significantly in a few cases (23). The remaining risk factors studied did not influence survival. The authors concluded that smokers appeared to have a worse prognostic compared to nonsmokers, and a family history of thyroid cancer had a non-significant negative effect on survival (23). Our data on micropapillary carcinomas showed a similar trend (13). Although associated nodular goiter was observed in 54% of autopsies and 26% of surgical specimens, and Hashimoto's thyroiditis was observed only in surgical material (15% of the cases), we were not able to correlate risk of malignancy with any concomitant lesion and we could not find risk factors for clinical evolution evaluation (13). However, we observed that the smallest papillary microcarcinomas appeared most frequently as nonencapsulated nonsclerosing tumors without inflammatory infiltrate or fibrosis, suggesting that they could represent the early stages of development thereby raising questions concerning the role of the immune system in delaying the progression of these tumors (13). There is evidence that oncogene-induced cytokine secretion is important for the development and progression of thyroid carcinomas in genetically permissive hosts (24). We demonstrated earlier that the odds for patients with a previous history of thyroid autoimmune disease ($p< 0.02$) or with thyroid autoantibodies ($p< 0.001$) having a worse outcome were lower than for patients with no evidence of autoimmune activity, suggesting that autoimmune activity against the gland may exert a protective effect on the outcome of patients with differentiated thyroid

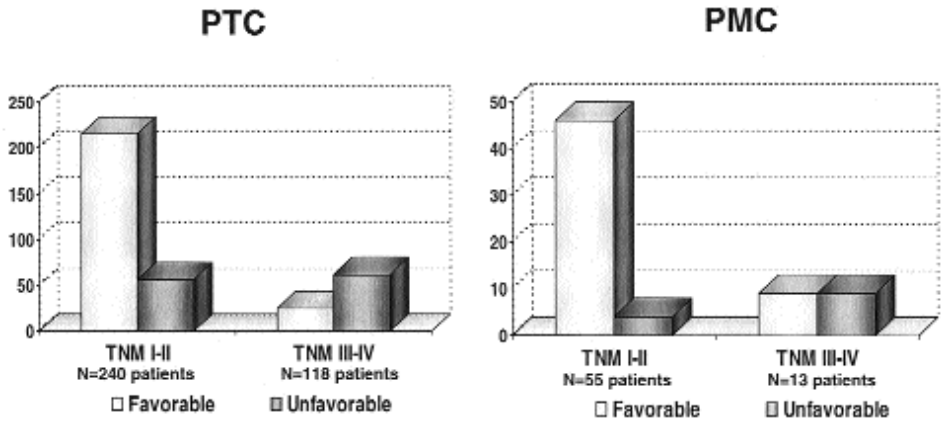

Figure 1. Graphical comparison between TNM classification system and outcomes, defined as favorable or unfavorable, in 68 papillary thyroid microcarcinomas with less than 1 cm in diameter (PMC), and in 358 papillary thyroid carcinomas larger than 1 cm (PTC non-PMC).

carcinoma (25). Markers of immune activation against the neoplastic tissue may be useful.

The literature is quite intriguing regarding the influence of smoking habits on thyroid cancer. Many epidemiologic observations were not able to associate cigarette smoking with thyroid cancer and some even found a lower risk for thyroid cancer among smokers. Twelve out of 13 case-control studies that examined the association between cigarette smoking and thyroid cancer found the risk of thyroid cancer to be decreased by 40% among smokers (26). Cohort studies, including a prospective Canadian one, were unable to demonstrate any association between smoking history and the risk of thyroid cancer (27). In addition, we were not able to find any correlation between smoking, clinical features, parameters of aggressiveness at diagnosis or during follow-up of 248 patients with thyroid nodules, including 67 benign goiters, 13 follicular adenomas, 136 papillary carcinomas and 32 follicular carcinomas, and 277 controls with similar ethnic backgrounds (28). Why would smoke influence the outcome of thyroid cancer patients in the Swedish study (23)? It is possible that other effects of the many toxic compounds existing in cigarette smoke, on other organs or systems, besides the thyroid, were responsible for this influence. Nevertheless, the role of smoke on thyroid cancer outcome remains to be elucidated. Likewise, the influence of the genetic profile and environmental factors on thyroid cancer biology remains largely unknown.

It has been proposed that incidental cancer found by histological examination of goiters would hold lower potential aggressiveness than cancers detected by fine-needle aspiration considering prognostic features, such as multifocality, lymph node metastasis and extracapsular invasion (29). On the other hand, there are many reports on the aggressive behavior of papillary microcarcinomas, which evolve not only with lymphatic regional metastases, but also with blood-borne lung, bone and brain metastases (30,31).

What do you know about differentiated thyroid cancer and how can you use this knowledge in order to define susceptibility profile and outcome for differentiated thyroid cancer?

ENVIRONMENTAL AND GENETIC RISK FACTORS FOR DIFFERENTIATED THYROID CARCINOMAS

Thyroid cancer is the most frequent endocrine cancer with a geographic variation in its incidence and manifestations (32). While it does not account for more than 1% of all human cancers in most countries, in other regions such as the Middle East, thyroid cancer is the second most common neoplasm among women (33). Brazilian data also show a highly distinctive incidence of thyroid tumors in different regions of the country (12). This great variation in incidence reflects differences not only in the access to health care and possibly diverse methods or thoroughness of thyroid examination, but also in environmental and genetic characteristics of the populations studied.

Exposure to ionizing radiation, especially during childhood, remains the only factor clearly associated with benign and malignant thyroid tumors in humans (34), although there is strong epidemiological evidence pointing towards the involvement of geographic, ethnic and dietary factors in the risk of sporadic thyroid cancer (32,34,35).

The most relevant genetic alterations identified so far in the different progression stages of thyroid tumors include *RAS* mutations in follicular tumors (36), *RET* gene rearrangements and *BRAF* mutations in papillary carcinomas (37,38), *PPARγ-PAX8* mutations in follicular carcinomas (39), and *p53* mutations in poorly differentiated and anaplastic carcinoma (40). There is compelling epidemiologic, experimental and clinical evidences that exposure to ionizing radiation may trigger a series of abnormalities related to these genes' activation or inactivation, aside from producing genetic instability (41). A low-level genomic instability may even be a feature of papillary thyroid carcinoma (42). However, we still do not understand why some individuals exposed to ionizing radiation develop thyroid cancer whereas others do not. The age at exposure has been shown to be a critical risk factor for developing thyroid carcinoma after exposure to fallout from Chernobyl, with those under the age of 1 showing a much greater risk than older children. There is a rapid decline to a level of relatively low risk for young adults (43,44). A similar situation was portrayed in Nagasaki and Hiroshima, where atomic bomb survivors displayed one of the highest solid tumor risk estimates (45). The likely reasons for this age-related risk include iodine radioisotope intake, radioiodine uptake, and biological sensitivity factors. The existence of biological sensitivity factors is confirmed by the finding of a similar, although less marked age-related, sensitivity to thyroid carcinomas after exposure to external radiation (46). Two main reasons for this sensitivity could be either the mitotic rate at the time of radiation exposure or the number of mitoses that occur in the progeny of the mutated cell, or perhaps both (47).

What other environmental factors may also contribute to the increasing incidence of cancer, not only in regions affected by fallout but in other countries as well?

Iodine has long been recognized as an important factor in thyroid cancer pathogenesis. Iodine-deficient thyroid glands will of course show a high radioactive iodine uptake, leading to the prediction that risk of developing thyroid carcinoma after exposure to radiation would be greater in areas with greater iodine deficiency. In fact, the type and consequent aggressiveness of thyroid tumors seem to be related to the population's iodine intake, although a clear relationship between iodine supply to the population and sporadic thyroid cancer incidence, as well as its alleged physiopathology are still unclear (48). This issue is discussed by Nobel and Medeiros-Neto in another article of this same journal.

Some of the genes related to thyroid carcinogenesis have been related to tumor aggressiveness and, hence, to prognostics. Many studies, using both immunocytochemical and genetic analyses, have shown that *p53* mutations are highly prevalent in poorly differentiated and undifferentiated thyroid carcinomas, as well as in thyroid cancer cell lines (40,49). However, these mutations are not found in benign tumors and are infrequent in well-differentiated cancers, suggesting that mutational inactivation of *p53* occurs at a late stage of thyroid tumor progression and may represent a key event in the progression from differentiated to anaplastic carcinomas (50,51). Preliminary data of our group using immunohistochemistry for p53 in 34 papillary carcinomas, including 21 cases of the classical histological type, 7 tall cell variants and 6 of the follicular variant type; 16 follicular carcinomas; 4 medullary and 3 anaplastic carcinomas did not confirm a relationship between the expression of p53 and prognostics. However, the Kaplan-Meier survival curve of these patients, displayed in figure 2, suggests that a longer follow-up and/or a larger cohort may prove p53 to be a reliable immunohistochemical, and perhaps even a immunocytochemical, prognostic marker. Furthermore, we tested the gene *MUC*, which was described as overexpressed in aggressive thyroid carcinomas (52). MUC1 overexpression is a key molecular event in the pathogenesis of aggressive thyroid tumors (53). In malignancy, MUC1 looses its polarized expression, redistributes, and is expressed on the whole cell surface. This redistribution shields other cell surface molecules from their ligands and interferes with integrin-mediated adhesion to the extracellular matrix and with cadherin-mediated cell-cell adhesion. Increased MUC1 expression thereby promotes cellular dissociation and oncogenic progression (53). MUC1 upregulation also protects tumor cells from immune recognition and destruction by the cellular arm of the immune system. Moreover, it has been shown to inhibit human T-cell proliferation, thereby contributing to cancer-propagated immunosuppression (53). Previous studies from Wreesmann et al. identified MUC1 expression in thyroid slides (52). We were able to reproduce this technique, but in the 50 differentiated thyroid tumors studied in a preliminary protocol, we were not able to associate MUC expression with the patients' outcome (figure 3).

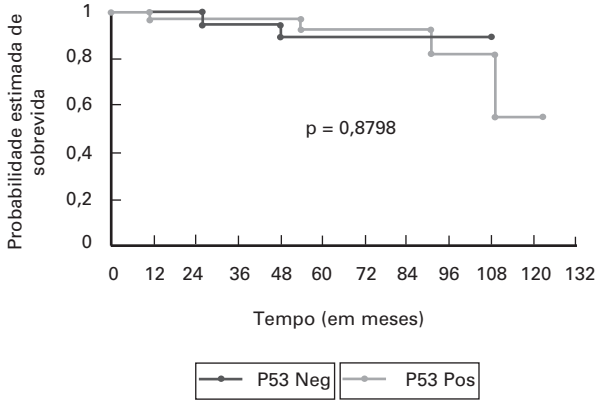

Figure 2. Kaplan-Meier survival curve (estimated probability of survival) of 50 differentiated thyroid cancer patients that were either immunohistochemically p53 positive or negative followed-up for 120 months.

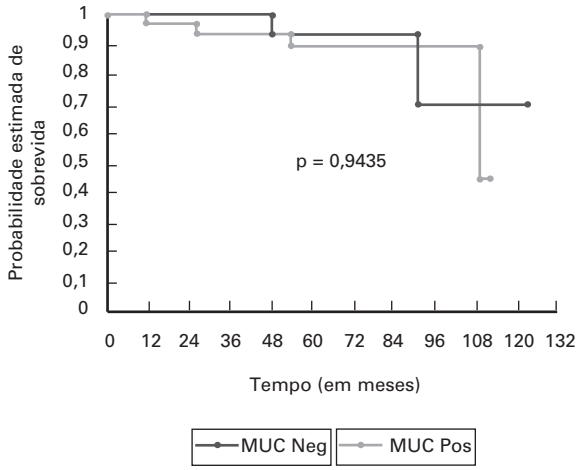

Figure 3. Kaplan-Meier survival curve (estimated probability of survival) of 50 differentiated thyroid cancer patients that were either immunohistochemically MUC positive or negative followed-up for 120 months.

We also investigated a gene that encodes a key protein for thyroid cancer detection and treatment and therefore related to patients' outcome: *NIS*. The sodium iodide symporter (NIS) protein expression and functional integrity is essential to assure a radioiodine uptake high enough to detect and destroy any tumoral thyroid tissue. The absence of radioiodide uptake is associated with high recurrence rate and reduced survival (54). We previously demonstrated that a low expression of NIS mRNA levels, quantified by real-time PCR, identifies aggressive thyroid tumors (55). Unfortunately, we were not able to find any relationship between NIS protein expression and patients' outcome in the same cohort mentioned above. Once again, the Kaplan-Meier survival curve of these patients, displayed in figure 4, suggested that a longer follow-up and/or a larger cohort may prove NIS to be a useful immunohistochemical and perhaps even immunocytochemical prognostic marker, although our preliminary data were not statistically significant in terms of outcome prediction.

Other genes directly related to thyroid tumorigenesis have actively been investigated as potential prognostic markers. One of the most exciting is BRAF. In vitro and in vivo models have demonstrated that overexpression of activated BRAF induces malignant transformation and aggressive tumor behavior, further discussed in another article of this issue. BRAF and other RAF kinases are frequently activated by other thyroid oncogenes and are important mediators of their biological effects, including dedifferentiation and proliferation. Two large studies showed that BRAF mutation predicts a poorer clinical prognosis for papillary thyroid cancer (56,57). However, a recent study from the Pisa group challenged these studies. A review of 61 papillary thyroid carcinoma patients with BRAF mutations, followed-up for 6 years, did not show evidence of any prognostic utility for BRAF (58).

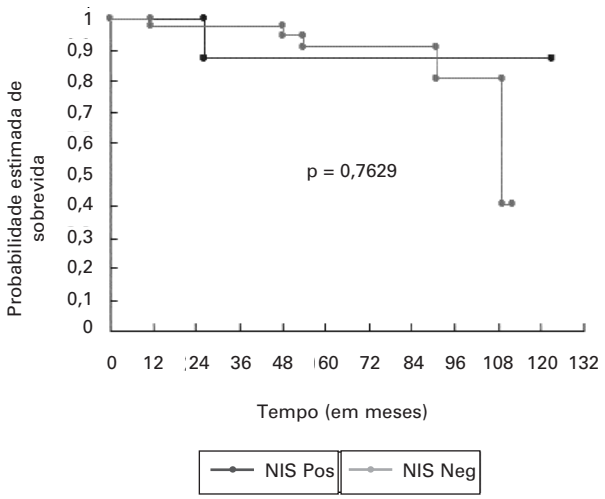

Figure 4. Kaplan-Meier survival curve (estimated probability of survival) of 50 differentiated thyroid cancer patients that were either immunohistochemically NIS positive or negative followed-up for 120 months.

In addition to the genes directly related to thyroid follicular cell differentiation, low-penetrance genes or rather polymorphisms in such genes could be of great significance to understand the tumorigenic processes of thyroid carcinomas.

DETOXIFICATION SYSTEMS AND THYROID CARCINOMA SUSCEPTIBILITY

As discussed, the thyroid cancer etiology is markedly uncertain. A wide variety of drugs, pesticides, goitrogenic xenobiotics and chemicals have been shown to increase the incidence of thyroid tumors in rodents (59-61). However, chemicals have seldom been associated with human thyroid cancer, in contrast to lung, bladder, and many other cancers. No increase in the human thyroid cancer risk has ever been consistently observed with any drug (62,63). On the other hand, there is compelling evidence that environmental factors, besides ionizing radiation, influence cancer incidence. Thyroid cancer is the fastest growing type of cancer in the USA with a 6.3% increase during the period of 1997–2003 and we have data suggesting that in Brazil it is not different (12,64). However, the rate of increase as well as the incidence of thyroid cancer varies in different geographic and ethnic group regions, including individuals exposed to well-defined factors, such as ionizing radiation (44-47,64-67). The question may be posed as to why some individuals are more susceptible to environmental aggressions? The biochemical basis for this susceptibility is related to genetic polymorphisms that normally occur in the general population regarding genes involved in the predisposition to a specific cancer, in the metabolic activation or detoxification of environmental genotoxins, as well as in controlling DNA repair or cellular damage (67-72).

Several polymorphic genes, encoding for enzymes involved in the biotransformation of carcinogens, have been studied as possible cancer risk modifiers. One of the most primitive defense mechanisms against environmental carcinogens is a supergene family of dimeric enzymes that are expressed in probably all life forms, the glutathione S-transferase (73). These enzymes catalyze the conjugation of glutathione to a variety of electrophiles, including arene oxides, inorganic arsenic, unsaturated carbonyls, organic halides and other substrates or oxida-

tive stress products (74). We demonstrated that the inheritance of null forms of genes codifying some of these enzymes (75), as well as of polymorphic gene variants that produce less active forms of enzymes (76), increase the susceptibility to thyroid differentiated cancer. Other polymorphic gene systems of defense may be involved in thyroid cancer risk. Preliminary data from our group showed that N-acetyl transferase 2 genes as well are involved in thyroid cancer risk (77).

Among the genes involved in cellular repair, p53 polymorphism is one of the most interesting. This polymorphism has been shown to have varying ethnic and geographical distribution, like most genetic human polymorphisms. It has been reported to be a potential genetic risk factor for many types of cancer, and we as well as others have demonstrated that it is also involved in thyroid cancer risk (78,79).

Many other systems may possibly be involved in the complex interaction between human beings and environment that ultimately leads to cancer. A series of promising reports identifying polymorphisms in germline DNA have been published in an effort to delineate polygenic models of thyroid cancer susceptibility and prognosis (80-82). Such models are particularly interesting since they may help select individuals for specific chemopreventive interventions and determine which patients with nodules or with microcarcinomas are most likely to benefit from specific therapies. Unfortunately, these studies have produced inconsistent results mostly derived from small samples but also from population stratification secondary to ethnic diversity, heterogeneity of the therapeutic measures employed as well as consideration being limited to only one rather than combinations of polymorphisms. In addition, an important limitation in case-control studies is the fact that some population controls may have undetected thyroid lesions that could mislead the interpretation of the observed associations. Our studies have the advantage of using the Brazilian population, which presents a highly heterogeneous ethnic composition. Nonetheless, larger cohorts and metanalysis are needed. Hopefully, a panel of molecular markers will be able to identify early on life those at risk for thyroid cancer, hence helping define preventive strategies for these individuals in a near future.

PREDICTORS OF THYROID OUTCOME

The thyroid cancer patient needs to be reevaluated at each clinical appointment. As stated in the Guidelines also published in this issue of the ABEM, this evaluation outlines the patient's outcome and defines further strategies.

Laboratory data

Thyroid hormone suppression lessens disease progression in high-risk papillary thyroid carcinoma (83). Recent data indicate that thyroid hormone suppressive therapy that yields serum TSH levels in the subnormal range also improves overall survival of patients with stage II differentiated thyroid cancer, but no additional improvement is associated with further degrees of thyroid hormone suppression (84). These data reinforce our Guideline recommendations: more aggressive thyroid hormone suppressive therapy is warranted in high-risk patients, whereas less aggressive thyroid hormone suppressive therapy aimed to maintain TSH levels slightly below normal is indicated in low-risk patients. Moreover, TSH levels may be considered of prognostic significance, and add value to thyroglobulin (Tg) measurement, which remains the most important predictor of outcome currently available for operated thyroid cancer patients (85-87).

Images

Nodule appearance on ultrasonography, together with ultrasound-guided fine-needle aspiration cytology, and neck exam during thyroid cancer follow-up are cornerstones of the current management of thyroid lesions. As more comprehensively discussed elsewhere in this issue of the ABE&M, ultrasonography is superior to other imaging methods, such as magnetic resonance imaging or computed tomography scan, except in particular cases (88,89).

More recently, ^{18}F-FDG PET/CT has emerged as a promising imaging modality to evaluate thyroid malignancies, as extensively discussed in another manuscript of this issue of the ABE&M. PET and PET/CT are highly sensitive for Tg and/or radioiodine negative-scan thyroids, primary, poorly differentiated or anaplastic tumors, and metastatic lesions (90). However, their role as predictors of thyroid tumor aggressiveness remains to be evaluated.

Fractal dimension

Cell structural and biochemical features change with increasing aggressiveness of a neoplasia. Morphometric and texture analyses of whole tissue sections or individual cells are simple, reproducible and inexpensive. Staining can be done on routine slides. Pathologists have proposed the fractal dimension of nuclear chromatin as a prognostic factor in some tumors, such as lymphoblastic leukemia (91), myelodysplastic syndromes (92) and laryngeal carcinoma (93). This is a promising method that may estimate patients' outcome early on a cytological fine-needle aspirate, since

it has also been used for fine-needle aspirates from breast cytology (94). Recent data suggest that this technique may be applied to thyroid and possibly be useful in predicting aggressiveness (95).

CONCLUSION

Screening tools designed to identify individuals at risk for thyroid nodule cancer are of extreme necessity. Some clinical and epidemiologic features, among other factors, have proved to be important in the identification of individuals at risk. However, an appropriate management algorithm should balance sensitivity in detecting malignancy against the costs of an additional evaluation, which poses considerable costs to the patient and to society (96,97). Likewise, the management of patients diagnosed with thyroid tumors demands the understanding of the factors that drive follicular cells towards a non-return path of dedifferentiation. New promising tools have been developed and hopefully we will soon be able to sort out aggressive cases from the vast majority of indolent thyroid cancers that, perhaps in a not so distant future, will be merely observed without invasive intervention.

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J Cancer Res Clin Oncol (2006) 132:521–528
DOI 10.1007/s00432-006-0099-3

ORIGINAL PAPER

Role of glutathione-*S*-transferase and codon 72 of *P53* genotypes in epithelial ovarian cancer patients

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Received: 12 December 2005 / Accepted: 12 April 2006 / Published online: 19 May 2006

Abstract *Purpose*. A series of polymorphisms in germ-line DNA have been investigated in an effort to delineate polygenic models of cancer susceptibility and prognosis. As low-penetrance susceptibility genes may combine additively or multiplicatively and contribute to cancer incidence and to the response to chemotherapy, we studied *GSTT1, GSTM1, GSTO2, GSTP1* and codon 72 of *p53* genotype profiles in ovarian cancer patients. *Methods*. We compared 69 ovarian cancer patients with 222 control healthy women paired for ethnic and life-style characteristics. Outcome was evaluated in 29 stage III and IV patients submitted to a platinum-based chemotherapy followed-up for 6–29 months (17 ± 9 months). *Results*. *GSTT1, GSTM1, GSTO2* and *GSTP1* genes presented a similar genotype distribution, but codon 72 of *p53* gene wild-type variant was less frequent in ovarian cancer patients than in controls (χ^2; $P = 0.0004$). *Conclusions*. We were unable to demonstrate any association between the GST genotypes studied and the risk of ovarian cancer but the inheritance of a heterozygous Arg/Pro genotype of *p53* increased the risk of ovarian cancer more than 2.5 times (OR = 2.571; 95% CI = 1.453–4.550). There was no association of the studied genes to any clinical or pathological feature of the patients or to their response to chemotherapy.

Keywords Susceptibility · Response to treatment · Germline polymorphic inheritance

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Introduction

Genetic polymorphisms in genes involved in DNA repair, cell cycle control, apoptosis or metabolic enzymes may determine individual susceptibility to cancer and the response to treatment (Beckman et al. 1994). A common germline single nucleotide polymorphism in the proline-rich domain of exon 4 of *p53* gene produces an arginine to proline change at aminoacid position 72. The resulting codon 72 variants have been reportedly associated with various types of cancer, like cervico-uterine (Koushik et al. 2004), breast (Buyru et al. 2003), thyroid (Granja et al. 2004), head and neck cancer (Shen et al. 2002) among others. However, not all investigations have been consistent and this hypothesized association remains controversial (Oren 2003; Drummond et al. 2002). Also, there is disagreement on which of the haplotypes represent a risk factor. For instance, *p53* Arg homozygosity is considered a risk factor for cervical cancer (Qie et al. 2002), while proline homozygotes were related to a higher risk of nasopharyngeal carcinomas (Tsai et al. 2002), lung (Wang et al. 1999a; Granja et al. 2004) and hepatocellular carcinomas (Yu et al. 1999), and the Arg/Pro genotype was associated with an increased susceptibility for

smoke-induced lung adenocarcinoma (Fan et al. 2000). These divergent findings have been attributed, at least in part, to the fact that distribution of the three genotypes (Arg/Arg, Arg/Pro and Pro/Pro) depends largely on the ethnic population (Harris et al. 1986; Weston et al. 1994; Birgander et al. 1995; Buller et al. 1997; Weston and Godbold 1997; Yung et al. 1997; Rosenthal et al. 1998; Klaes et al. 1999; Ngan et al. 1999; Zehbe et al. 1999; Wang et al. 1999a, b). An important drawback in these studies is the fact that most surveys are not able to gather a number of patients large enough to demonstrate a change in risk that might be small.

The role of codon 72 of *p53* polymorphism in the prognosis of cancer patients is even more conflicting. Dong et al., studying pancreatic cancer, concluded that the polymorphism at codon 72 did not show any significant effect on the pathology, prognosis and efficacy of adjuvant chemotherapy of the pancreatic cancers (Dong et al. 2003). Wu et al. also did not find any particular correlation between codon 72 polymorphism and testicular or prostate cancer grade or stage of each type of tumor (Wu et al. 1995). However, Wang et al. demonstrated *p53* polymorphism to be related to the prognosis of lung cancer (Wang et al. 1999a).

There are relatively few reports of the influence of *p53* codon 72 variants in ovarian cancer risk and treatment response. Most reports did not find any difference between the distribution of the three allelic types between control and patients (Buller et al. 1997; Hogdall et al. 2002; Wang et al. 2004). However, Buller et al. found the median survival for the Arg/Arg genotype to be significantly longer than Arg/Pro and Pro/Pro genotypes (Buller et al. 1997). More recently, Wang et al. did not find any correlation between TP53 codon 72 polymorphism and any clinico-pathological parameter; neither could he determine any influence of this polymorphism in the susceptibility to ovarian cancer or the outcome of the patients (Wang et al. 2004).

Besides *p53*, several other polymorphic genes have been studied as possible ovary cancer risk modifiers. Genes encoding for enzymes involved in the biotransformation of carcinogens are particularly interesting as susceptibility factors since their activity is essential for cell protection. Phase II glutathione-*S*-transferase (GST) system consists of a large multigenic group of detoxifying enzymes that catalyze the conjugation of toxic and mutagenic compounds with glutathione (Mannervik 1985). Deletion variants or null alleles exist for the GSTT1 and GSTM1 genes and these present biochemically as a failure to express protein (Board 1981; Seidegard et al. 1988; Pemble et al. 1994). Individual or combined null inheritance for GSTT1 and GSTM1 have been related to an increased risk to a long list of carcinogens and, consequently, to the susceptibility to various tumors (Mannervik 1985; Clapper 2000; Knudsen et al. 2001). Another important gene of the GST family, GSTP1, presents a single nucleotide polymorphism at exon 5 that has been demonstrated to produce a variant enzyme with lower activity and lesser capability of effective detoxification. This variant allele has been associated with a propensity to several neoplasms (Hu et al. 1997; Johansson et al. 1998). Because GSTP1 is also involved in the metabolism and subsequent removal of anticancer drugs, high levels of GSTP1 in tumors may contribute to drug resistance in several different cancers (Gilbert et al. 1993; Russo et al. 1994; Gaffey et al. 1995). Also, a recently characterized gene, *GSTO*, has been related to the ability of cells containing ryanodine receptors (RyR) to resist apoptosis induced by Ca+2 mobilization (Dulhunty et al. 2001). *GSTO2* presents a polymorphism involving an A to G transition at nucleotide position 424 in exon 4 that might be associated to a lower activity of the variant enzyme in a substrate-dependent manner that may help explain the variation between individuals in their susceptibility to oxidative stress and inorganic arsenic (Tanaka-Kagawa et al. 2003; Whitbread et al. 2003). Arsenic is a well-known environmental carcinogen and contamination of drinking water with inorganic arsenic is a worldwide health problem; but few studies have looked for the relationship of *GSTO* polymorphisms to cancer so far (Granja et al. 2005).

In addition, GSTs are implicated in the resistance to a number of drugs, including some that may be used to treat ovarian cancer, like platinum-based compounds, adriamycin, cyclophosphamide and etoposide (Ban et al. 1996). Reports on the effects of *GST* polymorphisms in the response to treatment and ovarian cancer survival have been contradictory (Howells et al. 1998; Lallas et al. 2000; Medeiros et al. 2003). An early study found that the combined null inheritance for *GSTM1* and *GSTT1* was associated with survival and mediated through *p53* expression in ovarian cancer (Howells et al. 2001).

We hypothesized that *GST*s and *p53* could combine additively or multiplicatively and contribute to cancer incidence and to its response to chemotherapy. Hence, the aim of this study was to investigate the combined effect of these genes in ovarian cancer, their association to hormones and other clinical, epidemiological and pathological markers of susceptibility. We also aimed to evaluate a possible utility of polymorphism genotyping of these genes in the prediction of the ovarian cancer patient's response to chemotherapy and outcome.

Materials and methods

Subjects

The study was approved by the Ethics Committee of the University Hospital – School of Medicine of the State University of Campinas–Sao Paulo, and informed written consent was obtained from all individuals. A total of 69 unrelated women with histologically confirmed invasive epithelial ovarian tumors followed-up in our outpatient clinic at the University Hospital were enrolled in the study. All patients had undergone primary surgery and chemotherapy between 1998 and 2004 according to a same-standard protocol that includes extended total hysterectomy, bilateral salpingo-oophorectomy, omentectomy and resection of metastatic tumors. Systematic pelvic and para-aortic lymph node dissection was employed when the optimal cytoreduction (residual tumor < 2 cm) was achieved. All data, including stage and grade of differentiation of the tumors, were obtained from surgical and pathological reports from the patients' records. Experienced pathologists of the University Hospital confirmed all diagnoses. Grade of tumor was determined according to architectural pattern and mitosis index.

A control group of 222 healthy women was selected from the general population of our region. There were 150 blood donors and 72 individuals recruited among co-workers and volunteers from the State University of Campinas. Individuals with history of previous ovarian cancer or any other neoplasia were excluded as well as women with a family history of gynecological cancers. Data on general health conditions, gynecological and medical history with emphasis on the use of estrogens, reproductive life and previous and/or current malignant diseases were obtained through interviews, using a structured questionnaire that included a lifestyle inquiry on occupational and professional history; cigarette smoking habit; dietary habits including red meat, vegetables and fat intake; and alcohol and drug consumption. Cigarette smoking habit was recorded and the patients were grouped into never-smokers and ever-smokers categories. Color was determined according to each individual's description of her own skin color. All patients were classified as whites and, therefore, the control group was composed only of white individuals. Disease stage was determined according to the International Federation of Gynecologists and Obstetricians (FIGO) staging scheme and included 17 stage I, 8 stage II, 35 stage III and 9 stage IV patients.

Outcome evaluation

In order to evaluate the response to therapy and survival, we focused on a more homogeneous group of 29 patients with epithelial ovarian carcinoma stages III and IV who were found to have residual tumor over 2 cm after the first laparotomy, and/or had high serum levels of the tumor marker CA125 (> 35 U/ml) after surgery. These patients did not present any other concomitant neoplasm, were followed-up for 6–29 months (17 ± 9 months) and received a platinum-based (cisplatin or carboplatin) three-agent chemotherapy. Additional agents were cyclophosphamide ($n = 19$), epirubicin ($n = 10$), taxol ($n = 7$) and adriamycin ($n = 5$). Response to chemotherapy was determined by the disappearance of clinically detectable tumor and a 50% or more reduction in CA125 level maintained for over 6 months after the last chemotherapy cycle. Patients were considered responsive to chemotherapy when the tumor disappeared completely on image methods or at a second-look laparotomy and a 50% of more reduction in the CA125 levels was maintained for over 6 months. Non-response was defined as the need of chemotherapy drugs changing due to clinical recurrence or disease-related death during the period of 6 months after chemotherapy. Patients with stable disease were also considered non-responsive. Survival was defined as the time interval from diagnosis to death. The cause of death was determined from the patients' records.

Polymorphism analysis

Genomic DNA was extracted from a peripheral blood sample using a standard proteinase K and phenol–chloroform protocol. A multiplex-PCR assay was used to simultaneously amplify the GSTT1, GSTM1 and beta-globin genes as an internal positive control as described previously (Morari et al. 2002). GSTP1 variants were studied using a PCR–restriction fragment length polymorphism analysis (PCR-RFLP). The primers used were forward 5′ CCA GGC TGG GGC TCA CAG ACA GC 3′ and reverse 5′ GGT CAG CCC AAG CCA CCT GAG G 3′. In brief, PCR was performed in 25 μl volumes of a mixture containing 100 ng DNA, 50 nM of each primer, 10 mM Tris–HCl (pH 8.0), 100 μM of each dinucleotide triphosphate, 2.0 mM $MgCl_2$ and 0.5 U Taq DNA polymerase. Amplifications were carried out for 35 cycles of 94°C for 45 s, annealing temperatures 62.4°C for 50 s and 72°C for 1 min, with an initial denaturation step of 94°C for 2 min and a final extension step of 72°C for 7 min using

a MJ PTC–200 PCR system. Thirteen microliter of each PCR product was subsequently submitted to Alw26I overnight digestion. The digests were eletrophoresed in 3% agarose gels stained with ethidium bromide and photographed under ultraviolet light. The identity of the allele was established based on the restriction patterns. The transition polymorphism A–G in the GSTO2 (asn142asp) codon 142 was demonstrated using the following primers: 5′ ACT GAG AAC CGG AAC CAC AG 3′ and 5′ GTA CCT CTT CCA GGT TG 3′; annealing temperature was 62°C (45 s). PCR products were digested with the restriction enzyme MboI at 37°C for 16 h and visualized on 3% agarose gel stained with ethidium bromide. The digestion products reveal three different patterns: The A/A homozygote of normal sequence was 280 bp fragment, whereas A/G heterozygosity was 280, 250 and 30 bp, and G/G homozygote of polymorphic sequence 242 and 48 fragments bp.

For the determination of the polymorphism at codon 72 of the *p53* gene, two sets of primers were used, one to amplify the Arg allele and the other to amplify the Pro allele, according to the procedure described by Storey et al. (1998) and previously employed in our laboratory (Granja et al. 2004).

Statistical analysis

Statistical analysis was conducted using SAS statistical software (Statistical Analysis System, Version 8.1, Cary, NC, USA, 1999–2000). Chi-square (χ^2) and Fisher's (F) exact tests were used to examine homogeneity between cases and controls regarding color, previous diseases, use of estrogen, number of pregnancies and cigarette smoking. Kruskal–Wallis (KW) test was used to compare age among groups. Pearson-χ^2 test was also used to assess association between genotypes and clinical variables. Univariate analysis using two-sided chi-square tests was performed to compare the genotype and allele frequencies between cases and controls. The observed genotype frequencies were compared with those calculated from Hardy–Weinberg disequilibrium theory ($p^2 + 2pq + q^2$; where p is the frequency of the variant allele and $q = 1\text{-}p$). The crude odds ratio (OR) and the 95% confidence intervals (CI) were calculated by logistic regression analysis. Multiple and univariate logistic regressions were used to evaluate the effect of genotypes, after adjusting for other potential confounders like age, tobacco and estrogen consumption. Comparison between overall survival rates in the patients with stages III and IV were calculated using the Kaplan–Meier survival curve and the log-rank test. The Cox proportional hazard regression model for multivariate analysis was used to analyze the individual effects of factors on survival. Survival analysis was done using Kaplan–Meier method. All tests were conducted at the $P = 0.05$ level of significance.

Results

There were no differences between the controls and the ovarian cancer patients regarding age (32–98 years old, 54.1 ± 15.4 years *vs.* 24–73 years old, 52.6 ± 11.9 years), demographic and lifestyle characteristics, including alcohol consumption, red meat, vegetables and fat intake, education, and exercise – data not shown.

Regarding histology, the majority of the tumors were classified as serous ($n = 37$, 53.6%), 13 (18.8%) were mucinous, 11 (15.9%) endometrioid and 8 (11.5%) undifferentiated. The median overall survival was 2 years (mean ± standard error: 83.48 ± 13.20 months). Gynecological and medical history, contraception, number of pregnancies, smoking habits and family history were not associated with survival. Neither was the type of primary chemotherapy or any of the subsequent chemotherapy schemes.

The proportion of GSTP1, GSTO2 and codon 72 of *p53* different haplotypes Hardy–Weinberg equilibrium was tested in the population of 291 individuals genotyped in this study. GSTP1 and GSTO2, but not codon 72 of *p53* variants, were in equilibrium.

Table 1 shows the overall distribution of genotypes in both control and patients groups. There was no association between GSTT1 and GSTM1 null genotypes, either considered individually or in combination, and the risk of ovarian cancer. Also, there was no difference between GSTP1 and GSTO2 variants' incidence in the control population and the ovarian cancer patients. However, the wild-type homozygous Arg/Arg variant of codon 72 of *p53* gene was underrepresented in ovarian cancer patients (33.33%) compared to the control population (52.70%) (χ^2; $P = 0.0004$). Indeed, 46 out of the 69 ovarian cancer patients (66.67%) presented an Arg/Pro haplotype that appeared in only 40.99% of the controls. Also, the Pro/Pro haplotype was not found among ovarian cancer patients although it was detected in 14 control individuals (6.31% of the control population). Univariate logistic regression analysis showed that the Arg/Pro genotype was an independent factor of risk for ovarian cancer ($P = 0.0012$) and this risk was confirmed to be 2.571 times higher in individuals with this genotype, by a multivariate logistic regression analysis (OR = 2.571; 95% CI = 1.453–4.550).

Table 1 Comparison among the distribution of the different studied genotypes in the control population ($n = 222$ individuals) and the ovarian cancer cases ($n = 69$ patients)

Gene	Genotype	Cases		Controls		Odds ratio	*P*-value
		n	%	*n*	%		
GSTP1	Ile 105 val						
	Ile/Ile	33	47.8	98	44.1		
	Ile/val	26	37.7	94	42.3	0.821	0.5110
	Val/val	10	14.5	30	13.5	0.990	0.9806
GSTO2	Asn142asp						
	Asn/asn	24	34.8	87	39.2		
	Asn/asp	37	53.6	104	46.8	1.290	0.3960
	Asp/asp	8	11.6	31	14.0	0.936	0.8844
GSTT1/ GSTM1	Pos/pos	26	37.7	98	44.1		
	Pos/neg	35	50.7	81	36.5	0.1133	1.551
	Neg/pos	5	7.2	24	10.8	0.1239	0.531
	Neg/neg	3	4.3	19	8.5		
p53	Arg72Pro						
	Arg/arg	23	33.3	117	52.7		
	Arg/pro	46	66.7	91	41.0	2.571	0.012
	pro/pro	0	0	14	6.3		

Among the group of 29 patients submitted to chemotherapy, 16 received six cycles of chemotherapy after the primary surgery, four patients received three cycles after the primary surgery followed by a second-look laparotomy and nine patients received neoadjuvant chemotherapy. None of the GST or the *p53* polymorphisms, either alone or in combination, were found to be associated with patient age, disease stage, tumor grade or histological type. We were unable to demonstrate any significant association between any of the genetic parameters studied, either alone or in combination, or in analysis adjusted for patient age, stage, histological grade or histological type and the patients' outcome or their response to treatment. Additional analysis also did not demonstrate any association between genotypes and outcome with the different therapeutic regimens used. None of the studied polymorphisms were found to be predictive of response to treatment. Also, we were not able to find any association between GSTP1 variant alleles and the combined or isolated null genotype for GSTM1 and GSTT1 or any codon 72 of *p53* haplotypes.

Discussion

Although ovarian cancer is the main cause of death among women with gynecological malignancies, other than the involvement of hormonal and reproductive factors, little is known of its etiology. Epidemiologic studies found a correlation to eggs, milk and diary products in general to the incidence of ovarian cancer (Kushi et al. 1999) suggesting an influence of the estrogen and progesterone contents of animal-derived food we consume (Ganmaa and Sato 2005). Also, asbestos, talc (Langseth and Kjaerheim 2004) and suspended particulate matter (Iwai et al. 2005) were hypothesized to influence the development of ovarian cancer.

Genes that are involved in a person's propensity toward carcinogenic exposure and/or modulation of therapeutic outcome may be implicated in both cancer risk and its prognostics. Therefore, at the level of risk assessment, germline polymorphisms in glutathione genes are very interesting candidate genes. Also, polymorphisms affecting genes with important effects on tumor behavior are candidates for both tumor susceptibility and outcome markers. It is conceivable that low-penetrance susceptibility genes combine additively or multiplicatively and contribute to cancer incidence and to its response to chemotherapy. However, the study of low-penetrance genes has produced inconsistent results mostly derived from small samples and also from population stratification secondary to ethnic diversity, and consideration limited to only one rather than combinations of polymorphisms. Ovarian cancer is a relatively rare malignancy, and our study, like most previous reports, has a relatively small sample. However, we studied the three variants of codon 72 of *p53* gene. In addition, we examined the association of this gene haplotypes to the genes encoding for GSTT1, GTM1, GSTP1 and GSTO2 enzymes in the Brazilian population whose highly heterogeneous composition might help dilute ethnic bias. Response to treatment and outcome were analyzed only in a homogeneous sample of grade III and IV patients treated with a three-agent platinum-based chemotherapy.

Our results concur with almost all previous studies that did not observe any association between the GSTT1 and GSTM1 null variants and ovarian cancer (Coughlin and Hall 2002). Concurring with the Spurdle et al. data, we also did not find any difference in the distribution of GSTP1 gene haplotypes between ovarian cancer cases and controls (Spurdle et al. 2001). More recently, a study of 215 patients followed-up for a median of 31 months found patients with GSTM1 and GSTP1 variant alleles to have a reduction in the risk of disease progression and a better overall survival (Beeghly et al. 2005). Also, we were not able to reproduce Howells' data on GSTP1 variants and the patients' outcomes, perhaps because we studied a small but more homogeneous group of patients (Howells et al. 2001).

Considered together with previous reports, our data provide compelling evidence against any important role of *GSTT1, GSTM1, GSTPO1* and *GSTO2* as susceptibility factors for ovarian cancer, with the possible

exception of *GSTM1* and *GSTT1* in endometrioid and endometrioid/clear cell ovarian cancers (Baxter et al. 2001; Spurdle et al. 2001). We demonstrated that Arg/Pro genotype of *p53* was an independent factor of risk for ovarian cancer although it was not related to the response to treatment. A recent report found no difference in the genotype or allele prevalence between 68 ovarian cancer patients and 95 healthy control Japanese women (Ueda et al. 2005)**.** In contrast, Agorastos et al. have found the Arg/Arg to increase the risk for Greek ovarian cancer patients than in healthy controls (odds ratio 4.16 at $P = 0.0058$) (Agorastos et al. 2004). Other authors also advocate a role for codon 72 of *p53* in the susceptibility and/or the aggressiveness of ovarian tumors (Hogdall et al. 2002; Pegoraro et al. 2003; Wang et al. 2004)

TP53 protein with Pro is structurally different from TP53 protein with Arg (Matlashewski et al. 1987). Although both polymorphs of *p53* are endowed with both apoptosis and DNA-repair properties, they have different efficiencies (Siddique and Sabapathy, 2006). TP53 variants enhance the binding of *p*73 and neutralize *p*73-induced apoptosis (Marin et al. 2000; Bergamaschi et al. 2003). The Arg variant has a greater ability to localize to the mitocondria; this localization is accompanied by release of cytochrome c into cytosol (Dumont et al. 2003). Recently, *p53* Pro/Pro homozygotes were showed to increase significantly the risk of estrogen receptor (ER) positive breast cancer (adjusted OR = 2.04, $P = 0.04$) as compared with Arg/Arg homozygotes (Noma et al. 2004). Regarding ovarian cancer, Buller et al. reported that Arg/Arg Midwestern American patients developed ovarian cancer at an earlier age than the others. In addition, they found Pro allele loss to be preferred in the tumors, and any tumor with a retained Pro allele to be more prone to *p53* sequence variants (Buller et al. 1997). Wang et al. found no indications of the codon 72 polymorphism influencing the susceptibility of developing ovarian cancer (Wang et al., 1999a, b). However, in agreement with Buller et al., they confirmed a preferential tendency of Pro tumor genotype to harbor TP53 sequence variants, and samples with the Pro tumor genotype had more severe changes like deletion, insertion, nonsense or splice variants (Buller et al. 1997). These findings suggest that the polymorphism at codon 72 may have a role in ovarian cancer susceptibility, but this effect may be dependent on the properties of genotoxic or cytotoxic agents.

We could not demonstrate any correlation between the frequency of the three genotypes (Pro/Pro, Arg/Arg and Arg/Pro) and clinical data, stage or histological type of the tumor, concurring with the few studies that have analyzed the relationship between the distribution of codon 72 polymorphisms and clinicopathological parameters (Hogdall et al. 2002; Wang et al. 2004). Also, there was no correlation between any of the studied genotypes; neither did they show any association with the response to chemotherapy or the patients' outcomes.

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Clinical Endocrinology (2007) doi: 10.1111/j.1365-2265.2007.03121.x

ORIGINAL ARTICLE

Genetic polymorphisms associated with cigarette smoking and the risk of Graves' disease

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Summary

Objective Cigarette smoking is a well-recognized risk factor of Graves' disease and, particularly, Graves' ophthalmopathy. Hence, germline polymorphisms of detoxification genes and genes belonging to the major DNA repair–apoptosis pathways might have an important role in disease susceptibility. In addition, as some of these genes are regulated by thyroid hormones, they may affect the patients' outcomes. We aimed to assess the influence of the *GST*, *CYP* and *TP53* gene polymorphisms in the risk of Graves' disease and its outcome.

Design Prospective case-control study.

Patients A PCR-based strategy was used for *GSTT1, GSTM1*, *GSTP1*, *CYP1A1* and *TP53* codon 72 genotypes in a group of 400 Graves' disease patients, and to compare them to 574 control individuals with similar environmental exposure features.

Results *GSTM1* and *GSTT1* genotypes were equally distributed in cases and controls, repectively. However, *GSTP1* ($P < 0{\cdot}0001$), *CYP1A1* ($P < 0{\cdot}0033$) and *Pro/ProTP53* ($P < 0{\cdot}0035$) variants appeared more frequently in Graves' disease patients than in controls. A multivariate analysis indicated that cigarette smoking and inheritance of *GSTP1*, *CYP1A1* and *Pro/ProTP53* variants were important risk factors for Graves' disease, but only smoking appeared as an independent risk factor for Graves' ophthalmopathy. There was no association between clinical features, including ophthalmopathy or treatment outcome, and the studied genotypes.

Conclusion We concluded that *GSTP1*, *CYP1A1* and *TP53*, but not *GSTT1* and *GSTM1* germline polymorphisms, may be associated with smoking-related Graves' disease susceptibility and configure a risk profile for the disease. However, these polymorphisms do not influence the patients' response to treatment.

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(Received 19 July 2007; returned for revision 11 August 2007; finally revised 20 September 2007; accepted 18 October 2007)

Introduction

It is widely accepted that Graves' disease is an autoimmune disorder, involving a complex interchange of multiple genetic, environmental exogenous and endogenous elements required for the right combination to trigger thyroid autoimmunity.[1–5] Environmental factors such as smoking, stress, bacterial and viral infections, and a large variety of drugs may be responsible for as much as 21% of the susceptibility of developing Graves' disease.[1–5]

The biochemical basis for genetic susceptibility to environmental hazards or endogenously produced toxins is related to genetic polymorphisms that normally occur in the general population, and involves a series of genes: genes implicated in the predisposition to a specific disease, in the metabolic activation or detoxification of genotoxins and in controlling DNA repair or cellular damage.[6] Polymorphic genes encoding for enzymes involved in the biotransformation of xenobiotics have been extensively investigated, in particular regarding their role as cancer risk modifiers. Recently, a series of studies have been focusing on the importance of these genes in the inflammatory reaction process and in various autoimmune conditions, including multiple sclerosis, systemic lupus erythematosus, Guillain–Barré syndrome and rheumatoid arthritis.[7–11]

Cigarette smoking is a well-recognized risk factor for the development of Graves' disease and even more so for Graves' ophthalmopathy.[12] Cytochrome P4501A1 (*CYP1A1*) enzymes play a key role in phase I metabolism of polycyclic aromatic hydrocarbons and other toxic components of cigarette smoke which are subsequently detoxified by phase II enzymes such as the glutathione S-transferases (GST). When these enzymes are not able to avoid cellular damages, the *TP53* gene plays an important role by recognizing and repairing DNA injuries.

The GST supergene family encodes a series of dimeric enzymes that catalyze the conjugation of glutathione with a variety of

electrophilic compounds, including oxidized lipid, DNA and catechol products generated by reactive oxygen species-induced damage to intracellular molecules and other potentially detrimental substrates.[13,14] Genetic polymorphisms have been demonstrated for *GSTM1*, *GSTT1* and *GSTP1* genes are localized on chromosomes 1p13.3, 22q11.2 and 11q13, respectively. Homozygous deletion individuals, who are classified as *GSTM1* null or *GSTT1* null, exhibit absence of enzymatic activity and are hypothesized to be at increased risk for toxic effects of a wide variety of environmental or endogenous exposures.[15] The *GSTP1* gene is located on chromosome 11 and presents an A to G transition resulting in a codon Ile105Val amino acid substitution (RS1695), which modifies heat stability and specific activity of the Val-containing isoform.[16] These gene variants produce enzymes with lower activity and possess less effective capability of detoxifying toxins than the wild-type *GSTP1*.[17] GST enzymes are subject to regulation by several hormones including the thyroid hormones.[18] Both T3 and T4 have been shown to reduce the GST activity, raising the possibility that the state of hyperthyroidism may exert an influence on the response to antithyroid drugs or even to radioiodine treatment.[18]

Cytochrome P450 1A1 (*CYP1A1*) gene, located on chromosome 15q22 encodes for the enzyme aryl hydrocarbon hydroxylase, which plays a key role in phase I metabolism of polycyclic aromatic hydrocarbons, transforming them into carcinogens.[19] A thymine–cytosine transition in the *CYP1A1 MspI* restriction site has been shown to be associated with a more inducible form of *CYP1A1*.[20] The higher levels of the corresponding enzymes would result in an increased capacity to activate polycyclic aromatic hydrocarbons, producing highly reactive electrophilic intermediates that might damage DNA.[21]

The *TP53* gene is located on chromosome 17p13·1 and its encoding p53 protein participates in the processes of cell-cycle arrest and apoptosis.[22,23] Apoptosis not only prevents tumour generation, but also normally regulates the maturation and control of both T- and B-cell immune responses, the basic pathogenetic components of autoimmune thyroid diseases, by removing autoreactive or nonreactive immune elements.[24] A common germline single nucleotide polymorphism in the proline-rich domain of exon 4 of p53 gene produces an arginine to proline change at amino acid position 72 (RS1042522). The resulting codon 72 variants reduce p53 ability to activate apoptosis and have been reported to be associated with some autoimmune diseases, including systemic lupus erythematosus and rheumatoid arthritis.[25,26]

Although the role of many polymorphisms of genes related to toxins' metabolism has been extensively investigated regarding thyroid cancer susceptibility, their influence on thyroid autoimmune disease risk is still largely unknown.[27–29] Hence, this study was designed to assess the influence of *GSTT1*, *GSTM1*, *GSTP1*, *CYP1A1* and *72TP53* polymorphic inheritance on Graves' disease susceptibility and response to treatment.

Materials and methods

This prospective case-control study was approved by the Research Ethics Committee of the School of Medical Sciences – State University of Campinas (FCM – UNICAMP), Santa Casa of Sao Paulo Hospital (Santa Casa), and the Pontifical Catholic University of Campinas Teaching Hospital (PUCC), Brazil. The informed written consent was obtained from all 974 enrolled individuals. Individuals with history of previous specific environmental or occupational exposure risks, and antecedents of autoimmune diseases other than Graves' disease were excluded. We also excluded individuals, who were taking medications that could interfere with thyroid function evaluation; who came from areas of suspected iodine deficiency; who had a history of radiation exposure; who had a recent history (2 months) of viral and bacterial infections. All controls that had any history or clinical suspicion of thyroid dysfunction were excluded.

Patients

Four-hundred Brazilian outpatients diagnosed with Graves' disease from the FCM – UNICAMP, Santa Casa and PUCC were enrolled in the study. All patients presented with clinical evidence of thyrotoxicosis with suppressed serum TSH, high serum free T4 and/or T3 levels, elevated 24-h radioactive iodine (24-h RAIU) or pertechnetate (Tc) uptake with homogeneous tracer distribution and diffuse gland enlargement. Patients were carefully examined and either treated with antithyroid drugs (102 cases with methimazole and 56 cases with propylthiouracil) or assigned to radioiodine therapy (232 cases) or surgery (10 cases). Antithyroid drug therapy was maintained for at least 12 months. Serum TSH and fT4 levels were assessed every 30–60 days during antithyroid drugs therapy, and at least three times during the 12 months after radioiodine, surgical or antithyroid drug treatment. Thyroid function was assessed at least once a year during the subsequent follow-up. One hundred and sixty-nine patients presented with clinical evidence of eye involvement. Ocular examinations were carried out every 2–3 months by a single examiner (RBS), whose examination included evaluation of soft-tissue changes and measurements of proptosis. The activity of ophthalmopathy was graded using a clinical activity score, which took into account seven manifestations of disease (spontaneous retrobulbar pain, pain with eye movement, eyelid erythema, eyelid oedema, conjunctival injection, chemosis and swelling of the caruncle). One point was given for each manifestation, and the score ranged from 0 (no activity) to 7 (very high activity).[30]

Cure for hyperthyroidism was defined as clinical and laboratory evidence of stable euthyroidism or hypothyroidism in the absence of antithyroid drugs at least 12 months (average 17 months) after ^{131}I therapy, surgery or one or more courses of antithyroid drug therapy discontinuation.

Controls

We included a control group of 574 healthy individuals selected from the general population of our region, expected to have normal iodine ingestion. Considering that Graves' disease is more frequent in women, we selected three females for each male. Unfortunately, we were neither able to collect blood from children nor match control and patients' groups for ethnicity, as most of the control individuals who agreed to be enrolled were white.

Both patient and control individuals were submitted to a physical examination and answered a structured questionnaire that included

demographic and ethnic background, lifetime occupational history, smoking and dietary habits, physical exercises, drug and medicine use, general health conditions and previous disease information. Skin colour was determined by the interviewer, in accordance with the Brazilian Institute of Geography and Statistics; however, due to the difficulty in classifying our highly heterogeneous population, we grouped individuals into white and nonwhite. Cigarette smoking habit was recorded but, due to the lack of reliability of the data obtained during the smoking period, age when smoking started, quantity smoked and years as smoking stopped, the patients were grouped in never-smoker and ever-smoker categories.

Genotypes identification

Blood was collected into EDTA-containing tubes, and DNA was extracted from frozen specimens and from leucocytes separated from whole blood using a standard phenol–chloroform protocol. Genotyping was conducted with blinding to case-control status.

GSTT1, *GSTM1*, *GSTP1* and *CYP1A1* genotyping was performed using the previously described PRC-RFLP methods.[27,29,31] In order to determine the polymorphism at codon 72 of the *TP53* gene, we also used a PCR-RFLP method. Primers for *TP53* were *TP53*F (5′-ATC TAC AGT CCC CCT TGC CG-3′) and *TP53*R (5′-GCA ACT GAC CGT GAC AGT CA-3′), which generated a 296-bp fragment. PCR was performed in 25 μl volumes of a mixture containing 100 ng DNA, 50 nM of each primer, 10 mM Tris–HCl (pH 8·0), 100 μM of each dinucleotide triphosphate, 2·0 mM $MgCl_2$ and 0·5 U Taq DNA polymerase. Amplifications were carried out for 35 cycles of 94 °C for 50 s, annealing temperatures were 65 °C for 50 s and 72 °C for 1 min, with an initial denaturation step of 94 °C for 2 min and a final extension step of 72 °C for 10 min, using a MJ PTC-200 PCR system. The PCR fragments were analysed by electrophoresis in a 2% agarose gel stained with ethidium bromide and visualized on a UV-light transilluminator. The *Bsh1236I* restriction enzyme was used to identify the *TP53* codon 72 (Arg/Pro) polymorphism, according to the manufacturer's protocol (Fermentas UAB, Vilnius, Lithuania). The wild-type (Arg/Arg) allele yielded two fragments of 169 and 127 bp. The homozygote variant (Pro/Pro) generated a single band, representing the entire 296 bp fragment and the heterozygote (Arg/Pro) variant presented the three bands. The restricted product was analysed by electrophoresis in 3% agarose gel containing ethidium bromide. RFLP results of *TP53* codon 72 were confirmed by DNA sequencing of PCR products, using the ABI prism big dye sequencing kit (Perkin-Elmer, Warrington, Cheshire, UK) with an automated sequencer (ABI PRISM 377; Perkin-Elmer).

Statistical analysis

The statistical analysis was conducted using the SAS statistical software (Statistical Analysis System, version 8·1, Cary, NC, 1999–2000). Associations were assessed using 2×2 or $2 \times n$ contingency table analysis; and the χ^2 or Fisher's (F) exact test was used to examine homogeneity between cases and controls regarding gender, ethnicity, medication use, cigarette smoking habit and genotypes. The Kruskal–Wallis (KW) test was used to compare the ages among the groups. The Mann–Whitney or Wilcoxon test was used to compare the age and goiter size among the different genotype groups. The observed genotype frequencies were compared to those calculated using the Hardy–Weinberg disequilibrium theory. The sample size and power calculation for each gene were calculated using the PS software version 2·1·31. The odds ratio (OR) and 95% CI provided the measure of association strength, for example, indicating the increase in odds of a given thyroid nodule, demonstrating a particular genotype compared to the control population. Variables that were significantly associated with Graves' disease incidence or outcome by univariate analysis were entered into a multiple logistic regression model, in order to evaluate the effect of all genotypes and clinical risk factors, including sex, age and cigarette smoking as independent predictors. A stepwise regression analysis was applied to further identify risk factors of Graves' disease or Graves' ophthalmopathy. All tests were conducted at the $P = 0{\cdot}05$ significance level.

Results

Table 1 summarizes patients and controls clinical characteristics. There were no differences regarding dietary habits, physical exercises,

Table 1. Percentage distribution of GD patients (cases) and controls according to smoke habits comparing clinical characteristics, including age ($X \pm$ SD in years), gender, ethnicity (white, nonwhite), ophthalmopathy and cure rate

		Cases			Controls		
Clinical Characteristics		Smoking	Nonsmoking	Total	Smoking	Nonsmoking	Total
Age		40·1 ± 10·2	37 ± 12·60	35·5 ± 13·7	42·03 ± 14·35	43·40 ± 17·79	43·3 ± 15·7
Gender	Female	73·53	81·71	75	57·5	66·45	69·52
	Male	26·47	18·29	25	42·5	33·55	30·48
Ethnics	White	67·65	68	68·75	74·8	82·74	84·50
	Nonwhite	32·35	32	31·25	25·2	17·26	15·50
Ophthalmopathy	Positive	46·77	27·48	48·63	–	–	–
	Negative	53·23	72·52	51·37	–	–	–
Rate of cure	Cure	37·06	39·29	77·87	–	–	–
	Noncure	62·94	60·71	22·13	–	–	–

Table 2. Distribution of *GSTM1* and *GSTT1* genotypes inheritance (present = positive; absent = negative); *GSTP1* and *CYP1A1m1* genotypes (wild-type, heterozygous and homozygous variants) and *TP53* codon 72 (wild-type = Arg/Arg, heterozygous = Arg/Pro, homozygous = Pro/Pro) among 400 Graves' disease cases and 574 control individuals. The odds ratio (OR) was calculated in relation to the positive or to the wild-type genotype adjusted for sex, age and ethnicity. The power calculation (PC) is presented in percentage

All cases

		Control		Cases					
		N	(%)	*N*	(%)	PC (%)	OR	95% CI	*P*
GSTM1	Positive	275	56·35	239	59·75	16	0·895	0·675–1·186	0·4390
	Negative	213	43·65	161	40·25				
GSTT1	Positive	385	78·89	320	80·00	0·5	0·955	0·677–1·349	0·7951
	Negative	103	21·11	80	20·00				
GSTP1	Wild-type	290	59·43	169	42·25	99	2·074	1·559–2·760	< 0·0001
	Heterozygous	153	31·35	186	46·50				
	Homozygous	45	9·22	45	11·25				
CYP1A1m1	Wild-type	173	62·45	52	44·44	87	1·971	1·254–3·098	0·0033
	Heterozygous	96	34·66	61	52·14				
	Homozygous	8	2·89	4	3·42				
TP53 codon 72	Arg/Arg	139	44·98	121	44·65	97	2·984	1·433–6·217	0·0035
	Arg/Pro	158	51·13	115	42·44				
	Pro/Pro	12	3·88	35	12·92				

or gender ($P = 0{\cdot}0612$); however, GD patients and controls differed regarding age ($P < 0{\cdot}0001$) and ethnicity ($P < 0{\cdot}0001$). Cigarette smoking habits were also different between control individuals and patients (29·26% ever-smokers and 70·74% never-smokers *vs.* 36·82% ever-smokers and 63·18% never-smokers) ($P = 0{\cdot}0397$; OR = 1·409; 95% CI = 1·023–1·940). By the end of the minimum 12-month period of follow-up, 39 patients were still under treatment; 274 (75·9%) individuals considered cured, and 87 patients were still hyperthyroid.

Table 2 summarizes data of the overall proportions of the *GSTM1*, *GSTT1*, *GSTP1*, *CYP1A1* and *72TP53* genotypes in the control population and in the patients with Graves' disease. Unfortunately, the power calculation for *GSTM1* and *GSTT1* genes was very low. A multivariate logistic regression analysis corrected for sex, age and ethnicity showed that the inheritance of a variant *GSTP1* allele increased the risk of Graves' disease by over two times. When data were also corrected for smoking, the odds of developing Graves' disease were increased: OR = 2·125, 95% CI = 1·521–2·969 ($P < 0{\cdot}0001$). *CYP1A1m1* variants inheritance also increased the Graves' disease susceptibility in almost two times. Correction for smoking slightly reduced the odds (OR = 1·884; 95% CI = 1·187–2·990; $P = 0{\cdot}0072$). The inheritance of a *Pro/Pro* allele of *72TP53* increased the risk of Graves' disease in almost three times, and even more when the multivariate logistic regression analysis was adjusted for smoking: OR = 4·685; 95% CI = 1·707–12·861; $P = 0{\cdot}0027$.

In order to further examine the role of the investigated genotypes and other susceptibility factors to Graves' disease, we applied a stepwise regression analysis that confirmed the inheritance of *GSTP1* variants (OR = 1·881; 95% CI = 1·201–2·946; $P = 0{\cdot}0058$) and *Pro/Pro* allele of *72TP53* (OR = 5·784; 95% CI = 1·688–19·816; $P = 0{\cdot}0052$) as significant risk factors.

Considering that Graves' disease is typically more frequent in young adults, we compared the genotypes of patients under and over 25 years of age. A multiple logistic regression analysis adjusted for age, sex and ethnicity identified the *GSTP1* genotype as a risk factor in individuals under 25 years old (OR = 17·798; 95% CI = 6·125–51·719; $P < 0{\cdot}0001$); while in individuals over 25 years, *GSTP1* (OR = 1·684; 95% CI = 1·244–2·279; $P = 0{\cdot}0007$), *CYP1A1* (OR = 2·096; 95% CI = 1·283–3·424; $P = 0{\cdot}0031$) and *Pro/Pro 72TP53* (OR = 2·609; 95% CI = 1·207–5·638; $P = 0{\cdot}0147$) appeared as susceptibility factors to the disease.

A multivariate logistic regression analysis corrected for sex identified smoking habits as an independent risk factor that increased the susceptibility to Graves' ophthalmopathy by over two times (OR = 2·243; 95% CI = 1·325–3·797; $P = 0{\cdot}0026$). None of the studied genotypes were related to the development of Graves' ophthalmopathy risk, intensity as measured by the clinical activity score, or outcome.

There was no association among *GSTM1*, *GSTT1*, *GSTP1*, *CYP1A1m1* and *TP53* codon 72 genotypes. Moreover, we were unable to find any association between any of the investigated genotypes and the patients' clinical features – including goiter size, thyroid hormone levels and the presence of antithyroid antibodies – or the cure rate.

Discussion

Graves' disease is considered a multifactorial condition in which the development of the autoimmune response against thyroid antigens is facilitated by a particular, but still largely known, polygenic background. Environmental factors probably trigger the development of the disease in genetically susceptible individuals, as indicated

by the low concordance rate in monozygotic twins, among other evidences.[1–5]

Some chemicals of environmental concern have been demonstrated to alter the immune system in different species.[32,33] In humans, exposure to environmental xenobiotics, including smoking, has been proposed as one of the initiating factors that lead to the loss of tolerance to self-proteins in genetically susceptible hosts.[1,7–11,25,26,34–37] Our data are in accordance with the current literature, indicating that smoking plays an important role in the risk of developing Graves' disease, and even more so, Graves' ophthalmopathy.

GST enzymes are specific substrates, but their mechanism for acting is still largely unclear.[14,38] Our data suggest that individuals with *GSTP1* variants may be more susceptible to the effect of autoimmunity-inducing environmental or endogenous products that depend on *GSTP1* but not *GSTM1* or *GSTT1* substrate detoxification. In addition to a reduced ability in the detoxification of damaging materials capable of producing oxidative stress, the *GSTP1* polymorphism affects cellular response to DNA damage, and hence, may modify individual sensitivity to genotoxins.[14,38] The fact that cigarette smoking increased the odds of *GSTP1* associated with the risk for Graves' disease suggests that some of more than 4000 toxic compounds aspirated or ingested during cigarette smoking may be involved in the development of this disease.

CYP1A1m1 variants were also found to be related to susceptibility to Graves' disease. Whereas over 3·5 billion years ago P450 enzymes were undoubtedly designed as necessary components of signal transduction pathways, during the past billion years most P450 enzymes have usually been responsible for the detoxification of numerous toxic compounds. During such oxidative metabolism, these enzymes are also capable of working in an ambivalent manner, generating toxic intermediates. We recently demonstrated that the *CYP1A1* genotype might be associated with the reduced risk of papillary carcinomas reported among smokers.[29] Our present results suggest that the inheritance of *CYP1A1* variants increases the susceptibility to Graves' disease, but that OR is decreased among smokers, reinforcing our previous observation in thyroid cancer.[29] CYP enzymes, together with *Pro/Pro 72TP53*, may participate in the metabolism of some toxins of cumulative effects, as they appear to be more important in individuals over 25-years-old.

The role of the Pro homozygous variant of *TP53* in Graves' disease susceptibility is more difficult to interpret. Increased DNA damage and apoptosis may be associated with autoimmune thyroid disease, as well as the finding that anti-p53 antibodies may be detected in the sera from approximately 4% of patients suspected of having autoimmune thyroid disease.[39] The role of programmed cell death in autoimmune thyroid diseases is still poorly understood, but it is reasonable to assume that apoptosis contributes, at least partially, in regulating the maturation and control of the immune response mediated by both T- and B-lymphocytes.[24] Proline is a hydrophobic amino acid that reduces the ability of p53 to activate apoptosis, and also increases the susceptibility to thyroid cancers.[40] The role of *TP53* variants in smoke-related tumours has been widely investigated, and p53 polymorphisms have been even implicated in vascular disease severity, such as atherosclerosis.[41] Our data indicate that *ProTP53* homozygous inheritance also plays an important role in the risk of Graves' disease, especially among smokers.

As we found no association among clinical features, ophthalmopathy, cure rate and the studied genotypes, we assumed that this polymorphism profiling might not be useful as an indicator of disease severity or outcome. On the other hand, considering that Graves' disease is one of the most widely spread and common autoimmune illnesses, our data may contribute to delineate a polygenic model of susceptibility to this disease and, hopefully, for prevention. Furthermore, the inheritance of these enzymes may help explain the observed relationship between smoking habits and this autoimmune disease.

Acknowledgements

This study was supported by the grants numbers 0601651-1 and 470317/2006-0 from the State of São Paulo Research Foundation (FAPESP) and the National Council for Scientific and Technological Development (CNPq), respectively.

We thank the statistical team of the School of Medical Sciences – FCM/UNICAMP for their useful discussions and important suggestions. We gratefully recognize Drs Denize Zantut Wittman and Elizabeth J. Pavin for providing some of the patients used in this study, and Helio A. Kitagaki, Ana Carolina Longato Assuncão, Juliana Alves Camargo and Aline Castaldi Sampaio for their technical contributions.

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